IDENTIFYING MEDIATORS OF ANDROGEN-INDEPENDENT PROSTATE CANCER USING MASS SPECTROMETRY-BASED PROTEOMICS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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ABSTRACT

Androgen-deprivation remains the principal therapy for advanced and metastatic prostate cancers. However, some cancer cells are able to survive this treatment and transform themselves to a more aggressive androgen-independent prostate cancer (AIPC). An understanding of the molecular alterations that occur during the progression to androgen-independence is of utmost importance in order to generate effective targeted therapies.

Using two different in vitro approaches coupled to high through-put mass spectrometry, we were able to identify numerous potential mediators of AIPC and aggressive prostate cancer. Our first approach using an in vitro cell line model of androgen-independence allowed us to identify enzymes of the ketogenic pathway as elevated in cases of aggressive prostate cancer. Specifically, ACAT1, an enzyme in this pathway, was further validated and shown to be substantially elevated in clinical cases of castration-resistant metastatic prostate cancer. In addition, ACAT1 was found to be an independent tissue-based prognostic marker of biochemical recurrence-free survival.
In our second approach we compared the secreted proteomes of androgen independent cell lines (PC3, DU145, PPC1, LNCaP-SF, 22Rv1) to androgen-dependent (LNCaP, VCaP) and normal prostate epithelial (RWPE) cell lines. Of the over 3000 proteins identified in the secretomes, we found more than 100 proteins that were differentially secreted in the androgen independent cell lines. Of these, Protein S (PROS1) was elevated in the secretomes of all the AIPC cell lines and was not detected in the normal or androgen-dependent prostate cancer cell lines. Next, using RT-PCR and immunohistochemistry, we observed significantly higher tissue expression levels of PROS1 in localized high-grade and castrate-resistant metastatic prostate cancer samples compared to normal and low-grade prostate cancer, further indicating its importance in prostate cancer progression. Finally, functional validation revealed that PROS1 increases cell proliferation, migration and viability and thus may play a direct role on prostate cancer biology.

Taken together, the results of this research will help in not only increasing the understanding of key molecular alterations and mechanisms by which prostate cancer cells can utilize to gain androgen-independence, and more importantly, aid in the development of new targeted therapies by potentially focusing on blocking or altering specific critical signalling cascades.
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The past five years have been a challenging, yet very rewarding experience for which I have grown immensely as an individual. Although this thesis is the end product and what will be most remembered from the past five years, equally as most important for me was the actual journey leading up to this point. There have been many people that have guided me throughout this journey to make this thesis possible.

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List of Abbreviations

ACAT1, Acetyl-Coenzyme A acetyltransferase 1
AIPC, androgen-independent prostate cancer
AGR2, Anterior gradient protein 2 homolog
AKT, V-Akt Murine Thymoma Viral Oncogene Homolog
ALDH6, Aldehyde dehydrogenase 6
AR, androgen receptor
ARG2, Arginase 2
AUC, area under curve
AXL, AXL Receptor Tyrosine Kinase
BDH1, D-beta-hydroxybutyrate dehydrogenase
BPH, benign prostate hyperplasia
CM, conditioned medium
DHRS2, Dehydrogenase/Reductase (SDR Family) Member 2
DHT, dihydrotestosterone
ERG, Ets related gene
FSH, Follicle-stimulating hormone
GAS6, Growth arrest-specific 6
GBA, Glucosidase Beta Acid
GS, Gleason score
HPLC, high pressure liquid chromatography
HMGCL, 3-hydroxymethyl-3-methylglutaryl-CoA lyase
HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2
HR, hazard ratio
HRPC, hormone-refractory prostate cancer
HSD11B2, Hydroxysteroid (11-Beta) Dehydrogenase 2
HSP, heat shock protein
IHC, immunohistochemistry
IPA, ingenuity pathway analysis
LC-MS/MS, liquid chromatography-tandem mass spectrometry
LH, Luteinizing Hormone
LHRH, luteinizing-hormone releasing hormone
LTBP1, Latent Transforming Growth Factor Beta Binding Protein 1
LTQ, linear ion trap
MAOA, Monoamine oxidase A
MAOB, Monoamine oxidase B
MERTK, C-Mer Proto-Oncogene Tyrosine Kinase
MRI, Magnetic resonance imaging
NF-kB, Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells
OXCT1, Succinyl-CoA:3-ketoacid-coenzyme A transferase 1
PAM, Peptidylglycine Alpha-Amidating Monooxygenase
PCA3, Prostate cancer antigen 3
PI3K, Phosphoinositide 3-kinase
PROS1, Protein S
PSA, prostate-specific antigen
PTEN, Phosphatase and tensin homolog
SD, standard deviation
SILAC, stable isotope labeling of amino acids in cell culture
SCX, strong cation exchange chromatography
SQSTM1, Sequestosome 1
TBP, Tata-binding protein
TMA, tissue microarray
TRUS, Transrectal ultrasound
TYMP, Thymidine Phosphorylase
TWSG1, Twisted Gastrulation BMP Signaling Modulator 1
TGFβ, Transforming growth factor beta
TNF, Tumor necrosis factor
TYRO3, TYRO3 Protein Tyrosine Kinase
CHAPTER 1:

INTRODUCTION

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**Mechanisms of Androgen-independent Prostate Cancer.**

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1.1 Prostate Cancer

Prostate cancer is the most commonly diagnosed and second leading cause of cancer-related deaths among men in North America [1]. Statistically, one in six men will develop some form of prostate cancer in their lifetime, and interestingly, almost 50% of men have tumors within their prostate upon autopsy. This indicates that prostate cancer is a slow growing cancer that may not directly lead to morbidity. However, there are aggressive forms of the disease that ultimately lead to fatal outcomes. Prostate cancer is initially diagnosed with a physical digital rectal examination followed by a serum prostate-specific antigen (PSA) test [2, 3]. PSA is one of the best known cancer biomarkers available; however, it has its own limitations. Serum PSA is also elevated in other pathological conditions of the prostate including benign prostate hyperplasia (BPH) and prostatitis. In addition, PSA does not provide powerful prognostic potential, as it is unable to discriminate between indolent and aggressive forms of prostate cancer [2, 3]. Patients presenting with positive PSA tests undergo a prostatic biopsy, where histological assessment of prostatic tissue is analyzed to determine whether cancer is present or not (Figure 1.1) [2, 3]. Not surprisingly, 75% of positive PSA cases do not present with cancer, indicating the lack of specificity of the marker. It is for these reasons, that active research is necessary to understand prostate cancer pathobiology, in order to identify additional biomarkers that either complement serum PSA and/or discriminate between indolent and aggressive forms of the disease.
Figure 1.1. Prostate cancer diagnosis and treatment. After an initial physical digital rectal examination followed by a positive PSA test, a prostatic biopsy is examined. Based on histology, the biopsy will either confirm no cancer or cancer, and based on the Gleason scoring system, prostatic cancerous cells will be assigned a Gleason score. Gleason score 6 and less cancers do not require any curative treatments and undergo active surveillance, whereas Gleason 7 or higher cancers are normally treated with radical prostatectomy and androgen deprivation therapy. Patients often regress to androgen-independent prostate cancer, where there are no effective targeted therapies available.
### 1.1.1 Anatomy and Histology of the Prostate

The prostate is an organ found within the human urogenital system that consists of exocrine glands that are found surrounding the proximal urethra. It is located beneath the bladder, where the urethra from the bladder passes through the prostate before it enters the penis. The part of the urethra within the prostate is referred to the prostatic urethra.

Anatomically, the glandular tissue within the prostate can be separated into four distinct regions or lobes of the prostate: the anterior, middle, posterior, and two lateral lobes. The prostate can also be subdivided into two zones: the centrally located transition zone, and the posterior and laterally located peripheral zone. Most cases of BPH are commonly found in the transition zone, whereas most malignant tumors are found in the peripheral zone [4, 5].

In addition to secretions from the other organs of the male urogenital tract, primarily the seminal vesicles and testes, the prostate produces and secretes a fluid that combines to form seminal plasma. Seminal plasma arises from secretions from seminal vesicles (~65% semen volume), prostate (~25% semen volume), testis and epididymis (~10%) and bulbourethral and periurethral glands (~1%) [6-8]. The prostatic secretions into seminal plasma consist of proteolytic enzymes, citrate and lipids [9, 10]. In addition, the prostate secretions are alkaline in nature, which is important for the survival of sperm cells in the acidic vaginal environment [9, 10]. The most abundant proteins secreted by the prostate gland into the seminal plasma include kallikreins, prostatic acid phosphatase, zinc-α2-glycoprotein, and β-microseminoprotein. These proteins are involved in the proteolytic cleavage of semenogelins, hydrolysis of phosphomonoesters, lipid mobilization and protection against fungal infections [11-14]. Not surprisingly, the concentration of PSA (kallikrein 3) is 300,000-fold higher in seminal plasma than in blood serum.
The histology of the prostate consists of ducts and acini which are lined by a double epithelial layer. The ducts consist of luminal cells and basal cells and beneath these two layers is a basal membrane. There are also a small number of neuroendocrine cells scattered around the basal cell layers. The glands are supported by a dense stroma of smooth muscle fibres and fibrous connective tissue that exerts its major function during ejaculation when the prostatic secretions are combined into the seminal plasma.

The majority of the prostatic secretions come from the luminal cells, which are able to secrete various proteins within the ducts that are then later combined into the seminal plasma. Basal cell function is unclear; however, it is suspected that these cells act in a stem-cell like manner to replenish luminal cells, or act as a barrier between the luminal cells and the stroma [15]. The neuroendocrine cells of the prostate produce peptides such as chromogranin A, serotonin and progastrin-releasing peptide. The functions of these cells are unclear, but rare tumors derived from these neuroendocrine cells have been identified and these peptides are often useful as markers in the diagnosis [16-18].

1.1.2 Methods of Diagnosis and Prognosis

Prostate cancer is diagnosed based on an abnormal digital rectal examination (DRE) or a serum PSA test. Following these, cancer is confirmed by performing a transrectal ultrasound (TRUS)-guided biopsy, and histological examination of the tissue is assessed for whether cancer is present. In North America, it has become common practise for men over the age of 50 to have routine DRE and PSA tests conducted. Men who are deemed high risk for the disease based on family background are assessed even earlier.
A DRE is performed by a physician to assess the physical features of the prostate to see if there are any extensions to the area surrounding the prostate. However; this test is highly subjective and the results often do not correlate well with disease progression. It does nevertheless aid in determining if a tumour has spread outside of the prostate or remains localized.

In conjunction with a DRE, a serum test is also administered to assess the presence of elevated PSA levels, which is a highly prostate-specific protein produced in high quantities by prostatic cells. Interestingly, at a cellular level, it has been found that both normal and BPH tissue produce more PSA than prostate cancer tissue; however, serum levels of PSA are elevated in cases of prostate cancer and BPH because of a disruption of the basement membrane surrounding the glandular epithelial cells [19]. This facilitates the entry PSA protein into surrounding blood vessels, resulting in increased levels of this marker in serum. Normal concentrations of serum PSA are less than 4 ng/mL; however, there is continuous expression of this marker in benign conditions and prostate cancer, which reduces its specificity. In addition, PSA is also elevated in other benign conditions of the prostate including BPH and prostatitis. Thus, approximately 75% of the patients that present with a positive PSA test don’t actually have prostate cancer, again indicating that PSA is not a very specific marker for cancer itself [20]. In lieu of this, elevated PSA is still a good marker of aberrant prostatic pathology.

Following abnormal results of the DRE and/or serum PSA test, a TRUS is performed on the prostate to determine staging, prostate volume and deduce areas that needle biopsies can be performed on [21-23]. Based on the TRUS results, a needle biopsy is performed transrectally, and areas that are suspected to be cancerous or abnormal are sampled with at least 10 to 12 cores. Biopsies are normally taken from the peripheral or transition zones of the prostate. Each of these
cores is then assessed by a pathologist to deduce whether there is cancer tissue present. One of the best prognostic indicators for prostate cancer is Gleason score (GS), which characterizes the glandular architecture of the prostate based on a score that represents the level of cancer ‘de-differentiation’ [24, 25]. The Gleason score is comprised of two numbers, each representing the most common Gleason patterns ranging from 1 to 5, where 1 represents a highly differentiated carcinoma and 5 represents an aggressive de-differentiated one. It is now accepted that the transition from pattern 3 to pattern 4 represents disease progression from low-grade to high-grade. Gleason 7 cancers, which are comprised of pattern 3 and pattern 4, are considered an intermediate state, requiring definitive treatment such as prostatectomy or radiotherapy. In addition, a proportion of patients with Gleason score 6 prostate cancers are eligible for active surveillance instead of immediate curative therapy. Following pathological assessment, further imaging of the surrounding tissue may be utilized using ultrasound, computed tomography, magnetic resonance imaging in combination with contrast agents to determine the spread of the tumour to distal sites including bone and lymph nodes. Based on these results the tumour may be staged based on the Tumour Node Metastasis (TNM) staging system, where appropriate prognosis and treatment options are deduced.

### 1.1.3 Current Treatments

Treatment options for prostate cancer depend on whether the cancer is clinically localized or has spread to distal sites. Clinically localized tumours present challenges for treatment, as a large portion of these cases remain in an indolent state, not requiring immediate treatment. Most of these tumours are slow growing; however, 15% of these cases are known to be aggressive in nature, requiring some form of direct treatment [26, 27]. A major problem is defining high-risk
disease. Although the literature on ‘high-risk’ prostate cancer is extensive, a set classification scheme that enables outcomes for patients with high-risk prostate cancer to be determined in a reliable manner remains elusive. This is also confounded by the multiple methods of diagnostics used to classify patients, and by variations in the treatment modalities themselves, based primarily on surgery or radiotherapy.

Treatment options vary based upon the Gleason score. It is well accepted that patients who present with a Gleason grade of 6 or less are considered to be ‘low grade’ or indolent. For these patients, a management approach referred to as “active surveillance” is undertaken. Here patients enter a clinical follow-up program in which the tumour size and progression is measured by routine serum PSA tests [28, 29]. If there is suspicion of elevated serum PSA and tumour progression, subsequent biopsies will be performed and the resulting tissue will be assessed to deduce whether the tumour has become more aggressive, which in turn would require direct treatments. For many older patients that present with localised and low grade/indolent tumours, active surveillance approach may seem to be a better option in order to avoid over treatment. It was found that patients that undertook conservative management of localised prostate cancer using active surveillance protocols had a 10-year survival of 94% for Gleason 2 to 4 tumours compared to 45% for Gleason 8 to 10 [30]. However, a major issue with solely relying on Gleason grade as a measure of disease prognosis and aggressiveness is that 20% of patients that present with Gleason 6 or less cases (which are deemed as indolent), actually have aggressive disease. For this reason, additional biomarkers that can aid in distinguishing aggressive from non-aggressive disease are required. One such approach could be with the use of MRI in conjunction with biopsy, termed MRI-targeted biopsy. It was found in a series of patients who underwent extensive systematic biopsy and MRI-targeted biopsy that 24% had their Gleason
score upgraded from the initial biopsy [31, 32]. In addition, many groups are also trying to identify various biological molecules such as proteins and RNA markers in tumours from diagnostic biopsy samples to further refine risk assessment [33]. These include cell proliferation markers such as Ki-67, alterations in specific pathways like the PI3K/PTEN signalling cascade, and copy number alterations at the DNA level. In addition, PCA3, a prostate cancer-specific noncoding mRNA is overexpressed in prostate cancer compared to benign normal tissue and detected in urine, has also been used effectively in an active surveillance setting [34, 35]. Although PCA3 levels correlate well with Gleason grade and tumour volume in radical prostatectomy cases, its use to define disease risk are still uncertain. For patients that present with high-risk prostate cancer, immediate treatment options are required. Various treatment options for aggressive prostate cancers include radical prostatectomy, radiation therapy, androgen deprivation therapy, and chemotherapy.

**Radical Prostatectomy**

Since the advent of PSA testing, prostate cancer diagnostic rates have been elevated in clinically localised disease, many of which are clinically relevant [36]. Radical prostatectomy is the complete removal of the prostate gland to treat high-risk localized prostate cancer or urinary obstruction due to BPH. An extended pelvic lymph-node dissection is also normally conducted to remove potential cancerous tissue that has invaded surrounding areas. The survival benefit of radical prostatectomy will vary by patient, as the patient may die of prostate cancer or other pathological conditions most notably cardiovascular disease or old age. Two large independent studies assessed whether men that underwent radical prostatectomy versus watchful waiting had benefitted from this surgical procedure. The first study known as the Prostate Cancer Intervention Versus Observation Trial (PIVOT) randomly assigned 731 men with newly
diagnosed prostate cancer to either watchful waiting or radical prostatectomy. Surprisingly, no overall benefit of surgery was observed, although bone metastasis-free survival was higher in the radical prostatectomy group [37]. The second study was the Scandinavian SPCG-4 study, which also randomised patients into receiving radical prostatectomy or watchful waiting [38]. This study showed that after 15 years, radical prostatectomy decreased prostate cancer-specific mortality, and the incidence of bone metastases.

*Radiation Therapy*

Another treatment option that is typically utilized to treat patients with localized prostate cancer is radiation therapy. The two most commonly used radiation therapies for prostate cancer are external beam radiation and brachytherapy. Radiation therapy works on the concept that radiation directed towards a specific malignant region of the prostate will cause DNA damage and genetic instability, resulting in the destruction of the targeted cells. Cancer cells normally have a higher susceptibility to radiation as they typically have defects in cellular repair mechanisms, typically resulting in higher cell death compared to normal cells.

External-beam radiation has been utilized for many years for the treatment of localized prostate cancer [39]. Typically a patient is administered daily with either X-rays or gamma rays externally for a 6 to 10 week duration. The implementation of image-guided radiation therapy has allowed for increased accuracy and precision of the target localization, in turn, reducing the amount of healthy tissue in the treatment field [40]. Three-dimensional images of the prostate and surrounding tissues are generated using computed images to produce high resolution images. From here, optimal delivery and doses of radiation are determined and administered [41]. External-beam radiation is often administered in patients with a rising or detectable PSA after radical prostatectomy.
Unlike external beam radiation, brachytherapy is a form of radiotherapy whereby a radiation source is placed inside or next to the area requiring treatment [42, 43]. It is often advantageous as the radiation is confined to malignant areas of the prostate, where there is little leakage of radiation to the surrounding tissue. The survival rate of brachytherapy is similar to that of external-beam radiation or radical prostatectomy. It is also often administered in conjunction with external-beam radiation. The procedure is also completed more quickly and the patients are able to go home on the same day of treatment. In addition, the use of permanent metal seed implants is often a less invasive option compared to radical prostatectomy [44]. Some of the common side-effects of external-bean radiation and brachytherapy include incontinence, impotence, infertility, bowel problems and fatigue.

*Androgen Deprivation Therapy*

Both normal and malignant prostate cells require androgens for their growth and development. The discovery that prostate cancer growth relied on factors such as androgens resulted in the awarding of Dr. Charles Brenton Huggins with the Nobel Prize in 1966 [45]. Since then, it is known that prostate cancer cells deprived of androgens have decreased proliferation and elevated apoptosis rates, whereas normal prostate cells undergo atrophy resulting in a reduction of the overall size of the prostate gland. Consequently, methods to block the actions of androgens have become a very efficient treatment for prostate cancer, especially metastatic disease.

There are various methods used to decrease overall androgen levels, including orchiectomy (surgical castration) and chemical castration. The gold standard for metastatic prostate cancer has been bilateral orchiectomy. By this method, 95% of serum testosterone levels are eliminated, resulting in a permanent rise in the hormones LH and FSH, both of which
are integral in the feedback mechanism to produce testosterone. In addition to surgical castration, there also exists a method to reduce androgen synthesis via chemical castration. Here, one of two different treatments can be utilized: LHRH agonists or antagonists, both of which lower the levels of testosterone made by the testicles. Both essentially work by inhibiting the formation of LH in the pituitary gland. Some of the most common LHRH agonist and antagonists used in the clinic include leuprolide, goserelin, triptorelin, histrelin and degarelix. These drugs are normally injected into the blood, and achieve similar results of surgical castration. Another method for androgen deprivation is the use of anti-androgens. In the next section, we will go into detail on androgen action; however, briefly, androgens are a substrate for the androgen receptor protein (AR), a nuclear transcription factor that regulates the expression of many genes [46]. Many molecules that can inhibit the activity of AR have also been used, and are known as anti-androgens. Anti-androgen molecules can enter prostate cancer cells and prevent the binding of testosterone to the AR, due to higher affinity to this protein than androgens themselves. This effectively inhibits the protein function and prevents it from performing its normal downstream roles as a transcription factor. The most commonly used anti-androgens include flutamide, bicalutamide, and nilutamide, all of which are taken orally.

*Chemotherapy for Advanced and Aggressive Prostate Cancer*

Although many of the aforementioned treatments are effective, patients who present with aggressive/androgen independent prostate cancer (AIPC) often do not have definitive treatment options available other than conventional chemotherapies. Current chemotherapeutic agents used for the treatment of aggressive/AIPC have not shown strong survival benefits, but provide palliative care and improve overall quality of life. The current chemotherapeutic agents available for advanced prostate cancer include: mitoxantrone, prednisone, docetaxel and
paclitaxel. The vast majority of these agents target fast growing cells and alter important cellular machinery mechanisms. For example, docetaxel and paclitaxel both function by inhibiting microtubule spindle formation, resulting in defects in mitosis. Although they all appear to have a slight benefit on overall survival (usually months), they are non-specific, affecting other non-cancerous cells as well.

1.2 Pathobiology of Prostate Cancer

1.2.1 Progression Model of Prostate Cancer

A normal prostate requires basal levels of androgen for growth and survival. Likewise, during prostate cancer development, the cancerous cells are initially dependant on androgens. Figure 1.2 illustrates a simplified model of prostate cancer progression. At first, multiple carcinogenic processes occur, whereby some cells are altered and begin to proliferate out of control. If detected early, androgen ablation can be used for therapy either via chemical castration using anti-androgens, or surgical removal of the testicles, the major producers of androgens. This therapy is very effective in the destruction of androgen-dependent cells. However, over time, this continuous androgen ablation results in the selection of cell subpopulations that can survive in the absence of androgens, leading to an emergence of androgen-independent cancer. Further evolution of these androgen-independent cells can result in increased angiogenesis, whereby the cells metastasize and migrate to distant sites, primarily the bone and lymph nodes. The specific molecular alterations that govern these changes still remain unknown, but one thing remains clear; the androgen receptor is a major player involved in this process.
Figure 1.2 Progression to androgen-independence in prostate cancers. 1) Various carcinogenic processes occur whereby some prostate cells proliferate out of control. 2) Prostate cancer cells are initially androgen-dependent; therefore, androgen-deprivation therapy is successful in destroying these cancer cells. 3) Some cells are able to survive this treatment and continue proliferating. 4) Cells are now androgen-independent and gain subsequent changes resulting in increased angiogenesis. 5) AIPC begins to metastasize to distant sites.
1.2.2 Androgen Receptor Signalling – a Key Regulator of Prostate Cancer Progression

Testosterone, the main androgen produced by the body, is predominantly released by the Leydig cells of the testes [46]. Small amounts are also produced in the adrenal glands. Free circulating testosterone, which is not bound to steroid hormone binding protein (SHBP), can enter prostate cells, where it can be converted to its more potent metabolite dihydrotestosterone (DHT), which, in turn, binds to the androgen receptor (AR) protein (Figure 1.3) [46]. Testosterone itself can bind to the androgen receptor as well, but DHT has a much higher affinity [46]. The androgen receptor is a nuclear transcription factor that can activate and regulate the expression of many genes involved in growth and proliferation. The AR protein consists of three domains: a central DNA binding domain, ligand binding C-terminal domain, and an N-terminal transactivation domain. The N-terminal domain plays a crucial role in the transcriptional transactivation activity of the AR, as inhibitory studies of this domain result in decreased AR transcriptional activity [47]. The ligand-binding domain is where androgens bind to result in a conformational change in the AR where it dissociates from heat shock proteins in the cytoplasm, and localizes to the nucleus [48]. In the nucleus, AR binds to specific DNA sequences called androgen responsive elements (ARE) via the DNA-binding domain, promoting further association of factors into a complex, which leads to gene transcription [49]. Various genes are regulated by the AR, including kallikrein-3 (KLK3) formerly known as prostate-specific antigen (PSA).

As alluded to earlier, most prostate cancers begin in an androgen-dependent state, where androgens are required for the AR to be activated and regulate its downstream effectors. However, during androgen deprivation, the AR is not activated as prominently, resulting in decreased growth and proliferation of cancerous cells [50]. The androgen receptors, as well as
factors of the androgen receptor signalling pathway, have been found to be aberrantly expressed or mutated in many prostate cancers, leading to speculation that these proteins remain as key players in prostate cancer progression [51-55].
Figure 1.3 Mechanisms of the androgen action and androgen receptor signalling in prostate cells. T, testosterone; DHT, dihydrotestosterone; HSP, heat-shock protein; AR, androgen receptor; ARE, androgen responsive element. For explanation of pathways please see text.
1.2.3 Androgen Independent/Castration-Resistant Prostate Cancer

Approximately 70% of prostate cancer patients respond well to initial androgen deprivation therapy; however, most tumours eventually become androgen-independent/castration-resistant [56]. These tumours are able to grow even when sub-optimal levels of androgens are available. The prognosis of these patients is poor with median survival time of less than 10 months.

1.3 Mechanisms of Androgen-independent/Castration-Resistant Prostate Cancer

There are many proposed models describing how the AR signalling cascade may be altered including AR gene amplification, AR mutation, changes in AR co-regulator levels, alterations in steroidogenic pathways and activation in a ligand-independent manner via alternative ‘outlaw’ pathways (Figure 1.4).
Figure 1.4 Mechanisms of androgen-independence in prostate cancers. AIPC can arise due to many cellular changes. The AR signalling pathway is by far the most commonly studied pathway in the context of AIPC. This pathway has been shown to be aberrantly regulated at various levels including: gene amplifications, mutations, changes in the levels of AR co-regulators or steroidogenic enzymes. The AR protein has also been shown to be activated in ligand-independent manner via ‘outlaw’ pathways by a number of different proteins. Various AR-independent ‘bypass’ mechanisms/pathways have also been implicated in the development of AIPC. AR, androgen receptor; CR, co-regulator; T, testosterone.
Androgen Receptor Gene Amplifications

Androgen receptor over-expression has been implicated in many AIPC both in vitro and in vivo. Together, gene and protein expression data show that the AR is overexpressed at the mRNA and protein level, respectively [57-59]. Studies have found that approximately 25-30% of androgen-independent tumours have AR amplifications [59]. Interestingly, AR amplification has not been found in any untreated prostate cancer samples, suggesting that AR amplification is one by-product of hormone therapy leading to AIPC. Gene amplifications of the AR loci have also been found in many clinical prostate cancer samples that were in an androgen-independent state, indicating that gene amplification may lead to AR protein over-expression, and subsequently to increased AR signalling. Recently, it was found that increased AR expression sensitized prostate cancer cells to lower than normal levels of androgens [60]. High AR protein expression help cancer cells sustain and continue proliferating in environments with minimal androgen concentrations, which can explain the evolution of AIPC during androgen deprivation [60]. Furthermore, AR over-expression at the mRNA and protein level has also been observed in the absence of AR gene amplification, suggesting gene amplification-independent regulators such as epigenetic and miRNA factors [49]. It appears that tumors have selective pressures for continued AR signalling to allow for survival and further evolution, therefore therapies that are more efficient at blocking this crucial signalling pathway are promising to prevent or slow cancer progression.
1.3.2 Androgen Receptor Mutations

Androgen receptor mutations are another means for prostate cancer cells to gain androgen-independent properties. The AR gene is located on the X chromosome, and a loss of function of the gene results in androgen-insensitivity syndrome. The frequencies of genetic mutations in the AR loci are typically rare in early stage prostate tumors (0-4%) (24), but become more frequent in advanced and recurrent tumors [61]. AR mutations have been reported in 10-20% of patients with androgen-independent tumors, strengthening the model that particular mutations in the AR gene help cells to survive and proliferate in androgen deprived conditions [61]. The ‘McGill Androgen Receptor Gene Mutation Database’(http://androgendb.mcgill.ca/) website provides an extensive list of all reported AR mutations, as well as the specific domains within the protein that have been altered. In this review we will only focus on the most common AR mutations present in the context of AIPC.

The first reported AR mutation was described in the hormone-dependent LNCaP human prostate cancer cell line derived from a lymph node metastasis [62]. This cell line contains a missense mutation at codon 877 of the AR mRNA, resulting in an amino acid substitution of threonine to alanine [62]. This mutation occurs in the ligand binding domain, and results in decreased ligand specificity, whereby other hormones such as progesterone, estrogens and many anti-androgens can bind and activate the transcription factor. During androgen ablation, such a mutation becomes beneficial for the cancer cells, as it broadens the specificity of the for activation, leading to growth advantage and survival over cells that lack this mutation. In an early study, Gaddipati et al. [63] found that 6 out of 24 metastatic tumor samples harboured this T877A mutation, indicating that the mutation is commonly found in patients with AIPC. Furthermore, the frequency and nature of AR mutations in prostate cancers appears to be
dependent upon the stage and selective pressures exerted on the cancers. Localized cancers appear to have a lower frequency of mutations, whereas tumors that have metastasized have increased incidence of AR mutations [61]. A study by Marcelli et al. showed that point mutations were found in 8 of 38 patients with lymph node metastasis on hormone therapy, whereas no mutations were found in 99 prostatectomy-removed glands of patients with no hormone therapy [64].

As is the case with the T877A mutation, most of the clinically relevant AR mutations are found in the ligand-binding domain of the protein, resulting in either broadened ligand specificity or constitutive protein activity. These mutations also include H874Y, V715M, L701H+T877A, and Y741C [61, 65-67]. Another subset of mutations that have been observed in a small number of patients is loss of function mutations of the AR. Although poorly understood, it is speculated that loss of function mutations can confer growth advantage to cells that have already surpassed the need for androgens.

AR splice variants have also been identified recently, and were found to be over-expressed in AIPC. Guo et al. discovered 3 novel variants (AR3, AR4, AR5) in AIPC, all lacking the ligand-binding domain [68]. Although studies on these variants are premature, they nevertheless present an interesting mechanism through which cancer cells may survive androgen ablation. Cells harbouring these variants can by-pass the need for androgens, since AR can become constitutively active. Also, considering that these isoforms are not inhibited by currently available anti-androgens, the development of new drugs targeting these isoforms may provide another effective treatment for AIPC.
1.3.3 Changes in Expression of AR Co-regulators

Considering that the AR is a nuclear transcription factor, it relies on many interactions with various co-regulatory proteins, to form a productive transcriptional complex. These co-regulators can either enhance (co-activators) or reduce (co-repressors) AR transactivation, resulting in altered transcription rates. As of 2007, approximately 170 proteins have been identified as AR co-regulators [69]. Alterations in the balance of these co-regulatory proteins can perhaps provide growth advantages to prostate cancer cells; thus, these co-regulators are becoming very interesting targets for therapeutics. Due to the sheer number of AR co-regulator proteins that have been identified, it would not be plausible to discuss each one. For the sake of this review, we will focus on some of the main co-regulators that have been thoroughly studied in the context of AIPC.

A. Co-activators

AR co-activators can be classified into distinct groups. SRC/p160 co-activators such as TIF2, GRIP1 and SRC1 share common structural components and are able to recruit other transcription factors to initiate transactivation of AR regulated genes [70]. These co-activators are also able to recruit other co-activators that contain histone acetyl-transferase activity such as Tip60, CBP/p300, p/CAF, which are involved in the acetylation of specific histone residues resulting in chromatin remodelling [71]. Gregory et al. found that levels of TIF2 and SCR1 increased in AIPC samples that had increased AR expression [72]. In another study, Tip60 was also found to be over-expressed in AIPC [73]. Another broad group of AR-associated (ARA) proteins have been described as being potent AR co-activators. These ARA proteins (ARA24, ARA 54, ARA55, ARA70, ARA170, ARA267) do not share structural or functional similarities, but are potent co-activators of the AR [74].
**B. AR Co-repressors**

Unlike co-activators, AR co-repressor proteins can form complexes with AR and inhibit the transcription of AR-regulated genes. Any alterations in the expression of these co-repressor proteins can also play an important role for the development of AIPC. Two well-characterized AR co-repressors are nuclear receptor co-repressor (NCoR) and its homologue silencing mediator for retinoid and thyroid hormone receptors (SMRT) [74]. Both these co-repressors can recruit histone deacetylases, which promote chromatin packing, resulting in reduced transcriptional activity.

During androgen deprivation, changes in the levels of these co-activators and co-repressors can promote the expression of androgen-regulated genes [49, 50, 52, 53]. Increases in the expression of various co-activators, or decreases in expression of co-repressors can provide a means for increased AR activity, promoting prostate cancer cell survival in androgen-deprived conditions.

### 1.3.4 Increased Steroidogenic Signalling Pathways

As it has become apparent, androgens are the mediators for AR activation. Following androgen binding to the AR, a conformational change occurs whereby the AR protein dissociates from HSP in the cytosol, translocating to the nucleus. Therefore, any alterations in the production of these androgens, or their precursors could have an effect on the development of AIPC.

Although LHRH agonists are effective at blocking androgen production by the testes at supra-physiological concentrations, the adrenal glands are also capable of producing circulating
androgens, albeit at much lower concentrations. Along with endogenous production from the adrenal glands, recent studies have shown that *de novo* synthesis of androgens within the tumour may also play a critical role to maintain levels that can activate AR [54]. Furthermore, over-expression of enzymes involved in the steroid biosynthetic pathway has been observed in AIPC samples [54].

In the prostate, free testosterone is converted to its more active metabolite DHT, and this reaction is catalyzed by the enzymes 5alpha-reductase type 1 and 2 (5α-reductase). Comparative analysis of testosterone and DHT in the serum and prostate tumor samples, showed higher DHT concentrations in the tumors than the serum, suggesting that the cancer cells might be up-regulating the 5α-reductase enzymes to provide sufficient DHT for AR signalling [75]. Montgomery *et al.* aimed to determine whether prostate cancer metastases were capable of synthesizing androgen *de novo*, by quantifying the transcripts encoding enzymes involved in steroid biosynthesis [76]. They found that castration-resistant metastases expressed higher levels of many enzymes responsible for the synthesis of adrenal androgens. They also observed high levels of intratumoral androgens in castrate-resistant xenografts lacking the adrenal *CYP17* gene, which is critical for the production of androgens by the adrenal glands.

More recently, Hofland *et al.* conducted a study showing that intratumoral steroid biosynthesis contributes less androgen to the tumors than circulating adrenal androgens, suggesting the blockade of *CYP17* as an interesting therapeutic target in AIPC patients [77]. Further studies using abiraterone acetate, a potent inhibitor of *CYP17*, have also shown a positive effect on survival.
1.3.5 “Outlaw” Pathways

So far we have been discussing the various ways AR signalling can be altered in the presence of its androgen ligands. However, various steroid hormone receptors, including AR, can also be activated in a ligand-independent manner referred to as “outlaw” pathways. The AR also has non-genomic roles where it can interact with various other signalling pathways and alter them. Many growth factors, cytokines, kinases and other proteins have been demonstrated to activate the AR at low or even near-zero levels of androgens.

A. Growth Factor-Induced Activation

Growth factors such as insulin-like growth-factor-1 (IGF1), keratinocyte growth factor (KFG) and epidermal growth factor (EGF) can activate the AR, allowing the induction of transactivation of AR target genes in low androgen conditions [65]. IGF1 has been the most widely studied of the growth factors, and was shown to potentiate the signalling of AR, even in the absence of androgens. Interestingly, when prostate cancer cells are subjected to anti-androgen treatment, IGF1 is no longer able to activate the AR, suggesting a direct interaction between these proteins [65]. IGF1 has also been shown to promote AR signalling by increasing the expression of various AR co-activators such as TIF2 and insulin degrading enzyme (IDE) [78]. Likewise, EGF signalling activates the AR in a ligand-independent manner and induces the transcription of AR-regulated genes [65]. These growth factors are all ligands for receptor tyrosine kinases (RTK), which just illustrates the importance of RTK signalling in prostate cancer progression.
B. Cytokine Activation

Along with growth factors, various cytokines can activate the AR. Interleukin-6 (IL6) and interleukin 8 (IL8) are cytokines that are regulated by the NF-kB signalling pathway, which has been shown to be upregulated in many AIPC. Increased NF-kB signalling results in increased AR activation in LNCaP cells, and by blocking this signalling pathway, AR activation is inhibited [79]. Likewise, IL6 and IL-8 are known to stimulate the AR activity and enhance the expression of AR regulated genes and, much like IGF1, when cells are treated with anti-androgens, the AR signalling is inhibited even in the presence of these cytokines, suggesting a direct interaction.

C. Receptor Tyrosine Kinase-Induced Activation

Receptor tyrosine kinases are important signalling molecules that are aberrantly expressed in a wide variety of pathologies, especially cancers. One of the major RTKs that have been implicated with AIPC is HER-2/neu (ERBB2). HER-2/neu has been shown to be over-expressed in androgen-dependent cell lines that have been converted to androgen-independent cell lines, as well as sublines that have been xenografted into castrated mice [80, 81]. Over-expression of HER-2/neu in prostate cancer cells can activate AR-induced genes in the absence of androgens, but unlike IGF1, IL6 and IL8, treatment with anti-androgens does not block the AR signalling pathway, indicating that this pathway is independent of the AR-ligand binding domain [80]. Along with HER-2/neu, other RTK pathways that have been implicated in AR signalling activation are IGFR and EGRF, both of which are the receptors for IGF1 and EGF, respectively. These receptors are known to induce downstream activation of essential growth and survival pathways, including AKT, MAPK and STAT pathways, many of which are also aberrantly expressed during AIPC.
D. Wnt Signalling Activation

The canonical Wnt signalling pathway has also been implicated with AIPC, as β-catenin, a main downstream effector in this pathway has been shown to interact with the AR and modulate its function [82, 83]. Interestingly, during AIPC, Wnt has been shown to activate the AR, which can then act as co-activator for β-catenin activation of Wnt-related genes as well as AR-induced genes. Studies *in vivo* have also shown that β-catenin and AR both colocalize and interact in castrate-resistant tumors in mice, but surprisingly not in tumors harvested from non-castrated mice [84]. Taken together, the Wnt/AR crosstalk is another important interaction that can promote AIPC in a ligand-independent manner.

1.3.6 Non-AR Related Pathways: “Bypass” Pathways

The mechanisms discussed so far are dependent upon AR and its signalling cascade for the development of AIPC. However, alternative pathways can also be involved in the progression to AIPC, regardless of AR signalling. These pathways have been deemed “bypass” pathways, as they are defined as pathways that are completely independent of AR signalling. It is interesting to note that many of the outlaw pathways we mentioned previously, which activate AR signalling in a ligand-independent manner, can also act as bypass pathways as well. Growth factors such as IGF1 and their respective tyrosine kinase receptors can activate a signal transduction cascade inducing the expression of various genes responsible for promoting cell growth and survival. Many of these pathways activate critical kinases such as MAPK/Ras/Raf/PKC, which influence cell-cycle regulation and increase cell proliferation by activating various transcription factors such as AP1, c-MYC and NFkB [85]. For example,
Weber et al. have demonstrated that prostate cancer specimens recurring in castrated mice have increased MAPK expression and activity [86].

Similarly, the Akt signalling cascade is also important for the development of AIPC in an AR-independent manner. As mentioned previously, Akt signalling can activate AR signalling via outlaw mechanisms; however, it also has key roles in the control of apoptosis and proliferation in prostate cancer cells [87]. Interestingly, PTEN, a pro-apoptotic protein that inhibits the Akt signalling cascade, is frequently found mutated or functionally inactive in AIPC, further demonstrating the importance of Akt signalling in cancer cell survival [88]. Akt signalling can also alter cell cycle regulation by decreasing protein expression of p27, an important cell-cycle regulator [89].

During androgen deprivation, apoptotic pathways are induced, indicating that anti-apoptotic factors become an important way to circumvent programmed cell death due to the absence of AR signalling. Bcl-2 is a critical anti-apoptotic protein that can help prostate cancer cells block apoptosis. In normal prostatic epithelial cells, Bcl-2 is not normally expressed; however, it is expressed in prostatic intraepithelial neoplasia (PIN), as well as in AIPC [90]. Liu et al. were able to detect Bcl-2 expressing prostate cancer cells in xenografts in castrated mice, and by down-regulating Bcl-2 with siRNA in LNCaP cells, there was a delay in the onset of AIPC in xenograft models [91].

Both bypass and outlaw pathways have been shown to interact with one another as they can induce AR signalling as well as other pathways, however, the exact molecular mechanisms of this interaction warrants further investigation. Upregulation of oncogenes, such as Bcl-2, and decreases in tumor-suppressor genes can play prominent bypass roles in the development of AIPC.
1.3.7 Epigenetic Alterations

Epigenetic regulation plays a critical role in normal cellular development, so it is not surprising that many genes have aberrant epigenetic signatures during cancer development. A number of epigenetic alterations, including methylation and histone modification being the most prominent, have been reported in prostate cancer progression, and specifically AIPC; however, discussing each one is beyond the scope of this review. We will focus on the most relevant epigenetic alterations that occur during AIPC.

Hypermethylation has been linked to the AR gene in a subset of metastatic and AIPC cancers. Previously, we pointed out that AR over-expression is a mechanism by which AIPC compensates for decreased androgen levels; however, 20-30\% of AIPC, and the two highly studied DU145 and PC3 cell lines, do not express AR [92]. The loss of AR is due to promoter hypermethylation, and it has been observed to be more prevalent in AIDP tumors than primary androgen-dependent cancers [93]. Gene silencing of the AR via DNA methylation suggests an alternative mechanism leading to androgen-independence in a subset of patients with advanced prostate cancer.

Furthermore, epigenetic modulation of various genes involved in cell cycle control, cell invasion, cellular architecture, DNA damage repair, tumor-suppressors and oncogenes have been shown in many prostate cancer samples and cell lines [94, 95]. The major genes that have been shown to be epigenetically altered in prostate cancers include CDKN2A, E-cadherin (CDH1), CD44, MGMT, RASSF1A, MDR1, APC and GSTP1. Of all these genes, GSTP1 promoter methylation is the most frequently detected epigenetic alteration with a frequency of 70-100\% in prostate cancer DNA samples [96][97]. Methylation of this gene has been detected in prostate cancer cells or DNA isolated from blood, plasma or serum, prostate secretions, voided urine and
prostate biopsy specimens [94, 95]. However, further investigation needs to be conducted in this field, as it is seems to be a promising way of not only identifying aberrantly expressed genes that are involved in prostate cancer progression, but also providing potential useful clinical biomarkers of aggressiveness based on specific epigenetic signatures.

1.3.8 miRNA Regulation

MicroRNA regulation is another mechanism that can have an impact on the progression to AIPC. These small non-coding RNAs can regulate gene expression in a post-transcriptional manner. They have critical functions during development, and many disease pathologies including cancers, exploit miRNA regulation to promote tumor formation and progression. Just as various proteins can be classified as oncogenes and tumor-suppressor genes, miRNAs can act in a similar fashion. Up-regulated miRNAs that inhibit tumor-suppressor genes in cancer cells have been termed oncogenic miRNAs or oncomirs. On the other hand, miRNAs that are down-regulated to promote cancer progression are known as tumor-suppressor miRNAs. Many studies have been conducted with the aim of elucidating various miRNAs that are dysregulated during prostate cancer progression. At present, there is still much controversy in the literature regarding miRNA regulation in prostate cancer, as results from one study, at times, contradicts another. Nonetheless, for the sake of this review, specific miRNAs that have been studied in the context of AIPC include miR-221, miR-222, mir-125b and miR-146. For a comprehensive analysis of many other miRNAs implicated in prostate cancer, several reviews are available [98, 99].

Two of the most frequently over-expressed miRNAs in many cancers include miR-221 and miR-222 and have been identified as oncomirs because they target p27, an important cell
cycle inhibitory gene [100]. Interestingly, these miRNAs have been found to be over-expressed in AIPC, but down-regulated during androgen-dependent prostate cancers. Sun et al. determined that by over-expressing both miRNAs in the androgen-dependent LNCaP cell line, there was amplified androgen-independent growth [101]. On the contrary, knocking down miR-221 and miR-222 in an androgen-independent LNCaP clone, resulted in decreased growth rates of these cells and increased reliance and sensitivity to DHT.

Furthermore, unlike miR-221 and miR222, the miR146 has been consistently shown to be down-regulated in androgen-independent cell lines when compared to androgen dependent cell lines [102]. Androgen-independent PC3 cells exogenously over-expressing miR-146 had decreased cell proliferation and survival, which implies that miR-146 may act as a tumor-suppressor which is therefore down-regulated during progression to AIPC.

The miR-125b has also been shown to be over-expressed in AIPC, as androgen-independent LNCaP cells have increased expression compared to parental cells [103]. Shi et al. found that the pro-apoptotic protein Bak1 is a target to miR-125b, and interestingly, this protein has been shown to be decreased in hormone-refractory tumors [103]. The overall role of miR125b as an oncomir in prostate cancer progression needs to be further investigated, considering that some studies have demonstrated its down-regulation during AIPC [104]. Additionally, miR-125b has been described as a tumor-suppressor in breast cancers, where it can negatively regulate the expression of HER-2/neu [105]. This is particularly interesting because Her-2/neu, as previously described, has been found to be over-expressed during AIPC, supporting the idea that miR-125b may be decreased during AIPC, in order to promote increased expression of HER-2. Further studies need to be conducted to elucidate the exact roles of miR-
125b during AIPC, as it appears to act as both an oncomir and tumor-suppressor based on different cellular environments.

In a recent study by Narayanan et al, the identification of feedback loops between various miRNAs, the AR and its co-repressors was assessed [106]. They found that the AR could regulate gene expression through a three step pathway which includes miRNA activation, corepressor suppression and DNA interaction [106].

1.4 Prostate Cancer Model Systems

The development and utilization of various mouse and cell culture models of prostate cancer has enabled the study and understanding of disease progression. These models are especially useful to study the mechanistic role of various genes and proteins, as well as studying potential therapies for the disease. Here we discuss the commonly used mouse models and cell lines with respect to understanding prostate cancer pathobiology.

1.4.1 In vivo Mouse Models

The use of mouse models has been of enormous aid in understanding prostate cancer pathobiology. The most commonly used mouse model in prostate cancer is the transgenic adenocarcinoma of the prostate (TRAMP) mouse, where Simian virus SV40 large and small T antigens are expressed under the prostate specific probasin promoter [107]. In these mice, prostate cancer typically develops within 12 weeks of birth with initial prostatic intraepithelial neoplasia (PIN), a malignant precursor phenotype. By 30 months, metastatic lesions within these mice are observed. Another mouse model utilized in prostate cancer research is the LADY
mouse, which is more commonly used as a model for early prostate cancer progression. In these mice, the SV40 antigen is also utilized, and PIN lesions are seen by 20 months; however, no metastatic spread is observed [108]. Two additional commonly used mouse models are the c-myc mice [109] and the heterozygous PTEN mice [110]. PTEN is a common deletion observed in many prostate cancers. The PTEN these mice lack one copy of this gene, and have been shown to recapitulate many features of prostate cancer progression. Xenograft models are also often utilized, where human prostate cancer cells from patients or cell lines are injected into immunocompromised mice to assess for malignant growth [111, 112]. In these studies, specific proteins are usually knocked down or over-expressed in the initial human cells before being injected into the mice. The resulting growth potential in the immunocompromised mice would give further knowledge as to whether the manipulated protein plays an important role with respect to prostate cancer pathobiology.

When it comes to studying aggressive AIPC prostate cancer, unique xenograft models have been developed by many groups where mice are either castrated or non-castrated and injected with human primary prostate cancer cells. In castrated mice, the resulting tumours were considered to be androgen-independent, whereas in the non-castrated mice, the tumours were deemed androgen-dependent [113]. These xenografts have been widely used and shown to thoroughly recapitulate AIPC progression. A rat model of prostate cancer known as the Dunning rat model was one of the first to be developed and used [114]. This model has also been useful to studying AIPC growth as well as understanding prostate cancer metastasis. Overall, a variety of mouse models are available for studying prostate cancer; however, no single model is perfect, so it is imperative that in addition to in vivo studies, other studies including in vitro cell lines also be conducted as well.
1.4.2 *In vitro* Cell Culture Models

The use of cell lines derived from human tumours allows for a more controlled and simplified model to study prostate cancer pathobiology. There are many prostate cancer cell lines available with most of these having been generated from metastatic tumours [115]. The most commonly used prostate cancer cell lines include the LNCaP (lymph node metastasis), PC3 (bone metastasis), DU145 (brain metastasis), 22Rv1 (localized primary tumor), VCaP (bone metastasis) and MDA PCa 2b (bone metastasis). RWPE cells are an immortalized non-malignant prostate epithelial cell line. These cell lines can be further sub-classified into their responsiveness to androgens, as LNCaP, VCaP, MDA PCa 2b and RWPE are all androgen-dependent, whereas PC3, DU145, and 22Rv1 are all androgen-independent.

There are many advantages and disadvantages of using cell lines when compared to *in vivo* mouse models. One of the major benefits of using cell lines is that they are easier to maintain and grow compared to animal models, and as a result, are less expensive to use. In addition, cell lines are versatile in the types of molecular studies that can be conducted on them. They can readily be genetically manipulated, and also be treated in culture to assess the effects of various stimulants. Also, as mentioned previously, cell lines can be xenografted into mice to assess their *in vivo* growth potential. However, as with any model system, cell lines have their disadvantages and limitations. One of the major limitations of cell lines is that they do not capture the heterogeneity of the tumour microenvironment or the tumour itself. Cell lines represent for the most part, a homogenous group of cells that act in a similar fashion. Cancer is a complex disease that involves not only the cancer cells themselves, but also other cell types that comprise the tumor microenvironment. Studies conducted on cell lines are not able to address this major interplay within cancer biology. Another drawback of cell lines is that they are no
longer within their native environment, and have adapted to grow in a culture-based system. This results in the cell lines themselves having genetic alterations and phenotypes that allow them to adapt to their artificial environment, which then leads to question whether or not they are indeed representative of the original tumor they were extracted from. Many of the available prostate cancer cell lines have been in existence for decades, and many of them have already been shown to have genetic alterations as they continue to be passaged. However, regardless of these issues, cell lines remain a valuable tool in prostate cancer research, as they provide a strong starting point for many studies trying to understand the role of various genes with respect to prostate cancer pathobiology.

1.5 Rationale and Objectives of the Present Study

1.5.1 Rationale

Understanding the molecular alterations that occur during the progression of prostate cancer to an androgen-independent state remains of importance in order to identify potential biomarkers of aggressive prostate cancer and effective therapeutic targets. High-throughput proteomics using mass spectrometry is a growing field that has the capability to provide information on the ‘proteomes’ of virtually any biological material. Studies have been conducted with respect to finding novel prostate cancer biomarkers as well as key molecules involved in cancer progression using various cell lines and biological materials such as tissues and fluids; however, an individual marker or a panel of markers with sufficient clinical utility has yet to be transferred into the clinic [116-119].

With respect to understanding the progression of AIPC, there has not been a comprehensive proteomics based study to find differential protein expression between androgen-
dependent and androgen-independent cells. To address this, we have chosen to use two-independent cell line based models for identifying differential protein expression for our discovery approach. The first approach assesses the differential protein expression of the whole cell lysate of an *in vitro* cell line model of prostate cancer progression. Briefly, I collected lysates from LNCaP (androgen-dependent) cells and its androgen-independent generated subline, LNCaP-SF, and performed quantitative proteomics analysis to deduce differential protein expression between the two cell lines. In the second approach, I collected the conditioned medium from various prostate cancer cell lines that are non-malignant (RWPE), androgen-dependent (LNCaP and VCaP), or androgen-independent (PC3, DU145, 22Rv1, PPC1 and LNCaP-SF), and compared differential protein expression among all these cells.

From each of these approaches, the proteins identified were investigated for their potential as candidate tumour markers. Using immunohistochemistry analysis on various clinical specimens, I verified and validated our top candidates to see whether they are associated with aggressive prostate cancer. In addition, I also assessed the functional/mechanistic role of the most interesting candidates using multiple molecular biology techniques including knock-down experiments on various prostate cancer cell lines.

1.5.2 Hypothesis

During androgen deprivation, many prostate cancer cells undergo cell death, resulting in substantially reduced tumour volume. However some cells survive and are able to grow in near the absence of androgens, evolving into more aggressive androgen-independent prostate cancer. The molecular mechanisms that cause these transitions remain largely unknown. Key modulators involved in this process could serve as important biomarkers to distinguish
aggressive disease. Here we hypothesize that during the progression to androgen-independence, various molecular perturbations occur whereby the cells are able to survive in sub-optimal conditions. Using an in vitro cell culture model system to depict this transition, we performed global quantitative proteomic assessment to identify critical modulators of this process with the aim of finding potential proteins that could be used as biomarkers to predict an indolent vs. aggressive course of the disease.

1.5.3 Objectives

Using high-throughput quantitative proteomic approaches, we addressed the need to identify mediators of androgen-independence using two approaches. The first approach utilized an in vitro model of prostate cancer progression. The LNCaP cell line, which is initially androgen sensitive, has been cultured in androgen-deprived conditions for a prolonged period to generate the androgen insensitive LNCaP-SF cell line. Using quantitative high through-put proteomics, we performed a complete global proteomic signature of these cell lines using stable isotope labelling in cell culture (SILAC). Briefly, the SILAC procedure relies on the metabolic incorporation of a given ‘light’ or ‘heavy’ isotope form of the amino acids arginine and lysine into proteins of the cell lines. In this case, we labeled LNCaP cells with ‘light’ amino acid containing media, and LNCaP-SF cells with ‘heavy’ amino acid containing media. Cell lysates were mixed from each condition and LC-MS/MS was performed to identify differential protein expression. The second approach used Mass Spectrometry, to compare the ‘secretomes’ (secreted proteins) of androgen independent cell lines (PC3, DU145, PPC1, LNCaP-SF, 22Rv1) to androgen-dependent (LNCaP, VCaP) and normal prostate epithelial (RWPE) cell lines. The most interesting candidates from both of these approaches were then further validated in clinical
specimens and functionally validated to assess if they play a mechanistic role in prostate cancer pathobiology.
CHAPTER 2:
QUANTITATIVE PROTEOMICS REVEALS THAT ENZYMES OF THE KETOGENIC PATHWAY ARE ASSOCIATED WITH PROSTATE CANCER PROGRESSION

The work presented in this chapter was published in Molecular and Cellular Proteomics

Quantitative Proteomics Reveals that Enzymes of the Ketogenic Pathway are Associated with Prostate Cancer Progression

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2.1 Introduction

Prostate cancer is the most commonly diagnosed cancer, and the second leading cause of cancer-related deaths among men in North America [1]. In the early stages of cancer development, prostate cancer cells rely on androgens for their growth and survival. Androgen deprivation remains one of the most widely used therapies for metastatic and recurring prostate cancers [120]. However, patients often regress to the more lethal androgen-independent prostate cancer (AIPC) or hormone-refractory prostate cancer (HRPC) which is characterized by poor prognosis [121, 122]. Effective therapeutic management is unavailable for androgen-independent prostate cancer. Hence, studying the molecular changes that occur during progression to androgen independence remains of utmost importance both to gain a better understanding of this process and to generate biomarkers and targeted therapies.

The androgen receptor (AR) signalling cascade has been demonstrated to be an important pathway, which is activated during androgen independence. Its activation has been documented to occur through AR gene amplifications, AR gene mutations, changes in co-regulators or steroidogenic enzymes, or through alternative proteins via outlaw pathways [121-127]. Although the AR pathway appears to be a key player in the development of androgen-independence, it is important to note that numerous ‘bypass pathways’, the so-called AR-independent pathways, have also been suggested to be involved in this process [87].

LNCaP, a hormone-dependent prostate cancer cell line, has often been used to model the progression of prostate cancer to androgen-independence [62]. Many groups have been able to generate androgen-independent sub lines of LNCaP, by culturing them for extended periods in androgen-deprived media, a process that selects for clones that have gained the ability to grow in the absence of androgens [128-132]. Many of these androgen-independent LNCaP sublines,
such as LNCaP-SF, have similar features to clinical cases of hormone-refractory prostate cancer, including increased AR expression, and represent excellent model systems for the investigation of AIPC progression [128-134].

Proteomics using mass spectrometry is a maturing analytical technique, which has provided the opportunity to characterize the proteome of virtually any biological specimen. Such studies have been conducted to identify novel candidate prostate cancer biomarkers, as well as implicated proteins in cancer progression, using various cell lines and biological samples, specifically tissues and fluids; however, markers with sufficient clinical relevance have yet to be introduced into practice [118, 119, 135-139]. PSA, a widely utilized biomarker for prostate cancer diagnosis, lacks specificity, as it becomes elevated in a variety of prostatic diseases, including benign prostate hyperplasia and prostatitis [2]. The tissue specificity of PSA has also been questioned, as it has been found in the sera of women with breast cancer and well as in nipple aspirate fluid [140].

In this study, we utilized a high-throughput quantitative proteomics approach to delineate important modulators implicated in the development of AIPC, using the LNCaP/LNCaP-SF model system. Stable isotope labeling of amino acids in cell culture (SILAC) coupled to mass spectrometry was employed to comparatively quantify over 3300 proteins in LNCaP and LNCaP-SF cells. Several proteins of the ketogenesis pathway (HMGCS2, ACAT1, BDH1, HMGCL, OXCT1), were up-regulated in LNCaP-SF cells. These ketogenic proteins were over-expressed in high-grade human prostate cancer samples, with ACAT1 displaying the greatest change in expression. In addition, ACAT1 expression was also elevated in castration-resistant metastatic lesions from prostate cancer patients. These results demonstrate that enzymes
involved in the ketogenesis pathway could serve as potential biomarkers for high-grade prostate cancers, and identify new targets for potential therapeutic intervention.
2.2 Materials and Methods

2.2.1 Cells and reagents

The human prostate cancer LNCaP cell line and the androgen-independent subline LNCaP-SF cells were kindly provided by Dr. Atsushi Mizokami and maintained in DMEM (Wisent, St- Bruno, Quebec) medium supplemented with either 10% (v/v) FBS (Hyclone) for LNCaP cells or 10% charcoal stripped FBS (Hyclone) for LNCaP-SF cells at 37°C with 5% CO₂ in a humidified incubator.

2.2.2 Stable isotope labelling with amino acids in cell culture (SILAC)

LNCaP and LNCaP-SF cells were seeded at low confluency (~25%) in T75 flasks. SILAC media was prepared from customized DMEM lacking two essential amino acids: L-arginine and L-lysine (Athena ES, Baltimore MD). Heavy amino acids, L-Arg6 (¹³C) and L-Lys8 (¹³C and ¹⁵N), were supplemented to the medium to generate the ‘heavy’ medium (Cambridge Isotope Laboratories, Andover MA). For control or ‘light’ medium, L-arginine and L-lysine were supplemented into the medium (Sigma). Additionally, both ‘heavy’ and ‘light’ media were further supplemented with the same dialyzed FBS (Gibco). LNCaP cells were metabolically labelled with ‘light’ medium and LNCaP-SF cells were metabolically labelled with ‘heavy’ SILAC conditioned medium in T25 flasks. Three independent biological replicates were used for both LNCaP and LNCaP-SF cells. A minimum of five doubling times was ensured, and growth medium was changed every two to three days. Cells were grown for additional 48 hours and were then detached non-enzymatically, washed twice in PBS, and centrifuged at 1500 x g for 5 minutes. Cell pellets were kept at -80°C, until further processing.
2.2.3 Sample preparation

Cell pellets were lysed using 250μl of 0.1% RapiGest (Waters Inc, Milford MA) in 25mM ammonium bicarbonate and were subsequently sonicated three times for 30 seconds. The resulting cell lysates were then centrifuged for 15 min, at 15 000 x g, at 4°C. Protein lysates from both LNCaP and LNCaP-SF cells were quantified using a BCA assay (Thermo Scientific) and mixed in a 1:1 ratio, to obtain a total of 250μg of total protein in each replicate (125μg protein from heavy isotope-labelled and 125μg from light-labelled condition). Proteins were then heat-denatured at 85°C for 15 minutes, reduced with 10mM DTT (Sigma-Aldrich) for 10 min at 70°C, alkylated with 20mM iodoacetamide (Sigma-Aldrich) for 60 min with shaking in the dark, and trypsin-digested (Promega) at a ratio of 1:50 (trypsin:protein concentration) overnight at 37°C. The resulting tryptic peptides were reconstituted in 200μl of 0.26M formic acid in 5% acetonitrile (mobile phase A) buffer.

2.2.4 Strong cation exchange (SCX) on a high performance liquid chromatography (HPLC) system

To reduce sample complexity, samples were subjected to SCX using an Agilent 1100 High Performance Liquid Chromatography (HPLC) system. Tryptic peptides were initially diluted to 500μl with strong cation exchange (SCX) mobile phase A (0.26 M formic acid in 5% acetonitrile; pH 2–3) and loaded directly onto a 500μl loop connected to a PolySULFOETHYL A™ column with a 2μm pore size and a diameter of 5μm (The Nest Group Inc., Southborough MA). Peptides were eluted using an elution buffer that contained all components of mobile phase A with the addition of 1M ammonium formate, which was introduced at 10 min, and
further increased to 20% at 30 min and then to 100% at 45 min. A total of 14 fractions were collected to perform mass spectrometric analysis. The HPLC fractions were C\textsubscript{18}-extracted using ZipTipC\textsubscript{18} micropipette tips (Millipore, Billerica MA) and eluted in 5µl of Buffer B (90% acetonitrile, 0.1% formic acid, 10% water and 0.02% trifluoroacetic acid). An additional 80µl of Buffer A (95% water, 0.1% formic acid, 5% acetonitrile, and 0.02% trifluoroacetic acid) was added to the samples.

2.2.5 Mass Spectrometry (LC-MS/MS)

Forty microliters of each fraction were injected into an autosampler on the EASY-nLC system (Proxeon Biosystems, Odense, Denmark). Firstly, the peptides were collected onto a 3cm C18 column (inner diameter of 200µm), and were then eluted onto a resolving 5-cm analytic C18 column (inner diameter of 75µm) with an 8µm tip (New Objective). This HPLC system was coupled online to an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer, using a nano-Electro Spray Ionization (ESI) source (Proxeon Biosystems), in data-dependent mode. The fractions were run on a 60 min gradient consisting of 3 min at 1-14% Buffer B (100% acetonitrile, 0.1% formic acid), 44 min at 14-40% Buffer B, 5 min at 40-65% Buffer B, 3 min at 65-100% Buffer B and 5 min at 100% Buffer B. The eluted peptides were subjected to one full MS1 scan (450-1450 m/z) in an Orbitrap at 60 000 resolution, followed by six data-dependent MS2 scans in the linear ion trap. The following parameters were enabled: monoisotopic precursor selection, charge state screening and dynamic exclusion. In addition, charge states of +1, >4 and unassigned charge states were not subjected to MS2 fragmentation.
2.2.6 Protein Identification and Quantitation using MaxQuant Software

The resulting mass spectra were analyzed using MaxQuant Software (version 1.1.1.25), which generates the extracted ion current-based quantitation for SILAC pairs. Raw MS files were loaded directly into MaxQuant, and identification and quantitation of individual peptides were generated in protein groups. The MaxQuant searches were executed against the International Protein Index (IPI) human protein database (version 3.62, 167,894 forward and reverse protein sequences) and decoy database. All entries were filtered using a false positive rate of 1%, and all false positives were removed. The following search parameters were used: 1 missed and/or non-specific cleavages permitted, carbamidomethylation (57m/z) on cysteine fixed modification, and oxidation (methionine) and acetal (N-terminus proteins) variable modifications. The mass tolerance for precursor ions was initially 20 ppm, and then adjusted to 6 ppm followed by recalibration in MaxQuant. The mass tolerance for fragment ions was 0.5 Da. Quantification of proteins was based on the normalized heavy/light (H/L) ratios, as determined by MaxQuant.

2.2.7 Data Analysis

Protein groups from MaxQuant were exported to excel files, which displayed the results of three independent SILAC runs with their corresponding H/L ratios. The normalized average H/L ratio for each protein was the final quantitative value used to filter and select for candidates. To visualize and assess networks of over-expressed and under-expressed candidates, Ingenuity Pathway Analysis (IPA; Ingenuity Systems, CA, USA) software was used. Using this software,
pathway analysis was performed, obtaining information on canonical pathways and molecular networks that have been altered were determined by Fisher’s exact test.

2.2.8 Reverse-transcription and Quantitative Polymerase Chain Reaction

Total RNA was isolated from LNCaP and LNCaP-SF cells using an RNeasy Kit (Qiagen). cDNA was generated from 1µg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was conducted using 1X SYBR reagent (Applied Biosystems) and transcript levels of HMGCS2, ACAT1, BDH1, HMGCL, OXCT1, DHRS2, AGR2, HSD11B2, ALDH6, MAOA, MAOB, TYMP ARG2 and SQSTM1 were measured on a 7500 ABI system. All qPCR data were normalized to tata-binding protein (TBP) expression. Sequences of all primers used are shown in Table 2.1.

For clinical validation, the TissueScan Prostate Cancer cDNA Array II was used (Origene). Quantitative PCR was conducted on these samples using the same SYBR green reagent as mentioned above.
<table>
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<th>Gene Name</th>
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<th>Reverse Sequence</th>
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2.2.9 **Protein Extraction**

LNCaP and LNCaP-SF cell pellets were washed with phosphate buffered saline (PBS-pH 7.4) three times and cells were lysed using 1% SDS solution. The samples were then sonicated three times on ice in 30 second intervals using a MISONIX immersion tip sonicator (Q SONICA LLC, CT, USA), and centrifuged at 15 000 x g for 15 minutes at 4°C. Protein concentrations were quantified using a BCA protein assay kit (Thermo Scientific).

LuCaP96 and LuCaP96AI xenograft tissues, as previously described [76], were frozen in liquid nitrogen, and then ground into a fine powder using a mortar and pestle. The resulting powdered tissue was lysed using 1% SDS solution followed by sonication on ice for three 30 second intervals. The samples were then centrifuged at 14 000 x g for 15 minutes at 4°C, and the resulting supernatant was used for further analysis.

2.2.10 **Western blotting**

Protein expression of ACAT1, HMGCS2, BDH1, OXCT1, and HMGCL was assessed using western blot analysis. Roughly, 30µg of total protein from each sample was loaded onto an SDS-PAGE gel (4-15%, BioRad), and transferred onto PVDF membranes (BioRad). Membranes were then incubated with 5% blocking solution (2.5g skim milk powder in tris-buffer solution containing 0.1% Tween (TBST)) overnight at 4°C. Membranes were incubated with rabbit polyclonal antibody against ACAT1, BDH1, OXCT1, HMGCL (Sigma) or HMGCS2 (Protein Tech) for 1 hour at room temperature. The membranes were then washed six times (three 15 minute washes followed by three 5 minute washes) with TBST. Membranes were then incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase.
(Jackson Laboratories) for 1 hour at room temperature. After washing with TBST, proteins were detected using the ECL detection reagent (Siemens). The expression of GAPDH (Cell Signalling Technology) or β-actin (Abcam) was used as an internal standard.

2.2.11 Beta-hydroxybutyrate Detection Assay

LNCaP and LNCaP-SF cells were grown in T25 flasks in serum-free media for 48 hours, and the resulting cell supernatants were collected and assayed for β-hydroxybutyrate levels using a β-hydroxybutyrate (Ketone Body) Colorimetric Assay Kit (Cayman Chemical), according to the manufacturers protocol. Total β-hydroxybutyrate values were normalized to total protein levels in the supernatants.

2.2.12 Immunohistochemistry

Prostate cancer tissue microarrays (TMA) consisting of 8 normal and 40 cancer cores were purchased from US BioMax (Rockville, MD). The metastatic prostate cancer tissue microarray was developed and provided by the GU Cancer Research laboratories at the University of Washington, Seattle, USA. Human tissue microarrays of fixed paraffin-embedded metastatic tissues from 23 rapid autopsy patients who died of prostate cancer (consisting of 3 tissue microarray blocks with 2 replicate cores per metastatic site) were used for immunohistochemical (IHC) analyses. All patients had castrate resistant prostate cancer at the time of autopsy, defined by the presence of a rising serum PSA following medical or surgical castration.
TMAst were deparaffinized in xylene and rehydrated using ethanol. Endogenous peroxidase was reduced using hydrogen peroxide for 10 minutes and washed with PBS. Antigen retrieval was then performed using citrate buffer in a microwave for 10 minutes. Slides were then blocked for 5 min in casein and incubated overnight with the following primary antibodies: ACAT1 (1:1000), BDH1 (1:2000), HMGCL (1:1200), HMGCS2 (1:600), and OXCT1 (1:1000). Rabbit IgG was used on a duplicate slide to serve as a negative control. Following 10 min of PBS washing, slides were placed in secondary antibody for 30 minutes using the BGX kit (Biogenex, Fremont, CA). After a 10 min wash in PBS, slides were developed with the addition of DAB for 5 min. Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

2.2.13 Statistical Analysis

All gene expression studies on cell lines and xenografts consisting of normalized expressions were compared using a two tailed t-test (GraphPad Prism Software). Gene expression studies on human prostate cancer and normal tissue were compared using a non-parametric Mann-Whitney Test (GraphPad Prism Software). Finally, chi-squared tests were used to compare different groups from the immunohistochemistry data. Differences were considered significant if P value was less than 0.05. All data are expressed as mean ± standard error of the mean.
2.3 Results

2.3.1 Quantitative Proteomic Profiling of LNCaP vs. LNCaP-SF cells

To identify potential modulators of androgen-independent prostate cancer, we performed global proteomic profiling of the LNCaP cell line, and its androgen-independent counterpart, LNCaP-SF, using SILAC. This approach provided a robust and comprehensive method to detect differential proteomic expression between both cell lines, as each was grown in distinct amino acid isotopic media (Figure 2.1A). Using MaxQuant software, we were able to identify and quantify 3416 proteins with one peptide hit. Based on the heavy-to-light ratio for each protein, a numerical value representing the differential protein expression between LNCaP-SF cells (grown in heavy media), and LNCaP cells (grown in light media) was assigned. The majority of proteins (85%) fell within one standard deviation of the total average ratio (H/L ratio between 0.67-1.5) amongst the three replicates, indicating there were no significant differences with respect to their protein levels between the two cell lines (Figure 2.1B). Among the three biological replicates, we observed a reproducibility of 80-90% with respect to the H/L ratio between independent experiments. In addition, H/L ratios of various housekeeping proteins, including β-actin (H/L=1.37), GAPDH (H/L=0.85), HSP90 (H/L=0.92), and α-tubulin (H/L=1.06), all remained within one standard deviation of their average ratio.

In order to identify candidates that were either up or down-regulated in LNCaP-SF cells, in comparison to LNCaP cells, we used H/L values that were two standard deviations from the mean H/L ratio of all proteins identified, which corresponded to H/L ratios > 2.5 as over-expressed hits and H/L ratios < 0.4 as under-expressed. Each of the candidates must have met these cut-offs in all three independent biological replicates to be further considered. After implementing these stringent criteria, 42 (Figure 2.1C, Table A1) and 46 proteins (Table A2)
were found to be up and down-regulated, respectively, in LNCaP-SF cells compared to LNCaP cells.

2.3.2 Analysis of Candidate Proteins

After assessing the candidate lists, we identified proteins that were previously studied in the context of prostate cancer progression; including AGR2, ALDH1A3, S100P MAOA, AADACL1 and SOD2, among the over-expressed and IGFBP2, STMN1, DNMT1, ATAD2, KPNA2, and ADII within the under-expressed ones [141-152]. We next subjected our candidate list to pre-clustering pathway analysis using Ingenuity Pathway Analysis (IPA). This analysis revealed that the top molecular networks of the over-expressed candidates were cancer and cellular development (Figure 2.1D). Specifically, “lipid metabolism” and “small molecular biochemistry” were the top molecular function clusters. The over-expressed candidates had central nodes in the NF-κB, p53, ESR1, TGFβ, TNF and SP1 signalling cascades, all of which have been previously documented to be associated with prostate cancer progression [153-157]. With regards to our under-expressed candidates, the top molecular networks were: 1) DNA replication, recombination, and repair, cell cycle, cellular assembly and organization as well as 2) cell death, cellular growth and proliferation. Our top under-expressed candidate was SEMG1, with a 13-fold down-regulation in LNCaP-SF cells (H/L of 0.08) and its functionally related protein, SEMG2, was also under-expressed.

The top over-expressed candidate, HMGCS2, which had a 9-fold increase in LNCaP-SF cells, is an enzyme involved in ketogenesis, a metabolic pathway that provides lipid-derived energy during times of carbohydrate deprivation [158]. We identified another protein in this
pathway, OXCT1, with more than 2-fold increase in LNCaP-SF cells, which led us to investigate the remainder of the proteins involved in this pathway. We were able to identify all five proteins within the ketogenic pathway (HMGCS2, HMGCL, BDH1, ACAT1, OXCT1), where four of them, HMGCS2, OXCT1, BDH1, and ACAT1 exhibited increased protein expression in LNCaP-SF cells based on our initial SILAC experiments. The normalized H/L ratio for each protein was 9.24 for HMGCS2, 2.15 for OXCT1, 1.88 for ACAT1, 1.75 for BDH1 and 1.37 for HMGCL (Figure 2.3A). These preliminary results suggest that the ketogenesis pathway may be up-regulated during progression of prostate cancer to androgen-independence.
Figure 2.1 SILAC-based quantitative proteomic profiling of prostate cancer progression to androgen-independence. A, flow-chart depicting the steps involved in quantitative proteomic profiling of LNCaP and LNCaP-SF cells. LNCaP cells were grown in medium containing $^{12}\text{C}$-Arg/Lys, whereas LNCaP-SF were grown in $^{13}\text{C}-^{15}\text{N}$-Arg/Lys medium and both cell lines were allowed to grow for a minimum of 5 doubling times. Cells were lysed and total protein from LNCaP and LNCaP-SF cell lysates was mixed in a 1:1 ratio. Following trypsin digestion and fractionation with strong cation exchange chromatography (SCX), peptides were identified using...
MS/MS and the resulting data were analyzed with MaxQuant Software. B, following protein identification using MaxQuant, over 3400 proteins were quantified, with the majority having a ‘1:1’ ratio, indicating no change between protein expression between the two cell lines. C, 42 proteins were found to be up-regulated in LNCaP-SF cells using stringent criteria (greater than 2.5 fold H/L ratio in 3 experimental replicates). D, Ingenuity Pathway Analysis revealed alterations in central nodes such as p53, ESR1, SP1, TGFβ and TNF signalling pathways.

2.3.3 In vitro Validation of Candidates

The gene expressions of our top candidates were further validated in vitro using real time PCR. Validation of the proposed overexpressed genes revealed that seven of the top 10 over-expressed candidates (HMGCS2, DHRS2, AGR2, HSD11B2, ALDH6, MAOA, MAOB) had increased gene expression in LNCaP-SF cells (Figure 2.2), whereas three candidates (TYMP, ARG2, SQSTM1) did not show significant changes in gene expression. Considering that we used RNA to verify our protein discovery results, it is important to note that several post-transcriptional factors may play a role in the differences observed between RNA and protein expression.

Additionally, we performed qPCR validation of the genes associated with the ketogenesis pathway. Four genes (HMGCS2, OXCT1, ACAT1 and BDH1) had increased expression in LNCaP-SF cells, whereas HMGCL displayed relatively similar expression levels among the two cell lines (Figure 2.3B). Protein validation of the ketogenic pathway enzymes using Western blotting supported our preliminary data, demonstrating an up-regulation of our identified enzymes in LNCaP-SF cells, with HMGCS2, OXCT1 and ACAT1 having the greatest changes in protein expression (Figure 2.3C). Finally, since this pathway is responsible for the generation of ketone bodies, we assessed the secretion levels of β-hydroxybutyrate, the most common
ketone body, in these cell lines. We found a statistically significant two-fold increase in β-hydroxybutyrate levels in the conditioned medium of LNCaP-SF cells, compared to LNCaP cells (Figure 2.3D). These preliminary in vitro results imply that the ketogenic pathway enzymes identified in our preliminary analysis, as well as the associated ketone bodies, exhibit elevated expression in LNCaP-SF cells.
Figure 2.2. Validation of the top over-expressed candidates using real-time PCR in LNCaP and LNCaP-SF cells. Seven of the ten candidates (HMGCS2, DHRS2, AGR2, HSD11B2, ALDH6, MAOA, MAOB) showed an up-regulation in transcript levels in LNCaP-SF cells, while three (TYMP, ARG2, SQSTM1) displayed no significant changes in mRNA expression (*, P < 0.05, two tailed t-test).
Figure 2.3. Analysis of gene and protein expression levels of enzymes involved in the ketogenic pathway (ACAT1, BDH1, OXCT1, HMGCL, HMGCS2) in LNCaP and LNCaP-SF cells. A, enzymes of ketogenic pathway, quantified in our analysis. B and C, gene and protein expression validation using real-time PCR and Western blotting confirmed the significant over-expression of HMGCS2, OXCT1, ACAT1 and BDH1 at the mRNA level (*, P < 0.05, two tailed t-test) with all enzymes displaying elevated expression at the protein level. D, β-hydroxybutyrate secretion was found significantly elevated in the conditioned medium of LNCaP-SF cells (*, P < 0.05, two tailed t-test)
2.3.4 Ketogenic Pathway Gene Transcript and Protein Levels are Increased During High Grade Prostate Cancer

To determine if the ketogenesis pathway genes play a role during prostate cancer progression, we measured their transcript levels in normal and human tumor tissue samples. Using real-time PCR, we found that \textit{HMGCS2} (p=0.048), \textit{OXCT1} (P=0.04), \textit{BDH1} (p=0.0008) and \textit{ACAT1} (P=0.009) have significantly increased gene expression in prostate cancer compared to normal tissue (Figure 2.4). In particular, \textit{ACAT1} and \textit{BDH1} displayed the most prominent changes in gene expression, with 3-fold and 4-fold increases, respectively. \textit{HMGCL} expression was slightly elevated in prostate cancer; however, this was not statistically significant (P=0.075). Interestingly, of the 36 prostate cancer patients analyzed, 20 had at least a two-fold increase in expression of at least 2 ketogenic enzymes, compared to the 8 normal patients analyzed. Among these 20 patients, four had increases in 2 ketogenic genes, twelve had increases in 3 genes, three had increases in 4 genes and one patient had increases in all 5 ketogenic genes.

In addition, using immunohistochemistry, we assessed the protein expression levels of the ketogenesis enzymes in various normal and prostate cancer samples. We devised a scoring system to assess protein expression, whereby each core was scored with a 0, 1, 2, or 3, which corresponds to no staining, low staining, moderate staining or high staining, respectively. All ketogenic enzymes had higher staining patterns in high-grade prostate cancers (Gleason grade ≥ 8). The expression profiles of each enzyme followed an increasing pattern, going from normal prostate samples to low-grade prostate cancer (Gleason ≤7), and to high-grade prostate cancer (Figure 2.5). In \textit{ACAT1}, normal cores had little positive staining (13% stained with a score of 2 or greater), moderate levels of staining were observed in low-grade prostate cancer cores (46%), and almost all high-grade prostate cancer cores had intense staining (86%). For \textit{BDH1}, there
was moderate or high staining in 63% of normal cores, 77% in low-grade prostate cancer, and 87% in high-grade prostate cancer samples. HMGCL also had similar staining patterns, with 50% of normal cores having moderate or high staining, 70% in low-grade cancer cores and 93% in high-grade prostate cancers. Both BDH1 and HMGCL proteins, did not exhibit sharp changes in expression, as in the case of ACAT1. OXCT1 also displayed increased staining intensity in high-grade prostate cancers (66%), compared to normal (25%) and low-grade samples (27%). Finally, the top candidate from our initial proteomic experiments, HMGCS2, had similar staining intensities in both normal and low-grade cancers (50% in both), but also displayed increased staining in high-grade prostate cancer samples (65%). These results indicate that enzymes involved in the ketogenic pathway appear to be over-expressed during development of high-grade prostate cancers, and may therefore play a role in the progression of prostate cancer to an advanced stage disease.
Figure 2.4. Gene expression profiling of ketogenic pathway enzymes in normal and prostate cancer human tissue. Using the TissueScan Prostate Cancer cDNA Array II consisting of 8 normal and 36 prostate cancer specimens, the gene expression profiles of HMGCS2, OXCT1, BDH1 and ACAT1 all showed significantly elevated mRNA expression in cancer compared to normal specimens (*, P < 0.05; **, P < 0.01, two tailed t-test).
Figure 5. Protein expression of ketogenic pathway enzymes in human prostate cancer specimens of varying grade. A, representative immunohistochemistry images of HMGCS2, HMGCL, BDH1, OXCT1 and ACAT1 expression in normal, low grade (Gleason ≤ 7) and high grade (Gleason ≥ 8) prostate cancer specimens, under light microscopy (x20). B,
immunohistochemical staining was quantified using a scoring scale of 0, 1, 2, and 3 corresponding to no staining, low staining, moderate staining and high staining, respectively, as blindly determined by a pathologist.

2.3.5 The Ketogenic Pathway is Activated in the LuCaP 96AI Androgen-Independent Prostate Cancer Xenograft Model

To evaluate whether ketogenic enzymes play a role during the progression to androgen-independent prostate cancer, LuCaP 96, and its androgen-independent counterpart, LuCaP 96AI, xenografts were utilized. The transcript and protein levels of the 5 ketogenic enzymes were assessed using qPCR and western blotting, respectively. It was found that \( HMGCS2 \) (\( p=0.014 \)), \( BDH1 \) (\( p=0.003 \)), \( ACAT1 \) (\( p=0.024 \)) and \( HMGCL \) (\( p<0.001 \)) all displayed statistically significant increases in expression in the LuCaP 96AI xenograft-derived cells (Figure 2.6A). \( ACAT1 \) and \( BDH1 \) had the most prominent gene expression differences, exhibiting almost 3-fold increases in their transcript levels in the androgen-independent xenograft. Both \( HMGCS2 \) and \( HMGCL \) displayed roughly 2-fold increases in expression within LuCaP 96AI cells. Surprisingly, our results indicate that \( OXCT1 \) exhibited decreased expression in the LuCaP 96AI xenograft. Protein expression profiles corresponded well with our gene expression data, where \( ACAT1 \), \( BDH1 \), \( HMGCL \) and \( HMGCS2 \) all displayed increased protein expression in LuCaP 96AI cells (Figure 2.6B). Taken together, these results further support the involvement of altered ketogenic enzyme profiles, in the development of AIPC.
Figure 2.6. Expression of ketogenic pathway enzymes in in vivo LuCaP 96 xenograft and its androgen-independent xenograft LuCaP 96AI. A, Gene expression profiling reveals statistically significant up-regulation of HMGCS2, ACAT1, HMGCL and BDH1 (*, P < 0.05, two tailed t-test, n=3) at the transcript level. B, Protein expression profiles using western blot analysis, corresponded well with gene expression data, where ACAT1, BDH1, HMGCL and HMGCS2 all displayed increased protein expression in LuCaP 96AI cells.
2.3.6 ACAT1 is highly expressed in Castration-Resistant Metastatic Prostate Cancers

Based on our observations, ACAT1 was identified to be the most promising candidate, since its expression was found to be almost absent in normal samples, and gradually increased in advanced grade prostate cancers. Increased ACAT1 transcript and protein expression were also demonstrated in both, prostate cancer samples and an androgen-independent xenograft model. Therefore, we aimed to examine ACAT1 expression in various castration-resistant metastatic prostate cancer samples, to identify whether it is dysregulated during hormone-refractory prostate cancer. Using our reported scoring system in a tissue microarray, containing metastatic lesions to the bone, lymph nodes, lung and liver, we demonstrated increased ACAT1 staining in all metastatic sites, with bone metastases presenting with highest expression (Figure 2.7). Specifically, we observed 36.8% of lung and liver metastases containing none or low staining, 31.6% staining moderately, and 31.6% of cores having high staining. With respect to the lymph node metastasis cores, we found 32.1% with low staining, 46.4% staining moderately and 21.4% with high staining. Bone metastatic lesions had the most prominent ACAT1 expression patterns, as 13.5% of cores had low staining, 47.3% had moderate staining, and 39.2% had high staining. In contrast, in normal prostate cores, ACAT1 staining was very low, as the majority of the samples (87.5%) had little or no staining, and a small proportion (12.5%) having moderate ACAT1 expression. Following statistical analysis, ACAT1 expression was found significantly up-regulated in lung and liver (p=0.0329), lymph node (p=0.0121), and bone (p=0.0001) prostate cancer metastatic lesions compared to normal prostate samples (Table 2.2). Based on these results, ACAT1 expression is up-regulated in castration-resistant prostate cancer metastases, with bone metastatic lesions having the most prominent expression patterns.
Figure 2.7. Expression of ACAT1 in castration-resistant metastatic prostate cancer specimens to the bone, lymph node, liver and lungs.  

A, representative immunohistochemistry images of ACAT1 staining in normal, liver metastasis, lung metastasis, lymph node metastasis and bone metastasis of the prostate are shown. Images were taken under light microscopy (x20).

B, immunohistochemical staining was quantified using a scoring scale of 0, 1, 2, and 3 corresponding to no staining, low staining, moderate staining and high staining, respectively, as blindly determined by a pathologist.
Table 2.2. ACAT1 Expression in Castrate Resistant Metastatic Prostate Cancer Specimens

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Number of Positive Samples*</th>
<th>Staining Percentage</th>
<th>p value compared to Normal**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Prostate</td>
<td>1/8</td>
<td>12.5%</td>
<td>NA</td>
</tr>
<tr>
<td>Lung and Liver Metastasis</td>
<td>12/19</td>
<td>63.2%</td>
<td>0.0329</td>
</tr>
<tr>
<td>Lymph Node Metastasis</td>
<td>19/28</td>
<td>67.9%</td>
<td>0.0121</td>
</tr>
<tr>
<td>Bone Metastasis</td>
<td>64/74</td>
<td>86.5%</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* positive staining is defined as a score of 2 or higher, based on pathologists score assessment
**p value was calculated using a chi-squared test

2.4 Discussion

Prostate cancer is a curable disease upon early detection, since the tumor is most often localized to the prostate. However, once the cancer has spread outside of the prostate gland, effective treatments are limited, with radiation therapy, chemotherapy, and the gold standard, hormonal therapy, usually failing in the long run. Although hormonal therapy is initially very effective in reducing tumor growth and volume, cancer cells eventually gain resistance and grow in the absence of circulating androgens. This is the stage where the disease becomes fatal, since no other targeted therapies are available. Therefore, studies focusing on understanding the development and progression of androgen-independent prostate cancer are crucial, in order to develop targeted therapeutic strategies.

LNCaP is a widely used cell line for the investigation of androgen-dependent prostate cancer, and has been utilized by numerous groups to develop an androgen-independent clone [128-134]. However, several limitations exist in using this model. For instance, LNCaP cells carry a mutation in the ligand-binding domain of the AR, making it more ‘promiscuous’, whereby the AR can be activated by molecules other than DHT, including various anti-
androgens. However, during prostate cancer progression, mutations within the AR gene, specifically the ligand binding domain, become more common, so this mutation reflects one mode of gaining a survival advantage for cancer cells. Also, phenotypic characteristics of the various LNCaP androgen-independent sublines generated by others, accurately represent common features usually presented in clinical cases of AIPC, including increased AR expression. Therefore, this model remains quite valuable for studying the progression of prostate cancer.

In this study, by performing a powerful, high-throughput, global proteomic profiling of an *in vitro* LNCaP cell line model of androgen-independent prostate cancer progression, we identified over 3400 proteins, and specified over 80 proteins to be differentially regulated between LNCaP/LNCaP-SF. To our knowledge, this is the most comprehensive quantitative proteomics study to date, aiming to understand the mechanisms of prostate cancer progression. To internally validate our approach, we observed among our candidates (42 over-expressed; 46 under-expressed), a plethora of proteins previously studied, or implicated in prostate cancer progression. Six of the up-regulated genes (*AGR2, ALDH1A3, S100P MAOA, AADACL1* and *SOD2*) have been previously deemed to be involved in promoting prostate cancer progression [141, 142, 146, 147, 150, 151]. For example, AGR2, which exhibited almost a 3-fold increase in LNCaP-SF cells, has been previously shown to be involved in prostate cancer metastasis. Likewise, six (*IGFBP2, STMN1, DNMT1, ATAD2, KPNA2, and ADI1*) of the under-expressed candidates have also been previously implicated in prostate cancer progression [143-145, 148, 149, 152]. Interestingly, two of our top under-expressed candidates, SEMG1 and SEMG2, have been previously shown to be decreased in expression in two androgen-independent cell lines (PC3 and DU145), which corresponds well with our results [159].
Our top over-expressed candidate, HMGCS2, which was 9-fold elevated, is an enzyme involved in the ketogenic pathway. This significant dysregulation prompted us to investigate additional candidates of the ketogenesis pathway. Performing clinical validation on a variety of samples, we observed that these enzymes were over-expressed during prostate cancer, specifically in high-grade prostate cancer. The most interesting of these enzymes, ACAT1, was also found to be highly expressed in castration-resistant metastatic prostate cancer specimens.

In order for cancer cells to proliferate and survive, they have a high energy demand, to carry out integral cellular processes. Cell growth, proliferation and migration require large amounts of energy in the form of ATP, and by using alternative energy-producing pathways, cancerous cells gain a survival advantage. The ketogenic pathway is such an alternative energy producing pathway, primarily responsible for the production of ketone bodies from fatty acids via the breakdown of acetyl-CoA, a key molecule formed during fatty acid metabolism [160]. Acetyl-CoA, under normal high glucose conditions, is oxidized, resulting in the formation of the high energy molecules NADH and FADH$_2$ in the citric acid cycle. However, when levels of acetyl-CoA are higher than required for the citric acid cycle, then, it is used for biosynthesis of ketone bodies, through the aid of five cellular enzymes. From our initial proteomics data, we identified all five of these enzymes to be up-regulated in LNCaP-SF cells, and also found an increase in β-hydroxybutyrate, the most common ketone body, in the secretome of these cells, as well. These observations suggest that the ketogenesis pathway may be an alternate energy-producing mechanism through which prostate cancer cells gain a survival advantage, to become increasingly aggressive and gain androgen-independent properties. During androgen deprivation, prostate cancer cells are losing a critical signalling cascade via AR activation, resulting in decreased expression of AR-regulated genes. A recent study by Massie et al,
identified key energy producing networks, including glucose uptake and glycolysis, to be regulated by AR signalling [161]. A decrease in the activity of the glycolytic pathway places prostate cancer cells under stress to generate energy in a quick manner, in order to carry out necessary cellular functions. One avenue, through which such an effect can be alleviated, is to increase energy production through the break-down of fatty acids via the β-oxidation pathway [162].

Fatty acid oxidation has been widely studied with respect to prostate cancer progression, specifically by providing an important source of bioenergy [162, 163]. Various proteins involved in the metabolism of fatty acids have been determined to be altered during prostate cancer [162, 163]. Fatty acid synthase (FASN) has been one of the most widely studied of these proteins, as it has been found overexpressed at both the mRNA and protein levels in prostate cancer [164]. Interestingly, the highest levels of FASN are associated with androgen-independent bone metastatic lesions [164]. Other frequently described fatty acid oxidation pathway protein alterations observed in prostate cancer include loss of stearoyl-CoA desaturase (SCD) expression, and increase in D-functional protein (DBP) and α-methylacyl-CoA racemase (AMACR) expression [165-167]. AMACR expression, in particular, has been associated with increased prostate cancer risk. [165, 168].

For the first time, we identified the ketogenic pathway, as a novel bioenergetic pathway potentially involved in the progression of prostate cancer from low-grade to high-grade disease, followed by androgen-independence. With the exception of ACAT1, the ketogenesis pathway has not been investigated in prostate cancer. Specifically, ACAT1 was shown to be involved in the androgen-mediated cholesterol metabolism in prostate cancer cell lines [169]. In another recent study, ACAT1 protein expression was found to be elevated in LNCaP androgen-
independent xenografts, further implicating its importance during prostate cancer progression [170]. Both studies focused on ACAT1, and its involvement with cholesterol metabolism, an important metabolic pathway for cholesterol biosynthesis. Cholesterol metabolism has also been found to be altered during the progression of prostate cancer to AIPC, as free cholesterol from increased biosynthesis or uptake, is a potential source for intratumoral de novo androgen synthesis. Thus, an alternative hypothesis rationalizing the observed overexpression of ketogenic enzymes during high-grade prostate cancer and AIPC is to provide more efficient production of cholesterol, which, in turn, can act as a precursor to generate androgens. In order to better understand the mechanism of action of these ketogenic enzymes in prostate cancer progression, further studies, using RNA interference technology in prostate cancer cell lines and mouse models, need to be conducted. In addition, to further support our results, expression studies on a larger number of samples, including more diverse clinical samples, such as hormone naïve and hormone-refractory specimens, are needed to provide additional insight into the importance of these enzymes in prostate cancer. ACAT1 is of particular interest, as it was found to be significantly elevated in prostate cancer metastatic lesions. ACAT1 inhibitors have been previously investigated for various other diseases [171, 172], and present an interesting avenue of therapeutic intervention to potentially treat high-grade and metastatic prostate cancers.

In recent years, the field of cancer bioenergetics has enjoyed a resurgence, specifically, pertaining to alterations of metabolic pathways during carcinogenesis. Initially described as the Warburg effect, tumor cells were observed to switch from oxidative phosphorylation to anaerobic glycolysis, even in the presence of oxygen [173]. As a result of the identification of mutations in genes that encode for enzymes for specific metabolic pathways, it has become apparent that malignant cells will gain a survival advantage, if they are able to produce higher
amounts of energy to carry out important cellular tasks, such as rapid cellular growth and proliferation.

Overall, in this study, through the use of quantitative proteomics and validation on clinical samples, we demonstrate that: (1) many proteins become altered during the progression of prostate cancer to androgen-independence, (2) the ketogenic pathway enzymes become over-expressed during high-grade prostate cancers, and (3) ACAT1 becomes highly elevated in metastatic prostate cancer. Going forward, the complete understanding of the function of these ketogenic pathway proteins with respect to prostate cancer pathophysiology will depend on the development of relevant in vitro and in vivo models. However, our present findings provide sufficient evidence that the ketogenic pathway-associated enzymes play an important role during prostate cancer pathogenesis and may provide an interesting area for therapeutic intervention for a disease that, to date, lacks targeted treatments.
CHAPTER 3:
EVALUATION AND PROGNOSTIC SIGNIFICANCE OF ACAT1 AS A MARKER OF PROSTATE CANCER PROGRESSION

The work presented in this chapter was published in the Prostate:
Saraon P, Trudel D, Kron K, Dimitromanolakis A, Bapat B, van der Kwast T, Jarvi KA, Diamandis EP.
Evaluation and Prognostic Significance of ACAT1 as a Marker of Prostate Cancer Progression
Prostate, 2014; 74(4): 372-80

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3.1 Introduction

Prostate cancer is the most common solid organ tumor and the second leading cause of death due to cancer among men in North America [1]. Prostate-specific antigen (PSA) is a protein predominantly secreted by prostatic epithelial cells and is one of the best serum cancer biomarkers available [2, 3]. Since the introduction of the PSA test in the late 1980’s, prostate cancer diagnosis increased substantially; however, a reduction in mortality is not matched by the improved diagnosis. A major issue with PSA is its lack of specificity, as other non-malignant diseases of the prostate also display elevated serum PSA levels, including benign prostate hyperplasia (BPH) and prostatitis, which can lead to over-diagnosis [2]. In addition, PSA levels are a poor indicator of aggressiveness, leading to potential over-treatment of many patients.

It is well-established that the majority of prostate cancer cases are slow growing and often indolent; however, a subset of patients develop more aggressive cancers which are usually fatal [122, 127]. Therefore, markers that are able to discriminate between the various forms of the disease are of utmost importance. One of the best prognostic indicators for prostate cancer is Gleason score (GS), which characterizes the glandular architecture of the prostate based on a score that represents the level of cancer ‘de-differentiation’ [24, 25]. The Gleason score is comprised of two numbers, each representing the most common Gleason patterns ranging from 1 to 5, where 1 represents a highly differentiated carcinoma and 5 represents an aggressive de-differentiated one. It is now accepted that the transition from pattern 3 to pattern 4 represents disease progression from low-grade to high-grade [174]. Gleason 7 cancers, which are comprised of pattern 3 and pattern 4, are considered an intermediate state, requiring definitive treatment such as prostatectomy or radiotherapy. In addition, a proportion of patients with
Gleason score 6 prostate cancers are eligible for active surveillance instead of immediate curative therapy.

Many studies focusing on genomics, epigenomics, and proteomics have been conducted to identify biomarkers that complement PSA or correlate with disease aggressiveness [136, 137, 175-177]. However, these studies have come-up short on yielding useful biomarkers for diagnosis or prognosis. For example, recent interest on gene fusions, specifically the TMPRSS2-ERG fusion, which has been found in approximately 50% of prostate cancer cases, has been evaluated for its prognostic potential [178, 179]. There have been many conflicting reports of TMPRSS2-ERG as a prognostic marker, as some studies correlated it with several clinicopathological characteristics such as disease stage and biochemical recurrence, whereas others found no association [180-182]. Since TMPRSS2-ERG fusion is a very early event in prostate carcinogenesis, ERG positive and negative prostate cancers may represent two disparate lineages of prostate cancer with their own biomarker repertoire.

Recently, using an in-vitro quantitative proteomics approach, we identified enzymes of the ketogenic pathway to be elevated during prostate cancer progression [183]. After validation on a small clinical cohort, we found that ACAT1 in particular, a key enzyme within this pathway, was strongly associated with high-grade (GS≥8) and castration-resistant metastatic prostate cancer. In this study, we assessed the diagnostic/prognostic potential of ACAT1 expression in relationship with ERG expression status using immunohistochemistry on a large cohort of prostate cancer patients, and analyzed the relationship between ACAT1 expression and clinicopathological features of prostate cancer.
3.2 Materials and Methods

3.2.1 Patient Cohort and Pathology

A total of 251 patients who were diagnosed with localized prostate cancer and underwent radical prostatectomy between 1998 and 2001 at the University Health Network (UHN) in Toronto were included in this study. All samples along with their clinical and pathological follow-up data were obtained according to protocols approved by the Research Ethics Board at Mount Sinai Hospital, Toronto and UHN, Toronto.

The complete set of hematoxylin and eosin (H&E)-stained slides were reviewed by an expert pathologist (T. van der Kwast) and were assigned a Gleason score (WHO/ISUP 2005 criteria) [184], pathological stage (TNM), and surgical margin status.

3.2.2 Tissue Microarray Construction

Between 3 and 13 cores were taken from each of the 251 cases, to have representation of each primary, secondary, and occasionally, tertiary Gleason pattern present within the case. Matched benign tissue adjacent to the tumor was also taken from every patient. This resulted in a total of 1438 cores within 7 tissue microarray (TMA) blocks. For each TMA, five-micrometer serial sections were used for H&E staining, to verify the presence of tumor vs. normal cells.

3.2.3 Immunohistochemical staining

TMA 4µm-slides were deparaffinized in xylene, dehydrated and blocked in hydrogen peroxide in methanol for 10 minutes. Antigen retrieval was then performed using 10 mM citrate
buffer, pH 6.0, in a microwave for 10 minutes. Slides were then blocked for 5 min in casein and incubated overnight with an ACAT1 polyclonal antibody (1:500 in PBS) (Sigma). Following 10 min of phosphate buffer saline (PBS) washing, slides were placed in secondary antibody for 30 min using the Polymer-HRP Immunohistochemistry kit (Biogenex, Fremont, CA) according to the manufacturer’s instructions. After a 10 minute wash in PBS, slides were developed with the addition of 3,3-Diaminobenzidine (DAB) for 5 min. Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

3.2.4 Evaluation of immunohistochemistry staining

Immunostained slides were scanned and analyzed with the Aperio system at objective x 20. An in-house positive pixel count algorithm was developed to measure ACAT1 expression applied to individual cores using the TMA lab application. The positive pixel count algorithm provided information on intensity and percentage of positive pixels, which was used to calculate the H-score [185] using the following formula:

\[
100 \times \left(\% \text{ of pixels with weak intensity} \times 1 + \% \text{ of pixels with moderate intensity} \times 2 + \% \text{ of pixels with high intensity} \times 3\right) / \text{Total number of pixels}
\]

When assessing ACAT1 expression for each case (251 cases in total), the H-scores of all cancerous cores were averaged to determine an overall H-score for each case. In addition, each of the cores was independently reviewed by a pathologist to verify the positive pixel count results. When needed, cases were manually annotated to remove portions of the cores that had a different positivity level than the tissue of interest. Cores that presented with less than 15% total
measured areas were excluded from analysis. Also, to ensure accuracy, 10% of all cores were individually assessed by a pathologist, strictly analysing the tissue of interest on each core.

3.2.5 Biochemical recurrence

Biochemical recurrence was defined as either if (a) two or more consecutive increases in serum PSA resulting in at least doubling of initial PSA, or (b) any increase in PSA resulting in values exceeding 0.2 ng/ml, or (c) there was at least a ten-fold increase in serum PSA between two consecutive samples. Patients not meeting any of these criteria were categorized as exhibiting “stable disease” [186].

3.2.6 Statistical analysis

Analysis of the relation between ACAT1 expression and Gleason score and pathological stage was done using two-tailed t-tests. Pearson correlation analysis was performed comparing ACAT1 expression, age and preoperative PSA. Cox proportional hazards regression model was used to evaluate whether Gleason score, stage, ACAT1 staining, ERG staining, age, preoperative PSA or margin status had a relationship with biochemical recurrence. Each factor was coded as a binary variable, with the exception of Gleason score, which was categorized into the 3 groups: GS≤6, GS7 and GS≥8. Univariate disease-free survival was assessed using the Kaplan-Meier curve and log-rank tests. All tests were conducted with SPSS software.
3.3 Results

3.3.1 Associations between ACAT1 Expression and clinicopathological variables

We assessed ACAT1 expression based on H-score intensity, in 251 prostate cancer specimens along with their adjacent benign matched tissues. In total, there were 1541 cores; however, after excluding cores based on total area and improper tissue type, a total of 1438 cores were used for the analysis (103 cores were excluded). Table 3.1 shows the clinicopathological characteristics of the patient cohort analyzed. Also, to ensure accuracy, 10% of all cores were individually assessed by a pathologist, strictly analyzing the tissue of interest on each core. The correlation coefficient after independent assessment was 0.89, indicating strong positive correlation of the two independent analyses. Representative cores displaying ACAT1 expression are shown Figure 3.1A. Using an in-house quantitative algorithm, we assessed ACAT1 expression between benign and cancer cores. We observed significantly elevated ACAT1 expression in cancer cores (mean H-score=40.3, SD=27.3, n=1174) compared to adjacent benign cores (mean H-score=16.9, SD=10.5, n=264) (P<0.0001) (Figure 3.1B).

Next, we assessed ACAT1 expression and its association with Gleason score (GS). After analysis, ACAT1 expression was observed to be higher in cases of high-grade (GS≥8) prostate cancer (Figure 3.1C). The distribution of ACAT1 expression according to GS was significantly different between GS≤6 (mean H-score=37.9, SD=18.3, n=128), GS7 (mean H-score=40.2, SD=19.3, n=103), and GS≥8 (mean H-score=59.4, SD=38.2, n=20) compared to benign tissues (P<0.0001) (Table 3.2). In addition, there was a significant difference between GS≤6 and GS≥8 cases (P<0.0001), as well as between GS7 and GS≥8 cases (p=0.001) (Table 3.3). However, there was no difference of ACAT1 expression between GS≤6 and GS7 cases (P=0.372). When
we assessed GS7 (3+4) and GS7 (4+3) cases, we again, did not find a significant difference of ACAT1 expression.

We also assessed the relationship between ACAT1 expression and pathological stage (Figure 3.1D). Due to the small number of patients with pT4 stage tumors, we decided to combine pT3 and pT4 patterns to represent locally aggressive tumors. In organ-confined pT2 tumors, ACAT1 displayed moderate expression patterns (mean H-score=37.3, SD=19, n=163), whereas pT3/4 tumors displayed higher expression (mean H-score=46.6, SD=24.7, n=88) (Table 3.2). There was a significant difference between pT2 and pT3/pT4 cases compared to benign controls (P<0.0001; Table 3.3). In addition, there was a significant difference between organ-confined pT2 tumors and the advanced pT3/pT4 tumors (P=0.001).

Next, we tested the association between ACAT1 expression and other variables including age, preoperative PSA, and surgical margin status. However, we did not find a significant correlation between ACAT1 expression and any of these variables (Table 3.3).

Using previous ERG protein expression data conducted on the study cohort [187], we next assessed whether there was an association between ACAT1 and ERG staining. We did not find a statistically significant association; however, there was a trend for elevated ACAT1 expression in ERG positive cases (P=0.133).
Figure 3.1. ACAT1 staining vs. clinicopathological parameters of prostate cancer. A, Representative prostate samples stained for ACAT1 expression at various Gleason patterns. B, Average H-score between normal and cancerous tissues. C, ACAT1 staining vs. Gleason score. D, ACAT1 staining vs. pathological stage. Immunohistochemical staining was quantified using Aperio imaging Software. Error bars represent the standard deviation. For more details see text. Statistical comparisons between groups are shown in Table 3.3.
Table 3.1. Cohort clinicopathological characteristics

Clinicopathological characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>251</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
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<tr>
<td>6</td>
<td>87</td>
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<td>7</td>
<td>103</td>
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<td>8</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Pathological stage</td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>163</td>
</tr>
<tr>
<td>pT3</td>
<td>83</td>
</tr>
<tr>
<td>pT4</td>
<td>5</td>
</tr>
<tr>
<td>Surgical margin status</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>58</td>
</tr>
<tr>
<td>Negative</td>
<td>193</td>
</tr>
<tr>
<td>Average preoperative PSA (range)</td>
<td>8.6 (0.1-55.4)</td>
</tr>
<tr>
<td>Average follow-up time (range), y</td>
<td>4.43 (0.17-9.48)</td>
</tr>
<tr>
<td>Number of biochemical recurrences (%)</td>
<td>83 (33.7)</td>
</tr>
<tr>
<td>Median age (range), y</td>
<td>62 (32-75)</td>
</tr>
</tbody>
</table>
Table 3.2. ACAT1 expression stratified by clinical characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>Mean ACAT1 H-Score</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign Cores</td>
<td>264</td>
<td>16.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Cancerous Cores</td>
<td>1174</td>
<td>40.3</td>
<td>27.3</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>163</td>
<td>37.3</td>
<td>19</td>
</tr>
<tr>
<td>pT3/pT4</td>
<td>88</td>
<td>46.6</td>
<td>24.7</td>
</tr>
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<td><strong>Gleason Score</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤ 6</td>
<td>128</td>
<td>37.9</td>
<td>18.3</td>
</tr>
<tr>
<td>7</td>
<td>103</td>
<td>40.2</td>
<td>19.3</td>
</tr>
<tr>
<td>≥ 8</td>
<td>20</td>
<td>59.4</td>
<td>38.2</td>
</tr>
<tr>
<td><strong>Surgical Margin Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>58</td>
<td>39.9</td>
<td>22.4</td>
</tr>
<tr>
<td>Negative</td>
<td>193</td>
<td>42.7</td>
<td>18.8</td>
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<tr>
<td><strong>ERG Expression</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Positive</td>
<td>109</td>
<td>38.3</td>
<td>24</td>
</tr>
<tr>
<td>Negative</td>
<td>102</td>
<td>42.7</td>
<td>19</td>
</tr>
</tbody>
</table>

1. For statistical comparisons see Table 3.3
Table 3.3. P values for ACAT1 Expression by clinical characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. Cancer</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>Normal vs. pT2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal vs. pT3/pT4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pT2 vs. pT3/pT4</td>
<td>0.001</td>
</tr>
<tr>
<td>pT3a vs. pT3b</td>
<td>0.707</td>
</tr>
<tr>
<td><strong>Gleason Score</strong></td>
<td></td>
</tr>
<tr>
<td>Normal vs. ≤ 6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal vs. 7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal vs. ≥ 8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≤ 6 vs. 7</td>
<td>0.372</td>
</tr>
<tr>
<td>≤ 6 vs. ≥ 8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>7 vs. ≥8</td>
<td>0.001</td>
</tr>
<tr>
<td>GS7 (3+4) vs. GS7 (4+3)</td>
<td>0.783</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>0.43</td>
</tr>
<tr>
<td>Preoperative PSA</td>
<td>0.763</td>
</tr>
<tr>
<td>ERG Postive Cases</td>
<td>0.133</td>
</tr>
<tr>
<td>Surgical Margin Status</td>
<td>0.396</td>
</tr>
</tbody>
</table>
3.3.2 ACAT1 Expression and Biochemical Recurrence-Free Survival

We next evaluated whether there was a relationship between ACAT1 staining and other clinicopathological variables with biochemical recurrence. Before performing the analysis, we categorized all cases as either having low or high ACAT1 expression based on the median H-score of all cases. Any case below the median was considered low for ACAT1 expression, and any case higher than median H-score was considered high. Univariate Cox regression analysis showed that high ACAT1 expression (HR, 1.81, CI=1.13-2.9, p=0.0128), GS7 (HR, 1.97, CI=1.17-3.31, p=0.0103), GS≥8 (HR, 3.99, CI=1.93-8.28, p=0.0002), pathological stage (pT3/4 tumors) (HR, 3.02, CI=1.91-4.81, p<0.0001), preoperative PSA (HR, 1.03, CI=1.01-1.05, p=0.0061) and positive surgical margin status (HR, 2.20, CI=1.37-3.54, p=0.0011), were all associated with shorter disease-free survival (Table 3.4). Univariate Kaplan-Meier/log-rank analysis revealed that high ACAT1 staining was associated with a significant decrease in biochemical recurrence-free survival (log rank P=0.0014; Figure 3.2A).

We also conducted multivariate Cox regression analysis, which revealed that ACAT1 expression (HR, 1.69, CI=1.01-2.81, p=0.0431), GS≥8 (HR, 2.69, CI=1.2-6.04, p=0.017), pathological stage (pT3/4 tumors) (HR, 3.42, CI=2.02-5.78, p<0.0001), preoperative PSA (HR, 1.02, CI=1.01-1.06, p=0.0461) and positive surgical margin status (HR, 2.31, CI=1.37-3.88, p=0.0015), were significant predictors of biochemical recurrence (Table 3.4). In addition, when combining ACAT1 expression and pathological stage, both these variables were able to further stratify the likelihood of biochemical recurrence. Among the subset of more advanced pT3/pT4 tumors, those with a high ACAT staining had a significantly increased likelihood of biochemical recurrence (log rank P<0.0001; Figure 3.2B). Furthermore, after performing a similar analysis with the GS≤6 group, any case that also displayed high ACAT1 expression had increased
likelihood of biochemical recurrence compared to GS≤6 and low ACAT1 expression (log rank P=0.023; Figure 3.2C).

We next looked at whether biochemical recurrence could be further stratified based on ERG and ACAT1 expression. We observed that cases that were negative for ERG but displayed high ACAT1 expression, had a significantly increased likelihood of biochemical recurrence compared to ERG negative and low ACAT1 expression cases (log rank P=0.0025; Figure 3.2D). In contrast, ACAT1 lost its prognostic impact in the subset of ERG positive cases (Figure 3.2D).
Figure 3.2. Prognostic potential of ACAT1 staining as a marker of biochemical recurrence. A, Biochemical recurrence-free survival vs. high ACAT1 and low ACAT1 cases. B, Univariate Kaplan-Meier/log-rank analysis of biochemical recurrence-free survival vs. ACAT1 staining and pathological stage. C, Univariate Kaplan-Meier/log-rank analysis of biochemical recurrence-free survival vs. ACAT1 staining and GS≤6 cases. D, Univariate Kaplan-Meier/log rank analysis of biochemical recurrence-free survival vs. ACAT1 staining and ERG expression. For statistical comparisons see Table 3.4.
Table 3.4. Associations between ACAT1 expression and biochemical recurrence

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
</table>

**Univariate Analysis**

- High ACAT1: 1.81 (1.13-2.9) 0.0128
- Gleason Grade 7: 1.97 (1.17-3.31) 0.0103
- Gleason Grade $\geq 8$: 3.99 (1.93-8.28) 0.0002
- Tumor Stage (pT3/4): 3.02 (1.91-4.81) <0.0001
- ERG Positivity: 0.87 (0.56-1.38) 0.5714
- Positive Surgical Margin: 2.20 (1.37-3.54) 0.0011
- Age: 1.02 (0.99-1.06) 0.1604
- Preoperative PSA: 1.03 (1.01-1.05) 0.0061

**Multivariate Analysis**

- High ACAT1: 1.69 (1.01-2.81) 0.0431
- Gleason Grade 7: 1.17 (0.67-2.05) 0.5643
- Gleason Grade $\geq 8$: 2.6 (1.2-6.04) 0.0167
- Tumor Stage (pT3/4): 3.42 (2.02-5.78) <.0001
- ERG Positivity: 0.65 (0.38-1.11) 0.114
- Positive Surgical Margin: 2.31 (1.37-3.88) 0.0015
- Age: 1.00 (0.96-1.04) 0.9327
- Preoperative PSA: 1.02 (1.0-1.06) 0.0461

1. CI, Confidence interval
3.4 Discussion

Previously, we identified enzymes of the ketogenic pathway to be associated with prostate cancer progression. Of these enzymes, ACAT1 displayed the most interesting expression patterns, as it was found to be highly elevated in primary high-grade and in castration-resistant metastatic prostate cancer specimens [183]. In the current study, we validated the expression of ACAT1 by immunohistochemistry on a separate cohort of hormone-naïve prostate cancers and correlated the ACAT1 findings with various clinicopathological parameters and clinical outcomes, to evaluate its potential as a prognostic biomarker.

Our results show that ACAT1 expression is elevated in high-grade (GS≥8) prostate cancers compared with low and intermediate grade GS≤7 tumors. We also observed elevated ACAT1 expression in more advanced pT3/pT4 tumors compared to organ-confined pT2 tumors. These results are of importance, as patients who present with GS≥8, harbour tumors displaying properties that are usually associated with poor prognosis [188].

After performing Kaplan Meier survival analysis, ACAT1 expression was an independent prognostic marker for biochemical recurrence. When we performed survival analysis looking at both ACAT1 staining and tumor stage, it was observed that patients who displayed high ACAT1 expression along with having a tumor stage of pT3/pT4, had significantly worse prognosis (increased likelihood of biochemical recurrence) than other groups. In addition, ACAT1 was also able to discriminate between pT2 tumors, as pT2 cases that displayed high ACAT1 expression had worse prognosis than pT2 cases with low ACAT1 staining. After performing a similar analysis with Gleason score, we found that in cases of low-grade cancer (GS≤6), patients that also displayed high ACAT1 expression had a significantly higher risk of biochemical recurrence than those with low ACAT1 expression. These results suggest that ACAT1 staining
could be of clinical value as a prognostic marker at the time of biopsy diagnosis, if these findings could be confirmed in prostate biopsies.

After performing similar Kaplan-Meier survival analysis comparing ERG and ACAT1 expression, we found that in ERG negative cases, patients that had high ACAT1 expression had an increased likelihood of biochemical recurrence. Such a trend was not observed when we performed the analysis on ERG positive cases, indicating that ERG expression might distinguish two distinct subsets of prostate cancers with their own biomarker profile. Recent studies have demonstrated such an effect, as different biomarkers displayed differential expression profiles in ERG negative and positive cases [187, 189]. One of the major caveats of immunohistochemistry analysis is the reproducibility due to variability and the semi-quantitative nature of the approach. To address these, we decided to use a software that detects positive ACAT1 staining using an in-house generated algorithm for positive pixel count. Such an automated approach reduces variability of the scoring system, as the algorithm will always detect the same amount of ACAT1 positivity after each run. In addition, each of the cores was independently reviewed by a pathologist to verify the positive pixel count results detected by the software, to ensure the analysis was as accurate as possible.

The role of ACAT1 with respect to prostate cancer pathophysiology has yet to be elucidated. A recent study showed that ACAT1 was involved in androgen-mediated cholesterol metabolism in prostate cancer cell lines [169]. In another study, ACAT1 was found to be elevated in androgen-independent xenografts, further demonstrating its importance during prostate cancer progression [170]. Both these studies considered ACAT1 and its role with respect to cholesterol biogenesis. Interestingly, an alternative hypothesis as to why prostate cancer cells may over-express ACAT1 is to accelerate biosynthesis of cholesterol precursor
molecules, as cholesterol has been shown to be involved in intratumoral de novo androgen biosynthesis [190]. Essentially, prostate cancer cells may be utilizing an alternate pathway to produce endogenous androgens to activate the AR signalling cascade, during times of androgen deprivation.

The ketogenic pathway is an alternate energy producing pathway that results in the formation of high energy ketone bodies such as beta-hydroxybutyrate [191]. Two recent studies looked at the importance of the ketogenic pathway with respect to tumor growth and progression in a human breast cancer cell line model [192, 193]. In these studies, it was found that ACAT1, along with other ketogenic pathway enzymes, behaved functionally as a metabolic oncogene, as breast cancer cells over-expressing these enzymes had increased tumor growth and metastatic potential [192, 193]. Interestingly, in these studies, it was observed that both BDH1 and HMGCS1/2 were rate-limiting with respect to ketone body formation, whereas ACAT1, HMGCS1/2 and OXCT1 were integral for ketone body re-utilization [192, 193]. In order to better understand the mechanism of action of ACAT1 in prostate cancer progression, further studies, using RNA interference technology in prostate cancer cell lines and animal models, need to be conducted. Such functional studies can provide insight into whether ACAT1 has a direct mechanistic role on prostate cancer progression, which in turn can be utilized as a potential area of therapeutic intervention. ACAT1 inhibitors have been previously investigated for various other diseases including atherosclerosis [171, 172], and may present and interesting avenue of therapeutic intervention for aggressive prostate cancers if this protein is indeed implicated in prostate cancer pathobiology. In addition, assessing the expression of ACAT1 on other diverse clinical samples, such as hormone-naïve and hormone-refractory specimens, will provide better
overall interpretation of the importance of this protein with respect to the development and progression of aggressive prostate cancer.

In conclusion, we assessed ACAT1 expression, using an automated scoring system, in a large series of prostate cancer cases and analyzed its relationship with the most relevant clinicopathological parameters. Overall, we have shown that ACAT1 expression is significantly elevated in 1) prostate cancer versus benign prostatic glandular tissue 2) high-grade vs. low/intermediate grade prostate cancer, and 3) advanced pT3/pT4 vs. organ-confined pT2 tumors. In addition, ACAT1 expression is also an independent indicator of reduced biochemical recurrence-free survival. Going forward, further studies need to be conducted to assess the clinical utility of ACAT1 and its functional role during prostate cancer progression.
CHAPTER 4:
PROTEOMIC PROFILING OF ANDROGEN-INDEPENDENT PROSTATE CANCER CELL LINES REVEALS A ROLE FOR PROTEIN S DURING THE DEVELOPMENT OF HIGH GRADE AND CAstrate-RESISTANT PROSTATE CANCER

The work presented in this chapter was published in the Journal of Biological Chemistry:

Proteomic Profiling of Androgen-Independent Prostate Cancer Cell Lines Reveals a Role for Protein S during the Development of High Grade and Castrate-Resistant Prostate Cancer

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4.1 Introduction

Prostate cancer is the most commonly diagnosed solid organ tumor and the second leading cause of death due to cancer among men in North America [1]. It is observed as either a slow-growing indolent tumor, or as a more advanced aggressive form; however, the current screening methods are not able to differentiate between these two forms. Prostate-specific antigen (PSA) is the most widely used biomarker for prostate cancer. However, due to documented lack of specificity, it may also be elevated in other non-cancerous pathologies, including benign prostate hyperplasia and prostatitis [2, 3]. This inevitably leads to over-diagnosis and ultimately, to over-treatment.

Current treatments depend on whether the tumour is localized or has metastasized. Localized prostate cancers are usually treated with either targeted radiation or radical prostatectomy. For more advanced and metastatic cancers, the mainstay treatment is androgen deprivation therapy, which is initially effective at reducing tumour volume and growth [122, 127]. However, persisting androgen deprivation often results in the selection and accumulation of cancerous cells that have acquired resistance, leading to the development of androgen-independent or castration-resistant prostate cancer [122, 127]. This genotypic and phenotypic alteration is accompanied by increased mortality rates, since there are no alternative targeted therapies. Thus, the understanding of molecular alterations during the progression to androgen-independence becomes of utmost importance for catalyzing development of targeted and efficient therapeutic options.

The androgen receptor (AR) signalling cascade has been extensively studied with respect to the progression of androgen-independent prostate cancer. Overall, AR signalling resistance has been attributed to AR gene amplifications, AR gene mutations, changes in co-regulators or
steroidogenic enzymes, or through alternative proteins via outlaw pathways [121-127]. However, recent interest has shifted towards the identification of novel ‘bypass’ pathways, the so-called AR-independent pathways, which can play a role in this process, as well [58, 87].

High-throughput proteomics is a growing field that can virtually be used to study the proteomic signature of any biological material [194]. Numerous studies have been conducted using proteomics to identify biomarkers for prostate cancer using cell lines, tissues, and biological fluids, including expressed prostatic secretions [118, 119, 135, 136, 138, 139, 195, 196].

Previously, we performed proteomic analysis of the conditioned media of three prostate cancer cell lines (LNCaP, PC3 and 22Rv1), and identified over 1000 proteins that could serve as potential biomarkers for prostate cancer diagnosis [118]. In the current study, we performed proteomic analysis of the conditioned media (secretome analysis) of five androgen independent cell lines (PC3, DU145, PPC1, LNCaP-SF, 22Rv1), two androgen-dependent (LNCaP, VCaP) and one normal prostate epithelial (RWPE) cell line. In total, we identified over 3000 proteins, with over 100 proteins being differentially secreted between the AIPC and non-AIPC cell lines. Of these, Protein S (PROS1) was elevated in all five AIPC cell lines, with no observed secretion in the normal and androgen-dependent prostate cancer cell lines. Subsequently, we observed PROS1 overexpression in high-grade prostate cancer tissue and seminal plasma. In addition, PROS1 expression was highly elevated in castrate-resistant metastatic prostate cancer specimens. These results demonstrate that PROS1 could serve as a potential biomarker for high-grade prostate cancers, as well as provide new clues of therapeutic intervention for the treatment of AIPC patients.
4.2 Materials and Methods

4.2.1 Cells and reagents

The human prostate cancer cell lines PC3, DU145, LNCaP, 22Rv1, VCaP and the normal prostate epithelial cell line RWPE-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The LNCaP-SF cells were kindly provided by Dr. Atsushi Mizokami and the PPC-1 cell line was provided by Dr. Aaron Schimmer. Cell culture media specified by the ATCC for each of the cell lines were used as follows: Dulbecco’s modified Eagle’s medium (DMEM)(ATCC) with 10% fetal bovine serum (Thermo Scientific) was used for PC3, DU145 and VCaP; Roswell Park Memorial Institute (RPMI) (ATCC, Manassas, VA) with 10% FBS was used for 22RV1, PPC-1 and LNCaP cells. The normal RWPE cell line was grown in keratinocyte serum-free media (ATCC) supplemented with bovine pituitary extract and recombinant epidermal growth factor. The LNCaP-SF cells were grown in DMEM media supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen). All cells were maintained at 37°C with 5% CO₂ in a humidified incubator. All experiments were performed within the first 5 passages from the initiation of all cultures.

4.2.2 Cell Culture

Cells were cultured in six T-175cm² flasks and allowed to grow in 30ml of their respective media until they reached 70% confluence. Generally, cells were allowed to grow for 48 hours for them to achieve this confluence. The media was then removed, and cells were washed three times with 20ml of PBS (Invitrogen). Following the washes, 30ml of Chinese hamster ovary serum-free medium (Invitrogen) were added to each of the flasks, which were then cultured for two days. After further growth, the conditioned medium was collected and...
centrifuged at 2000 x g for 5 minutes to remove cellular debris. The resulting supernatant was transferred to a fresh tube and total protein was measured using a Coomassie (Bradford) blue total protein assay. Each flask contained approximately 500ug of total protein, of which two were combined to generate a total of 1mg. Since we initially grew six T-175cm² flasks, we were able to combine them in three replicates containing a total protein level of approximately 1mg. The triplicates were then further processed following the sample preparation protocol below.

4.2.3 Sample Preparation

Each sample containing 1mg of total protein was initially dialyzed using a 3.5 kDa molecular weight cut-off membrane (Spectrum Laboratories, Inc, Compton CA) in 5L of 1mM ammonium bicarbonate buffer solution at 4°C overnight. The buffer solution was changed twice and the sample was then frozen at -80°C and lyophilized to dryness using a Modulyo Freeze Dryer (Thermo Electron Corporation). The resulting dry protein product was denatured in 8M urea and reduced with 200mM dithiothreitol at 50°C for 30 min. Samples were then alkylated in 500mM iodoacetamide while shaking in the dark for 1 hour. Each replicate was then further desalted using a NAP5 column (GE Healthcare), frozen, and lyophilized again to complete dryness. Finally, the samples were then digested with trypsin (Promega, sequencing-grade modified porcine trypsin) at 37°C overnight using a 1:50 trypsin to protein concentration ratio. The samples, now containing tryptic peptides, were acidified with formic acid before strong cation exchange (SCX).
4.2.4 Strong cation exchange (SCX) on a high pressure liquid chromatography (HPLC) system

To reduce sample complexity, the samples were subjected to SCX using an Agilent 1100 High Performance Liquid Chromatography (HPLC) system. The HPLC system was connected to a PolySULPHOETHYL A™ column with a 200 Å pore size and a diameter of 5 µm (The Nest Group Inc., Southborough MA). A total of 12 fractions per replicate containing the greatest number of peptides based on peak intensity were eluted and collected to perform mass spectrometric analysis. The HPLC fractions were C\textsubscript{18}-extracted using ZipTipC\textsubscript{18} micropipette tips (Millipore, Billerica MA) and eluted in 5µl of Buffer B (90% acetonitrile, 0.1% formic acid, 10% water and 0.02% trifluoroacetic acid). An additional 80µl of Buffer A (95% water, 0.1% formic acid, 5% acetonitrile, and 0.02% trifluoroacetic acid) were added to each zipped fraction.

4.2.5 Mass Spectrometry (LC-MS/MS)

40µl of each fraction were injected into an autosampler on the EASY-nLC system (Proxeon Biosystems, Odense, Denmark). Peptides were first collected onto a 3cm C18 column (inner diameter of 200µm), and were then eluted onto a resolving 5-cm analytic C18 column (inner diameter of 75µm) with an 8µm tip (New Objective). This HPLC system was coupled online to an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer, using a nano-Electro Spray Ionization (ESI) source (Proxeon Biosystems), in data-dependent mode. The fractions were run on a 55 minute gradient, and eluted peptides were subjected to one full MS\textsubscript{1} scan (450-1450 m/z) in the Orbitrap at 60,000 resolution, followed by six data-dependent MS\textsubscript{2} scans in the linear ion trap. The following parameters were enabled: monoisotopic precursor
selection, charge state screening and dynamic exclusion. In addition, charge states of +1, >4 and unassigned charge states were not subjected to MS2 fragmentation.

4.2.6 Protein Identification and Data Analysis

RAW files were uploaded onto Mascot Daemon (v.2.2) and extract_msn was used to generate DAT and Mascot Generic Files (MGFs). MGF files were further searched on GPM (Global Proteome Machine Manager, version 2006.06.01) against the concatenated nonredundent IPI.Human v.3.62 database with parent and fragment tolerances of 7 ppm to generate X!Tandem files. The DAT and X!Tandem files were then merged and searched with Scaffold (Proteome Software Inc., Portland, OR; v2.0) to generate a file containing protein lists. The final Scaffold files contained normalized spectral counts for each of the cell lines, which were subsequently used as a semi-quantitative measure to compare protein secretion among each cell line.

ProteinCenter (Thermo Fisher Scientific) was used to retrieve Gene Ontology annotations and pathway analyses from the Kyoto Encyclopedia of Genes and Genomes (KEGG). To visualize and assess networks of over-expressed and under-expressed candidates, Ingenuity Pathway Analysis (IPA; Ingenuity Systems, CA, USA) software was used. Using this software, pathway analysis was performed, obtaining information on canonical pathways and molecular networks that have been altered, which were determined by Fisher’s exact test.

4.2.7 RNA Extraction and qPCR

Total RNA was isolated from cells using an RNeasy Kit (Qiagen). cDNA was generated from 1µg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). LuCaP 96 and LuCaP 96AI xenograft tissues were frozen in liquid nitrogen, and then ground into a fine powder.
using a mortar and pestle. RNA was extracted from the resulting powdered tissue using an RNeasy Kit (Qiagen) and the final RNA product was used for further cDNA synthesis.

Quantitative PCR was conducted using 1X SYBR reagent (Applied Biosystems) and transcript levels of **PROS1 TWSG1, LTBPI, GBA, PAM** and **GAS6** were measured on a 7500 ABI system. For clinical validation, the TissueScan Prostate Cancer cDNA Array II was used (Origene). Quantitative PCR was conducted on these samples using the same SYBR green reagent as mentioned above. The following primer sequences were used:

**PROS1 Forward**- GGCTCCTACTATCCTGGTTCTG,
**PROS1 Reverse**- CAAGGCAAGCATAACACCAGTGC
**GAS6 Forward**- CCTTCCATGAGAAGGACCTCGT
**GAS6 Reverse**- GAAGCACTGCATCCTCGTTG
**TBP Forward**- TGTATCCACAGTGAATCTTGGTG
**TBP Reverse**- GGTTCGTGGCTCTTTATCCTC
**TWSG1 Forward**- CTTTGGACGAGTGCTGTGACT
**TWSG1 Reverse**- GAGAAGGGATCGGTTCATGCAG
**LTBP1 Forward**- TGAATGCCAGCACCGTCATCTC
**LTBP1 Reverse**- CTGGCAAAACACTCTTTGCCTCC
**GBA Forward**- TGCTGCTCTCAACATCCTTGCC
**GBA Reverse**- TAGGTGCGGATGGAGAAGTCAC
**PAM Forward**- TGAAGGCAACCTGGGAACCAGA
**PAM Reverse**- CTCTGTGGAAAATCACCAGGTTAT
4.2.8 Western blotting

Protein expression of PROS1, TWSG1, LTBP1, GBA and PAM was assessed using Western blot analysis. Roughly, 30µg of total protein from LuCaP 96 and LuCaP 96AI were loaded onto an SDS-PAGE gel (4-15%, BioRad), and transferred onto PVDF membranes (BioRad). Membranes were then incubated with 5% blocking solution (2.5g skim milk powder in tris-buffer solution containing 0.1% Tween (TBST)) overnight at 4°C. Membranes were incubated with rabbit polyclonal antibody against PROS1 (1:1000), TWSG1 (1:500), LTBP1 (1:100), GBA (1:1000, Sigma), or PAM (1:1000, Protein Tech) for 1 hour at room temperature. The membranes were then washed six times (three 15 minute washes followed by three 5 minute washes) with TBST. Membranes were then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (1:3000, Jackson Laboratories) or goat anti-mouse secondary antibody conjugated to alkaline phosphatase (1:3000, Jackson Laboratories) for 1 hour at room temperature. After washing with TBST, proteins were detected using the ECL detection reagent (Siemens). The expression of GAPDH (Cell Signalling Technology) was used as an internal standard.

4.2.9 Immunohistochemistry

Prostate cancer tissue microarrays (TMA) consisting of 8 normal and 40 cancer cores were purchased from US BioMax (Rockville, MD). The metastatic prostate cancer tissue microarray was developed and provided by the GU Cancer Research laboratories at the University of Washington, Seattle, USA. Human tissue microarrays of fixed paraffin-embedded metastatic tissues from 23 rapid autopsy patients who died of prostate cancer (consisting of 3 tissue microarray blocks with 2 replicate cores per metastatic site) were used for
immunohistochemical (IHC) analyses. All patients had castrate resistant prostate cancer at the
time of autopsy, defined by the presence of a rising serum PSA following medical or surgical
castration.

TMAs were deparaffinized in xylene and rehydrated using ethanol. Endogenous
peroxidase was inactivated using hydrogen peroxide for 10 minutes and washed with PBS.
Antigen retrieval was then performed using citrate buffer in a microwave for 10 minutes. Slides
were then blocked for 5 minutes in casein and incubated overnight with the following antibodies:
PROS1 (1:500, Epitomics), TWSG1 (1:50, Abgent), LTBP1 (1:200, Abgent). Rabbit IgG was
used on a duplicate slide to serve as a negative control. Following 10 min of PBS washing,
slides were placed in secondary antibody for 30 min using the BGX kit (Biogenex, Fremont,
CA). After a 10 min wash in PBS, slides were developed with the addition of DAB for 5 min.
Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.

4.2.10 Seminal Plasma and ELISA

Semen was collected from suspected prostate cancer patients prior to biopsy, allowed to
liquefy at room temperature for 1 hour, and centrifuged at 13 000 rpm for 15 minutes to separate
seminal plasma from cells and cellular debris. The seminal plasma was aliquoted into 1.5mL
Eppendorf tubes and frozen at -80°C until further use.

PROS1 protein levels in seminal plasma were assessed using an enzyme-linked
immunosorbent assay (American Diagnostica). Briefly, seminal plasma samples were diluted 10
times and the ELISA was performed according to the manufacturer’s instructions.
4.2.11 Wound Repair/Scratch Assay

To assess wound repair and cell migration, LNCaP cells were grown to full confluence in 6-well plates in RPMI with 10% FBS. Upon reaching full confluence, the cells were incubated with 10µg per ml of mitomycin-C for 2 hours to inhibit cell proliferation, and a standardized scratch was made down the middle of each well using a 200µl pipette. The media was removed and cells were washed three times with PBS to remove any debris caused by the initial scratch. Scratch-induced cells were then either treated with 2µg/ml human purified PROS1 (Enzyme Research Laboratories) or left untreated. Wound closure was measured by counting the distance of cells at the ends of the wound 24 and 48 hours post-scratching. After 48 hours, cells were fixed and stained with Crystal Violet dye, and the number of migrating cells was measured by counting the amount of cells found within the middle of the wound.

4.2.12 Statistical Analysis

All gene expression studies on cell lines and xenografts consisting of normalized expressions were compared using a two-tailed t-test (GraphPad Prism Software). Gene expression studies on human prostate cancer and normal tissue were compared using a non-parametric Mann-Whitney Test (GraphPad Prism Software). Finally, chi-squared tests were used to compare different groups from the immunohistochemistry data. Differences were considered significant if P values were less than 0.05. All data are expressed as mean ± standard error of the mean.
4.3 Results

4.3.1 Proteomic profiling of prostate cancer cell line conditioned media

To identify modulators of androgen-independent prostate cancer that could serve as potential biomarkers of aggressive disease, we performed an in-depth proteomic analysis of the conditioned media of five androgen-independent prostate cancer cell lines (DU145, PC3, LNCaP-SF, PPC-1, 22Rv1), two androgen-dependent cell lines (LNCaP, VCaP), and one ‘near normal’ prostate epithelial cell line (RWPE). Briefly, cells were grown in serum-free media and the conditioned media were collected, reduced, alkylated and trypsin-digested, and peptides were subjected to two-dimensional liquid chromatography (LC), which combined SCX chromatography on an HPLC system, followed by reverse-phase (RP)-LC, prior to tandem mass spectrometry (MS/MS). After performing the analysis, we identified with a minimum of two peptides, 1974 proteins in DU145 cells, 1448 proteins in PC3 cells, 1146 proteins in LNCaP-SF cells, 1426 proteins in PPC-1 cells, 885 proteins in 22Rv1 cells, 1199 proteins in LNCaP cells, 1344 proteins in VCaP cells and 943 proteins in RWPE cells, with adequate reproducibility among the triplicates (Figure 4.1A). In total, we identified 3,110 non-redundant proteins with at least 2 peptides, in the conditioned media of the cell lines combined. Seven-hundred and twenty-three proteins (out of 3110; 23.2%) were common to all cell lines. Amongst the androgen-independent cell lines, 1180 proteins were found to be unique, whereas 335 and 39 proteins were unique to the androgen-dependent cell lines and normal RWPE cell line, respectively (Figure 4.1B). These data are summarized in Table 4.1.

To identify gene ontology classifications, which include molecular function, biological process, and cellular components, we utilized the Protein Center database. The top cellular localization annotations of the proteins identified within each of the cell lines were cytoplasmic,
membrane bound or nuclear. Over 67% of proteins were annotated as being either cell surface, extracellular or membrane-bound. The majority of proteins were functionally annotated as either being protein binding or as having catalytic activity. Finally, the top biological processes of the proteins were metabolic processes, followed by regulation of biological processes and response to stimuli.
Figure 4.1. Total non-redundant proteins identified and candidate selection for markers of AIPC. 
A, Venn diagrams displaying protein identification with a minimum of 2 peptides in each of the cell line conditioned medium in triplicate. B, Overlap of 3110 total non-redundant proteins identified in five androgen-independent cell lines (DU145, PC3, LNCaP-SF, 22Rv1, PPC-1), two androgen-dependent cell lines (LNCaP, VCaP, and a normal prostate epithelial cell line (RWPE)). C, Heat map depicting the top proteins specifically found secreted in AIPC cell lines (red), but without secretion in either the androgen-dependent or normal cell lines (black). Scale represents the average spectral counts observed in triplicate experiments (full list of candidates can be found in Table A3). D, Ingenuity Pathway Analysis revealed alterations in two main networks: 1) Cellular Movement, and 2) Cellular Function and Development. Alterations in central nodes (depicted in orange) within ERK, AKT, NFkB, TGFB and TNF signalling pathways were observed.
<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>RWPE</th>
<th>LNCaP</th>
<th>VCaP</th>
<th>22Rv1</th>
<th>PPC1</th>
<th>DU145</th>
<th>PC3</th>
<th>LNCaP-SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Non-Redundant Proteins [with ≥ 2 peptides]</td>
<td>943</td>
<td>1199</td>
<td>1344</td>
<td>885</td>
<td>1426</td>
<td>1974</td>
<td>1448</td>
<td>1146</td>
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<td>Number of Peptides Identified with…*</td>
<td></td>
<td></td>
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<td>643</td>
<td>227</td>
<td>615</td>
<td>964</td>
<td>588</td>
<td>535</td>
</tr>
</tbody>
</table>

* Based on the average triplicate spectral count value
4.3.2 Prioritization of candidate markers of androgen-independence

In order to identify candidates of androgen-independent and aggressive prostate cancers, we compared the differential protein expression based on normalized spectral counts between our androgen-independent, androgen-dependent and/or normal RWPE cell lines. Since we were interested in finding proteins that are elevated during AIPC, we decided to set as cut-offs, proteins that were found with a minimum of two peptides within our androgen-independent cell line proteomes, and with less than two peptides in either the androgen-dependent or normal prostate cell line proteomes. This filter resulted in the selection of 1180 proteins that were unique to at least one AIPC cell line. The top candidates included proteins that were expressed in multiple AIPC cell lines, with minimal or no spectral counts in the androgen-dependent or normal cells (Figure 4.1C). To further prioritize the candidate list of proteins, a final dataset of 57 proteins was derived, consisting of proteins that were secreted in at least three AIPC cell lines with a minimum of 2 spectral counts, and with less than 1 spectral count in all androgen-dependent and the normal cell line. Table A3 summarizes these candidates with respect to the observed spectral counts in each of the cell lines as well as the number of AIPC cell lines that displayed positive results. The list contains proteins that were previously studied in the context of prostate cancer progression; including MGAT5, PAM, GBA, ROBO1, CD59, MMP1, IGFBP4, CDH2, TGFβ2, ICAM1, EPHA2, and IGFBP5 [197-208], thus providing further confirmation for the robustness of our quantification method.

We next subjected our candidate list to pre-clustering pathway analysis using Ingenuity Pathway Analysis (IPA). This analysis revealed two major networks that the candidates were enriched for: 1) cellular movement and, 2) cellular function and development (Figure 4.1D). The candidates had central nodes in the NF-kB, AKT, ERK, p38 MAPK, TGFβ and TNF
signalling cascades, all of which have been previously documented to be associated with prostate cancer progression [156, 209-213].

### 4.3.3 Protein S is elevated in androgen-independent prostate cancer cell lines and is activated in the LuCaP 96AI Androgen-Independent Prostate Cancer Xenograft Model

Based on our initial discovery results, the vitamin-K dependent Protein S was our most interesting candidate, as it was found to be secreted in all five androgen-independent cell lines, with no detectable secretion in any of the androgen-dependent or normal prostate epithelial cell lines (Table 4.2). The highest secretion was found in LNCaP-SF cells, with an average spectral count of 23, followed by 20 in DU145 cells, 20 in PPC-1 cells, 8 in 22Rv1 cells and 3 in PC3 cells. To verify the discovery results, we performed real-time PCR of PROS1 as well as other top candidates (TWSG1, LTBP1, GBA, PAM) on various prostate cancer cell lines to investigate if the gene expression correlated with the protein expression data. As expected, we observed significantly elevated transcript levels of each of the candidates in androgen-independent cell lines (PC3, DU145), compared to the normal (RWPE) and androgen-dependent cells (LNCaP, VCaP) (Figure 4.2A).

To determine whether PROS1 and other top candidates may play a role during the progression of androgen-independent prostate cancer, LuCaP 96, and its androgen-independent counterpart, LuCaP 96AI, xenografts were utilized. The transcript and protein expression of PROS1, TWSG1, LTBP1, PAM and GBA were assessed using qPCR and western blot, respectively. It was shown that all the candidates displayed significant increases in their gene expression in the LuCaP 96AI xenograft-derived cells (P<0.05) (Figure 4.2B). Protein validation of these candidates using western blotting supported our data, demonstrating an up-
regulation of our identified enzymes in LuCaP 96AI cells (Figure 4.2C). Taken together, our cell line and xenograft data clearly demonstrates that PROS1 and other top candidates are elevated at both the protein and transcript level in androgen-independent prostate cancer cells, and could potentially serve as biomarkers and/or therapeutic targets for aggressive prostate cancer.
Figure 4.2. Analysis of gene and protein expression levels of PROS1, TWSG1, LTBPI, GBA and PAM on prostate cancer cell lines and LuCaP96/96AI xenografts. A, gene expression profiles of top candidates on normal (RWPE), androgen-dependent (LNCaP, VCaP, and androgen-independent (DU145, PC3) cell lines. B-C, gene and protein expression levels of top candidates are increased in the LuCaP 96AI androgen-independent xenograft model (*, P < 0.05, Mann-Whitney Test).
4.3.4 Protein S expression is elevated in high-grade prostate cancer

We then sought to investigate whether PROS1 was also overexpressed in prostate cancer patients. To examine this, we measured its transcript levels in normal and human tumor tissue samples. Using real-time PCR, we found that PROS1 transcript levels were significantly elevated (p=0.017) by over 2-fold in prostate cancer compared to normal tissue (Figure 4.4A). In addition, using immunohistochemistry, we assessed the protein expression level of PROS1 in normal (n=8) and clinically localized prostate cancers of varying grade (n=40). We devised a scoring system to assess protein expression, whereby each core was scored with a 0, 1, 2, or 3, which corresponds to no staining, low, moderate or high staining, respectively. After analysis, we observed a stair-wise expression pattern of PROS1 staining, with minimal staining in normal, moderate staining in low grade prostate cancer (Gleason ≤7), and increased staining in high grade prostate cancer (Gleason grade > 8) (Figure 4.3A). Normal cores depicted very little positive staining, with only 13% of cores displaying a score of 2 or greater, whereas low-grade prostate cancer cores had intermediate expression, staining positively in 25% of cases. Finally, high-grade prostate cancer cores displayed the greatest expression levels, with 43% of cores staining positively (Figure 4.3B). Similarly, we also assessed the expression of TWSG1 and LTBP1 on these samples. Both TWSG1 and LTBP1 displayed elevated expression in cases of high-grade prostate cancer; however, the expression pattern was not as prominent at PROS1 (Figure 4.3). These results indicate that PROS1 is over-expressed in high-grade prostate cancers, and may therefore play a role in the progression of prostate cancer to an advanced stage disease.
Figure 4.3. Protein expression of PROS1, TWSG1, and LTBP1 in human prostate cancer tissues. A, representative immunohistochemistry images of cases of normal, low grade (Gleason \( \leq 7 \)) and high grade (Gleason \( \geq 8 \)) prostate cancer specimens, under light microscopy (x20). B, immunohistochemical staining was quantified using a scoring scale of 0, 1, 2, and 3 corresponding to no staining, low, moderate and high staining, respectively, as blindly determined by a pathologist. Positive cores were determined to be ones that stained with an intensity of 2 or greater.
4.3.5 **Protein S is elevated in the seminal plasma of intermediate and high grade prostate cancer patients**

After observing increased gene and protein expression of PROS1 in high grade prostate cancer tissue specimens, we wanted to determine if elevated PROS1 expression could also be reflected with elevated seminal plasma levels. We chose to analyze seminal plasma since it represents the proximal fluid in which prostatic secretions should be enriched for. We assessed PROS1 levels using ELISA in a variety of seminal plasmas taken from control (n=8), prostatitis (n=8), low-grade (Gleason ≤ 6, n=8), and intermediate/high-grade (Gleason ≥ 7, n=13) prostate cancer patients. Based on our analysis, we observed a statistically significant (P<0.05) elevation of PROS1 in seminal plasma from intermediate and high-grade prostate cancer patients (**Figure 4.4B**). The area under the curve (AUC) of PROS1 being able to distinguish benign (negative biopsy and prostatitis) and low-grade prostate cancers from intermediate/high-grade prostate cancer patients in seminal plasma was 0.875 (CI=0.744-1.0, p<0.001) (**Figure 4.4C**). These results suggest a potential role of PROS1 as a biomarker, to assist in the differential diagnosis of high-grade from low-grade prostate cancer and/or benign conditions.
Figure 4.4. Expression of PROS1 in seminal plasma specimens of varying Gleason Grade.
A, Using the TissueScan Prostate Cancer cDNA Array II consisting of 8 normal and 36 prostate cancer specimens, the gene expression profiles of PROS1 showed significantly elevated mRNA expression in cancer compared to normal specimens (*, P < 0.05, Mann-Whitney Test). B, ELISA analysis of PROS1 protein levels in seminal plasma from negative biopsy (N=8) (positive PSA test), prostatitis (N=8), low grade prostate cancer (Gleason ≤ 6) (N=8) and intermediate and high grade prostate cancer (Gleason ≥ 7) (N=13) patients (*, P < 0.05, Mann-Whitney Test).
4.3.6 PROS1-stimulated prostate cancer cells have increased migratory potential

To explore whether PROS1 has a role in prostate cancer growth or migration, we performed in vitro scratch assays, in which LNCaP cells were grown to full confluence, treated with mitomycin-C for 2 hours, and scratched to induce wounding. Cells were then either treated with 1-2µg/ml of human purified PROS1 or left untreated to serve as controls, and the amount of wound closure as well as the number of migrating cells was assessed. We observed that 24 and 48 hours post-scratch, there was a statistically significant (p<0.05) increase in wound closure, in PROS1-treated LNCaP cells (Figure 4.5A). Specifically, over 40% of the original wound was healed in PROS1-treated cells compared to less than 20% wound closure in non-treated control cells (Figure 4.5B). In addition, to assess cell migration during PROS1-stimulation, we fixed and stained cells after 24 and 48 hours of inducing the wound and counted cells that were found within default squares within the original wound gap. After analysis, we observed a significant increase (p<0.05) in the number of migrating cells during PROS1 stimulation (Figure 4.5B). Taken together, this data is indicative that PROS1 has a direct role on prostate cancer cellular processes, including growth and migration.

4.3.7 PROS1 transcript levels increase after continuous growth in androgen-deprived conditions

During androgen-deprivation, prostate cancer cells undergo apoptosis due to the absence of key growth stimuli, in particular androgens. To assess the effect of androgen-deprivation on PROS1 expression, we grew LNCaP cells in androgen-deprived conditions for varying time points including 1, 2 and 5 days, and extracted total RNA to examine the expression levels of PROS1. Interestingly, PROS1 gene expression levels were increased in a time dependent
manner (**Figure 4.5C**). We also observed an increase in PROS1 transcript level in PC3 androgen-deprived cells (data not shown).

Previously we assessed PROS1 expression in the LuCaP 96/LuCaP 96AI xenograft model and observed elevated expression in the androgen-independent xenograft. We measured transcript levels of GAS6, a known homologue of PROS1 which has previously been linked to prostate cancer progression, on these xenografts and found its elevation in LuCaP 96AI cells (P<0.05). Taken together, these results further support the involvement of PROS1 and GAS6 in the development of AIPC.
Figure 4.5. LNCaP cells were treated with human purified PROS1, and a wound repair scratch assay was performed. A, PROS1 treated cells (1ug/ml or 2 ug/ml) had significantly increased wound repair and, B, an increase in migrating cells compared to untreated cells 24 and 48 hours post scratch (*, P < 0.05, Mann-Whitney Test). C, LNCaP cells were grown in androgen depleted conditions for a 5 day period. PROS1 gene expression levels increased the longer the cells were grown in androgen depleted conditions (*, P < 0.05, Mann-Whitney Test). A similar expression profile was observed with PC3 cells (data not shown). D, PROS1 (Figure 4.2B) and its homologue GAS6 transcript levels were increased in the LuCaP 96AI androgen-independent xenograft model (*, P < 0.05, Mann-Whitney Test).
4.3.8 PROS1 is highly expressed in Cases of Castrate-Resistant Metastatic Prostate Cancers

Thus far, we have demonstrated that PROS1 is elevated: (a) *in vitro*, in androgen-independent cell lines, (b) in localized high grade disease, both at the tissue and seminal plasma level, and (c) in an androgen-independent xenograft model. Based on these findings, we aimed to examine PROS1 expression in various castrate-resistant metastatic prostate cancer human samples, to identify whether it is dysregulated during prostate cancer metastasis as well. Using our previous scoring system in a tissue microarray, containing castrate-resistant metastatic lesions to the bone (n=72), lymph nodes (n=28), lung and liver (n=19), we demonstrated substantially increased PROS1 staining in all metastatic sites (Figure 4.6). Specifically, we observed 15.8% of lung and liver metastases containing none or low staining, and 84.2% of cores having intense staining. With respect to the lymph node metastasis cores, we found 25% with low staining, and 75% with intense staining. Within bone metastatic lesions, 29% of cores displayed low staining, while 71% had intense staining. In contrast, in normal prostate cores, PROS1 staining was very low, as the majority of the samples (87.5%) had little or no staining, and a small proportion (12.5%) having moderate PROS1 expression. Following statistical analysis, PROS1 expression was found significantly up-regulated in lung and liver (p>0.001), lymph node (p=0.002), and bone (p=0.002) prostate cancer metastatic lesions compared to normal prostate samples (Table 4.2). In conclusion, PROS1 expression is up-regulated in castrate-resistant prostate cancer metastases, and thus, could serve as a potential biomarker and therapeutic target for aggressive disease.
Figure 4.6. Expression of PROS1 in castrate-resistant metastatic prostate cancer to the bone, lymph node, liver and lungs. Representative immunohistochemistry images of PROS1 staining in, A, normal prostate, B, liver metastasis, C, bone metastasis, D, lymph node metastasis and E, lung metastasis of the prostate are shown. Images were taken under light microscopy (x20). F, immunohistochemical staining was quantified using a scoring scale of 0, 1, 2, and 3 corresponding to no staining, low, moderate and high staining, respectively, as blindly determined by a pathologist. Positive cores were determined to be ones that stained with an intensity of 2 or greater.
Table 4.2. PROS1 Expression in Castrate Resistant Metastatic Prostate Cancers

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Number of Positive Cores*</th>
<th>Staining Percentage</th>
<th>p value compared to Normal**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Prostate</td>
<td>1/8</td>
<td>12.5%</td>
<td>NA</td>
</tr>
<tr>
<td>Lung and Liver Metastasis</td>
<td>16/19</td>
<td>84.2%</td>
<td>0.0009</td>
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<tr>
<td>Lymph Node Metastasis</td>
<td>21/28</td>
<td>75.0%</td>
<td>0.0026</td>
</tr>
<tr>
<td>Bone Metastasis</td>
<td>51/72</td>
<td>70.8%</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

* positive staining means a score of 2 or higher, based on pathologists score (see text)

**p value was calculated using a chi squared test vs. normal prostate tissue

4.4 Discussion

In the present study, we aimed to delineate the proteomes of several prostate cancer and a near normal prostate cell line conditioned media to identify proteins that are elevated during androgen-independent prostate cancer. Specifically, by using LC-MS/MS, we performed proteomic analysis on five androgen-independent (DU145, PC3, 22Rv1, PPC1, LNCaP-SF), two androgen-dependent (LNCaP, VCaP) and one near normal (RWPE) prostate epithelial cell line. After performing experiments in triplicates, and using various protein identification search engines (X!Tandem, Mascot, Scaffold), we were able to identify between 885 and 1974 proteins with at least two peptides in each of the cell line CM. In total, we identified a 3110 non-redundant protein dataset, which, to our knowledge, is the most comprehensive one to date. To internally validate our approach, we observed among our top candidates, twelve (MGAT5, PAM, GBA, ROBO1, CD59, MMP1, IGFBP4, CDH2, TGFb2, ICAM1, EPHA2, and IGFBP5) which have previously been studied, or implicated in prostate cancer progression (22-33). For example,
MGAT5, has been shown to mediate enhanced invasion and metastatic potential for prostate cancer cells through many *in vitro* invasion assays and xenograft animal models.

After analyzing our candidates, we decided to further investigate the anticoagulation factor PROS1, since it was found secreted in only the androgen-independent cell lines, with no detectable secretions in androgen-dependent or normal prostate epithelial cell lines. After performing clinical validation on a variety of tissue and seminal plasma samples, we observed elevation of PROS1 specifically in high grade cases, in addition to its elevation in castrate resistant metastatic prostate cancer cases, which supported our initial goal of identifying markers of aggressive prostate cancer.

To our knowledge, this is the first study documenting a role for PROS1 with respect to prostate cancer pathogenesis. In fact, after performing multiple searches, the only other study that we could find assessing PROS1 in any form of cancer was a study evaluating various coagulation factor expressions during colorectal cancer development. In the aforementioned studies, PROS1 staining in colorectal cancer cells was performed, however, the investigators did not go into further details aside from their immunohistochemistry analysis [214]. Although PROS1 has been highly studied with respect to the coagulation cascade, recent studies have shown that PROS1 could act a ligand for a family of receptor tyrosine kinases, consisting of Tyro3, Axl, and Mer (TAM receptors) [215-218]. Interestingly, GAS6, which shares 40% amino acid identity with PROS1, is a known ligand for these TAM receptors [215]. Interestingly, we also observed GAS6 secretion in two AIPC cell lines (DU145 and LNCaP-SF), with no detectable secretions in either the androgen-dependent or normal prostate cell lines. Previously, GAS6 has been shown to have increased affinity for the Axl protein [215]. In regards to prostate cancer pathology, Sainaghi *et al*, demonstrated that Axl could be activated by GAS6 in DU145
and PC3 cells, resulting in the phosphorylation of the MEK protein, leading to increased cell survival and proliferation [219]. Shiozawa et al, went a step further, and observed that upon GAS6/Axl stimulation, prostate cancer cells had increased metastatic and invasive properties, particularly to bone [220]. Both these studies demonstrate that TAM receptors, specifically Axl, and their corresponding ligands (GAS6), are able to promote prostate cancer tumorogenesis. The question whether PROS1 is able to act in a similar way has yet to be elucidated. However, from our analysis we also observed increased cell migration upon stimulation with PROS1, as well as its elevated expression in high-grade and metastatic disease, further providing evidence that it may be providing survival advantage for prostate cancer cells. However, the mechanistic role of PROS1 and its potential downstream signalling cascade still requires further exploration.

PROS1 shares 40% amino acid identity with GAS6, and both proteins have identical structural domains including a gamma-glutamic acid domain, which is integral for vitamin-K binding, four epidermal growth factor (EGF)-like modules, and two tandem laminin G domains that are structurally related to those of the sex hormone binding globulin [215]. PROS1, but not GAS6, contains a unique thrombin-cleavage domain, which is important for its functions within the coagulation cascade [215]. Interestingly, two recent studies conducted on mouse neuronal cells showed that upon chemically induced cell injury, PROS1 was able to activate AKT and induce an anti-apoptotic cascade, resulting in reduced cell death [221, 222]. The response was specific to PROS1 binding to the TYRO3 receptor, and not Axl or Mer. These results marked a novel role for PROS1 outside of the coagulation cascade as a signalling molecule. Also, it was observed that the laminin G domains in particular, were integral for the binding of PROS1 and subsequent activation of its downstream signalling pathway [221, 222].
The novel role of PROS1 as a signalling molecule in the neuronal mouse model provides an interesting explanation as to why it may possibly become activated in high-grade and aggressive prostate cancer. During aggressive prostate cancer, androgen-deprivation is usually the gold-standard therapy. Many cells undergo apoptosis during this treatment, however, some are able to evade the therapy and continue growing in the absence of androgens. A possible hypothesis for our observed increase in PROS1 expression in aggressive prostate cancer could be due to its potential involvement in activating downstream anti-apoptotic pathways, which in turn, provide survival advantage for cancer cells and promote their progression to AIPC. Further experimentation needs to be conducted to determine more precisely the functional role of PROS1 and its potential downstream signalling in aggressive disease. In addition, PROS1 as a therapeutic target becomes of interest, as the development of potential molecules that can possibly inhibit PROS1 signalling function without altering its coagulation properties presents an interesting avenue of therapeutic intervention.

Overall, in this chapter, we demonstrate that PROS1 is a novel marker of high-grade and castrate resistant metastatic prostate cancer, which warrants further functional validation through the use of relevant in vitro and in vivo models. Our present findings provide sufficient evidence that PROS1 plays an important role in prostate cancer pathogenesis and suggest an interesting area for therapeutic intervention for a disease that lacks targeted treatments.
CHAPTER 5:

INVESTIGATING THE ASSOCIATION OF PROS1, GAS6 AND TAM RECEPTOR PROTEINS (TYRO3, AXL, MERTK) AS MARKERS OF AGGRESSIVE PROSTATE CANCER
5.1 Introduction

Protein S (PROS1) is an anti-coagulant factor involved in the coagulation cascade. Its role is to act a co-factor to activate Protein C, which inhibits coagulation processes [216, 223]. PROS1 has been implicated to have roles outside the coagulation pathways, specifically, as a putative ligand for a unique family of receptor tyrosine kinases known as TAM receptors [215-218]. The TAM receptor family consists of 3 members: Tyro3, Axl and Mer. Interestingly, GAS6, which shares 40% amino acid identity with PROS1, is the only other known ligand for these TAM receptors.

TAM signalling has been shown to have important regulatory roles in vascular smooth-muscle homeostasis [224-226], platelet function and thrombosis stabilization [227-229], in erythropoiesis [230], and cancer development and progression [231-235]. The primary signal transduction pathway associated with TAM activation appears to involve the PI3K-AKT pathway, although other pathways have also been shown to be activated after TAM receptor activation [236-238]. Aside from PROS1, which has not been widely studied outside of its role in the coagulation cascade, the other TAM signalling molecules have been linked to various pathological conditions, including cancer [239]. With respect to prostate pathology, GAS6 has been previously been linked to activating Axl, resulting in increased survival and metastatic potential of cells, especially to the bone marrow niche [219, 220].

Previously, we identified PROS1 expression to be elevated in local high-grade and castration-resistant metastatic prostate cancer tissues (Chapter 4) [137]. Based on these initial findings, and the observation that GAS6 and AXL have also been explored with respect to prostate cancer pathobiology, we hypothesize that PROS1 and the other TAM signalling molecules (GAS6, AXL, TYRO3 and MERTK) could be associated with prostate cancer
progression. There have been no comprehensive studies in the past that have looked at the association of TAM receptor and ligand expression with clinical cases of prostate cancer. Here we aimed to examine the expression of TAM signalling molecules in various cases of normal, primary and metastatic prostate cancer samples to assess whether there expression is associated with prostate cancer progression.
5.2 Materials and Methods

5.2.1 Reverse-transcription and qPCR

LuCaP96 and LuCaP96AI xenograft tissues, as previously described [76], were frozen in liquid nitrogen, and then ground into a fine powder using a mortar and pestle. Total RNA was isolated using an RNeasy Kit (Qiagen). cDNA was generated from 1µg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was conducted using 1X SYBR reagent (Applied Biosystems) and transcript levels of PROS1, GAS6, TYRO3, AXL, MERTK were measured on a 7500 ABI system. All qPCR data was normalized to tata-binding protein (TBP) expression. The following primer sequences were used:

PROS1 Forward- GGCTCCTACTATCCTGGTTCTG,
PROS1Reverse– CAAGGCAAGCATAACACCAGTG
TBP Forward– TGTATCCACAGTGAATCTTGGTTG
TBP Reverse- GGTTCGTGGCTCTTATCCTC
GAS6 Forward- CCTTCCATGAGAAGGACCTCGT
GAS6 Reverse- GAAGCACTGCATCCTCGTG
MERTK Forward- CAGGAAGATGGGACCTCGT
MERTK Reverse- GGCTGAAGTCTTTCATGCACGC
TYRO3 Forward- GCAAGCCTTTGACAGTGTCATGG
TYRO3 Reverse- GTTCATCGCTGATGCCAAGCT
AXL Forward- GTTTGGAGCTGTGATGGAAGGC
AXL Reverse- CGCTTCACTCAGGAATCCTCC
5.2.2 Immunohistochemistry

Prostate cancer tissue microarrays (TMA) which consisted of 30 normal and 60 primary cancer cores, were developed and provided by the GU Cancer Research laboratories at the University of Washington, Seattle, USA. The metastatic prostate cancer tissue microarray used was developed and provided by the GU Cancer Research laboratories at the University of Washington, Seattle, USA. Human tissue microarrays of fixed paraffin-embedded metastatic tissues from 23 rapid autopsy patients who died of prostate cancer (consisting of 3 tissue microarray blocks with 2 replicate cores per metastatic site) were used for immunohistochemical (IHC) analyses. All patients with metastatic disease had castrate resistant prostate cancer at the time of autopsy, defined by the presence of a rising serum PSA following medical or surgical castration.

TMAs were deparaffinized in xylene and rehydrated using ethanol. Endogenous peroxidase was reduced using hydrogen peroxide for 10 min and washed with PBS. Antigen retrieval was then performed using citrate buffer in a microwave for 10 minutes. Slides were then blocked for 5 min in casein and incubated overnight with the following primary antibodies: PROS1 (1:1000; Epitomics), GAS6 (1:2000; R and D), AXL (1:1200, R and D), TYRO3 (1:600, Cell Signalling Technology), and MERTK (1:1000, Epitomics). Rabbit IgG was used on a duplicate slide to serve as a negative control. Following 10 min of PBS washing, slides were placed in secondary antibody for 30 min using the BGX kit (Biogenex, Fremont, CA). After a 10 min wash in PBS, slides were developed with the addition of DAB for 5 min. Slides were then counterstained with hematoxylin, dehydrated, and coverslipped.
5.2.3 Statistical Analysis

All gene expression studies on xenografts consisting of normalized expressions were compared using a student’s two tailed t-test (GraphPad Prism Software). Chi-square tests were used to compare different groups from the immunohistochemistry data. Differences were considered significant if P value was less than 0.05. All data are expressed as mean ± standard error of the mean.
5.3 Results

5.3.1 TAM Receptors and Ligands have Increased Expression in the LuCaP96AI Androgen-Independent Prostate Cancer Xenograft Model

To evaluate whether TAM receptor and ligands play a role during the progression to androgen-independent prostate cancer, LuCaP 96, and its androgen-independent counterpart, LuCaP 96AI, xenografts were utilized. The transcript and protein levels of the 5 proteins were assessed using qPCR. It was shown that \( PROS1 \) (p<0.001), \( GAS6 \) (p<0.001), \( TYRO3 \) (p<0.001) AXL (p<0.001) and MERTK (p<0.001) all displayed statistically significant increases in expression in the LuCaP 96AI xenograft-derived cells (Figure 5.1). \( GAS6 \) and AXL had the most prominent gene expression differences, exhibiting almost 43-fold and 10-fold increases in their transcript levels in the androgen-independent xenograft, respectively. \( PROS1 \), \( TYRO3 \) and MERTK also displayed elevations of 1.9-fold, 2.3-fold and 2.7 fold increases in expression within LuCaP 96AI cells, respectively. Taken together, these results support the association of altered TAM receptor and ligand profiles, with the development of AIPC/aggressive prostate cancer.
Figure 5.1 Expression of TAM signalling genes in *in vivo* LuCaP 96 xenograft and its androgen-independent xenograft LuCaP 96AI. Gene expression profiling reveals statistically significant up-regulation of PROS1, GAS6, TYRO3, AXL and MerTK (p < 0.05, two-tailed test, n=3) in LuCaP 96AI xenografts.
5.3.2 TAM Regulators are Increased in Localized Prostate Cancer, but only the Ligands are Elevated in Castration-Resistant Metastatic Prostate Cancer

Using immunohistochemistry, we assessed the protein expression levels of PROS1, GAS6, AXL, MERTK and TYRO3 in various normal (n=30), primary prostate cancer (n=60), and castration-resistant metastatic prostate cancer (n=120) samples. We devised a scoring system to assess protein expression, whereby each core was scored with a 0, 1, 2, or 3, which corresponds to no staining, low staining, moderate staining or high staining, respectively. All of these proteins had preferentially higher staining patterns in primary and metastatic prostate cancers compared to normal cores. However, only PROS1 and GAS6 displayed elevated expression in metastatic specimens compared to primary prostate cancer. Representative staining of each of the proteins are displayed in Figure 5.2. In PROS1, normal cores had very little positive staining (13% stained with a score of 2 or greater), moderate levels of staining were observed in primary prostate cancer cores (35%), and almost all castration-resistant metastatic prostate cancer cores had intense staining (74%) (Figure 5.3). Similarly, for GAS6, there was low positive staining in normal cores (21%), moderate staining in 29% of primary prostate cancer cores, and 75% positive staining in metastatic prostate cancer cores. For TYRO3, there was positive staining in 7% of normal cores, 35% of primary prostate cancer cores, and 34% of metastatic prostate cancer cores. AXL displayed similar expression profiles, as there was positive staining in 18% of normal cores, 40% of primary prostate cancer cores, and 39% of metastatic prostate cancers. Finally, MERTK displayed positive staining in 28% of normal cores, 41% of primary prostate cancer cores and 43% of metastatic prostate cancer cores. These results indicate that all the TAM enzymes appear to be over-expressed during development of primary prostate cancers, however, only the two ligands, PROS1 and GAS6 are
further elevated in castration resistant metastatic prostate cancer. Therefore, these enzymes may play a role in the progression of prostate cancer to an advanced stage disease.

**Figure 5.2.** Protein expression of TAM signalling enzymes in human prostate cancer specimens. Representative immunohistochemistry images of PROS1, GAS6, AXL, TYRO3 and MerTK expression in normal prostate, primary prostate cancer, and castration-resistant metastatic prostate cancer specimens under light microscopy (20X).
Figure 5.3. Protein expression of TAM signalling enzymes in human prostate cancer specimens. Immunohistochemical staining was quantified using a scoring scale of 0, 1, 2, and 3 corresponding to no staining, low staining, moderate staining, and high staining, respectively, as blindly determined by a pathologist. A total of 30 normal cores, 60 primary prostate cancer, and 120 castration-resistant metastatic prostate cancer cores were examined.
5.4 Discussion

In the previous chapter, we identified PROS1 to be associated with the development of high grade prostate cancer through immunohistochemistry and gene expression analysis. Now established as a ligand for TAM receptors, PROS1-TAM receptor signalling may represent a novel mechanism for prostate cancer progression to an aggressive state. Previous studies have already linked two other members, Gas6 and Axl, as being associated with prostate cancer progression (219, 220). Specifically, these studies showed that upon activation of Axl by the Gas6 ligand, prostate cancer cells displayed more malignant phenotypes including increased growth and proliferation (219). Another study was able to go one step further and found that the Gas6-Axl signalling cascade was activated in prostate cancer cell metastasis, specifically to the bone marrow (220). In that study, Axl immunohistochemistry was conducted on primary prostate cancer specimens and it was observed that Axl expression increased in a step-wise manner as the Gleason grade of the patient increased. This indicates that Axl expression seems to have an association with prostate cancer aggressiveness. In this chapter, we aimed at assessing the expression of all five TAM receptor/ligands in clinical cases of normal, primary and castration-resistant metastatic prostate cancer specimens using immunohistochemistry.

We first assessed the expression of TAM signalling molecules on a xenograft model of prostate cancer progression and observed significant elevation of gene transcript levels of all five genes in the more aggressive LuCaP 96AI xenograft. This provided initial support that TAM signalling molecules may be associated in the development of aggressive prostate cancer. Next, after performing immunohistochemistry on various clinical specimens, we observed increased expression of all 5 TAM signalling molecules in cancerous tissue compared to normal prostate. However, when we assessed the expression in castration-resistant metastatic prostate
cancer samples, only the two ligands, PROS1 and GAS6, displayed elevated expression, whereas the three receptors displayed similar expression in these metastatic specimens as primary prostate cancer.

Although this study only provides an association of TAM signalling molecules with prostate cancer progression, we postulate that these proteins have a direct effect on prostate cancer pathobiology as previous studies have already shown that Gas6-Axl signalling have been implicated with increased prostate cancer malignant phenotypes. It is also likely the other molecules, PROS1, MerTK and TYRO3, also have a direct role on prostate cancer pathobiology. To address this, functional and mechanistic studies are required to delineate the exact role of TAM signalling in prostate cancer. Specifically, knock-down and overexpression experiments in both in vitro and in vivo models would be required. However, from our studies, we hypothesize that during prostate cancer progression, all TAM receptors and ligands have a preferential for elevated expression in localized disease, though only the ligands become further activated in aggressive metastatic disease. It is possible that dysregulated ligand expression, through an unknown mechanism, is the driving point for this signalling cascade and cancerous cells are utilizing increased expression of these ligands to activate TAM signalling to activate pathways involved in growth and survival. Based on previous studies of TAM signalling on various other cancer types, direct phenotypic roles of this signaling cascade have been shown to influence malignant phenotypes. So it would not be surprising to see similar outcomes with respect to prostate cancer. Again, all this needs to be addressed using the appropriate model systems.

Overall, in this chapter we assessed the association of TAM signalling molecules with respect to prostate cancer progression. We showed that all five of these proteins are elevated in aggressive xenografts and primary prostate cancer specimens, but only the ligands are further
elevated in castration-resistant metastatic prostate cancer samples. Subsequent functional validation through the use of relevant *in vitro* and *in vivo* models is required to assess whether this signalling cascade could serve as an important driving force for the development of prostate cancer and potentially be utilized as a therapeutic target.
CHAPTER 6:

INVESTIGATING THE FUNCTIONAL ROLE OF PROS1 DURING PROSTATE CANCER PROGRESSION
6.1 Introduction

Protein S (PROS1) is a plasma protein secreted predominately by hepatocytes, where it functions as an anti-coagulant; however other cell types including megakaryocytes, endothelial cells, Leydig cells, Sertoli cells, osteoblasts, dendritic cells, T-cells, vascular smooth muscle cells, and tumour cells also synthesize and secrete PROS1 [218]. Interestingly, in these cells, PROS1 appears to have non-coagulation cascade functions, specifically acting as a ligand for a unique family of receptor kinases made up of the three members Tyro3, Axl, and MerTK, referred to as TAM receptors [218, 240-242]. As a ligand for TAM receptors, PROS1 appears to have unique functions outside its role as a coagulation factor, including cell proliferation, survival/apoptosis, regulation of inflammatory cytokine release, atherosclerosis, vasculogenesis and cancer development [218, 243-248].

In the previous chapter, we aimed at exploring whether TAM receptor/ligand expression associated with clinical cases of prostate cancer. We found that while all five proteins were elevated in primary prostate cancer specimens only the two ligands (PROS1 and GAS6) were further elevated in cases of castration-resistant metastatic prostate cancer. This demonstrated for the first time that TAM signalling molecules are elevated in clinical cases of prostate cancer, and warrants further investigation as to whether they play a direct functional role on prostate cancer pathobiology. Previously, Gas6 and Axl have been studied in the context of prostate cancer progression [219, 220, 235, 249]. Specifically, upon activation of Axl by Gas6, it was observed that prostate cancer cells had increased proliferation and metastatic potential particularly to a bone marrow niche [220]. The role of PROS1 with respect to prostate cancer progression has yet to be explored. In this chapter, we aimed at studying whether PROS1 plays a direct role on prostate cancer pathobiology using in vitro models. We observed that PROS1-knock down cells
displayed reduced cell proliferation, migration and viability, indicating that PROS1 plays a direct functional role on prostate cancer phenotypes. These results demonstrate that PROS1 and its downstream signalling cascade could serve as a novel area for the therapeutic intervention for the treatment of patients with aggressive prostate cancer.
6.2 Materials and Methods

6.2.1 Cells and Reagents

The human prostate cancer cell lines PC3 and DU145 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)(ATCC) with 10% fetal bovine serum (Thermo Scientific) as specified by ATCC. All cells were maintained at 37°C with 5% CO₂ in a humidified incubator. All experiments were performed within the first 5 passages from the initiation of all cultures.

6.2.2 Generation of stable PROS1 knockdown cells

Four PROS1-specific shRNAs along with a scrambled shRNA were obtained from Origene. DU145 and PC3 cells were grown to 70% confluence, and were transfected with either scrambled shRNA (shScr) or PROS1-specific shRNA (shPROS1) using Turbofectin 2.0 reagent (Origene). Cells stably expressing shScr or shPROS1 were established by selection with Puromycin (Sigma-Aldrich). Confirmation of knock-down was conducted using qPCR and western blotting. The clones with the greatest reduction in PROS1 expression were used for further study.

6.2.3 RNA Extraction and Quantitative PCR

Total RNA was isolated from cells using an RNAeasy Kit (Qiagen). cDNA was generated from 1μg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was conducted using 1X SYBR reagent (Applied Biosystems) and transcript levels of PROS1, Bcl-2, Bcl-xl, Bax, Bak and TBP were measured on a 7500 ABI system. The following primer sequences were used:
PROS1 Forward– GGCTCCTACTATCCTGGTTCTG,
PROS1Reverse– CAAGGCAAGCATAACACCAGTG
TBP Forward– TGTATCCACAGTGAATCTTGGTTG
TBP Reverse– GGTTCGTGCTCTCTTATCCTC
Bcl-2 Forward– ATCGCCCTGTGGATGACTGAGT
Bcl-2 Reverse– GCCAGGAGAAATCAAACAGAGGC
Bcl-xl Forward– GCCACTTACCTGAATGACCACC
Bcl-xl Reverse– AACCAGCGGTTGAAGCGGTTCCT
Bax Forward– TCAGGATGCCTCCACCAAGAAG
Bax Reverse– TGTGTCCACGGCGGCAATCATC
Bak Forward– TTACCGCCATCAGCAGGAACAG
Bak Reverse– GGAACCTCTGAGTCATAGCGT

6.2.4 Western Blotting

Protein expression of PROS1, Bcl-2, Bcl-xl, Akt, pAkt, Bad and pBad was assessed using western blot analysis. Roughly, 30µg of total protein from cells was loaded onto an SDS-PAGE gel (4-15%, BioRad), and transferred onto PVDF membranes (BioRad). Membranes were then incubated with 5% blocking solution (2.5g skim milk powder in tris-buffer solution containing 0.1% Tween (TBST)) overnight at 4°C. Membranes were incubated with rabbit polyclonal antibody against PROS1 (1:1000, Epitomics), Bcl-2 (1:1000, Cell Signalling Technology), Bcl-xl (1:100, Cell Signalling Technology), Akt (1:1000, Cell Signalling Technology), pAkt (1:1000, Cell Signalling Technology), Bad (1:1000, Cell Signalling Technology), or pBad (1:500, Cell Signalling Technology) for 1 hour at room temperature. The membranes were then washed six times (three 15 minute washes followed by three 5 minute washes) with TBST. Membranes were then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (1:3000, Jackson Laboratories) or goat anti-mouse secondary antibody conjugated to alkaline
phosphatase (1:3000, Jackson Laboratories) for 1 hour at room temperature. After washing with TBST, proteins were detected using the ECL detection reagent (Siemens). The expression of Beta-actin (Abcam) was used as an internal standard.

6.2.5 Wound Repair/Scratch Assay

To assess wound repair and cell migration, cells were grown to full confluence in 6-well plates in DMEM with 1% FBS. Upon reaching confluence, the cells were incubated with 10µg per ml of mitomycin-C for 2 hours to inhibit cell proliferation, and a standardized scratch was made down the middle of each well using a 200µl pipette tip. The media was removed and cells were washed three times with PBS to remove debris caused by the initial scratch. Wound closure was measured by counting the distance of cells at the ends of the wound 24 and 48 hours post-scratching. After 48 hours, cells were fixed and stained with Crystal Violet dye, and the number of migrating cells was measured by counting the amount of cells found within the middle of the wound.

6.2.6 Cell Migration and Invasion Assay

Cell migration assays were performed using the Cultrex Cell Migration Assay (Cultrex). Briefly, this assay uses a Boyden chamber format as 50000 cells were seeded on the top chamber consisting of an 8 micron polyethylene terephthalate membrane. Cells were allowed to migrate to the bottom chamber, and the number of migrated cells was quantified using Calcein-AM. Cell invasion assays were performed using the Cultrex Cell Invasion Assay (Cultrex). The top chamber of this assay was coated with a basement membrane extract, and 50000 cells were seeded and allowed to migrate through the membrane to a bottom chamber. After 24 hours, the
number of cells that were able to reach the bottom chamber was quantified using Calcein-AM.

6.2.7 Cell Viability Assay

DU145 and PC3 cells were plated into triplicate 96-well plates at a concentration of 10000 cells per well (100 ul per well) in growth medium with 0.1% FBS. After 24 hours, cells were treated with either docetaxel or paclitaxel. As a control, cells were also treated with DMSO. Thereafter, the cultures were incubated in these conditions for another 24 hours and cell viability was measured and quantified using Alamar blue reagent (Sigma-Aldrich). Optical intensities were read on a multiwell scanning spectrophotometer (Roche).

6.2.8 Statistical Analysis

All gene expression studies on cell lines consisting of normalized expressions were compared using a student’s two-tailed t-test (GraphPad Prism Software). All cell-based assays including migration, invasion, and viability were compared using two-tailed t-test (GraphPad Prism Software). Differences were considered significant if P values were less than 0.05. All data are expressed as mean ± standard error of the mean.
6.3 Results

6.3.1 Generation of Stable PROS1-Knockdown DU145 and PC3 cells

Previously, we observed that PROS1 expression was elevated in androgen-independent prostate cancer cells lines compared to androgen-dependent and normal prostate cells [137]. To explore the role of PROS1 on prostate cancer pathobiology, we stably knocked down PROS1 expression by shRNA (shPROS1), or used a scrambled control shRNA (Scr) in the two androgen-independent cell lines PC3 and DU145. We chose two shPROS1 clones for each of the cell lines showing the greatest reduction in PROS1 expression levels. We observed a greater than 80% knock-down of PROS1 in two shPROS1 constructs in the DU145 cell line, and over 60% knock-down in PC3 cells using qPCR (Figure 6.1A and B).

6.3.2 PROS1 Knockdown Cells have Reduced Migratory Potential

To explore whether PROS1 has a role in prostate cancer migration, we performed in vitro scratch assays, in which DU145 or PC3 PROS1 knock-down or scrambled control cells were grown to full confluence, treated with mitomycin-C for 2hr, and scratched to induce wounding. In the DU145 cell line, we observed that after both 24 and 48 hours post-scratch, the two shPROS1 construct cells had significantly reduced wound closure (p<0.05) (Figure 6.1C). Specifically, over 60% of the wound was healed in the DU145 scrambled cells, whereas less than 35% was observed in either the shPROS1 cells. Similarly in the PC3 cells, after 24 hours, the scrambled control had over 80% wound closure compared to less than 50% wound closure in either of the shPROS1 constructs (Figure 6.1D). After 48 hours, the PC3 Scr cells completely closed the wound, whereas the PC3 shPROS1 cells still did not (data not shown).
together, these data are indicative that PROS1 has a direct role on prostate cancer cellular processes, including migration.

**Figure 6.1. Generation of stable PROS1 knock-down cells.**  A-B, Using qPCR, PROS1 gene transcript levels were reduced in PROS1 shRNA transfected DU145 and PC3 cells (*p < 0.05, two-tailed t-test). C-D, PROS1-knockdown cells displayed reduced migratory phenotypes using an in vitro scratch assay to assess wound repair in both DU145 and PC3 cells (*p < 0.05, two-tailed t-test).
6.3.3 **PROS1 Knockdown Cells have Reduced Adhesive and Invasive Potential**

Next we assessed the adhesive potential using cell adhesion assays. Briefly, cells were seeded in serum-free media for 24 hours and the number of cells that were able to adhere to the culture dish was quantified. It was observed that PROS1 knockdown cells have significantly reduced adhesion compared to scrambled control (p<0.05) (**Figure 6.2A**). Subsequently, we measured the cell migratory and invasion potential of the cells using Boyden chamber assays. Both cell migration and cell invasion assays showed that PROS1 knockdown cells have significantly reduced migratory and invasive potential (p<0.05), indicating that PROS1 plays a role in the migration and invasion properties for prostate cancer cells (**Figure 6.2B-C**).
Figure 6.2. PROS1 knock-down cells have reduced adhesive and invasive properties. A, cell adhesion assays display reduced adhesive properties in PROS1 knock-down cells. B-C, cell migration and invasion assays using Boyden chamber format display reduced cell migratory and invasive properties in PROS1 knock-down cells. All work was conducted on PC3 cells with a minimum of 6 replicates. (p < 0.05, two-tailed t-test)
6.3.4 PROS1 Knockdown Cells have Decreased Cell Viability

To assess whether PROS1 may play a cell survival role, we performed cell viability assays. Briefly, we decided to induce cell apoptosis using two commonly used prostate cancer chemotherapeutic agents, docetaxel and paclitaxel, on DU145 and PC3 cells, and assessed cell viability with Alamar blue after 24 hours treatment. We observed that in both DU145 and PC3 cells, there was significantly reduced cell viability in PROS1 knock-down cells compared to Scrambled controls (p<0.05) (Figure 6.3). Specifically, in DU145 cells there was over 20% reduced viability in the knock-down cells with either docetaxel or paclitaxel treatment. Similarly, in PC3 cells there was also over 20% reduced viability in the knock-down cells in either of the two chemotherapeutic agents. These data suggest that PROS1 plays a direct role on prostate cancer cell survival.
Figure 6.3. PROS1 Knock-down cells have reduced cell viability. A-B, after treatment with two commonly administered chemotherapeutic agents, docetaxel and paclitaxel, cell viability was measured. In both DU145 and PC3 cells, PROS1 knock-down cells displayed reduced cell viability after 24 hours, indicating that PROS1 plays a direct role on cell survival (p < 0.05, two-tailed t-test).
6.3.5 PROS1 Knockdown Cells have Reduced Expression of Anti-apoptotic Factors Bcl-2 and Bcl-xl

Previously, PROS1 has been revealed to specifically activate Tyro3 in injured neuronal cells, to induce an anti-apoptotic cascade, resulting in reduced cell death (222). Specifically, it was observed that two anti-apoptotic proteins, Bcl-2 and Bcl-xl, were elevated in expression after PROS1 stimulation. Here, we wanted to assess whether the observed reduction in cell survival in PROS1 knock-down cells may be attributable to expression of these anti-apoptotic proteins. We found that both Bcl-2 and Bcl-xl (anti-apoptotic genes) were decreased in gene and protein expression in PROS1 knock-down cells, whereas the expression of pro-apoptotic factors Bax and Bak remained unchanged (Figure 6.4A-B). Going one step further, we assessed whether PROS1 could impact the Akt signalling pathway. We observed that in PROS1 knockdown cells, there was a reduction in pAkt levels compared to scrambled controls, indicating that PROS1 activates the Akt pathway (Figure 6.4C). In addition, we observed reduced pBad levels in PROS1 knockdown cells as well, which is a downstream target in the Akt signalling pathway that controls the expression of Bcl-2 and Bcl-xl (Figure 6.4C). These findings further link the pro-survival phenotypes of PROS1 signalling with respect to activation of anti-apoptotic factor expression.
Figure 6.4. PROS1 knock-down cells display reduced expression of the anti-apoptotic factors Bcl-2 and Bcl-xl. A, gene transcript levels of two anti-apoptotic factors (Bcl-2 and Bcl-xl) and two pro-apoptotic factors (Bax and Bak) was assessed using qPCR. In PROS1 knockdown cells, there was a reduction in the expression of Bcl-2 and Bcl-xl (p < 0.05, two-tailed t-test). B, protein expression of levels of Bcl-2 and Bcl-xl was assessed using Western blot analysis, and also displayed decreased protein expression of these two proteins in PROS1 knockdown cells. C, Western blot analysis displays decreased activation of AKT (reduced pAKT) and BAD (reduced pBAD) in PROS1 knock-down cells, providing a potential mechanism of action for PROS1 within these cells.
6.4 Discussion

Previously we identified PROS1 to be associated with the development of high-grade prostate cancer (Chapter 4 and 5). The functional role, if any, of PROS1 with respect to prostate cancer pathobiology has not been previously studied, so in this chapter we aimed at exploring if PROS1 plays any role during prostate cancer progression.

We generated PROS1 knockdown cells in the high PROS1 expressing cell lines DU145 and PC3 cells. We observed that there was reduced cell proliferation, migration, adhesion, and invasion properties in the PROS1 knockdown cells. We also observed decreased cell viability of PROS1 knockdown cells after treatment with commonly used chemotherapeutic agents. Going one step further, we attributed the reduction in cell survival through decreased expression of two anti-apoptotic factors, Bcl-2 and Bcl-xl, in the PROS1 knock-down cells, and both Akt and Bad activation was also decreased, providing a potential mechanism of action for PROS1 within the cells. Collectively, all these data suggest that PROS1 plays a direct role on prostate cancer pathobiology.

This was the first study assessing the functional role of PROS1 with respect to not only prostate cancer, but any cancer for that matter. Limited studies have been conducted looking at the role of PROS1 outside of the coagulation cascade. Recently, it was found that PROS1 had a direct role on survival properties. In the study, injured mouse neuronal cells were treated with PROS1, and it was observed that there was reduced apoptotic rates and increased cell survival [221, 222]. It was found that upon activation of TYRO3, and downstream signalling cascades involving the PI3K/Akt signalling pathway, that two anti apoptotic proteins, Bcl-2 and Bcl-xl, became elevated to induce a pro-survival state [221, 222]. In our current study, we also observed that PROS1 activated the Akt pathway. Specifically, we observed that in PROS1 knockdown
cells, there was a reduction in pAkt expression. In addition, there was also a reduction in pBad levels, a protein that is a downstream target of Akt and when un-phosphorylated, is bound to both Bcl-2 and Bcl-xl. To see a reduction in pBad levels in PROS1 knockdown cells would indicate that less Bcl-2 and Bcl-xl protein would be available to perform their anti-apoptotic function, which in turn would give the cells less survival advantage.

As a continuation of this study, further work needs to be conducted on the exact mechanism of action of PROS1 signalling. In the current study, we have shown that the Akt-Bad signalling pathways become activated when PROS1 is present; however, we did not look into the upstream receptor that activates these signalling cascades. Based on previous studies we hypothesize that TAM receptors, specifically Tyro3, becomes activated upon PROS1 stimulation, resulting in a downstream signalling cascade involving Akt and Bad to induce the expression of anti-apoptotic factors [221, 222]. Subsequent studies need to be conducted to confirm whether this is the case or not. In addition, our current study assessed the function of PROS1 strictly within in vitro cell lines, so future in vivo studies need to also be conducted. Specifically, xenograft models can be utilized in addition to conditional PROS1 knock-down mice. Complete PROS1 knock-out mice are embryonic lethal, so the generation of tissue-specific PROS1 knockdown specifically within prostate cells would be ideal [245].

Based on our data, we propose a novel mechanism for which prostate cancer cells can gain androgen-independent properties. During the development of aggressive prostate cancer, androgen deprivation is commonly administered. Although initially very efficient at reducing cancerous growth and proliferation, some cells are able to gain resistance and grow in sub-optimal conditions. We propose that some prostate cancer cells elevate their expression of PROS1, resulting in increased local secretion of the protein. Thereafter, in both a paracrine and
autocrine fashion, PROS1 is able to bind to specific receptors, which we suspect to be Tyro3 based on literature, and activate a downstream signalling cascade that involves the PI3K-Akt pathway. Upon activation of Akt, the downstream effector Bad becomes phosphorylated, allowing it to dissociate from Bcl-2 and Bcl-xl, enabling these anti-apoptotic factors to block apoptosis (Figure 6.5). Overall, PROS1 and its downstream signalling cascade provides a survival advantage for cancer cells and promotes progression to AIPC. Again, further experimentation needs to be conducted to determine more precisely the functional role of PROS1 and its potential downstream signalling in aggressive disease. In addition, PROS1 as a therapeutic target becomes of interest, as the development of potential molecules that can possibly inhibit PROS1 signalling function without altering its coagulation properties presents an interesting avenue of therapeutic intervention.

Overall, in this chapter, we demonstrate that PROS1 plays a direct functional role on prostate cancer pathobiology including roles in cell proliferation, migration and viability. Our present findings provide enough evidence that PROS1 plays an important role in prostate cancer pathogenesis and suggest an interesting area for therapeutic intervention for a disease that lacks targeted treatments.
Figure 6. Proposed model of PROS1 action within prostate cancer cells. We propose that some prostate cancer cells elevate their expression of PROS1, resulting in increased local secretion of the protein. Thereafter, in both a paracrine and autocrine fashion, PROS1 is able to bind to specific receptors, which we suspect to be Tyro3 based on literature, and activate a downstream signalling cascade that involves the PI3K-Akt pathway. Upon activation of Akt, the downstream effector Bad becomes phosphorylated, allowing it to un-bind with Bcl-2 and Bcl-xl, which in turn allows these anti-apoptotic factors to block apoptosis from occurring. Overall, PROS1 and its downstream signalling cascade provides a survival advantage for cancer cells and promotes their progression to AIPC.
CHAPTER 7:

DISCUSSION AND FUTURE DIRECTIONS
7.1 Summary and Conclusions

7.1.1 Brief Overview

This thesis presents a comprehensive analysis on the identification of mediators of androgen-independent/castration-resistant prostate cancer. Using two different in vitro approaches coupled to high through-put mass spectrometry, we were able to identify numerous potential mediators of AIPC and aggressive prostate cancer. Our first approach using an in vitro cell line model of androgen-independence allowed us to identify enzymes of the ketogenic pathway to be elevated in cases of aggressive prostate cancer (Chapter 2). Specifically, ACAT1, an enzyme in this pathway, was further validated and shown to be substantially elevated in clinical cases of castration-resistant metastatic prostate cancer. In addition, ACAT1 was found to be an independent tissue-based prognostic marker of biochemical recurrence-free survival (Chapter 3). Our second approach utilized global ‘secretomics’ analysis of various prostate cancer cells lines identified PROS1 to be elevated in high-grade and castration-resistant metastatic prostate cancer specimens (Chapter 4 and 5). Further functional validation revealed that PROS1 affects cell proliferation, migration and viability (Chapter 6).

7.1.2 Key Findings of Approach 1

Using the parental LNCaP cells and its androgen-independent LNCaP-SF counterpart, we performed global quantitative proteomics analysis to identify differentially regulated proteins between the two cell lines (Chapter 2). Many differentially regulated proteins were identified which included members of the ketogenic pathway. Among these ketogenic enzymes, we performed extensive immunohistochemistry-based validation of ACAT1 on various clinical
cases of prostate cancer (Chapter 3) to assess whether it could provide prognostic potential. The major points from the first approach are summarized below:

- To identify mediators involved during the progression of androgen-independent prostate cancer, we used an in-vitro cell line model of androgen-independent prostate cancer growth, and conducted an in-depth quantitative proteomic analysis of identifying mediators of AIPC.
- Using strict selection criteria, we identified 42 and 46 proteins that were up and down-regulated in LNCaP-SF cells compared to parental LNCaP cells, respectively.
- The top over-expressed candidate, HMGCS2, was an enzyme within the ketogenic pathway. We found the other enzymes of the ketogenic pathway (ACAT1, HMGCL, OXCT1, BDH1), to be preferentially elevated in LNCaP-SF cells as well.
- Subsequent validation studies on a variety of clinical samples showed that protein expression of the ketogenic enzymes is elevated during high-grade prostate cancer (Gleason ≥ 8), as observed by immunohistochemistry analysis. ACAT1 in particular, displayed the most prominent expression patterns, thus, we examined its expression in a sub-set of castrate-resistant metastatic prostate cancer tissues, and observed its elevated expression in these specimens.
- We assessed the diagnostic and prognostic potential of ACAT1 by analyzing its expression using immunohistochemistry on a tissue microarray consisting of 251 clinically localized prostate cancer samples from patients who have undergone radical prostatectomy. Using quantitative digital imaging software, we found that ACAT1 expression was significantly elevated in high-grade prostate cancer (GS≥8 and pT3/4 tumors).
• In addition, ACAT1 predicted biochemical recurrence in univariate and multivariate models including pre-operative PSA level, Gleason score and pathological stage. In univariate time-to-recurrence analysis, ACAT1 expression predicted recurrence in ERG negative cases, whereas ERG positive cases did not display differences.

7.1.2 Key Findings of Approach 2

Using global ‘secretomics’ analysis using mass spectrometry-based proteomics on various prostate cancer cell lines (1 normal, 2 androgen-dependent, 5 androgen-independent), we identified various proteins that were differentially expressed between AIPC cells and androgen-dependent and normal cells (Chapter 4). Among the many dysregulated proteins, we validated PROS1 as a marker of aggressive prostate cancer (Chapter 4 and 5). In addition, we performed functional mechanistic studies on the role of PROS1 on prostate cancer pathobiology (Chapter 6). The major points from the second approach are summarized below:

- We performed proteomic analysis of the conditioned media (secretome analysis) of five androgen independent cell lines (PC3, DU145, PPC1, LNCaP-SF, 22Rv1), two androgen-dependent (LNCaP, VCaP) and one normal prostate epithelial (RWPE) cell line.

- In total, we identified over 3000 proteins, with over 100 proteins being differentially secreted between the AIPC and non-AIPC cell lines. Among these, 57 proteins were found to be uniquely elevated in androgen-independent cell lines, and hence, could serve as potential mediators/markers of high grade prostate cancer.

- Of these, Protein S (PROS1) was elevated in all five AIPC cell lines, with no observed secretion in the normal and androgen-dependent prostate cancer cell lines. Subsequently, we observed PROS1 overexpression in localized high-grade prostate cancer tissue and
seminal plasma. In addition, PROS1 expression was highly elevated in castrate-resistant metastatic prostate cancer specimens

- Assessed the expression of TAM signalling molecules (PROS1, GAS6, TYRO3, AXL, MERTK) on a xenograft model of prostate cancer progression and observed significant elevation of gene transcript levels of all five genes in the more aggressive LuCaP 96AI xenograft. Immunohistochemistry analysis on various clinical specimens, showed increased expression of all 5 TAM signalling molecules in cancerous tissue compared to normal prostate. However, only the two ligands, PROS1 and GAS6, displayed elevated expression in castration-resistant metastatic prostate cancer samples.

- Functional assays revealed that PROS1 knock-down cells have reduced cell proliferation, migration, adhesion, invasion and viability properties.

- The reduction in cell survival was attributed through decreased expression of two anti-apoptotic factors, Bcl-2 and Bcl-xl, in the PROS1 knock-down cells, and both Akt and Bad activation was also decreased, providing a potential mechanism of action for PROS1 within the cells.

### 7.2 Future Perspectives

This body of work has demonstrated the identification of many potential novel mediators of AIPC and aggressive prostate cancer. Using two independent approaches, we identified unique markers that could play an important role in prostate cancer pathobiology. From each approach we ended up further focusing two specific proteins, ACAT1 from approach 1, and PROS1 from approach 2, to see if they are involved in prostate cancer pathogenesis.
The role of ACAT1 with respect to prostate cancer pathophysiology has yet to be elucidated. Recent studies have found that ACAT1 is involved in androgen-mediated cholesterol metabolism in prostate cancer cell lines [169]. Another study showed that ACAT1 was elevated in androgen-independent xenografts, which coincides well with our data and hypothesis [170]. Both of these studies focused on the role of ACAT1 with respect to cholesterol biogenesis, as this enzyme is involved within this pathway. So in addition to its role in the ketogenic pathway which produces high energy ketone bodies, ACAT1 also has been shown to be involved in the generation of cholesterol precursor molecules. The generation of excess cholesterol within prostate cancer cells becomes of importance, as subsequent enzymes within the cells can convert these cholesterol precursor molecules into androgens, which in turn can activate the androgen receptor signalling cascades in an intratumoral de novo androgen biosynthesis mechanism. Essentially, prostate cancer cells may be utilizing an alternate pathway to produce endogenous androgens to activate the AR signalling cascade, during times of androgen deprivation.

The ketogenic pathway is an energy producing pathway that produces high energy ketone bodies such as beta-hydroxybutyrate [191]. Studies looking at the importance of the ketogenic pathway with respect to tumor growth and progression in a human breast cancer cell line model found that the enzymes within this pathway acted as metabolic oncogenes, as cells overexpressing these enzymes have increased tumor growth and survival [192, 193]. Our data is the first to show that members of the ketogenic pathway, specifically ACAT1, are preferentially elevated in clinical cases of aggressive high grade prostate cancer. We also are the first to show that ACAT1 expression can be used as an independent prognostic tissue based marker of biochemical-recurrence. In order to better understand the mechanism of action of ACAT1 in prostate cancer progression, subsequent mechanistic studies using RNA interference technology
in prostate cancer cell lines and animal models, need to be conducted. Such functional studies can provide important information into whether ACAT1 has a direct mechanistic role on prostate cancer progression, which further can be utilized as a potential area of therapeutic intervention. ACAT1 inhibitors have been previously investigated for various other diseases including atherosclerosis [171, 172], and may present and interesting avenue of therapeutic intervention for aggressive prostate cancers if this protein is indeed implicated in prostate cancer pathobiology.

In addition, assessing the expression of ACAT1 on other diverse clinical samples, such as hormone-naïve and hormone-refractory specimens, will provide better overall interpretation of the importance of this protein with respect to the development and progression of aggressive prostate cancer.

Our second approach allowed us to identify PROS1 as a potential marker of AIPC and aggressive prostate cancer. This was the first study that assessed the role for PROS1 with respect to prostate cancer pathogenesis. PROS1 has been highly studied in the context of the coagulation cascade, specifically as a negative regulator of this process, however recent studies have shown that it can act as a ligand for a unique family of receptor tyrosine kinases, consisting of Tyro3, Axl, and Mer [215-218]. Interestingly, GAS6, which shares 40% amino acid identity with PROS1, is the only other known ligand for these TAM receptors [215]. Previous studies have shown that that Axl could be activated by GAS6 in DU145 and PC3 cells, resulting in the phosphorylation of the MEK protein, leading to increased cell survival and proliferation [219]. Another study went a step further, and observed that upon GAS6/Axl stimulation, prostate cancer cells had increased metastatic and invasive properties, particularly to bone [220]. Both these studies demonstrate that TAM receptors, specifically Axl, and their corresponding ligands (GAS6), are able to promote prostate cancer tumorogenesis. We wanted to see if PROS1 also
acts in a similar manner, so we performed various mechanistic studies to study the potential role of PROS1 with respect to prostate cancer pathobiology. We observed that PROS1 and its downstream signalling cascade have direct roles in cell proliferation, survival, migration, adhesion, and invasion properties of prostate cancer cells. This was the first study assessing the functional role of PROS1 with respect to any cancer. Subsequently, we also observed that PROS1 activated the PI3K-Akt pathway, which in turn activated the protein Bad. When Bad is activated, it unbinds to the anti-apoptotic factors Bcl-2 and Bcl-xl, and allows them to perform their downstream actions in pro-survival pathways. We found that PROS1 signalling was also able to elevate the expression of these two anti-apoptotic factors, which give cells a survival advantage. The results from our study coincide very nicely with a recent study looked at injured mouse neuronal cells that were treated with PROS1. In this study, it was observed that there was a reduction in apoptotic rates and increased cell survival upon activation of these injured neuronal cells with PROS1. [221, 222]. It was found that upon activation of TYRO3, and downstream signalling cascades involving the PI3K/Akt signalling pathway, that two anti-apoptotic proteins, Bcl-2 and Bcl-xl, became elevated to induce a pro-survival state [221, 222]. Our results also demonstrated the same mechanism to be activated in prostate cancer cells however; we still did not look into the receptor that becomes activated by PROS1. Based on the previous studies we hypothesize that TAM receptors, specifically Tyro3, becomes activated upon PROS1 stimulation, which results in the downstream signalling cascade that activates both Akt and Bad, allowing the anti-apoptotic factors Bcl-2 and Bcl-xl to be expressed and active. Subsequent studies need to be conducted to confirm whether this is the case or not. In addition, our study assessed the function of PROS1 only in an in vitro setting, so future in vivo studies need to also be conducted. Finally, PROS1 as a therapeutic target becomes of interest, as the
development of potential molecules that can possibly inhibit PROS1 signalling function without altering its coagulation properties presents an interesting avenue of therapeutic intervention.

Overall, in this thesis, we have identified many potential novel mediators of AIPC and aggressive prostate cancer. We specifically focused on two of these candidates through extensive clinical and functional validation; however, most of the remaining candidates still need to be assessed for their roles in prostate cancer pathobiology. Future studies on all these potential candidates need to be conducted as they may provide an interesting area for therapeutic intervention for a disease that, to date lacks targeted treatments.
CHAPTER 8:

REFERENCES


86. Schweizer, L., et al., The androgen receptor can signal through Wnt/beta-Catenin in prostate cancer cells as an adaptation mechanism to castration levels of androgens. BMC Cell Biol, 2008. 9 : p. 4.


APPENDIX
Table A1. List of 42 proteins up-regulated in LNCaP-SF Cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Average H/L Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCS2</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A synthase</td>
<td>9.2394</td>
</tr>
<tr>
<td>ALDH1A3</td>
<td>aldehyde dehydrogenase 1 family, member A3</td>
<td>8.1484</td>
</tr>
<tr>
<td>C6orf115</td>
<td>chromosome 6 open reading frame 115</td>
<td>6.6899</td>
</tr>
<tr>
<td>HSD11B2</td>
<td>hydroxysteroid (11-beta) dehydrogenase 2</td>
<td>5.4227</td>
</tr>
<tr>
<td>AGR2</td>
<td>anterior gradient homolog 2 (Xenopus laevis)</td>
<td>5.0377</td>
</tr>
<tr>
<td>S100P</td>
<td>S100 calcium binding protein P</td>
<td>4.92</td>
</tr>
<tr>
<td>DHRS2</td>
<td>dehydrogenase/reductase (SDR family) member 2</td>
<td>4.5974</td>
</tr>
<tr>
<td>MAOB</td>
<td>monoamine oxidase B</td>
<td>4.3792</td>
</tr>
<tr>
<td>TYMP</td>
<td>thymidine phosphorylase</td>
<td>3.8776</td>
</tr>
<tr>
<td>MAOA</td>
<td>monoamine oxidase A</td>
<td>3.8563</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>sequestosome 1</td>
<td>3.8075</td>
</tr>
<tr>
<td>TPM1</td>
<td>tropomyosin 1 (alpha)</td>
<td>3.5117</td>
</tr>
<tr>
<td>CTSD</td>
<td>cathepsin D</td>
<td>3.3074</td>
</tr>
<tr>
<td>GDF15</td>
<td>growth differentiation factor 15</td>
<td>3.2564</td>
</tr>
<tr>
<td>ARG2</td>
<td>arginase, type II</td>
<td>3.2251</td>
</tr>
<tr>
<td>GLS</td>
<td>glutaminase</td>
<td>3.2099</td>
</tr>
<tr>
<td>AADACL1</td>
<td>arylacetamide deacetylase-like 1</td>
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<tr>
<td>ALDH4A1</td>
<td>aldehyde dehydrogenase 4 family, member A1</td>
<td>3.0362</td>
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<tr>
<td>SLC12A7</td>
<td>solute carrier family 12 (potassium/chloride transporters), member 7</td>
<td>3.0201</td>
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<tr>
<td>SPATS2L</td>
<td>spermatogenesis associated, serine-rich 2-like</td>
<td>2.9743</td>
</tr>
<tr>
<td>ASAH1</td>
<td>N-acylsphingosine amidohydrolase (acid ceramidase) 1</td>
<td>2.9654</td>
</tr>
<tr>
<td>CAPN2</td>
<td>calpain 2, (m/II) large subunit</td>
<td>2.9387</td>
</tr>
<tr>
<td>ACOT9</td>
<td>acyl-CoA thioesterase 9</td>
<td>2.9016</td>
</tr>
<tr>
<td>KRT18</td>
<td>keratin 18</td>
<td>2.8839</td>
</tr>
<tr>
<td>EPHX1</td>
<td>epoxide hydrolase 1, microsomal (xenobiotic)</td>
<td>2.8828</td>
</tr>
<tr>
<td>GSTK1</td>
<td>glutathione S-transferase kappa</td>
<td>2.8356</td>
</tr>
<tr>
<td>AKR1C2</td>
<td>aldo-keto reductase family 1, member C2</td>
<td>2.8239</td>
</tr>
<tr>
<td>ACSL3</td>
<td>acyl-CoA synthetase long-chain family member 3</td>
<td>2.8157</td>
</tr>
<tr>
<td>MANBA</td>
<td>mannosidase, beta A, lysosomal</td>
<td>2.813</td>
</tr>
<tr>
<td>VAMP8</td>
<td>vesicle-associated membrane protein 8 (endobrevin)</td>
<td>2.7515</td>
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<td>RETSAT</td>
<td>retinol saturase (all-trans-retinol 13,14-reductase)</td>
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<td>OCLN</td>
<td>occludin</td>
<td>2.7082</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Expression</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>SQRDL</td>
<td>sulfide dehydrogenase like</td>
<td>2.7062</td>
</tr>
<tr>
<td>ACADS</td>
<td>acyl-CoA dehydrogenase, C-2 to C-3 short chain</td>
<td>2.6711</td>
</tr>
<tr>
<td>MICAL1</td>
<td>microtubule associated monoxygenase, calponin and LIM domain containing 1</td>
<td>2.6382</td>
</tr>
<tr>
<td>HIBCH</td>
<td>3-hydroxyisobutyryl-CoA hydrolase</td>
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<tr>
<td>ABHD11</td>
<td>abhydrolase domain containing 11</td>
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<tr>
<td>ACSL4</td>
<td>acyl-CoA synthetase long-chain family member 4</td>
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<tr>
<td>SOD2</td>
<td>superoxide dismutase 2, mitochondrial</td>
<td>2.536</td>
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<tr>
<td>FUCA1</td>
<td>fucosidase, alpha-L- 1, tissue</td>
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<tr>
<td>SFXN3</td>
<td>sideroflexin 3</td>
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<tr>
<td>CPT2</td>
<td>carnitine palmitoyltransferase 2</td>
<td>2.5117</td>
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Table A2: List of 46 proteins down-regulated in LNCaP-SF Cells

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<tr>
<th>Gene</th>
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<th>Average H/L Ratio</th>
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<tr>
<td>SEMG1</td>
<td>semenogelin I</td>
<td>0.076735</td>
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<tr>
<td>CDC2</td>
<td>Cyclin-dependent kinase 1</td>
<td>0.081925</td>
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<tr>
<td>TK1</td>
<td>thymidine kinase 1, soluble</td>
<td>0.083392</td>
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<td>SEMG2</td>
<td>semenogelin II</td>
<td>0.10883</td>
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<td>BANF1</td>
<td>barrier to autointegration factor 1</td>
<td>0.17609</td>
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<tr>
<td>cDNA FLJ16186</td>
<td>cDNA FLJ16186</td>
<td>0.19531</td>
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<td>PLK1</td>
<td>polo-like kinase 1</td>
<td>0.20362</td>
</tr>
<tr>
<td>CDK2</td>
<td>cyclin-dependent kinase 2</td>
<td>0.22407</td>
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<td>IGFBP2</td>
<td>insulin-like growth factor binding protein 2, 36kDa</td>
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<td>STMN1</td>
<td>stathmin</td>
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<td>RRM2</td>
<td>ribonucleotide reductase M2</td>
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<td>S100A13</td>
<td>S100 calcium binding protein A13</td>
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<td>GINS3</td>
<td>GINS complex subunit 3 (Psf3 homolog)</td>
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<td>NCAM2</td>
<td>neural cell adhesion molecule 2</td>
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<td>C13orf33</td>
<td>chromosome 13 open reading frame 33</td>
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<td>KPNA2</td>
<td>karyopherin alpha 2 (RAG cohort 1, importin alpha 1)</td>
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<td>NASP</td>
<td>Nuclear autoantigenic sperm protein (histone-binding)</td>
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<td>acireductone dioxygenase 1</td>
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<td>PDZ binding kinase</td>
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