Abstract

The Effect of Diet, Exercise and Metformin on the Progression of Prostate Cancer

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Prior research has suggested that lifestyle factors, such as diet and physical activity, influence prostate cancer (PCa) progression. Metformin intake has been shown to be associated with decreased cancer risk in type II diabetic patients. We hypothesize that a low carbohydrate diet, prolonged aerobic exercise and metformin treatment can all independently slow prostate tumor development and a combination regimen will have an additive benefit. We used LNCaP xenografts to test this hypothesis. Results revealed that a diet low in carbohydrate reduced food consumption and a combination with exercise significantly reduced animal body weights. Ten weeks of metformin did not significantly alter tumor growth rate compared to control animals. Ten weeks of exercise significantly inhibited tumor growth. Our results suggest that dietary carbohydrate alteration or the administration of metformin alone cannot significantly influence prostate tumor progression. A suitable sustained exercise regimen may offer a more protective effect against PCa progression.
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This work would not have been possible if not for the kind help of many individuals:

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<td>Acetyl-CoA Carboxylases 1</td>
</tr>
<tr>
<td>ACF</td>
<td>Aberrant Crypt Foci</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen Deprivation Therapy</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Activator Protein</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-Triphosphate</td>
</tr>
<tr>
<td>BCa</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>BCR</td>
<td>Biochemical Recurrence</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
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<tr>
<td>BUPA</td>
<td>British United Provident Association</td>
</tr>
<tr>
<td>CAPB</td>
<td>Cancer of the Prostate and Brain</td>
</tr>
<tr>
<td>CaPSURE</td>
<td>Cancer of the prostate Strategic Urologic Research Endeavor</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>ED</td>
<td>Erectile Dysfunction</td>
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<tr>
<td>EGIR</td>
<td>European Group for the Study of Insulin Resistance</td>
</tr>
<tr>
<td>EFR</td>
<td>Early Growth Response Protein</td>
</tr>
<tr>
<td>ERG</td>
<td>ETS-Related Gene</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinases</td>
</tr>
<tr>
<td>ETS</td>
<td>E-Twenty Six</td>
</tr>
<tr>
<td>ETV</td>
<td>ETS-Translocation Variant</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>4EBP1</td>
<td>4E Binding Protein 1</td>
</tr>
<tr>
<td>Gl</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GPS</td>
<td>Genomic Prostate Score</td>
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<tr>
<td>GPCR</td>
<td>G protein Coupled Receptor</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin-Releasing Hormone</td>
</tr>
<tr>
<td>GTPase</td>
<td>Hydrolase Enzymes Hydrolyze Guanosine Triphosphate (GTP)</td>
</tr>
<tr>
<td>HCD</td>
<td>High Carbohydrate Diet</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HF-HC</td>
<td>High Fat-High Carbohydrate</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-Inducible Factor 1</td>
</tr>
<tr>
<td>HPC</td>
<td>Hereditary Prostate Cancer</td>
</tr>
<tr>
<td>HPCX</td>
<td>HPC X-linked</td>
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<tr>
<td>HR</td>
<td>Hazard Ratio</td>
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<tr>
<td>HUNT2</td>
<td>Nord Trøndelag Health Study 2</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
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<tr>
<td>IGF-1</td>
<td>Insulin Like Growth Factor - 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>IGF-BP</td>
<td>IGF Binding Protein</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>i. p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>IRS-I</td>
<td>Insulin Receptor Substrate 1</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LKB1</td>
<td>Liver Kinase B1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>LCD</td>
<td>Low Carbohydrate Diet</td>
</tr>
<tr>
<td>MeS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MMA</td>
<td>Massachusetts Male Aging</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PCAP</td>
<td>Predisposing for Prostate Cancer</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCPT</td>
<td>Prostate Cancer Prevention Trial</td>
</tr>
<tr>
<td>PCR</td>
<td>Pathologic Complete Response</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-Kinases</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>PLCO</td>
<td>Prostate, Lung, Colorectal and Ovarian Cancer</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-Myristate-13-Acetate</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>Rheb</td>
<td>Ras Homolog Enriched in Brain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RP</td>
<td>Radical Prostatectomy</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex Hormone Binding Globulin</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 Kinase</td>
</tr>
<tr>
<td>SREBP-I</td>
<td>Sterol Regulatory Element-Binding Protein-I</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist to Hip Ratio</td>
</tr>
</tbody>
</table>
Introduction

1) Prostate Cancer overview

1.1 Epidemiology

Prostate cancer (PCa) is the most common form of noncutaneous malignancy and the second leading cause of cancer death affecting men in North America (Siegel, 2013). It has been estimated that about one in six men will be diagnosed with PCa during lifetime, and one in 36 will die from the disease (American Cancer Society, 2013). The incidence and mortality of PCa varies considerably across countries. This reflects genetic, environmental, and dietary influences.

1.1.1 Incidence

In 2008, there were 903,500 new incidences of PCa worldwide, which accounted for 14% of new cancer cases (Jemal, 2011). The age-standardized incidence rate was 37.0 per 100,000, and a 5-fold variation between developing and more developed areas existed, with the developed nations having a much higher incidence (Jemal, 2011). Generally, the highest incidences are found in Northern America and Western Europe, and the lowest incidences are in Eastern Asia, particularly China, Japan and India (Nelen, 2007). For instance, in the United States, 186,320 men developed PCa in 2008 (Ferlay, 2010), with an incidence rate of 85.6 (Jemal, 2011). In contrast, in the same year, only 33,802 Chinese men were diagnosed with PCa (Ferlay, 2010), the incidence rate being 14.5 (Jemal, 2011). The differences may be attributed to genetics, diet and lifestyle, as well as differences in health care and under-reporting (Nelen, 2007). Screening
for PCa using prostate specific antigen (PSA) testing has become common practice in most developed countries. This has led to a rapid increase in the number of cases being diagnosed with PCa, leading to the increase in the incidence of PCa in Western countries and more recently in the Eastern countries as well (Schroder, 2010). The incidence of PCa is expected to continue increasing due to increased longevity and more commonly PSA screening, and therefore it will remain a large and growing health problem.

1.1.2 Mortality

Considering the high incidence, the prognosis for PCa is relatively good, with 258,400 deaths worldwide in 2008, which was the sixth leading cause of cancer death in men (Jemal, 2011). It accounts for approximately 3% of all cancer deaths and 6% of cancer deaths in men (Nelen, 2007). The age-standardized mortality rate was 8.1 per 100,000. The mortality rate was 2 fold higher in developed regions compared to developing areas, which was a smaller difference than the incidence (Jemal, 2011). Though burdened with a much higher incidence rate, the survival rate for PCa is better in developed countries. For example, in 2008, the mortality-to-incidence rate ratio was 0.12 and 0.44 for the United States and China respectively (Zhang, 2011). Mortality rates for PCa have been decreasing in many developed countries, such as Australia, the United Kingdom and the United States (Bouchardy, 2008). Since 1993, a 35% decrease in PCa mortality has been observed in the United State, with an average of 4% decrease per year (Schroder, 2010). The contributors to this decrease include early detection, improved treatment strategies and changes in lifestyle (Schroder, 2010).

1.1.3 Prevalence
PCa is the third most prevalent cancer worldwide, following breast and colorectal cancer (Parkin, 2005). In 2008, the world prevalence of PCa was 3200,000 cases (Ferlay, 2010). Since prevalence measures the total cases at a certain point of time, it depends on both incidence and mortality rates, and therefore, also differs considerably across different regions. Again, this is observed more in developed countries than in less developed nations, with a 3.5-fold disparity. For instance, the total cases of PCa in 2008 were 783,000 in the United States and 75,000 in China (Ferlay, 2010). With increasing incidence rate and decreasing mortality, PCa is expected to be more prevalent globally in the future.

1.2 Risk Factors

Although recent research has improved insight into the probable causes and risk factors for PCa, the specific causes still remain obscure. PCa results as a consequence of several different factors. Epidemiology studies have suggested that both genetic and environmental factors may contribute to the initiation and progression of this disease (Patel and Klein, 2009; Nelen, 2007). The following section will talk about some well-studied risk factors for PCa.

1.2.1 Age

Older men are at a higher risk for PCa, with the incidence increasing with age more rapidly for PCa compared to many other cancers (Nelen, 2007). PCa is rarely seen in men below the age of 40, and patients younger than 50 years of age account for less than 0.1% of all men affected with PCa (Gronberg, 2003). Incidence rate tends to rise fast after age 60 and 75% of PCas are diagnosed in men over the age of 65 (Spickett and Roberston, 2010). Autopsy data suggest that histological cancer also increases with age. Microscopic PCa lesions were found in 15% - 30% in
men older than 50 years of age and 60% - 70% in men older than 80 years of age (Pienta and Esper, 1993). These studies demonstrate that a larger population of men will die with PCa than from PCa (Dunn and Kazer, 2011).

**1.2.2 Ethnic Origin**

Another well-known risk factor for PCa is ethnicity. As depicted by the epidemiology data, the incidence rate of PCa varies greatly across countries and between different ethnic groups (Quinn and Babb, 2002). The highest rates of PCas are found in North America, Western and Northern Europe and Australia, with an annual incidence of around 100 new cases per 100,000 of population. The lowest rates are among Asians, specifically men living in China, India and Japan, where the incidence rates are less than 5.8 (Ferlay, 2010). Although incidence of clinically detected PCa varies, the histological cancer does not follow the trend: the incidence of histological cancer is the same in the United States and Japan (Nelen, 2007). More recent report compared PCa prevalence in Japanese population to Russian population, which both has a low penetrance in prostate specific antigen (PSA) screening. The study found that PCa is found on autopsy in a similar proportion of Russian and Japanese men (Zlotta, 2013). These data suggest that age is most likely the determinant factor for initiation of PCa and differences of progression to clinical cancer are related to other unknown external factors. Evidence from migratory studies are in line with this concept. For example, one study found that PCa incidence of Chinese and Japanese men increased noticeably from 1.8 and 5.1 to 14.9 and 16.5 respectively after relocating to North America (Prezioso, 2004).
In the United States, between 1999 to 2007, African American men had the highest incidence of PCa (>250 per 100,000), followed by Caucasians (about 160 per 100,000) and lastly the Asians who had the lowest incidence rate (<100 per 100,000) (Centers for Disease Control and Prevention, 2010). The impact of race and ethnicity in the development of PCa remains unclear, but may be related to genetic, environmental, lifestyle and socioeconomic factors (Dunn and Kazer, 2011).

1.2.3 Genetics

Genetics is another well-established risk factor for PCa. Studies have shown that risk of developing PCa increases among men with affected relatives (Nelen, 2007). Furthermore, the relative risk is positively related with number of affected relatives, degree of relatedness, and negatively related to age of diagnosis of the affected family members (Bratt, 2002). For example, men with a first-degree family history of PCa have a two-fold risk compared to others (Dunn and Kazer, 2011). Studies of PCa risk among twin population revealed that concordance rate was higher in monozygotic twins than in dizygotic twins (Gronberg, 1994; Page, 1997), which further confirms that genetics plays a role in PCa development. In addition, a study identified that family history was also a predictor for elevated risk of aggressive PCa (Klein, 1998).

During the last two decades, complex segregation analysis has identified several PCa susceptibility genes in multiple chromosomal regions, including hereditary prostate cancer 1 (HPC1) at 1q24-25, predisposing for PCa (PCAP) at 1q42-43, HPC X-linked (HPCX) at Xq27-28, cancer for the prostate and brain (CAPB) at 1p36, HPC20 at 20q13, a locus at 16q, and a locus at 8p22-23 (Gillanders, 2004). Zheng et al (2008) evaluated 16 single nucleotide
polymorphisms (SNPs) in five chromosomal regions (three at 8q24 and one each at 17q12 and 17q24.3). They identified the most significant SNP from each of the five regions and the five SNPs combined with family history were estimated to account for 46% of the cases of PCa in the investigated population. Xu et al (2008) systematically reviewed the association of 15 independent genetic variants with clinical characters of PCa. Although they have been implicated in PCa risk by recent genome-wide association studies, these SNPs were not associated with Gleason scores, pathologic stage, or age at diagnosis of PCa.

Studies have shown that gene fusions involving 5’-untranslated region of transmembrane protease serine 2 (TMPRSS2) and two members of the E-twenty six (ETS) family of transcription factors (ETV1 and ERG) are associated with PCa (Tomlins, 2005). ETS translocation variant 1 (ETV1) and ETS-related gene (ERG) are involved in oncogenic translocations in Ewing’s sarcoma and myeloid leukemia (Oikawa and Yamada, 2003). Expression of TMPRSS2 in PCa is induced by androgen stimulation. Studies have suggested that overexpression of ERG or ETV1 in subsets of PCa is due to fusion with TMPRSS2 (Tomlins, 2005). It has also been hypothesized that ETS gene fusion may be a genetic trigger for the transition of prostatic intraepithelial neoplasia (PIN) to adenocarcinoma (Morris, 2008). Fusion status is currently being developed as a biomarker for PCa detection (Perner, 2007). In addition, conflicting date supports the association of TMPRSS2: ERG fusion with both improved and worsened patient outcomes (Morris, 2008). Therefore, further understanding on TMPRSS2:ETS gene fusion may provide new insight for understanding PCa tumorigenesis and developing novel diagnostics tools (Tomlins, 2005).
Recently, genomic tests became available for PCa. The Oncotype DX test for prostate cancer, developed by Genomic Health, is a genomic test designed to help men with newly diagnosed, early stage PCa make the right treatment decision. This test is performed on a small tumor sample and analyzes the expression of the 17-gene Oncotype DX panel, which predicts cancer aggressiveness. It gives a Genomic Prostate Score (GPS), with a lower GPS predicting less aggressive disease and indicating more favorable tumor biology and a higher GPS predicting more aggressive disease and indicating less favorable tumor biology. Another genomic test to aid physicians in predicting PCa aggressiveness in conjunction with clinical parameters is Prolaris, developed by Myriad. This test looks at the RNA expression of a 46-gene panel related to the progression of prostate tumors. Prolaris has been proven to predict clinical progression in 4 different clinical cohorts, in both pre and post-treatment scenarios (Freedland, 2013; Cooperberg, 2013; Cuzick, 2012; Cuzick, 2011).

1.2.4 Androgen

Androgens are essential for the normal growth, development, maintenance and function of the prostate (Jadvar, 2011). The biosynthesis of androgens is performed in the testes and adrenal glands, as well as peripheral tissues such as the skin and the prostate (Hsing, 2001). The two important androgens of adult male are testosterone and its reduced metabolite dihydrotestosterone (DHT) (Hsing, 2001). Testosterone is the main type of androgen in circulation, and is essential for growth of muscle mass, maintenance of bone and cardiovascular health, regulation of spermatogenesis and sexual drive (Vis, 2009). DHT, on the other hand is the principal androgen in prostate tissue and mainly regulates intra-prostatic androgen-mediated
processes such as cellular proliferation and differentiation (Vis, 2009). In the prostate, most DHT develops from conversion of testosterone by the action of 5α reductase type II (Hsing, 2001).

There is mounting evidence to believe that androgens play an important role in prostate carcinogenesis and high level of circulating androgens is a risk factor for PCa (Hsing, 1996). Androgens were found to induce PCa cells proliferation in vitro (Shaneyfelt, 2000) and induce PCa in rodent models in vivo (Hsing, 1996). Most PCa’s respond to androgen deprivation therapy (ADT) by temporary remission, however, they eventually will relapse to a castration-refractory state (Bosland, 2000). Furthermore, a decreased risk of PCa was observed among diabetic patients and diabetes is associated with lower total and free testosterone levels (Giovannucci, 1998). Despite all these convincing observations, epidemiological studies investigating circulating androgen levels and risk of PCa have not yielded consistent results.

Some studies have demonstrated that men with high serum levels of androgens had an increased risk of developing PCa. For example, Gann et al (1996) analyzed plasma samples from Physicians’ Health Study, and observed a positive association between PCa risk and levels of plasma testosterone, when both hormone and sex hormone binding globulin (SHBG) levels were adjusted. Also, an inverse association was seen between risk of PCa and serum levels of SHBG. Shanefelt et al (2000) performed a meta-analysis on hormonal predictors of PCa, and their results showed that men with the highest quartile of total testosterone were 2.34 times more likely to develop PCa, when adjusted for levels of hormones, body mass index (BMI) and age.
However, a few studies demonstrate no association between endogenous sex hormones and PCa. For instance, Eaton et al (1999) did a quantitative review of 8 prospective studies on endogenous sex hormones and PCa risk. They found no significant differences in the levels of circulating hormones in men who developed PCa and those who remained disease free. More recently, Roddam et al (2008) evaluated 18 prospective studies on sex hormones and PCa and performed a meta-analysis. Their results showed that there was no association between the risk of PCa and serum concentrations of total or free testosterone, estradiol, DHT or other androgen metabolites. However, a modest inverse association was observed between serum concentration of SHBG and risk of PCa. Interestingly, outcome from a nested case-control study with the samples collected prospectively in the prostate, lung, colorectal and ovarian cancer (PLCO) Trial demonstrated that sex hormone levels could be related to aggressiveness of PCa. They also found that the ratio of testosterone and SHBG tended to be directly related to the aggressiveness of disease in older men (Weiss, 2008).

The inconsistency in demonstrating a direct relationship between serum androgen levels and PCa risk may be a consequence of several limitations. It could be methodological limitations such as inadequate statistical power in most studies or the relatively small number of incidence cases (Hsing, 2001). There is uncertainty as to whether a single measurement of androgens can represent the actual androgen status over the etiologically relevant span of life (Gann, 1996). The most imperative limitation is that there is no knowledge as to whether circulating androgens reflect androgenic activity in prostate tissue (Hsing, 2001). It is also hypothesized that androgens have a dual action in PCa: it stimulates growth of prostate cancer cells, and also induce differentiation and maintains androgen dependency of the tumor (Grossmann and Wittert, 2012).
Thus, men with low levels of testosterone have a lower risk of developing PCa in the first place, however, they have increased risk of developing high grade, poorly differentiated PCa.

As stated above, DHT is the principal androgen within the prostate and it is generated by conversion of testosterone by the enzyme 5α reductase type II. Therefore, it has also been hypothesized that 5α reductase activity is associated with risk of PCa. It is to be noted that, the Massachusetts Male Aging (MMA) study found that androstanediol glucuronide was significantly associated with the risk of PCa (Mohr, 2001), and the concentration of androstanediol glucuronide is a serum marker of 5α reductase activity (Roddam, 2008). The most important study discerning the relationship between 5α reductase activity and PCa is the Prostate Cancer Prevention Trial (PCPT). In this study men were randomized to treatment with finasteride, an inhibitor of 5α reductase, or to the placebo arm of the trial for seven years. Treatment with finasteride for seven years resulted in a 24.8% reduction in the incidence of PCa. However this reduction in PCa risk was coupled with more frequently reported sexual side effects and a small increased risk of high-grade PCa (Thompson, 2003). This increased risk of high-grade cancer has been attributed to bias associated with the cytoreduction of the prostate with 5α reductase inhibitors. Another possible explanation is a grading bias: histologic changes that mimic those of high-grade disease are caused by androgen deprivation therapy (ADT) (Thompson, 2003). These results suggest that a lower activity of 5α reductase prevents/delays incidence of PCa.

1.2.5 Insulin and IGF axis

Besides androgen, other growth factors can also regulate growth of PCa cells, such as insulin like growth factor 1 (IGF-1) and insulin. IGF-1 is a peptide hormone, which involves in the
promotion of DNA synthesis, stimulation of cell cycle and inhibition of cell apoptosis (Lima, 2009), and thus a potent mitogen for normal and cancer cells. Most of the circulating IGF-1 is synthesized in the liver, but there is also local production of IGF-1 within IGF-1 responsive tissue, including the prostate (Pollak, 2001). Therefore, IGF-1 is regulated by both autocrine and paracrine mechanisms, and the action of IGF-1 in tissue is determined by both the circulating level and local production (Shi, 2001). In circulation, less than 5% of IGF-1 is free, and the majority forms a complex with IGF binding proteins (IGF-BPs). To date at least six IGF-BPs have been identified and cloned (Chen, 2005). Among the six IGF-BPs, IGFBP-3 is the most prevalent one, which forms ternary complexes with IGF-1 and an additional protein known as the acid-labile subunit (Papatsoris, 2005). IGFBP3 impairs IGF-1 reaction by coupling with IGFs and also other IGF-independent mechanisms (Papatsoris, 2005). IGF mitogenic activity is higher under circumstances in which IGF-BP levels are low (Lima, 2009). The action of IGF-1 is through the binding to its receptor, IGF-1R. Binding of IGF-1 to IGF-1R can trigger the receptor’s tyrosine kinase activity, which further generates a cascade of cell signaling pathways to regulate cell proliferation and apoptosis (Lima, 2009).

Insulin may also act as growth factor and regulate cell proliferation, differentiation and apoptosis (Nandeesha, 2008). The insulin signaling pathways are very similar to IGF-1’s and their receptors resemble each other with more than 50% homology. Therefore, the two mitogens are able to bind to each other’s receptors. A hybrid receptor also exits, which is assembled by half of each receptor whereby both mitogens can bind to it (Giovanna, 2009). In the liver, insulin stimulates synthesis of IGF-1 and suppresses production of IGF-BP1 and IGF-BP2, consequently influencing the bioavailability of IGF-1 (Chen, 2004). Insulin also suppresses the production of
SHBG, resulting in a higher level of free testosterone, which may influence development of PCa (Barnard, 2002).

Since two decades ago, there is evidence to show that IGF-1 and insulin are associated to a variety of cancers, including PCa (Giovanna, 2009). IGF-1 and growth hormone are required for the normal development of the prostate (Ruan, 1999). It has been demonstrated that over-expression of IGF-1 in transgenic mice leads to the transformation of prostate epithelial cells (DiGiovanni, 1999). *In vitro* studies have revealed that IGF-1 and insulin promote the growth of PCa cell lines, such as LNCaP, PC3 and DU-145 (Chen, 2004). Studies conducted *in vivo* have shown that xenografts from PC3 cells grew much faster in IGF-1 expressing hosts than IGF-1 deficient hosts (Stattin, 2000). Both IR and IGF-1R are commonly expressed on the surface of human PCa cells, which suggests their role in initiation and progression of the disease (Cox, 2009). In addition, IGF-1 may also play a role in the transition of PCa cells from an androgen sensitive state to androgen independent condition. Significant changes of IGF-BP levels, including a rapid decrease of IGF-BP3 can be observed after castration or during progression (So, 2005).

The association between IGF family and risk of PCa is well established through many epidemiology studies (Giovanna, 2009). For example, Stattin *et al* (2000) conducted a nested case-control study within the Northern Sweden Health and Disease Cohort Study to evaluate the role of IGF-1, IGFBPs (BP-1, -2 and -3) and insulin in PCa risk. They found that PCa risk is increased in men with elevated plasma IGF-1 and this association was more profound in younger men. Similarly Chen *et al* (2004) performed a nested case-control study within the
Cardiovascular Health Study Cohort, whereby IGF-1 level was not positively associated with PCa risk, but IGF-BP3 was associated with modest increase in PCa.

There are also some meta-analyses assessing the association of IGF family and risk of PCa. Shi et al (2001) reviewed 14 case control studies and their results showed that circulating levels of IGF-1 and IGF-BP3 were both significantly higher in PCa patients than in controls. Renehas et al (2004) looked at 21 case control studies on a variety of cancers. Their meta-analysis suggested that high circulating IGF-1 is associated with an increased risk of PCa, whereas high IGF-BP3 is associated with a decreased risk. Morris et al (2006) analyzed the data from British United Provident Association study (BUPA) and did a meta-analysis of the prospective epidemiological studies. Their results showed that there was a modest association between IGF-1 levels and PCa, but no association was observed between risk of disease and IGF-2 or IGF-BP3. Roddam et al (2008) examined data from 12 prospective studies and they observed that increased level of IGF-1 in the serum was significantly associated with a moderately increased risk of PCa. More recently, Rowlands et al (2009) performed a systematic review and once again confirmed the positive association of circulating IGF-1 and PCa risk and negative association of IGF-BP3 and PCa. They suggested that world-wide literature is consistent with an average 21% increase risk of PCa per standard deviation increase in IGF-1, and an average 12% reduced risk of PCa per standard deviation increase in IGF-BP3. It was worth noting that although the association between IGF family and PCa is well established, the association is only modest and some authors claim these peptides are not useful as additional markers in PCa PSA screening (Rowland, 2009; Morris, 2006).
In contrast, the studies that investigated the association of insulin and risk of PCa have not appeared to be consistent (Lima, 2009). Hsing et al (2001) examined whether leptin and insulin were associated with prostate cancer in a population based study. They found out that higher serum insulin levels were associated with significantly higher PCa risk. On the contrary, Hubbard et al (2004) assessed the association of prostate risk with insulin and glucose levels in the serum and found out that neither fasting insulin nor glucose was related to prostate cancer risk. However, high insulin levels are associated with PCa aggressiveness. Lehrer et al (2002) examined the association of serum insulin levels and risk of recurrence in men diagnosed with localized PCa. Their result indicated that insulin levels of high-risk PCa patients were significantly higher compared to levels observed in medium and low risk patients. Hammarsten et al (2005) carried out a prospective study to test whether hyperinsulinaemia is a risk factor for lethal clinical PCa, and reported a significant relationship between fasting plasma insulin level and death from prostate cancer. Similarly, Ma et al (2008) also reported that men, whose C-peptide concentrations are in the highest quartile, had significant higher risk of PCa mortality compared with patients in the lowest quartile.

1.2.6 Obesity

As a consequence of unhealthy diet and lack of physical activity, obesity has become a major health problem in ‘Western’ countries. It is estimated that more than one third (35.7%) of the adult population are categorized as being obese in the United States (Ogden, 2012). Obesity is linked to multiple chronic medical conditions, such as coronary artery disease, hypertension, diabetes, asthma, and arthritis (Freedland, 2005). In addition, obesity has also been linked to the development and progression of several types of cancers, including colon, breast, pancreas and
PCa (Giovannucci and Michaud, 2007). In the United States it was estimated that obesity could account for 14% of all cancer related death in men, and 20% in women (Calle, 2003). The most common measure of obesity is BMI. According to standards of World Health Organization, a BMI $< 25 \text{ kg/m}^2$ is considered normal, $25 - 29.9 \text{ kg/m}^2$ is overweight and $\geq 30 \text{ kg/m}^2$ is obese (Joseph, 2005). However, BMI is a crude measurement for overall obesity, which does not reflect body fat distribution. Abdominal fat is metabolically more active and also more closely linked to insulin resistance. Waist circumference and waist-to-hip ratio (WHR) are generally used to measure central obesity (Hsing, 2007).

The epidemiological evidence has inconsistently shown a modest association between obesity and risk of total PCa, and a stronger association may exist for risk of advanced disease (Engeland, 2003). Many epidemiological studies demonstrated a positive relationship between obesity and PCa incidence. Andersson et al (1997) performed a large, retrospective cohort study among Swedish construction workers and established that all anthropometric measurements, including BMI and WHR were positively associated with risk of PCa. Engeland et al (2003) analyzed data from a large Norwegian cohort and observed a modest increase in risk of PCa with increasing BMI. Cerhan et al (1997) also observed a positive association between BMI and risk of PCa and discovered that the association was stronger for advanced or aggressive disease. Similarly, Freedland et al (2005) observed that a higher BMI was positively associated with being diagnosed with PCa. In addition, a higher BMI also increased the odds of high-grade disease among men with cancer. Hsing et al (2000) conducted a case-control study in China and found out that high levels of WHR were related to excess risk of PCa, but not high BMI, which suggested the importance of abdominal fat. Hafe et al (2004) used computed CT to measure
visceral fat and observed that participants with higher visceral fat had a significantly higher risk of developing PCa. However, some other population-based studies did not detect such a positive association (Friedenreich, 2004; Giovannucci, 1997; Jonsson, 2003). For example, a population-based case-control study observed no association between any measures of anthropology and PCa risk (Fridenreich, 2004). Moreover, several analyses reported a negative relationship between obesity and PCa risk (Giovannucci, 2003; Presti, 2004; Kane, 2005; Porter, 2005). For instance, Presti et al (2004) found out that a normal BMI is correlated with a higher cancer detection rate and larger cancers in men undergoing prostate biopsy. In 2006, MacInnis et al systematically reviewed 31 cohort and 25 case-control studies and concluded that obesity is weakly associated with an increased risk of PCa; the association is stronger with advanced stage tumors. Recent studies have examined the association of BMI with prostate cancer grade. There was evidence supporting a negative association between obesity and low-grade cancer but a positive association with high-grade cancer (Giovannucci and Michaud, 2007). Gong et al (2006) investigated the associations of obesity with low and high-grade PCa, and their data showed that compared with normal weight men, obese men had an 18% decreased risk of low-grade PCa and a 29% increased risk of high-grade disease. By analyzing the date collected from the Cancer Prevention Study II Nutrition Cohort, Rodriguez et al (2007) also found that BMI was inversely associated with risk of non-metastatic low-grade PCa but was positively associated with risk of non-metastatic high-grade and metastatic or fatal PCa.

Obesity may also have an impact on the outcome of surgical treatment of PCa. Several reports investigated the relationship between BMI and cancer control after radical prostatectomy (Presti, 2005). Amling et al (2004) showed that BMI was associated with a higher risk of biomedical
recurrence after radical prostatectomy. Similarly, Freedland et al (2004) also found out that obesity was associated with increased risk of positive surgical margins and higher biochemical failure rates after radical prostatectomy treatment. Further studies were carried out to determine if the higher PSA recurrence was solely due to surgical technical difficulties. They examined the relationship between obesity and biochemical failure among men with negative surgical margins and their results indicate that among men with BMI $\geq 35$ kg/m$^2$, BMI was a significant predictor of biochemical failure (Freedland, 2004, J Urol). BMI seems to predict treatment outcome despite the technical difficulty and surgical margin status.

The relationship between obesity and mortality from PCa is less obscure compared to the association between obesity and prostate cancer risk. The literature relatively consistently supports a significantly positive association between increased BMI and risk of PCa death (Freedland, 2005). In the large cohort study carried out by Andersson et al, anthropometric measurements were more strongly related to mortality than to incidence of PCa (Andersson, 1997). Rodriguez et al (2001) examined the data from the two large American Cancer Society Cohorts, and concluded that PCa mortality rate were significantly higher among obese than non-obese patients in both cohorts. A large prospective study in the United States also demonstrated a significant trend of increasing risk of death from PCa with higher BMI (Calle, 2003). In another population-based cohort study, researchers found out that among patients who were diagnosed with local or regional state disease, obesity was associated with an increased risk of developing metastasis and a significant increase in PCa mortality (Gong, 2007). Ma et al (2008) also observed that compared with normal weight men at baseline, overweight and obese men had significantly higher risk of PCa mortality. Some studies have also investigated the relationship
between obesity in young adulthood and subsequent cancer mortality. One study showed that BMI in adolescence was positively associated with risk of PCa mortality in later life (Okasha, 2002).

The mechanisms behind the complex epidemiologic pattern for obesity and PCa are not well established. These include changes in hormones and growth factors; biomarker dilution; clinical factors; and altered treatment responses. Obesity is associated with a lower testosterone and higher estrogen levels, which may help to elucidate the protective effect of higher BMI on non-aggressive PCa (Presti, 2004). Another possible explanation is that obese men have been reported to have lower serum PSA levels relative to normal-weight men in population-based studies (Oh, 2013). Other non-biological factors related to detection and treatment may also be relevant (Giovannucci and Michaud, 2007). For example, in obese men, PSA may be reduced because of lower androgen levels. Digital rectal examinations may also be impaired. In addition, radical prostatectomy could be avoided because of technical difficulty or comorbidity. Obesity may also be associated with a higher positive margin even if surgery is carried out (Giovannucci and Michaud, 2007). These factors may partially explain the association between obesity and reduced risk of low-grade PCa.

Several hormonal factors contribute to the promoting effect of obesity on PCa progression. Obesity is associated with hyperinsulinemia, insulin resistance, and higher circulating IGF-1 levels. As discussed above, IGF-1 and insulin may stimulate the growth of PCa. Recent studies have been focusing on the fact that adipose tissue is not only storage for energy but also an endocrine and paracrine organ (Baillargeon, 2005). Several adipose-derived cytokines, including
leptin, interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and adiponectin are recognized for their regulating effects on tumor development (Baillargeion, 2006). Leptin, which disrupts insulin signaling, is elevated in obese men. And higher serum leptin levels appear to be associated with higher grade and more advanced tumors in men with PCa (Amling, 2005). On the other hand, adiponectin, a cytokine promotes insulin sensitivity and believed to have protection effect on PCa, is reduced with increasing adiposity (Baillargeion, 2005).

1.2.7 Type II Diabetes

Obesity, especially central adiposity, is associated with insulin resistance, hyperinsulinemia and type II diabetes (Freedland, 2005). As discussed above, hyperinsulinemia might be predicted to increase the risk of several cancers through growth promotion. Epidemiological studies have demonstrated a positive relationship between type II diabetes and some cancers, including cancers of breast, colon and pancreas (Cannate, 2010).

However, contrary to other type of cancers, numerous studies have demonstrated a negative association between type II diabetes and risk of PCa (Giovannucci and Michaud, 2007). In a large, population-based cohort study in Sweden, researchers observed a small, but significantly decreased risk of PCa among patients who had been hospitalized for diabetes mellitus (Weiderpass, 2002). Gonzalez-Perez et al (2005) carried out a nested case-control study using the General Practitioner Research Database in the UK and found that diabetic patients had a decreased risk of PCa. However, this association was only observed among treated diabetics, not among untreated diabetics. In the PCa Prevention Trial, some researchers studied the association of diabetes with low-grade and high-grade PCa risk. The results suggested that diabetes was
associated with a 47% reduced risk of low-grade PCa and a 28% reduced risk of high-grade PCa. This effect was independent of patients’ BMI and abdominal obesity (Gong, 2006).

Some studies also investigated whether time since diagnosis of diabetes mellitus modifies the association between diabetes and PCa. Giovannucci et al (1998) examined this relationship in the Health Professionals Follow up Study and found that PCa risk was not reduced in the first five years after diagnosis of diabetes, but was lower in the next five years and lowest after 10 years of diagnosis. In the Cancer Prevention Study II Nutrition Cohort Study, authors found out that diabetes was overall associated with a lower incidence of PCa, but this association differed significantly by time since diagnosis of diabetes. During the first 3 years after diagnosis of diabetes, risk of PCa was slightly increased but was reduced among men who were diagnosed over 4 or more years (Rodriguez, 2005). A nested case-control study conducted within the US Physicians’ Health Study suggested an association between diabetes and decreased PCa risk despite time of diagnosis of the diabetes. For men with diabetes, relative to those without the disease, the odds ratios were estimated to be 0.63, 0.77, 0.59 and 0.59 for diabetes diagnosed 1-5, 6-10, 11-15 and >16 years prior to PCa diagnosis (Zhu, 2004).

Two meta-analyses highlighted the modest protective effect of diabetes on risk of PCa. Bonovas et al (2004) examined 14 population studies, 5 case-control and 9 cohort studies and their results showed that diabetic patients had a statistically significant (9%) decrease in risk of developing carcinoma of the prostate. A more recent meta-analysis included 19 studies, 12 cohort and 7 case-control studies. The meta-analysis showed that diabetic men have a statistically significant (16%) decreased risk of developing PCa (Kasper, 2006).
Despite the mounting evidences supporting the inverse association between diabetes and risk of PCa, other studies did not observe decreased prostate cancer risk among diabetic patients. Steenland et al (1995) observed cancer incidence in the National Health and Nutrition Survey I and found that cancer incidence was augmented in diabetic men and the elevated risk was particularly evident for colorectal and PCa. The Cancer Prevention Study conducted during 1959-1972 found little or no association between baseline diabetes and PCa incidence. However, men who had diabetes for 5 or more years had a higher incidence of PCa (Will, 1999). A more recent hospital-based case-control study showed no association between diabetes and PCa. Moreover, PCa risk was not related to time since diagnosis of diabetes (Tavani, 2005). Chan et al (2005) analyzed data from cancer of the prostate strategic urologic research endeavor (CaPSURE), and found that history of diabetes was not associated with aggressiveness at time of diagnosis or risk of recurrence in this population of men with PCa.

It is noteworthy that many studies did not distinguish between Type I and Type II diabetes (Cannata, 2010). Type I diabetes results from autoimmune destruction of pancreatic β-cells, which subsequently causes insulin deficiency and hyperglycemia. On the other hands, Type II diabetes is a metabolic disorder and develops from peripheral insulin resistant, resulting in high blood glucose levels. Therefore, Type II diabetes is usually characterized by β-cell hyperplasia and hyperinsulinemia (Cannata, 2010). However, the large majority of patients included in the studies were diagnosed with diabetes at an older age, and therefore most of the patients studied were Type II diabetic patients. Type II diabetes has been consistently associated with cancer, whereas Type I diabetes, a disease of insulin deficiency, has not (Cannata, 2010).
The inverse association between diabetes and PCa may be a result of hormonal alterations in diabetes patients. First of all, insulin and IGF-1 axis could be altered during the long natural history of diabetes. Initially, men with diabetes are characterized with higher concentrations of insulin as a result of insulin resistance. However, a long history of diabetes could subsequently damage pancreatic β-cells, which results in deficiency of insulin (Hsing, 2007; Gong, 2006). In addition, as previously discussed, decreased insulin levels also up-regulate IGF-BPs, which decreases the bioavailability of IGF-1. High levels of circulating insulin and IGF-1 are both potential risk factors for PCa; the findings of Giovannucci et al (1998) and Rodriguez et al (2005) particularly support this hypothesis that a reduction of these two factors may partially explain the decreased PCa risk among diabetes patients. Both studies reported a variable influence of diabetes on PCa risk along with the long history of diabetes, which are correspondent with the changes of insulin levels. Secondly, alteration in sex hormones may also contribute to the observed inverse association. Serum levels of testosterone are lower in diabetics than non-diabetic men (Rodriguez, 2005). For example, Ando et al (1984) found that free testosterone concentration was 0.29 nmol/L in diabetic patients, which is a smaller number compared to 0.39 nmol/L in normal men. In animal studies, diabetic mice have reduced number of testicular Leydig cells (Jackson, 1984), which provides a possible explanation as to why diabetics experience decreased testosterone levels. Although associated with obesity, type II diabetes is likely to be related to reductions in leptin, which is an adipose-derived cytokine associated with higher grade and more advanced prostate cancer as discussed above.
The health related effects of diabetes and the treatment of the disease might influence the risk of prostate cancer. (Gong, 2006; Gonzalez-Perez, 2005; Kasper, 2006). Diabetic patients take medications, which may have a protective effect for cancer. Metformin, a widely used drug for treatment of type II diabetes, may also have anti-tumor properties (Belda-Iniesta, 2011). Many studies observed that metformin decreased cancer incidence among diabetic patients, the details will be discussed under next section. Higher serum cholesterol level is a common complication in type II diabetes and patients often take statins for treatment of hypercholesterolemia (Gong, 2006). Many clinical and animal studies suggest that statins may decrease PCa incidence and risk of recurrence (Papadopoulos, 2011). In the study by Gonzalez-Perez (2005), the authors only observed an inverse correlation among treated diabetics, which implicate that anti-diabetic treatments warrant further research for their clinical implications on prostate management. Secondly, a patient may alter his life-style in order to control complications relating to diabetes. The patient might switch to a more healthy diet and include more physical activity in his daily life. These life-style changes may also influence their risk to develop PCa.

1.2.8 Metabolic Syndrome

Metabolic syndrome (MeS), also known as insulin resistance syndrome, is a cluster of conditions associated with increased risk of cardiovascular disease and diabetes. Components include insulin resistance, dyslipidemia, hypertension, central obesity, and proinflammatory and prothrombotic states (Grundy, 2004). There is evidence indicating that the prevalence of metabolic syndrome is increasing among US adults. Results from the National Health and Nutrition Examination Survey (1999-2000) showed that the prevalence of the metabolic syndrome was 26.7 percent among US adults (Ford, 2004).
There are four generally accepted definitions for MeS, which were put forth by World Health Organization (WHO) (Alberti, 1998), National Cholesterol Education Program Adult Treatment panel III (NCEP) (Miranda, 2005), the European Group for the Study of Insulin Resistance (EGIR) (Balkau, 1999), and International Diabetes Federation (IDF) (Alberti, 2005). Their definitions are similar, but none of them can be considered as a universal standard because they each emphasize different aspects of the MeS (Grundmark, 2010). Most of the studies regarding MeS, PCa risk and ADT use the NCEP definition. The guidelines suggest that a diagnosis of MeS is made where three or more of the risk factors are present: central obesity (waist circumference > 102 cm), elevated triglycerides (>= 1.7 mmol/L), Low high density lipoprotein (HDL) cholesterol (< 1.0 mmol/L), raised blood pressure (>= 130, >= 85 mmHg) and raised fasting glucose (>= 6.1 mmol/L) (Miranda, 2005).

Reaven first described MeS in 1988 and he called this syndrome X (Raven, 1988). The author also suggests that central obesity is the physical manifestation of this metabolic state and insulin resistance is the central component. Impaired insulin response in fat, muscle and liver cells all contribute to different components of MeS. Insulin resistance in fat cells results in hydrolysis of stored triglycerides, which elevates free fatty acids in circulation. The consequent absorption of fatty acids by liver cells result in increased triglycerides, low-density lipoprotein (LDL)-cholesterol and decreased HDL-cholesterol in serum. Insulin resistance in liver cells also reduces glucose storage, whereas insulin resistance in muscle cells reduces glucose utilization, both of which will cause elevated blood glucose levels (Nobes, 2009). As discussed in the previous
paragraph, insulin resistance is also a very important risk factor for the development of PCa, which may explain the recent observed associations of metabolic syndrome and PCa.

Overall, there is inconsistency in the current literature supporting the hypothesis that MeS, and its metabolic and endocrine perturbations are associated with prostate cancer incidence and aggressiveness (Hammarsten and Peeker, 2011). Some authors suggest that PCa could be regarded as a new aspect of the MeS (Barnard, 2002; Hammarsten, 2004). A prospective population based study carried out in Eastern Finland did a 13-year follow up in 1880 middle-aged men. Their results showed that middle-aged men with the MeS were nearly 2-fold more likely to develop PCa than those without, and controlling for potential confounding lifestyle or dietary factors did not attenuate the association (Laukkanen, 2004). Another study in 2004 found that prostate gland volume, BPH growth rate, hyperuriceamia and some components of MeS, such as hypertension, obesity, dyslipidaemia, and hyperinsulinaemia, are all associated with development of clinical PCa, as measured by stage and grade of the disease. They hypothesized that clinical PCa was also a component of the MeS (Hammarsten, 2004). The same group carried out a prospective study to assess if hyperinsulinaemia and other components of MeS are risk factors for lethal clinical PCa. Their results showed that hyperinsulinaemia, hypertension, obesity, dyslipidaemia and hyperuriceamia were all prospective risk factors for deaths that can be ascribed to PCa. They also suggested that hyperinsulinaemia could be used as a marker of PCa prognosis and tumor aggressiveness, regardless of the patient’s PCa stage, grade and PSA level (Hammarsten, 2005). A 27-year follow-up, prospective cohort study was carried out to investigate the association between MeS and incidence of PCa. The results suggest that any component of two or three factors of the MeS were predictive of PCa (Haheim, 2006). Another
case-control study exclusively among African Americans indicated that features of the MeS, specifically abdominal obesity and hypertension, were associated with PCa incidence in African-American men (Beebe-Dimmer, 2007). Finally, results from the prospective Uppsala Longitudinal Study of Adult Men indicated that the presence of the MeS at age 50 was a risk factor for clinically relevant or advanced stages of PCa over 34 years of follow-up (Grundmark, 2010).

However, it is important to note that some studies demonstrated conflicting results. For example, results from the second wave of the Nord Trøndelag Health Study (HUNT2) cohort showed that there was no significant association between obesity, central obesity, total or HDL-cholesterol, triglycerides, presence of the MeS, diabetes or cardiovascular disease (CVD), and incidence or fatal PCa (Martin, 2009). In another study based on the ARIC cohort, men with MeS were in fact found to be less likely to develop PCa than normal men, after adjusting for other risk factors (Tande, 2006). The authors hypothesized that this finding reflected a decrease in bioavailable testosterone associated with the MeS and a concomitant reduction in PCa risk.

ADT is a very important treatment option for locally advanced PCa, and it is widely used in a number of different settings (Nobes, 2009). The aim of ADT is to reduce the growth of PCa promoted by androgens, and includes therapies that abolish production of male hormones and therapies that prevent androgens from activating their receptor. Clinically, it is often accomplished surgically with orchiectomy or chemically with gonadotropin-releasing hormone (GnRH) agonists (Dunn and Kazer, 2011). Widespread PAS testing and aggressive treatment for PCa has been associated with increased usage of ADT, both as primary and neoadjuvant therapy (Collier, 2012). For example, a prospective population based cohort study showed that the prevalence of ADT among the studied population increased steadily from 1.8% in 1993 to 2.9%
in 2000 (Barry, 2006). Despite mounting evidence for ADT as an effective treatment for PCa, it has been associated with substantial treatment-related morbidities, such as an increase in body mass index, increased fat mass, reduced lean body mass and muscle strength, osteoporosis, sexual dysfunction, and reduced quality of life (Basaria, 2002; Smith, 2002). More recently, data regarding long-term treatment related morbidity have shown that CVD and MeS are potential consequences of this therapy (Nobes, 2009).

Oral estrogens were used as first line hormone treatment for PCa, until studies showed that they were associated with cardiovascular death in treated patients (Byar, 1973). At this period of time GnRH agonists, replaced estrogens as a standard modality of treatment (Albertsen, 2009). Though initially GnRH was thought to have negligible cardiovascular toxicity, recent studies have shown that long term ADT may be associated with adverse cardiovascular consequences (Collier, 2012). An observational study of a population-based cohort showed that the use of GnRH agonist was associated with increased risk of diabetes, coronary heart disease, myocardial infarction, and sudden cardiac death (Keating, 2006). In another study in 2007, D’Amico et al reported that men aged 65 years or older who received 6 months of androgen suppression therapy experienced shorter times to fatal myocardial infarctions compared with men in this age group who did not undergo androgen suppression. Another study based on a cohort of newly diagnosed men showed that patients who received ADT for at least one year were found to have a 20% higher risk of serious cardiovascular morbidity compared with similar group of men not receiving ADT (Saigal, 2007). Similarly, Tsai et al (2007) demonstrated that the use of ADT were associated with statistically significantly increased risks of death from cardiovascular causes in patients treated with radical prostatectomy, after controlling for age and CVD risk.
factors. These results suggest that potential cardiovascular risks should be considered when using ADT as a treatment option for PCa.

ADT induces MeS. This finding is not very surprising because male hypogonadism has been known as an independent risk factor in the development of metabolic syndrome (Braga-Basaria, 2006). Laaksonen et al (2004) found that low total testosterone and SHBG levels independently predict development of MeS and diabetes in middle-aged men. On the other hand, Muller et al (2005) demonstrated that high testosterone and SHBG levels in circulation are independently associated with higher insulin sensitivity and a reduced risk of MeS in aging men, independent of insulin levels and body composition measurements. Low serum testosterone levels are also found to be associated with hypertension (Philips, 1993), which is a component of MeS.

Long term ADT results in male hypogonadism and found to be associated MeS. A cross-sectional study indicated that men undergoing long-term ADT had significantly higher prevalence of MeS compared to patients who didn’t receive ADT and healthy males. The data suggested that MeS was present in more than 50% of the men treated with long-term ADT. Among the components of metabolic syndrome, ADT patients are more likely to develop abdominal obesity, hyperglycemia and elevated triglycerides (Braga-Basaria, 2006). A more recent study showed GnRH agonists increased subcutaneous fat mass, HDL cholesterol and adiponectin, but didn’t increase WHR, blood pressure or C-reactive protein level, compared to the classical metabolic syndrome. This suggests that the MeS associated with ADT differs in some important ways from the classical MeS, and may have less severe health risks associated with it (Smith, 2008).
Short-term prospective studies of ADT in PCa patients have shown that induced hypogonadism was associated with insulin resistance (Smith, 2001). As discussed above, insulin resistance is the underlying key factor for the development of metabolic syndrome. MeS is also a known risk factor for CVD (Ford, 2004), which might partly explain the association of ADT with CVD. Therefore, life-style interventions such as healthy diet and physical activity could be beneficial for patients who undergo ADT. Also, therapeutic drugs that improve insulin sensitivity, such as metformin, may also play a role in the management of these patients.

Figure 1 Risk factors for prostate cancer. Age, ethnic origin and genetics are three well-established risk factors for prostate cancer. Some circulating hormones are also linked to risk of prostate cancer, such as androgens, insulin, IGF-1 and IGFBPs. In addition, some common diseases are also associated with prostate cancer risk, including obesity, type II diabetes and metabolic syndrome.
2) Metformin and PCa

2.1 Overview

As we discussed above, physiologic concentrations of insulin play an important role in cancer development and type II diabetes. It is recognized that insulin resistance and hyperinsulinemia are associated with elevated risk of overall cancers. Based on this argument, it would be reasonable to hypothesize that insulin lowering agents which are used to treat type II diabetes may be associated with reduced risk of cancer. Recent interest has been focused on metformin, a biguanide derivative with insulin-sensitizing and anti-hyperglycemic properties. Recent epidemiological studies have shown that metformin treatment has been associated with reduced overall cancer risk when compared with other treatments in type II diabetic patients (Bost, 2012).

Metformin (1,1-dimethylbiguanide hydrochloride), widely prescribed around the world, is an orally administered drug used as first-line therapy for type II diabetes (Aljada, 2012). Its anti-hyperglycaemic effects occur by multiple mechanisms. The primary action of metformin is to inhibit hepatic glucose production (Shaw, 2005), but the precise mechanism of action remains poorly understood. Metformin may reduce glucose synthesis by activating the AMP-activated protein kinase (AMPK) pathway, however, recent genetic loss-of-function experiments suggest that there exists mechanisms that are independent of AMPK. Miller et al (2013) proposed that metformin antagonizes the action of glucagon, thus blocking glucose output from hepatocytes. Metformin also increases the sensitivity of peripheral tissues to insulin such as muscle and adipose tissue, leading to enhanced uptake and utilization of glucose, thereby reducing blood glucose level (Cazzaniga, 2009). Metformin also delays glucose absorption from the
gastrointestinal (GI) tract and stimulates insulin secretion via glucagon like-peptide-1 (Clements, 2011).

Beneficial effects of metformin have also been observed in the treatment of polycystic ovarian syndrome, nonalcoholic fatty liver disease, and premature puberty, all attributes to its insulin-sensitizing property (Aljada, 2012). Metformin is a relatively safe drug in its clinical use, with the main limited side effect as mild GI distress, and it is often transient. Lactic acidosis is a rare but serious adverse effect in metformin treated patients. However, in most cases it occurred because one or more contraindications were overlooked, predominantly renal insufficiency (Bailey, 1999). Long-term use of metformin may promote hirsuitism and vitamin B12 malabsorption (Cazzaniga, 2009).

More recently, metformin’s potential anti-neoplastic effects caught a lot of attention. Although the mechanism of action of metformin is still under intense investigation, current knowledge suggests that metformin may exert this effect through both direct and indirect pathways. At cellular level, metformin may directly activate the AMPK pathway in cancer cells, inducing cell cycle arrest. Activation of AMPK also results in the inhibition of the mammalian target of rapamycin (mTOR), which reduces protein synthesis and suppresses cellular proliferation (Cazzaniga, 2009). Metformin treatment is associated with a decreased insulin level and an increased IGFI/IGFBP1 ratio, which also indirectly suppresses cancer cell growth and signaling (Aljada, 2012).
2.2 Preclinical Studies

*In vitro* experiments suggest that metformin exhibits consistent anti-proliferative action on several cancer cell lines, including breast, colon, ovarian, pancreatic, lung and prostate cancer cells (Bost, 2012). Metformin may exert these anti-tumoral effects by multiple mechanisms: it promotes apoptosis and inhibits autophagic processes, induces cell-cycle arrest, activates AMPK pathway, which suppresses cell proliferation (Bost, 2012). Activation of AMPK also causes inhibition of mTOR pathway, which reduces global protein synthesis and therefore proliferation (Clements, 2011). Metformin may also indirectly reduce Akt activation; thereby blocking the proliferative and anti-apoptotic activity of the Akt pathway (Clements, 2011). More recently, other possible effects of metformin have been proposed, such as inhibiting TNF-α production and preventing metastasis (Bost, 2012). Finally metformin has been found to selectively kill cancer stem cells and therefore enhances anti-proliferative effects of several therapeutic agents, such as cisplatin, paclitaxel and tamoxifen (Hirch, 2013).

2.2.1 Metformin and the AMPK Pathway

The most established action of metformin is the activation of the AMPK pathway. In the cell, AMPK is the major cellular sensor of energy state and a master regulator of metabolic homeostasis. AMPK can be activated by a number of conditions that lead to alterations of the intracellular adenosine monophosphate/adenosine-5'-triphosphate (AMP/ATP) ratio, such as hypoxia and glucose deprivation. It also can be activated by the changes in calcium concentration, as well as the action various hormones, cytokines, and adipokines. Once activated AMPK is responsible for the phosphorylation of various downstream substrates, and the net effect is a change in local and whole-body energy utilization from an energy consuming state to
an energy producing state in order to restore energy balance (Zhang, 2009). Metformin is found to activate the AMPK pathway in an unclear manner. One proposed mechanism is that metformin may block the activity of complex I of oxidative phosphorylation, which raises the reduced nicotinamide adenine dinucleotide/ nicotinamide adenine dinucleotide (NADH/NAD) ratio. As a result of raised NADH/NAD ratio, fatty acid beta-oxidation is blocked, which activates AMPK by increasing the AMP/ATP ratio (Owen, 2000). Several studies showed that the inhibition of AMPK with siRNA or compound C (an AMPK inhibitor) reversed the anti-proliferative effects of metformin in breast and ovarian cancer cell lines, which emphasized the importance of AMPK activation in metformin’s action (Zakikhani, 2006; Gotlieb, 2008).

The activation of AMPK by metformin occurs in a liver kinase b1 (LKB1) dependent mechanism (Clements, 2011). LKB1 is a tumor suppressor encoded by the gene stk11, which harbors germ-line mutations in Peutz-Jeghers Syndrome. This syndrome is a predisposition for epithelial neoplasia, characterized by several gastrointestinal polyps and also by an increased risk of various epithelial cancers (Giardiello, 2000). AMPK is a direct substrate of LKB1. AMPK is a heterotrimer composed of a α catalytic subunit and two regulatory subunits. LKB1 is responsible for the phosphorylation of the α subunit at Thr172, which enhances AMPK activity (Alessi, 2006). Many in vitro and in vivo studies have suggested that loss of LKB1 results in complete loss of AMPK activity and the reduction of glucose output observed in metformin treated hepatic cell culture was not observed in lkb1−/− hepatic cells treated with metformin (Aljada, 2012). These findings suggest that LKB1 plays an essential role for the effect of metformin. However, Algire et al (2011) recently reported that metformin inhibited tumor growth in mice with diet-induced
hyperinsulinemia, independent of tumor LKB1 expression. These issues demonstrate that metformin triggers various responses, which may vary in *in vitro* vs. *in vivo* settings.

Activation of AMPK then activates tuberous sclerosis complex (TSC), a tumor suppressor that inhibits mTOR activity. TSC is made up of TSC1 and TSC2 complex and it is activated by AMPK by phosphorylating TSC2 at the Thr\textsuperscript{1227} and Ser\textsuperscript{1345} residues (Mamane, 2006). Phosphorylation of TSC stimulates the GTPase activity of TSC2 toward the G-protein Ras homolog enriched in brain (Rheb) and down regulates it, which in turn results in inhibition of mTOR activity (Mamane, 2006). mTOR is found in two biochemically and functionally discrete signaling complexes mTORC1 and mTORC2. mTORC1 is an important positive regulator of translation initiation and thereby cell proliferation. Growth factors such as insulin and IGF-1 enhance mTORC1 activity through the Phosphatidylinositide 3-kinases/Akt (PI3K/Akt) pathway and stimulates cell growth and proliferation (Hay, 2004). The PI3K/Akt/mTORC1 pathway is dysregulated in a number of cancers, which resulted in abnormally increasing of mTORC1 activity, thereby enhances global protein production and cellular proliferation (Aljada, 2012). Activated form of mTORC1 phosphorylates and activates key initiators of protein translation, S6 Kinase (S6K) and 4E binding protein 1 (4EBP1), and as a consequence enhancing mRNA translation and cell growth (Dowling, 2007). mTORC1 regulates the translation of many cell growth regulators including Cyclin D1, hypoxia-inducible factor 1 (HIF-1) and Myc, which in turn controls many cellular processes such as cell cycle progression, cell growth and angiogenesis, all of which are important in tumorigenesis (Inoki, 2003). In addition, an AMPK-independent pathway by which metformin inhibits mTORC1 has recently been reported. Kalender *et al* (2010) found that metformin inhibited mTORC1 signaling in the absence of
TSC1/2 and in the absence of AMPK. The ability of metformin to inhibit mTORC1 is through a rag GTPase dependent pathway. These results suggested that regardless of AMPK activation, inhibition of mTORC1 is certainly an important mechanism for metformin to exert its beneficial effects.

2.2.2 Responses to Activation of mTORC1

Metformin induced AMPK activation and downstream mTORC1 inhibition in tumor cells may trigger a large number of cellular responses that contribute to growth inhibition. For instance, metformin may indirectly reduce Akt activation and its downstream signaling, which promotes cell proliferation and growth. Metformin disrupts the crosstalk between insulin receptor and G protein coupled receptor (GPCR signaling) in pancreatic cancer cells. GPCR and their agonists are known autocrine/paracrine growth factors for multiple solid tumors (Kisfalvi, 2009). In breast cancer cells, metformin exposure decreases Akt activation by inhibiting insulin receptor substrate 1 (IRS-I) through an AMPK dependent manner. Activated AMPK phosphorylates IRS-I at Ser789, a site inhibiting its downstream signaling (Zakikhani, 2010). An in vivo study supported this finding by showing that metformin attenuated diet induced tumor growth in mice, which was accompanied by reduced IR/Akt signaling (Algire, 2008; Algire, 2010). Metformin may also inhibit prostate cancer cell proliferation by controlling lipogenesis via activation of AMPK/mTOR pathway. Several reports have shown that androgen biosynthesis and androgen receptor (AR) signaling in prostate cancer cells is intimately affected by lipogenesis. Sterol regulatory element-binding protein-I (SREBP-1) is a critical transcription factor for lipogenesis and its key target gene, fatty acid synthase (FASN), is reported to be a metabolic oncogene, which have been shown to be involved in prostate cancer progression (Huang, 2012). Our lab
found that metformin, in combination with dutasteride, decreased cellular proliferation of LNCaP cells, which was accompanied with reduced expression of cleaved SREBP-1 (active form of SREBP-1) and FASN (Besla, 2013). Treating hepatocytes with metformin alone also suppressed the expression of SREBP-1 (Zhou, 2001). In vivo study shows that metformin treatment decreased the diet-induced expression of FASN in mice xenografts (Algire, 2010). AMPK activation is also responsible for regulating the expression and phosphorylation of P53, a crucial regulator of cell metabolism. The role of P53 in metformin’s anti-proliferation effect is not clear and the results are controversial (Bost, 2012). Buzzai et al (2007) found that treatment with metformin selectively suppressed the tumor growth of P53 deficient colon cancer cell xenografts, but not P53 wild type colon cancer cell xenografts. Further in vitro work suggested that P53 induction following metformin treatment leaded to activation of autophagy in cancer cells, which might be responsible for p53 dependent cell survival in vivo. On the contrary Ben Sahra et al (2011) identified a P53 dependent mechanism responsible for the inhibition of prostate cancer cell lines by metformin. They showed that metformin increased expression of REDD1, a negative regulator of mTORC1, in a P53 dependent manner. Therefore P53 may be important for the sensitivity of cancer cells responding to metformin. On one hand, p53 deficiency may facilitate the anti-proliferative effect of metformin, and on the other hand, p53 may be required to mediate metformin action on cell cycle arrest and apoptosis (Bost, 2012).

It is interesting to note that AMPK activation, in keeping with its evolutionary role, is a mechanism that enhances cell survival under cellular stresses (such as nutrient deprivation), even if this requires a reduction in proliferation as a compensate (Pollack, 2012). Previous studies showed that LKB-1 or AMPK deficient cells are resistant to oncogenic transformation and
tumorigenesis (Bardeesy, 2002), which suggests AMPK exert important role to promote metabolic adaptation in tumor cells. For example, Jeon et al. (2012) showed that during energy stress, AMPK maintains tumor cell NADPH homeostasis by inhibiting acetyl-CoA carboxylases 1 (ACC1) and ACC2 activity, therefore promotes cell survival. As we discussed above, the fundamental action of metformin is to impair mitochondrial oxidative phosphorylation and therefore induce energy stress. This in turn triggers several mechanisms that serve to restore the energy balance. This includes the activation of the master energy sensor AMPK and P53 induced autophagy (Phonex, 2008), which inhibits cell proliferation but are cell survival actions in their essence. Therefore, cells that are defective in AMPK signaling and P53 are actually more sensitive to metformin (Pollack, 2012).

2.2.3 AMPK Independent Pathways

Some other studies suggest that mechanisms other than AMPK may also contribute to the anti-proliferative effect of metformin on cancer cells. First of all, metformin may target major regulators of cell cycle and induce cell cycle arrest. Ben Sahra et al. (2008) found that metformin inhibited the proliferation of several prostate cancer cell lines with a 50% decrease of cell viability, including DU145, PC3 and LNCaP. This inhibition was due to cell cycle arrest in G0/G1 phase and is independent of AMPK activation. Further analysis revealed that it was accompanied by a strong decrease of cyclin D1 protein level, pRP phosphorylation and an increase in p27kip1 protein expression. Interestingly, metformin showed modest effect on normal prostate epithelial cell line P69, which suggest that metformin might specifically target cancer cells. Also, metformin may selectively target cancer stem cells in breast cancer cell lines with unknown mechanisms. The cancer stem cell hypothesis suggests that a small portion of cells
within the tumor are cancer stem cells, which resist chemotherapeutic drugs and can regenerate the various cell types in the tumor, thereby causing relapse of the disease (Hirsch, 2009). Metformin was shown to inhibit cellular transformation and selectively kill cancer stem cells in different breast cancer cell lines. The combination of metformin and doxorubicin killed both cancer stem cells and non-stem cancer cells in culture. Further more, this combination was found to reduce tumor growth and prevent disease relapse more effectively than doxorubicin alone in xenografts generated with breast cancer (Hirsch, 2009), prostate and lung cancer cell lines (Iliopoulos, 2011). In fact, metformin may exert these effects when combined with a variety of standard chemotherapeutic agents, such as paclitaxel and carboplatin (Iliopoulos, 2011). These studies provide a rationale for using combination of metformin and existing chemotherapeutic agents to treat cancer patients.

2.2.4 In Vivo Studies

Many in vivo studies investigated the effect of metformin treatment on tumor growth and provide opportunity to study the indirect mechanism of metformin: by decreasing circulating IGF-1 and insulin levels. A very interesting experiment was carried out by Memmott et al (2010), where A/J mice were treated with metformin orally or by i.p. for tobacco carcinogen (NKK) induced lung cancer. Metformin treatment reduced lung tumor burden by 53% and 72%, by oral delivery and i.p. injection respectively. Further analyses revealed that metformin activated AMPK and inhibited mTOR in liver tissue, but did not activate AMPK in lung tissue. In lung tissue, metformin inhibited phosphorylation of IGF-1R and IR, Akt and mTOR, which might be due to the reduced IGF-1 and insulin circulation. This experiment emphasized the indirect mechanism of metformin, and some other studies also found similar results. Schneider et al (2001) showed
that metformin prevented the stimulatory effects of high fat diet on the development of carcinogen induced pancreatic cancer in hamster, which was accompanied with a marked decrease in insulinemia. Algire et al (2008) found out that the effect of high-energy diet promoted tumor growth was significantly attenuated by metformin in mice; however, the effect was not observed on tumor growth of the mice on the control diet. This high-energy diet promoted insulin resistance with hyperinsulinemia, and metformin significantly reduced the insulin receptor activation associated with the diet.

In contrast, several studies showed that metformin treatment did not affect insulinemia, suggesting an insulin-independent anti-tumoral action of the drug. Fonseca et al (2011) found that obese rats had a significantly higher rate of tumor development, and metformin treatment reduced it without the correcting of insulin resistance. Metformin increased the necrosis index in tumor tissues, which may cause the reduction of tumor development. In another study, metformin treatment significantly decreased the incidence and size of mammary adenocarcinomas in female Her2/neu transgenic mice; however, this effect was not coupled with significantly decreased insulin levels (Anisimov, 2005). Huang et al (2008) found that metformin activated AMPK pathway and significantly delayed tumor development in phosphatase and tensin homolog (PTEN) deficient mice. PTEN is an essential regulator for the IR/Akt pathway; therefore, their results suggest the direct effect of metformin through activation of AMPK. Similarly, metformin significantly reduced the number of large intestinal polyp formation in Apc\textsuperscript{Min/+} mice, without altering the index of insulin or serum lipid levels. In contrast, metformin activated AMPK/mTOR pathway in the intestinal polyps, which suggest the effect in due to the
direct drug action (Tominoto, 2008). Together, these results showed that both the indirect and direct mechanism of metformin contribute to the anti-tumoral effects of metformin.

2.2.5 Other Mechanism of Metformin

At last, some studies also reported other potential effects of this drug, including reducing reactive oxygen species (ROS), preventing inflammation and inhibiting cancer cell metastasis. Algire et al (2012) showed that metformin attenuated paraquat-induced ROS in mice, and also reduced ROS related DNA damage. Importantly, metformin also inhibited Ras-induced ROS production and DNA damage in ras-transfected human fibroblasts. The potential role of metformin in inflammation is not that clear. Various cytokines contribute to pathogenesis of inflammation and TNF plays an important role. A study demonstrated that metformin inhibited the inflammatory responses in human monocytes, by reducing TNF production through the inhibition of the extracellular signal regulated kinases 1/2 - early growth response protein-1 (Erk1/2-Egr-1) pathway (Arai, 2010). Furthermore, another study showed that metformin attenuated the TNF-α induced gene expression of pro-inflammatory markers in human vein epithelial cells, such as vascular cell adhesion molecule-I, E-selection and intercellular adhesion molecule-I (Hattori, 2006). More recently Hirsch et al (2013) found out that metformin inhibited cellular transformation via inhibition of activation of the inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). Furthermore, metformin-based combinational therapy was only effective in xenografts involving inflammatory cancer cell lines: DU-145 and MDA-MB-435, but was not effective in their non-inflammatory cell lines from these lineages (LNCaP and A375). Metformin may also inhibit tumor cell migration and invasion. In human fibrosarcoma cells, metformin inhibited cell migration and invasion. Metformin also
suppressed phorbol-12-myristate-13-acetate (PMA)-induced matrix metalloproteinases-9 (MMP-9) expression through suppression of activator protein -1 (AP-1) activation (Hwang, 2010). In another study, serum obtained from metformin treated PCOS woman significantly reduced cell invasion of human endometrial cells. This effect was associated with decreased MMP-2 and MMP-9 expression (Tan, 2011). Although not well established, these potential anti-tumoral effects of metformin offered new research aspect of this intensely studied drug.

Figure 2 A simplified view of proposed antineoplastic mechanisms of action of metformin. Primary action of metformin is to inhibit the complex I of oxidative phosphorylation in mitochondria, resulting in reduced ATP production and energetic stress. In hepatocytes, this effect suppresses liver gluconeogenesis, leading to declines in circulating insulin and IGF-1 levels. More directly, metformin induced energetic stress inhibits tumor cell growth, proliferation through various cellular pathways.
2.3 Clinical studies

Several population-based studies have investigated the association of metformin treatment and risk of cancer in type II diabetic patients. The results generally support that metformin usage is associated with reduced risk of overall cancer risk as well as cancer at specific sites, such as breast, colon and pancreas. Overall cancer mortality was also observed to decrease in type II diabetic patients who are exposed to metformin. Some studies have also shown that treatment of metformin is associated with improved response to anti-neoplastic treatments, especially chemotherapy. Because of the convincing results from the observational studies and mounting evidence from other in vitro and animal work, several planned prospective clinical trials have been initiated recently to test the hypothesizing therapeutic benefits of metformin on the treatment of cancer (Bost, 2012).

2.3.1 Metformin and Risk of All Cancers

Several epidemiological studies found that type II diabetic patients on metformin had a lower prevalence of malignancies and reduced cancer-specific mortality compared with patients on other treatments, such as insulin. Evans et al (2005) did a pilot case-control study based a cohort of residents between 1993-2001 living in Tayside. They identified 923 cases among diagnosed type II diabetes and generated 1846 random controls from the diabetic population without cancer. 336 (36.4%) of the cases had been exposed to metformin in the year before their index date, compared with 732 (39.7%) of the controls. The unadjusted odds ratio (OR) was 0.86 (95% CI, 0.73-1.02). The unadjusted OR for any exposure to metformin since 1993 was 0.79 (0.63-0.93). Their results showed that metformin usage was associated with reduced risk of cancer in type II diabetic patients.
A retrospective cohort study in UK examined the risk of developing solid tumors in relation to different treatments in type II diabetes patients (Currie, 2009). The cohort included 62,809 patients who were divided into four groups: monotherapy with metformin or sulfonylureas, combination of the two, and insulin treatment. The outcome was progression to any type of solid tumors or cancer of the breast, colon, pancreas or prostate. Overall, monotherapy using metformin was associated with the lowest risk of cancer. In comparison, the adjust hazard ratio (HR) for sulfonylureas was 1.36 (1.19-1.54), for combination therapy was 1.08 (0.96-1.21), for insulin based regimens was 1.42 (1.27-1.60). Analyses for specific cancer showed that metformin usage was associated with decreased risk of colon and pancreas cancer, but had no significant effect on breast or prostate cancer. In another observational cohort study conducted in UK, the researchers identified 4085 type II diabetes patients who were new to metformin in 1994-2003. They also identified patients who had never used metformin, and individually matched to the metformin users by year of diagnosis. Their results showed a significantly reduced risk of cancer associated with metformin exposure. The adjusted HR for cancer was found to be 0.63 (0.53-0.75). In a cross-sectional study, 7482 patients participated in a nationwide cross-sectional and prospective epidemiological study (Diabetes Cardiovascular Risk and Evaluation: Targets and Essential Data for Commitment of Treatment Study) were recruited from German primary care practices to examine the association between diabetes and cancer risk and mortality (Baur, 2011). Subjects with type II diabetes were found to have a higher prevalence of malignancies compared to non-diabetic subjects (OR 1.64, 1.12-2.41). However, patients on metformin had a lower incidence of cancer then diabetic patients overall, which was comparable with non-diabetic patients (OR 1.04, 0.46-1.39). Patients under any other treatment
excluding metformin had much higher cancer prevalence than that among non-diabetic population (OR 2.26, 1.24-4.13). Monami et al (2011) performed a nested case-control study in a cohort of 1,340 type II diabetic patients in Italy. After a median follow-up of 75.9 months, 112 patients with incidence of cancer were compared with 370 control subjects. Results showed a significantly lower proportion of case subjects were exposed to metformin and sulfonylureas, however, after adjustment for potential confounders, only exposure to metformin was associated with reduced incidence of cancer (OR 0.46, 0.25-0.85). In a prospective cohort study in Hong Kong, researchers showed that the protective effect of metformin against cancer might be particularly evident in type II diabetic patients with low HDL cholesterol (Yang, 2011). The study was based on a consecutive cohort of 2,658 Chinese Type II diabetic patients enrolled between 1996 and 2005 with a median follow-up of 5.5 years. Compared with non-metformin users, use of metformin was associated with reduced cancer risk in patients with low HDL cholesterol (<1.0 mmol/L) (HR 0.29, 0.13-0.61) and, to a lesser extent, in patients with high HDL cholesterol. Low HDL cholesterol plus nonuse of metformin had an adjusted HR of 5.75 (3.03-10.90) compared with high HDL cholesterol plus use of metformin.

2.3.2 Metformin and Cancer Mortality

A population-based cohort study was carried out in Saskatchewan to compare cancer mortality rate among metformin users, sulfonylureas users and insulin users in type II diabetes patients (Bowler et al, 2006). 10,309 new users of metformin or sulfonylureas were identified with an average follow-up of 5.4 years. After multivariate adjustment, both patients exposed to sulfonylureas and exogenous insulin were found to have a significantly higher risk of cancer-related mortality compared with metformin treated patients. The adjusted HR for sulfonylurea
monotherapy was 1.3 (1.1-1.6) and for insulin was 1.9 (1.5-2.4). In a prospective cohort study in Netherlands (Zwolle outpatient diabetes project integrating available care (ZODIAC)), 1,353 type II diabetes were enrolled in the year of 1998 and 1999 and followed on an average of 9.6 years (Landman, 2010). In general patients in this cohort were at an increased risk for cancer mortality, the standard mortality ratios for cancer mortality was 1.47 (1.22-1.76). The use of metformin was associated with lower cancer mortality compared with non-metformin users, the adjusted HR being 0.43 (0.23-0.80) and the HR with every increase of 1g of metformin calculated to be 0.58 (0.36-0.93).

### 2.3.3 Negative Reports

However, a report from Home et al, in 2010 did not support the view that metformin offered protection against malignancy. They extracted data for malignancies from the ADOPT and RECORD, two randomized control trials assessing the efficacy and safety of metformin compared with sulfonylureas and rosiglitazone. In ADOPT, 50 (3.4%) patients on metformin and 55 (3.8%) patients on the other two drugs developed malignancies, giving HR for metformin of 0.92 (0.63-1.35) compared to rosiglitazone and 0.78 (0.52-1.14) compared to glibenclamide. In RECORD, 69 (6.1%) patients on metformin and 56 (5.1%) patients on rosiglitazone developed malignancy, giving HR for metformin of 1.33 (0.94-1.88).

### 2.3.4 Systematic Review and Meta-analyses

DeCensi et al (2010) performed a systematic review and meta-analysis to assess the effect of metformin treatment on cancer incidence and mortality in diabetic patients. They reviewed 11 studies, which reported 4,042 cancer events and 529 cancer deaths. Their results suggested a 31%
overall reduction in subjects taking metformin compared with patients on other anti-diabetic drugs (RR=0.69). Analyses for specific cancers showed that the inverse association was significant for pancreatic and hepatocellular cancer, but not significantly for colon, breast and prostate cancer. A more recent meta-analysis on this subject was carried out by Noto et al (2012). They reviewed 4 cohort studies and 3 randomized control trials, reporting 11,117 cancer incidences and 994 cases of cancer death among 210,892 diabetic patients. The cancer incidence and mortality among metformin users were significantly lower than those among non-metformin users. The pooled RR was 0.67 (0.53-0.85) for cancer incidence and 0.66 (0.49-0.88) for cancer mortality. Another meta-analysis in 2012 reviewed 7 studies, including 37,632 cancer cases to investigate the association between metformin, sulfonylurea intake and the risk of cancer (Sarana, 2012). Their results showed that use of metformin was associated with significantly decreased RR of all cancer (0.61 (0.54-0.70)), colorectal cancer (0.64 (0.54-0.76)) and pancreatic cancer (0.38 (0.14-0.91)). However, they didn’t find any evidence to support that metformin affects the risk of breast and prostate cancer.

2.3.5 Metformin and Cancer Risk of Specific Sites

Some studies looked at metformin intake and incidence of specific cancers in diabetic populations. Bodmer et al (2010) conducted a nested case-control study among 22,621 female diabetic patients to see whether use of hypoglycemic agents is associated with reduced breast cancer (BCa) risk. Their results suggested that long-term of metformin intake (>5 years) was associated with reduced risk of BCa compared with no use of metformin. The adjusted OR was 0.44 (0.24-0.82). However, this inverse relationship was not observed in short term use of
metformin. Another nested case-control study also suggested protective effect of metformin against BCa (Bosco, 2011). Metformin users were less likely to be diagnosed with BCa than patients who didn’t take metformin. The OR was found to be 0.77 (0.61-0.99). Further adjustment for other predictors of BCa did not substantially alter the association. Jiralerspong et al (2009) carried out a study to determine whether metformin was associated with higher pathologic complete response (PCR) rates in diabetic patients with BCa receiving neoadjuvant chemotherapy. The patients were compared in groups: 68 diabetic patients taking metformin, 87 not taking metformin and 2,374. The rate of PCR in metformin group (24%) was significantly higher than rate in non-metformin group (8%) (p=0.007). Comparison of the PCR rates between the metformin and non-diabetic groups (16%) did not meet significance (p=0.1). This study suggested that metformin intake might improve the therapeutic responses of BCa to neoadjuvant chemotherapy.

In a population-based case-control study (Wright and Stanford, 2009), a total number of 1,001 cases of PCa and 942 controls were analyzed to explore the relationship between metformin use and PCa risk. Metformin use was found to be associated with a 44% risk reduction for PCa (adjust OR: 0.56 (0.32-1.0)) in Caucasian men. However, no association was found in African-American men. A nested case-control analysis was conducted in UK, which included 739 cases of PCa and 7,359 matched controls (Azoulay, 2011). Their results suggested metformin did not reduce the risk of PCa in type II diabetic patients (RR=1.23 (0.99-1.52)). And surprisingly, in secondary analyses, PCa risk was found to increase as a function of the number of metformin prescriptions received. However, He et al (2011) examined the effect of thiazolidinediones and metformin on the overall survival of diabetic patients with PCa and found that both drugs were
associated with improved overall survival. After analyzing a total of 233 cases, metformin usage remained as a significant predictor of favorable survival (HR=0.55 (0.32-0.96). Patel *et al* (2010) investigated the relationship between metformin intake and biochemical recurrence (BCR) after radical prostatectomy (RP) for clinically localized PCa. A total of 616 patients were evaluated. In diabetic population, the estimated 5-year BCR-free survival was 66.1% for metformin users and 59.3% for non-metformin users, which was not significantly different. Metformin was found to have no benefit, but diabetes, regardless of metformin use, was significantly associated with an increased likelihood of BCR. A recent retrospective cohort study (Margel, 2013) used a nested case-control approach to examine the relationship between metformin exposure and the risk of PCa and its grade among diabetic men in Ontario, Canada. There were 5306 case subjects and 26,530 matched controls. Their results showed that there was no association between metformin use and risk of any PCa (OR=1.03), high-grade cancer (OR=1.13), low-grade cancer (OR=0.94) or biopsy-diagnosed cancer (OR=0.98). Margel *et al* (2013) performed a population-based retrospective cohort study to evaluate the association between cumulative duration of metformin use after PCa diagnosis and all-cause and PCa-specific mortality among patients with diabetes. Their results suggested that cumulative duration of metformin treatment after PCa diagnosis was associated with a significant decreased risk of PC-specific (HR=0.76) and all-cause mortality (HR=0.76) in a dose-dependent fashion.

2.3.6 Clinical Trials

The observational studies described above provide strong evidence that metformin may have anti-tumor effect by itself, or may be used to enhance the efficacy of other therapeutic agents. This has led to several prospective clinical trials.
Hosono *et al* (2010) performed a pilot study to evaluate the chemo-preventive effect of metformin on rectal aberrant crypt foci (ACF) formation, which is a marker for colorectal cancer. 12 non-diabetic patients with ACF were randomized to the metformin group (250mg/day) and 14 patients in control. Their result showed that after 1 month of intervention, patients in metformin group had a significant decrease in the mean number of ACF, while there was no significant change of ACF number in control. Further immunohistochemistry (PCNA stain) showed a decreased proliferative activity in colonic epithelium in the metformin patients. An open label, randomized clinical trial investigated the effects of metformin on gene expression in primary BCa (Hadad, 2010). This trial consisted 2 arms: trial A included 8 patients who underwent tumor core biopsies; trial B included 47 patients who had paired core biopsies and venous blood for insulin measurements. In both arms, four genes were found to be consistently down regulated by metformin: PDE3B (critical regulator of cAMP), SSR3 (regulates retention of ER resident proteins), TP53 (well-known tumor suppressor) and CCDC14 (DNA damage repair). These findings suggest that metformin may exert its anti-cancer effects in several mechanisms. Niraula *et al* (2010) carried out a clinical trial to examine biological and clinical effect of metformin in early stage BCa. In this study, 15 of 40 non-diabetic women newly diagnosed with untreated BCa were treated with metformin until definitive surgery. After a median of 21 days of treatment, patients in the metformin group had increased insulin sensitivity. Their tumor tissue showed decreased ki67 index (IHC) and increased apoptotic index (TUNEL assay). These results suggest that metformin has potential physiologic and cellular effects that may be beneficial for BCa. Nobes *et al* (2012) investigated the effects of metformin and lifestyle changes on the development of ADT related metabolic syndrome. Patients with PCa who would be treated with
ADT were randomized into two arms: control arm received ADT alone (n=20); intervention arm received ADT in combination with six months of metformin, a low glycaemic index diet and exercise program (n=20). All patients were investigated for biochemical and physical parameters of metabolic syndrome after six months. Their results showed significant improvements in abdominal girth, weight, BMI and systolic blood pressure in the intervention arm. These results suggest that metformin may have a role in preventing metabolic syndrome induced by ADT, however, the individual effect of metformin other than combined with life-style intervention need to be evaluated in further studies.

There are many ongoing clinical trials investigating metformin and its potential role in PCa treatment and management (Table 1).
<table>
<thead>
<tr>
<th>Title</th>
<th>Phase</th>
<th>Main Objective</th>
<th>Status</th>
<th>Clinicaltrial.gov identifier code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castration Compared to Castration Plus Metformin as First Line Treatment for Patients With Advanced Prostate Cancer</td>
<td>II</td>
<td>Compare both cohorts of castrated men (metformin vs. placebo) with regard to metabolic consequences of castration therapy</td>
<td>Recruiting</td>
<td>NCT01620593</td>
</tr>
<tr>
<td>Open-Label Study Of Metformin In Combination With Simvastatin For Men With Prostate Carcinoma And A Rising Serum Prostate-Specific Antigen Level After Radical Prostatectomy And/OR Radiation Therapy</td>
<td>II</td>
<td>To find out whether the two drugs used in the study, metformin and simvastatin, can slow down the speed of rise of prostate specific antigen (PSA) or stop its rise or even bring the level down.</td>
<td>Recruiting</td>
<td>NCT01561482</td>
</tr>
<tr>
<td>Metformin Hydrochloride as First-Line Therapy in Treating Patients With Locally Advanced or Metastatic Prostate Cancer</td>
<td>II</td>
<td>To study the safety of giving metformin hydrochloride as first-line therapy in treating patients with locally advanced or metastatic prostate cancer.</td>
<td>Active, not recruiting</td>
<td>NCT01243385</td>
</tr>
<tr>
<td>Metformin-Docetaxel Association in Metastatic Hormone-refractory Prostate Cancer (TAXOMET)</td>
<td>II</td>
<td>To evaluate the biological efficacy of Metformin combination with TAXOTERE® in patients with metastatic hormone-refractory prostate cancer</td>
<td>Recruiting</td>
<td>NCT01796028</td>
</tr>
<tr>
<td>Metformin Hydrochloride in Treating Patients With Prostate Cancer Undergoing Surgery</td>
<td>II</td>
<td>To study how well metformin hydrochloride works compared to placebo in treating patients with prostate cancer undergoing surgery.</td>
<td>Recruiting</td>
<td>NCT01433913</td>
</tr>
<tr>
<td>Prospective Study of Metformin in Castration-Resistant Prostate Cancer</td>
<td>II</td>
<td>To study the safety and efficacy of metformin in treating patients who are receiving androgen deprivation therapy (ATD) for prostate cancer</td>
<td>Active, not recruiting</td>
<td>NCT01215032</td>
</tr>
<tr>
<td>The Metformin Active Surveillance Trial (MAST) Study</td>
<td>III</td>
<td>Aims to see if metformin can delay the time to progression in men with low risk prostate cancer when compared to a placebo.</td>
<td>Not yet recruiting</td>
<td>NCT01864096</td>
</tr>
<tr>
<td>Impact of the Addition of Metformin to Abiraterone in Metastatic Prostate Cancer Patients (MetAb-Pro)</td>
<td>II</td>
<td>To assess the impact of the addition of metformin to abiraterone on survival in patients with metastatic prostate cancer</td>
<td>Not yet recruiting</td>
<td>NCT01677897</td>
</tr>
<tr>
<td>Metformin in Preventing Androgen Deprivation Therapy Induced Insulin Resistance and Metabolic Syndrome (MVENT)</td>
<td>II</td>
<td>To assess the efficacy of metformin in abrogating androgen deprivation therapy (ADT) induced insulin resistance as measured by homeostasis model assessment (HOMAIR) in men with non-metastatic prostate cancer.</td>
<td>Active, not recruiting</td>
<td>NCT01077479</td>
</tr>
</tbody>
</table>
3) Diet and PCa

We have discussed that there is large international and interethnic variations in PCa incident rate. Dietary factors are believed to play an important role for the differences observed (Venkateswaran and Klotz, 2010). Some dietary agents may offer protective effects against PCa, including selenium (Vogt, 2003), vitamin E (Heinone, 1998), lycopene and tomato products (Giovannucci, 1995), cruciferous vegetables (Kristal, 2004), chili peppers (Sanchez, 2006), fish (Chan, 2006), and green tea polyphenols (Khan, 2013). On the contrary, some other dietary factors are associated with elevated risk of PCa, such as vitamin D and calcium (Tseng, 2005), red meat (Di Masco, 2013), dairy products (Allen, 2008), alcoholic drinks (McGregor, 2013), and total fat and carbohydrate intake. For this study, we will discussion the effects of dietary fat and refined carbohydrate on PCa. For a more detailed review about dietary factors and PCa, the following review articles are recommended. (Venkateswaran and Klotz, 2010; Marshall, 2012; Chan, 2005; Wilson, 2012).

3.1 Fat and PCa

Fat is a major macronutrient in diet. Fats can be found in many dietary agents including animal meats, plant oils and dairy products. The primary building blocks of fat are fatty acids, which are essential nutrients for membrane synthesis and cell growth. They are also important source of fuel and when metabolized, yield large quantities of ATP (Masko, 2013). A fatty acid consists a carboxylic acid with a long aliphatic tail. Depending on the presence of hydrocarbon double bonds in the tail, fatty acids can be classified as “saturated” or “unsaturated” (Masko, 2013).
Results from animal studies suggest that dietary fat intake is a risk factor for PCa. Ngo et al (2003) investigated effect of dietary fat on the primary tumor growth rate using Los Angeles prostate cancer (LAPC)-4 xenografts in severe combined immune-deficient (SCID) mice. Their results suggested that intake of a low fat diet was associated with slower tumor growth compared to mice fed on a high fat diet. Kobayashi et al (2008) evaluated the effect of fat consumption on PCa development using the Hi-Myc mouse transgenic model. They found that the number of mice that developed invasive adenocarcinoma at 7 months was significantly less in the low fat diet group relative to the high fat diet group. For both studies, the protective effect of low fat diet was mediated through modulation of the insulin/IGF axis (Ngo, 2003; Kobayashi, 2008).

The data from epidemiologic studies linking total fat consumption with PCa risk are controversial (Niclis, 2013; Marshall, 2012). Most of the case control studies have reported a positive association between total fat intake and risk of PCa (Deneo-Pellegrini, 1999; Graham, 1983; Hayes, 1999; Lee, 1998; Lophatananon, 2010; Pourmand, 2007; Sunny, 2005; Tzonou, 1999 and Whittemore, 1995). For example, a case-control study was conducted in 12 cities in China to evaluate the relationship between dietary fat and PCa risk (Lee, 1998). They found that cases were more likely than controls to consume food with high fat. The adjusted OR for total fat between highest quartile and lowest quartile was OR = 3.6 (95% C.I. 1.8-7.2); for saturated fat, OR = 2.9 (1.5-5.7); and for unsaturated fat OR = 3.3 (1.7-6.3). A more recent case-control study conducted in UK also reported a positive association between PCa risk and intake of total fat and fat subtypes (Lophatananon, 2010). The adjusted OR for the highest vs. lowest quartile of intake of total fat, saturated fat, mono-unsaturated fat and poly-unsaturated fat were OR = 2.53 (1.72-3.74); OR = 2.49 (1.69-3.66); OR = 2.69 (1.82-3.96); OR = 2.34 (1.59-3.46), respectively.
However, some other studies, counteract against these findings, did not report such a positive association (Szymanski, 2010; Darlington, 2007).

The findings from cohort studies that investigating effect of dietary fat and PCa risk are more divergent than results from case-control studies. Some studies support the observed positive association (Giovannucci, 1993; Kristal, 2010; Le Marchand, 1994) while others do not support it (Crowe, 2008; Park, 2007; Veierod, 1997; and Wallstrom, 2007). An early cohort study conducted in Hawaii investigated consumption of high-fat animal products in relation to subsequent occurrence of PCa (Le Marchand, 1994). They found that a summary variable for intake of high-fat animal products was associated with PCa (RR = 1.6; 95% C.I. 1.0-2.4). On the contrary, a large multicenter prospective study conduced in Europe did not observe such an association between fat intake and PCa risk (Crowe, 2008). The hazard ratio for PCa for the highest vs. lowest quintile of total fat intake was 0.96 (95% C.I. 0.84-1.09).

Epidemiological studies have shown that dietary fat intake is more consistently associated with more aggressive or metastatic PCa (Giovannucci, 1993; Whittemore, 1995; Kristal, 2002 and Kristal, 2010). An early prospective cohort study used data from the Health Professionals Follow-up Study and examined the relationship of fat consumption to the incidence of advanced PCa (Giovannucci, 1993). They found that total fat consumption was directly related to risk of advanced PCa. The relative risk for highest vs. lowest quintile of intake of fat was found to be 1.79 (95% C.I. 1.04-3.07). Another cohort study used data from the Prostate Cancer Prevention Trial to examine nutritional risk factors for PCa (Kristal, 2010). They found that high intake of
The evidence linking total dietary fat consumption and PCa risk is mixed, therefore, some studies focused on the role of types of dietary fat on PCa, not just total fat intake (Masko, 2013). Some studies have shown that high consumption of saturated fat or animal fat has a stronger association with risk of PCa (Bidoli, 2005; Giovannucci, 1993; Kolonel, 1988; and Kurahashi, 2008). An early case-control study performed in Hawaii suggested that intake of saturated fat was associated with elevated PCa risk (Kolonel, 1988). The adjusted odds ratio for highest vs. lowest quartile of saturated fat intake was OR = 1.7 (95% C.I 1.0-2.8). A population based prospective study has investigated the association between saturated fatty acid and PCa in Japan (Kurahashi, 2008). A statistically significant increase in risk was observed for saturated fat intake, especially some specific saturated fatty acids: myristic acid and palmitic acid. Relative risks on comparison of the highest with the lowest quartiles of myristic acid and palmitic acid were 1.62 (95% C.I. 1.15-2.29) and 1.53 (1.07-2.20), respectively. Study also showed that men who consumed high saturated fat diets were more likely to experience biochemical failure and had significantly shorter biochemical failure free survival than men with low saturated fat diets after prostatectomy (Strom, 2008). For poly-unsaturated fatty acids (PUFA), omega-6 PUFA has been found to be associated with elevated risk of PCa (Newhouser, 2007 and Kristal, 2010). For example, a prospective cohort study reported a positive association of omega-6 PUFA and risk of PCa (Newhouser, 2007). The hazard ratio for highest vs. lowest quartile of omega-6 PUFA intake was HR = 2.61 (95% C.I. 1.01-6.72). On the contrary, studies have reported a protective effect of omega-3 PUFA against PCa (Spencer, 2009; Gann, 1994; and Augustsson, 2003). Fish
oil is an important source of omega-3 fatty acids in human diets. A prospective cohort study investigated whether high consumption of fish and marine fatty acids reduced the risk of PCa in men (Augustsson, 2003). They found that men with high consumption of fish had a lower risk of PCa, especially for metastatic cancer. Each additional daily intake of 0.5g of marine fatty acid was associated with a 24% decreased risk of metastatic PCa. However, some other studies did not support such a protective effect from marine fatty acids (Terry, 2003 and Szymanski, 2010).

A high ratio of dietary omega-6/omega-3 PUFAs has been found to be associated with increased risk of PCa (Williams, 2011 and Aronson, 2011). For instance, a case-control study performed in Durham showed that the highest dietary ratio of omega-6/omega-3 was significantly associated with elevated risk of high-grade PCa (OR = 3.55, 95% C.I., 1.18-10.69).

### 3.2 Carbohydrates and PCa

One of the proposed mechanisms linking dietary fat and prostate cancer growth is through alterations in the insulin and IGF-1 endocrine axis. While dietary fat can induce hyperinsulinemia, one of the most potent stimulants for insulin production is glucose consumption. Therefore, some studies have investigated the role of dietary carbohydrates on risk of PCa (Freedland, 2008).

Carbohydrates, also called saccharides, are another important macronutrient in human diets. Carbohydrate rich foods are often highly processed foods made from plants, including sweets, candy, table sugar, fruit and grain products. Carbohydrates perform numerous roles in living organisms, including energy storage, structural composition, coenzyme function, and backbone of genetic material (Masko, 2013). The carbohydrates can be divided into four chemical
groupings: monosaccharides, disaccharides, oligosaccharides, and polysaccharides.

Monosaccharides and disaccharides are also called simple carbohydrates. The body more rapidly absorbs simple carbohydrates and high consumption of simple carbohydrates can lead to hyperinsulinemia and obesity. Alternatively, the body less rapidly metabolizes complex carbohydrates and the consumption of complex carbohydrates can avoid dramatic insulin spikes therefore protect against obesity (Masko, 2013).

Several animal studies have demonstrated the beneficial effects of a LCD on slowing tumor progression. Ho et al (2011) showed that several formulated low carbohydrate high protein diets slowed tumor progression in a mouse xenograft model compared to a ‘Western’ diet. This effect was also coupled with lower glucose, insulin and lactate levels in the serum of animals. Other studies investigated the effect of several low carbohydrate high fat diets on cancer progression in murine prostate cancer xenografts (Masko, 2010; Mavropouls, 2009; Freedland, 2008). Their results showed that these diets significantly reduced the growth of xenograft tumors and prolonged animal survival compared to mice administered a ‘Western’ diet. Meanwhile, mice on low carbohydrate high fat diets were characterized with lower serum insulin, IGF-I and higher IGF-I binding proteins. Using athymic nude mice to obtain prostate cancer xenografts, Venkateswaran et al (2007) also demonstrated that compared to mice on a high carbohydrate diet, mice placed on a low carbohydrate diet experienced slower tumor growth which was associated with decreased serum insulin and IGF-I levels.

There are currently limited published epidemiological studies assessing the role of dietary carbohydrates on the risk and progression of PCa, and the existing data are controversial. Some
studies suggest that dietary carbohydrates are associated with risk of PCa. In a multicenter case-control study, investigators assessed the relationship of glycemic index (GI) and glycemic loads (GL) with PCa risk. They found direct associations between dietary GI and GL and PCa risk. Compared to the lowest quintile of GI, the OR was 1.57 for the highest quintile. The corresponding OR for GL was found to be 1.41 (Augustin, 2004). In another case-control study carried out in Italy, a direct association with PCa was found for starch intake (Bidoli, 2005). The odds ratio was 1.4 in the highest vs. the lowest quintile of starch intake. More recently, a large cohort study examined the association between dietary intakes of carbohydrates, fiber and their food sources and risk of PCa (Drake, 2012). Their results suggest that high intake of refined carbohydrates, such as low-fiber cereals, cake, and pasta, is associated with increased risk of PCa. However, other studies suggest that exposure to a high carbohydrate diet does not affect PCa incidence. A cohort study investigated the association between dietary GI, GL, insulin index (II), fiber, and whole grains and risk of PCa in male participants of the Health Professionals Follow-up Study (Nimptsch, 2011). They found that dietary GI, GL, II, or fiber was not associated with risk of total or subgroups of PCa. Another cohort study investigated the associations between dietary carbohydrate, GI, GL, and incident PCa in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Their results suggest that dietary carbohydrate, GI and GL were not associated with incident PCa (Shikany, 2011).
4) Exercise and PCa

Another important aspect of lifestyle is exercise. Exercise has been increasingly recognized to play an important role in the primary prevention of various cancers, including PCa (Liu, 2011). Exercise exerts protective effects along the PCa continuum. It has been shown to be associated with deceased risk of PCa. Exercise has also been shown to slow disease progression in patients on active surveillance who are diagnosed with low-grade PCa (Ornish, 2005). Furthermore, exercise also reduces comorbidity and improves quality of life (QOL) after treatments for PCa (Antonelli, 2009). Exercise intervention has a pleiotropic effect, which influences many pathways relating to PCa pathogenesis. Chronic exercise stimulates endogenous antioxidant protection, reduces systematic inflammation, improves innate immune function and protects from obesity (Antonelli, 2009). Aerobic exercise also decreases serum levels of several metabolic and sex steroid hormones, including fasting insulin and IGF-I (Barnard, 2003; Barnard, 2007).

4.1 Animal studies

A few animal studies have investigated the effect of exercise on initiation and progression of PCa. However, the results are not consistent. Zheng et al (2008) investigated the effect of voluntary exercise on the formation and growth of human PC-3 tumors in male severe combined immunodeficient (SCID) mice. Exercise treatment was commenced one week before the subcutaneous injection of tumor cells. Results revealed that 9 weeks of voluntary running wheel exercise significantly suppressed the growth of PC-3 tumors. Further mechanistic studies showed that exercise inhibited proliferation and stimulated apoptosis in PC-3 tumors. In a second study
by Esser *et al* in 2009, the effect of voluntary wheel running on prostate cancer growth in C3(1) Tag mice that are predisposed to prostate cancer was investigated. It was found that 83% of the dorsolateral prostates were classified normal for mice that ran greater than 5 km/day, and only 43% were classified normal for the mice running less than 5 km/day. In addition, there was a relationship between average running distance and pathologic progression to high-grade PIN and local invasion. Also, none of the dorsolateral prostates from mice that ran more than 5 km/day was classified with advanced pathology as compared to 43% in mice that ran less than 5 km/day. More recently, Jones *et al* (2012) studied the effect of exercise on an orthotopic model of murine prostate cancer. They injected transgenic adenocarcinoma of mouse prostate C-1 cells orthotopically in C57BL/6 male mice and the exercise group was given voluntary access to a spinwheel 24 hours/day. They found that 8 weeks of voluntary exercise did not significantly alter primary tumor growth rate between the exercise and control groups. However, exercise was associated with improved tumor vascularization with a shift toward suppressed metastasis. This finding was very similar to a previous study where long-term voluntary wheel running significantly improved blood perfusion compared to their sedentary control (Jones, 2010). Our group found that 8 weeks of sustained aerobic exercise was associated with an increased tumor growth rate in an LNCaP xenograft model. However, exercise increased the consumption of a high-fat diet, which may have tumor-promotional effects (Vandersluis and Venior, 2013).

4.2 Exercise and risk of PCa

Many epidemiological studies have investigated the association between exercise and PCa, and the evidence is inconsistent. In all conducted studies, about 40% indicated a protective effect of exercise on PCa risk and none described a negative association (Rebillard, 2013). For a more
A recent meta-analysis investigated 19 eligible cohort studies and 24 eligible case-control studies and found that total physical activity was significantly associated with a small decreased risk of prostate cancer. The pooled relative risk was found to be 0.90 (95% C.I. 0.84-0.95). Furthermore, both occupational physical activity and recreational physical activity showed beneficial effects against PCa. The pooled relative risk for occupational and recreational physical activity were found to be 0.81 (0.73-0.91) and 0.95 (0.89-1.00), respectively (Liu, 2011).

Some studies have shown that occupational or professional physical activity is associated with reduced risk of PCa. For example, Norman et al (2002) conducted a large cohort study in Sweden and showed that the relative risk for PCa increased with decreasing level of occupational physical activity (P<0.001). The rate ratio was 1.11 for men with sedentary jobs as compared with those whose jobs have high activity levels (95% C.I. 1.05-1.17). A nested case-control study (Krishnadasan, 2008) in Southern California showed that high activity levels at work were inversely associated with PCa incidence among aerospace workers (odds ratio = 0.55, 95% C.I. 0.32-0.95). Johnsen et al (2009) examined the association between risk of PCa and physical activity at work and in leisure time in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Their data supported an inverse association between advanced PCa risk and occupational physical activity (P trend = 0.024), but they found no support for beneficial effects of physical activity in leisure time.
Other studies have also looked at the effects of recreational or leisure time physical activity on PCa risk. For example, Clarke and Whittemore (2000) showed that lower levels of recreational activity were associated with increased PCa risk among African-Americans. The relative risk for men who reported low levels of physical activity compared with very active men was 3.7 (95% C.I 1.7-8.4). Another large cohort study in the US indicated that recreational physical activity was associated with reduced risk of aggressive PCa (Patel, 2005). The relative risk for physically actively men (>35 metabolic equivalent hours/wk) compared with men who reported no physical activity was 0.69 (95% C.I. 0.52-0.92).

These studies demonstrated a link between exercise and PCa, and all men should be encouraged to increase their exercise in both occupational and recreational settings. However, the questions of what type, parameters, and time period are most beneficial for PCa reduction remain unclear (Rebillar, 2013). Some studies suggest that exercise intensity may play an important role. For example, Antonelli et al (2009) reported that specific moderate exercise (>3 metabolic equivalent hour/wk) was associated with a lower risk of PCa. However, this reduction was more significantly for men who had higher exercise intensity (>9 metabolic equivalent hour/wk).

4.3 Exercise and PCa Progression

Many studies have also demonstrated the beneficial effects of exercise on slowing disease progression among diagnosed PCa patients. Giovannucci et al (2005) assessed physical activity in relation to PCa incidence, mortality, and Gleason histologic grade. They found that vigorous activity could slow the progression of PCa and reduce PCa-specific death. The relative risks for highest category of vigorous activity for advanced PCa and Fatal PCa were 0.33 (95% C.I. 0.17-
0.62) and 0.26 (0.11-0.66), respectively. These results are in line with the study carried out by Antonelli et al (2009), which showed that the low-grade PCa is correlated to physical activity among men undergoing prostate biopsy.

Using active surveillance patients, the Ornish group has shown that exercise as part of a lifestyle modification program (consists moderate aerobic exercise, vegan diet and stress management) has a beneficial effect for patients with a low-grade PCa, as evidenced by decline in serum PSA and reduction in disease progression (Ornish, 2005; Frattaroli, 2008; Ornish, 2008). More recently, Burton et al (2012) also showed that PSA at age 50 years was 2.1% lower per unit increase in weighted exercise score in active surveillance patients.

Kenfield et al (2011) performed an observational study to determine whether higher physical activity after prostate cancer diagnosis decreases risk of overall and prostate specific death. They found that physically active men had lower risk of overall as well as prostate specific mortality. In addition, men with more than 3 hours per week of vigorous activity had a 61% lower risk of prostate cancer death compared with men with less than 1 hour per week of vigorous activity. One prospective study examined physical activity after prostate cancer diagnosis in relation to risk of prostate cancer progression. They found that men who walked briskly for more than 3 hours per week had a 57% lower rate of disease progression compared to men who walked at an easy pace for less than 3 hours per week (Richman, 2011).

These findings suggest that exercise can be incorporated as a comprehensive lifestyle
intervention for patients diagnosed with low-grade disease, which exert the potential effect on slowing disease progression and reducing PCa mortality.

4.4 Exercise and Quality of Life (QOL) During PCa Therapy

Except active surveillance, the most common treatments available to men diagnosed with PCa include surgery, radiation therapy, and hormonal therapy (Antonelli, 2009). Despite improvements in treatment regimes, all therapies are accompanied by numerous side effects. For instance, one of the most common complications caused by radical prostatectomy is urinary incontinence. Androgen deprivation therapy is often associated with side effects including missing libido, anemia, hot flushes and metabolic syndrome. Taken together these aspects strongly affect patients’ quality of life and bring about a social withdrawal of many patients (Baummann, 2012). Many studies have focused on exercise interventions in PCa patients and suggest that exercise demonstrate positive physiological and psychological effects and therefore, improve overall QOL of patients.

Exercise reduces comorbidity and improves quality of life (QOL) after surgical treatments (Antonelli, 2009). For patients who have a history of engaging in physical activity, they have a reduction in short-term morbidity and mortality associated with prostatectomy (Warburton, 2006). There is also evidence suggests that intensive exercise training interventions in the acute setting before surgery is also beneficial (Jones, 2007). Exercise also improves QOL after prostatectomy, such as reducing post-prostatectomy erectile dysfunction (ED) and incontinence (Park, 2012). Furthermore, exercise shows beneficial effects for men undergoing concomitant androgen deprivation therapy (ADT) or radiation therapy, including increased body strength,
decreased fatigue, higher aerobic fitness and increased QOL measurements (Segal, 2003; Windsor, 2004). An 8-week cardiovascular exercise program also improved cardiovascular fitness, flexibility, muscle strength, fatigue, and overall QOL in patients undergoing radiotherapy (Monga, 2007).

Although the underlying mechanisms for the favorable effects of exercise are still poorly understood, above evidences suggest that exercise should be actively applied to the management of PCa.
Hypothesis and Specific Aims

This thesis consists of two main studies. In the first study, we have examined the effect of sustained aerobic exercise on prostate cancer (PCa) progression on animals placed on diets formulated with varying carbohydrate concentrations. In the second study, we have used Metformin to combat hyperinsulinemia and study the combined effect of metformin and aerobic exercise on PCa progression.

We hypothesize that a low carbohydrate diet and exercise will slow prostate tumor development. Also we hypothesize that administration of metformin combined with sustained exercise will slow the growth of prostate tumor progression in a murine xenograft model.

To test this hypothesis, we have designed the following specific aims:

1) To investigate the effect of exercise on PCa progression in diets differing in their carbohydrate content.

2) To investigate the effect of metformin and exercise on animals placed on a high fat-high carbohydrate (HF-HC) diet on PCa progression.

3) To investigate the combined effect of metformin and physical activity on PCa tumor growth.
Materials and Methods

There were 2 studies that were conducted using the xenograft model of prostate cancer (PCa). Study one focused on the administration of high carbohydrate diet and an exercise regimen. This will be referenced as “The Carbohydrate (Carb) Study” in my entire thesis. Study two will focus on the effect of a high fat-high carbohydrate diet (HF-HC) with and without the administration of metformin as well as an exercise regimen. This aspect of the study will be referenced as “The Metformin Study” in my entire thesis.

1) The LNCaP Xenograft Model of Prostate Cancer

Animal ethical approval for this study was obtained from the University of Toronto Animal Research Ethics Board and the Sunnybrook Research Institute. All work was conducted in accordance with the institutional guidelines including the Care and Use of Experimental Animals Guidelines of the Canadian Council on Animal Care (CACC).

Six-week-old male nu/nu athymic nude mice were purchased from Harlan Laboratories (Mississauga, Ontario, Canada). All animals were maintained in a sterile, pathogen free facility and housed as five mice per cage. All animals were acclimatized for one week prior to tumor cell inoculation.

Human prostate cancer cells (LNCaP) (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen, Canada) with 10% fetal bovine serum (Sigma, USA), 0.3mg/ml l-glutamine and 100IU/ml penicillin and 100µg/ml streptomycin (Invitrogen, Canada). All cells were cultured at 37ºC in a 5% CO2 incubator. When cells arrive at 80%
confluency, they were trypsinized and 1 x 10^6 cells resuspended in 100µL matrigel solution (BD Biosciences, Franklin Lakes, NJ, USA). Cells in matrigel were inoculated subcutaneously, unilaterally, into the right flank of each mouse, under inhalational general anaesthesia (isofluorane).

In study 1 the “Carbohydrate Study”, about two weeks (Day 16) after inoculation, all mice, with palpable tumors, were randomly assigned into four experimental groups (n = 10 per group):

a) High carbohydrate diet with exercise (HC-Ex)

b) High carbohydrate diet without exercise (HC-noEx)

c) Low carbohydrate diet with exercise (LC-Ex),

d) Low carbohydrate diet without exercise (LC-noEx).

In the second study termed the “Metformin Study”, after tumor inoculation, all mice were commenced on a high carbohydrate-high fat (HC-HF) diet and randomly assigned into four experimental groups (n = 10 per group) right away:

a) Metformin Alone

b) Exercise Alone

c) Combination of metformin and exercise

d) Control group

After two weeks of injection, some mice didn’t develop palpable tumors. In metformin group (n = 9), all mice developed tumors, but one died. In exercise group (n = 8), two mice didn’t develop tumors. In the combination group (n = 9), 1 mouse didn’t develop tumor. In the control group (n = 7), three mice didn’t develop tumors.
2) Diet Formulation and Treatment

The high carbohydrate diet included 70.1% carbohydrate, 19.9% fat, and 10% protein. The low carbohydrate diet consisted of 10.1% carbohydrate, 19.9% fat, and 70.1% protein. The high carbohydrate-high fat diet was as in our previously published studies (Venkateswaran, 2007) and included 40% carbohydrate, 45% fat, and 15% protein (Table 1). All diets were formulated by Purina Mills Test Diets (Richmond, IN) and irradiate for sterilization. Diets were stable for six months, as determined by the manufacturer and stored at 4°C before administration. Mice had ad libitum access to food and water throughout the treatment period.

In the Carb Study, mice fed on the low carbohydrate diet were only on this diet from Day 16 to Day 26. Started on day 16, mice that were randomized to LCD (both exercise and non-exercise groups) had a significantly reduced food intake compared to mice that were randomized to HCD. Consequently mice that fed on the LCD started to lose their body weight continually. Canadian Council on Animal Care and Cancer Endpoint Guidelines require mice to be sacrificed if they lose 20% of their body weights. In order to continue the study, on Day 26 mice placed on the low carbohydrate diet were switched to the high carbohydrate diet as well. In the Metformin Study, all mice were placed on the high carbohydrate-high fat diet soon after tumor inoculation, randomized and continued on the same diet until the termination of the experiment.
Table 1: Composition of diets for Carbohydrate Study and Metformin Study

<table>
<thead>
<tr>
<th>Dietary Parameters</th>
<th>High Carbohydrate</th>
<th>Low Carbohydrate</th>
<th>High Carbohydrate-High Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition of Diet, % Weight</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Carbohydrate (dextrin + Sucrose)</td>
<td>73.5</td>
<td>9.8</td>
<td>47.5</td>
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<td>Fat</td>
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<td>23.8</td>
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<tr>
<td>Protein</td>
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<tr>
<td>Others (fiber, minerals and vitamins)</td>
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<td>14.3</td>
<td>10.8</td>
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<tr>
<td><strong>Energy contributions, %</strong></td>
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<tr>
<td>Carbohydrate (Dextrin + Sucrose)</td>
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<td>10.1</td>
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<tr>
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<td><strong>Caloric Density</strong></td>
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<tr>
<td>Energy (Kcal/gram)</td>
<td>4.2</td>
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<td>4.76</td>
</tr>
</tbody>
</table>

3) Assessment of Body Weight, Tumor Volume and Food Consumption

Body weight, tumor volume and food consumption were recorded three times per week. An electronic vernier caliper was used to measure the longest (L) and shortest (W) tumor diameters and tumor volume was calculated by the formula: \( \pi/6 \times W^2 \times L \). Quantity of food consumed was
measured and was converted into energy consumption using established caloric density (kcal/g) values for the respective diets.

All animals were sacrificed between 10-11 weeks of active treatment. Upon sacrifice, all the tumors were under 17 mm, the Canadian Council on Animal Care and Cancer Endpoint Guidelines maximum permissible tumor diameter.

Note: in the Metformin Study, the high carbohydrate-high fat diet was very soft and easily became powder and fell in the cages, which made the measurement not accurate. Therefore, although food consumption was recorded, the results will not be discussed.

4) Exercise Treatment

Mice were exercised using the Forced Exercise/Walking Wheel Bed (Lafayette Instruments, USA). Mice were trained to run in the running wheel starting with low exercise intensity (3 m/min) with the intensity gradually being increased. Mice that were not in the exercise groups were place in a running wheel and the wheel was rotated briefly to control for the induced stress by exercise.

In the Carb Study, mice were exercised 5 times/week, 45 minutes/day (3 sessions, 15 minutes/session and 2 minutes break between sessions), over 11 weeks. Exercise intensity was gradually increased from 3 m/min to 10 m/min. In the Metformin Study, mice were exercised 3 times/week, 45 minutes/day (3 sessions, 15 minutes/session and 2 minutes break between sessions), over 10 weeks. Exercise intensity was gradually increased from 3 m/min to 6 m/min.
5) Metformin Treatment

Metformin was dissolved in cold PBS and was administered at a dose of 50 mg/kg b.w. It was administered three times per week, on alternative days of exercise training, by intraperitoneal injection, with a total volume of 200 uL. Mice in the other two groups that didn’t require metformin treatment was injected with 200 uL cold PBS alone.

6) Blood and Tissue Samples

Blood samples were obtained by saphenous vein bleeding before active treatment (first blood draw) and during active treatment (second blood draw). Mice’s blood from the same group was pooled together. At termination of the experiments, serum samples were collected by direct cardiac puncture (third blood draw). Collected blood was centrifuged and serum was separated, aliquot, and stored at -80°C.

Tumors were excised, weighed, and cut into two portions using a sterile razor blade. One portion was processed for histopathology studies and hence fixed in 10% v/v buffered formalin and embedded in paraffin. Sections (5-µm thick) were cut from the paraffin-embedded tissue, mounted on slides, and stained with hematoxylin and eosin (H&E). Stained tumours were analyzed by a blinded pathologist to confirm the presence of prostate cancer and to determine the percentage of tumor necrosis. The remaining portion of the tumors was snap frozen in liquid nitrogen and stored at -80°C for western blot analysis as described below.
7) Plasma Insulin Measurements

Serum insulin was measured in duplicate using a rat insulin ELISA kit (Mercodia AB, Sweden), according to the manufacturer’s instructions. Mercodia Mouse Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. 10 µL each of Calibrators (provided by the manufacturer) and samples were incubated with 100 µL enzyme conjugate solution (provided by the manufacturer) in microplate wells for 2 hours at room temperature. During incubation insulin in the sample would react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate wells. After incubation, the plate was washed 6 times with 700 µL wash buffer solution (provided by the manufacturer) per well to remove unbound enzyme labeled antibody. 200 µL Substrate TMB (provided by manufacturer) was added into each well and the bound conjugate was detected by reaction with 3,3’,5,5’-tetramethylbenzidine. The reaction was stopped by adding 50 µL Stop Solution (provided by manufacturer) to give a colorimetric endpoint that was read spectrophotometrically (450 nm). Results were calculated according to the manual provided by manufacturer.

8) Plasma IGF-1 Measurements

Serum IGF-1 was measured in duplicate using a Mouse/Rat IGF-1 Immunoassay (Quantikine ELISA, R&D Systems, Inc.), according to the manufacturer’s instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. All reagents, standard dilutions, control and samples were prepared according to the manufacturer’s guidance. 50 µL of Calibrator Diluent RD5-38 (provided by the manufacturer) and 50 µL of Standard, Control
(provided by the manufacturer) or sample were incubated in wells of the microplate pre-coated with a monoclonal antibody specific for mouse/rat IGF-1 for 2 hours at room temperature. After incubation, each well was washed with 400 µL Wash Buffer (provided by manufacturer) for a total of 5 times. 100 µL of Conjugate Solution (provided by manufacturer) was added to each well and incubated for 2 hours at room temperature followed by the wash step described above. 100 µL of Conjugate Solution (provided by manufacturer) were then added to each well and incubated for 30 minutes at room temperature, the plate were protected from light. The reaction was stopped by adding 100 µL Stop Solution (provided by manufacturer) to each well. The optical density of each well was determined within 30 minutes, using a macroplate reader set to 450 nm. Results were calculated from the standard curve according to the manual of the manufacturer.

9) Plasma C-peptide Measurement

Serum C-peptide was measured in duplicate using a Mouse C-peptide ELISA kit (ALPCO Immunoassays, USA), according to the manufacturer’s instructions. This assay is a sandwich type immunoassay. All reagents, standard dilutions, control and samples were prepared according to the manufacturer’s guidance. 100 µL of Working Strength Conjugate (provided by the manufacturer) and 10 µL of Standard, Control (provided by the manufacturer) or sample were incubated in wells of the microplate pre-coated with a monoclonal antibody specific for C-peptide for 2 hours at room temperature. After incubation, each well was washed with 350 µL Working Strength Wash Buffer (provided by manufacturer) for a total of 3 times. 100 µL of TMB Substrate (provided by manufacturer) was added to each well and incubated for 10 minutes at room temperature followed by adding to each well 100 µL Stop Solution (provided by manufacturer) to stop the reaction. The optical density of each well was determined within 30
minutes, using a macroplate reader set to 450 nm. Results were calculated from the standard curve according to the manual of the manufacturer.

10) Western Blotting

Tumor tissues that were stored at -80°C were thawed on ice. Tumors (3 mice in each group) were cut into small pieces and homogenized in ice-cold RadioImmuno Precipitation Assay (RIPA) buffer (50 mM Tris-HCL, PH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA) with a mixture of protease and phosphatase inhibitors being added (1 mM phenylmethylsulfonylfluoride and 0.02 mg/mL each of aprotinin, leupeptin, and pepstatin; Sigma Chemical Company, St. Louis, MO). Protein quantification was determined by Bradford method. Proper amount of tumor lysate (volume that contains 40 ug protein) suspended in lysis buffer was loaded in lanes of solium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) gels and electrophoresed followed by overnight transferring to membranes (Immonilon transfer membrane, Millipore, Bedford, MA). Transferred membranes then were blocked with 5% non-fat milk (non-fat milk powder dissolved in phosphate-buffered saline containing 0.2% Tween 20). Blotted membranes were subsequently incubated with primary antibodies and secondary antibodies. Primary antibodies used were: β-actin mouse monoclonal antibody (Sigma) at 1: 20000 dilution; mouse anti-insulin receptor (β-subunit) monoclonal antibody (Chemicon, Temecula, CA) at 1:200 dilution and mouse anti-IGFI receptor monoclonal antibody (Chemicon, Temecula, CA) at 1:200 dilution.

Secondary antibodies used were horseradish peroxidase-labeled anti-mouse IgG, 1:5000 dilution. Antibody protein complexes were visualized by electrochemiluminescence. Protein expression
levels, relative to $\beta$-actin, were determined using image quantification software (ImageJ, US National Institute of Health, USA).

11) **In Vitro Mitogenicity Assay**

LNCaP cells were plate seeded in 96-well plates with 5000 cells per well. Cell counts were performed in triplicates using a hemocytometer, with trypan blue exclusion to identify viable cells. Cells were incubated for 24 hours and washed with phosphate-buffered saline (PBS) twice to remove serum followed by treatment of serum-free media for an additional 24 hours to synchronized cell cycling. Then cells were treated for 72 hours with appropriate media supplemented with 5% mouse serum or fetal bovine serum (control). The 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was then used to measure cell proliferation. 20 µl of MTS solution was added to each well and incubated for an additional period of 2 h at 37°C in a humidified incubator. The absorbance at 490 nm was recorded using a 96-well plate reader. The 490 nm absorbance reading is directly proportional to the number of cells normally used in the proliferation assay.

12) **Statistical Analyses**

Analyses of between-group variations at specific time points in the *in vivo* experiments was performed using Student’s t test. For differences between whole treatment groups over time, repeated-measures analysis of variance was used. Analyses of between-group variations for all *in vitro* experiments were assessed using student’s t test (SPSS, IBM, USA).
Results

1) The Carbohydrate Study: Effect of a Low Carbohydrate Diet and Exercise on Prostate Cancer (PCa) Progression

1.1 The Low Carbohydrate Diet (LCD) Significantly Reduced Food Intake and Reduced Body Weights

On Day 16, all mice showed signs of tumor development and hence all animals were switched to their treatment diets according to the randomization. Starting on day 16, mice that were randomized to LCD (both exercise and non-exercise groups) showed significantly reduced food consumption compared to mice that were randomized to HCD (both exercise and non-exercise groups) (Figure 2). Consequently mice that were fed on the LCD started to lose their body weight continually (Figure 1). Although we had initially inferred that the loss of body weight was due to the mice adjusting to the LCD, after careful monitoring of the mice we found that the LCD mice were consuming less food and hence demonstrating loss of their body weights. After they were on this diet for 10 days, they consumed an average of 40% less calories compared to mice fed on HCD and their body weights were reduced an average of 13% compared to their body weights before the diets were switched (Day 15). In order to continue the study, we modified the experimental design and changed the diets in the group where mice fed on LCD started to receive a HCD on day 26. Hence this group of animals remained on the LCD only for a period of 10 days. After the diets were switched, mice originally fed a LCD started to rapidly consume more food and their body weights increased considerably. From Day 26 to Day 30, the mice consumed an average of 35% more calories compared to their food intake previously and their average body weight increase was 11%. After the initial spike, their food intake and body
weights started to stabilize gradually and remained relatively constant from day 40 to the end of the study (D 96).

The food consumption of the mice fed on the HCD remained relatively constant, consuming an average of 13 kcal/mouse/day, and their body weights did not change significantly across the entire treatment time (Figure 1 and 2).

Despite the changes in the experimental design, the repeated-measures analysis of variance (RANOVA) analysis showed that there was a significant difference in body weight gain among various treatment groups (p=0.002). Compared to “HC-NoEx” group, mice in “LC-Ex” and “LC-NoEx” groups had a significantly lower body weight gain (p = 0.002 and 0.018 respectively). Compared to mean body weight-gain in “HC-NoEx” group the body weight gain in the “LC-Ex” and “LC-NoEx” groups were 2.7g and 3.5 g lower. The “HC-Ex” groups also had a higher average body weight gain across the study compared to “LC-Ex” and “LC-NoEx” groups. However, the difference was not significant (p = 0.077 and 0.397 respectively). Compared to “HC-NoEx” group, “HC-Ex” group showed a non-significant trend towards a reduced gain in body weight. Similarly, “LC-Ex” group also showed a non-significant trend towards decreased body weight gain compared to “LC-NoEx” group (Figure 1).

Figure 2 depicts the food consumption presented as energy intake (Kcal) per mice per day for various groups across the whole experiment. There were significant differences among the four treatment groups in food intake (p < 0.001). Despite the initial energy restriction during the period while animals were on the LCD, compared to “LC-NoEx” group, mice on “HC-Ex”,
“HC-NoEx” and “LC-Ex” groups consumed significantly less amount of food. Their mean consumption of food (per day per mouse) was 1.05 kcal, 0.83 kcal and 1.79 kcal lower, respectively (p = 0.001, 0.015 and <0.001 respectively). Interestingly, compared to “LC-Ex” group, mice in “HC-Ex”, “HC-NoEx” and “LC-NoEx” groups consumed a significantly higher quantity of food. Their mean consumption of food was 0.73 kcal, 0.96 kcal and 1.79 kcal higher, respectively (p = 0.040, 0.003 and <0.001 respectively). Mice in “HC-Ex” group also had a lower food intake than mice in “HC-NoEx” group. However, the difference was not significant.

Figure 1. Variation in body weight gain over time in the four different treatment groups: Body weight measurements were subtracted from initial body weight (day 0) for each mouse to get body weight gain on the different days of measurement. Using repeated-measures analysis of variance (RANOVA) to assess variation in body weight gain across the whole time period of the study, we found that overall there were significant differences among groups (P=0.002). Further post hoc pairwise analyses (Tukey’s honestly significant difference post hoc test) showed that compared to “HC-NoEx” group, mice in “LC-Ex” and “LC-NoEx” groups had a significantly lower body weight gain (p = 0.002 and 0.018 respectively). Error bars represent +/- 1 standard error.
Figure 2. Effect of exercise and diet on food consumption over the treatment period: Data presented was the energy intake (Kcal) per mice per day (it was assumed that each mouse in the same cage consumed same amount of food). Using analysis of variance (ANOVA) to assess the food intake, we found that there were significant differences among the four treatment groups (P<0.001). Follow up post hoc pairwise comparisons (Tukey’s HSD) revealed that compared to “LC-NoEx” group, mice in “HC-Ex”, “HC-NoEx” and “LC-Ex” groups consumed significantly less amount of food (p = 0.001, 0.015 and <0.001 respectively). Compared to “LC-Ex” group, mice in “HC-Ex”, “HC-NoEx” and “LC-NoEx” groups consumed significantly greater quantity of food (p = 0.040, 0.003 and <0.001 respectively).

1.2 Effect of diet and exercise on the growth of prostate LNCaP tumors in nude mice

As discussed under Materials, animals placed on different diets were exercised for 11 weeks. Figure 3 shows the mean standardized tumor volume in the different treatment groups over the entire treatment period. Overall, there were no significant differences in tumor progression among the four treatment groups (p = 0.151). However, the two groups fed on LCD for 10 days had a non-significant trend towards slower tumor growth compared to the two groups fed on
HCD, suggesting that the 10 days of energy starvation may have had an effect on tumor growth and development. Compared to the “HC-NoEx” and “LC-NoEx” groups, “HC-Ex” and “LC-Ex” also showed a non-significant trend towards tumor progression respectively. This suggests that exercise could play a role in slowing tumor growth over time. However, the number of animals included in our experiment was not powerful enough to detect these effects.

Figure 4 shows the mean tumor wet weight for different treatment groups at the time of sacrifice: Similar to tumor volume, there was no significant difference in tumor wet weight among different treatment groups (p = 0.571). At sacrifice, the mean tumor wet weights depicted from the highest to the lowest group were as follows: HC-NoEx, HC-Ex, LC-NoEx and LC-Ex. A similar trend was also observed for tumor volume.

Figure 3. Effect of diet and exercise on the growth of prostate LNCaP tumors in nude mice: Tumor volumes for the individual animals were divided by their initial tumor volume (tumor volumes on day 30, where all mice developed measurable tumors) to get the standardized tumor volume. Compared to “HC-NoEx” and “LC-NoEx” groups, “HC-Ex” and “LC-Ex” groups had a non-significant trend towards a reduction in tumor volume. Compared to mice fed on the HC diet, mice fed on the LC diet had a non-significant trend towards reduced tumor volume. RANOVA analysis revealed that there was no significant difference in tumor volume among the different groups across the treatment period (P = 0.151). Error bars represent +/- 1 standard error.
Figure 4. The mean tumor wet weight for different treatment groups upon sacrifice: The mean tumor wet weight, depicted from the highest group to the lowest group were HC-NoEx, HC-Ex, LC-NoEx, LC-Ex. Using ANOVA analysis, we found there were no significant differences in tumor wet weight at sacrifice among the different treatment groups ($p = 0.571$). The error bars represent $\pm 1$ standard error.

### 1.3 The effect of exercise on body weight gain in animals placed on a high carbohydrate diet

Mice in the “LC-Ex” and “LC-NoEx” groups were on the LCD for only 10 days and consumed the HCD for the majority of the treatment period. We combined the animal data from “LC-Ex” and “HC-Ex” and renamed this new group as “Ex” and also combined the animal data from “LC-NoEx” and “HC-NoEx” to form the new group “NoEx” to assess the effect of exercise alone on body weights, food consumption and tumor development.
Figure 5 shows the alteration in body weight gain with exercise: RANOVA analysis suggests that mice without exercise had a higher mean body weight than mice with exercise across the study and the differences were close to significance (p = 0.150)

![Graph showing body weight gain with exercise](image)

**Figure 5.** Alterations in body weight gain with exercise: Data from “HC-Ex” group and “LC-Ex” group were combined and the new group was depicted as the “Ex” group. Evaluation of results from “HC-NoEx” group and “LC-NoEx” group were combined to get “NoEx” group. Body weight measurements were subtracted from their initial body weight (day 0) for each mice to get body weight gain on different days. Using RANOVA to assess variation in body weight gain across the whole time period of the study, we found that mice in exercise group had a lower mean body weight than mice in the non-exercise group; however, the differences was not significant (p = 0.150). Error bars represent +/- 1 standard error.

### 1.4 The effect of exercise on food consumption

As illustrated in Figure 6, exercise significantly reduced food consumption as evaluated during the entire treatment period. Compared to mice on exercise, mice not exercising consumed an average of 1 kcal more amount of food per mouse per day (P<0.001)
Figure 6. Effect of exercise on food consumption over the treatment period: Data presented is the energy intake (Kcal) per mice per day. Data from “HC-Ex” group and “LC-Ex” group were combined to get “Ex” group. Date from “HC-NoEx” group and “LC-NoEx” group were combined to get “NoEx” group. Using student’s t-test, we found that mice in the exercise group consumed significantly less amount of food compared to mice in the non-exercise group (P<0.001).

1.5 The effect of exercise on tumor development over time

Two weeks after tumor inoculation, it was observed that all mice started to have sign of tumor development. On day 30, all mice developed measurable tumors.

Figure 7 shows the mean standardized tumor volume over time for mice with and without exercise: Compared to mice in the non-exercise group, mice in the exercise group had a non-
significant trend towards reduced tumor volume. RANOVA analysis revealed that there was no significant difference in tumor volume between the two groups ($p = 0.535$).

Figure 8 shows the mean tumor wet weight for exercise and non-exercise groups at sacrifice: Mice in the exercise group had a lower mean tumor wet weight compared to mice in the non-exercise group. However, this difference was not statistically significant ($p = 0.738$).

Figure 7. Effect of exercise on the growth of prostate LNCaP tumors in nude mice: All tumor volumes were divided by the initial tumor volumes to get the standardized tumor volume. Data from “HC-Ex” group and “LC-Ex” group were combined to get “Ex” group. Data from “HC-NoEx” group and “LC-NoEx” group were combined to get “NoEx” group. Compared to mice in the non-exercise group, mice in the exercise group had a non-significant trend towards reduced tumor volume. RANOVA analysis revealed that there was no significant difference in tumor volume between the two groups across the treatment period ($P = 0.535$). Error bars represent +/- 1 standard error.
Figure 8. The mean tumor wet weight for exercise and non-exercise groups upon sacrifice: Data from “HC-Ex” group and “LC-Ex” group were combined to get “Ex” group. Date from “HC-NoEx” group and “LC-NoEx” group were combined to get “NoEx” group. Mice in the exercise group had a lower mean tumor wet weight compared to mice in the non-exercise group. Using student’s t-test, we found there was no significant differences in tumor wet weight at sacrifice between the two groups (p = 0.738). The error bars represent +/- 1 standard error.
2) The Metformin Study: Effect of metformin and exercise on tumor growth of mice placed on a high fat-high carbohydrate diet

2.1 Effect of metformin and exercise on body weight gain

Right after tumor inoculation (Day 0), all mice were switched to the high fat – high carbohydrate (HF-HC) diet and the mice were randomized into 4 groups: metformin alone, exercise alone, combination and control. Figure 9 shows the variation in body weight gain over time after the initiation of treatment for the four different groups. Overall, there was no significant difference among various groups (p = 0.549). Compared to control, all the other three groups had a lower mean body weight gain over time, but the difference did not reach significance (p = 0.479, 0.803 and 0.866 for group of metformin, exercise and combination respectively) (Figure 9)

![Figure 9](image.png)

Figure 9. Variation in body weight gain over time for four different treatment groups: Body weight measurements were subtracted by the initial body weight (day 0) for each mice to get body weight gain on different days. Compared to control group, all the other three groups had a non-significant trend towards decreasing body weights. Using RANOVA to assess variation in body weight gain across the whole time period of the study, we found that there was no significant difference among groups (P=0.549). Error bars represent +/- 1 standard error.
2.2 Effect of metformin and exercise on tumor growth

Two weeks after tumor inoculation (Day 0), not all mice had developed tumors. The overall tumor development rate was 85% (34/40). In the control group, 7/10 mice developed tumors. In the metformin group, 10/10 developed tumors, but 1 mouse died, probably from an impact of i.p injection. In the exercise group, 8 out of 10 animals developed tumors and in the combination group, 9/10 developed tumors. Tumor volumes were assessed three times per week and the mean standardized tumor volume in various groups over time is displayed in Figure 10. RANOVA analysis revealed that there were significant differences in tumor volume (p = 0.005). Compared to control mice, mice in both exercise and combination groups had significantly slower tumor development (p = 0.012 and 0.014 respectively). Mice in metformin groups displayed a trend towards slower tumor growth; however, it was not significant (p = 0.685).

Figure 10. Effect of metformin and exercise on the growth of prostate LNCaP tumors in nude mice: All tumor volumes were divided by the initial tumor volume (tumor volumes on day 26, where all mice developed measurable tumors) to get the standardized tumor volume. RANOVA analysis revealed that there was significant difference in tumor volume among the different groups across the treatment period (P = 0.005). Post hoc pairwise comparisons (Tukey’s HSD) revealed that compared to control mice, mice in the “Exercise” and “Combination” groups had significantly slower tumor growth (P = 0.012 and 0.014 respectively). Although mice in “metformin” group had a trend towards reduced tumor volume compared to control mice, the p value was not significant (P = 0.685). Error bars represent +/- 1 standard error.
We measured the tumor wet weight at sacrifice for all mice that developed tumors. Figure 11 displays the mean tumor wet weight for the different groups. Similar to tumor volume, control mice showed the greatest tumor wet weight, followed by the mice in metformin groups. Mice in exercise and combination groups had a smaller mean tumor weight than the other two groups, while mice in exercise group had the smallest tumor weight at sacrifice. However, it has to be noted that there was a fair amount of variation in tumor volume within each group; hence the comparison was not significant under ANOVA analysis (p = 0.184).

Figure 11. The mean tumor wet weight for different treatment groups upon sacrifice: The mean tumor wet weight, from the highest group to the lowest group were “Control”, “Metformin”, “Combination” and “Exercise”. Using ANOVA analysis, we found there was no significant differences in tumor wet weight at sacrifice among the different treatment groups (p = 0.184). The error bars represent +/- 1 standard error.
2.3 Effect of metformin and exercise on tumor necrosis on H&E stained tumor tissue slides

Tumor tissues was extracted, a portion of the tumor taken and fixed in 10\% formalin. Sections were cut, mounted on slides and stained with hematoxylin and eosin. Pathological studies were conducted in a blinded fashion. Stained slides were analyzed to determine the percentage of tumor necrosis. Figure 12 shows the percentage of tumor necrosis in different groups. The pattern is very similar to tumor wet weight. Control groups displayed the highest percent of tumor necrosis, followed by the metformin and combination groups. Exercise group showed the least amount of tumor necrosis as evaluated by a pathologist. Figure 13 depicts representative pictures of tumor necrosis from the various groups.

Figure 12. The mean tumor necrosis for different treatment groups upon sacrifice: The H & E stained tumor tissue slides were scored for the percentage of the tumor necrotic areas on the slides. The mean percentage of tumor necrosis, from the highest group to the lowest group was “Control”, “Metformin”, “Combination” and “Exercise”. Using ANOVA analysis, we found there was significant difference in tumor necrosis among the different treatment groups (p = 0.038). Post hoc pairwise comparisons showed that compared to tumor in control, tumors in exercise group had significantly less area of tumor necrosis (P = 0.05). The error bars represent +/- 1 standard error. (* means P < 0.05)
Figure 13 Histological representation of tumor necrosis after treatment with metformin and exercise: Formalin fixed tumor tissues were cut, mounted on slides and stained with H&E staining. One representative tumor from each group was selected. Hematoxylin stains the nuclei of cells blue and eosin stains other structures in various shades of red or pink. Therefore, pink or red areas represent tumor necrosis.

2.4 Mitogenecity of mouse serum *in vitro*

We hypothesized that metformin and exercise would alter the level of circulating hormones in serum and a consequential effect on tumor development. Therefore, we investigated if serum obtained from mice can alter the growth of human prostate cancer cells *in vitro*. LNCaP cells were grown in media containing 5% mice serum for 72 hrs and cell proliferation was measured by MTS assay as described in Materials and Methods. Mice serum obtained from first (before treatment) and second (during treatment) blood draw were pooled together by group. At sacrifice, blood was obtained from direct heart puncture representing the last blood draw and serum from individual mouse was used to treat LNCaP cells in culture.
Serum obtained from the first blood draw did not alter the growth of LNCaP cells when compared between the various groups (p = 0.777, Figure 14). Pooled serum from the second blood draw, in the exercise group had a significant stimulatory effect on LNCaP cell growth compared to the other three groups (p = 0.012, 0.001 and 0.016 for comparison with metformin, combination and control respectively. Figure 15). LNCaP cells treated with the serum from mice from the final blood draw in the metformin group showed a higher rate of proliferation compared to the other three groups. However, this difference was close to but did not reach significance (p = 0.054. Figure 16).

Figure 14. Serum mitogenicity assay showing the effect of serum from first blood draw on LNCaP cell proliferation. Blood samples were obtained by saphenous vein bleeding before active treatment and serum was pooled within each group. LNCaP cells in culture were treated for 72 hours with appropriate media supplemented with 5% mouse serum. MTS assay and the absorbance at 490 nm was recorded using a 96-well plate reader, which is directly proportional to the number of cells in the media. ANOVA analysis showed that there was no significant difference in LNCaP cell proliferation among different groups (P = 0.777).
Figure 15. Serum mitogenicity assays showing the effect of serum from second blood draw on LNCaP cell proliferation. Blood samples were obtained by saphenous vein bleeding during active treatment, and mice’s blood was pooled together in each group. LNCaP cells were treated for 72 hours with appropriate media supplemented with 5% mouse serum. MTS assay and the absorbance at 490 nm was recorded using a 96-well plate reader, which is directly proportional to the number of cells in the media. ANOVA analysis showed that there was a significant difference in LNCaP cell proliferation among different groups (P = 0.001). Compared to serum from “Exercise” group, serum from all the other three groups (Control, Metformin and Combination) had a reduced proliferative effect on LNCaP cell growth. The p values were 0.016, 0.012 and 0.001 respectively (Tukey’s HSD) (* means P < 0.05)

Figure 16. Serum mitogenicity assays showing the effect of serum from last blood draw on LNCaP cell proliferation. Blood samples were obtained by direct cardiac puncture at sacrifice. LNCaP cells were treated for 72 hours with appropriate media supplemented with 5% mouse serum from each mouse. MTS assay and the absorbance at 490 nm was recorded using a 96-well plate reader, which is directly proportional to the number of cells in the media. ANOVA analysis showed that there was a close to significant difference in LNCaP cell proliferation among different groups (P = 0.054). Compared to serum from “Metformin” group, serum from all the other three groups (Control, Exercise and Combination) had a less proliferative effect on LNCaP cell growth.
2.5 Serum insulin, IGF-1 and C-peptide levels

At the outset it was obvious that both metformin and exercise may influence circulating levels of insulin and IGF-1; therefore, we measured the serum levels of insulin, C-peptide and IGF-1 for each mouse at sacrifice using the ELISA assay.

Compared to serum of the control mice, prolonged treatment with metformin and exercise significantly reduced serum levels of insulin (p = 0.002 and 0.001 respectively, Figure 17). However, there was no significant change in the insulin levels in the combination group compared to control group (Figure 17). Compared to serum of the control mice, the other three groups were characterized with lower mean IGF-1 levels in the serum. However, the differences were not significant among various groups (Figure 18). At sacrifice, serum levels of C-peptide in the metformin and exercise groups were lower compared to C-peptide levels in serum in the Control and Combination groups, similar to the distribution of insulin measurements. Post Hoc comparisons showed that compared to control group, animals in the exercise group showed a significantly lower level of circulating C-peptide (p = 0.018). Serum C-peptide level in the combination groups was also significantly higher than the level in Exercise group (p = 0.039).

We also analyzed the insulin receptor and IGF-1 receptor expression in the tumor lysates. The expression of insulin receptor or IGF-1 receptor was not significantly different among the different groups (p = 0.180 and 0.231 respectively). (Figure 20)
Figure 17. Serum insulin levels at sacrifice for various treatment groups: Serum insulin was measured in duplicate using a rat insulin ELISA kit. ANOVA analysis showed that there was significant difference in serum insulin levels among different treatment groups (p < 0.001). Post Hoc analyses (Tukey’s HSD) showed that serum insulin level in “control” group was significantly higher than “Metformin” and “Exercise” groups (P = 0.004 and 0.001 respectively). Serum insulin level in “Combination” group was also significantly higher than “metformin” and “Exercise” groups (P = 0.002 and 0.001 respectively). (* means P < 0.05)

Figure 18. Serum IGF-1 levels at sacrifice for various treatment groups: Serum IGF-1 was measured in duplicate using a rat IGF-1 ELISA kit. The “Control” group had a higher mean IGF-1 level in the serum compared to the other three groups. However, ANOVA analysis showed that there was no significant difference in serum IGF-1 levels among different treatment groups (p = 0.126).
Figure 19. Serum C-peptide levels at sacrifice for various treatment groups: Serum C-peptide was measured in duplicate using a rat insulin ELISA kit. ANOVA analysis showed that there was significant difference in serum C-peptide levels among different treatment groups (p = 0.012). Post Hoc analyses (Tukey’s HSD) showed that serum C-peptide level in “control” group and “Combination” group was significantly higher than “Exercise” groups (P = 0.018 and 0.039 respectively). (* means P < 0.05)

Figure 20 The expression of insulin receptor and IGF-1 receptor in the tumor lysates. Tumor tissues of three randomly selected mice from each group were analyzed by western blotting. Relative density was measured using Image J. ANOVA analysis showed that there was no significant difference in the expression of insulin receptor (p = 0.180) or IGF-1 receptor (p = 0.231).


Discussion

Prostate cancer (PCa) is expected to be the leading cancer type for the estimated new cancer cases in US men. In 2013, PCa alone will account for 28% of incident cases in men (Siegel, 2013). Although PCa will remain the number one cancer diagnosed in North American men, many prostate tumors are estimated to have a protracted natural history and pose little or low threat to the patients during their lifetime (Dall’Era, 2012). Although one in six men will be diagnosed with PCa during their life span, only one in thirty six will die from the disease. The long latency period of PCa thus provides an opportunity for lifestyle-based interventions such as dietary changes and physical activity.

Androgens are the primary growth factors for growth of the prostatic epithelium. However, other non-androgenic growth factors have also been instrumental in regulating the development of prostate cancer (Cunha, 1987). Insulin and Insulin-like growth factor I (IGF-1) family are associated with various cancer types including PCa and have been investigated intensely since the year of 1900 (Lima, 2009). These hormones are believed to play integral roles across the whole PCa continuum. As discussed in Introduction, elevated serum IGF-1 and insulin are associated with increased PCa risk as well as heightened disease progression. They are also vital in androgen-independent progression of PCa following androgen deprivation therapy (ADT) (So, 2005). One of the proposed mechanisms linking lifestyle intervention and PCa prevention is that, both healthy diets and physical activity decrease the circulating levels of insulin and IGF-1.

Energy balance and dietary factors have been associated with PCa risk and mortality rate (Pollak, 2009). Caloric restriction has been shown to reduce the incidence of prostate cancer. High total
energy intake, independent of body mass index (BMI), is associated with increased risk of fatal 
PCa (Blando, 2011). Most reviews on diet and PCa have suggested that dietary saturated fat is 
linked with higher PCa progression (Meyerhardt, 2010). PCa patients with higher saturated fat 
consumption are associated with higher disease specific mortality and greater biochemical 
recurrence after prostatectomy (Meyer, 1999; Strom, 2008). A high intake of refined 
carbohydrates has been associated with increased risk of PCa (Drake, 2012). Several animal 
stoudies have also demonstrated that consumption of a diet that is low in carbohydrates has a 
protective effect against PCa progression compared to diets containing high levels of 
carbohydrates (Mavropoulos, 2009; Masko, 2010). Alteration in the insulin and IGF-1 axis is one 
of the proposed mechanism through which diet may influence PCa risk and progression (Kaaks, 
2004).

Another important aspect of lifestyle is physical activity. Physical activity/exercise has been 
increasingly recognized to play an important role in the primary prevention of various cancers, 
including PCa (Liu, 2011). It has been shown to be associated with deceased risk of PCa. 
Exercise has also been shown to slow disease progression in patients on active surveillance who 
are diagnosed with low-grade PCa (Ornish, 2005). Exercise intervention has a pleiotropic effect, 
which influences many pathways relating to PCa pathogenesis. Chronic exercise stimulates 
endogenous antioxidant protection, reduces systematic inflammation, improves innate immune 
function and protects from obesity (Antonelli, 2009). For this project specifically, aerobic 
exercise lowers serum levels of several metabolic and sex steroid hormones, including fasting 
insulin and IGF-1 (Barnard, 2003; Barnard, 2007).
As discussed in Introduction, metformin has been found to be associated with reduced cancer risk in type II diabetic patients. The indirect mechanism of metformin is through the alteration of circulating levels of insulin and IGF-1. Therefore, this project aims to investigate the effect of lifestyle intervention (altering dietary carbohydrate or fat content as well as exercise) and metformin use on serum insulin and IGF-1 levels, and to explore how this effect influences prostate progression in a LNCaP xenograft model of PCa.

1) Effect of Low Carbohydrate Diet (LCD) on body weight and food consumption

In order to investigate the effect of dietary carbohydrate on prostate cancer growth, we formulated two special diets that differed in their carbohydrate content (70% vs. 10%). However, we found that the LCD significantly reduced food consumption and reduced body weight of the animals. This was significant enough that they consumed an average of 40% less calories compared to mice placed on a high carbohydrate diet (HCD) with their body weights being reduced by approximately 13% within 10 days. For this reason we modified the experimental design and switched animals on the LCD to a HCD as well, after being on the LCD for 10 days. Low carbohydrate - high protein diets have been frequently adopted by people for short-term weight control (Floegel, 2012). Several trials also showed that obese subjects on carbohydrate-restricted diet lost more weight than on a calorie and fat-restricted diet (Samaha, 2003; Sacks, 2009), which has been shown to be associated with significant increase in insulin sensitivity (Mehrabani, 2012). Our study has been able to confirm these findings wherein animals on a low carbohydrate-high protein diet lost significant body weight. However, this weight loss was coupled with significantly reduced food consumption in mice, which should be considered when using such a diet for weight control.
Ho et al. (2011) investigated the effect of low carbohydrate-high protein diets on tumor initiation and growth in colon cancer xenografts. Their use of a 10% carbohydrate diet (10% carbohydrate and 64% protein) is very close in composition to the LCD used in our present study. However, they observed that mice on this diet consumed the same amount of food compared to control mice fed on a ‘Western’ diet. Mice on the 10% carbohydrate diet also gained weight throughout the study, although their body weights were slightly (7%) lower than control mice. Our observation on the loss of weight of animals on the LCD was much more severe than any previously reported. This could be a potential consequence of variation in the mice strain or models used in our study compared to earlier published studies (nude mice vs. Rag2M mice) or due to the type of carbohydrate used for the dietary composition (dextrin vs. amylose).

The feeding of a LCD for 10 days can also be viewed as a short-term calorie restriction, for mice on LCD only consumed 60% of calorie compared to the mice fed on HCD. As stated earlier mice fed a LCD lost an average of 13% of their body weights within 10 days, which was consistent with many animal studies that have shown that short-term food restriction can effectively reduce body weights (Grasl-Kraupp, 1994; Sylvester, 1981; Zhu, 2002). After the mice were switched to HCD, they started to consume more food and their body weights increased very rapidly. Despite the 10 days of food restriction, animals on the low carbohydrate-no exercise group (LC-NoEx) showed the highest food consumption across the entire study period. However, their average body weights were lower than the mice in the HCD groups. This indicates that short-term energy restriction may hold a long-term effect on reducing body weights. This observation was not consistent with the other studies, which have shown that after the short-term food restriction, when animals were placed on *ad libitum* food intake, they would gradually reach body weights
equal to that of the control animals (Sylveste, 1981; Zhu, 2002). This difference is likely due to the different animal model systems under study.

2) Effect of Exercise on Food Consumption and Body Weights

Our results showed that exercise has the potential to reduce food consumption and thereby result in a reduction in the body weights of the animals. As shown in Figure 6 in Results, exercising animals consumed significantly less food compared to non-exercising animals. Body weight gain of exercising animals was much lower than non-exercising animals (Figure 5). The observations from previously published work investigating the role of exercise and cancer are in line with our present result (Lane, 1991; Thorling, 1994). Thorling et al (1994) found that moderate exercise i.e., running (2 km/day, 5 days/week, 38 weeks) reduced calorie intake among rats and the body weight gain with exercising male rats was slower in these animals, than in sedentary reference rats. Lane et al (1991) showed that moderate exercise (60 min/day, 5 days/week, 34 weeks) significantly reduced food consumption coupled with a non-significant reduction in body weight gain, which was very similar to our observed results. However, some other experiments have conflicting results (Thompson, 1988; Colbert, 2000; Vandersluis, 2013). Thompson et al (1988) observed that moderate exercise (15 min/day; 15 days/week; 18 week) stimulated calorie intake and exercising animals weighted more. Similarly, Vandersluis et al (2013) showed that sustained aerobic exercise (45 min/day, 3 days/week, 8 weeks) increased animal food consumption, however, significant alterations in body weight gain was not observed. Lastly, Colbert et al (2000) exercised animals for 7 weeks (10-18 m/min, 15-60 min/day) and did not observe any effect on body weight or food intake.
The effect of exercise on food consumption and body weights can be influenced by several factors. First of all, exercise regimens varied amongst studies; exercise intensity, frequency and duration may all have an additive impact on outcome. Secondly, the diets used in different studies are not the same and many are specially formulated. Given that the exercise regimen and administered diets are totally different, exercise may or may not have an influence on food intake. Thirdly, even when food intake was altered, this may or may not be reflected in body weight changes. Different models and differences in gender may have an influence. For example, given the same conditions, female rats were capable of maintaining body weights during exercise as opposed to male rats, whose body weight gain was slower (Thorling, 1994).

3) Effect of LCD on tumor progression

Mice that were initially placed on LCD showed a non-significant trend towards reduced tumor volume, even though they were only on this diet for a short duration of 10 days. More than 80 years ago, Otto Warburg discovered that cancer cells rely more on glycolysis than oxidative phosphorylation to generate energy even when enough oxygen is present (Warburg, 1927). Because glycolysis is less efficient in generating ATP, cancer cells require higher glucose levels to meet their high rate of proliferation (Ho, 2011). Thus, whether a diet low in carbohydrate content could sufficiently reduce tumor growth is a very intriguing question. Several animal studies have demonstrated the beneficial effects of a LCD on slowing tumor progression. Ho et al (2011) showed that several formulated low carbohydrate high protein diets (8% carb or 10% carb or 15% carb) (see table 1) all slowed tumor progression in a mouse xenograft model compared to a ‘Western’ diet (55% carb). This effect was also coupled with lower glucose, insulin and lactate levels. Other studies investigated the effect of several low carbohydrate high
fat diets (0% carb or 10% carb or 20% carb) on cancer progression in murine prostate cancer xenografts (Masko, 2010; Mavropouls, 2009; Freedland, 2008). Their results showed that these diets significantly reduced the growth of xenograft tumors and prolonged animal survival compared to mice administered a ‘Western’ diet. Meanwhile, mice on low carbohydrate high fat diets were characterized with lower serum insulin, IGF-1 and higher IGF-1 binding proteins. Using athymic nude mice to obtain prostate cancer xenografts, Venkateswaran et al (2007) also demonstrated that compared to mice on a high-carbohydrate diet, mice placed on a low-carbohydrate diet experienced slower tumor growth which was associated with decreased serum insulin and IGF-1 levels. Our current results are in line with these observations. However, we are unable to provide any additional evidence on the benefit of low carbohydrate diet due to lack of significance.

**Table 1** Composition of Different Low-Carbohydrate Diets

<table>
<thead>
<tr>
<th>Investigator (Year)</th>
<th>Carbohydrate %</th>
<th>Fat %</th>
<th>Protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ho, 2011</td>
<td>8</td>
<td>22.6</td>
<td>69.4</td>
</tr>
<tr>
<td></td>
<td>10.6</td>
<td>25.9</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>26.2</td>
<td>58.2</td>
</tr>
<tr>
<td>Masko, 2010</td>
<td>10.5</td>
<td>73.6</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>20.2</td>
<td>64</td>
<td>15.8</td>
</tr>
<tr>
<td>Mavropouls, 2009 &amp; Freedland, 2008</td>
<td>0</td>
<td>82.8</td>
<td>17.2</td>
</tr>
<tr>
<td>Venkateswaran, 2007</td>
<td>10</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Western Diet, present study</td>
<td>44.3</td>
<td>40</td>
<td>15.7</td>
</tr>
</tbody>
</table>
As discussed before, the 10 days of LCD feeding was also a short-term calorie restriction, which could contribute to tumor growth inhibition. Dietary restriction has been found to be a very robust intervention for extension of life span (Klebanov, 2007). Many caloric restriction regimens almost uniformly extend life span in different model systems, from yeast to non-human primates (Kemnitz, 2011). A major contributor to the anti-aging effect of calorie restriction is believed to be the inhibition of spontaneous carcinogenesis (Klebanov, 2007). Using animal models a few studies have looked at the effect of dietary restriction on the development of prostate cancer. Some studies have showed that calorie restriction delayed tumor incidence in mouse models (Pollard, 1989; Bonorden, 2009; Blando, 2011; Buschemeyer, 2010). For instance, Blando et al (2011) demonstrated that 30% calorie restriction significantly reduced the incidence of in situ adenocarcinomas at 3 months and 6 months compared with overweight controls in male Hi-Myc mice. This was associated with reduced Akt/mTOR signaling, which suggested serum insulin and IGF-1 axis mediated the effect of calorie restriction. However, a few other investigators were unable to observe such beneficial effects of food restriction (Thomas, 2010; Huffman, 2007; McCormick, 2007). It has to be noted that the degree and the duration of dietary restriction were different in these studies, which may in part explain the inconsistent findings. No studies have until now investigated the long-term effect of short-term energy restriction on the growth of prostate cancer. Although in our present study we did not see a significant reduction in tumor volume, the trend may provide a rationale that warrants further investigation.

4) Effect of Metformin and Exercise on Animal Body Weights

Accessing the results of our Metformin Study, we found that there was no significant difference in body weight gain among the various groups. However, compared to control mice, mice in all
the other groups had a non-significant trend towards a reduction in body weight gain. In humans, metformin intake leads to weight loss in patients either due to suppression of appetite or gastrointestinal distress (Kirphichnikov 2002). Many animal studies have also shown that metformin administration reduces food consumption, which may or may not cause a significant reduction in body weight gain (Algire, 2008; Anisimov, 2005; Fonseca, 2011; Hou, 2010; Schneider, 2001). For example, Anisimov, et al (2005) showed that chronic treatment of female transgenic HER-2/neu mice with metformin (100 mg/kg b.w. in drinking water) decreased energy consumption of these animals, but failed to reduce body weight gain. Similarly, in another study, 6 weeks of metformin treatment (50 mg/kg b.w. in drinking water) did not cause a significant reduction in body weight gain in mice (Algire, 2008). However, Borst et al (2011) showed that metformin administration (320 mg/kg b.w. in drinking water) significantly reduced both food intake and animal body weight gain. Similarly, Schneider et al (2001) showed hamsters administered metformin for 2 weeks (320 mg/kg b.w.) consumed lesser amount of food and water, and consequently had a lower weight gain. Hou et al (2010) administered metformin to CD 1 mice with three different doses: 50, 100 and 250 mg/kg b.w. in drinking water. Metformin at all three doses significantly reduced food and water consumption, but only a dose at 250 mg/kg showed a significant reduction in body weight gain. These results are suggestive of the fact that the dosage of metformin may have a significant influence on altering body weights.

As discussed above, exercise may also reduce food consumption thereby decreasing body weight of animals. In our study, however, the combination of exercise and metformin did not cause a further decrease in animal body weights. This observation was not consistent with Borst et al, (2011), who showed that metformin and exercise training in combination caused a greater
inhibition of weight gain than did either treatment alone. Not many studies have investigated the long-term combined effect of metformin and exercise. Hence, more studies need to be conducted with a larger sample size to fully understand this issue.

5) Effect of Metformin on Prostate Tumor Development

We established that 10 weeks of metformin treatment (50 mg/kg b.w., 3 times per week) did not alter tumor growth significantly compared to control in the LNCaP xenograft model of prostate cancer. However, mice in the group that was administered metformin alone experienced a non-significant trend toward reduction in tumor growth (p = 0.685). Many in vitro and in vivo studies have looked at the effect of metformin treatment on prostate cancer progression. Metformin has been found to consistently inhibit the proliferation of several prostate cancer cells lines, such as LNCaP, PC-3 and DU-145 (Colquhoun, 2012; Ben Sahra, 2008; Ben Sahra, 2010). However, this inhibitory effect of metformin may or may not be recapitulated in in vivo studies. Colquhoun et al (2012) showed that millimolar doses of metformin caused a significant dose-dependent reduction in the clonogenicity of LNCaP and PC3 cells. Subsequent in vivo experiments demonstrated that although treatment with metformin significantly reduced IGF-1 levels in comparison with untreated control mice, metformin treatment alone (50 mg/kg b.w.) did not significantly impact tumor growth. Our results also demonstrated that metformin significantly reduced circulating insulin and non-significantly reduced circulating IGF-1 levels, but didn’t cause a significant reduction in tumor volume over time. However, Ben Sahra et al (2008), established that metformin not only inhibited the proliferation of DU-145, PC-3 and LNCaP cancer cells with a 50% decrease of cell viability, but also reduced tumor volume in vivo. The same group also demonstrated that oral (200 ug/mL water) and intraperitoneal treatment with
metformin (1 mg per day) in mice bearing xenografts from LNCaP cells for 5 weeks resulted in a 50% and 35% reduction of tumor volume, respectively. However, these results are inconsistent with our findings because LNCaP tumors were induced in female nude mice, these animals potentially bear a different hormonal background compared to male mice. Also, due to the small number of animals in each group (5/group) they were unable to arrive at a statistically significant difference. In addition, the dose of metformin used was different compared to the dosage use in our present study (50 mg/Kg b.w.). Among the several factors that may contribute to variability in results, the type of cells under study plays a major role. Hirch et al (2013) showed that 9 weeks of administration of metformin (200 ug/mL drinking water) significantly slowed the growth of DU-145 xenograft tumors, but had little effect on the growth of LNCaP xenograft tumors. Lastly, a study conducted with 12 weeks of metformin treatment (750 mg/Kg b.w., in drinking water) reduced systemic IGF-1 levels, and significantly improved tumorigenic progression in a breast cancer xenograft model (Phoenix, 2009). Taken together our observation suggest that although metformin can systematically reduce serum insulin or IGF-1 levels, this is not sufficient to inhibit tumor growth in a xenograft model. Other mechanisms, such as the inflammatory pathway (Hirch, 2013) or angiogenesis (Phoenix, 2009) may also have a role to play. Further investigation is needed to fully understand the mechanism underlying metformin treatment on tumor progression.

6) Effect of Exercise on Prostate Tumor Progression

Exercise has been associated with reduced risk as well as reduction in disease progression of various types of cancers, including prostate cancer. For example, a recent meta-analysis investigated 19 eligible cohort studies and 24 eligible case-control studies and found that total
physical activity was significantly associated with a small decreased risk of prostate cancer (Liu, 2011). Kenfield et al. (2011) performed an observational study to determine whether higher physical activity after prostate cancer diagnosis decreases risk of overall and prostate specific death. They found that physically active men had lower risk of overall as well as prostate specific mortality. In addition, men with more than 3 hours per week of vigorous activity had a 61% lower risk of prostate cancer death compared with men with less than 1 hour per week of vigorous activity. One prospective study examined physical activity after prostate cancer diagnosis in relation to risk of prostate cancer progression. They found that men who walked briskly for more than 3 hours per week had a 57% lower rate of disease progression compared to men who walked at an easy pace for less than 3 hours per week (Richman, 2011).

In our present study, we are trying to discern the relationship between exercise and prostate cancer progression using the LNCaP xenograft model of prostate cancer. Our results from the Metformin Study showed that ten weeks of exercise (3 times/ per week) significantly inhibited prostate tumor growth, which was associated with reduced insulin levels in serum. However, the Carb Study showed that 11 weeks of exercise (5 times/week) did not significantly slow tumor growth compared to control mice. Different factors may explain the divergent results. Firstly, the background diet was different. In the Carb Study, the diet used was a high carbohydrate diet and in the Metformin study, the background diet was a high carbohydrate-high fat diet. Secondly, exercise regimens were different. In the Carb Study, the exercise was 5 days/week, and lasted for 11 weeks, with an average intensity being 7.5 m/min. The exercise in the Metformin Study was 3 days/week, for only 10 weeks, and was less intense with an average intensity of 4.5 m/min.
Studies on exercise and prostate cancer progression in animal models conducted by Zheng et al (2008) investigated the effect of voluntary exercise on the formation and growth of human PC-3 tumors in male severe combined immunodeficient (SCID) mice. Exercise treatment was commenced one week before the subcutaneous injection of tumor cells. Results revealed that 9 weeks of voluntary running wheel exercise significantly suppressed the growth of PC-3 tumors. Further mechanistic studies showed that exercise inhibited proliferation and stimulated apoptosis in PC-3 tumors. In a second study by Esser et al in 2009, the effect of voluntary wheel running on prostate cancer growth in C3(1) Tag mice that are predisposed to prostate cancer was investigated. It was found that 83% of the dorsolateral prostates were classified normal for mice that ran greater than 5 km/day, and only 43% were classified normal for the mice running less than 5 km/day. In addition, there was a relationship between average running distance and pathologic progression to high-grade PIN and local invasion. Also, none of the dorsolateral prostates from mice that ran more than 5 km/day was classified with advanced pathology as compared to 43% in mice that ran less than 5 km/day.

More recently, Jones et al (2012) studied the effect of exercise on an orthotopic model of murine prostate cancer. They injected transgenic adenocarcinoma of mouse prostate C-1 cells orthotopically in C57BL/6 male mice and the exercise group was given voluntary access to a spinwheel 24 hours/day. They found that 8 weeks of voluntary exercise did not significantly alter primary tumor growth rate between the exercise and control groups. However, exercise was associated with improved tumor vascularization with a shift toward suppressed metastasis. This finding was very similar to a previous study where long-term voluntary wheel running significantly improved blood perfusion compared to their sedentary control (Jones, 2010).
From our results and other published work, the effect of exercise on prostate cancer progression is not conclusive. It is evident that there is a plethora of different factors that may contribute to the beneficial and/or added effect of exercise, such as differences in diets, time of exercise initiation and model systems. In addition, different exercise regimens may also have an influence, such as different style (forced or voluntary), duration, intensity and frequency.

In the Metformin Study, exercise significantly decreased tumor growth, which was coupled with decreased insulin and IGF-1 levels. However, this needs to be viewed with caution because in the metformin alone group, the decreased level of circulating insulin was not associated with reduced tumor growth. Exercise may exert a beneficial effects on reducing tumor volume by several different mechanistic changes, including modulation of circulating host levels of hormones (insulin, IGF-1, androgen, leptin, etc), improvements in immune surveillance, reduced systemic inflammation and improved host anti-oxidative defense (McTiernan, 2008). Hence, it is likely that the beneficial effect of exercise seen in our metformin study is potentially multifactorial.

Our results from serum mitogenicity assay showed that serum from exercising mice did not significantly reduce the proliferation of LNCaP cells in vitro. This finding is not consistent with a previously reported study, where serum from men with regular aerobic exercise (5 days/week) reduced LNCaP cell proliferation in vitro and induced tumor cell apoptosis. The inhibitory effect observed in the study was associated with a reduced level of insulin and IGF-1 in the serum from exercising men (Barnard, 2003; Barnard, 2007). In our present mitogenicity study, we used
growth medium containing 5% mouse serum, compared to medium containing 10% human serum observed by Barnard et al (2007). Also, Barnard demonstrated that exercising men participated in the exercise program for at least 10 years, which consists of 40-45 min of continuous, strenuous exercise, 5 days/week. Our present study utilizing a mouse model of prostate cancer clearly utilizes a much shorter and less intense exercise program. To further illustrate the effect of exercise on the alterations of host serum and its subsequent effects on tumor cell growth, more studies on the underlying mechanisms need to be executed.

7) The Combined Effect of Metformin and Exercise

Since Metformin and Exercise can both alter the circulating levels of insulin and IGF-1, we hypothesized that the combination of metformin and exercise will cause a further decrease in both insulin and IGF-1 levels to a greater extent than either monotherapy. Such a combination would likely be reflected by a slower tumor growth in the LNCaP xenograft model. However, our results did not support the combinatorial effect of metformin and exercise. Exercise and metformin independently reduced serum levels of insulin and non-significantly reduced IGF-1 levels; however, the combination did not appear to have an enhanced effect. Exercise alone significantly reduced tumor development over time; however, the combination of exercise and metformin caused no further slowing down of tumor growth compared with exercise treatment. Since metformin alone did not slow tumor growth significantly, these results suggest that exercise alone but not metformin exert an inhibitory effect on tumor growth, which can in part, but not fully be explained by alterations in serum insulin and IGF-1 levels.

A few studies have investigated the combined effect of metformin and exercise and their results are supportive to our finding. Malin et al (2012) found that exercise alone, metformin alone or
the combination increased insulin sensitivity relative to the control group. However the highest mean rise in insulin sensitivity was observed in exercise alone group, which suggested that the addition of metformin blunted the full effect of exercise training. Similarly, a second study by Sharoff et al (2010) found that short-term metformin treatment and an acute bout of exercise did not enhance insulin sensitivity, and the addition of metformin may attenuate the effects of exercise. A third study also found that swimming training and metformin therapy independently decreased serum glucose levels in db/db mice, and these effects were not synergistic (Tang, 2001). As previously discussed, metformin is a mild inhibitor of Complex I in the mitochondrial electron transport chain and, therefore, it is possible that metformin constrains the cellular adaptations to exercise training, which might be an explanation for the non-synergistic effects of the combination (Malin, 2012). On the other hand, exercise influences the disposition and pharmacokinetics of metformin, which reduces metformin serum levels (Chien, 2012). This may impact the full effect of metformin.

Other studies suggest possible metabolic benefits from the combined therapy. Smith et al (2007) found that exercise and metformin produced additive effects on GLUT4 protein expression in skeletal muscle of Zucker diabetic fatty rats. Jenkins et al (2013) showed that exercise training and metformin had additive influences on adipose tissue secretion, where leptin secretion was reduced and IL-10 was increased. More studies need to be carried out to gain a better understanding of this combinatorial therapy.
Conclusion

In the Carbohydrate Study utilizing diets varying in carbohydrate content, we found that the low carbohydrate diet (LCD) significantly reduced food consumption using the nude mice xenograft model of prostate cancer (PCa), to an extent that this diet was not tolerable to the mice. As a consequence, animal body weights also dropped significantly. Although the mice were only on this diet for 10 days, the short period of calorie restriction had a long-term effect on reducing animal body weights and was comparable to the results in mice that had 11 weeks of exercise (5 days/week). The mice in this group also had reduced food consumption and body weight gain. Mice that were initially placed on LCD showed a non-significant trend towards reduced tumor volume compared to mice in the group that consumed the high carbohydrate diet (HCD) throughout the study. Whether this observation is due to the low carbohydrate content or a lower calorie intake warrants further investigation.

In the Metformin Study, treatment with 10 weeks of metformin (50 mg/kg b.w., 3 days/week) did not alter animal food consumption significantly and there was no significant effect on animal body weight gain. We also found that 10 weeks of metformin treatment did not alter the tumor growth rate significantly compared to control mice in the LNCaP xenograft model, despite the fact that metformin reduced serum insulin levels significantly. Further experiments need to be carried out to fully understand the mechanisms underlying metformin treatment in vivo. Our results, together with the inconsistent observations in other studies, suggest that further investigation is needed for metformin as a mono-therapy to treat PCa.
Our results from “The Metformin Study” showed that ten weeks of exercise (3 times/week) significantly inhibited prostate tumor growth; however, the use of varying carbohydrate content from the Carbohydrate Study showed that 11 weeks of exercise (5 times/week) did not significantly slow tumor growth compared to control mice. These results suggest that a few things need to be considered while investigating the relationship between exercise and prostate cancer progression, including the background diet, length of exercise, as well as the intensity and frequency of exercise. The effect of exercise on prostate cancer progression remains inconclusive.

Finally, exercise alone and metformin alone significantly reduced serum levels of insulin and non-significantly reduced IGF-1 levels; however, the combination did not have a greater effect. Thus, our results do not support the combination effect of metformin and exercise. It is possible that metformin restrains the cellular adaptations to exercise training, which might be a possible explanation for the non-additive effects observed in the combination treatment.
Future Directions

1) Improve the Experimental Design

In the Carbohydrate Study, the formulated low carbohydrate diet (LCD) was not tolerable to the nude mice. Mice placed on this diet refused to eat the food and their body weights dropped very quickly. To solve this issue, two methods could be incorporated. First, increase the carbohydrate content and decrease the protein content of the LCD slightly. For example, from 10% carbohydrate 70% protein to 15% carbohydrate 65% protein. In this way, there would still be a large gap in carbohydrate content between the two diets, and the diet could be more tolerable to the mice. Secondly, mix the LCD with normal diet at first to let mice get used to the diet. In this experiment, we changed the diet completely in one day and the mice may need more time to adjust to the treatment food. If the experiment was carried out again, we could mix the LCD with normal food and slightly increased the percentage of LCD in the mixture over time. In this way, mice might be able to get used to the food before tumor inoculation.

In the Metformin Study, the formulated high fat-high carbohydrate (HF-HC) diet was very soft and when mice ate the food, the food became powders and fell into the cage, which made the food measurement very inaccurate and was not discussed in this experiment. To solve this problem, better food packaging to allow more firmly pellet formation is needed.

In both experiments, we observed several non-significant trends in body weights, tumor volume over time, and serum hormone levels, which indicates that both experiments might be underpowered. In order to get better results, more animals per group might be needed, such as 20
mice per group. Also, it might be better to look at one factor at a time. In the Carbohydrate Study, we investigated the effect of dietary carbohydrate and exercise at the same time. In the Metformin Study, we looked at the effect of metformin and exercise at the same time. It could be better to investigate one factor at a time to have a better understanding of the specific effect and mechanism, and then study the combination effects.

2) Diet and Prostate Cancer

A comparison of the patterns of incidence and mortality across populations can provide clues about the role of dietary factors in PCa etiology. Dietary changes are appealing as a means of disease prevention as well as intervention post-diagnosis to improve clinical course. Most studies indicate that dietary fat and carbohydrate intake is associated with altered prostate cancer risk and disease progression. As we discussed in this study, elevated circulating insulin and IGF-1 levels could offer a possible explanation. However, the exact mechanisms underlying these complicated interactions of diet and prostate health still demands a clear explanation. In future studies, another interesting question to look at is how the obesity related cytokines are related to prostate health, since obesity in adulthood has been associated with worst PCa outcomes in most studies. Some potential adipokines are adiponectin, leptin, vasfatin (Luhn, 2013) and resistin (Kim, 2011).

Another interesting question to ask would be what type of fat and carbohydrate is related to prostate cancer risk and progression. Heber et al (2002) proposed that a low amount of fruits and vegetables and a high amount of energy from animal fats in Western Diet, might cause the higher risk of benign prostatic hyperplasia (BPH) in Western Counties. Very recently Richman et al
(2013) published an interesting epidemiological study. They examined post-diagnostic fat intake in relation to lethal prostate cancer and all-cause mortality. They found that among men with non-metastatic PCa, replacing carbohydrate and animal fat with vegetable fat could reduce the risk of all-cause mortality. The benefit of vegetable fat for prostate cancer progression could be a future question to ask.

Studies have shown that consumption of polyunsaturated fatty acids modulates the development and progression of PCa. Although omega-3 and omega-6 are both essential fatty acids, high amounts of omega-6 fatty acids have been linked with increased prostate cancer risk (Hughes-Fulford, 2006), whereas omega-3 fatty acids have been shown to inhibit PCa growth (Akinsete, 2012). Furthermore, diet with a low ratio of omega-6 to omega-3 fatty acids is associated with reduced risk of prostate cancer, such as the Asian diet. A recent study has shown that a low omega-6 to omega-3 fatty acids ratio can delay the progression of LNCaP cells towards castration resistance by suppressing pathways involves in PCa progression, such as the Akt/mTOR pathway (Apte, 2013). The effect of type of fat and the ratio of dietary components on prostate health merits further research.

Micronutrients may also play a role in PCa risk and disease outcome. Calcium intake has been consistently associated with risk of advanced or fatal prostate cancer in epidemiologic studies (Tseng, 2005). Our body needs vitamin D to absorb calcium. There is evidence that vitamin D also plays a role in PCa progression. For example, genetic variants of vitamin D receptors are associated with Gleason score (Chen, 2009), risk of recurrence and PCa specific mortality (Holt, 2010). Vitamin E intake has also been associated with reduced risk of PCa. A trial in Finland
revealed that those assigned to alpha tocopherol supplementation experienced a 40% decrease in PCa risk (heinone, 1998). Selenium is another micronutrient that has been studied for the prevention of PCa. A large case control study, comparing serum selenium levels in subjects, showed a 30% decrease in PCa risk at higher selenium levels (Vogt, 2003). Another clinical trial revealed a statistically significant, 50% decrease in PCa risk among subjects assigned to selenium supplementation (Duffield-Lillico, 2003). Some other natural compounds generated interests are isothiocyanates, compounds found in cruciferous vegetables (Kristal, 2004); capsaicin, from chili peppers (Sanchez, 2006); and lycopene, the chief carotenoid in tomatoes (Giovannucci, 1995). These compounds may all exert potentials for prevention of PCa. The large randomized, placebo-controlled trial, Selenium and Vitamin E Cancer Prevention trial (SELECT), did not show any beneficial effect of selenium or Vitamin E in prevention of PCa. However, I think it is not likely that one or two of these micronutrients can have a profound effect on risk of PCa, but rather a precise combination of them may exert the effect. Thus, to study a panel of micronutrients on prostate health might be a further approach.

3) Exercise and Prostate Cancer

Physical activity/exercise has potential beneficial effects for patients along the PCa continuum (Antonelli, 2009). First of all, exercise has been inconsistently associated with decreased risk of PCa, with about 40% of the conducted studies indicating a protective effect of exercise (Rebillard, 2013). Secondly, exercise can be incorporated as a comprehensive lifestyle intervention during PCa therapy. For active surveillance, the Ornish group has shown that exercise as part of a lifestyle modification program (consists moderate aerobic exercise, vegan diet and stress management) has a beneficial effect for patients with a low-grade PCa, as
evidenced by decline in serum PSA and reduction in disease progression (Ornish, 2005; Frattaroli, 2008; Ornish, 2008). More recently, Burton et al (2012) also showed that PSA at age 50 years was 2.1% lower per unit increase in weighted exercise score in active surveillance patients. Exercise also reduces comorbidity and improves quality of life (QOL) after surgical treatment (Antonelli, 2009). For patients who have a history of engaging in physical activity, they have a reduction in short-term morbidity and mortality associated with prostatectomy (Warburton, 2006). There is also evidence suggests that intensive exercise training interventions in the acute setting before surgery is also beneficial (Jones, 2007). Exercise also improves QOL after prostatectomy, such as reducing post-prostatectomy erectile dysfunction (ED) and incontinence (Park, 2012). Furthermore, exercise shows beneficial effects for men undergoing concomitant androgen deprivation therapy (ADT) or radiation therapy, including increased body strength, decreased fatigue, higher aerobic fitness and increased QOL measurements (Segal, 2003; Windsor, 2004). Although the underlying mechanisms for the favorable effects of exercise are still poorly understood, above evidences suggest that exercise should be actively applied to the management of PCa.

These results demonstrate a link between exercise and PCa. However, the question of what activity parameters (in terms of type, intensity, frequency, and time) are most beneficial for PCa reduction remains unanswered. The inconsistent effects of exercise observed in our two different experiments also suggest the importance of this question. To design an experiment aiming to find the perfect exercise parameter is not easy, however, a detailed systematic review of previous clinical as well as animal studies should give us clues on how to design the study.
It is also imperative to determine the molecular mechanisms involved in the beneficial effects of physical activity. During exercise, humoral factors are released from working muscles for endocrical signaling to other organs. The serum level of myokines drops quickly after exercise and may have a role in cancer protection (Hojman, 2011). These myokines may offer an explanation for why our serum mitogenecity assay did not capture the full humoral effects of exercise, for the serum were not obtained right after an exercise bout. Hojman et al (2011) showed that oncostatin M (OSM), an exercise induced myokine, inhibited breast cancer cell proliferation, increased caspase activity and induced cancer cell apoptosis. Another interesting myokine to look at is interleukin 6 (IL-6). Although high serum concentration of IL-6 is associated with negative prognosis in various cancer types (Lippitz, 2013), contracting human skeletal muscles are found to produce IL-6 during exercise (Ostrowski, 1998; Steensberg, 2000). Thus, how exercise induced myokines influence cancer biology is an intriguing future research question.

4) Metformin and PCa

Recent understanding of cancer cell heterogeneity in tumors has led to the concept of cancer stem cells (CSCs). CSCs are distinguished by some major properties that include, self-renewing ability by asymmetric division, ability to differentiate into diverse phenotypes, ability to initiate tumors from minute numbers and high chemo-resistance (Rattan, 2012). It is believed that CSCs resist chemotherapeutic drugs and can regenerate the various cell types in the tumor, thereby causing relapse of many cancers (Hirsch, 2009). Recently, metformin has been shown to selectively target CSCs. Hirsch et al (2009) showed that low doses of metformin (<0.2 mmol/L) inhibits cellular transformation and selectively targets CSCs in various breast cancer cell lines.
They also showed that the combination of metformin and doxorubicin killed both CSCs and non-stem cancer cells \textit{in vitro} and this combination prevented the relapse of tumor more effectively than either drug alone in mice xenograft model. Similarly, metformin synergistically interacts with the anti-HER2 monoclonal antibody Trastuzumab, and suppresses self-renewal and proliferation of CSCs in HER-2 positive carcinomas (Vazquez-Martin, 2010). More recently, low concentrations of metformin (<0.2 mmol/L) were found to selectively inhibit the proliferation of CD133\textsuperscript{+} pancreatic cancer cells, which considered being pancreatic CSCs (Gou, 2013). One proposed mechanism of this effect of metformin is that metformin induces metabolic stress, which triggers a signal transduction pathway that inhibits NF-kB and its subsequent inflammatory responses (Hirsch, 2013). Therefore, an interesting future research is to see how metformin influences CSCs in PCa.

Metformin’s primary activity is the inhibition of complex I of the mitochondrial electron-transport chain. This inhibition of oxidative phosphorylation leads to lower ATP levels and reprogramming of cellular energy metabolism in favor of conserving energy and restoring ATP levels (Pollak, 2012). Furthermore, when mitochondrial respiration is impaired by metformin, cancer cells compensate by boosting glycolysis to improve bioenergetics (Choi, 2013). Therefore, cancer cells that have lost these compensation pathways are more sensitive to metformin (Buzzai, 2007) and targeting these compensation systems together with metformin administration could be more effective. For instance, a study found that metformin decreased glucose oxidation and increased dependency on reductive glutamine metabolism in cancer cells. The inhibition of glutamine anaplerosis in the presence of metformin further attenuated cancer cell proliferation (Fendt, 2013). In another report, metformin worked synergistically with 2-deoxyglucose (2-DG,
hampers cell glycolysis) to impair survival of pTEN deficient lymphoma cells (Rosilio, 2013). Leukemic cells with high basal Akt phosphorylation, glucose consumption or glycolysis were found not to exhibit a markedly reduction in proliferation in response to metformin and were resistant to metformin-induced apoptosis. However, glucose starvation or treatment with 2-DG or an Akt inhibitor induced sensitivity to metformin treatment (Scotland, 2013). Therefore, interfering with energy compensation pathways in response to metformin may be an interesting research, which may synergize with metformin induced energy stress and improve therapeutic effects of this drug.


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