Metformin: A Potential Chemosensitizing Agent for Improving Castration-Resistant Prostate Cancer Treatment

by

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Abstract
Symptomatic metastatic castration-resistant prostate cancer (CRPC) is treated with docetaxel chemotherapy which provides a survival benefit but is associated with significant toxicity. A novel therapeutic approach is combining docetaxel with an agent that enhances its efficacy (i.e. a chemosensitizer). Metformin, an oral biguanide used to treat Type 2 diabetes, has been shown to have anti-neoplastic activity, and more specifically, enhance the effect of chemotherapy in multiple types of solid tumors. We hypothesized that metformin would act as a chemosensitizing agent when combined with docetaxel chemotherapy in CRPC. A retrospective cohort study of CRPC patients in Ontario indicated metformin did not have a chemosensitizing effect. However, in vitro experiments completed using CRPC cells indicated metformin may act as a chemosensitizing agent when combined with docetaxel in PC3 cells but not in DU145 cells. Therefore, metformin may be a novel chemosensitizing agent for CRPC and further investigation of cellular mechanisms is warranted.
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Contributions

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2DG: 2-Deoxy-D-Glucose
ADP: Adenosine diphosphate
ADT: Androgen deprivation therapy
Akt: Protein kinase B
AMP: Adenosine monophosphate
AMPK: Adenosine monophosphate-activated protein kinase
AR: Androgen receptor
ATP: Adenosine triphosphate
BAX: BCL2-associated X protein
BCL2: B-cell lymphoma 2
BMI: Body mass index
BPH: Benign prostatic hyperplasia
CARNs: Castration-resistant Nkx3.1-expressing cells
CRPC: Castration-resistant prostate cancer
CYP17: Cytochrome P450 17alpha hydrolase/17,20-lyase
DHT: Dihydrotestosterone
DRE: Digital rectal exam
ETS: Erythroblast transformation-specific
GSTP1: Glutathione-S-Transferase P1
HGPIN: High grade prostatic intraepithelial neoplasia
HMG-CoAR: 3-hydroxy-3-methylglutaryl-CoA reductase
IGF-1: insulin-like growth-factor-1
IGF-1R: Insulin-like growth factor 1 receptor
IL-4: Interleukin 4
IL-6: Interleukin 6
KGF: keratinocyte growth factor KGF
LKB1: Liver kinase B1
mCRPC: Metastatic castration-resistant prostate cancer
mTOR: Mammalian target of rapamycin
OCT1: Organic cation transporter 1
P-4EBP1: Phosphorylated 4E-binding protein 1
p53: Protein 53
PAP: Prostatic acid phosphatase
PCa: Prostate cancer
PI3K: PI3 kinase
PSA: Prostate-specific antigen
PTEN: Phosphatase and tensin homolog deleted on chromosome 10
REDD1: Regulated in development and DNA damage responses 1
ROS: Reactive oxygen species
TMPRSS2: Transmembrane protease serine 2
TRAMP mice: Transgenic adenocarcinoma model of the mouse prostate
TRUS: Transrectal ultrasound
TSC1: Tuberous sclerosis protein 1
TSC2: Tuberous sclerosis protein 2
VEGF-A: Vascular endothelial growth factor-A
VEGF-B: Vascular endothelial growth factor-B
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Chapter 1
Literature Review

1.1 Prostate Cancer Epidemiology

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men and fifth leading cause of cancer death in the world, with 1.4 million incident cases of PCa diagnosed and 293,000 PCa-related deaths occurring globally (Torre et al 2015 Global Cancer Stats 2012; Torre et al 2015 Global cancer incidence; Global Burden of Disease Cancer Collaboration 2015). Incidence rates vary by more than 25-fold worldwide, with the highest rates of PCa occurring in Australia/New Zealand, North America, Northern and Western Europe, and some Caribbean nations, whereas the lowest rates are found in South, East, and Central Asia (Torre et al 2015 Global Cancer Stats 2012; Global Burden of Disease Cancer Collaboration 2015; Ferlay et al 2015).

The variation in international incidence rates of PCa reflects differences in the use of prostate-specific antigen (PSA) testing for diagnosing latent PCa (Torre et al 2015 Global Cancer Stats 2012; Torre et al 2015 Global cancer incidence; Center et al 2012). In countries where PSA testing was quickly adopted in the late 1980s and early 1990s, such as Australia, Canada, and the United States, a distinct pattern emerged (Torre et al 2015 Global Cancer Statistics 2012). There was a rapid increase in incidence rates as more new case of PCa were detected, but the incidence rates then quickly declined as the pool of detectable cases decreased significantly (Torre et al 2015 Global cancer incidence; Center et al 2012; Torre et al 2015 Global Cancer Statistics 2012; Baade et al 2009). In countries that adopted PSA testing more gradually (Western Europe), or PSA testing was adopted at a much later time and is still uncommon (Japan, United Kingdom), the incidence rates of PCa are increasing (Torre et al 2015 Global Cancer Statistics 2012; Center et al 2012).

Mortality rates for PCa vary less worldwide (10-fold) compared to incidence, and the mortality rates are generally high in populations of African descent such as the Caribbean and Sub-Saharan
Africa, intermediate in the Americas and Oceania, and very low in Asia (Torre et al 2015 Global Cancer Stats 2012; Ferlay et al 2015 GLOBOCAN 2012) The mortality rate trends are a greater reflection of different prostate cancer treatment approaches throughout the world, as well as underlying risk factors, and are less affected by diagnostic practices (Center et al 2012). The three most well-established risk factors for PCa include age, family history, and ethnic origin (Heidenreich et al 2014; Leitzmann and Rohrmann 2012; Damber and Aus 2008; Attard et al 2016). However, there are a range of other potential risk factors including genetics, obesity, Type II diabetes, inflammation, infection, and lifestyle factors such as smoking and diet (Leitzmann and Rohrmann 2012). The pathophysiology of prostate cancer will be discussed in more detail in another section of the literature review.

1.2 Prostate Structure and Development
The prostate is a walnut-sized exocrine gland comprised of 2 lobes that surround the urethra at the base of the bladder and produces important components of the seminal fluid, providing nutrients and fluid to sperm (Shen and Abate-Shen 2010). The prostate gland has three defined morphological zones reflecting three distinct sets of ducts present in the human prostate: the peripheral zone, the transition zone, and the central zone (Abate-Shen and Shen 2000; McNeal 1969, 1988; Marker et al 2003). The peripheral zone is the most posterior, occupies the greatest volume, and is where 80-85% of prostate cancer cases arise (Shen and Abate-Shen 2010; American Joint Committee on Cancer 2010). The transition zone is located anterior to the peripheral zone and enlarges with age, potentially leading to benign prostatic hyperplasia (BPH) later in life (James 2014). Approximately 10-15% of cancer originate in the transition zone (American Joint Committee on Cancer 2010). The central zone is the most anterior since it is located in front of the transition zone and only 5-10% of prostate cancers arise here (American Joint Committee on Cancer 2010).The entire prostate is enclosed by the prostatic capsule which is composed of collagen, elastin, and smooth muscle (Campbell-Walsh Urology 2012).

The mature ducts of the prostate are comprised of three distinct populations of prostate epithelial cells. Luminal epithelial cells are responsible for maintaining barrier integrity and the
production of prostatic secretion (Campbell-Walsh Urology 2012). Luminal cells are tall, polarized, columnar secretory cells that line the prostate lumen and are the predominant cell type of the prostate epithelium (Schrecengost and Knudsen 2013; Abate-Shen and Shen 2000). Luminal cells express high levels of androgen receptor (AR), CK8, CK18, CK19, CD57, and produce proteins such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) that are secreted into the luminal space of the prostate (Abate-Shen and Shen 2000; Shen and Abate-Shen 2010; Schrecengost and Knudsen 2013; Mayer et al 2015). Basal epithelial cells are a small population found along the basement membrane of each prostatic duct and express low or undetectable levels of AR as well as CK5, CK14, CD44, CD133, p63, GSTP1 and Bcl-2 (Abate-Shen and Shen 2000; Shen and Abate-Shen 2010; Schrecengost and Knudsen 2013; Mayer et al 2015). A rare population of neuroendocrine cells is dispersed throughout the basal layer which is AR-negative, expresses chromogranin A, and secretes both neuroendocrine peptides and luminal cell growth factors (Abate-Shen and Shen 2000; Shen and Abate-Shen 2010; Schrecengost and Knudsen 2013; Mayer et al 2015).

Another unique population of cells within the prostatic epithelium are prostate stem cells. The primary survival of basal cells following androgen deprivation led to the hypothesis that prostate stem cells reside within the basal layer (Chen et al 2013; Lawson et al 2007; Mayer et al 2015). Collins et al (2001) identified a small population of basal prostate epithelial stem cells which expressed high levels of $\alpha_2\beta_1$. This was further corroborated by the identification of a small (1%) population of human prostate basal stem cells which expressed CD133 and were restricted to the $\alpha_2\beta_1$ population (Richardson et al 2004). Leong et al (2008) demonstrated that a single stem cell with the Lin-/Sca-1+/CD133+/CD44+/CD117+ phenotype was able to regenerate a prostate after transplantation in vivo. Lineage tracing has shown that multipotent basal stem cells give rise to basal, luminal, and neuroendocrine cells, as well as unipotent luminal progenitors, which cause the epithelial expansion observed during postnatal prostate development (Ousset et al 2012). Wang et al (2014) also demonstrated that basal cells and luminal cells undergo different types of division. Basal cells can divide symmetrically to produce two daughter basal cells or asymmetrically to produce one basal cell and one luminal cell which contributes to both early and late prostate development, as well as adult prostate regeneration (Wang et al 2014). Luminal
cells, however, can only undergo symmetric divisions to produce two luminal cells and cannot produce any basal cells (Wang et al 2014).

Androgens, such as testosterone and dihydrotestosterone (DHT), and androgen receptor (AR) signaling is required for normal prostate cellular functions and architecture maintenance since the prostate is the primary target organ of androgens (Schrecengost and Knudsen 2013; Zhou et al 2015). AR signaling in normal prostatic tissue promotes a pro-differentiation gene signature and plays an important role in the differentiation of the prostate terminal end buds (Schrecengost and Knudsen 2013). Testosterone and DHT have also been shown to play an important role in specifying the development of the urogenital sinus (UGS) into the prostate via stimulation of the AR (Zhou et al 2015). Androgen ablation during the early stages of murine embryonic development prevents adult prostate development and lack of functional AR causes absence of the prostate (Schrecengost and Knudsen 2013). Homeostatic maintenance of the adult prostate epithelium is also dependent on AR signaling (Zhou et al 2015). Androgen deprivation therapy (ADT) in developed prostate glands have been shown to cause luminal cell apoptosis and lumen involution which can be reduced using testosterone supplementation (Schrecengost and Knudsen 2013; Zhou et al 2015). Epithelial AR function has been shown to maintain prostate homeostasis though suppression of basal cell proliferation and stimulation of luminal cell survival (Zhou et al 2015; Wu et al 2007).

It is important to note that although there is limited evidence suggesting that androgens can autonomously promote PCa development, if PCa is present androgens are significant mediators of cancer growth and progression (Schrecengost and Knudsen 2013). Therefore, there are important differences in androgen function in the developing or normal prostate compared to PCa (Schrecengost and Knudsen 2013).
1.3 Prostate Cancer Detection and Diagnosis

1.3.1 Prostate Cancer Detection

The primary tools used to diagnose prostate cancer include the digital rectal exam (DRE), serum concentration of prostate-specific antigen (PSA), and transrectal ultrasound (TRUS)-guided biopsy (Heidenreich et al 2014 Part 1). The DRE consists of a physician inserting a gloved, lubricated finger into the rectum of a patient and feeling the prostate through the wall of the rectum for any abnormalities. Prostate-specific antigen (PSA) is a glycoprotein that functions as a serine protease and an arginine esterase and is found primarily in the epithelial cells of the prostate (Campbell-Walsh Urology 2012). PSA is secreted by the prostate into the seminal fluid in high concentrations and small amounts of PSA can also be found circulating in the blood of healthy men in complexed and free forms, which allows serum levels of PSA to be evaluated using a simple blood test. There is no specific normal or abnormal level of PSA in the blood but generally the higher the PSA level, the more likely prostate cancer is present. For example, the prevalence of prostate cancer was shown to range from 6.6% among men with a PSA level of 0-0.5ng/mL to 26.9% in men with a PSA level of 3.1-4ng/mL (Thompson et al 2004).

PSA is an organ-specific but not a cancer-specific marker which has contributed to some of the challenges that have been associated with its use in screening for prostate cancer (Campbell-Walsh Urology 2012). Elevated PSA levels are associated with prostate cancer, but they can also be caused by other prostatic disease such as benign prostatic hyperplasia (BPH) or prostatitis. Furthermore, not all men with prostate cancer will in fact have an elevated PSA level. Screening also does not come without risk, since it is associated with minor harms such as bleeding, bruising, and test-related anxiety (Ilic et al 2013). PSA testing can also potentially lead to TRUS-guided biopsy associated harms such as infection, bleeding, and pain, as well as overdiagnosis and overtreatment (Ilic et al 2013).

Two significant trials which have investigated PSA screening are the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening trial from the United States and the European Randomized Study of Screening for Prostate Cancer (ERSPC) (Hayes and Barry 2014). The
PLCO trial showed that after 13 years of follow-up, there was no statistically significant difference in the cumulative prostate cancer mortality rates between the screening and control arm (Andriole et al 2012). This indicates no significant benefit for screening, but some limitations of the study must be considered such as a low proportion of men with a family history of prostate cancer and contamination of the control group with PSA testing which may have diluted true screening effects (Hayes and Barry 2014). The ERSPC trial demonstrated that after 11 years of follow-up, a 21% relative risk reduction of prostate cancer mortality was found for the PSA screening group but there was no difference between groups for overall mortality (Schroder et al 2012).

Recently, a number of significant medical societies have released recommendations about PSA screening for prostate cancer. The U.S. Preventive Services Task Force released a statement recommending against PSA-based screening for prostate cancer (Moyer et al 2012). The American Urological Association released guidelines indicated men aged 55-69 or over 70 years of age with a life expectancy longer than 10-15 years and average risk should use a shared decision-making approach with their physician regarding PSA screening (Carter et al 2013). The Canadian Task Force on Preventive Health Care strongly recommends no PSA screening be completed for men under age 55 or over age 70, and weakly recommends against PSA screening in men 55-69 years of age (Canadian Task Force 2014). There is concern among urologists that while limiting PSA screening may reduce the number of diagnoses of early prostate cancer, it could lead to an increase in diagnoses of more advanced, potentially metastatic prostate cancer which is much more difficult to treat and does not have the same curative options available compared to early stage disease. Since the introduction of the U.S. Preventive Services Task Force recommendation against PSA screening in 2011, Gaylis et al (2016) demonstrated men diagnosed with Gleason score ≥ 8 PCa on biopsy significantly increased from 21% in 2011 to 30% in 2014 and Gejerman et al (2016) showed high Gleason score (8-10) PCa diagnoses at biopsy significantly increased from 9% in 2011 to 19% in 2014. Further evaluation will be necessary in the coming years to determine the long-term impact of the recommendations to reduce PSA screening.
1.3.2 Prostate Cancer Diagnosis
The need for a TRUS-guided prostate biopsy is determined based on the results of a PSA test, a suspicious DRE result, as well as the patient’s biological age, potential comorbidities and therapeutic consequences (Heidenreich et al 2014 Part 1). A PSA level that is considered suspicious for prostate cancer should be measured a second time before requesting a prostate biopsy to eliminate the chance of PSA fluctuation causing a false-positive result (Eastham et al 2003). For a prostate gland volume of 30-40mL, at least 10-12 cores of prostate tissue should be sampled during a biopsy (Heidenreich et al 2014 Part 1). If there is suspicion of prostate cancer despite negative prostate biopsy results, multiparametric magnetic resonance imaging (MRI) may be used to investigate the possibility of a cancer located in the anterior portion of the prostate gland since this region is extremely difficult to sample during a biopsy (Heidenreich et al 2014 Part 1).

After obtaining the requisite tissue samples from the prostate biopsy, histologic examination is completed to determine the grade of the prostate tumor if one is present. In prostate cancer, the Gleason grading system is the most widely accepted tumor grading system (Gleason and Mellinger 1974). The Gleason system is based on the architectural pattern of a tumor observed at relatively low (10-40x) magnification with a standard light microscope (Humprey et al 2004). A grade is assigned to the primary (most dominant) pattern and the secondary (second most dominant) pattern present from a pattern grade of 1 being the most differentiated tumor architecture (least aggressive) to a pattern grade of 5 being the least differentiated (most aggressive) (Thompson et al 2007). The final Gleason score is determined by adding the primary pattern grade and secondary pattern grade together, or in some cases the Gleason score is reported as the most dominant pattern added with the highest grade pattern if the highest grade pattern present is not one of the most common (Thompson et al 2007). For example, a Gleason score 3 + 4 tumor has 3 as the most common pattern of the tumor and 4 as the second most common or highest pattern observed in the cores (Thompson et al 2007). The Gleason score can range from 2-10, however tumors with a Gleason score of 2-4 should be diagnosed rarely, if ever, and thus the range of Gleason scores often seen for prostate cancer are between 6 and 10.
A Gleason score of 3+3 is regarded as low grade PCa, 3+4 or 4+3 as intermediate grade PCa, and 8-10 as high grade PCa (James 2014).

Tumor staging is another important characteristic of prostate cancer diagnosis. The TNM system for tumor staging is the most common, where $T(TX,T0,T1-4)$ is the size and extent of the primary tumor, $N(X,0,1)$ is the number and location of regional lymph nodes infiltrated by tumor cells, and $M(0,1)$ is metastatic status of the tumor (i.e. has it spread) (American Joint Committee on Cancer 2010). An example of a risk stratification scheme for prostate cancer is included below from Thompson et al (2007):

- **Low risk:** PSA $\leq 10$ng/mL, Gleason score $\leq 6$, and clinical stage T1c or T2a
- **Intermediate risk:** PSA $> 10-20$ng/mL or Gleason score of 7 or clinical stage T2b
- **High risk:** PSA $> 20$ng/mL or a Gleason score of 8-10 or clinical stage T2c

1.4 Prostate Cancer Pathophysiology

When prostate cancer is identified at the clinically localized stage and is classified as a low grade tumor, it can be monitored with active surveillance. Higher grade PCa can be effectively treated by radical prostatectomy, whereby the prostate is surgically resected, or radiation therapy. If the cancer invades into the surrounding tissue outside the prostatic capsule, or if it recurs after local therapy, PCa becomes more difficult to treat. Advanced stage prostate cancer is usually treated with ADT, however most patients managed with this therapy eventually relapse with castration-resistant prostate cancer (CRPC) and this stage of the disease is incurable. A complex interplay of processes contribute to the initiation and progression of PCa to castration-resistant disease.

1.4.1 Prostate Cancer Initiation and Progression

Prostate cancer typically has a long natural history and is generally regarded as multifocal and heterogeneous since primary tumors often contain multiple, independent histologically defined foci that are genetically distinct (Shen and Abate-Shen 2010). Studies demonstrate that although prostate cancer is usually associated with older men, healthy men between 20 and 40 years of
age frequently show the presence of prostate cancer foci, indicating that cancer initiation may take place at a relatively early age (Shen and Abate-Shen 2010; Sakr et al 1994). However, despite evidence that indicates prostate cancer initiation may happen at a somewhat early age, prostate cancer can often remain latent for extended periods of time. The inherent heterogeneity and multifocality of prostate cancer may play a role in the distinction between latent and clinically detectable prostate cancer (Shen and Abate-Shen 2010). The prostate gland may be the site of multiple neoplastic transformation events which in many cases results in the development of latent prostate cancer but this may not progress to clinically detectable disease (Shen and Abate-Shen 2010). It may be possible that clinical prostate cancer develops from a different pathogenic program or latent prostate cancer foci may not undergo critical neoplastic events that lead to clinical disease and therefore these latent foci remain in a subclinical state (Shen and Abate-Shen 2010). PSA screening has led to an increased number of prostate cancer diagnoses, however it is still very difficult to distinguish latent prostate cancer from prostate cancer that will progress more aggressively (Shen and Abate-Shen 2010).

The presence of high grade prostatic intraepithelial neoplasia (HGPIN), characterized as a multifocal lesion with hyperplasia of luminal epithelial cells and a decrease in basal epithelial cells, represents in situ adenocarcinoma and is recognized to progress to clinically detectable prostate cancer (Schrecengost and Knudsen 2013). After progression from HGPIN to prostate cancer, the great majority of prostate cancers are classified as adenocarcinomas which express AR, with a small proportion (<2%) classified as neuroendocrine prostate cancer which is AR-independent and therefore castration-resistant (Grignon 2004). The wide range of factors which contribute to prostate cancer initiation and progression will be discussed in the following section.

1.4.1.1 Processes Contributing to Prostate Cancer Initiation
There are some additional processes aside from genetic and epigenetic alternations that can contribute to prostate carcinogenesis. Chronic prostate inflammation, due to hormonal alterations, infection by bacterial or viral agents, physical trauma or dietary factors, may contribute to prostate carcinogenesis (De Marzo et al 2007b; Shen and Abate-Shen 2010). An
age-associated influence on prostate carcinogenesis could be the cumulative impact of oxidative stress which can lead to DNA damage (Shen and Abate-Shen 2010; Khandrika et al 2009). An imbalance between reactive oxygen species (ROS) and detoxifying enzymes that control cellular ROS levels causes oxidative stress which leads to cumulative DNA, lipid, and protein damage (Shen and Abate-Shen 2010). This is particularly important for the prostate, which is especially vulnerable to oxidative stress, possibly as a consequence of inflammation, hormonal dysregulation, diet or genetic modifications. Nkx3.1, which protects against DNA damage in prostate cancer cell lines, is frequently down-regulated in early stage prostate cancer and its inactivation may contribute to prostatic vulnerability to oxidative stress and DNA damage associated with PCa initiation (Shen and Abate-Shen 2010; Bowen and Gelmann 2010). Telomere shortening has also been implicated in prostate cancer initiation, since high grade PINs and prostate carcinomas display increased telomerase activity (Shen and Abate-Shen 2010). Shortening of telomeres is also generally associated with DNA damage and may lead to chromosomal instability which could lead to the occurrence of cancer initiating events (Shen and Abate-Shen 2010; Meeker et al 2004).

1.4.1.2 Prostate Cancer Genetics
The genome of prostate cancer is characterized by a limited number of focal chromosomal gains or losses and overall has a low mutation rate compared with other cancers (Attard et al 2016; Barbieri and Tomlins 2014). Glutathione-S-Transferase P1 (GSTP1) is an enzyme that decreases oxidative damage in cells by catalyzing the reaction which conjugates toxic compounds to glutathione (Knudsen and Vasioukhin 2010). Silencing of GSTP1 expression due to promoter hypermethylation is an early event in prostate cancer initiation which is observed in 70% of HGPIN lesions and over 90% of prostate cancers (Henrique et al 2004; Lee et al 1994; Knudsen and Vasioukhin 2010). Decreased GSTP1 expression may predispose luminal epithelial cells to increased oxidative damage, which results in the accumulation of genetic changes that could cause cellular transformation and cancer (Knudsen and Vasioukhin 2010). Another early and frequent event in prostate cancer is the loss of heterozygosity at chromosome 8p21-22 and subsequent reduction of Nkx3.1 expression (Bethel et al 2006; Swalwell et al 2002). Nkx3.1, a homeobox transcription factor, is expressed in prostate luminal epithelial cells and is important
for normal prostate epithelial differentiation (Abate-Shen et al 2008). Decreased levels of Nkx3.1 may cause deficient prostate epithelial differentiation, promote a nondifferentiated phenotype and contribute to neoplastic transformation (Knudsen and Vasioukhin 2010).

Erythroblast transformation-specific (ETS) transcription factor family gene rearrangements are another common event in prostate cancer initiation. Approximately 50-60% of PSA-screened PCa have recurrent gene fusions, the most common being a fusion of the 5’ untranslated region of the androgen-regulated TMPRSS2 (transmembrane protease serine 2) gene to the coding sequence of ETS family member ERG (Attard et al 2016; Barbieri and Tomlins 2014). TMPRSS2:ERG fusion results in androgen regulation of ERG expression and therefore androgen-responsive elements that usually restrict TMPRSS2 expression to the prostate instead drive overexpression of 5’ truncated ETS oncogenes (Tomlins et al 2009). ETS fusions are associated with poor prognosis in population-based studies of active surveillance cohorts, however the association of ETS fusions with outcomes in radical prostatectomy cohorts is not clearly defined due to conflicting evidence (Barbieri and Tomlins 2014). Characterization of prostate cancer into ETS-positive (ETS+) or ETS-negative (ETS-) subclasses is important since tumors that are ETS+ are distinct biological entities compared to ETS- tumors (Barbieri and Tomlins 2014). Mutations and deletions in PTEN and p53 are occur more frequently in ETS+ tumors, while SPINK overexpression occurs exclusively in ETS- tumors (Barbieri and Tomlins 2014).

There are a number of other genetic changes that occur which are involved in the initiation and progression of higher grade prostate cancer. PTEN (Phosphatase and tensin homolog deleted on chromosome 10), a well-established tumor suppressor involved in a number of human cancers, is a lipid phosphatase which opposes PI3 kinase (PI3K) activity by dephosphorylating PIP3 and this abrogates PI3K/Akt signaling (Schrecengost and Knudsen 2013). 70% of primary PCa are PTEN haploinsufficient and complete loss is often described in metastatic PCa (Schrecengost and Knudsen 2013). *In vivo* models of PTEN loss closely follow human PCa characteristics based on disease progression, downregulation of Nkx3.1 and other similarities in gene expression and provides evidence for PTEN loss involvement in PCa initiation (Wang et al 2006;
Schrecengost and Knudsen 2013). Conditional inactivation of PTEN in the prostate epithelium results in high-grade prostate cancer, in some cases with small metastases appearing in distant organs (Wang et al 2003). It is suggested that a single PTEN allele loss is associated with early stage PCa and loss of the second PTEN allele occurs with other genetic alterations, such as loss of tumor suppressor p53, to promote more aggressive PCa (Schrecengost and Knudsen 2013). PTEN loss can also alter AR signaling and cellular response to ADT which is a key determining factor in CRPC formation and in fact almost one-third of castration-resistant metastatic prostate tumors display loss of PTEN (Shen and Abate-Shen 2007). Double-heterozygous Nkx3.1/PTEN mice have been shown to develop androgen-independent prostate cancer (Gao et al 2006). In human tumor biopsy samples, AR staining has been shown to be heterogeneous in PTEN null regions but AR staining was uniform in PTEN positive regions (Mulholland et al 2011; Schrecengost and Knudsen 2013). Therefore PTEN appears to play an important role in PCa initiation and progression to more advanced disease.

Another important genetic alteration that contributes to the development of more advanced PCa is overexpression of the proto-oncogene Myc. Myc, a transcription factor involved in a wide range of cellular processes including cell cycle progression and protein synthesis, is often upregulated in PCa (Knudsen and Vasioukhin 2010; Schrecengost and Knudsen 2013). Myc is located at chromosome 8q24, which is often found to be amplified in advanced PCa (Schrecengost and Knudsen 2013). Transgenic mice overexpressing Myc in the prostate epithelium develop invasive PCa and combining Myc overexpression with other genetic aberrations such as PTEN/p53 heterozygosity further contributes to development of invasive PCa (Ellwood-Yen et al 2003; Kim et al 2012). In human PCa, Myc overexpression has been shown to predict biochemical recurrence and is associated with higher Gleason score, earlier disease progression and earlier cancer death (Hawksworth et al 2010; Sato et al 2006).

1.4.1.3 Prostate Cancer Cell of Origin
The “cell of origin” refers to the initial cell that is the target of genetic alteration(s) that result in malignant transformation and this transformed cell then gives rise to prostate cancer (Mayer et al
This topic has received a significant amount of attention in the past decade, but the definitive cell of origin for prostate cancer is still under debate. Luminal cells were initially thought to be the target of oncogenic transformation due to the presence of predominantly luminal cell characteristics and an absence of basal cell characteristics in the majority of PCa. A rare population of luminal cells that are castration-resistant and express Nkx3.1 (CARNs) have bipotentiality, can self-renew in vivo and reconstitute prostate ducts in renal grafts (Wang et al 2009). Furthermore, inducible deletion of PTEN in CARNs resulted in rapid formation of HGPIN and carcinoma in castrated male mice after androgen-mediated regeneration of the prostate (Wang et al 2009). A lineage tracing study using luminal-specific and basal-specific inducible Cre systems demonstrated prostate luminal cells with PTEN deletion are more susceptible to direct oncogenic transformation but basal cells require oncogenic signaling-induced differentiation into luminal cells to become susceptible to oncogenic transformation (Choi et al 2012). However, additional studies have provided evidence that basal cells may in fact be the site of the cell of origin for PCa. Primary basal cells transduced with AKT, ERG, and AR can initiate prostate cancer in immunodeficient mice but this did not occur with similarly transduced luminal cells (Goldstein et al 2010). Furthermore, human basal cells transduced with Myc or AKT gave rise to benign glands or low-grade prostate intraepithelial neoplasia, whereas basal cells transduced with both Myc and AKT together gave rise to large tumors in immunodeficient mice in as little as 6 weeks (Stoyanova et al 2013). Transduced human luminal cells, however, did not give rise to any detectable epithelial structures (Stoyanova et al 2013). Therefore, more work needs to be completed before the debate between luminal and basal cells as the cell of origin for prostate cancer can be put to rest.

1.4.1.4 Cellular Metabolic Alterations

Another process which contributes to carcinogenesis is the reprogramming of metabolism by cancer cells (Hanahan and Weinberg 2011). Normal cells rely primarily on oxidative phosphorylation for energy production, however Otto Warburg discovered in the 1920s that cancer cells, even in the presence of sufficient oxygen, shift their energy metabolism to glycolysis despite it being a less efficient pathway for ATP production (Warburg 1956, Hsu and Sabatini 2008; Vander Heiden et al 2009). Glycolysis can only generate 2 ATPs per molecule of
glucose, compared to oxidative phosphorylation which generates up to 36 ATPs after completed oxidation of one glucose molecule (Vander Heiden 2009). The primary dependence on glycolysis despite presence of ample oxygen is called “aerobic glycolysis” and this shift in energy metabolism which occurs in cancer cells is known as the “Warburg effect” (Hsu and Sabatini 2008; Vander Heiden 2009). There are multiple mechanisms that may contribute to the Warburg effect. One possible explanation is that aerobic glycolysis confers a selective advantage for survival and proliferation in the hypoxic tumor microenvironment, since decreased dependence on aerobic respiration would be beneficial (Hsu and Sabatini et al 2008). Another factor may be that the distinguishing features of the Warburg effect, such as increased glucose consumption, decreased oxidative phosphorylation, and accompanying lactate production, are also features of oncogene activation (Hsu and Sabatini 2008). For example, the oncogene Ras promotes glycolysis when mutated and Myc upregulates the expression of multiple metabolic genes (Hsu and Sabatini 2008; Ramanathan et al 2005; Gordon et al 2007). Another possibility is that cancer cells may alter metabolic pathways to exploit the tumor microenvironment to support cancer-specific signaling (Hsu and Sabatini 2008).

1.4.2 Progression to Castration-Resistant Prostate Cancer (CRPC)

CRPC is the most advanced stage of prostate cancer and is currently incurable. CRPC is defined as progression of disease despite castrate levels of testosterone, and can present as a continuous rise in serum PSA levels, the progression of pre-existing disease, or the appearance of new metastases (Saad et al 2015). ADT is the most widely used first-line treatment for advanced prostate cancer and can be achieved by bilateral orchiectomy, chemical castration to reduce serum testosterone to a minimum, or blocking the AR (Zong and Goldstein 2013). Despite very high initial response rates to ADT, many patients progress to CRPC. Although CRPC cells proliferate effectively in an androgen-depleted environment, they have been shown to continue expressing AR and that activation of the AR is altered at this very advanced stage of PCa (Dutt and Gao 2009). There are two models, which are not mutually exclusive, that have been proposed to explain the cellular underpinning of CRPC development: the adaptation model and the selection model (Zong and Goldstein 2013). The adaptation model proposes that androgen-dependent prostate cancer cells undergo genetic or epigenetic changes which permit adaptation
to the castrated state and leads to CRPC (Zong and Goldstein 2013). The selection model proposes that there is a small pre-existing population of castration-resistant clones which will expand under the pressure of ADT leading to CRPC development (Zong and Goldstein 2013).

1.4.2.1 The Adaptation Model
The adaptation model, which proposes that genetic and epigenetic changes contribute to prostate cancer cells becoming resistant to ADT, shares some similarity with the development of drug resistance in patients with infectious diseases (Zong and Goldstein 2013). There are several mechanisms that confer resistance to ADT, including several AR-dependent mechanisms that effect the function of the AR and AR signaling pathways, as well as other AR-independent mechanisms.

The AR has been shown to remain active in CRPC (Yuan et al. 2014). One mechanism by which PCa cells adapt to the ADT environment is through increased sensitivity of the AR to the very low levels of androgens that are present after ADT. One mechanism that can increase sensitivity to androgens is increasing the expression of AR which permits enhanced ligand binding in the presence of reduced androgen concentration (Pienta and Bradley 2006; Feldman and Feldman 2001). Increased AR expression sensitizes cells to extremely low concentrations of androgens, can override inhibitory effects of AR antagonists such as bicalutamide, and also promotes altered androgen-dependent genetic signatures through increased AR-binding sites on chromatin (Schrecengost and Knudsen 2013; Waltering et al. 2009; Tran et al. 2009; Linja et al. 2001). Gene profiling in prostate cancer xenograft models shows upregulation of AR gene expression with progression from androgen-dependent to androgen-independent tumor growth (Chen et al. 2004). Genome-wide expression analysis of human PCa tissue samples shows expression patterns were more similar between CRPC and ADT-naïve primary cancers compared to ADT-treated tissue indicating a re-activation of androgen-responsive elements (Holzbeierlein et al. 2004; Zong and Goldstein 2013). Another mechanism for increasing AR sensitivity is through increased local production of androgens by PCa cells (Pienta and Bradley 2006). This localized androgen production is likely due to the increased activity of 5α-reductase allowing for an increased
conversion rate of testosterone to DHT (Pienta and Bradley 2006). Higher rates of PCa have been found in ethnic groups with increased 5α-reductase activity and genes involved in steroid biosynthesis are overexpressed in recurrent human PCa tissue (Makridakis 1997; Holzbeierlein et al 2004; Pienta and Bradley 2006). Increased expression of enzymes involved in steroidogenesis, such as CYP17A, may also contribute to de novo intratumoral androgen synthesis (Zong and Goldstein 2013; Cai et al 2011). Intratumoral levels of testosterone and DHT are higher in metastatic CRPC compared to localized PCa in untreated patients and the levels found in metastatic CRPC patients was in a range known to stimulate AR and promote PCa growth (Montgomery et al 2008; Karantanos et al 2015).

Another related mechanism involves genetic mutations, usually missense mutations in the AR gene, leading to aberrant activation of the androgen signalling axis (Feldman and Feldman 2001). The AR is among nine genes that are significantly mutated in metastatic CRPC (Grasso et al 2012). The wild-type AR is activated only by testosterone and DHT and mutations of the AR can expand its ligand binding specificity, allowing ligands other than DHT, such as nonandrogenic steroids and antiandrogens, to activate the AR (Pienta and Bradley 2006; Dutt and Gao 2009). Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice have AR mutations in the signature loop of the receptor, the flanking region where coactivators can bind, and the ligand-binding domain (Dutt and Gao 2009; Feldman and Feldman 2001). Coactivator levels are also elevated in castration-resistant prostate cancer, which enhances the sensitivity of AR to non-androgens (Grossmann et al 2001).

Furthermore, it is possible for activation of the AR or downstream signaling of the AR to occur through ligand-independent mechanisms (Pienta and Bradley 2006). Growth and proliferation of tumor cells are no longer solely under androgenic control but the AR machinery remains active (Pienta and Bradley 2006). Deregulated growth factors such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF), as well as cytokines IL-4 and IL-6, have been shown to phosphorylate and activate the AR (Pienta and Bradley 2006; Dutt and Gao 2009). AR-dependent genes can also be activated by the deregulation of signal transduction pathways (Pienta and Bradley 2006). For example, receptor
tyrosine kinases such as HER-2/neu, which are overexpressed in CRPC, can activate AR-dependent genes in the absence of a ligand (Pienta and Bradley 2006).

Although AR signaling does appear to play a role in progression of CRPC, there are also mechanisms which bypass the AR or AR signaling which have substantial roles in CRPC development (Zong and Goldstein 2013). The circumvention of the AR pathway can be accomplished by utilizing other pathways to stimulate prostate cancer cells to proliferate in a castrate environment without ligand-mediated or non-ligand-mediated AR activation (Dutt and Gao 2009; Pienta and Bradley 2006). Multiple human prostate cancer cell lines derived from metastases, such as DU145 and PC3 cells, lack AR expression but can still form castration-resistant xenograft tumors (Zong and Goldstein 2013; van Bokhoven 2003; Tran et al 2009). The most established bypass mechanism involves modulation of apoptosis (Pienta and Bradley 2006). Upregulation of anti-apoptotic molecules, such as apoptosis regulatory protein Bcl-2, has been shown in androgen independent tumors (McDonnell et al 1992; Zong and Goldstein 2013). The upregulation of these molecules would inhibit apoptosis and potentiate growth of prostate cancer cells in an androgen-independent manner (Zong and Goldstein 2013). Another potential mechanism is the neuroendocrine differentiation of prostate cancer cells (Pienta and Bradley 2006). Neuroendocrine prostate cancer cells have low rates of proliferation and secrete neuropeptides such as bombesin which enhance the proliferation of cells in a cancerous environment in the absence of hormones (Pienta and Bradley 2006; Dutt and Gao 2009).

1.4.2.2 The Selection Model
The selection model proposes that androgen deprivation leads to the selection and subsequent outgrowth of pre-existing castration-resistant prostate cancer cells (Zong and Goldstein 2013). Studies completed by Isaacs and Coffey in the 1980s demonstrated that rat prostate adenocarcinoma is composed of a heterogeneous population of androgen-dependent and androgen-independent tumor cells (Isaacs and Coffey 1981; Zong and Goldstein 2013). Serial-dilution studies using human prostate cancer xenograft cell lines LAPC4 and LAPC9 cells have shown that androgen-independent cells are present at a frequency of approximately one cell per
$10^5$-$10^6$ androgen-dependent cells and will expand under the selective pressure of androgen deprivation leading to the development of castration-resistant tumors (Craft et al 1999; Zong and Goldstein 2013). AR mutations have also been detected in high-grade prostate cancer or metastatic prostate cancer before initiation of ADT, which indicates that castration-resistant cancer cells are not necessarily a direct result of adaptation to ADT (Thompson et al 2003; Zong and Goldstein 2013). Furthermore, low AR expression has been found in hormone-naïve metastatic tumors which suggests small populations of prostate cancer cells can escape androgen dependence even in the presence of normal hormone levels (Fleischmann et al 2011; Zong and Goldstein 2013).

The very low frequency of androgen-independent cells found prior to ADT in the selection model is also consistent with the cancer stem cell (CSC) model which provides an explanation for the hierarchical organization of prostate cancer (Chen et al 2013; Zong and Goldstein 2013). CSCs are a small subpopulation of tumor cells which have the capacity to self-renew through symmetric or asymmetric division, high proliferative potential, and differentiate into multiple cell lineages (Mayer et al 2015). CSCs are also typically quiescent but can give rise to highly proliferative cell types (Mayer et al 2015). The CSC model postulates that CSCs are at the top of the hierarchy, and although CSCs themselves are typically quiescent, CSCs can give rise to prostate cancer progenitor cells which are highly proliferative (Mayer et al 2015). These prostate cancer progenitor cells can then go on to proliferate and differentiate into many different types of tumor cells which creates the heterogeneity observed in prostate cancer (Mayer et al 2015). The characteristics of prostate CSCs confer therapeutic resistance and therefore prostate CSCs are candidates to survive low-androgen conditions following ADT and then contribute to tumor recurrence and development of CRPC (Zong and Goldstein 2013). Prostate CSCs have been identified using side-population analysis, aldehyde dehydrogenase activity and combinations of cell-surface markers including CD44, $\alpha_2\beta_1$, and CD133 (Zong and Goldstein 2013; Mayer et al 2015). All identified subsets of prostate CSCs have been shown to lack AR expression or have low AR activity, which illustrates prostate CSCs are likely not dependent on AR signaling for survival and growth (Zong and Golstein 2013). Androgen depletion therefore provides selective pressure, killing the androgen-dependent cancer cells, while the androgen-independent prostate
CSCs survive and through their self-renewing and tumor propagating ability give rise to castration-resistant prostate cancer (Mayer et al 2015).

A: Adaptation Model

![Diagram showing the adaptation model of CRPC development]

B: Selection Model

![Diagram showing the selection model of CRPC development]

**Figure 1.1: The adaptation and selection models of CRPC development.** The adaptation model proposes that during androgen deprivation therapy (ADT) a small percentage of androgen-dependent prostate cancer cells will acquire genetic/epigenetic changes that confer resistance to ADT and these surviving ADT-resistant cells will continue proliferating and give rise to CRPC. (B) The selection model proposes that there is a small subpopulation of castration-resistant cells which are already present within the tumor and this subpopulation will survive ADT and continue proliferating to give rise to CRPC.
1.5 Castration-Resistant Prostate Cancer (CRPC) Treatment

Androgen deprivation therapy (ADT) is the standard systemic treatment for patients with metastatic hormone-sensitive disease (Quinn et al 2013). Despite initial response rates, however, resistance to ADT occurs in most patients after 18 months of treatment and this leads to the development of castration-resistant prostate cancer (CRPC) (Quinn et al 2013). The treatment of CRPC is complex and has evolved significantly in recent years. CRPC represents a spectrum of disease, ranging from asymptomatic non-metastatic cancer identified by rising PSA levels despite ADT, to aggressive metastatic tumors with a significant disease burden (Bishr and Saad 2013). Approximately 90% of patients with metastatic CRPC (mCRPC) have bone metastases which produce significant morbidity including pain, pathologic fractures, and spinal cord compression (Bishr and Saad 2013; Loriot et al 2012). Until 2010, docetaxel chemotherapy was the only agent that demonstrated a survival benefit for CRPC patients (Lorente et al 2015). However, improved understanding of the biological underpinnings of CRPC led to the development of several novel therapeutic agents including abiraterone acetate, enzalutamide, cabazitaxel, sipuleucel-T, and radium-223. Despite the development of these novel therapies, docetaxel is still the standard-of-care first-line chemotherapy for patients presenting with symptomatic mCRPC (Saad et al 2015). However, even with the larger variety of treatment options available for mCRPC patients, the survival for this advanced stage of prostate cancer is only approximately 19 months (Heidenreich et al 2013). The following section will focus primarily on the initial treatment of mCRPC based on the therapeutic agents used in Canada before progression post-docetaxel chemotherapy (Saad et al 2015).

1.5.1 CRPC Treatment Agents

1.5.1.1 Abiraterone Acetate

Abiraterone acetate is a highly selective and irreversible inhibitor of CYP17, a critical enzyme involved in androgen biosynthesis (Bishr and Saad 2013). Treatment with abiraterone acetate blocks CYP17 enzyme activity which blocks androgen synthesis in the adrenal glands, testes and within the prostate tumor itself (de Bono et al 2011). The regulatory body approval of abiraterone acetate for treating CRPC was initially established for the post-chemotherapy setting.
following the results of the COU-AA-301 trial. The COU-AA-301 trial included 1195 patients who were previously treated with docetaxel assigned randomly to receive 1000mg of abiraterone acetate plus prednisone or to receive placebo plus prednisone (placebo group) (de Bono et al 2011; Fizazi et al 2012). The abiraterone acetate-prednisone group had a median overall survival of 15.8 months (95% confidence interval 14.8-17.0) compared to 11.2 months (95% confidence interval 10.4-13.2) for the placebo-prednisone group (Hazard ratio = 0.74, 95% confidence interval 0.64-0.86, p < 0.0001) (Fizazi et al 2012). In addition, all other efficacy endpoints that were examined (PSA progression, radiographic progression-free survival, proportion of patients with a PSA response, objective response assessed by Response Evaluation Criteria in Solid Tumors) were improved with abiraterone acetate treatment compared to the placebo (Fizazi et al 2012).

Since its initial approval, abiraterone acetate has also been approved for chemotherapy-naïve CRPC patients following the COU-AA-302 trial. The COU-AA-302 trial included 1088 asymptomatic or mildly symptomatic mCRPC patients who had not received chemotherapy and were randomized to receive 1000mg abiraterone acetate once daily plus prednisone or placebo plus prednisone (Ryan et al 2015). Median overall survival was significantly longer in the abiraterone acetate group at 34.7 months (95% confidence interval 32.7-36.8) compared to 30.3 months (95% confidence interval 28.7-33.3) in the placebo group (Hazard ratio = 0.81, 95% confidence interval 0.70-0.93, p < 0.0033) (Ryan et al 2015).

1.5.1.2 Enzalutamide
Enzalutamide (formerly known as MDV3100) is an AR signaling inhibitor selected for clinical development based on its activity in prostate cancer models with overexpression of AR (Scher et al 2012). Enzalutamide inhibits nuclear translocation of the AR, DNA binding and coactivator recruitment (Scher et al 2012). Regulatory approval for enzalutamide was first completed in 2012 following the AFFIRM trial. The AFFIRM trial included 1199 patients with CRPC who received chemotherapy and were randomly assigned to receive 160mg of enzalutamine per day or placebo (Scher et al 2012). The median overall survival was 18.4 months for the enzalutamide
group compared to 13.6 months in the placebo group, with a 37% reduction in the risk of death for the enzalutamide group (Hazard ratio = 0.63, 95% confidence interval 0.53-0.75, p < 0.001) (Scher et al 2012). Enzalutamide was also superior compared to placebo for all secondary endpoints including PSA-level response rate, soft-tissue response rate, FACT-P quality of life response, and time to PSA progression, radiographic progression-free survival, and time to first skeletal-related event (Scher et al 2012).

Similar to abiraterone acetate, after approval in the post-chemotherapy setting enzalutamide was approved for use in chemotherapy-naive patients following the PREVAIL trial. The PREVAIL trial included 1717 patients diagnosed with metastatic CRPC but had not received chemotherapy and were randomly assigned to receive 160mg of enzalutamide daily or placebo (Beer et al 2014). The enzalutamide group had a 65% rate of radiographic progression-free survival at 12 months compared to a 14% rate among the placebo group (Hazard ratio = 0.19, 95% confidence interval 0.15-0.23, p < 0.001) (Beer et al 2014). There was also a 29% reduction in risk of death for the enzalutamide group (Hazard ratio = 0.71, 95% confidence interval 0.60-0.84, p < 0.001) and enzalutamide was shown to be superior for all secondary endpoints including time until initiation of chemotherapy, time until first skeletal-related event, complete or partial soft-tissue response, time until PSA progression, and a rate of decline of at least 50% in PSA (Beer et al 2014).

1.5.1.3 Radium-223
Radium-223 (radium-223 dichloride) is a targeted alpha emitter which emits high-energy alpha particles of short range (<100um) and selectively binds to areas of increased bone turnover in bone metastases (Parker et al 2013). Radium-223 is a bone-seeking calcium mimetic and is bound into newly formed bone stroma, especially within osteoblastic or sclerotic metastases (Parker et al 2013). The high-energy radiation from the alpha-particles induces double-stranded DNA breaks that result in a highly localized cytotoxic effect which also minimizes any toxic effects on adjacent healthy tissue and bone marrow (Parker et al 2013). The ALSYMPCA trial at the updated analysis included 921 patients who had received, were not eligible to receive, or
declined docetaxel and were randomized to receive six injections of radium-223 (at a dose intravenously of 50kBq/kg of body weight) or matching placebo with one injection every 4 weeks (Parker et al 2013). Patients receiving radium-223 had longer overall survival than the placebo group (14.9 vs 11.3 months) and had a 30% reduction in risk of death (Hazard ratio = 0.70, 95% confidence interval 0.58-0.83, p <0.001) (Parker et al 2013). All main secondary endpoints also provided support for the benefit of radium-223, including significantly prolonged time to first symptomatic skeletal event and prolonged time to an increase in the PSA level (Parker et al 2013). This led to the use of radium-223 for patients with pain due to bone metastases who do not have visceral metastases (Saad et al 2015).

1.5.1.4 Docetaxel Chemotherapy
Two landmark trials published in 2004 established the survival benefit of docetaxel (Lorente et al 2015). The SWOG 99-16 trial included 770 men diagnosed with metastatic CRPC who were randomly assigned to receive one of two treatments given in 21-day cycles: 280mg of estramustine 3 times daily on days 1-5, 60mg of docetaxel per square meter of body-surface area on day 2, and 60mg of dexamethasone in 3 divided doses before docetaxel or 12mg of mitoxantrone per square meter on day 1 plus 5mg of prednisone twice daily (Petrylak et al 2004). The group receiving docetaxel and estramustine had a significant 1.9 month median overall survival benefit compared to the mitoxantrone group (Hazard ratio = 0.8, 95% confidence interval 0.67-0.97) (Petrylak et al 2004; Lorente et al 2015). The TAX-327 trial included 1006 men with metastatic CRPC who were given 5mg of prednisone twice daily and were randomly assigned to receive 12mg of mitoxantrone per square meter of body-surface area every 3 weeks, 75mg of docetaxel per square meter every 3 weeks or 30mg of docetaxel per square meter weekly for 5 of every 6 weeks (Tannock et al 2004). The group receiving docetaxel every 3 weeks had a significant 2.9 month improvement in median overall survival compared to the mitoxantrone group (Hazard ratio = 0.76, 95% confidence interval 0.62-0.94) (Tannock et al 2004; Lorente et al 2015). There was also a significant reduction in pain, significant improvement in quality of life, and improvement in PSA response in the group receiving docetaxel compared to mitoxantrone (Tannock et al 2004). 75mg of docetaxel per square meter every 3 weeks with 5mg of prednisone twice per day was established as the standard of care due
to the toxic side effects and lack of additional efficacy when combined with estramustine (Lorente et al 2015). It is important to note that although a significant survival benefit was achieved with docetaxel chemotherapy, there are a large number of side effects experienced by patients including fatigue, nausea and/or vomiting, alopecia, diarrhea, nail changes, sensory neuropathy, anorexia, changes in taste, inflamed or sore mouth, dyspnea, tearing, peripheral edema, and bleeding from the nose (Tannock et al 2004).

Docetaxel is a semi-synthetic taxane analog derived from the European yew (*Taxus baccata*) (Li et al 2004). There are two proposed mechanisms of action of docetaxel with the first being the inhibition of microtubule depolymerization (Mackler and Pienta 2005). Microtubules are one of three types of protein filaments which form the cytoskeleton of eukaryotic cells, which is a scaffolding network that modulates a wide range of cellular signaling processes (Mollinedo and Gajate 2003; Mackler and Pienta 2005). Microtubules control the position of intracellular organelles and proteins and also pull apart the chromosomes during mitosis (Mackler and Pienta 2005). Each microtubule is composed of α- and β-tubulin heterodimers that self-assemble through non-covalent interactions in tandem from head to tail, each following the same direction which creates a distinct structural polarity (Mackler and Pienta 2005). Microtubules are highly dynamic and display treadmilling, which is the process of net growth at the plus end of the microtubule and net shortening at the minus end, as well as dynamic instability which is the process by which microtubule polymers undergo prolonged period of gradual assembly then followed by period of rapid disassembly (Mackler and Pienta 2005). Treating cells with docetaxel results in docetaxel binding to the β-tubulin subunits which inhibits microtubule depolymerization while microtubule polymerization continues to occur (Mackler and Pienta 2005). The stabilization of microtubule polymerization causes cell-cycle arrest and mitotic inhibition at the G2 and M phases leading to apoptosis and cell death (Mackler and Pienta 2005). Docetaxel can also target centromere organization which causes cell damage in the S, G2 and M phases of the cell cycle (Mackler and Pienta 2005). The second proposed mechanism of action of docetaxel involves the inhibition of BCL2 (Mackler and Pienta 2005). Proteins in the BCL2 family play important roles in the regulation of apoptosis (Mackler and Pienta 2005). BCL2 inhibits apoptosis and contributes to the neoplastic process and therefore enhances tumor
survival whereas BAX is pro-apoptotic (Mackler and Pienta 2005). The stabilization of microtubule structure by docetaxel causes phosphorylation and thus inactivation of BCL2 function which prevents binding of BAX (Mackler and Pienta 2005). Increased free BAX within the cell leads to activation of the caspase cascade and apoptosis (Mackler and Pienta 2005; Haldar et al 1996). The mechanism of BCL2 phosphorylation and inhibition appears to be unique to antimicrotubule agents (Mackler and Pienta 2005).

1.5.2 Docetaxel Chemotherapy and Chemosensitizing Agents
Since docetaxel was established as the first known agent to extend the survival of men with mCRPC, there have been ongoing efforts focusing on the development of novel agents which can be combined with docetaxel chemotherapy to enhance its efficacy (Antonarakis and Eisenberger 2013). These agents can be defined as chemosensitizers.

Two Phase III double-blind, randomized clinical trials have been completed evaluating the combination of docetaxel with antiangiogenic drugs. The CALGB 90401 trial evaluated the effect of combining docetaxel with bevacizumab, a humanized immunoglobulin G monoclonal antibody to all the isoforms of VEGF-A (vascular endothelial growth factor-A), since inhibition of VEGF may enhance docetaxel efficacy in mCRPC (Kelly et al 2012). Adding bevacizumab to docetaxel treatment did significantly extend progression-free survival, improve objective response rates, and led to more patients achieving $>50\%$ post-therapy PSA decline compared to patients treated with docetaxel only (Kelly et al 2012). However, combination bevacizumab-docetaxel treatment did not prolong median overall survival, which was the primary end point of the study, and the bevacizumab-docetaxel group also had greater number of adverse events, including maximum hematologic and nonhematologic adverse events, compared to the docetaxel only group (Kelly et al 2012). The VENICE trial evaluated the effect of combining docetaxel with aflibercept, a recombinant fusion protein which has high binding affinity to the isoform VEGF-A, as well as VEGF-B and platelet-derived growth factors, which results in inhibition of angiogenesis (Tannock et al 2013). There was no difference in median overall survival, or secondary outcomes such as progression-free survival, between the aflibercept-docetaxel and
docetaxel only groups and there was also greater toxicity recorded in the aflibercept-docetaxel group (Tannock et al 2013). Therefore the combinations of docetaxel and antiangiogenic drugs have not yet been successful.

Three Phase III clinical trials have been completed evaluating the combination of docetaxel with bone microenvironment agents. Managing bone metastases are particularly important for CRPC patients since more than 90% of men with fatal prostate cancer have bone metastases which are associated with significant morbidity such as pain, spinal cord compression, and fractures (Quinn et al 2013; Tannock et al 2004; Loriot et al 2012). The SWOG S0421 trial evaluated the effect of combining docetaxel with atrasentan, an antagonist of endothelin receptor A which is a component of the endothelin pathway which is crucial for the initiation and maintenance of bone metastases from prostate cancer (Quinn et al 2013). The trial was terminated in April 2011, on the basis of a recommendation provided after the review of the third interim futility analysis (Quinn et al 2013). The atrasentan-docetaxel group did not have improved progression-free survival or overall survival compared to the docetaxel group (Quinn et al 2013). Another Phase III trial investigating an inhibitor of the endothelin pathway evaluated the effect of docetaxel combined with zibotentan (ZD4054), a specific endothelin A receptor antagonist (Fizazi et al 2013). There was no difference in overall survival, secondary end points including time to pain progression or pain response between the zibotentan-docetaxel and docetaxel only groups (Fizazi et al 2013). The READY trial evaluated the combination of docetaxel and dasatinib, a tyrosine kinase inhibitor that inhibits Src family kinases that contribute to the development of bone metastases (Araujo et al 2013). There was no improvement in overall survival in the dasatinib-docetaxel treated group compared to docetaxel only (Araujo et al 2013).

There are some additional novel agents which have been combined with docetaxel in Phase III clinical trials. The MAINSAIL trial investigated the combination of docetaxel and lenalidomide, an immunomodulatory agent which has been shown to enhance the immune response and inhibit microvascularization (Petrylak et al 2015). The trial was discontinued for futility in November 2011 after an independent data monitoring committee concluded the study was unlikely to reach its primary end point and patients in the lenalidomide-docetaxel group had an increased
incidence of serious adverse events (Petrylak et al 2015). The docetaxel only group had significantly better overall survival and progression-free survival compared to the lenalidomide-docetaxel group and the group receiving lenalidomide also had higher toxicity, including more grade 3 or higher neutropenia and febrile neutropenia (Petrylak et al 2015).

A randomized, open-label Phase III trial investigated the combination of docetaxel with a high dose of calcitriol (DN-101), a form of vitamin D which has been shown to inhibit proliferation and induce apoptosis in a range of tumor types (Scher et al 2011). The patients included in the study were randomized to Androgen-Independent Prostate Cancer Study of Calcitriol Enhancing Taxotere (ASCENT; 45ug DN-101, 36mg/m² docetaxel, 24mg dexamethasone weekly for 3 of every 4 weeks) or control (5mg prednisone twice daily with 75mg/m² docetaxel and 24mg dexamethasone every 3 weeks) (Scher et al 2011). At interim analysis more deaths were noted in the ASCENT arm and the trial was halted and the ASCENT arm had shorter survival than the control, however the docetaxel dose given to this group likely affected the outcome (Scher et al 2011).

Another agent which held promise for combination with docetaxel therapy was custirsen (OGX-011). Clusterin is a stress-induced, cytoprotective chaperone which is elevated in prostate cancer and contributes to treatment resistance (Saad et al 2011). Custirsen (OGX-011) is an antisense oligonucleotide which has high affinity for clusterin RNA and this results in suppression of clusterin levels and improved chemosensitivity to docetaxel (Saad et al 2011; Sowery et al 2008). The Phase III SYNERGY trial evaluated docetaxel treatment combined with curstirsen (OGX-011) and the results showed adding custirsen to docetaxel chemotherapy did not significantly improve overall survival compared to the docetaxel only group (Chi et al 2015). However in an abstract submitted to ASCO 2015, it was shown that stratifying the patient population from the SYNERGY trial into poor and good prognosis revealed that patients with poor prognosis did have improved median overall survival compared to patients with good prognosis (Chi et al 2015). More detailed results will need to be published in order to fully evaluate this potential benefit of custirsen in mCRPC patients receiving docetaxel chemotherapy.
Table 1.1: Summary of Phase III clinical studies evaluating potential chemosensitizing agents combined with docetaxel chemotherapy. OS: Overall survival; PFS: Progression-free survival.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Chemosensitizing Agent</th>
<th>Function of Chemosensitizing Agent</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kelly et al 2012</td>
<td>Bevacizumab</td>
<td>Inhibits angiogenesis through inhibition of VEGF-A</td>
<td>Bevacizumab-docetaxel treatment did not prolong median OS, had a greater number of adverse events compared to docetaxel only</td>
</tr>
<tr>
<td>Tannock et al 2013</td>
<td>Aflibercept</td>
<td>Inhibits angiogenesis through inhibition of VEGF-A, VEGF-B, platelet-derived growth factors</td>
<td>Aflibercept-docetaxel treatment did not improve median OS, PFS and had greater toxicity compared to docetaxel only</td>
</tr>
<tr>
<td>Quinn et al 2013</td>
<td>Atrasentan</td>
<td>Interferes with bone metastasis formation by antagonizing endothelin receptor A</td>
<td>Atrasentan-docetaxel treatment did not improve PFS or OS compared to the docetaxel only</td>
</tr>
<tr>
<td>Fizazi et al 2013</td>
<td>Zibotentan</td>
<td>Interferes with bone metastasis formation by antagonizing endothelin receptor A</td>
<td>Zibotentan-docetaxel treatment did not improve OS, time to pain progression or pain response compared to docetaxel only</td>
</tr>
<tr>
<td>Araujo et al 2013</td>
<td>Dasatinib</td>
<td>Interferes with bone metastasis formation by inhibiting Src family kinases</td>
<td>Dasatinib-docetaxel treatment did not improve OS compared to docetaxel only</td>
</tr>
<tr>
<td>Petrylak et al 2015</td>
<td>Lenalidomide</td>
<td>Enhances the immune response and inhibits microvascularization</td>
<td>Lenalidomide-docetaxel treatment resulted in worse OS, PFS and had higher toxicity compared to docetaxel only</td>
</tr>
<tr>
<td>Scher et al 2011</td>
<td>Calcitrol (DN-101)</td>
<td>High dose vitamin D that inhibits proliferation, induces apoptosis of tumor cells</td>
<td>Calcitrol-docetaxel treatment resulted in shorter survival compared to docetaxel only (trial halted early)</td>
</tr>
</tbody>
</table>
The only paper which has investigated any effect of metformin use during docetaxel chemotherapy was conducted using data collected during the TAX 327 trial (Niraula et al. 2013). The TAX 327 trial demonstrated the significant survival benefit of docetaxel chemotherapy in men with metastatic castration-resistant prostate cancer and led to docetaxel becoming one of the standard-of-care therapies for metastatic CRPC (Tannock et al. 2004). This retrospective study demonstrated there was no additive or synergistic effect of metformin use during docetaxel chemotherapy and no improvement in survival was observed (Niraula et al. 2013). However, only 38 patients were included in the analysis and therefore the conclusion is based on a very small sample size (Niraula et al. 2013).

Given the lack of efficacy shown in the Phase III clinical trials conducted to date evaluating potential chemic sensitizing agents combined with docetaxel chemotherapy, there is still a need to develop novel agents that could be combined with docetaxel to improve the therapeutic options available to mCRPC patients.

### 1.6 Metformin and Cancer

#### 1.6.1 Metformin Overview

Metformin (N, N-dimethylbiguanide) belongs to the biguanide class of anti-diabetic drugs and is the most commonly prescribed oral hypoglycemic agent for the treatment of Type 2 diabetes worldwide (Foretz et al. 2014; Kourelis et al. 2012; Pernicova and Korbonits 2014). Metformin was originally derived from galegine (isoamylene guanidine), a compound found in the French lilac *Galega officinalis*, which was used to treat polyuria in medieval Europe before diabetes had even been identified (Foretz et al. 2014; Kourelis et al. 2012; Pollak 2010; Pernicova and Korbonits 2014). In the modern era, active fractions were identified from this natural product and drug discovery efforts led to the development of three biguanides for diabetes therapy:

<table>
<thead>
<tr>
<th>Chi et al. 2015</th>
<th>Custirsen (OGX-011)</th>
<th>Suppresses levels of cytoprotective chaperone clusterin</th>
<th>Custirsen-docetaxel treatment did not improve OS compared to the docetaxel only</th>
</tr>
</thead>
</table>

Chi et al 2015 Custirsen (OGX-011) Suppresses levels of cytoprotective chaperone clusterin Custirsen-docetaxel treatment did not improve OS compared to the docetaxel only

Chi et al 2015 Custirsen (OGX-011) Suppresses levels of cytoprotective chaperone clusterin Custirsen-docetaxel treatment did not improve OS compared to the docetaxel only
metformin, phenformin, and buformin (Foretz et al 2014; Kourelis et al 2012; Pollak 2010; Pernicova and Korbonits 2014). However, phenformin and buformin were withdrawn from use in the early 1970s due to risk of lactic acidosis and increased cardiac mortality, while metformin demonstrated an excellent safety profile and therapeutic index (Foretz et al 2014; Pollak 2010). Since metformin was approved for the treatment of diabetes in the 1970s in Europe and in 1995 in the United States, it has become the most widely used oral hypoglycemic agent and is recommended as first line therapy for all newly diagnosed Type 2 diabetes patients in the United States (Quinn et al 2013; Foretz et al 2014; Pollak 2010; American Diabetes Association 2014). The highly favourable safety profile of metformin has also led to its use for a number of other conditions including polycystic ovarian syndrome (Palomba et al 2009), metabolic syndrome (Bianchi et al 2007), gestational diabetes (Viollet et al 2012) and diabetes prevention in high risk patients (Knowler et al 2002).

1.6.2 Epidemiological Studies of Metformin and Cancer
The concept of using biguanides in oncology was first explored by Dilman (1971) who proposed using metabolic rehabilitation, consisting of combining biguanides with caloric restriction, for treating cancer patients (Quinn et al 2013). Patients with breast, colorectal, or gastric cancer treated with this metabolic rehabilitation approach had reduced primary tumor development and lower incidence of metastases (Dilman et al 1988; Quinn et al 2013; Kasznicki et al 2014). However, the amount of investigation into the anti-neoplastic effects of metformin did not become significant until Evans et al (2005) published a study demonstrating a reduced cancer burden in diabetic patients treated with metformin compared to those treated with other types of diabetic therapy. These results led to a significant increase in the amount of research devoted to further elucidating how metformin use may affect cancer risk and outcomes.

Libby et al (2009) demonstrated diabetic patients taking metformin had a significantly reduced cancer risk compared to the control group of type 2 diabetic patients who had no record of metformin use after adjusting for sex, age, BMI, A1C levels, smoking, and other drug use (HR = 0.63). A number of subsequent studies have also shown diabetic patients using metformin to
manage type II diabetes have a reduced cancer risk and mortality compared to diabetic patients using other types of diabetic therapy. A systematic review and meta-analysis conducted by DeCensi et al (2010) demonstrated 31% reduction in overall cancer risk for patients taking metformin compared to other antidiabetic medications and Noto et al (2012) demonstrated a lower risk ratio for cancer mortality (RR = 0.66) and all-cancer incidence (RR = 0.67) for metformin users compared to non-metformin users. A systematic review completed by Franciosi et al (2013) showed there was a significant association of exposure to metformin and a reduced risk of cancer death (OR = 0.65) and a reduced risk of all malignancies (OR = 0.73). Most recently, Gandini et al (2014) conducted a systematic review and meta-analysis which including adjusting for confounders such as BMI and time-related biases. Metformin use was associated with a 31% reduction in cancer incidence (SRR = 0.69), similar to the DeCensi (2010) study, and a 34% reduction in cancer mortality (SRR = 0.66) compared to other antidiabetic drugs (Gandini et al 2014). After adjusting for BMI, a significant reduction in cancer incidence for metformin users was still observed, although at a lower magnitude (SRR = 0.82), and a significant reduction in cancer mortality was maintained (SRR = 0.60). After adjusting for time-related biases, metformin use was still associated with a significant reduction in overall cancer incidence (SRR = 0.90) (Gandini et al 2014). Furthermore, metformin use in diabetic patients has been associated with a reduction in risk of pancreatic cancer (Li et al 2009, Wang et al 2014), colorectal cancer (Zhang et al 2011; Singh et al 2013, Mei et al 2014) and breast cancer (Bodmer et al 2010, Col et al 2012), although the association is stronger in long-term metformin users for reducing breast cancer risk. The epidemiological data has demonstrated a convincing anti-cancer effect of metformin use and has led to more detailed investigation of the characteristics of metformin that contribute to its anti-neoplastic effects.

1.6.3 Pharmacological Characteristics of Metformin
The pharmacological characteristics of metformin are important to consider for understanding its mechanism of action at both the systemic and cellular level. Due to the hydrophilic chemical nature of metformin, the passive diffusion of metformin through cell membranes is very limited (Graham et al 2011). Therefore, metformin must be actively transported into the cell by organic cation transporters (OCTs), such as OCT1, which affects the distribution, elimination and
bioavailability of metformin (Graham et al 2011; Gong et al 2012). Tissues, such as the liver, which have high levels of OCT transporters usually accumulate higher concentrations of metformin (Foretz et al 2014; Gong et al 2012). It is important to note that the expression of OCTs on tumor cells has not yet been fully elucidated and is still under investigation.

The optimal oral dose of metformin for many diabetic patients is 2000mg/day and the oral bioavailability of metformin is approximately 50-60% (Foretz et al 2014; Bailey et al 1996; Graham et al 2011). Absorption occurs predominantly in the small intestine, but relatively high metformin concentrations can also be found in the liver and kidney due to the high levels of OCT expression in these tissues which enhances cellular uptake (Foretz et al 2014; Pollak 2013; Gong et al 2012). The higher concentrations of metformin found in the liver are also likely due to the liver being supplied by the portal vein (Foretz et al 2014; Wilcock and Bailey 1994). The peak plasma concentration of metformin is achieved after approximately 2-3 hours and ranges from 1.0 to 1.6 ug/mL (6-10uM) after a 0.5g dose up to ~3ug/mL (~18uM) after a 1.5g dose (Bailey et al 1996; Tucker et al 1981). Metformin is not metabolized by the liver and is excreted unchanged in the urine with an elimination half-life of approximately 5 hours in patients with good renal function (Graham et al 2011; Gong et al 2012). When the human metformin dose of 20mg/kg/day is adapted to 250mg/kg/day in mice, according to the normalization of body surface area, the murine plasma levels of metformin achieved peaked at 1.6ug/mL (~10uM) and accumulation occurred primarily in the gastrointestinal tract, kidney, and liver (Memmott et al 2010; Foretz et al 2014; Wilcock and Bailey 1994).

The accumulation of metformin varies not only among tissues, but also within cells. Due to the positive charge on the biguanide moiety of metformin, it accumulates in the mitochondrial matrix in response to the proton-motive forces across the inner mitochondrial membrane, and this well-described physical phenomenon results in an intramitochondrial concentration of metformin that is far above the external concentration (Bridges et al 2014; Owen et al 2000). In vitro, a 100-fold accumulation of metformin in the mitochondria of cells compared to the external medium has been shown (Bridges et al 2014). Furthermore, in vivo the combined plasma and mitochondrial membrane potentials accumulate metformin by 1000-fold in the mitochondrial
matrix relative to the serum (Bridges et al 2014). This represents an increase from the serum concentrations of 10uM and 200nM to a mitochondrial concentration of 10mM and 200uM which can inhibit catalysis by 25% (Bridges et al 2014). Moving forward it will be extremely important to further elucidate the accumulation of metformin in not only different tissues, but in the mitochondria specifically.

1.6.4 Metformin Mechanism of Action
The anti-neoplastic effects of metformin can be divided into two categories: systemic effects that indirectly influence cancer and direct effects on cancer cells (Foretz et al 2014; Pollak 2012). Both mechanisms will be discussed in the following section.

1.6.4.1 Indirect Systemic Effect of Metformin on Cancer
Type 2 diabetes is characterized by insulin resistance in classic insulin target tissues such as liver, muscle, and fat, which leads to hyperglycemia and secondary hyperinsulinemia (Pollak 2012). Metformin is used to effectively treat Type 2 diabetes primarily due to its ability to decrease hepatic glucose production by suppressing gluconeogenesis and enhancing insulin suppression of endogenously produced glucose (Foretz et al 2014; Natali and Ferrannini 2006). The specific mechanism by which metformin reduces hepatic gluconeogenesis still needs to be fully elucidated since AMPK (AMP-activated protein kinase)-dependent, AMPK-independent, and mitochondrial glycerophosphate dehydrogenase-dependent mechanisms have all been investigated (Shaw et al 2005; Foretz et al 2010; Miller et al 2013; Madiraju et al 2014). Metformin can also reduce intestinal glucose absorption and improve glucose uptake and utilization in peripheral tissues, such as skeletal muscle, and this effect coupled with decreased gluconeogenesis contributes to a lower levels of circulating glucose (Foretz et al 2014; Pollak 2012; Natali and Ferrannini 2006; Turban et al 2012). The reduction in circulating glucose levels achieved with metformin treatment can also lead to secondary reduction of insulin secretion, and therefore reduction of hyperinsulinemia if present at baseline (Pollak 2012; Pollak 2013; Foretz et al 2014).
The mechanisms by which metformin can be used to manage type 2 diabetes also have an effect on cancer biology (Foretz et al 2014). Hyperinsulinemia has been identified as an adverse prognostic factor and/or risk factor in several types of cancer, including prostate cancer (Pollak et al 2012; Ma et al 2008). Metformin treatment could therefore slow the growth of insulin responsive tumors by reducing insulin levels (Pollak 2012; Pollak 2012). Other systemic effects of metformin may be due to effects on inflammatory mediators which could influence chronic inflammatory states which favor cancer development (Pollak 2013). Metformin has also been shown to antagonize glucagon signaling which reduces glucose output from hepatocytes and lowers fasting glucose levels (Miller et al 2013). For prostate cancer specifically, ADT has been shown to increase circulating insulin levels (Smith et al 2006). There is also evidence that insulin promotes local androgen synthesis by prostate cancer cells, which is a resistance mechanism to castration (Lubik et al 2011). Therefore, there is a strong rationale for using metformin in combination with androgen deprivation therapy to get the benefit of lowered hyperinsulinemia and reducing local androgen synthesis (Pollak 2012).

1.6.4.2 Direct Effect of Metformin on Cancer Cells

Metformin is hydrophilic and requires active transport into cells by membrane transport proteins, such as organic cation transporter 1 (OCT1) (Segal et al 2011). Metformin, being a positively charged molecule, then accumulates in high concentrations in the mitochondria due to the mitochondrial membrane potential and the mechanism of metformin action is mediated through direct effects on mitochondrial function (Pollak 2013). The initial studies which investigated the mechanism of metformin action were completed in 2000 and demonstrated metformin decreases oxidative phosphorylation through mild, specific inhibition of the mitochondrial respiratory-chain complex 1 (NADH:ubiquinone oxidoreductase) without affecting other components of mitochondrial machinery (Foretz et al 2014; El-Mir et al 2000; Owen et al 2000). Since then, specific inhibition of mitochondrial complex 1 by metformin was confirmed in a wide range of in vitro and in vivo models, including cancer cells (Foretz et al 2014; El-Mir et al 2000; Owen et al 2000; Bridges et al 2014; Wheaton et al 2014). The specific mechanism by which metformin inhibits mitochondrial complex 1 has not yet been fully elucidated, however it has been reported that metformin can directly inhibit the function of mitochondrial complex 1 in a purified form, as
well as in isolated mitochondria and in submitochondrial particles derived from the bovine heart (Foretz et al 2014; Andrzejewski et al 2014; Bridges et al 2014).

The inhibition of complex 1 causes a drop in cellular energy charge which results in a decreased cellular ATP concentration and an increase in both ADP/ATP and AMP/ATP ratios (Foretz et al 2014). The increased level of AMP and decreased level of ATP activates tumor suppressor LKB1, an upstream kinase which regulates AMP-activated protein kinase (AMPK), a critical sensor of cellular energy homeostasis (Hardie et al 2012). LKB1 then phosphorylates AMPK at Thr172 to activate catalytic subunit AMPKα (Shaw et al 2004; Shaw et al 2004b). In times of energetic crisis, AMPK activation directs cells toward an “energy-saving” phenotype by down-regulating ATP-consuming processes such as protein and fatty acid synthesis (Pollak 2012; Viollet et al 2012; Pollak 2013; Mayer et al 2015). More specifically, phosphorylated AMPK then phosphorylates and activates tumor suppressor genes tuberous sclerosis complex 1 (TSC1) and TSC2. TSC1 and TSC2 form an mTOR-inhibition complex which inhibits the mammalian target of rapamycin (mTOR) pathway and this disturbs protein synthesis and tumor cell proliferation (Pernicova and Korbonits 2014). The energetic stress caused by the reduction in oxidative phosphorylation following metformin treatment is considered an important component of its anti-neoplastic effect. Although most cancer cells increase glycolysis (i.e. the Warburg effect) mitochondrial ATP production is not inconsequential (Pollak 2013). Even with high levels of glycolysis mitochondrial oxidative phosphorylation contributes to ATP production and is involved in other metabolic functions, such as maintenance of the redox balance for citric acid cycle function (Pollak 2013; Pollak 2013). This mechanism is illustrated in Figure 1.2.

The specific mechanism of metformin acting in prostate cancer will be explored in the section focused on pre-clinical evidence of metformin in prostate cancer.
Figure 1.2: The direct effects of metformin on cancer cells. Metformin is actively transported into the cell by organic cation transporters (OCTs) and then accumulates in the mitochondria due to the mitochondrial membrane potential. Metformin then inhibits mitochondrial complex 1 activity which reduces mitochondrial oxidative phosphorylation and ATP production. A reduction in ATP results in increased ADP/ATP and AMP/ATP ratios. The increased AMP/ATP ratio activates LKB1 and LKB1 then phosphorylates AMPK. Activated AMPK then phosphorylates TSC1 and TSC2 which form an mTOR-inhibition complex which inhibits mTOR signaling and reduces protein synthesis and cell proliferation.

1.7 Metformin and Prostate Cancer

1.7.1 Clinical Studies of Metformin and Prostate Cancer

Epidemiological studies investigating the effect of metformin use on prostate cancer outcomes have produced more mixed results compared to overall cancer risk and mortality. A retrospective cohort study completed by Currie et al (2009) demonstrated that metformin did not have a significant effect on reducing prostate cancer risk compared to treatment with sulfonylureas, metformin plus sulfonylureas, or insulin-based therapies. However, a population-based case-control study by Wright et al (2009) showed metformin use was associated with a borderline significant decrease in risk of prostate cancer in Caucasian men (Odds ratio = 0.56, 95% confidence interval 0.32-1.00). A study evaluating the occurrence of malignancies in patients...
participating in the ADOPT (A Diabetes Outcome Progression Trial) and RECORD (Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycaemia in Diabetes) trials showed metformin treatment provided no significant protection against prostate cancer compared to rosiglitazone (Home et al. 2010). However, the number of prostate cancer cases was extremely small within each trial. For example looking at the ADOPT data, of the 1,454 patients using metformin only 10 developed a prostatic malignancy (1.2%), and the percentages were similar for the other groups within the ADOPT trial and these similar percentages extended to the RECORD trial as well (Home et al. 2010). Additional retrospective studies have also demonstrated that metformin use did not reduce the risk of prostate cancer in patients with Type 2 diabetes (Azoulay et al. 2011; Feng et al. 2015). A large retrospective cohort study conducted using health-care administrative databases in Ontario identified that in a cohort of 119,315 men with diabetes, of which 5,306 subjects developed prostate cancer, there was no association between metformin use and risk of any prostate cancer (Odds ratio = 1.03, 95% confidence interval = 0.96-1.1), low-grade prostate cancer (Odds ratio = 0.94, 95% confidence interval = 0.82-1.06), high-grade prostate cancer (Odds ratio = 1.13, 95% confidence interval = 0.96-1.32), or biopsy-diagnosed cancer (Odds ratio = 0.98, 95% confidence interval = 0.84-1.02) compared to other oral hypoglycemic agents (Margel et al. 2013). However, another retrospective nested case-control study completed in Denmark showed metformin use was associated with a reduced risk of prostate cancer diagnosis, while other antidiabetic medications did not reduce risk (Preston et al. 2014). Therefore, given the current evidence, the effect of metformin on prostate cancer risk is still under investigation.

The results regarding the use of metformin after prostate cancer diagnosis have also been mixed but provide some encouraging results. A number of studies have investigated the effect of metformin on biochemical recurrence of prostate cancer. Metformin has been shown to have no significant effect on biochemical recurrence of prostate cancer following radical prostatectomy (Allott et al. 2013; Kaushik et al. 2014; Raval et al. 2015). But a meta-analysis of 2,953 patients demonstrated a significant reduction in biochemical recurrence of prostate cancer was associated with metformin use (Yu et al. 2014). Another study which stratified metformin use based on the type of exposure showed that ever use of metformin or use of metformin only after diagnosis is
not associated with reduced PCa recurrence, but use of metformin both before and after diagnosis resulted in a decreased risk of PCa recurrence (Winters et al 2015). Furthermore, patients in the highest quartile of metformin dosage or cumulative duration of dosing had a significant 58% reduction in risk of biochemical recurrence compared to those who had never taken metformin (Winters et al 2015). Another very recent study showed metformin use was associated with significantly fewer deaths, recurrences, metastases and secondary cancers compared to non-metformin users (Chong et al 2016). A pilot study of neoadjuvant metformin use in localized prostate cancer demonstrated a significantly reduced Ki67 index, significantly reduced P-4EBP1 staining and a trend towards PSA reduction, indicating metformin is well-tolerated prior to radical prostatectomy and supports further investigation into using metformin in this context (Joshua et al 2014). This led to the Metformin Active Surveillance Trial (MAST) study (NCT01864096) which is currently recruiting patients to evaluate the effect of metformin compared to placebo on prostate cancer outcomes in active surveillance patients (Joshua et al 2014).

The effect of metformin in castration-resistant prostate cancer patients has produced some interesting results. In diabetic patients diagnosed with prostate cancer, metformin use was associated with significantly improved prostate-cancer specific survival, PSA-recurrence free survival, and distant metastases-free survival (Spratt et al 2013). Furthermore, metformin use was independently associated with decreased development of CRPC in patients with biochemical failure compared to diabetic patients not using metformin (Spratt et al 2013). A multicentre Phase 2 trial (SAKK 08/09) conducted in Switzerland investigated the effect of metformin on prostate cancer progression (Rothermundt et al 2014). 44 nondiabetic men with metastatic CRPC received 1000mg of metformin twice per day until disease progression was observed (Rothermundt et al 2014). Metformin use was also shown to be safe in nondiabetic patients and the primary end point of absence of disease progression at 12 weeks was reached with 36% of patients progression-free at 12 weeks and 9.1% progression-free at 24 weeks (Rothermundt et al 2014). 52.3% of patients experienced a prolongation of PSA doubling-time after starting metformin (Rothermundt et al 2014). These encouraging results should be interpreted with caution however due to the very small sample size of this study.
The effect of metformin use and prostate-cancer specific and overall survival has been evaluated in prostate cancer patients. Metformin use has shown a trend towards reducing the odds of high grade tumors and decreased risk of progression but this was not significant (Hitron et al 2012). A large population-based retrospective cohort study conducted in Ontario including 3,837 diabetic patients showed cumulative duration of metformin use after prostate cancer diagnosis was associated with a significant decreased risk of prostate-cancer specific and all-cause mortality in a dose-dependent manner (Margel et al 2013). Another retrospective study conducted using data from the United Kingdom showed metformin use after prostate cancer diagnosis was not associated with a decreased risk of prostate cancer-specific or all-cause mortality (Bensimon et al 2014). A meta-analysis investigating the effect of metformin on mortality following cancer in diabetic patients demonstrated metformin use was associated with a significant reduction in cancer-specific and all-cause mortality, but subgroup analysis for prostate cancer did not show significance (Lega et al 2014). However, another recent meta-analysis investigating the effect of metformin use on the treatment outcomes of prostate cancer demonstrated a significant increase in overall survival but no significant association with prostate cancer-specific survival (Stopsack et al 2016).

The studies discussed in this section are summarized in Table 1.1. The current clinical evidence for metformin use is somewhat mixed regarding the results, but the use of metformin after diagnosis with prostate cancer does indicate a novel use for this medication. Further investigation is needed in order to fully elucidate the effect of metformin in prostate cancer, particularly in later stages of the disease.
Table 1.2: Summary of clinical studies of metformin and prostate cancer. \( HR \): Hazard ratio; 95\% \( CI \): 95\% confidence interval; \( OD \): Odds ratio; \( RR \): Relative risk; \( BCR \): Biochemical recurrence; \( RP \): radical prostatectomy; \( PCSM \): Prostate cancer-specific mortality; \( PSA-RFS \): Prostate-specific antigen-recurrence-free survival; \( DMFS \): Distant metastases-free survival.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Type</th>
<th>Summary of Results</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>Currie et al 2009</td>
<td>Retrospective cohort (UK) study</td>
<td>Compared to metformin alone, treatment with sulfonylureas (HR: 1.07), metformin plus sulfonylureas (HR: 1.18) or insulin-based therapies (HR: 1.10) did not have a significant effect on risk of developing PCa</td>
<td>Metformin use was not associated with lower risk of PCa compared to other anti-diabetic therapies</td>
</tr>
<tr>
<td>Wright et al 2009</td>
<td>Population-based case-control study</td>
<td>Metformin use was associated with a borderline significant 44% reduction in relative risk of PCa (OR: 0.56, 95% CI: 0.32-1.00) in Caucasian men</td>
<td>Metformin use was associated with a reduction in relative risk of PCa in Caucasian men</td>
</tr>
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</table>
| Home et al 2010    | Retrospective cohort study                      |  \( ADOPT \) data: Metformin vs rosiglitazone HR: 0.92 and metformin vs glibenclamide HR: 0.78  

  \( RECORD \) data: On background sulfonylurea metformin vs rosiglitazone HR: 1.22 |
<p>|                    |                                                 |                                                                                       | Metformin provided no significant protection against development of PCa                              |
| Azoulay et al 2011 | Retrospective cohort study with nested case-control approach | Metformin users had a 1.23 adjusted risk ratio (95% CI: 0.99-1.52) of PCa compared to never users of metformin | Metformin does not reduce the risk of PCa in patients with Type II diabetes                           |
| Feng et al 2015    | Retrospective cohort study                      | Metformin use was not associated with total (OR: 1.19; ( p = 0.50 )), low-grade (OR: 1.01, ( p = 0.96 )), or high-grade (OR: 1.83, ( p = 0.19 )) PCa diagnosis | Metformin was not associated with a reduced risk of PCa diagnosis in diabetic patients               |
| Margel et al 2013a | Retrospective cohort study with nested case-control approach | No association between metformin use and risk of any prostate cancer (OR: 1.03; 95% CI: 0.96-1.1), high-grade PCa (OR: 1.13; 95% CI: 0.96-1.32), low-grade PCa (OR: 0.94; 95% CI: 0.82-1.06), or biopsy-diagnosed PCa (OR: 0.98; 95% CI: 0.84-1.02) | Metformin use was not associated with risk of PCa diagnosis in older men with diabetes               |</p>
<table>
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<tr>
<th>Study</th>
<th>Design</th>
<th>Findings</th>
<th>Conclusion</th>
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</thead>
<tbody>
<tr>
<td>Preston et al 2014</td>
<td>Retrospective cohort study with nested case-control approach</td>
<td>Metformin users had a decreased risk of PCa diagnosis compared with never users (OR: 0.84; 95% CI: 0.74-0.96). In the PSA-tested group metformin use was associated with decreased risk PCa compared to no use (OR: 0.66; 95% CI: 0.51-0.86)</td>
<td>Metformin use associated with decreased risk of PCa diagnosis</td>
</tr>
<tr>
<td>Allott et al 2013</td>
<td>Retrospective cohort study</td>
<td>Time to BCR showed no association with metformin use (HR: 0.93; 95% CI: 0.61-1.41), high metformin dose (HR: 0.96; 95% CI: 0.57-1.61) or duration of metformin use (HR: 1.00; 95% CI: 0.99-1.02)</td>
<td>Metformin use, dose, or duration of use was not associated with BCR in patients treated with RP</td>
</tr>
<tr>
<td>Kaushik et al 2014</td>
<td>Retrospective cohort study</td>
<td>Metformin use and BCR had a HR: 0.91 (95% CI: 0.67-1.24, p = 0.56). Metformin use and systemic progression had a HR: 0.83 (95% CI: 0.39-1.74, p = 0.62). Metformin use and all-cause mortality had a HR: 1.16 (95% CI: 0.73-1.86, p = 0.53).</td>
<td>Metformin use not associated with risk reduction of BCR, progression or all-cause mortality</td>
</tr>
<tr>
<td>Raval et al 2015</td>
<td>Systematic review and meta-analysis</td>
<td>Metformin use was marginally associated with reduced risk of BCR (HR: 0.82; 95% CI: 0.67-1.01, p = 0.06). Metformin use was not associated with metastases (HR: 0.59; 95% CI: 0.30-1.18, p = 0.14), all-cause mortality (HR: 0.86, 95% CI: 0.67-1.10, p = 0.23), and prostate cancer-specific mortality (HR: 0.76; 95% CI: 0.43-1.33, p = 0.33)</td>
<td>Metformin may reduce the risk of BCR in PCa</td>
</tr>
<tr>
<td>Yu et al 2014</td>
<td>Meta-analysis</td>
<td>Metformin use associated with significant decreased cancer risk (OR: 0.91; 95% CI: 0.85-0.97) and BCR of PCa (HR: 0.81; 95% CI: 0.68-0.98)</td>
<td>Metformin use associated with significant reduction in PCa risk and BCR</td>
</tr>
<tr>
<td>Winters et al 2015</td>
<td>Retrospective cohort study</td>
<td>Ever metformin use not associated with BCR (HR: 1.12; 95% CI: 0.77-1.65) but</td>
<td>Metformin use associated with reduced BCR after</td>
</tr>
<tr>
<td>Study</td>
<td>Study Design</td>
<td>Findings</td>
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<tr>
<td>Chong et al 2016</td>
<td>Retrospective cohort study</td>
<td>Metformin use among diabetic PCa patients was associated with significantly (p &lt; 0.004) fewer deaths (10% vs 23%), recurrences (8% vs 15%), metastases (0% vs 5%), and secondary cancers (6% vs 17%) compared to non-diabetic PCa patients</td>
<td>Metformin use significantly reduced deaths, recurrences, metastases, and secondary cancers</td>
</tr>
<tr>
<td>Joshua et al 2014</td>
<td>Pilot “window of opportunity” neoadjuvant study</td>
<td>Metformin significantly reduced Ki67 proliferation index by 29.5% (p = 0.0064) per patient and significantly reduced P-4EBP1 staining (p &lt; 0.001). There was a trend towards PSA reduction (p = 0.08). Treatment was well tolerated with only 3 patients experiencing G3/4 toxicities</td>
<td>Neoadjuvant metformin well tolerated prior to RP and has promising effects on metabolic, proliferation and signaling</td>
</tr>
<tr>
<td>Spratt et al 2013</td>
<td>Retrospective cohort study</td>
<td>Metformin use significantly reduced prostate cancer-specific mortality (p ≤ 0.001). Metformin improved PSA-RFS (HR: 1.99, 95% CI: 1.24-3.18, p = 0.004), DMFS (HR: 3.68, 95% CI: 1.78-7.62, p &lt; 0.001), PCSM (HR: 5.15, 95% CI: 1.53-17.35, p = 0.008). Metformin use decreased development of CRPC in patients experiencing BCR (OR: 14.81, 95% CI: 1.83-119.89, p = 0.01)</td>
<td>Metformin use may improve PSA-RFS, DMFS, PCSM, OS and reduce CRPC development</td>
</tr>
<tr>
<td>Rothermundt et al 2014</td>
<td>Single arm Phase II prospective trial</td>
<td>Among non-diabetic patients taking metformin 36% were progression-free at 12 weeks and 9.1% were progression-free at 24 weeks. 52.3% of patients taking metformin had a prolongation of PSA doubling-time</td>
<td>Metformin treatment safe in nondiabetic patients and results in objective PSA responses and may stabilize disease</td>
</tr>
<tr>
<td>Study</td>
<td>Study Type</td>
<td>Findings</td>
<td>Comments</td>
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<tr>
<td>Hitron et al 2012</td>
<td>Retrospective cohort study with nested case-control approach</td>
<td>Metformin use trended towards decreased odds of high grade PCa (OR: 0.61, 95% CI: 0.23-1.62) and decreased PCa progression (OR: 0.58, 95% CI 0.22-1.53) but neither result was significant.</td>
<td>Use of metformin trended towards decreased odds of high-grade PCa and decreased progression of PCa</td>
</tr>
<tr>
<td>Margel et al 2013</td>
<td>Retrospective cohort study</td>
<td>Cumulative duration of metformin treatment after PCa diagnosis was associated with a significant decrease in PCa-specific mortality (HR: 0.76, 95% CI: 0.64-0.89). Metformin was associated with a significant decrease of all-cause mortality in the first 6 months (HR: 0.76; 95% CI: 0.70-0.82)</td>
<td>Increased cumulative metformin exposure after PCa diagnosis associated with decreased all-cause and PCa-specific mortality</td>
</tr>
<tr>
<td>Bensimon et al 2014</td>
<td>Retrospective cohort study with nested case-control approach</td>
<td>Use of metformin post-PCa diagnosis was not associated with a decreased risk of cancer-specific mortality (RR: 1.09; 95% CI: 0.51-2.33) or all-cause mortality (RR: 0.79; 95% CI: 0.50-1.23)</td>
<td>Metformin use after PCa diagnosis not associated with decreased risk PCa-specific or all-cause mortality</td>
</tr>
<tr>
<td>Lega et al 2014</td>
<td>Systematic review and meta-analysis</td>
<td>Metformin use was associated with a reduction in all-cause mortality (HR: 0.73; 95% CI: 0.64-0.83) and cancer-specific mortality (HR: 0.74; 95% CI: 0.62-0.88). However, there was no significant effect on PCa</td>
<td>Metformin use associated with reduced all-cause and cancer-specific mortality but no significant effect on PCa</td>
</tr>
<tr>
<td>Stopsack et al 2016</td>
<td>Systematic review and meta-analysis</td>
<td>Metformin use associated with improved overall survival (HR: 0.88, 95% CI: 0.86-0.90) but not PCa-specific mortality (HR: 0.76, 95% CI: 0.44-1.31)</td>
<td>Metformin use associated with significant increase in overall survival but not PCa-specific survival</td>
</tr>
</tbody>
</table>
1.7.2 Pre-clinical Studies of Metformin and Prostate Cancer

Metformin was initially shown to significantly inhibit the proliferation of prostate cancer cell lines in a dose-dependent manner (Sahra et al 2008). Metformin injected daily intraperitoneally at 1mg per day or added to drinking water led to significant inhibition of tumor growth in LNCaP xenografts (Sahra et al 2008). The mechanism of this effect was shown to be due to metformin blocking the cell cycle in G0/G1 by inhibiting cyclin D1 expression and retinoblastoma protein (Rb) phosphorylation independently of AMPK (Sahra et al 2008). Cyclin D1 regulates the cell cycle machinery by binding to and activating cyclin dependent kinase 4/6 which then phosphorylates Rb (Sahra et al 2008). Rb then releases the transcription factor E2F which then activates the transcription of genes required for G1/S phase transition (Sahra et al 2008). However, these results were obtained using LNCaP cells which were derived from a lymph node metastasis and is one of the less aggressive PCa cell lines (Sahra et al 2008). However, in PC3 cells, which are aggressive androgen-independent PCa cells, metformin was shown to inhibit growth through the phosphorylation and activation of AMPK in an LKB1-dependent manner (Zakikhani et al 2008). Metformin was then shown to increase REDD1 (negative regulator of mTOR) expression in a p53-dependent manner which mediated mTOR inhibition and decreased cyclin D1 expression independently of AMPK (Sahra et al 2011). However, these results were again obtained using LNCaP cells which express functional p53. In DU145 prostate cancer cells which have non-functional mutated p53 and PC3 prostate cancer cells which are p53-null, REDD1 expression did not change but phosphorylation of AMPK did occur (Sahra et al 2011). This indicates an AMPK-dependent effect of metformin in these cells (Sahra et al 2011). Metformin has also been shown to down-regulate AR mRNA levels, which reduces AR protein levels and represses AR signaling, and this leads to inhibition of prostate cancer cell growth and induction of apoptosis in androgen-sensitive PCa cell lines (Wang et al 2015). Metformin treatment has been shown to inhibit proliferation, migration, and invasion of PC3 prostate cancer cells and inhibit tumor growth in a PC3 cell xenograft mouse model by reducing IGF-1R (insulin-like growth factor 1 receptor) expression (Kato et al 2015). Therefore, the precise mechanism of action of metformin in prostate cancer cells is still under investigation.

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A number of pre-clinical studies of metformin in prostate cancer have investigated the effect of metformin in combination with other agents. Metformin combined with 2-deoxyglucose (2DG), an inhibitor of glucose metabolism, functioned synergistically to induce a large amount of ATP depletion in prostate cancer cells which significantly inhibited cell viability compared to either metformin or 2DG alone (Sahra et al 2010). The reduced cell viability was due to the combination of metformin and 2DG causing induction of apoptosis via AMPK activation (Sahra et al 2010). Metformin combined with bicalutamide, a non-steroidal antiandrogen which blocks the AR, resulted in a significant reduction in cell survival, particularly in PCa cells expressing a functional AR (Colquhoun et al 2012). The anti-proliferative effect was mediated by the phosphorylation and activation and AMPK and subsequent inhibition of downstream mTOR signaling along with G1/S cell cycle arrest (Colquhoun et al 2012). In vivo experiments using LNCaP xenografts showed metformin combined with bicalutamide significantly reduced tumor volume and may be linked to the significant reduction in IGF1 (insulin-like growth factor 1) levels (Colquhoun et al 2012). Metformin has also been used in combination with rapamycin, a macrolide used clinically as an immunosuppressant in organ transplant patients and has been shown to have potent cancer chemopreventive properties (Saha et al 2015). In HiMyc mice, a model which overexpresses c-Myc in the prostate which results in the development of prostatic lesions that share both molecular and histopathological features with human prostate tumors, metformin combined with rapamycin significantly decreased in situ adenocarcinomas and completely inhibited locally invasive adenocarcinomas (Saha et al 2015). The inhibition of prostate cancer progression was associated with significant reduction in mTOR signaling (Saha et al 2015). Metformin combined with doxorubicin has been shown to reduce growth of PC3 xenograft tumors and inhibit tumor relapse (Iliopoulos et al 2011). Furthermore, the combination of metformin and doxorubicin has been shown to be particularly effective in inflammatory PCa xenografts (DU145 cells) compared to noninflammatory PCa xenografts (LNCaP cells) indicating metformin may inhibit inflammatory pathways which contributes to its anti-cancer effect (Hirsch et al 2013). Metformin combined with simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR) which is involved in cholesterol production, has been shown to significantly and synergistically reduce viability and metastatic potential in CRPC cell lines compared to docetaxel chemotherapy through the phosphorylation of AMPK and inhibition of downstream anabolic pathways (Babcock et al 2014a). The cell death mechanism
induced by combined metformin and simvastatin treatment functioned through to significant cell cycle arrest in the G1-phase and a reduced percentage of cells in the S-phase, as well as induction of necrosis (Babcock et al 2014b). Furthermore, metformin and simvastatin given daily by oral gavage for 9-weeks significantly inhibited tumor formation, cachexia, bone metastases and biochemical failure compared to docetaxel (Babcock et al 2014a).

The studies discussed in this section are summarized in Table 1.2. Therefore, although there has been investigation pre-clinically into the action of metformin alone and in combination with novel agents, the combination of metformin and docetaxel chemotherapy has not been evaluated.

Table 1.3: Summary of preclinical studies of metformin and prostate cancer.

<table>
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<th>Reference</th>
<th>Study Type</th>
<th>Summary of Results</th>
<th>Conclusion</th>
</tr>
</thead>
</table>
| Sahra et al 2008   | In vitro: LNCaP, DU145, PC3 (human PCa cell lines) In vivo: LNCaP xenograft | **In vitro:** Metformin treatment inhibited proliferation of PCa cells. Metformin treatment blocked cell cycle in the G0/G1 phase, reduced cyclin D1 levels and activated AMPK in LNCaP cells  
**In vivo:** Oral and intraperitoneal treatment of LNCaP xenografts with metformin significantly reduced tumor growth and reduced cyclin D1 levels | Metformin significantly inhibited PCa cell proliferation (especially LNCaP cells) and LNCaP xenograft tumor growth by decreasing cyclin D1 |
<p>| Zakikhani et al 2008 | In vitro: PC3 cells | Metformin treatment inhibited PC3 cell growth and increased expression of phosphorylated AMPK which resulted in a decline in protein synthesis in an LKB1-dependent manner. siRNA targeting AMPKα1 reduced phosphorylated AMPK levels | Metformin inhibited growth of PC3 cells in a dose-dependent manner through the activation of AMPK |
| Sahra et al 2011   | In vitro: LNCaP, DU145, PC3 cells | In LNCaP cells metformin treatment increased REDD1 expression which inhibited mTOR. REDD1 was regulated by p53 and required for cell cycle arrest caused by metformin. siRNA targeting | The p53/REDD1 axis may be a new molecular target of metformin that contributes to its anti-cancer effect |</p>
<table>
<thead>
<tr>
<th>Authors</th>
<th>In vitro:</th>
<th>Metformin effects</th>
<th>In vivo:</th>
<th>Metformin represses PCa cell viability and enhances apoptosis by targeting AR signaling. Demonstrates potential to combine metformin and anti-AR agents</th>
</tr>
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<tbody>
<tr>
<td>Wang et al 2015</td>
<td>LNCaP, 22Rv1 cells (human PCa cell lines)</td>
<td>Metformin treatment significantly increased apoptosis and reduced cell viability in LNCaP and 22Rv1 cells. Metformin combined with bicalutamide showed additive effects in LNCaP cells</td>
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<tr>
<td>Kato et al 2015</td>
<td>PC3 cells</td>
<td>In vitro: Metformin treatment significantly reduced proliferation, migration and invasion. Metformin treatment also significantly decreased insulin growth factor-1 receptor expression</td>
<td>In vivo: High dose metformin treatment significantly reduced tumor growth and insulin growth factor-1 receptor mRNA expression</td>
<td></td>
</tr>
<tr>
<td>Sahra et al 2010</td>
<td>LNCaP, DU145, PC3 cells</td>
<td>Metformin combined with 2-deoxyglucose (2DG) inhibited mitochondrial respiration and depleted ATP and significantly reduced cell viability in LNCaP cells. Metformin and 2DG induced p53 dependent apoptosis via AMPK. Restoring p53 in DU145 and PC3 cells restored caspase-3 activity. Metformin and 2DG also caused G2/M arrest independent of p53</td>
<td></td>
<td>Metformin combined with 2DG significantly reduced cell viability by inducing apoptosis in a p53 dependent manner</td>
</tr>
<tr>
<td>Colquhoun et al 2012</td>
<td>LNCaP, DU145, PC3, PC3AR2 cells</td>
<td>In vitro: Metformin combined with bicalutamide significantly reduced clonogenicity, with a greater effect in AR+ cells. LNCaP cells had decreased phosphorylated mTOR expression and G1/S cell cycle arrest. PC3 cells showed enhanced apoptosis</td>
<td>In vivo: LNCaP xenografts</td>
<td>Metformin combined with bicalutamide significantly reduced PCa cell growth and tumor growth, with AR+ cells displaying reduced proliferation and AR- cells displaying apoptosis</td>
</tr>
<tr>
<td>Study</td>
<td>In vitro:</td>
<td>In vivo:</td>
<td>Metformin combined with bicalutamide significantly reduced tumor growth and significantly reduced insulin growth factor-1 and PSA levels in the serum</td>
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<tr>
<td>Saha et al 2015</td>
<td>LNCaP human PCa cells, HMVP2 murine PCa cells</td>
<td>HiMyc mice</td>
<td>Metformin treatment alone significantly activated AMPK but not mTORC1 in both LNCaP and HMVP2 cells. Combined treatment of metformin and rapamycin activated both AMPK and mTORC1. Metformin combined with rapamycin treatment reduced PCa progression and significantly inhibited mTORC1 signaling as well as tissue inflammation in HiMyc mice.</td>
<td></td>
</tr>
<tr>
<td>Iliopoulos et al 2011</td>
<td>PC3 xenografts</td>
<td>Metformin combined with doxorubicin significantly suppressed growth of PC3 xenograft tumors and inhibited relapse</td>
<td>Metformin combined with doxorubicin suppressed growth of PC3 xenograft tumors.</td>
<td></td>
</tr>
<tr>
<td>Hirsch et al 2013</td>
<td>LNCaP and DU145 xenografts</td>
<td>Metformin combined with doxorubicin effectively reduced tumors generated by inflammatory DU145 cells but did not affect tumors created by noninflammatory LNCaP cells</td>
<td>Inflammatory prostate cancer cells are more susceptible to metformin-based combinatorial therapy.</td>
<td></td>
</tr>
<tr>
<td>Babcock et al 2014a</td>
<td>LNCaP, C4-2B3, B4, B5 human PCa cells</td>
<td>C4-2B4 orthotopic mouse model</td>
<td>Metformin combined with simvastatin acts synergistically to inhibit cell proliferation. Combined treatment also reduces tumor growth and progression of PCa more effectively compared to docetaxel treatment.</td>
<td></td>
</tr>
<tr>
<td>Babcook et al 2014b</td>
<td><em>In vitro</em>: C4-2B3, C4-2B4 cells</td>
<td><em>In vivo</em>: Metformin combined with simvastatin inhibited tumor formation, cachexia, bone metastasis, and biochemical failure more effectively than docetaxel treatment.</td>
<td>Metformin combined with simvastatin causes significant G1-phase cell cycle arrest and decrease in DNA-replicating cells in S-phase in C4-2B cells. Combined treatment led to Ripk1 and Ripk3-dependent necrosis.</td>
<td>Metformin combined with simvastatin significantly inhibits metastatic CRPC cell viability and metastatic properties by inducing cell cycle arrest and necrosis.</td>
</tr>
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Chapter 2
Research Aims and Hypothesis

2.1 Rationale
Advanced stage PCa is usually treated with androgen-deprivation therapy (ADT). However, most patients with metastatic disease managed with ADT eventually relapse with castration-resistant prostate cancer (CRPC) and die of the disease (Feldman and Feldman 2001). The standard of care first-line therapy chemotherapy for symptomatic metastatic CRPC is docetaxel chemotherapy, which provides significant survival benefits, but is associated with a large number of side effects and the survival of metastatic CRPC patients is only approximately 19 months (Heidenreich et al 2013). Thus, there is still a need to improve the therapeutic options available for advanced stage prostate cancer patients. One novel therapeutic approach is combining docetaxel chemotherapy with an agent that enhances its effectiveness which can be defined as a chemosensitizer.

One potential chemosensitizing agent is metformin, a commonly prescribed and well-tolerated oral biguanide used to treat type II diabetes, which has been shown to exert anti-neoplastic effects in multiple types of cancer (Foretz et al 2014). Metformin can be used to manage Type 2 diabetes due to its ability to reduce hepatic gluconeogenesis, increase glucose uptake in peripheral tissue such as skeletal muscle, and increase insulin sensitivity (Pollak 2012; Kourelis et al 2012). The first paper to demonstrate an anti-cancer effect of metformin was published by Evans et al (2005) and showed a reduced cancer burden in diabetic patients treated with metformin compared to those treated with other diabetic therapies. Since the publication of the Evans paper, metformin has been shown to have anti-neoplastic properties in several types of cancer including breast cancer (Zakikhani et al 2010; Alimova et al 2009), ovarian cancer (Rattan et al 2011), pancreatic cancer (Kisfalvi et al 2013), and prostate cancer (Sahra et al 2008).

In prostate cancer, metformin inhibits the proliferation of LNCaP, DU145, and PC3 human prostate cancer cell lines and also reduced tumor growth in LNCaP xenografts (Sahra et al 2008).
More specifically, metformin has been shown to improve the efficacy of chemotherapy in breast cancer and colon cancer models. Metformin has been shown to enhance the tumor suppressing effect of doxorubicin in four different types of breast cancer cell lines and prolong remission in breast cancer xenograft models (Hirsch et al 2009). Furthermore, metformin combined with a 4-fold lower dose of doxorubicin was shown to be as effective as the standard dose of doxorubicin treatment alone in breast cancer xenografts (Iliopoulos et al 2011). Metformin has also been shown to enhance the effect of paclitaxel and carboplatin in breast cancer xenografts at doses comparable to the dose/kg used in type II diabetes patients (Iliopoulos et al 2011). In colon cancer cells, treatment with metformin combined with 5-fluorouracil enhanced the antiproliferative effect of 5-fluorouracil (Zhang et al 2013). In addition, metformin acts synergistically with 5-fluorouracil and oxaliplatin to inhibit cell proliferation and tumor growth of chemo-resistant colorectal cancer cells by reducing the viability of colorectal CSCs (Nangia-Makker et al 2014).

In prostate cancer patients, metformin use is associated with a decreased risk of PCa diagnosis while other oral diabetes medications are not (Preston et al 2014). Increased cumulative metformin exposure after PCa diagnosis has been associated with decreased all-cause and PCa-specific mortality in diabetic patients (Margel et al 2013). A meta-analysis by Yu et al (2014) showed that metformin was associated with a significant reduction in cancer risk and biochemical recurrence. Metformin treatment has also been associated with reduced rate of CRPC development (Spratt et al 2013). Only one trial has been completed to date exploring the combination of metformin and docetaxel in prostate cancer patients. The effect of metformin combined with docetaxel therapy was evaluated in patients who participated in the TAX 327 trial, which was crucial to establishing docetaxel chemotherapy for metastatic castration-resistant prostate cancer (Niraula et al 2013). Metformin did not have a statistically significant additive or synergistic effect with docetaxel, however there were only 38 patients included in the analysis (Niraula et al 2013).
2.2 Overall Hypothesis
Given the very limited data examining the effect of metformin combined with docetaxel chemotherapy both in vitro and in prostate cancer patients, and current literature indicates a potential chemosensitizing effect of metformin, the objective of this thesis is to evaluate the chemosensitizing effect of metformin when combined with docetaxel chemotherapy in castration-resistant prostate cancer.

2.3 Specific Aims and Hypotheses
Two independent studies were conducted to evaluate the chemosensitizing effect of metformin combined with docetaxel chemotherapy in castration-resistant prostate cancer.

2.3.1 Clinical Approach

_Aim 1: Determine if metformin acts as a chemosensitizer when combined with docetaxel chemotherapy in metastatic castration-resistant prostate cancer patients_

We hypothesize that diabetic patients with metastatic castration-resistant prostate cancer administered metformin during docetaxel chemotherapy will have improved prostate cancer-specific and overall survival compared to diabetic metastatic castration-resistant prostate cancer patients on diet control, diabetic patients taking other types of antidiabetic medication, and non-diabetic patients receiving docetaxel chemotherapy. Improved prostate cancer-specific and overall survival in the group taking metformin would indicate a chemosensitizing effect for metformin that would enhance docetaxel treatment efficacy.

2.3.2 In vitro Approach

_Aim 2: Determine if metformin enhances the effect of docetaxel in prostate cancer cell lines_
_Aim 3: Evaluate the mechanism of action for metformin chemosensitization_

We hypothesize that metformin will act as a chemosenzitizing agent and therefore a combination of metformin and docetaxel will be more effective than docetaxel alone at eradicating prostate cancer cells.
2.4 Research Impact

If metformin is shown to have a chemosensitizing effect when combined with docetaxel and improves the chemotherapeutic efficacy of docetaxel, it would provide evidence for a novel therapeutic application for this medication in metastatic castration-resistant prostate cancer patients. This finding would warrant further investigation into the specific molecular mechanisms contributing to the combinatorial treatment effect and also provide support for the development of prospective clinical trials to provide more robust evidence for the chemosensitizing effect of metformin combined with docetaxel chemotherapy and its impact on prostate cancer-specific survival and overall survival in diabetic metastatic castration-resistant prostate cancer patients. In the future, administration of metformin during docetaxel therapy and the effect on prostate cancer-specific and overall survival may also be investigated in non-diabetic metastatic CRPC patients depending on the results obtained in the aforementioned initial studies in diabetic metastatic CRPC patients.
Chapter 3
Retrospective cohort study evaluating the prostate cancer-specific and overall survival of diabetic mCRPC patients taking metformin while receiving docetaxel chemotherapy

3.1 Introduction
The first-line chemotherapy for symptomatic metastatic castration-resistant prostate cancer (mCRPC) is docetaxel (Basch et al 2014). Although it provides significant survival benefits, docetaxel is associated with significant toxicity and at conventional doses is not tolerated by frail patients. Furthermore, despite the development of new treatment agents such as abiraterone and enzalutamide, the average survival of metastatic CRPC patients is only approximately 19 months (Heidenreich et al 2013). Thus, there is a need to develop novel therapeutic approaches for mCRPC. One approach could be combining chemotherapy with other agents that enhance its effectiveness or permit dosage reduction to reduce toxicity (i.e. chemosensitizers).

Metformin, a well-tolerated oral biguanide commonly used to treat type II diabetes has been shown to exert anti-neoplastic effects in several types of solid tumors, including prostate cancer (Pollak 2012; Pollak 2013; Mayer et al 2015). Metformin has been shown to inhibit mitochondrial complex 1 which reduces mitochondrial ATP production, causing cellular energetic stress (Pollak 2012). This energetic stress leads to the activation of AMPK which directs the cell towards an anti-proliferative phenotype that inhibits tumor growth (Pollak 2012; Mayer et al 2015). One unique aspect of the anti-neoplastic activity of metformin that has been explored is its ability to act as a chemosensitizer. Metformin has been shown to enhance the tumor suppressing effect of chemotherapy in breast cancer cell lines and both enhance the effect of chemotherapy and prolong remission in breast cancer xenograft models (Hirsch et al 2009; Iliopoulos et al 2011). In colon cancer cells, metformin enhances the effect of 5-fluorouracil and oxaliplatin (Zhang et al 2013; Nangia-Makker et al 2014). Diabetic breast cancer patients using metformin while receiving neoadjuvant chemotherapy had significantly increased pathologic complete response compared to diabetic patients not using metformin (Jiralerspong et al 2009).
Metformin use during chemotherapy has also been shown to improve survival in diabetic patients with advanced nonsmall cell lung cancer and advanced endometrial cancer (Tan et al 2011; Ezewuiro et al 2016).

In prostate cancer patients, metformin use is associated with a decreased risk of prostate cancer diagnosis and progression, and increased cumulative metformin exposure after PCa is associated with both decreased all-cause and prostate cancer-specific mortality in diabetic patients (Preston et al 2014; Hitron et al 2012; Margel et al 2013). Metformin has also been associated with reduced CRPC development (Spratt et al 2013). However, the chemosensitizing effect in metformin has not been explored in prostate cancer patients. Given the current literature indicating a potential chemosensitizing effect of metformin, we hypothesized that diabetic patients with metastatic CRPC administered metformin during docetaxel chemotherapy would demonstrate improved prostate-cancer specific and overall survival.

3.2 Methods
A retrospective cohort study was approved by the Research Ethics Board at Sunnybrook Health Sciences Centre in Toronto, Ontario, Canada.

3.2.1 Data Source
In Ontario, the Institute for Clinical Evaluative Sciences (ICES) maintains an inventory of coded and linkable health data sets encompassing many publicly funded administrative health services records. Our study required the use of several ICES databases. The Ontario Cancer Registry (OCR) contains data on cancers in Ontario that is more than 95% complete (Robles et al 1988) and has been shown to have a sensitivity of 95% and specificity of 88% regarding cause-of-death information for breast cancer (Brenner et al 2009). The Ontario Diabetes Database (ODD) is a validated registry of diagnosed diabetics in Ontario (Hux et al 2002). The Ontario Drug Benefit (ODB) database contained information on all outpatient pharmaceutical prescriptions filled by residents in Ontario over 65 years of age who are eligible for prescription drug coverage (Levy et al 2003). The New Drug Funding Program
(NDFP) database includes information on the administration of injectable cancer drugs, such as docetaxel. Additional databases used include the Ontario Health Insurance Plan (OHIP) which tracks claims paid to physicians, laboratories, and out-of-province providers (Williams et al 1996); the Canadian Institute for Health Information Discharge Abstract Database (CIHI DAD) which includes hospital stay records (Juurink et al 2006); the National Ambulatory Care Reporting System (NACRS) which includes ambulatory care records; and the Registered Persons Database (RPDB) which contains demographic information.

3.2.2 Cohort Identification and Stratification
The Ontario Cancer Registry (OCR) was used to identify patients diagnosed with prostate cancer and then the New Drug Funding Program (NDFP) database was used to identify prostate cancer patients treated with docetaxel chemotherapy from January 1st 2005 - December 31st 2012. We restricted the cohort to patients treated from 2005 onward because docetaxel became standard-of-care treatment for metastatic castration-resistant prostate cancer in 2004. The last date for docetaxel treatment was selected as the end of 2012 because our follow-up period could only continue until December 31st 2014 in order to capture all the death certificate records available. Another important note regarding the NDFP database is that the administration of docetaxel chemotherapy was used as a surrogate for identifying metastatic CRPC patients since coding information for metastatic CRPC is not available. We then cross-referenced these records with the Ontario Diabetes Database (ODD) to identify patients diagnosed with diabetes receiving docetaxel chemotherapy for metastatic CRPC. Finally, this data was linked with the Ontario Drug Benefit (ODB) to identify patients using metformin or other antidiabetic medications, statins, and COX-2 inhibitors during docetaxel chemotherapy treatment. The antidiabetic medications included in the study other than metformin were: sulfonylureas, meglitinides, thiazolidinediones, glucosidase inhibitors, DPP-4 inhibitors, GLP-1 receptor agonists, SGLT2 inhibitors. Insulin users were identified and excluded due to the greater amount of comorbidities associated with more severe diabetes and the evidence which indicates higher levels of insulin can contribute to prostate cancer progression and worse outcomes. Only patients 65 years and older were included since prescription drug coverage records are only available for patients in this age bracket. The RPDB, NACRS, OHIP, and CIHI DAD were used to further identify the
cohort and assess comorbidities. Data was collected and linked at ICES by authorized analysts and a research-ready dataset was prepared. Linkage was enabled by a unique confidential identifier attached to each data record and all data was anonymized. Four different groups of patients were stratified: 1) Metastatic CRPC patients receiving docetaxel but not diagnosed with diabetes, 2) Metastatic CRPC patients receiving docetaxel, diagnosed with diabetes, using metformin + other antidiabetic therapies (excluding insulin), 3) Metastatic CRPC patients receiving docetaxel, diagnosed with diabetes, using other antidiabetic therapies only (excluding insulin), 4) Metastatic CRPC patients receiving docetaxel, diagnosed with diabetes, no medical management for diabetes (diet-control). A flow chart illustrating cohort stratification is shown in Figure 3.1. Metformin use alone could not be evaluated due to a very small sample size for this group (n = 36) which prevented the release of this data from ICES. This is due to privacy protection regulations that prevent the release of data which could compromise anonymization.

**Figure 3.1: Cohort Stratification Flowchart.** Data was obtained from the Institute for Clinical Evaluative Sciences (ICES) in Ontario from the following databases: Ontario Cancer Registry (OCR), New Drug Funding Program (NDFP), Ontario Diabetes Database (ODD) and Ontario Drug Benefit (ODB). Patients were matched for: age, use of statins and/or COX-2 inhibitors, Johns Hopkins Adjusted Clinical Groups (ACG) score, socioeconomic status, and urban/rural designation.
3.2.3 Outcomes
We measured prostate cancer-specific survival using the OCR database cause-of-death records and overall survival using death certificate records from the RPDB.

3.2.4 Statistical Analysis
All analyses were conducted using SAS Version 9.4. Prostate cancer-specific and overall survival was estimated and plotted using Kaplan Meier survival curves and survival was compared between groups with the logrank test. Survival times were censored at the date of last follow-up if the patients was still alive. The effect of metformin use during docetaxel chemotherapy on prostate cancer-specific and overall survival was assessed using the multivariate Cox proportional hazards regression model adjusting for the following covariates: age, use of statins and COX-2 inhibitors, Johns Hopkins Adjusted Clinical Groups (ACG) score, socioeconomic status, and urban/rural designation.

3.3 Results

3.3.1 Cohort Characteristics
Excluding insulin users, 2,832 male patients 66 years and older who received docetaxel chemotherapy for metastatic CRPC were identified. Patient characteristics are summarized in Table 1. 1,226 patients were non-diabetic and were categorized as the control group. 359 patients were taking metformin with or without other antidiabetic medications during docetaxel therapy, 1,102 patients were taking other anti-diabetic medications only during docetaxel therapy, and 145 patients were diagnosed with diabetes but not on any medical management during docetaxel therapy (considered the “diet-control” diabetic group). The median follow-up time for prostate cancer-specific mortality was 398.5 days (IQR 195-598.5) for the diet-control diabetic group, 432 days (IQR 200-655) for the group taking metformin with or without other antidiabetic drugs, 400 days (IQR 205-648) for the group taking other anti-diabetic drugs only, and 412 days (IQR 210-722) for the non-diabetic group. The median follow-up time for all-cause mortality was 420 days (IQR 195-624) for the diet-control diabetic group, 440 days (IQR 203-693) for the
metformin use group, 411 days (213-687) for the group using other anti-diabetic drugs only, and 412 days (IQR 212-717) for the non-diabetic group. During the follow-up period, 2,062 patients died and of those 1,065 died of prostate cancer.

Table 3.1: Baseline characteristics of patients receiving docetaxel chemotherapy for prostate cancer at the date of first dose (i.e. index date) by patient subgroup. ACG: Johns Hopkins Adjusted Clinical Groups aggregated score.

<table>
<thead>
<tr>
<th></th>
<th>Has diabetes, not taking any diabetes drugs</th>
<th>Taking Metformin with or without other diabetes drugs</th>
<th>Taking other diabetes drugs only taking any diabetes drugs</th>
<th>No diabetes, not taking any diabetes drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=145</td>
<td>N=359</td>
<td>N=1,102</td>
<td>N=1,226</td>
<td></td>
</tr>
<tr>
<td>Age group (years), n(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66 to 70</td>
<td>30 (20.7%)</td>
<td>91 (25.3%)</td>
<td>228 (20.7%)</td>
<td>321 (26.2%)</td>
</tr>
<tr>
<td>71 to 75</td>
<td>38 (26.2%)</td>
<td>120 (33.4%)</td>
<td>339 (30.8%)</td>
<td>368 (30.0%)</td>
</tr>
<tr>
<td>76 to 80</td>
<td>43 (29.7%)</td>
<td>93 (25.9%)</td>
<td>283 (25.7%)</td>
<td>324 (26.4%)</td>
</tr>
<tr>
<td>81+</td>
<td>34 (23.4%)</td>
<td>55 (15.3%)</td>
<td>252 (22.9%)</td>
<td>213 (17.4%)</td>
</tr>
<tr>
<td>Rural, n(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1-5 (0.09-0.2%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>No</td>
<td>120 (82.8%)</td>
<td>318 (88.6%)</td>
<td>940 (85.3%)</td>
<td>1,034 (84.3%)</td>
</tr>
<tr>
<td>Yes</td>
<td>25 (17.2%)</td>
<td>41 (11.4%)</td>
<td>157-161 (14.2-14.6%)</td>
<td>192 (15.7%)</td>
</tr>
<tr>
<td>Resource Utilization Band (RUB) Score, n(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7-11 (4.8-7.6%)</td>
<td>20-24 (5.6-6.7%)</td>
<td>83 (7.5%)</td>
<td>94 (7.7%)</td>
</tr>
<tr>
<td>1</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1-5 (0.09-0.2%)</td>
<td>1-5 (0.08-0.4%)</td>
</tr>
<tr>
<td>2</td>
<td>1-5 (0.7-3.4%)</td>
<td>1-5 (0.3-1.4%)</td>
<td>1-5 (0.09-0.2%)</td>
<td>1-5 (0.08-0.4%)</td>
</tr>
<tr>
<td>3</td>
<td>36 (24.8%)</td>
<td>92 (25.6%)</td>
<td>210 (19.1%)</td>
<td>338 (27.6%)</td>
</tr>
<tr>
<td>4</td>
<td>39 (26.9%)</td>
<td>109 (30.4%)</td>
<td>352 (31.9%)</td>
<td>392 (32.0%)</td>
</tr>
<tr>
<td>5</td>
<td>58 (40.0%)</td>
<td>133 (37.0%)</td>
<td>452 (41.0%)</td>
<td>396 (32.3%)</td>
</tr>
<tr>
<td>ACG Aggregate Score Category for Two Years Prior to Index Date, n(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 5</td>
<td>35 (24.1%)</td>
<td>71 (19.8%)</td>
<td>243 (22.1%)</td>
<td>343 (28.0%)</td>
</tr>
<tr>
<td>6 to 8</td>
<td>32 (22.1%)</td>
<td>106 (29.5%)</td>
<td>271 (24.6%)</td>
<td>340 (27.7%)</td>
</tr>
<tr>
<td>9 to 11</td>
<td>37 (25.5%)</td>
<td>99 (27.6%)</td>
<td>292 (26.5%)</td>
<td>296 (24.1%)</td>
</tr>
<tr>
<td>12+</td>
<td>41 (28.3%)</td>
<td>83 (23.1%)</td>
<td>296 (26.9%)</td>
<td>247 (20.1%)</td>
</tr>
<tr>
<td>Other Cancers, n(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous non-prostate cancer</td>
<td>15 (10.3%)</td>
<td>28 (7.8%)</td>
<td>123 (11.2%)</td>
<td>117 (9.5%)</td>
</tr>
<tr>
<td>Subsequent non-prostate cancer</td>
<td>---------</td>
<td>8 (2.2%)</td>
<td>17 (1.5%)</td>
<td>17 (1.4%)</td>
</tr>
</tbody>
</table>
3.3.2 Prostate Cancer-Specific and Overall Survival

Survival analysis (Figure 3.2) as determined by the Kaplan Meier survival curves for prostate cancer-specific mortality revealed no statistically significant difference in survival between non-diabetic patients, patients taking metformin with or without other antidiabetic drugs, patients taking other diabetes drugs only, and patients diagnosed with diabetes but not taking any antidiabetic medications. Similar results were observed for all-cause mortality as no statistically significant differences were found between groups (Figure 3.3).

![Figure 3.2: Kaplan Meier survival curve of prostate cancer-related mortality.](image)

Probability of survival is along the Y-axis and time to prostate cancer death or censoring is along the X-axis. There was no statistically significant difference in survival between groups (Logrank test $p = 0.94$).
Figure 3.3: Kaplan Meier survival curve of all-cause mortality. Probability of survival is along the Y-axis and time to prostate cancer death or censoring is along the X-axis. There was no statistically significant difference in survival between groups (Logrank test p = 0.99).

The Cox proportional hazards regression model results are presented in Tables 3.2 and 3.3. For prostate cancer-specific mortality (Table 3.2), using metformin with or without other antidiabetic drugs (HR: 0.96, 95% CI, 0.79-1.16, p = 0.66), using other antidiabetic drugs alone (HR 0.99, 95% CI, 0.86-1.13, p = 0.83), or having diabetes but not using any antidiabetic medication (HR 0.91, 95% CI, 0.67-1.23, p = 0.53) did not have a significant effect. For overall mortality (Table 3.3), the results were similar since using metformin with or without other antidiabetic drugs (HR: 0.94, 95% CI, 0.82-1.08, p = 0.39), using other antidiabetic drugs alone (HR 0.96, 95% CI, 0.87-1.06, p = 0.44), or having diabetes but not using any antidiabetic medication (HR 0.94, 95% CI, 0.76-1.16, p = 0.54) did not have a significant effect.
Table 3.2: Results of Cox proportional hazards regression model for prostate cancer-related mortality. There was no statistically significant effect of patients taking metformin with or without other antidiabetic medications, or patients taking other antidiabetic medications only on prostate-cancer specific survival.

<table>
<thead>
<tr>
<th>Group (reference= No diabetes, not taking any diabetes drugs)</th>
<th>Hazard Ratio</th>
<th>95% LCL for Hazard Ratio</th>
<th>95% UCL for Hazard Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has diabetes, not taking any diabetes drugs</td>
<td>0.91</td>
<td>0.67</td>
<td>1.23</td>
<td>0.5354</td>
</tr>
<tr>
<td>Taking Metformin with or without other diabetes drugs</td>
<td>0.96</td>
<td>0.79</td>
<td>1.16</td>
<td>0.6648</td>
</tr>
<tr>
<td>Taking other diabetes drugs only</td>
<td>0.99</td>
<td>0.86</td>
<td>1.13</td>
<td>0.8343</td>
</tr>
</tbody>
</table>

Table 3.3: Results of Cox proportional hazards regression model for all-cause mortality. There was no statistically significant effect of patients taking metformin with or without other antidiabetic medications, or patients taking other antidiabetic medications only on overall survival.

<table>
<thead>
<tr>
<th>Group (reference= No diabetes, not taking any diabetes drugs)</th>
<th>Hazard Ratio</th>
<th>95% LCL for Hazard Ratio</th>
<th>95% UCL for Hazard Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has diabetes, not taking any diabetes drugs</td>
<td>0.94</td>
<td>0.76</td>
<td>1.16</td>
<td>0.5423</td>
</tr>
<tr>
<td>Taking Metformin with or without other diabetes drugs</td>
<td>0.94</td>
<td>0.82</td>
<td>1.08</td>
<td>0.3976</td>
</tr>
<tr>
<td>Taking other diabetes drugs only</td>
<td>0.96</td>
<td>0.87</td>
<td>1.06</td>
<td>0.443</td>
</tr>
</tbody>
</table>

3.4 Discussion

The results of this retrospective cohort study indicate that metformin use during docetaxel chemotherapy did not improve prostate cancer-specific or overall survival in diabetic metastatic CRPC patients.

Previous retrospective studies have investigated the effect of metformin on prostate cancer-related outcomes. The results have been mixed. Metformin has been associated with significantly decreased risk of prostate cancer diagnosis and progression, (Preston et al 2014;
Hitron et al 2012), as well as increased prostate-cancer specific and overall survival (Spratt et al 2013). However, some of these studies had the methodological issue of immortal time bias which may have resulted in the benefit of metformin being somewhat overestimated (Suissa and Azoulay 2012). Margel et al (2013) demonstrated cumulative metformin use after a prostate cancer diagnosis reduced prostate cancer-specific and all-cause mortality and by modeling cumulative metformin as a time-varying covariate minimized the issue of immortal time bias. Other studies have shown no effect of metformin use on prostate cancer risk, incidence, or prostate cancer-specific and overall survival (Margel et al 2013; Azoulay et al 2011; Bensimon et al 2014; Gandini et al 2014). Although the use of metformin in prostate cancer has been evaluated to a certain extent, one area that has not yet been explored is the potential use of metformin as a chemosensitizer in advanced prostate cancer.

The only study that assessed the effect of metformin use during docetaxel chemotherapy was conducted by Niraula et al (2013) by retrospectively evaluating data from patients who participated in the TAX 327 trial. This trial established docetaxel as a treatment agent for metastatic CRPC (Tannock et al 2004). Metformin did not have a statistically significant additive or synergistic effect when combined with docetaxel, however there were only 38 patients included in the analysis (Niraula et al 2013). Given the extremely limited evidence exploring metformin as a chemosensitizer in prostate cancer, and the very favourable safety profile of metformin, our study was conducted to evaluate this novel application of metformin. However, our results indicate metformin may in fact not be effective in enhancing the response to docetaxel.

This study has several strengths. The evidence available for the role of metformin as a chemosensitizer in CRPC is extremely limited. This study evaluated a novel role of metformin use in advanced prostate cancer. We had a large sample size (N=359) with which to evaluate the effect of combining metformin with docetaxel compared to the only other study by Niraula et al (2013) (N=38). The ODD uses diagnostic codes to capture diabetes diagnosis instead of medication use which allowed us to identify a proportion of patients who were diagnosed with diabetes but not medically managed and are therefore a diet-control group (N = 145). This allowed us to reduce the effect of healthy user bias since we were able to
compare diabetic patients using metformin to diabetic patients on diet-control only. We were also able to adjust for statin use, a potential confounder since statins have been shown to reduce risk of metastatic prostate cancer and cancer-related mortality (Platz et al 2006; Nielsen et al 2012). This reduced the possibility that the anti-cancer effects of statins could have been erroneously attributed to metformin. The generalizability of our results is broader than some previous observational studies since we compared the effect of metformin use in diabetic metastatic CRPC patients to both diet-controlled diabetic patients and non-diabetic patients. However, it is relevant to note that the proportion of metastatic CRPC patients is quite small compared to all diagnosed cases of prostate cancer.

This study has several limitations. First, since it is a retrospective study the use of metformin could not be randomly assigned. Docetaxel treatment for prostate cancer was used as the identifier for metastatic CRPC patients since coding for this was not available in the OCR. It is possible that some patients who had metastatic CRPC but did not receive docetaxel were not included in the cohort or some patients that were given neoadjuvant chemotherapy but did not in fact have metastatic CRPC were included. It is not possible to verify how many patients could have been misclassified due to the absence of CRPC status coding in the OCR. However, the number of patients that would have been excluded for these reasons is likely not very significant. We were not able to include patients younger than 65 years old since the Ontario Drug Benefit database includes information on prescriptions filled using the universal drug program provided in Ontario to individuals 65 years of age and older. Therefore, patients under 65 diagnosed with CRPC could not have been included since it would not be possible to evaluate the medications they were using. Given the long natural history of prostate cancer, the number of patients excluded based on age is likely not very high. The group of diabetic patients on diet-control and not using any anti-diabetic medications was small, which reduced the power of this group and may have affected the results. Another consideration is that the ICES databases lack some relevant co-variates such as performance status, severity of diabetes, smoking, body mass index, laboratory data, exercise, prostate-specific antigen and prostate cancer staging information, which prevented these factors from being included in the multivariate analyses. Despite their absence in the databases, we were able to include a
number of important confounders in our analysis such as patient comorbidities, which were determined by using the Johns Hopkins Adjusted Clinical Groups system.

In conclusion, this is the first study completed to evaluate metformin as a chemosensitizing agent in CRPC patients. The results obtained from our study revealed that metformin use during docetaxel chemotherapy in diabetic metastatic CRPC patients did not improve prostate cancer-specific or overall survival. However, due to the limitations of our study we cannot rule out that metformin is not an effective chemosensitizer for docetaxel chemotherapy. Given the retrospective nature of this study, further studies, particularly prospective trials, are warranted to evaluate metformin use during docetaxel chemotherapy treatment for metastatic CRPC, to further elucidate the potential effect of metformin in this patient population.
Chapter 4
Effect of combining metformin and docetaxel chemotherapy in human castration-resistant prostate cancer cells in vitro

4.1 Introduction
Advanced stage PCa is usually treated with androgen-deprivation therapy (ADT). However, most patients with metastatic disease managed with ADT eventually relapse with castration-resistant prostate cancer (CRPC) and die of the disease (Feldman and Feldman 2001; Li and Tang 2011). The first-line chemotherapy for symptomatic metastatic CRPC is docetaxel, which provides significant survival benefits, but is also associated with significant toxicity and the average survival of metastatic CRPC patients is only approximately 19 months (Heidenreich et al 2014; Heidenreich et al 2013). Thus, there is still a need to improve the therapeutic options available for advanced stage prostate cancer patients.

One novel therapeutic approach is combining docetaxel chemotherapy with an agent that enhances its effectiveness, known as a “chemosensitizer”. One such potential chemosensitizing agent is metformin, a commonly prescribed and well-tolerated oral biguanide used to treat type II diabetes. Metformin has been shown to exert anti-neoplastic effects in several types of cancer (Foretz et al 2014). Metformin reduces hepatic gluconeogenesis, increases glucose uptake in peripheral tissue such as skeletal muscle, and increases insulin sensitivity (Pollak 2012; Kourelis et al 2012). These processes are able to partially reverse hyperglycemia and insulin resistance and therefore help manage diabetes. Evans et al (2005) showed a reduced cancer burden in diabetic patients treated with metformin compared to those treated with other diabetic therapies (Pollak 2012; Evans et al 2005). Since the publication of the Evans paper, metformin has been shown to have anti-neoplastic properties in breast cancer (Zakikhani et al 2010; Alimova et al 2009), ovarian cancer (Rattan et al 2011), pancreatic cancer (Kisfalvi et al 2013), and prostate cancer (Sahra et al 2008).

In prostate cancer, metformin inhibits the proliferation of LNCaP, DU145, and PC3 human prostate cancer cells in addition to reducing tumor growth in LNCaP xenografts (Sahra et al
More specifically, metformin has been shown to improve the efficacy of chemotherapy in breast cancer, lung cancer and colon cancer cells and breast and lung cancer xenografts.

Metformin has been shown to enhance the tumor suppressing effect of doxorubicin in four different types of breast cancer cell lines and prolonged remission breast cancer xenograft models (Hirsch et al 2009). Furthermore, metformin combined with a 4-fold lower dose of doxorubicin was shown to be as effective as the standard dose of doxorubicin treatment alone breast cancer xenografts (Iliopoulos et al 2011). Metformin has also been shown to enhance the effect of paclitaxel and carboplatin in breast cancer xenografts at doses comparable to the dose/kg used in type II diabetes patients (Iliopoulos et al 2011). In breast cancer and lung cancer xenografts, treatment with both metformin and paclitaxel was more effective at reducing tumor growth and increasing apoptosis than either metformin or paclitaxel treatment alone (Rocha et al 2011). In colon cancer cells, treatment with metformin combined with 5-fluorouracil enhanced the antiproliferative effect of 5-fluorouracil (Zhang et al 2013). In addition, metformin acts synergistically with 5-fluorouracil and oxaliplatin to inhibit cell proliferation and tumor growth of chemo-resistant colorectal cancer cells (Nangia-Makker et al 2014).

Although metformin has been shown to enhance the anti-cancer effect of bicalutamide and simvastatin in advanced stage prostate cancer cell lines, there is very limited evidence for the use of metformin as a chemosensitizing agent in prostate cancer (Colquhoun et al 2012; Babcook et al 2014; Babcock et al 2014b). One of the only studies published shows that metformin combined with doxorubicin treatment can suppress the growth of PC3 xenograft tumors (Iliopoulos et al 2011). Although this indicates a chemosensitizing effect of metformin in a prostate cancer xenograft model, docetaxel, not doxorubicin, is used to treat castration-resistant prostate cancer patients. Evaluating doxorubicin in this context is therefore not very clinically relevant and it would be more relevant to evaluate docetaxel chemotherapy combined with metformin in prostate cancer. Investigating the use of metformin as a chemosensitizing agent in combination with docetaxel chemotherapy in prostate cancer has not been thoroughly investigated and is therefore a novel line of inquiry. Given the evidence in the literature demonstrating a chemosensitizing effect of metformin in other types of solid tumors, but very
limited evidence for metformin chemosensitization in prostate cancer, we hypothesize that metformin will act as a chemosensitizing agent in castration-resistant prostate cancer cell lines when combined with docetaxel chemotherapy.

4.2 Methods

4.2.1 Cell Culture
Two established human prostate cancer cell lines (PC3 and DU145) were purchased from the American Type Culture Collection (Rockville, MA, USA). Both PC3 and DU145 cells do not express a functional androgen receptor and are therefore androgen-independent. Cells were cultured in Dulbecco’s minimal essential medium/F12 (DMEM/F12) with 10% fetal bovine serum FBS (Invitrogen) and supplemented with 100IU/mL penicillin, 100ug/mL streptomycin, and 0.3mg/mL L-glutamine (Invitrogen). Cells were maintained at 37°C in a humidified 5% CO₂ incubator. PC3 and DU145 cells were selected for these experiments since they are among the most commonly used and well characterized human prostate cancer cells which represent advanced prostate cancer. PC3 cells are derived from a prostate cancer bone metastasis and DU145 cells from a prostate cancer brain metastasis, and along with the androgen-independent status of both cell types, they are representative of metastatic CRPC. Therefore, since docetaxel is used to treat patients with symptomatic metastatic CRPC, we decided that these cells would be appropriate for the evaluation of the effect of metformin combined with docetaxel treatment.

Table 4.1: Characteristics of PC3 and DU145 human prostate cancer cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Androgen Receptor</th>
<th>Androgen Response</th>
<th>PSA</th>
<th>LKB1</th>
<th>AMPK</th>
<th>p53 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>Lumbar metastasis</td>
<td>-</td>
<td>Androgen Independent</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Deleted</td>
</tr>
<tr>
<td>DU145</td>
<td>Brain metastasis</td>
<td>-</td>
<td>Androgen Independent</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mutated non-functional</td>
</tr>
</tbody>
</table>
4.2.2 Chemicals
Metformin (Sigma, Oakville, Ontario, Canada) was generously supplied by Dr. Michael Pollak, McGill University, Montreal, QC, Canada. Working concentrations (100mM and 1mM) were created by dissolving metformin in cell culture medium. Docetaxel (Santa Cruz Biotechnology, CA, USA) was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, UA) and diluted with a cell culture medium at a final concentration of 0.01% DMSO.

4.2.3 MTS Cell Viability Assay
The CellTiter 96® AQeuous One Solution Cell Proliferation (MTS) assay was used to evaluate cell viability (Promega, Madison, WI) of PC3 and DU145 cells. 4x10^3 cells were plated per well in a 96-well plate in triplicate and left to adhere for 24 hrs. After adherence, cells were treated with a range of metformin concentrations (0.01, 0.1, 1, 10, 100mM) or a range of docetaxel concentrations (0.001, 0.01, 0.1, 1uM) for 24, 48, or 72 hrs to establish dose standardization for each cell line. After evaluating the effect of metformin or docetaxel alone the following combinations were selected for evaluation in PC3 cells: 1mM metformin + 0.01uM docetaxel, 10mM metformin + 0.01uM docetaxel, 1mM metformin + 0.1uM docetaxel, 10mM metformin + 0.1uM docetaxel. The metformin and docetaxel combinations selected were slightly different for the DU145 cells: 1mM metformin + 0.001uM docetaxel, 10mM metformin + 0.001uM docetaxel, 1mM metformin + 0.01uM docetaxel, 10mM metformin + 0.01uM docetaxel. Media and 0.01% DMSO vehicle controls with cells were used since metformin was dissolved in cell culture medium and docetaxel was prepared in a final concentration of 0.01% DMSO. Blank wells containing only media were also used to establish a background colourimetric signal. After treatment for 24, 48, or 72 hrs, 20uL of MTS dye was added to each well and plates were placed in the incubator for 1.5-2 hrs. The absorbance was recorded at 490nm using a plate reader.

4.2.4 Wound-Healing/Scratch Assay
Cell motility was assessed using a wound-healing, or scratch, assay with PC3 cells. 2x10^5 cells were plated per well in a 24-well plate in triplicate. Cells were allowed to adhere for 48 hrs and then a wound was created using a 200uL pipette tip. Reference marks for imaging were made on
the bottom of each well using a fine-tipped marker. The cells were then washed twice with PBS and treated with metformin, docetaxel or one of the following combinations: 1mM metformin + 0.01uM docetaxel, 10mM metformin + 0.01uM docetaxel, 1mM metformin + 0.1uM docetaxel, 10mM metformin + 0.1uM docetaxel. Media and 0.01% DMSO vehicle controls with cells were used since metformin was dissolved in cell culture medium and docetaxel was prepared in a final concentration of 0.01% DMSO. A computer-based Axiovert microscope was used to capture images at 3 different reference points along the wound in each well at 0hrs of treatment to establish a baseline measurement. After capturing the images, cells were returned to the incubator and allowed to migrate for 24hrs. The computer-based microscope was then used again 24hrs after treatment to capture images at the same 3 reference points along the wound in each well. AxioVision SE64 Rel. 4.9.1 software was used to measure the images and then the percentage of wound closure occurring between 0hrs and 24hrs was calculated.

4.2.5 Complex 1 Activity Assay
The Colorimetric Complex 1 Enzyme Activity Microplate Assay Kit (abcam, Cambridge Science Park, UK) was used to evaluate the activity of the mitochondrial oxidative phosphorylation Complex 1 enzyme in PC3 and DU145 cells. A 10cm dish was prepared for each control and treatment condition by plating 1x10^6 cells per dish and allowing cells to adhere for 24hrs. After 24 hours of adherence cells were treated in the following manner: PC3 cells were treated with 1mM metformin, 0.1uM docetaxel, or 1mM metformin + 0.1uM docetaxel and DU145 cells were treated with 1mM metformin, 0.01uM docetaxel, or 1mM metformin + 0.01uM docetaxel. Treatments were applied for 48hrs. Metformin and 0.01% DMSO vehicle control dishes were also included since metformin was dissolved in cell culture medium and docetaxel was prepared in a final concentration of 0.01% DMSO. Kit components were prepared ahead of time and before use were allowed to equilibrate to room temperature. After 48hrs of treatment the instruction booklet provided for the kit by abcam was followed in detail. Cells were harvested by scraping each dish while on ice and transferring the cells to an appropriately labelled 2mL Eppendorf tube. Cells were washed twice with PBS, centrifuged, and then resuspended after each wash in PBS. After the second PBS wash, cells were centrifuged again, the supernatant was aspirated and the remaining cell pellet was resuspended in PBS. Proteins were extracted by
adding 10x Detergent solution to each sample and then samples were incubated on ice for 30 minutes to allow for solubilisation. Samples were centrifuged for 20 minutes at 4°C at 14,000g. Supernatant was collected and transferred to a clean tube at a concentration of 5mg/mL. Samples were diluted to 250ug/200uL and then 200uL of sample were loaded into the pre-coated 96-well plate provided in duplicate wells. The following controls were also included: media and 0.01% DMSO vehicle controls, 1X Buffer only, cells with no assay solution added. The microplate was then incubated at room temperature for 3 hours. The wells were emptied by turning the plate over, shaking out any remaining liquid and blotting the plate face down on a paper towel. 300uL of 1X buffer solution was added to each well. The wells were emptied again following the same procedure described above. Assay solution which included 1X buffer, 20X NADH and 100X dye was prepared fresh for each experiment as described. The wells were rinsed again with 300uL of 1X buffer and then emptied. 200uL of assay solution was added to each well, carefully avoiding the production of bubbles and any bubbles created were popped as quickly as possible with a 21 1/2G needle. The plate was then taken to a plate reader to measure the Complex 1 activity by using the kinetic program setting for 30 minutes of readings at 1 minute intervals.

4.2.6 ATP Concentration Assay
The Luminescent ATP Detection Assay Kit (abcam, Cambridge Science Park, UK) was used to evaluate total levels of cellular ATP in PC3 and DU145 cells. 4x10^3 cells were plated per well in a black, opaque 96-well plate in triplicate wells for each condition. Cells were allowed to adhere for 48hrs and were then treated in the following manner: PC3 cells were treated with 1mM metformin, 0.1uM docetaxel, or 1mM metformin + 0.1uM docetaxel and DU145 cells were treated with 1mM metformin, 0.01uM docetaxel, or 1mM metformin + 0.01uM docetaxel. Treatments were applied for 48hrs. Metformin and 0.01% DMSO vehicle controls were also included since metformin was dissolved in cell culture medium and docetaxel was prepared in a final concentration of 0.01% DMSO. Kit components were allowed to equilibrate to room temperature before use. All steps involving kit components were completed in subdued lighting to prevent plate phosphorescence which would artificially increase the background signal. After 48hrs of treatment 50uL of detergent was added to each well to lyse the cells and stabilize the ATP. The plate was then shaken on an orbital shaker at 160rpm for 5 minutes inside a box to
limit light exposure. While the plate was shaking, one vial of the lyophilized substrate was
reconstituted using 5mL of substrate buffer. 50uL of the reconstituted substrate solution was
added to each well and then the plate was shaken on an orbital shaker at 160rpm for 5 minutes
inside a box to limit light exposure. The plate was then dark adapted for 10 minutes before being
taken to a plate reader to measure the luminescent signal.

4.2.7 Western Blotting
Cells were prepared for lysate collection by plating 1x10⁶ cells per 10cm dish for each control
and treatment condition and allowing cells to adhere for 24hrs. After 24hrs of adherence cells
were treated in the following manner: PC3 cells were treated with 1mM metformin, 0.1uM
docetaxel, or 1mM metformin + 0.1uM docetaxel and DU145 cells were treated with 1mM
metformin, 0.01uM docetaxel, or 1mM metformin + 0.01uM docetaxel. Metformin and 0.01%
DMSO vehicle control dishes were also included since metformin was dissolved in cell culture
medium and docetaxel was prepared in a final concentration of 0.01% DMSO. Treatments were
applied for 48hrs. Cell lysis was completed using RIPA buffer with protease and phosphatase
inhibitor cocktails. Protein levels were quantified using the Pierce BCA Protein Assay Kit
(Thermo Fischer Scientific) before loading into a 15-well, 4-12% Bis Tris NuPage gel (Thermo
Fischer Scientific) to complete electrophoresis. Transfer to a nitrocellulose membrane was
completed and protein detection was completed using the following antibodies from Cell
Signaling Technology Inc.: AMPK α (Thr 172) [Cat. No. 2603] and Phospho-AMPK α (Thr172)
[Cat. No. 2535]. ImageJ software (US National Institute of Health, Bethesda, MA, USA) was
used to complete densiometry for the bands observed. Western blot experiments were completed
in duplicate.

4.2.8 Statistical Analysis
Experiments were completed with three technical replicates and three biological replicates. An
exception was for Western blotting in which two technical and biological replicates were
completed. Student’s t test was used to analyze between-group differences for all in vitro
experiments with a significance level of $p < 0.05$. The data shown represents the mean ± standard deviation.

4.3 Results

4.3.1 Dose standardization for metformin and docetaxel treatment on PC3 and DU145 cells

The MTS cell proliferation assay was used to evaluate the effect of metformin and/or docetaxel treatment on cell viability. Dose standardization experiments were completed for both PC3 and DU145 cells. PC3 cells were treated with metformin concentrations of 0.01mM, 0.1mM, 1mM, 10mM and 100mM for 24, 48, and 72 hours to determine the ideal metformin concentration to be used (Figure 4.1). The metformin treatments selected were 1mM and 10mM and 48 hours of treatment since these concentrations at this time point significantly reduced cell viability compared to the media control. PC3 cells were also treated with docetaxel concentrations of 0.001uM, 0.01uM, 0.1uM and 1uM for 24, 48, and 72 hours to determine the ideal docetaxel concentration to be used (Figure 4.2). The docetaxel treatments selected were 0.01uM and 0.1uM and 48 hours of treatment since these concentrations at this time point significantly reduced cell viability compared to the 0.01% DMSO control.

DU145 cells were treated with the same range of metformin and docetaxel concentrations and time points that were used to treat the PC3 cells (Figures 4.6 and 4.7). However, the concentrations selected were slightly different. The metformin treatment selected to move forward with was 1mM since although it did not reduce cell viability, it is a more physiologically relevant concentration to evaluate in additional experiments. The docetaxel treatment selected was 0.01uM of docetaxel for 48 hours of treatment since this concentration and time point significantly reduced cell viability (Figure 4.7).
4.3.2 Metformin combined with docetaxel significantly reduces cell viability in PC3 cells but not DU145 cells

The MTS cell proliferation assay was used to evaluate the effect of combinations of metformin and docetaxel treatment on cell viability to determine if metformin has any chemosensitizing effect. In PC3 cells after 48 hours of treatment, first evaluating the combination of 1mM or 10mM of metformin with 0.01uM docetaxel (Figure 4.4), 10mM metformin combined with 0.01uM docetaxel resulted in a significant reduction in cell viability compared to 0.01uM docetaxel treatment alone, but 1mM metformin combined with 0.01uM docetaxel was not significant compared to 0.01uM docetaxel alone. Then again in PC3 cells after 48 hours of treatment, evaluating the combination of 1mM or 10mM of metformin with 0.1uM docetaxel (Figure 4.4), both the 1mM metformin and 0.1uM docetaxel combination and the 10mM metformin and 0.1uM docetaxel combination significantly reduced cell viability compared to 0.1uM docetaxel treatment alone. However in DU145 cells, the results were significantly different. Treating DU145 cells with metformin alone, docetaxel alone, and multiple combinations of metformin and docetaxel concentrations did not have a significant effect on cell viability (Figures 4.8, 4.9, 4.10).
Figure 4.1: The effect of metformin treatment on the viability of PC3 cells. PC3 cells were treated with a range of concentrations of metformin for 24, 48, or 72 hours and the optical density at 490nm was evaluated to determine the number of viable cells present. These results were used to establish dose standardization for metformin treatment. Experiments were completed in triplicate and graphs represent the mean (*: p < 0.05, **: p < 0.001).
**Figure 4.2:** The effect of docetaxel treatment on the viability of PC3 cells. PC3 cells were treated with a range of concentrations of docetaxel for 24, 48, or 72 hours and the optical density at 490nm was evaluated to determine the number of viable cells present. These results were used to establish dose standardization for docetaxel treatment. Experiments were completed in triplicate and graphs represent the mean (*: p < 0.05, **: p < 0.001).
Figure 4.3: The effect of 24 hours of combined metformin and docetaxel treatment on the viability of PC3 cells. PC3 cells were treated with 1mM or 10mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 24 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean.
Figure 4.4: The effect of 48 hours of combined metformin and docetaxel treatment on the viability of PC3 cells. PC3 cells were treated with 1mM or 10mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 48 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean.
Figure 4.5: The effect of 72 hours of combined metformin and docetaxel treatment on the viability of PC3 cells. PC3 cells were treated with 1mM or 10mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 72 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean.
Figure 4.6: The effect of metformin treatment on the viability of DU145 cells. DU145 cells were treated with a range of concentrations of metformin for 24, 48, or 72 hours and the optical density at 490nm was evaluated to determine the number of viable cells present. These results were used to establish dose standardization for metformin treatment. Experiments were completed in triplicate and graphs represent the mean (*: p < 0.05, **: p < 0.001).
**Figure 4.7:** The effect of docetaxel treatment on the viability of DU145 cells. DU145 cells were treated with a range of concentrations of docetaxel for 24, 48, or 72 hours and the optical density at 490nm was evaluated to determine the number of viable cells present. These results were used to establish dose standardization for docetaxel treatment. Experiments were completed in triplicate and graphs represent the mean (*: p < 0.05, **: p < 0.001).
Figure 4.8: The effect of 24 hours of combined metformin and docetaxel treatment on the viability of DU145 cells. DU145 cells were treated with 1mM or 10mM metformin alone, 0.001uM or 0.01uM docetaxel alone, or a combination of metformin and docetaxel for 24 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean. No significant effect was observed for any treatment condition.
Figure 4.9: The effect of 48 hours of combined metformin and docetaxel treatment on the viability of DU145 cells. DU145 cells were treated with 1mM or 10mM metformin alone, 0.001uM or 0.01uM docetaxel alone, or a combination of metformin and docetaxel for 48 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean. No significant effect was observed for any treatment condition.
Figure 4.10: The effect of 72 hours of combined metformin and docetaxel treatment on the viability of DU145 cells. DU145 cells were treated with 1mM or 10mM metformin alone, 0.001uM or 0.01uM docetaxel alone, or a combination of metformin and docetaxel for 72 hours. The optical density at 490nm was evaluated to determine the number of viable cells present. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean. No significant effect was observed for any treatment condition.
4.3.3 Metformin combined with docetaxel does not significantly reduce cell migration in PC3 cells compared to docetaxel alone

The effect of metformin and/or docetaxel treatment on cell migration was assessed using a wound-healing assay in PC3 cells. The percent of wound closure, which is considered a way to evaluate the amount of cell migration occurring, was evaluated for metformin alone, docetaxel alone, and combinations of metformin and docetaxel. 1mM and 10mM of metformin compared to the media control significantly reduced cell migration (Figure 4.11). 0.01uM and 0.1uM of docetaxel compared to the 0.01% DMSO control significantly reduced cell migration (Figure 4.11). For the combination treatments, combining 1mM or 10mM of metformin with 0.01uM of docetaxel did not have a significant effect on cell migration compared to 0.01uM docetaxel alone (Figure 4.11). Furthermore, combining 1mM or 10mM of metformin with 0.1uM of docetaxel also did not have a significant effect on cell migration compared to 0.1uM docetaxel treatment alone (Figure 4.11). A selection of representative images have also been included which were the source of the measurements used for percent wound closure quantification (Figure 4.12).

**Metformin Combined with 0.01uM Docetaxel PC3 Cells**

![Graph showing the effect of various treatments on cell migration in PC3 cells.](image-url)

* statistically significant (p < 0.05) compared to media control
& stat. sig. compared to 0.01% DMSO control
# stat. sig. compared to metformin alone
Figure 4.11: The effect of 24 hours of combined metformin and docetaxel treatment on PC3 cell migration. A wound was created in each well of a 24-well plate following PC3 cell plating and adherence. Following wound creation, PC3 cells were treated with 1mM or 10mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 24 hours. Light microscopy was then used to image the wound and the percent wound closure was calculated using measurements obtained from the images. A lower percent wound closure indicates a greater reduction in cell migration. Experiments were completed in triplicate and graphs represent the mean.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media Control</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>0.01% DMSO Control</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>1mM Metformin (MET)</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>10mM Metformin</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>0.01uM Docetaxel (DTX)</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 4.12: Representative images of the effect of 24 hours of combined metformin and docetaxel treatment on PC3 cell migration. A wound was created in each well of a 24-well plate following PC3 cell plating and adherence. Following wound creation, PC3 cells were treated with 1mM or 10mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 24 hours. Light microscopy was then used to image the wound and those images are included in this figure for each treatment condition. Experiments were completed in triplicate.
4.3.4 Metformin combined with docetaxel does not significantly reduce ATP concentrations in PC3 or DU145 cells compared to docetaxel alone

The luminescent ATP detection assay was used to evaluate the effect of metformin and/or docetaxel treatment on total cellular ATP levels. In PC3 cells, 48 hours of treatment with 1mM metformin or 0.1uM of docetaxel alone significantly reduced total cellular ATP levels compared to their respective controls (Figure 4.13). However, the combination of 1mM of metformin with 0.1uM docetaxel did not have a significant effect on total ATP levels compared to 0.1uM of docetaxel alone (Figure 4.13). In DU145 cells, 48 hours of treatment with 1mM metformin or 0.01uM docetaxel alone significantly reduced total cellular ATP levels compared to their respective controls (Figure 4.13). However, the combination of 1mM of metformin with 0.01uM of docetaxel did not significantly reduce total cellular ATP levels compared to 0.01uM docetaxel treatment alone (Figure 4.13).

![ATP Concentration Assay PC3 Cells 48hrs Treatment](image)

* statistically significant (p < 0.05) compared to media control
& stat. sig. compared to 0.01% DMSO control
# stat. sig. compared to metformin alone
Figure 4.13: The effect of 48 hours of combined metformin and docetaxel treatment on cellular ATP concentration. PC3 (top) and DU145 (bottom) cells were treated with treated with 1mM metformin alone, 0.01uM or 0.1uM docetaxel alone, or a combination of metformin and docetaxel for 48 hours. The amount of luminescence was measured following the addition of the experimental reagent to determine the concentration of cellular ATP. The effect of combination treatments were compared to both metformin alone and docetaxel alone. Experiments were completed in triplicate and graphs represent the mean.

4.3.5 Metformin treatment increases AMPK expression in PC3 cells but not in DU145 cells

Western blotting was used to evaluate the effect of metformin and/or docetaxel treatment on AMPK and P-AMPK expression in PC3 and DU145 cells. In PC3 cells, 1mM metformin treatment increased AMPK expression slightly but increased phosphorylated-AMPK expression more significantly compared to the media control (Figure 4.14). 0.1uM of docetaxel treatment alone decreased AMPK expression compared to the 0.01% DMSO control, but the 0.01% DMSO control and 0.1uM docetaxel treatment both increased phosphorylated-AMPK expression compared to the media control (Figure 4.14). There was an increase in phosphorylated-AMPK expression with 0.1uM docetaxel treatment alone compared to the media control. The combination of 1mM metformin and 0.1uM of docetaxel decreased AMPK expression compared to 1mM metformin treatment alone and 0.1uM docetaxel treatment alone (Figure 4.14). The
A combination of 1mM metformin and 0.1uM docetaxel also decreased phosphorylated-AMPK expression compared to 1mM metformin treatment alone and 0.1uM docetaxel treatment alone.

In DU145 cells, 1mM metformin treatment did not increase AMPK or phosphorylated-AMPK expression compared to the media control (Figure 4.15). The 0.01% DMSO control significantly increased the expression of both AMPK and phosphorylated-AMPK (Figure 4.15). 0.01uM of docetaxel treatment alone did not change AMPK expression compared to the media control but AMPK expression was decreased with 0.01uM docetaxel treatment compared to the 0.01% DMSO control (Figure 4.15). Phosphorylated-AMPK expression was increased with 0.01uM of docetaxel treatment compared to the media control but was slightly decreased compared to the 0.01% DMSO control (Figure 4.15). The combination of 1mM metformin and 0.01uM docetaxel did not change AMPK expression compared to metformin or docetaxel treatment alone (Figure 4.15). The combination of 1mM metformin and 0.01uM docetaxel increased phosphorylated-AMPK expression compared to 1mM metformin alone, however it decreased phosphorylated-AMPK expression compared to 0.01uM docetaxel alone (Figure 4.15). Quantification of the Western blot results are provided in Figures 4.14 and 4.15.
Figure 4.14: The effect of metformin combined with docetaxel treatment on the expression of AMPK and phosphorylated AMPK (P-AMPK) in PC3 cells. PC3 cells were treated with 1mM metformin alone, 0.1uM docetaxel alone or a combination of 1mM metformin and 0.1uM of docetaxel for 48 hours. Cell lysates were then harvested and expression of AMPK and P-AMPK was evaluated using Western blotting. GAPDH was used as a loading control. The graphs (right) represent the average quantification of the protein expression of duplicate experiments.

Figure 4.15: The effect of metformin combined with docetaxel treatment on the expression of AMPK and phosphorylated AMPK (P-AMPK) in DU145 cells. DU145 cells were treated with 1mM metformin alone, 0.01uM docetaxel alone or a combination of 1mM metformin and 0.01uM of docetaxel for 48 hours. Cell lysates were then harvested and expression of AMPK and P-AMPK was evaluated using Western blotting. GAPDH was used as a loading control. The graphs (right) represent the average quantification of the protein expression of duplicate experiments.
4.4 Discussion

The current literature indicates an anti-neoplastic effect of metformin treatment in multiple types of cancer cells, including prostate cancer cells (Zakikhani et al 2008; Alimova et al 2009; Rattan et al 2011; Sahra et al 2008). Metformin has been shown to enhance the effect of bicalutamide and simvastatin in prostate cancer models, and improve the efficacy of chemotherapy in breast cancer, lung cancer, and colon cancer models (Colquhoun et al 2012; Babcook et al 2014; Babcook et al 2014b; Hirsch et al 2009; Rocha et al 2011; Zhang et al 2013; Nangia-Makker et al 2014). Metformin has been shown to enhance the tumor suppressing effect of doxorubicin in breast cancer cell lines and prolong remission breast cancer xenograft models (Hirsch et al 2009). In breast and lung cancer xenografts metformin combined with paclitaxel more effectively reduced tumor growth and increased apoptosis than either treatment alone (Rocha et al 2011). In colon cancer cells, metformin combined with 5-fluorouracil alone or both 5-fluorouracil and oxaliplatin enhanced the antiproliferative effect of these chemotherapeutic agents (Zhang et al 2013; Nangia-Makker et al 2014). Despite evidence indicating metformin has a chemosensitizing effect in other types of solid tumors, there is extremely limited evidence for use of metformin as a chemosensitizing agent in prostate cancer and our study was designed to address this knowledge gap. Collectively, our results revealed that metformin treatment or docetaxel treatment alone can effectively reduce cell viability and cell migration in PC3 cells, however neither treatment had an effect on the viability of DU145 cells. Combining metformin with docetaxel did significantly reduce cell viability in PC3 cells. Furthermore, metformin treatment or docetaxel treatment alone can effectively reduce intracellular ATP levels in both PC3 and DU145 cells, however combining metformin and docetaxel had the same efficacy as docetaxel treatment alone in PC3 cells and the combined treatment was in fact less effective than docetaxel treatment alone in DU145 cells. Metformin treatment resulted in increased phosphorylated-AMPK expression in PC3 cells, however this was not the case in DU145 cells. Furthermore, docetaxel treatment decreased AMPK and phosphorylated-AMPK expression in PC3 cells, but docetaxel only increased the phosphorylated-AMPK expression in DU145 cells. A summary of the in vitro results is included in Tables 4.2 and 4.3.
Table 4.2: Summary of *in vitro* effects of combined metformin and docetaxel treatment compared to single agent metformin treatment or docetaxel treatment in PC3 cells.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Metformin + Docetaxel vs. Metformin</th>
<th>Metformin + Docetaxel vs. Docetaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Viability</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
<tr>
<td>Cell Migration</td>
<td>Decrease</td>
<td>No change</td>
</tr>
<tr>
<td>ATP Concentration</td>
<td>Decrease</td>
<td>No change</td>
</tr>
<tr>
<td>AMPK Expression</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
<tr>
<td>Phosphorylated-AMPK Expression</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

Table 4.3: Summary of *in vitro* effects of combined metformin and docetaxel treatment compared to single agent metformin treatment or docetaxel treatment in DU145 cells.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Metformin + Docetaxel vs. Metformin</th>
<th>Metformin + Docetaxel vs. Docetaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Viability</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Cell Migration</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ATP Concentration</td>
<td>Decrease</td>
<td>Increase</td>
</tr>
<tr>
<td>AMPK Expression</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Phosphorylated-AMPK Expression</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

The different response to metformin between PC3 and DU145 cells is likely due to the mechanism of metformin action. The mechanism of action of metformin is mediated by inhibition of mitochondrial respiratory complex 1 (Pollak 2013). The inhibition of mitochondrial complex 1 inhibits mitochondrial oxidative phosphorylation and this reduces ATP production (Mayer et al 2015). This decline in cellular ATP and increase in both ADP/ATP and AMP/ATP ratios results in a cellular energy crisis (Foretz et al 2014). The increase in AMP and decrease in ATP activates tumor suppressor kinase LKB1, which then phosphorylates AMPK at Thr172 which activates the catalytic subunit of AMPK, AMPKα (Shaw et al 2004; Rocha et al 2011). Phosphorylated AMPK then activates tumor suppressor genes tuberous sclerosis complex 1.
(TSC1) and TSC2 which forms an mTOR-inhibition complex which then in turn inhibits the mammalian target of rapamycin (mTOR) pathway (Pernicova and Korbonits 2014). mTOR inhibition causes disruption of protein synthesis and this results in disturbing tumor cell proliferation (Pernicova and Korbonits 2014). It has been shown that LKB1 is expressed in PC3 cells but not in DU145 cells (Zadra et al 2014; Sun et al 2016). The presence of LKB1 in PC3 cells and absence of LKB1 in DU145 cells supports the LKB1-dependent phosphorylation mechanism of AMPK since our results show a reduction in cell viability in LKB1-expressing PC3 cells but no change in cell viability in LKB1-null DU145 cells. Metformin treatment also decreases cell migration in PC3 cells which is likely mediated through the LKB1-dependent mechanism. Furthermore, metformin treatment increased the expression of phosphorylated-AMPKα at Thr172 in PC3 cells, however there was no change in expression of phosphorylated AMPK observed in DU145 cells.

The decline in ATP concentration following metformin treatment in PC3 cells is likely due to its effectiveness in reducing mitochondrial oxidative phosphorylation and thereby reducing intracellular ATP levels. This would then lead to LKB1 activation, AMPK phosphorylation and activation which would inhibit mTOR and therefore reduce cell viability. However, in DU145 cells, metformin may have reduced mitochondrial oxidative phosphorylation and reduced intracellular ATP levels but since LKB1 is absent in these cells, AMPK was not phosphorylated and activated which resulted in no effect of metformin treatment on cell viability (Figure 4.13). The decline in ATP level caused by docetaxel treatment is likely due to the activation of the DNA damage response which would require large amounts of ATP to repair any damage caused by the chemotherapeutic agent.

The significant increase in phosphorylated AMPK in PC3 and DU145 cells caused by docetaxel treatment may be due to the phosphorylation of AMPK by the kinase ATM (Sanli et al 2014). The cytotoxicity of chemotherapeutic agents is mediated by the creation of permanent DNA lesions that trigger ATM activation and DNA damage response signaling (Sanli et al 2014). Prostate cancer cells treated with topoisomerase II inhibitor etoposide showed phosphorylation of ATM and in turn ATM-dependent phosphorylation of AMPK (Luo et al 2013). This ATM-
dependent phosphorylation of AMPK may be the mechanism through which docetaxel treatment alone is causing AMPK phosphorylation in PC3 and DU145 cells. This can also be linked to the significant reduction in cell viability observed for multiple docetaxel concentrations in the dose standardization for each cell line since ATM-dependent AMPK activation would lead to inhibition of mTOR signaling and a reduction in cell viability.

The lack of chemosensitizing effect of combining metformin with docetaxel treatment in DU145 cells is likely due to the absence of LKB1 expression which prevents the phosphorylation of AMPK following metformin treatment. It may also be possible that there is some kind of interference occurring when treating DU145 cells with metformin and docetaxel together which causes the reduced expression of phosphorylated AMPK and less effective intracellular ATP reduction, however this would need to be investigated further. In PC3 cells, metformin combined with docetaxel does significantly reduce cell viability compared to docetaxel treatment alone, but this is not the case for cell migration, intracellular ATP concentration, and phosphorylation of AMPK. This may be due to the high efficacy of docetaxel treatment alone reducing cell migration and intracellular ATP concentration, therefore adding metformin may not create an extreme enough energy crisis to enhance the effect of chemotherapy. Another possibility could be due to the saturation of AMPK activation since docetaxel treatment alone can effectively phosphorylate AMPK and if adding metformin ultimately activates AMPK as well, it may not be possible to enhance the effect of chemotherapy further through this mechanism. The decline in total AMPK expression with docetaxel treatment alone may also contribute to the possibility of AMPK activity saturation since lower levels of total AMPK expression would lead to a smaller pool of AMPK available for phosphorylation.

There has been some discussion in the literature regarding the mechanism of action of metformin in prostate cancer cells since it has been shown that metformin can act through both AMPK-dependent (Zadra et al 2014; Sahra et al 2010) and AMPK-independent mechanisms (Sahra et al 2008; Sahra et al 2011). However, the AMPK-independent action of metformin has been shown to function in a p53-dependent manner (Sahra et al 2011). PC3 cells are p53-null and DU145 cells have a mutated p53 which results in lack of p53 function in both cell lines (Carroll 1993).
Therefore, metformin action may be more dependent on the LKB1-AMPK signaling pathway since the AMPK-independent, p53-dependent mechanism is not available in these cell lines.

In conclusion, our study is the first to investigate the use of metformin as a chemosensitizer in combination with docetaxel treatment in castration-resistant prostate cancer cells. Our results indicate that metformin may be an effective chemosensitizer for castration-resistant prostate cancer cells. Further investigation is needed to fully elucidate the specific mechanism by which metformin combined with docetaxel chemotherapy exerts its effects in castration-resistant prostate cancer cell lines and provide explanation for the difference in response between cell lines. Some possible aspects to investigate include the AMPK signaling pathway, as well as the ATM-dependent AMPK phosphorylation pathway and expression of p53 which will be discussed further in Chapter 5.
Chapter 5  
General Discussion and Future Directions

5.1 General Discussion

The treatment of castration-resistant prostate cancer is extremely complex and despite the development of several novel therapeutics agents including abiraterone acetate, enzalutamide, and radium-223, the average survival for metastatic CRPC patients is only approximately 19 months (Heidenreich et al 2013). Docetaxel chemotherapy is still the first-line chemotherapeutic agent for patients with symptomatic metastatic castration-resistant prostate cancer, and although it does provide a significant survival benefit to patients, there are considerable side effects and associated toxicity. Therefore, there is a need to improve the therapeutic options available to metastatic castration-resistant prostate cancer patients.

One unique approach to improve docetaxel chemotherapy treatment is to combine it with agents that can enhance the efficacy of chemotherapy. Agents that are able to improve chemotherapeutic efficacy are defined as chemosensitizers. Since the establishment of docetaxel chemotherapy as standard-of-care in 2004, several agents have been combined with docetaxel to improve its efficacy in Phase 3 trials (reviewed in Chapter 1). However, none of these agents have yet been demonstrated to be effective chemosensitizers for docetaxel and there is still a need to identify novel chemosensitizing agents.

Metformin, a commonly prescribed oral biguanide used for treating Type II diabetes has been shown to not only have anti-neoplastic effects but also improve the efficacy of chemotherapy in lung cancer, breast cancer, and colon cancer models (reviewed in Chapter 1). However, the potential chemosensitizing effect of metformin has not been investigated thoroughly in prostate cancer. Therefore, the objective of this dissertation, specifically Chapter 3 and Chapter 4, was to investigate whether metformin acts as a chemosensitizer when combined with docetaxel in metastatic castration-resistant prostate cancer patients and in human prostate cancer cells.
The hypothesis for Chapter 3 was that diabetic metastatic castration-resistant prostate cancer patients taking metformin during docetaxel chemotherapy would have improved prostate cancer-specific and overall survival compared to diabetic metastatic castration-resistant prostate cancer patients on diet control, diabetic metastatic castration-resistant prostate cancer patients taking other types of antidiabetic medications, and non-diabetic metastatic castration-resistant prostate cancer patients all of whom were receiving docetaxel chemotherapy. An improvement in prostate cancer-specific and overall survival in the group taking metformin would indicate a chemosensitizing effect for metformin that enhanced docetaxel treatment efficacy. The results from our retrospective cohort study demonstrated that, contrary to our hypothesis, diabetic metastatic castration-resistant prostate cancer patients taking metformin during docetaxel chemotherapy did not in fact have improved prostate cancer-specific or overall survival compared to the other groups including patients on diet control, patients taking other types of antidiabetic medications, and non-diabetic patients. Our results indicate metformin may not be an effective chemosensitizer when combined with docetaxel for treatment of metastatic castration-resistant prostate cancer patients. However, due to the limitations of our study there is still a possibility of metformin acting as a chemosensitizing agent when combined with docetaxel chemotherapy.

The hypothesis for Chapter 4 was that metformin will act as a chemosenzitizing agent and therefore a combination of metformin and docetaxel will be more effective than docetaxel alone at eradicating prostate cancer cells. We used PC3 and DU145 castration-resistant human prostate cancer cells to investigate the effect of metformin combined with docetaxel chemotherapy on cell viability, migration, intracellular ATP levels and the potential mechanism for the effect of combination treatment. Our results indicate the metformin may be an effective chemosensitizing agent in PC3 cells which likely functions in an LKB1-AMPK dependent manner, however metformin was not effective as a chemosensitizer in LKB1-deficient DU145 cells. Therefore, more investigation is needed to determine an explanation for the different effects observed in each cell line.
Therefore, this dissertation provides new evidence regarding the novel approach of using metformin as a chemosensitizing agents in prostate cancer. The retrospective clinical study included in this dissertation showed that metformin use during docetaxel chemotherapy did not improve the prostate cancer-specific or overall survival of diabetic metastatic castration-resistant prostate cancer patients compared to the other groups including patients on diet control, patients taking other types of antidiabetic medications, and non-diabetic patients who all received docetaxel chemotherapy. However, due to some of the limitations of the retrospective study and the results obtained in Chapter 4, the possibility of metformin acting as a chemosensitizing agent in CRPC cannot be ruled out. In Chapter 4, metformin combined with docetaxel did significantly reduce the viability of PC3 cells compared to docetaxel treatment alone which indicates a chemosensitizing effect. Therefore, this dissertation provides a rationale for new lines of inquiry into metformin use in castration-resistant prostate cancer both in the pre-clinical and clinical setting. The following section will address some of the potential future directions to be taken from the work presented in this dissertation.

5.2 Future Directions

5.2.1 Pre-clinical Approach

5.2.1.1 Potential role of ATM, androgens, or p53 in metformin chemosensitization

There are a few different ways to expand on the in vitro work completed in Chapter 4. One possibility is extending the mechanistic work that was started in Chapter 4 to determine more precisely which mechanisms are involved in the different response to metformin, docetaxel, and combined metformin and docetaxel treatment in PC3 and DU145 cells. Investigating the AMPK pathway in more detail by completing Western blotting for the expression of downstream signaling components such as tuberous sclerosis complex 1 (TSC1) and TSC2, mTOR, and phosphorylated-mTOR after treating cells with metformin, docetaxel, and a combination of metformin and docetaxel would provide additional information about the response to treatment in PC3 and DU145 cells. Another pathway to investigate further would be the effect of docetaxel treatment on the ATM signaling pathway. ATM is activated in response to DNA damage and can phosphorylate and activate AMPK (Luo et al 2013). Therefore, it would be worth investigating
whether ATM-dependent activation of AMPK is occurring in these cell lines. There is also no consensus in the literature regarding the role of LKB1 in the ATM-dependent activation of AMPK and this could be investigated more thoroughly in PC3 and DU145 cells since PC3 cells express LKB1, while DU145 cells do not. Therefore, Western blotting for the expression of LKB1, ATM, AMPK, phosphorylated AMPK following metformin, docetaxel and combined metformin-docetaxel treatment would be another novel approach for addressing the difference in response between cell lines.

Another possibility for further experiments is completing the same set of experiments in different prostate cancer cell lines. Although PC3 and DU145 cells are well established prostate cancer models, there are some additional models that represent advanced stages of prostate cancer with different characteristics. C4-2 prostate cancer cells were derived from LNCaP cells which were co-inoculated with tissue-specific or bone-derived mesenchymal or stromal cells in castrated mice and could metastasize to the lymph nodes and bone (Russell and Kingsley 2003). C4-2 cells are androgen independent but still maintain sensitivity to androgens and have low levels of functional p53 which represents an earlier stage of castration-resistant prostate cancer (Russell and Kingsley 2003). Another potential cell line is 22Rv1, which was derived from a xenograft which was serially propagated in mice after castration-induced regression and relapse of the parental androgen-dependent CWR22 prostate cancer xenograft model (Sramkoski et al 1999; Russell and Kingsley 2003). 22Rv1 cells are androgen independent but still maintain sensitivity to androgens and have wild-type p53 (van Bokhoven et al 2003). 22Rv1 cells also represent an earlier stage of castration-resistant prostate cancer. These cell lines are in contrast to PC3 cells which are androgen independent and have a deletion mutation of p53, and DU145 cells which are also androgen independent but have mutated non-functional p53 (Russell and Kingsley 2003).

The p53 status and androgen sensitivity of C4-2 and 22Rv1 cell lines would allow for multiple avenues of future research. Metformin combined with paclitaxel has been shown to have significant antitumor effects in vitro and in vivo in breast and lung cancer models (Rocha et al 2011). The suggested mechanism of action was due to metformin causing AMPK activation due
to declines in ATP levels, and paclitaxel functioned through activation of p53 and sestrin 1 (SESN1) and sestrin 2 (SESN2) which also activated AMPK signaling and reduced downstream mTOR signaling (Rocha et al 2011). Therefore, evaluating the effect of metformin, docetaxel or combined metformin-docetaxel treatment in cell lines with functional p53 may potentially provide insight into the role of p53 in a chemosensitization response for metformin combined with docetaxel. The androgen sensitivity of each cell line is also important since this may provide an avenue for evaluating metformin combined with abiraterone or enzalutamide, especially since metformin has been shown improve the efficacy of bicalutamide (Colquhoun et al 2012). Investigating metformin combined with these hormonal agents that are used during the initial stages of treating castration-resistant prostate cancer would be yet another novel approach for metformin use in sensitizing prostate cancer cells to anti-cancer treatments.

5.2.1.2 Targeting prostate cancer stem cells
Another possible future direction for the investigation of metformin as a chemosensitizing agent in castration-resistant prostate cancer could be evaluating a more specific component of its anti-neoplastic effect.

As discussed in the Chapter 1 literature review, CSCs are a small subset of cells within a tumor that have self-renewing, tumor-initiating, and tumor-propagating abilities and these cells are believed to contribute to recurrence and therapeutic resistance (Bao et al 2013; Chen et al 2013; Clarke et al 2006; Ning et al 2013; Tang et al 2007; Chen et al 2013). CSCs have been found in several solid tumors including cancers of the breast, pancreas, colon, brain, and the prostate (Al-Hajj et al 2003; Li et al 2009; Ricci-Vitiani et al 2007; Singh et al 2004; Collins et al 2005). CSCs contribute to the development of castration-resistant prostate cancer and may also play a significant role in prostate cancer progression.

Primary prostate carcinomas have been shown to contain a subset of CSCs which play a role in local invasion, more specifically seminal vesicle invasion and bone metastases (Colombel et al 2012; Mayer et al 2015). Furthermore, the percentage of CSCs has a prognostic impact on the
risk of progression of bone metastases (Colombel et al 2012). The percentage of cells expressing a stem cell phenotype in bone marrow metastases in prostate cancer patients is predictive of progression of bone metastases (Mayer et al 2015; Ricci et al 2013). Additionally, there is a small population of docetaxel-resistant cells, likely CSCs, that has been shown to be present in primary and metastatic clinical prostate cancer samples but the size of this population was significantly greater in metastatic patients treated with docetaxel compared to untreated patients (Domingo-Domenech et al 2012). Furthermore, a greater percentage of cells expressing the docetaxel-resistance phenotype in primary samples was associated with prognostic factors and shorter time to biochemical relapse (Domingo-Domenech et al 2012; Mayer et al 2015). Prostate CSCs are also inherently resistant to conventional anti-cancer therapies such as androgen deprivation and chemotherapy (Mayer et al 2015). This is likely due to their quiescent and androgen-independent characteristics and if prostate CSCs give rise to castration-resistant tumors following the selection model (reviewed in Chapter 1), targeting this subpopulation using chemosensitizing agents would likely improve therapeutic efficacy of chemotherapy.

One unique aspect of the anti-neoplastic effect of metformin is its ability to target CSCs. Metformin has been shown to be cytotoxic to radio-resistant breast CSCs and metformin also increased the efficacy of radiation in suppressing tumor growth in xenografts, likely due to a reduction in the population of CSCs (Song et al 2012; Mayer et al 2015). Metformin has been shown to target breast CSCs in four different cell lines and in xenografts metformin combined with doxorubicin enhanced the tumor-suppressing effect of doxorubicin and prolonged remission (Hirsch et al 2009). Furthermore, metformin can enhance the effect of paclitaxel and carboplatin by targeting CSCs in breast cancer xenografts at doses comparable to those used in Type 2 diabetes patients (Iliopoulos et al 2011). In pancreatic cancer cells, metformin was shown to significantly decrease cell survival, clonogenicity, wound-healing, sphere-forming capacity, and CSC markers (Mayer et al 2015; Bao et al 2012; Gou et al 2013). In colon cancer cells, metformin treatment was shown to significantly reduce the population of CD133+ CSCs and it also enhanced the anti-proliferative effect of 5-fluorouracil on CSCs (Mayer et al 2015; Zhang et al 2013). In colon cancer, metformin has been shown to act synergistically when combined with 5-fluorouracil and oxaliplatin by inhibiting proliferation and tumor growth by reducing viability
of colorectal CSCs (Mayer et al 2015; Nangia-Makker et al 2014). However, the effect of metformin on prostate CSCs and combining metformin and docetaxel to target prostate CSCs has not been investigated.

Metformin acts directly on mitochondrial complex 1 to inhibit the production of energy by oxidative phosphorylation which forces cells to compensate by increasing the rate of glycolysis (Andrzejewski et al 2014). This causes an energy crisis in cells that rely more heavily on oxidative phosphorylation and these cells become more sensitive to additional stresses, such as anti-cancer therapy (Andrzejewski et al 2014). CSCs are thought to rely heavily on oxidative phosphorylation and would therefore be more sensitive to metformin treatment. Pancreatic CSCs have been shown to have a highly mitochondrial-dependent metabolic profile which is in contrast to the majority of cancer cells that rely on aerobic glycolysis (Mayer et al 2015; Lonardo et al 2013). If CSCs have functional AMPK, the activation of AMPK would prevent CSCs from shifting to aerobic glycolysis and this would render CSCs incapable of compensating for an energy crisis and make them more sensitive to metformin treatment (Mayer et al 2015). If CSCs are deficient in AMPK, they would be inherently unable to compensate for an energy crisis and this would make CSCs sensitive to metformin treatment (Mayer et al 2015). This sensitivity to metformin treatment and subsequent sensitivity to additional stressors would make CSCs a good candidate for combinatorial therapy with metformin and docetaxel chemotherapy (Mayer et al 2015). The potential mechanism for this effect is shown in Figure 5.1.
We have completed some preliminary experiments evaluating the potential chemosensitizing effect of metformin on prostate CSCs. Given that metformin has been shown to improve the effectiveness of chemotherapy in multiple types of solid tumors, we hypothesize that metformin will act as a chemosensitizing agent on prostate cancer stem cells and improve the efficacy of docetaxel chemotherapy.

The presence of CSCs in DU145 and PC3 prostate cancer cell lines was evaluated using flow cytometry to identify the following markers for prostate CSCs: CD44+/α2β1\text{high}/CD133+. This panel of prostate CSC markers was selected based on the current evidence in the literature. A population of CD44+/α2β1\text{high}/CD133+ cancer stem cells was identified in human prostate tissue samples (Collins et al 2001; Richardson et al 2004; Collins et al 2005). The CD44+/α2β1\text{high} subpopulation has also been shown to be enriched in CSCs in LAPC-9 cells (Patrawala et al 2007; Mayer et al 2015). Populations of CD44+ prostate CSCs have also been shown to be more proliferative, tumorigenic and metastatic which are all characteristics of CSCs (Patrawala et al 2006; Hurt et al 2008). Furthermore CSCs in LNCaP and DU145 cells have been shown to have enhanced expression of CD133 and CD44 (Jeter et al 2011).
Our preliminary results show that in PC3 cells, the majority of the population is expressing CD44 and $\alpha_2\beta_1$, but approximately 4% of the population appears to highly express these markers (Figure 2). There was also no expression of CD133, a key CSC marker, in PC3 cells (Figure 2). In DU145 cells, the entire population was expressing CD44 and $\alpha_2\beta_1$ but there was no subpopulation with higher expression that could be identified (Figure 3). Similar to PC3 cells, DU145 cells did not show any expression of CD133 (Figure 3). Therefore, no definitive population of prostate CSCs could be identified in these cell lines.

**Figure 5.2:** Flow cytometry isolation of prostate cancer stem cells from a monolayer of PC3 cells. The graph labels correspond to the following: EF450-A: CD44, APC-A: CD49b, PE-A: CD133. Experiments were completed in triplicate.

**Figure 5.3:** Flow cytometry isolation of prostate cancer stem cells from a monolayer of DU145 cells. The graph labels correspond to the following: EF450-A: CD44, APC-A: CD49b, PE-A: CD133. Experiments were completed in triplicate.
Next Steps:
One of the possible next steps would be to try and identify a prostate CSC population in these cell lines using a new set of markers. Unlike other types of solid tumors such as breast cancer which have a more defined set of CSC markers (CD24\textsubscript{low}/CD44\textsubscript{high}), there is no set of definitive markers which have been established for prostate CSCs. ALDH+ (aldehyde dehydrogenase) prostate cancer cells have been shown to exhibit several CSC characteristics including enhanced clonogenicity, migration, tumorigenicity, and readily form metastases \textit{in vivo} (Li et al 2010; van den Hoogen et al 2010; Mayer et al 2015). ABCG2+ (ATP-binding cassette transporter) prostate CSCs have also been identified (Foster et al 2013). Increased expression of stemness markers such as Nanog, Sox2, and Oct4 have also been shown to potential prostate CSCs (Guzel et al 2014). A collection of the different markers used to identify prostate CSCs is included in Table 1. Therefore, using a different set of markers and then repeating flow cytometry to identify a distinct prostate CSC population would be the next step that could be taken.

Table 5.1: Markers for isolation of prostate cancer stem cells (CSCs). Originally published in \textit{Prostate Cancer and Prostatic Diseases} (Mayer et al 2015).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression level</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH</td>
<td>High</td>
<td>Enzyme oxidizes aldehydes</td>
<td>Li et al., Qin et al., Finones et al.</td>
</tr>
<tr>
<td>ABCG2</td>
<td>High</td>
<td>ATPase transporter</td>
<td>Foster et al., Gangavasupu et al.</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>Cell adhesion and signaling</td>
<td>Patrawala et al., Patrawala et al., Collins et al., Hurt et al.</td>
</tr>
<tr>
<td>CD133 (Prominin-1)</td>
<td>+</td>
<td>Marker normal stem cells and CSCs</td>
<td>Richardson et al., Collins et al.</td>
</tr>
<tr>
<td>c-Kit (CD117)</td>
<td>+</td>
<td>Receptor tyrosine kinase</td>
<td>Finones et al.</td>
</tr>
<tr>
<td>Integrin α2β1 (CD49b)</td>
<td>High</td>
<td>Collagen receptor</td>
<td>Patrawala et al., Guzman-Ramirez et al.</td>
</tr>
<tr>
<td>CD49f</td>
<td>High</td>
<td>Laminin binding</td>
<td>Guzman-Ramirez et al.</td>
</tr>
<tr>
<td>CD166</td>
<td>+</td>
<td>Cell adhesion</td>
<td>Jiao et al.</td>
</tr>
<tr>
<td>PSA</td>
<td>−/lo</td>
<td>Glycoprotein</td>
<td>Qin et al.</td>
</tr>
<tr>
<td>CK5/14</td>
<td>+</td>
<td>Cytokeratin</td>
<td>Tokar et al.</td>
</tr>
<tr>
<td>CK8/18</td>
<td>+</td>
<td>Cytokeratin</td>
<td>Tokar et al.</td>
</tr>
<tr>
<td>Nestin</td>
<td>+</td>
<td>Intermediate filament protein</td>
<td>Guzman-Ramirez et al.</td>
</tr>
<tr>
<td>SCA-1</td>
<td>+</td>
<td>Cell surface marker</td>
<td>Guzman-Ramirez et al.</td>
</tr>
<tr>
<td>SMO (Smoothened)</td>
<td>+</td>
<td>G-protein-coupled receptor</td>
<td>Lawson et al., Xin et al.</td>
</tr>
<tr>
<td>Sox2</td>
<td>+</td>
<td>Transcription factor (self-renewal)</td>
<td>Patrawala et al., Rybak and Tang</td>
</tr>
<tr>
<td>Oct4</td>
<td>+</td>
<td>Transcription factor (self-renewal)</td>
<td>Patrawala et al., Jeter et al.</td>
</tr>
</tbody>
</table>

Abbreviation: ALDH, aldehyde dehydrogenase; CSC, cancer stem cell.

After identifying a distinct prostate CSC population in DU145 and PC3 cell lines, it would then be possible to treat cells with metformin alone, docetaxel alone, or a combination of metformin and docetaxel and then complete flow cytometry to determine if the percentage of CSCs has changed due to treatment. Another way to evaluate the effect of treatment on prostate CSCs
would be conducting a sphere-forming assay. The sphere-forming assay would consist of seeding single cell suspensions into non-adherent plates using serum free media (Allan 2011). Cells with self-renewing capacity survive and then proliferate over the course of 5-7 days to create spheres of cells which can then be counted (Allan 2011). A reduction in sphere formation capacity would indicate treatment was effective. Then the mechanism of action which allows metformin to target CSCs would be evaluated by Western blotting using antibodies for AMPK pathway components. Western blotting of stem cell markers including CD44, CD133, α2β1, Nanog, Sox2, and Oct4 could also be completed to evaluate the effect of metformin, docetaxel, and combined metformin-docetaxel treatment on stem cell markers.

After completing in vitro experiments, the next step would be serial transplantation of the isolated CSCs into immunodeficient mice. This is the current gold standard for confirming the characterization and identification of CSCs since this method allows for the evaluation of self-renewal and tumor propagation abilities, which are crucial characteristics of CSCs (Li et al 2011; Clarke et al 2006). Prostate CSCs would be isolated using flow cytometry and then nude mice would be inoculated only with the isolated prostate CSCs. Tumor initiation, as well as tumor growth and progression, would be monitored. The effect of treatment with metformin, docetaxel and combination metformin-docetaxel treatment on tumor growth would also be evaluated.

Evaluating the effect of metformin on prostate CSCs would be addressing an important gap in the literature and taking it a step further to determine whether it can act as a chemosensitizing agent on prostate CSCs when combined with docetaxel chemotherapy adds to the novel aspect of this approach.

5.2.2 Potential Phase II Clinical Trial
The current literature contains some encouraging results regarding the use of metformin in men with castration-resistant prostate cancer regarding disease progression and survival (reviewed in Chapter 1). However, the majority of these studies have been retrospective analyses and there is a need for prospective trials evaluating the effect of metformin in castration-resistant prostate
cancer in order to validate the retrospective study results. There are five studies which are active or currently recruiting patients registered on clinicaltrials.gov evaluating metformin use in castration-resistant prostate cancer patients. One of the active trials is the Metformin in castration-resistant prostate cancer: a multicenter Phase II trial (NCT01243385) which is evaluating the safety of giving metformin as first-line therapy for patients with locally advanced or metastatic prostate cancer with the primary outcome measure being progression-free survival. The other active trial is a Phase II randomized, placebo-controlled, double-blind study comparing castration alone to castration plus metformin as first-line treatment for patients with advanced prostate cancer with the primary outcome measure being metabolic syndrome evaluation (NCT01620593). The three trials recruiting patients are evaluating the combination of metformin with abiraterone acetate or enzalutamide. The IMPROVE trial (NCT02640534) is a randomized, open label, Phase II trial evaluating whether the combination of enzalutamide and metformin is more effective than enzalutamide alone in castration-resistant prostate cancer patients with the primary outcome of disease control at 15 months. A Phase I trial (NCT02339168) is also being conducted to evaluate the side effects and best dose of metformin to be combined with enzalutamide in patients with prostate cancer that has not responded to previous hormonal therapy. The MetAb-Pro Phase II pilot study (NCT01677897) is assessing the impact of metformin combined with abiraterone on the survival of patients with metastatic castration-resistant prostate cancer prior to docetaxel.

There are no active trials evaluating the effect of metformin combined with docetaxel therapy. There is one Phase II trial (NCT01478308) which was withdrawn due to lack of accrual, that was designed to evaluate the safety and efficacy of metformin combined with docetaxel treatment in metastatic castration-resistant prostate cancer patients. However no other studies have been registered to evaluate this combination. Therefore one potential future direction extending from the retrospective work completed in Chapter 3 would be conducting a prospective trial evaluating the effect of metformin use during docetaxel chemotherapy on survival of metastatic castration-resistant prostate cancer patients. The hypothesis would be that patients taking metformin during docetaxel chemotherapy would have improved prostate cancer-specific and overall survival compared to patients receiving docetaxel without metformin.
5.3 Conclusions

In conclusion, this dissertation provides novel evidence on the use of metformin as a chemosensitizing agent in castration-resistant prostate cancer. Clinically, diabetic castration-resistant prostate cancer patients taking metformin during docetaxel chemotherapy did not have improved prostate cancer-specific or overall survival when compared to diabetic metastatic castration-resistant prostate cancer patients on diet control, diabetic metastatic castration-resistant prostate cancer patients taking other types of antidiabetic medications, and non-diabetic metastatic castration-resistant prostate cancer patients all of whom were receiving docetaxel chemotherapy. Pre-clinically, metformin was shown to be a potential chemosensitizing agent when combined with docetaxel chemotherapy in PC3 cells due to the significant reduction in cell viability observed when metformin was combined with docetaxel treatment. Therefore, metformin may be a chemosensitizing agent for castration-resistant prostate cancer. The results included in this dissertation provide a base from which we could further investigate the mechanism of action of metformin’s chemosesitization potential in castration-resistant prostate cancer cells with varying characteristics. It also opens up avenues for potentially pursuing a prospective clinical trial to better understand the potential benefit of metformin use during docetaxel chemotherapy in castration-resistant prostate cancer patients.
References


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