

The role of diet, capsaicin and TRPV6 expression in the prostate gland

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Table of Contents

Abstract	3
Keywords.....	4
Declaration	5
List of Abbreviations:	6
List of Tables:.....	7
List of Figures:	7
Chapter 1: Introduction and literature review.....	8
1.1 Prostate cancer incidence and mortality	8
1.2 Prostate gland biology	9
1.3 Prostate cancer tumourigenesis	10
1.3 Genetic changes driving the development of prostate cancer.....	16
1.4 Impact of diet on prostate cancer development.....	18
1.5 Diet and inflammation	20
1.6 Inflammation and prostate cancer	20
1.7 Phytochemicals and cancer prevention.....	22
1.8 Capsaicin	24
Knowledge gaps and future directions	27
Project hypothesis:.....	29
Project aims:.....	29
Chapter 2: Materials and Methods.....	30
2.1 Animal Protocol.....	30
2.2 RNA extraction	30
2.3 Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)	30
2.4 Western Blotting	31
2.5 Immunohistochemistry	32
2.6 Statistical analysis	33
Chapter 3: Results	34
3.2 Gene expression in prostatic tissue	34
3.3 Prostate Histology.....	40
3.4 Western Blot	41
Chapter 4: Discussion	42
Chapter 5: Conclusion	49
References.....	50

Abstract

Prostate cancer (PCa) is the second most common cancer in men worldwide, however the prevalence in developed countries is significantly higher than in developing countries. In Australia prostate cancer is expected to be the most common cancer diagnosed in 2017, as one in five men are at risk of developing prostate cancer before 85 years of age. While the five-year survival for prostate cancer is at 95%, men in rural or regional areas have a 20% lower survival rate than those residing in the major cities. The high rate of diagnosis in developed countries, including Australia, has been linked to lifestyle, specifically diet. The Western diet is typically high in refined carbohydrates, fat and red meat and this diet has been shown to cause inflammation in various tissues. Inflammation in the prostate gland has been linked to the development of precancerous states and eventually cancer. Furthermore, alterations in gene expression pathways in the prostate in response to the inflammatory state are also associated with cancer progression. Capsaicin, a dietary phytochemical from chilli, has been shown to have anti-cancer and anti-inflammatory properties. The effectiveness of capsaicin dietary supplementation in preventing or reducing the inflammation and cancer-causing gene expression changes induced by a high carbohydrate and high fat diet has not been explored in the prostate gland. If effective, this may represent an inexpensive mechanism for men, living in both cities and in regional areas to decrease their risk of developing prostate cancer by supplementing their diet with capsaicin.

This study investigated the effect of a Western diet and the addition of capsaicin to the diet on gene expression in tumourigenic and inflammatory pathways. In response to the diet, the expression of the transient receptor potential vanilloid 6 (TRPV6) was significantly increased, a response that was prevented in the capsaicin treated rats receiving the same diet. Additionally, the histomorphology of the prostate demonstrated early abnormal cellular changes, with increased epithelial cell height and reduced glandular infolding.

These results suggests that after the treatment period the Western diet has induced molecular and cellular changes resulting in early alterations in cell function and appearance. The prevention of the

increased expression of TRPV6, suggests that the addition of capsaicin to the diet may be able to prevent changes associated with tumourigenesis. Further exploration into the effects of capsaicin in the prostate and the ability to prevent pre-cancerous stages are recommended.

Keywords

Prostate cancer, prostatic intraepithelial neoplasia, proliferative inflammatory atrophy, androgen receptor, Western diet, inflammation, capsaicin

Declaration

I certify that the work reported in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not previously been submitted for assessment in any other course of study, at any other University.

Signature of candidate:

Date:

Endorsement

Supervisor signature:

Date:

List of Abbreviations:

ADT	Androgen deprivation therapy
AR	Androgen receptor
AP-1	Activator protein 1
CC	Corn-starch plus 0.015% capsaicin fed rats
CCL4	C-C motif chemokine ligand 4
CS	Corn-starch fed rats
CVD	Cardiovascular disease
HC	High-carbohydrate, high-fat plus 0.015% capsaicin fed rats
HF	High-carbohydrate, high-fat fed rats
HGPIN	High grade prostatic intraepithelial neoplasia
hK3	Human kallikrein 3
IL-1	Interleukin-1
IL-6	Interleukin-6
NKX3.1	NK3 homeobox 1
PCa	Prostate cancer
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PMEPA1	Transmembrane prostate androgen-induced protein
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
RT-qPCR	Quantitative (real time) reverse transcriptase polymerase chain reaction
SIRT1	Sirtuin 1
SR-BI	Scavenger receptor class B type I
TMN	Tumour, node, metastasis
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRPV1	Transient receptor potential vanilloid 1
TRPV6	Transient receptor potential vanilloid 6

List of Tables:

Table 1: American Joint Cancer Commission (AJCC) stage grouping system	14
Table 2: Primers used for Real Time Quantitative RT-PCR Analyses	31

List of Figures:

Figure 1: Normal and cancerous prostate gland	9
Figure 2: Progression of normal prostate to precancerous state to cancer	10
Figure 3: The Gleason pattern classification	14
Figure 4: Androgen receptor signalling in the prostate, typical and atypical activation	17
Figure 5: Phytochemicals in Capsicum species	25
Figure 6: Change in body weight	34
Figure 7a: TRPV6 expression	35
Figure 7b: Mean TRPV6 expression	35
Figure 8a: NKX3.1 expression	36
Figure 8b: Mean NKX3.1 expression	36
Figure 9a: AR expression	37
Figure 9b: Mean AR expression	37
Figure 10a: PMEPA1 expression	38
Figure 10b: c-Myc expression	38
Figure 10c: Ki-67 expression	38
Figure 10d: Mean expression of Ki-67, PMEPA1 & c-Myc	38
Figure 11a: NF-kB1 expression	39
Figure 11b: NF-kB2 expression	39
Figure 11c: Mean expression of NF-kB1 & NF-kB2	39
Figure 12: Effect of diet on the prostate gland	40
Figure 13: Western blot of protein samples with target GAPDH (reference) and Mek1	41

Chapter 1: Introduction and literature review

1.1 Prostate cancer incidence and mortality

Cancer is a significant burden in developed, developing and undeveloped regions worldwide, with approximately 14.1 million cancer diagnoses and 8.2 million cancer related deaths in 2012. Prostate cancer (PCa) is the second leading cancer diagnosed and the fifth highest cause of cancer related death globally. In developed or so-called Western countries including Australia, United States, United Kingdom, Canada, New Zealand, Argentina, Chile and most European countries, the incidence of PCa is even greater (Torre et al. 2015). In Australia, it is estimated that in 2017 PCa will be the most common cancer, with one in five men diagnosed and is the second leading cause of cancer related deaths (Australian Institute of Health and Welfare 2017a). In 2011 it was the third highest cause of cancer burden in Australia, particularly among those aged 65 years and older (Australian Institute of Health and Welfare 2017b). The use of prostate specific antigen (PSA) screening for PCa has resulted in an increase in diagnosis and reduction of mortality from the disease, a trend also seen in Australia with a 95% five year survival rate in men diagnosed with PCa, although mortality is higher in rural and regional communities with a 20% lower survival rate reported (Australian Institute of Health and Welfare 2017a; Schröder et al. 2009). The health care cost of PCa in Australia in 2008-2009 was approximately \$349 million, 16% of the total cost of cancer related health care (Australian Institute of Health and Welfare 2013). There is no single cause of prostate cancer however the greater incidence of PCa in developed countries has been linked to increasing rates of obesity, the Western diet and an inactive lifestyle (Center et al. 2012). Although higher rates of diagnosis in Western countries can be partially attributed to the implementation of PSA testing detecting more cancers that would have previously gone undiagnosed, the Western diet also appears to be a major factor (Kimura 2012).

1.2 Prostate gland biology

The prostate is a gland located inferior to the urinary bladder, encircling the urethra. In humans, the prostate has three sets of ducts that serve three regions called the central, transition and peripheral zones. The morphology of the prostate gland in rodents differs from the human as it has distinct anterior and ventral lobes (Cunha et al. 2004). The gland is composed of an epithelium, consisting of basal cells, luminal tall columnar cells (secretory cells) and neuroendocrine cells, and supportive stroma formed from connective tissue, immune cells and blood vessels as shown in Figure 1 (Krušlin, Ulamec & Tomas 2015). The androgen hormones, testosterone and the more active dihydrotestosterone, bind to the androgen receptor (AR) which then regulates the expression of genes necessary for prostate function (Koochekpour 2010). Prostate cell survival and proliferation is dependent on AR signalling (Chang et al. 2013). The physiological function of the prostate is to produce a fluid added to semen, containing proteins, including prostate specific antigen (PSA), zinc and sugars (Murashima et al. 2015).

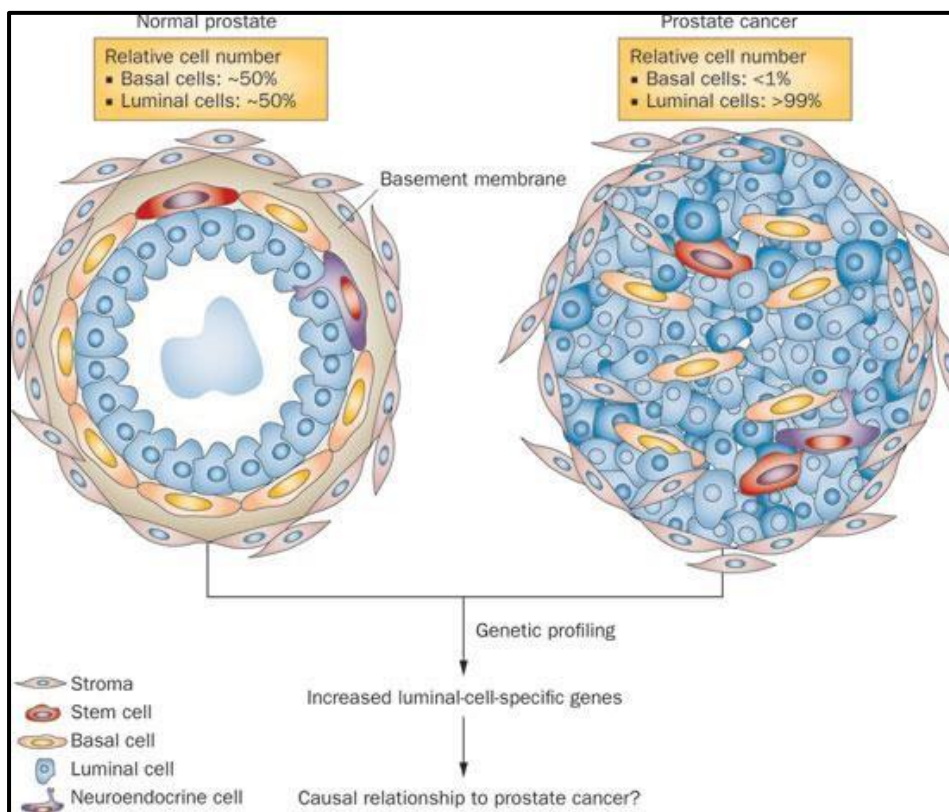


Figure 1: Normal and cancerous prostate gland (Rane, Pellacani & Maitland 2012)

1.3 Prostate cancer tumourigenesis

Cancer is the uncontrolled proliferation of malignant cells which are resistant to programmed cell death (apoptosis). Malignancy in cancer cells is initiated by a series of genetic mutations or alterations in gene expression (epigenetics) causing downregulation of tumour suppressor genes, and upregulation of oncogenes (genes promoting tumour growth). These changes facilitate the growth of cancer within its tissue of origin and as the cancer cells undergo further mutations, they gain the ability to invade nearby tissue, blood vessels and lymph nodes and metastasise (spread) throughout the body (Hanahan & Weinberg 2011; Sarkar et al. 2013).

Most often, PCa arises in the epithelium, which is composed of basal cells, luminal cells and neuroendocrine cells (Lee & Shen 2015). Cancer arising in the luminal cells are termed acinar adenocarcinomas and this is the most common form of PCa (Stoyanova et al. 2013).

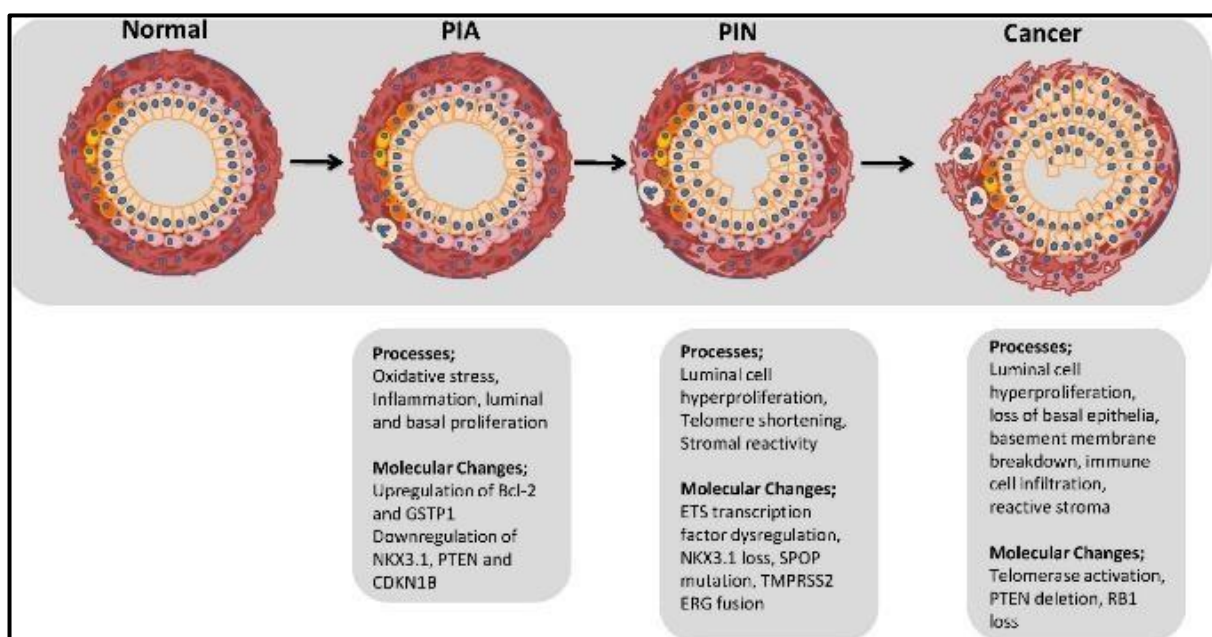


Figure 2: Progression of normal prostate to precancerous state to cancer (Packer & Maitland 2016)

The development of PCa is preceded by changes in the prostate tissue, referred to as proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) as illustrated in Figure 2. These

changes typically occur in the same area of tissue that most PCas occur, in the luminal cells of the peripheral zone (Packer & Maitland 2016). The characteristics of PIA include focal lesions of inflammation in the epithelium containing cells that are abnormally reduced in size (atrophic) and are undergoing increased proliferation. Inflammation is a key factor in the development of PIA, and greater severity of inflammation is strongly associated with increasing incidence of these focal lesions (Benedetti, Bettin & Reyes 2016). The presence of PIA lesions in prostate biopsies has been associated with an increased risk of PCa development and an increased risk of death, suggesting an association between both inflammation and increased proliferation of atrophic cells, and PCa (Davidsson et al. 2011; Kryvenko et al. 2012). Other cellular changes include oxidative stress and alterations in gene expression which are associated with carcinogenesis (cancer development). The gene coding for NK3 homeobox 1 (NKX3.1) protein is affected in the majority of early PCa, with one allele being deleted resulting in reduced protein production (Bethel et al. 2006). NKX3.1 is primarily expressed in the prostate in both humans and rodents and as a homeobox gene is essential for prostate gland development in utero and continued maintenance, but also appears to have tumour suppressor activity (Abate-Shen, Shen & Gelmann 2008; Zhang et al. 2007). A decrease in expression, without genetic alteration, was observed in PIA (Bethel et al. 2006). As this protein may also be involved in reducing oxidative stress, reduced expression may cause an increase in oxidative stress increasing cellular injury, including DNA damage. These effects are associated with carcinogenesis, so the early changes in gene expression in PIA may contribute to cancer development.

Another precancerous state is PIN, which is characteristically more similar to prostate cancer than PIA, with abnormal malignant cell proliferation (neoplasia) and abnormal cellular features, however unlike cancer, the basement membrane (a layer separating epithelium from other tissue) has not been degraded in PIN (Packer & Maitland 2016). While it's possible that PIA may transform to cancer, there is evidence that PIA may transition to high grade-PIN (HGPIN), a form of PIN more closely resembling cancer than any other precancerous lesion. In some PIA lesions there are atrophic epithelial cells which have developed abnormal characteristics more typical of cells within HGPIN lesions, and these

particular lesions likely represent a transition state between PIA and HGPIN (Wang, Bergh & Damber 2009). Additionally, PIA and HGPIN have been observed to arise within the same location and frequently merge also suggesting a developmental relationship (Putzi & De Marzo 2000). There is a very strong association between HGPIN and PCa. Approximately 40% of patients with HGPIN develop cancer within three years and approximately 80% within eight years (Park et al. 2014). Investigation of the alterations occurring between HGPIN and PCa found that HGPIN exhibits genome changes associated with tumour development, however the transition to cancer would necessitate further genetic alterations. The carcinogenic process involves many genetic alterations, such as mutations or changes in copy number, which all contribute to cancer progression. Many of the genome alterations are likely to occur in the late stages of HGPIN or early stages of cancer (Jung et al. 2016). Changes in NKX3.1 expression is also affected in PIN, where there is reduced expression without chromosomal deletion in low grade-PIN, whereas reduced expression in HGPIN may be due to genomic deletion (Bethel et al. 2006). While a chromosomal deletion of NKX3.1 is observed in only some HGPIN lesions, the occurrence in PCa is significantly higher, with one study observing the deletion in all cancer samples (Jung et al. 2016).

After a diagnosis of PCa, staging of the disease is important to determine appropriate treatment and the risk of death varies depending on the stage and grade. Stage is represented by the tumour, node and metastasis classification (TNM) and grade is classified by the Gleason grading system (Table 1). PSA levels can also be informative (as outlined in Table 2) and these levels are used to measure treatment effectiveness and evidence of reoccurrence (Paller & Antonarakis 2013). PCa biopsies and in some cases, lymph node (groin) biopsies are examined by a pathologist to determine the tumour (T) and the node (N) and various scanning modalities (such as ultrasound, X-ray, bone scans and computer tomography) are used to determine the metastasis (M). The clinical staging of primary tumours (T) begins with clinically apparent tumours in the T1 stage, progressing from T1a with histological evidence of the tumour in less than 5% of the tissue tested to T1c, with raised PSA and evidence of a tumour cells in the biopsy. T2 stage tumours are localised to the prostate, affect less

than one half of a lobe in T2a to affecting both lobes in T2c. Invasion beyond the connective tissue surrounding the prostate occurs in stage T3 cancer, on either one side or both sides of the prostate in T3a cancer, and invasion of the seminal vesicles in the T3b stage. The T4 stage involves invasion of tumour cells into adjacent tissue and organs including the urinary bladder, rectum or pelvic wall. Clinical staging of cells in lymph nodes defines NX and N0 as not assessed and no lymph node invasion respectively, and N1 as invasion of the cancer into regional lymph nodes in the groin. Metastases are classified as no metastasis into distant tissues or organs in M0 or metastasis occurring in M1. M1 is subclassified as M1a affecting lymph nodes other than regional nodes: M1b involves invasion of prostate cancer cells into the bone: and M1c as metastasis or presence of cancer cells in other tissues (Cheng et al. 2012).

The Gleason grading system for PCa is based on histological examination of biopsies from the prostate, observing the characteristics of the tissue, illustrated in Figure 3 (Gleason & Mellinger 1974). The lowest pattern of 1 represents normal cells that are well differentiated and the highest pattern of 5 represents cells that are anaplastic, which are poorly differentiated and display abnormal morphology. The Gleason score is calculated by adding two patterns observed together, therefore if Gleason 3 and Gleason 4 patterns are observed in a biopsy this forms a Gleason score of 7 (Gleason & Mellinger 1974). The Gleason score is important in staging PCa as it is strongly associated with the aggressiveness of the cancer (Chen & Zhou 2016). PSA, also called human kallikrein 3 (hK3), is produced by prostate luminal cells and is used for PCa screening, based on the ratio of free PSA and bound PSA (bound to molecules in the serum). PSA levels are also useful in staging PCa (Bryant & Lilja 2014).

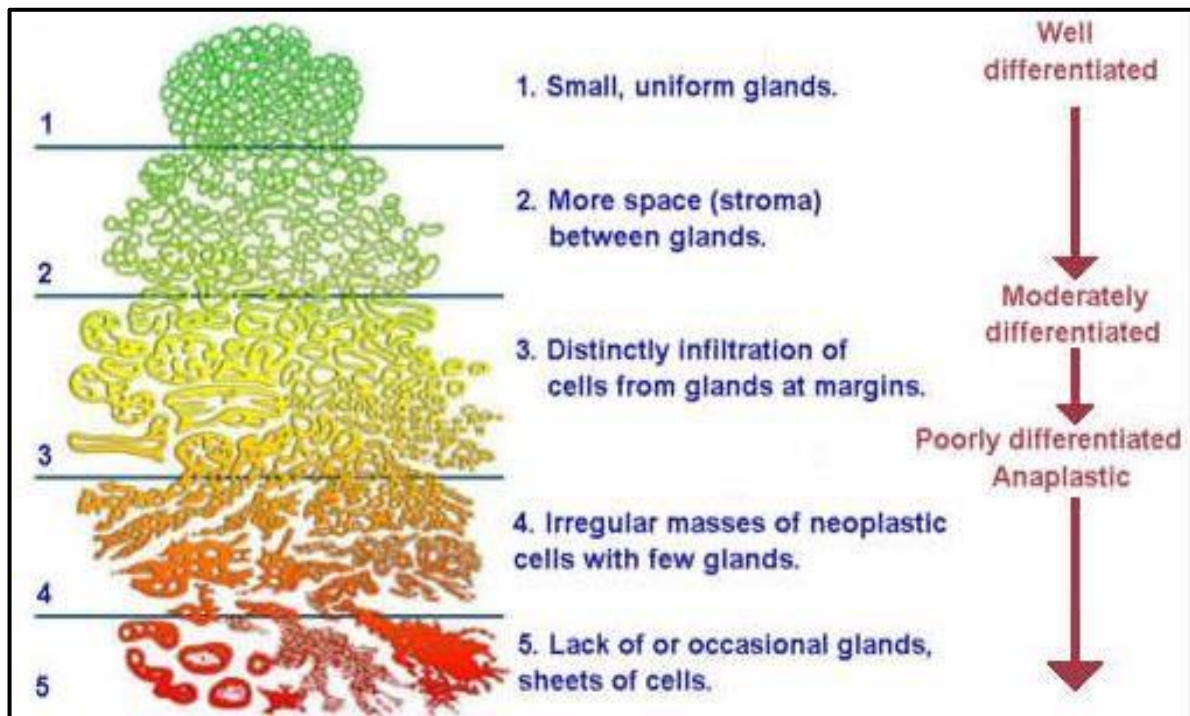


Figure 3: The Gleason pattern classification (*Gleason Score 2017*)

Table 1: American Joint Cancer Commission (AJCC) stage grouping system (*Clinical staging 2014*)

AJCC Prostate Cancer Stage Groupings														
	Stage I		Stage IIa					Stage IIb			III	Stage IV		
Jewett-Whitmore stage	A1		A2, B0-2								C1-3	D1	D2	
TNM stage	T1a-c N0M0	T2a N0M0	T1a-c N0M0	T1a-c N0M0	T2a N0M0	T2a N0M0	T2b N0M0	T2c N0M0	T1-2 N0M0	T1-2 N0M0	T3a-b N0M0	T4 N0M0	Any T N1M0	Any T Any N M1
Gleason score	≤ 6	≤ 6	7	≤ 6	≤ 6	7	≤ 7	Any	Any	≥ 8	Any	Any	Any	Any
PSA level (ng/ml)	< 10	< 10	< 20	10-19.9	10-19.9	< 20	< 20	Any	≥ 20	Any	Any	Any	Any	Any

Treatment of PCa depends on the risk classification which is determined by the stage and grade of the tumour. Low risk patients have T1c to T2a tumours, low PSA, and biopsy Gleason score 6. The biopsy Gleason score is calculated based on histological appearance of the cells. Moderate PSA, Gleason score 7 and cancer staging of T2b-c is considered intermediate risk cancer and high PSA, Gleason score 8 or higher and cancer staging of T3a or higher is high risk PCa (Heidenreich et al. 2014). Patients with low risk cancer may not receive treatment initially but be under active surveillance, as some cases of PCa

are indolent and will not pose a risk of death within the life expectancy of the patient, particularly in individuals over 70 years of age. In cases where the cancer is not indolent and therefore active surveillance is not appropriate, radical prostatectomy (surgical removal of the prostate) is a common treatment for localised PCa (Heidenreich et al. 2014). This treatment is associated with complications, as incontinence and impotence may occur after surgery, and may have a significant impact on quality of life even for patients in remission (Bianco, Scardino & Eastham 2005).

Radiation therapy or low dose brachytherapy, where the radiation is provided by an implant inserted into the prostate can also be used. In intermediate and high-risk cases, radical prostatectomy and androgen deprivation therapy (ADT), which prevents androgen stimulated tumour growth, can be used in conjunction with radiation therapy in intermediate and high-risk cases (Heidenreich et al. 2014). Side effects are also observed in radiation therapy with urogenital effects, including inflammatory obstruction of the urethra causing painful urination and increased urgency, sexual dysfunction, and gastrointestinal irritation causing increased urgency, bleeding and diarrhoea (Michaelson et al. 2008). These complications of therapy are important considerations for the quality of life of prostate cancer patients.

While ADT is a mainstay treatment for high risk and invasive PCa, its use is associated with metabolic syndrome in patients (Rezaei et al. 2016). The defining features of metabolic syndrome are insulin resistance or Type II diabetes, obesity, dyslipidaemia (abnormal triglyceride and cholesterol levels), and hypertension, which are associated with cardiovascular disease (CVD) (Huang 2009). Metabolic syndrome has been observed to occur in over half of the patients being treated with long term ADT, compared with only one in five in those not treated with ADT. The main metabolic changes in patients with metabolic syndrome were high blood sugar levels and increased fat deposition in the abdominal region, which were not influenced by other factors such as age or race, therefore hypogonadism was proposed as the cause of metabolic syndrome in ADT treated patients (Braga-Basaria et al. 2006). Intermittent ADT is still associated with an increased risk of metabolic syndrome, however the risk is

significantly lower than that of long term therapy, further supporting the role of ADT in metabolic syndrome (Rezaei et al. 2016). As metabolic syndrome is associated with CVD, the development of this syndrome caused by ADT would further increase risk of death, due to both CVD and cancer related mortality.

1.3 Genetic changes driving the development of prostate cancer

In the prostate, normal function is reliant on androgen signalling via the AR, located intracellularly as shown in Figure 4. Without androgen signalling there is reversible regression of the epithelium, which recovers upon reintroduction of the hormones. The interactions between stromal and epithelial cells, and androgen signalling is complex and well regulated. AR activation in the supportive stromal cells results in the release of paracrine growth factors, called andromedins, which stimulate epithelial proliferation and differentiation, thereby forming the secretory luminal cells (Antony et al. 2014). This AR mediated response in epithelial cells results in terminal differentiation where the cell has reached maturation and will no longer undergo proliferation. Thus AR signalling in the epithelium suppresses growth and does so via downregulating expression of a transcription factor, c-Myc.

In PCa however, AR signalling in the epithelium induces growth of the tumour as the AR in tumour cells upregulates the expression of c-Myc (Vander Griend, Litvinov & Isaacs 2014). Stromal AR activation is necessary for carcinogenesis in the prostate as deletion of stromal AR prevented cancer development and advancement, while cancer progression still occurred with epithelial AR deletion (Ricke et al. 2012). Changes in the AR signalling pathway have been observed to occur in over half of all primary tumours and all metastatic cancers, although mutations and increased copy numbers of the AR gene itself typically only occurs in advanced metastatic cancer. AR signalling induces proliferation via c-Myc, therefore changes downstream may be a mechanism involved in carcinogenesis (Taylor et al. 2010). c-Myc overexpression is common in many cancers, including breast, colon and PCa. There are often extra copies of c-Myc in advanced PCa, however in early stages this amplification rarely occurs. Despite this, c-Myc overexpression appears to be an important factor in carcinogenesis as c-Myc is involved in

many cancer cell processes, but in PCa its upregulation leads to proliferation but prevents terminal differentiation, promoting carcinogenesis (Hawksworth et al. 2010).

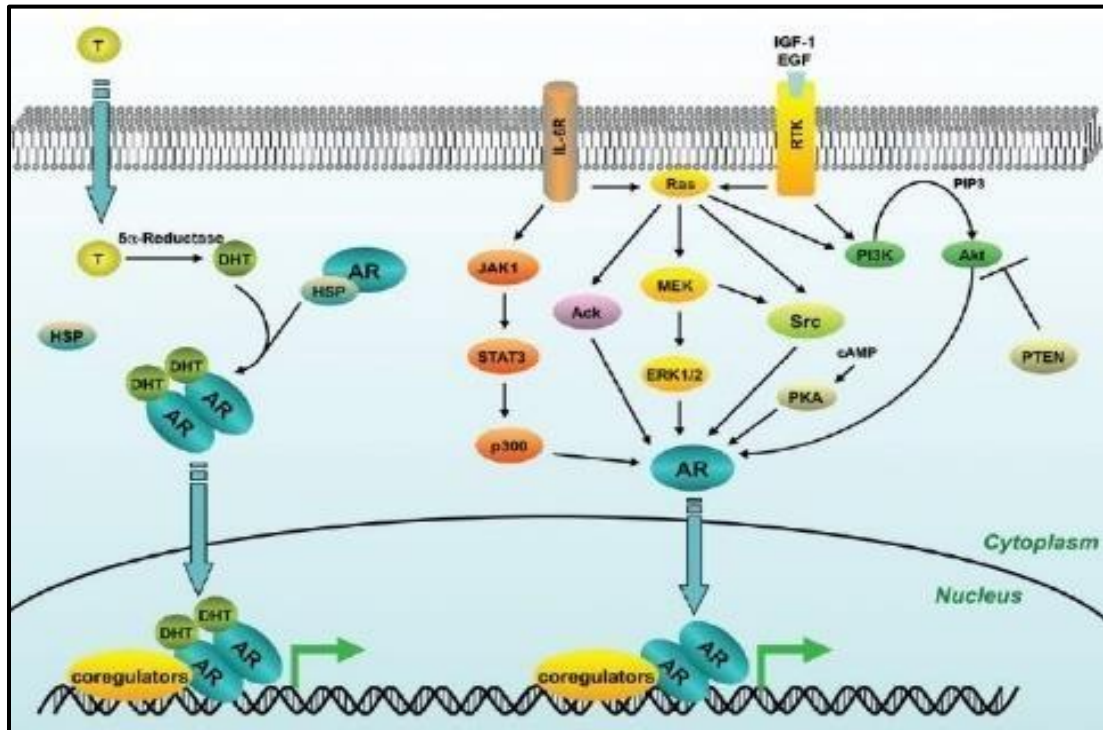


Figure 4: Androgen receptor signalling in the prostate, typical and atypical activation (Lonergan & Tindall 2011)

The transmembrane prostate androgen-induced protein precursor (PMEPA1) is produced in response to AR activated transcription. It functions as a regulatory protein, as it binds directly to the AR and initiates degradation of the AR protein, thereby creating a negative feedback loop (Li et al. 2008). Reduced expression of PMEPA1 has been observed in PCa, which has been associated with an increased AR function. Methylation (the addition of methyl groups to a gene promoter region) of the PMEPA1 gene, has been linked to gene silencing in PCa and is suggested to be an important step in AR driven cancer development (Li et al. 2015; Sharad et al. 2014). PMEPA1 silencing has also been linked to metastasis in mouse models and humans, and associated with reduced survival (Fournier et al. 2015).

NF- κ B is another transcription factor involved in PCa. It is usually inactive in the cytoplasm of normal epithelial cells and in the nuclear membrane of basal cells. Upregulation of cytoplasmic NF- κ B was seen in precancerous PIN lesions suggesting a role in early carcinogenesis. Nuclear translocation and overexpression of NF- κ B is observed in more advanced disease and is linked with a poorer outcome (Domingo-Domenech et al. 2005). NF- κ B is also involved in inflammation where it promotes expression of inflammatory cytokines and recruitment of immune cells, and is also itself upregulated by other cytokines, including interleukin-1 (IL-1). The increased expression in PIN is likely associated with the inflammation occurring in the lesion and eventually as the cancer progresses, NF- κ B becomes overexpressed (Nguyen et al. 2014).

1.4 Impact of diet on prostate cancer development

The reports of higher rates of PCa in Western countries compared to developing countries, has been attributed to the high fat, high carbohydrate 'Western Diet,' which includes a high consumption of meats, both red and processed, and many other fatty, sugary foods (Fabiani et al. 2016). Although a portion of the higher risk in Western countries can be attributed to better screening, the lifestyle and diet appears to be a significant factor (Kimura 2012). Investigations into whether specific foods, such as red meats are associated with increased risk of PCa have not yielded strong evidence of a relationship in meta-analyses and pooled analyses (Bylsma & Alexander 2015; Wu et al. 2016) so it is likely that the Western diet as a whole may have a greater influence on PCa development and progression compared to individual food types. A meta-analysis performed by Fabiani et al. (2016) compared the trends occurring in healthy, Western, and carbohydrate dietary patterns. As expected, the healthy diet was not associated with PCa and had a small, but not significant, reduction in risk. The Western diet was associated with a significant increased risk of PCa from an analysis of 12 studies. Additionally, analysis of the dose response, found that the risk of PCa also increases as the intake of the diet adheres more to the Western type foods (Fabiani et al. 2016).

Deep-frying food is a cooking method common in the Western diet, which increases dietary fat content. Additionally, the high heat conditions while cooking alters the structure of the fats, transforming unsaturated fats to trans fatty acids and causes formation of carcinogens, such as aldehydes, hydrocarbons, and heterocyclic and polycyclic amines (Stott-Miller, Neuhouser & Stanford 2013). Frequent intake of deep-fried foods was associated with an increased risk of PCa, while there was also a greater association with high risk PCa. The increased risk was attributed mainly to the regular consumption of high fat, high carbohydrate deep-fried fast food, while not finding a strong link between carcinogen formation and PCa risk (Stott-Miller, Neuhouser & Stanford 2013). High cholesterol is also a common feature of the Western diet and associated with metabolic disorder. A large prospective cohort study investigated the link between plasma cholesterol and PCa finding no increased occurrence of the disease, but did find a heightened risk of an aggressive form of PCa (Shafique et al. 2012). These results suggest that cholesterol may be more important in advanced progression of cancer, which would require a constant source of cholesterol for plasma membrane formation in aggressive cancer growth.

There is also evidence that the Western diet, which results in increased fat deposition and cholesterol levels, promotes cancer progression in a mouse model of PCa. It was also found that a plasma membrane receptor which selectively takes up high density lipoprotein (HDL)-cholesteryl ester, scavenger receptor class B type I (SR-BI), is increased in the PCa cells which would support tumour growth as cholesterol is essential for forming the plasma membrane of cells (Llaverias et al. 2010). As the Western diet is high in cholesterol, the cancer would have a generous supply of cholesterol for the plasma membranes of proliferating tumour cells. Furthermore, there was an increase in blood vessel growth (angiogenesis) in the tumour, an essential feature in cancer growth and invasion, and also supplying the tumour with essential nutrients including cholesterol (Llaverias et al. 2010). The occurrence of metastasis to the lungs was also significantly higher in mice fed the Western diet, a likely outcome of increased blood vessel formation and cholesterol uptake (Llaverias et al. 2010). There is also evidence of the Western diet impacting survival rates in humans. In patients who consume a

Western diet after diagnosis with nonmetastatic cancer, there was an overall increased risk of death, whereas individuals who consumed a healthier diet after being diagnosed appeared to lower the overall risk of death (Yang et al. 2015).

1.5 Diet and inflammation

There is a strong association between obesity and inflammation, with macrophage infiltration into adipose tissue and proinflammatory cytokine release from adipose tissue occurring in obesity. Obesity is often associated with a hypercaloric, red meat-based, high fat and high carbohydrate diet, common features of the Western diet (Johnson & Makowski 2015). The excessive fat accumulation in adipocytes leads to activation of pathways promoting insulin resistance and inflammation. A high fat diet in mice has been shown to lead to activation of an inflammasome, a multiprotein complex, which causes downstream activation of many inflammatory cytokines and cleavage and deactivation of sirtuin 1 (SIRT1), the loss of which is associated with metabolic dysfunction (Chalkiadaki & Guarente 2012). A rat study has also shown that elevated consumption of beef, a red meat, even for a short term resulted in an increase in inflammation and oxidative stress (Van Hecke et al. 2016).

There is also a strong link between PCa and diabetes which further supports a role for diet and metabolism in PCa development. A meta-analysis of PCa patients found that pre-existing type 1 or type 2 diabetes were both associated with an increased risk of death, and type 2 diabetes doubled the risk (Lee, Giovannucci & Jeon 2016). The likelihood of advanced cancer is increased in patients with diabetes as high blood sugar levels promote tumour growth, and the effectiveness of radiation therapy may be reduced in patients with this condition (Lee, Giovannucci & Jeon 2016).

1.6 Inflammation and prostate cancer

Inflammation has been proposed as a contributing factor in various epithelial cancers including PCa. The presence of inflammation in prostate biopsies is associated with the development of PCa, and a greater level of inflammation is linked to high-risk PCa (Gurel et al. 2014). Inflammation has been

shown to induce oncogenic alterations in epithelial basal cells, leading to differentiation to luminal cells, the phenotype seen in adenocarcinoma. Basal cells possess the capability for differentiation to the luminal lineage, however in normal tissue this has rarely been observed. Genomic alterations are also more common in basal than luminal cells, and apoptosis occurs less frequently in basal cells. Inflammatory signalling is a likely trigger for the change in differentiation, and may also contribute to genetic and epigenetic changes, suggesting a role in inflammatory induced basal origin of adenocarcinoma (Kwon et al. 2014). Inflammation in the prostate is associated with subpopulations of luminal cells that have low expression of the luminal marker CD38. These cells display PIA like characteristics and also have increased expression of NF- κ B, which is suggested to predispose the cells to tumorigenesis and development of aggressive cancer (Liu et al. 2016). While NF- κ B overexpression is important in tumorigenesis as previously discussed, it is not linked to cancer development when occurring alone, however a synergistic effect has been observed with c-Myc overexpression (Jin et al. 2014).

Inflammatory signalling is important in the link between inflammation and tumorigenesis. Interleukin6 (IL-6), is a proinflammatory cytokine released by immune cells, cells lining blood vessels and tumour cells. Increased IL-6 production in the prostate in an inflammatory setting is associated with immune cell infiltration in the prostatic tissue and surrounding adipose tissue. Continuous production of IL-6 in chronic inflammation initiates epigenetic changes which promotes tumorigenesis and can cause development of a neoplasm. Additionally, it can also induce autocrine (affecting the cell that produced the cytokine) IL-6 signalling, insulin-like growth factor (IGF) paracrine signalling, and further promotes inflammation (Liu et al. 2017). IGF upregulates cellular proliferation and enhances cell survival, and human studies have found that increased IGF levels in serum is linked to increased risk of PCa (Roddam et al. 2008; Weroha & Haluska 2012). IL-6 is also an alternative activator of the AR in PCa cells, as first reported by Hobisch et al. (1998). IL-6 activation of the AR promotes the growth of prostatic tumours and metastasis often occurs in organs in which IL-6 is produced. NF- κ B can also induce IL-6 expression,

demonstrating the complex interaction of transcription factors and cytokines in inflammatory related tumourigenesis (Malinowska et al. 2009).

Infiltration of immune cells also appears to be an important factor in PCa and inflammation. Macrophages have been observed promote a cancerous effect via tumour necrosis factor, as well as promote inflammation and progression of cancer in the prostate. The presence of tumour associated macrophages is associated with advanced cancer and poor outcomes, as well as recurrence of cancer (Nonomura et al. 2011). Macrophages are also involved in tumourigenesis, as shown by Fang et al. (2013) in a culture of macrophages and normal prostate epithelial cells. The culture was found to increase expression of various chemokines including C-C Motif Chemokine Ligand 4 (CCL4) and its receptors, which was found to inhibit expression of the tumour suppressor genes, tumour protein p53 and phosphatase and tensin homolog (PTEN). This chemokine and its receptors are upregulated by macrophage AR expression, as indicated by an inhibition of this effect when the macrophage AR is silenced (Fang et al. 2013). Infiltration of macrophages and these epigenetic changes may be important in the development of the precancerous PIN lesions and subsequent tumourigenesis.

1.7 Phytochemicals and cancer prevention

An area of investigation is the prevention of PCa using various dietary phytochemicals, including lycopene, curcumin and resveratrol, though in some cases these studies have yielded mixed results. Lycopene is a carotenoid (fat-soluble coloured pigment) occurring in high concentrations in processed tomato products, such as tomato sauce. It is an effective antioxidant, as such has been investigated for its cancer preventative effects (Giovannucci et al. 2002). A prospective cohort study of over 50 000 individuals examined the relation of dietary items to PCa and found a significantly decreased risk of PCa associated with lycopene intake, in men over 65 years of age (Giovannucci et al. 2002). A more recent follow up of the same individuals suggested that long-term intake of lycopene appeared to reduce the risk of lethal PCa, but was less important in reducing total cases (Zu et al. 2014). Other studies have found contradictory results, including a prospective study and a case controlled study,

both investigating the same population of nearly 30 000 men. These two studies found no association between lycopene intake and risk of PCa (Kirsh et al. 2006; Peters et al. 2007). Similar findings were also reported in a Prostate Cancer Prevention Trial involving around 10 000 men, where dietary nutrients including lycopene were found to have no impact on risk on PCa, including high-grade cancer (Kristal et al. 2010).

The spice turmeric contains an active compound curcumin, another potent antioxidant with potential as an anticarcinogenic agent (Sandur et al. 2007). Human trials of curcumin in patients at high risk of colorectal and other types of cancers have observed an anticarcinogenic effect in some patients, including histological improvements of some precancerous lesions (Carroll et al. 2011; Cheng et al. 2001). Curcumin also has many cancer and antitumorigenic effects on prostate cells, involving inflammatory pathways and alterations in expressions of genes related to carcinogenesis. Studies have shown that curcumin can antagonise the alternate activation of the AR by the inflammatory cytokine IL-6, and inhibit activation of NF- κ B, both pathways observed in inflammation and PCa (Sandur et al. 2007; Tsui et al. 2008). Curcumin has also been shown to downregulate the Hedgehog signalling pathway, which is vital for normal cell proliferation and differentiation but often becomes dysregulated and overexpressed in PCa (Ślusarz et al. 2010).

Resveratrol is found in some fruits and red wine as a phytoalexin, a compound produced by plants in response to environmental stress, and has antioxidant, anti-inflammatory and antitumorigenic effects (Benitez et al. 2009). The prevention of cancer by resveratrol has been observed in a reduction of oesophageal tumour formation as well as inhibition of angiogenesis, a hallmark of cancer, in lung carcinoma tumours in rodent models (Kimura & Okuda 2001; Li et al. 2002). In PCa cells resveratrol has been shown to inhibit AR activation, which results in an upregulation of PTEN, with tumour suppressor activity, overall reducing PCa growth (Wang et al. 2010). It has also been shown to reduce activation of NF- κ B (Benitez et al. 2009). A phase I trial in humans determined that resveratrol taken

orally was safe and produced biologically relevant plasma concentration, though the anticarcinogenic effects would have to be further investigated in humans (Boocock et al. 2007).

1.8 Capsaicin

Capsaicin is a phytochemical found in *Capsicum* species. It is a type of capsaicinoid (shown in Figure 5), an amide produced in the fruit that causes the spicy, pungent sensation when eaten. Capsaicin is the main capsaicinoid occurring in chilli extract, comprising approximately 69% of the total content (Wahyuni et al. 2013). Capsaicin activates the transient receptor potential vanilloid 1 (TRPV1), a receptor belonging to a family of nonselective cation channels, causing influx of calcium and sodium ions into the cell, and the sensation of heat and pain. TRPV1 is also activated by acidic conditions, high temperatures and some toxins (Yang & Zheng 2017). Capsaicin has been shown to have anti-cancer activity caused by binding and activation of TRPV1 in urothelial cancer cells via activation of proapoptotic proteins leading to cell death (Amantini et al. 2009). An important mechanism of apoptosis, as observed in microglial cells, is the influx of calcium caused by TRPV1 activation by capsaicin, which causes mitochondrial damage causing leakage of cytochrome c, a molecule which forms an apoptotic complex and irreversibly initiates apoptosis (Kim et al. 2006). Human prostate tissue expresses TRPV1, including the epithelial cells confirmed by increases in intracellular calcium concentrations in response to capsaicin, and expression of TRPV1 increases in PCa (Czifra et al. 2009; Sánchez et al. 2005). It is important to note that capsaicin activation of TRPV1 is followed by desensitisation of the receptor, during which it can no longer be activated for a period of time, depending on the dose and location. Prolonged capsaicin activation has been observed to lead to removal of the receptor from the plasma membrane, causing long term desensitisation (Sanz-Salvador et al. 2012).

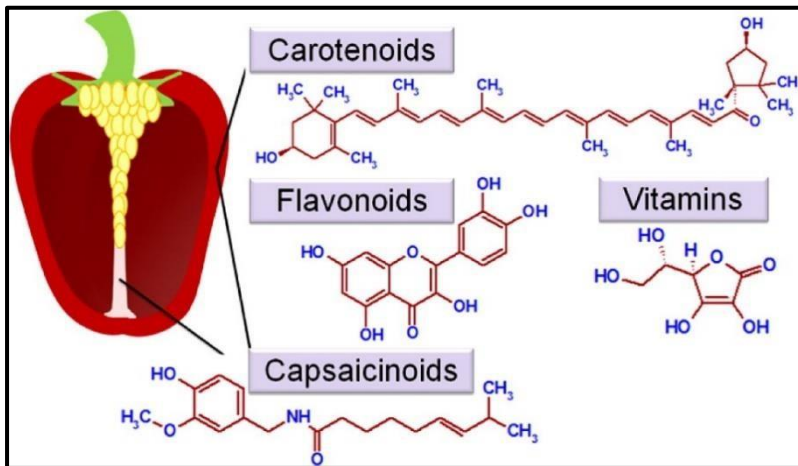


Figure 5: Phytochemicals in *Capsicum* species (Wahyuni et al. 2013)

Evidence also suggests that the mechanisms of capsaicin's cancer activity is partially TRPV1 independent, as the use of a TRPV1 antagonist was unable to prevent apoptosis in PCa cells in response to capsaicin treatment. Capsaicin inhibits pathways important in generating antioxidants and increases reactive oxygen species (ROS) production, thereby increasing oxidative stress. There is an initial peak of ROS production, occurring within 15 minutes of the addition of capsaicin to PCa cells, and a later peak of ROS production at 10 hours associated with disruption of the mitochondrial membrane. In mice grafted with human PCa cells both capsaicin and capsazepine, a TRPV1 antagonist, caused apoptosis at similar rates, indicating a TRPV1 independent mechanism of cell death (Sánchez et al. 2006).

Another receptor in the TRPV family TRPV6, a highly selective calcium ion channel, like TRPV1 is present in many tissues and has also been linked to capsaicin cancer activity in some cancer types. In gastric cancer the calcium influx and resulting induction of apoptosis is due to TRPV6 activation and is prevented by silencing of TRPV6 expression with RNA interference, whereas inhibiting TRPV1 had no effect (Chow et al. 2007). Capsaicin has also been observed to cause apoptosis via TRPV6 activation in small cell lung cancer by calcium influx but also by the calcium dependent calpain pathway, a group of enzymes which degrade proteins and are important in regulation of cell differentiation and apoptosis (Lau et al. 2014). TRPV6 is also highly expressed in PCa and increasing expression is associated with

high risk cancer. A moderate increase in calcium uptake by TRPV6 may promote cell growth and survival in cancer, whereas a continuous increase in calcium uptake, such as what occurs when capsaicin activates the receptor, causes mitochondrial membrane injury and promotes apoptosis (Lehen'Kyi et al. 2007).

There are various proteins and pathways which exhibit changes in expression in precancerous states and mutations or copy number alterations in PCa, and capsaicin has been observed to affect many of the targets. In androgen sensitive cells, low concentrations of capsaicin (0.01 to 20 μ M) have been shown to increase cell proliferation via an increase in AR expression which was dependent on TRPV1 activation, however at higher concentrations of capsaicin (50 to 100 μ M) an antiproliferative effect was observed (Malagarie-Cazenave et al. 2009; Zheng et al. 2015). At higher concentrations (100 μ M), capsaicin caused overexpression of a small non-coding microRNA which caused an inhibition of expression and degradation of the AR, preventing cell growth in the AR dependent cells (Zheng et al. 2015). In androgen independent cells, capsaicin caused an increase in TNF and IL-6 concentration which was associated with inducing cell death (Malagarie-Cazenave et al. 2011). Considering the dose dependent desensitisation of capsaicin on TRPV1, the higher doses may also be preventing TRPV1 activity after the initial activation.

Although capsaicin has been shown to promote inflammatory cytokine production in cancer cells, it appears to have an anti-inflammatory effect on macrophages, representing a possible anti-inflammatory effect on tissues with macrophage infiltrations. Capsaicin was observed to both prevent production of TNF and cause inactivation of NF- κ B in macrophages (Kim et al. 2003; Park et al. 2004). A more recent study showed that capsaicin had an inhibitory effect on induced inflammation in mice, corresponding with lower concentrations of markers of inflammation, however the infiltration of immune cells remained normal, suggesting the protective immune response against infections is not affected by capsaicin but excessive inflammation is downregulated (Jolayemi & Ojewole 2013).

Capsaicin has previously been shown to prevent NF- κ B expression and the expression of another oncogene, complex activator protein 1 (AP-1), in mouse skin (Surh et al. 2000). The mechanisms involve protection of I κ B- α , a protein which is normally bound to and therefore inhibits degradation of NF- κ B, and prevents binding of AP-1 to DNA, inhibiting its oncogenic effect. The inhibition of activity of both NF- κ B and AP-1 by capsaicin suggests an antitumorigenic effect (Han et al. 2001). Capsaicin has also been shown to be beneficial in obesity associated inflammation, by normalising cytokine release from adipocytes which are dysregulated in obesity. Adipokines, including IL-6, are cytokines released from adipocytes causing obesity associated inflammation and the addition of capsaicin to the diet of obese mice reduced inflammatory adipokine release, decreased macrophage infiltration in the adipose tissue and increased the release of the anti-inflammatory hormone, adiponectin (Kang et al. 2007).

A mouse model has investigated the anticarcinogenic properties of capsaicin in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, where prostate adenocarcinoma is induced by a mutation (probasin- SV40 T-antigen). The long-term administration of capsaicin (5 mg/kg body weight) resulted in reduced progression to metastatic cancer, with no indications of toxicity (Venier et al. 2015).

Knowledge gaps and future directions

The current evidence suggests that a high fat and high carbohydrate diet (Western diet) can induce inflammation in many tissues and organs in the body, including the prostate gland. Additionally, inflammation can promote carcinogenesis and cancer progression via several mechanisms. Capsaicin has been shown to reduce inflammation in multiple tissues and organs, therefore can it reduce inflammation in the prostate and prevent inflammation induced carcinogenesis in the prostate? Currently there has not been research published into whether capsaicin can prevent changes in the expression of inflammation and PCa associated genes and protein levels that are caused by a high fat and high carbohydrate diet in the prostate gland. As the incidence of prostate cancer increases

worldwide, less expensive and lower toxicity approaches for treatment and prevention are urgently required. Supplementing the diet with an inexpensive and readily available phytochemical such as capsaicin may provide a solution.

In developed countries, the consumption of a high fat, high carbohydrate Western diet is associated with inflammation in the prostate gland. Precancerous lesions, PIA and PIN, develop in association with inflammation, and display changes in expression of various genetic pathways which eventually lead to the tumourigenesis of PCa. Previous research has shown anti-inflammatory properties of capsaicin in numerous tissues in the human body, therefore this study has investigated whether the addition of capsaicin to a high fat, high carbohydrate diet in male Wistar rats could prevent or reverse the inflammation associated with the diet and also investigated if capsaicin could prevent or reverse the alterations in gene expression associated with the early development of PCa.

Project hypothesis:

The hypothesis of this study is that the addition of capsaicin to a high fat and high carbohydrate diet would prevent or reverse the gene and protein expression changes that drive diet-stimulated PCa tumourigenesis.

Project aims:

Using a male Wistar rat model of diet-induced obesity:

1. Measure the relative expression of genes in the prostate gland of rats fed either a balanced, control diet (CS), a diet representative of the Western diet (high in fat from mammalian sources and high in fructose, HCHF), a CS diet supplemented with (0.015%) capsaicin or the HCHF diet supplemented with (0.015%) capsaicin. This includes genes in the androgen receptor pathway, NF- κ B cell signalling pathway and TRPV pathway.
2. Determine the effects of the HCHF diet and capsaicin supplementation on protein levels of the proliferation marker MEK1 in the prostate gland.
3. Determine the histological changes, specifically the presence of inflammatory cells in the prostate gland in response to the HCHF diet.

Chapter 2: Materials and Methods

2.1 Animal Protocol

The animal protocol was completed by Edward Bliss and the Functional Foods Group at the University of Southern Queensland, Toowoomba campus. Briefly, male Wistar rats (eight to nine weeks old; 338 ± 7 g) were acclimatised for three weeks before being randomly divided into four experimental diet groups. The diets consisted of either a corn starch-based (**CS**) diet or high-carbohydrate high-fat (Western Diet, **HF**) diet for 16 weeks, or a corn starch-based diet for eight weeks with the addition of 0.015% capsaicin for the following 8 weeks (**CC**) or a high-carbohydrate high-fat diet for eight weeks with the addition of 0.015% capsaicin for the following 8 weeks (**HC**).

At the end of the protocol, rats were euthanised and prostate samples dissected for quantitative RTPCR were stored in RNA Later (QIAGEN) and samples for Western Blotting were stored in RIPA buffer (25mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, Protease inhibitors) at -80°C.

2.2 RNA extraction

Prostate samples were homogenised using GentleMacs Dissociator (Miltenyi Biotec) and Qiashredder columns (QIAGEN). Total RNA from 30 mg of tissue was then extracted using RNeasy Mini Kit (QIAGEN).

2.3 Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was quantified and RNA integrity determined using the 2100 Bioanalyzer (Agilent) and equivalent amounts (100 nanograms) converted to complementary DNA (cDNA) with iScript™ Reverse Transcription Supermix (Bio Rad). One microlitre of cDNA was amplified using Bio Rad CFX 384 real

time instrument, with SsoAdvanced™ Universal SYBR® Green Supermix using UBC or HPRT1 as a reference gene.

Table 2: Primers used for Real Time Quantitative RT-PCR Analyses

Primer Target	Unique Assay ID	Chromosome Location
UBC	qRnoCED0057022	12:38519875-38519992
HPRT1	qRnoCED0057020	X:153250276-153250384
Androgen Receptor	qRnoCID0006751	X:68693047-68699818
PMEPA1	qRnoCED0005395	3:177360138-177360237
TRPV1	qRnoCID0003147	10:59545607-59548162
TRPV6	qRnoCID0011754	4:135712922-135721028
IL-6	qRnoCID0053166	4:3095863-3097287
NF-kB1	qRnoCID0003698	2:259350292-259351746
NF-kB2	qRnoCED0007287	1:273490070-273490162
Myc	qRnoCID0007760	7:103160322-103161438
NKX3.1	qRnoCED0001679	15:54797829-54797927
Ki-67	qRnoCID0001488	1:214941606-214943149

2.4 Western Blotting

Samples stored in RIPA buffer were homogenised using liquid nitrogen and sonication and left for two hours before being centrifuged for 20 minutes at 12 000 rpm at room temperature. The supernatant was aliquoted to storage tubes. Protein lysates were quantified using BCA protein assay (Thermo Fisher).

Equal quantities of protein lysates (20µg) were separated using SDS-PAGE (Bio Rad) at 110 mV for approximately one hour, then proteins were transferred onto PVDF membranes (Bio Rad Trans-Blot® Turbo™ Transfer System). The PVDF membranes were blocked in 0.1% Tween 20 Tris Buffered Saline (TBST) with 5% skim milk powder (Woolworths) on a plate shaker for one hour at room temperature.

Using reagents from the Pierce™ Fast Western Blot Kit (Thermo Fisher), the membranes were washed in wash buffer (Thermo Fisher) before being incubated in the primary antibody, GAPDH (1:1000) or MEK1 (1:1000) (both Precision Antibodies from Bio Rad) at room temperature for 30 minutes on a plate shaker. The membranes were then incubated in Optimised HRP Reagent Working solution

(secondary antibody) (Thermo Fisher) for five minutes at room temperature. Chemiluminescence was detected using SuperSignal West Dura Working Solution (Thermo Fisher) and viewed using Fusion Fx (Vilber).

2.5 Immunohistochemistry

Rat prostate tissue samples were fixed in 10% formalin for four weeks and embedded in paraffin wax. Samples were cut into 5 µm sections and adhered to a slide. Sections were deparaffinised in two xylene washes, sequentially decreased concentrations of ethanol and 50mM Tris-HCL wash buffer. For heat induced antigen retrieval, slides were heated in a decloaking chamber in either Tri sodium citrate buffer or Tris-EDTA buffer for five minutes at 95°C then washed twice in 50mM Tris-HCL. Delineated sections were incubated in 3% hydrogen peroxide for five minutes then washed three times with 50mM Tris-HCL. Biocare Medical Background SNIPER was pipetted onto sections for 10 minutes at room temperature to block before adding the primary antibody (against the macrophage and monocyte cell marker CD68, Precision Antibodies, Bio-Rad) diluted to 1:100 and 1:200 in Dako antibody diluent and IgG control for one hour. Slides were then washed three times in 50 mM TrisHCL then incubated with the secondary antibody (DAKO Envision+ Dual Link System) for 45 minutes. 1:50 DAB solution (DAKO) was incubated on slides and washed in distilled water when colour developed then washed once in 50mM Tris-HCL. Slides were counter stained with Mayer's Haematoxylin for two minutes, rinsed in distilled water and 0.1% ammonium hydroxide added for approximately 30 seconds before being rinsed off with distilled water. Slides were then dehydrated with sequentially increasing concentrations of ethanol and then two xylene washes before mounting with a coverslip (Eukitt quick hardening mounting media). Photographs of the slides were taken on an Olympus BX41 microscope with a microscope-mounted Nikon Coolpix 4500 digital camera (MicroPublisher 3.3 RTV, Olympus, Q-Imaging).

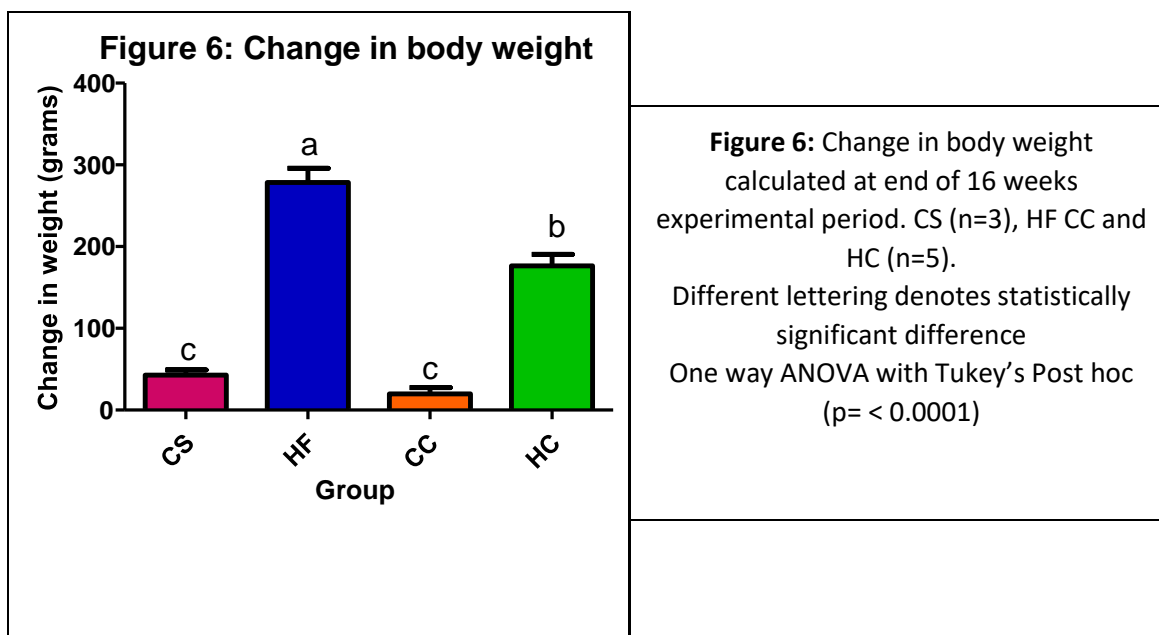
2.6 Statistical analysis

All data presented as mean +/- standard error of mean. Statistical significance ($p > 0.05$) as determined using one way ANOVA with Tukey's correction. Analysis was performed in GraphPad Prism.

Chapter 3: Results

3.1 Physiological Parameters:

After the 16 weeks of protocol, the rats in the HF group had gained a statistically significant amount of weight in comparison to the control CS group. There was also a significantly greater weight gain in the HC group compared to the CC group. Most notably, the weight gain of the HF group was significantly higher in the HF rats compared to the capsaicin treated HC rats. These changes are illustrated in figure 6.

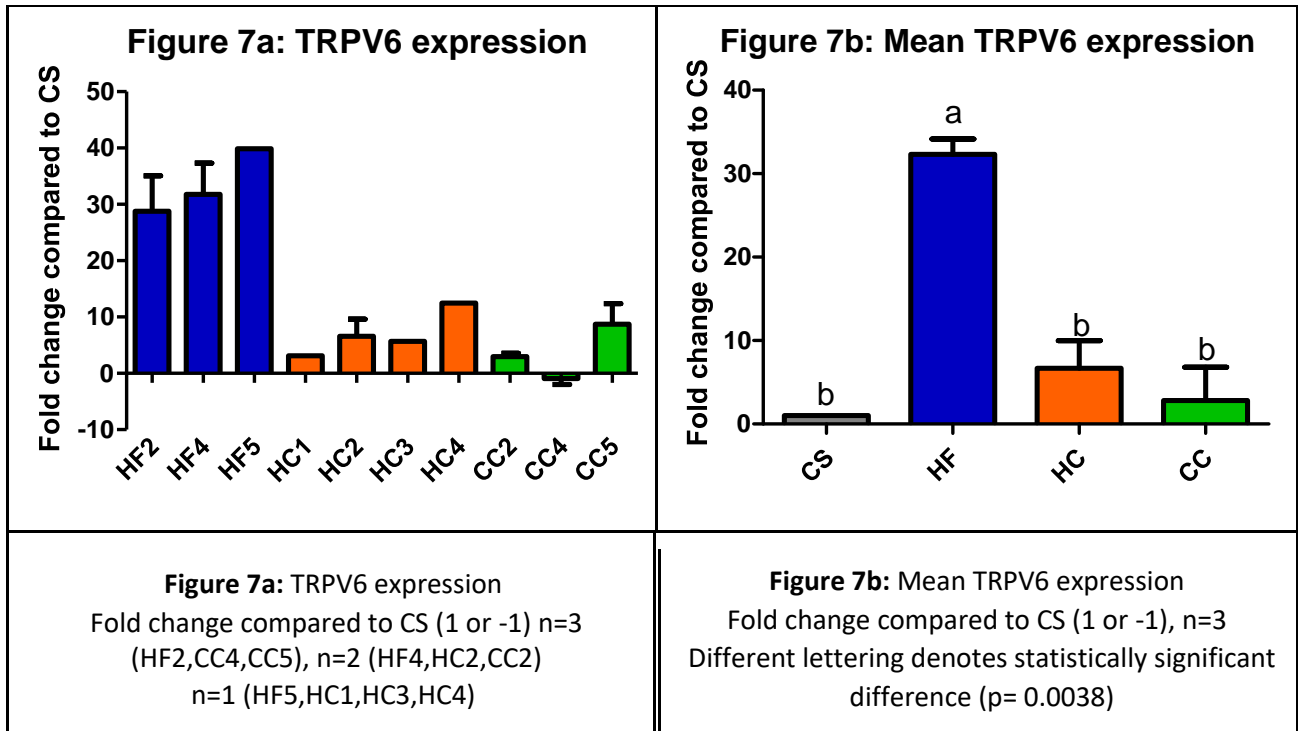


3.2 Gene expression in prostatic tissue

TRPV1 was excluded from the study as the expression in the prostate was too low to be reliable. IL-6 was also excluded from the study due to unreliable results.

Figure 7a illustrates the change in expression of TRPV6 compared to the CS group (as control CS = 1 or -1). There was a statistically significant increase in the HF group as shown in figure 7b. This effect was

not observed in the HC group, receiving the same diet with the addition of capsaicin for the last 8 weeks of protocol. The small increases observed in the CC and HC group weren't significantly higher than the control.



The prostate specific gene NKX3.1 demonstrated an increase in expression in three of four HF and CC animals (figure 8a), with larger increases observed in the CC group. Conversely three of four animals in the HC group showed a decrease in expression. There is a general trend towards an increase in NKX3.1 expression in the CC group (figure 8b), however the mean response of the HF and HC group is no different to the CS control (as control CS = 1 or -1).

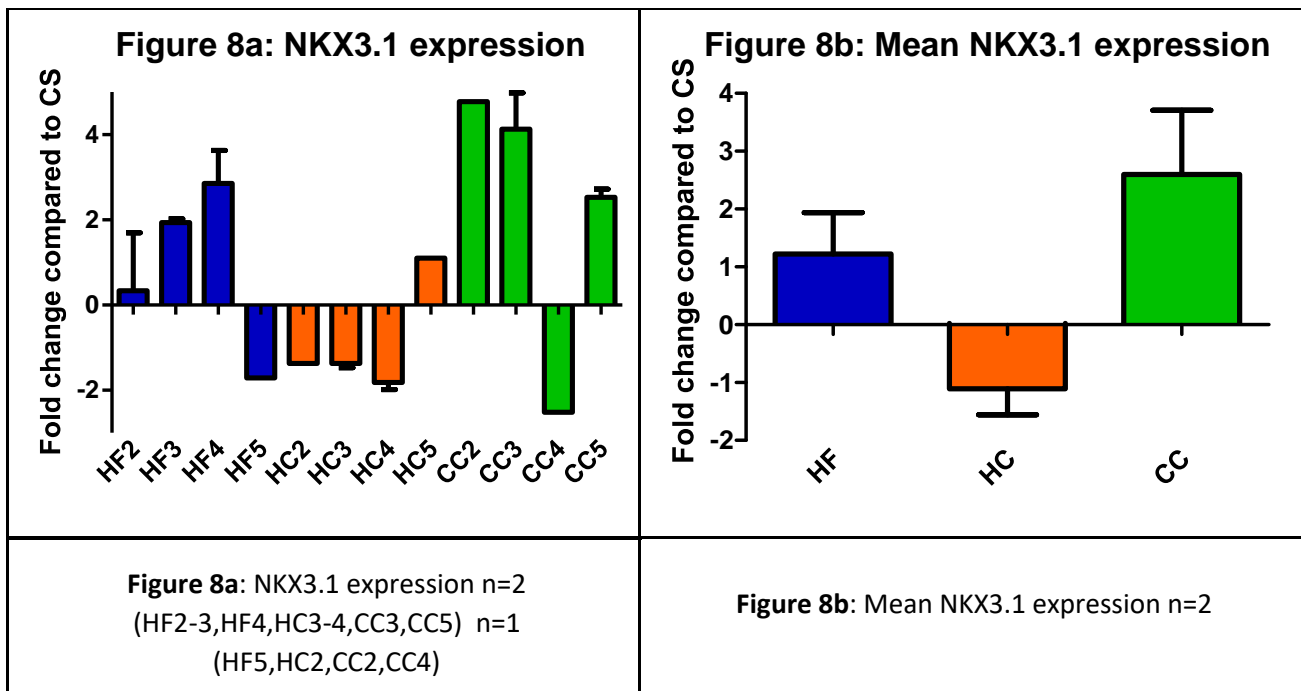
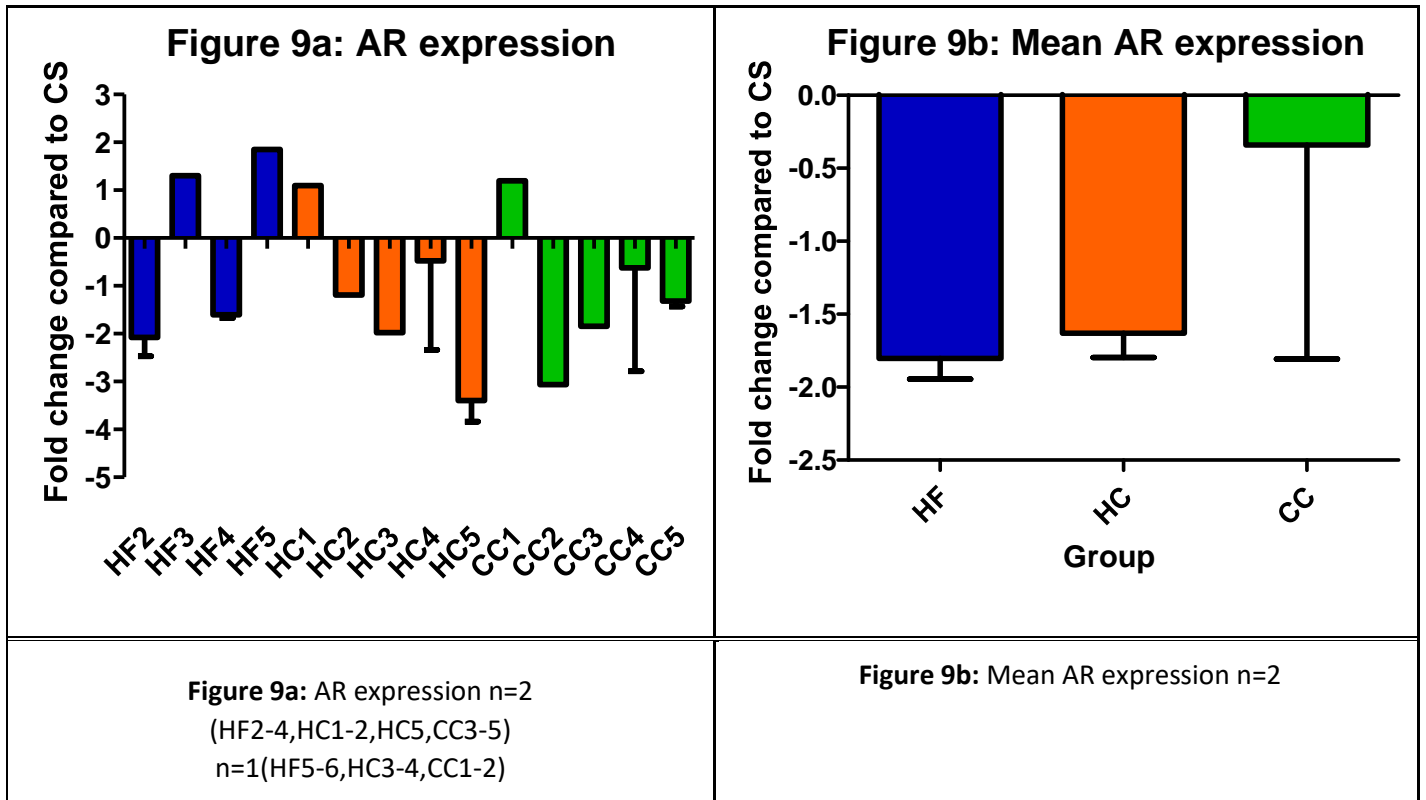


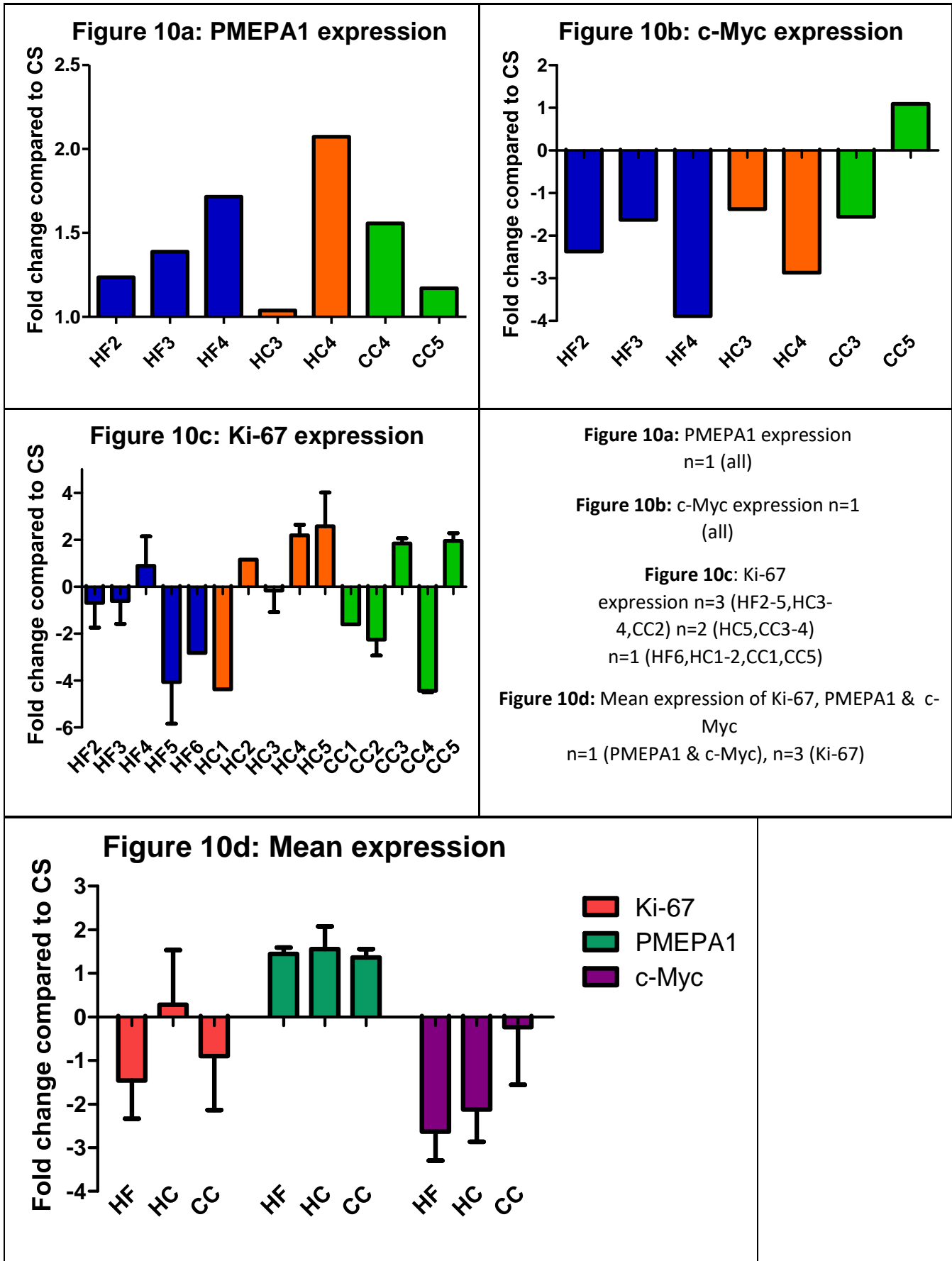
Figure 9a shows the relative change of expression of the AR in the HF, CC and HC animals. There is a high degree of biological variation between the individuals ranging from a two-fold increase to a twofold decrease in HF, and from no change compared to CS (as control CS = 1 or -1) to approximately a three-fold decrease in HC and CC. The mean fold change (figure 9b) shows a small decrease in the HF and HC group compared to the CS group and the CC group showed no overall change compared to CS in response to either the diet or capsaicin.



In figure 10a PMEPA1 showed only a slight increase in all animals compared to the CS group. Due to variation between the individual animals no overall trend is discernible. The greatest variation was seen in the HC group with very little change in HC3, and a two-fold change in HC4. As shown in figure 10d the mean expression change demonstrates no difference between the groups, suggesting there is no effect of either the diet or capsaicin.

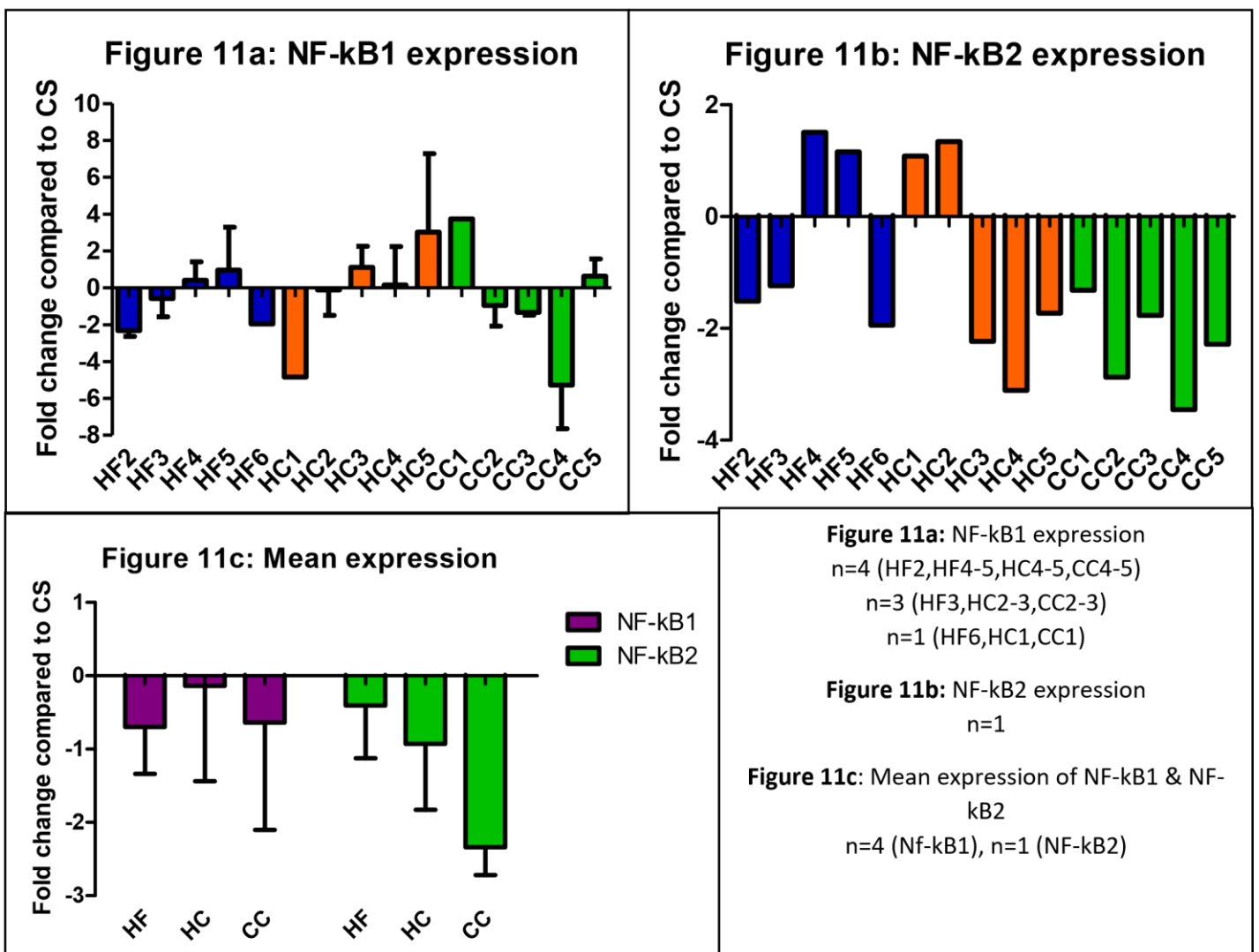
The expression of the c-Myc (figure 10b) demonstrated a decrease in all individuals in the HF and HC group. There was no significant difference between the mean expression of the groups (figure 10d).

In figure 10c the expression of the cellular proliferative marker Ki-67 demonstrates substantial biological diversity. In all groups there are both increases and decreases in expression however the mean expression change (figure 10d) shows no significant change in expression compared to CS (as control CS = 1 or -1).



The expression of NF-kB1 in figure 11a shows no observable trends, with large variations between individuals of the same group and little change compared to CS (as control CS = 1 or -1), also shown by the mean expression (figure 11c).

In figure 11b, the expression of Nf-kB2 in the CC group showed a consistent decrease in all individuals. Both HF and HC group showed greater variation with an increase in two individuals and a decrease in three individuals. The decrease in expression in the HC group animals is slightly greater than in the HF group as shown in figure 11c.



3.3 Prostate Histology

Immunohistochemistry was performed on sections of rat prostate using CD68 (figure 12), a cell marker of inflammation by macrophages and monocytes (Bio Rad), however the protocol required further optimisation. The staining of CD68 cells could not be determined however the differences in histology of the prostate gland in the CS and HF rats demonstrated morphological differences with a slight increase in height of the epithelial luminal cells in the HF prostate compared to the CS and reduced glandular infolding in the HF prostate suggesting some cellular irregularities.

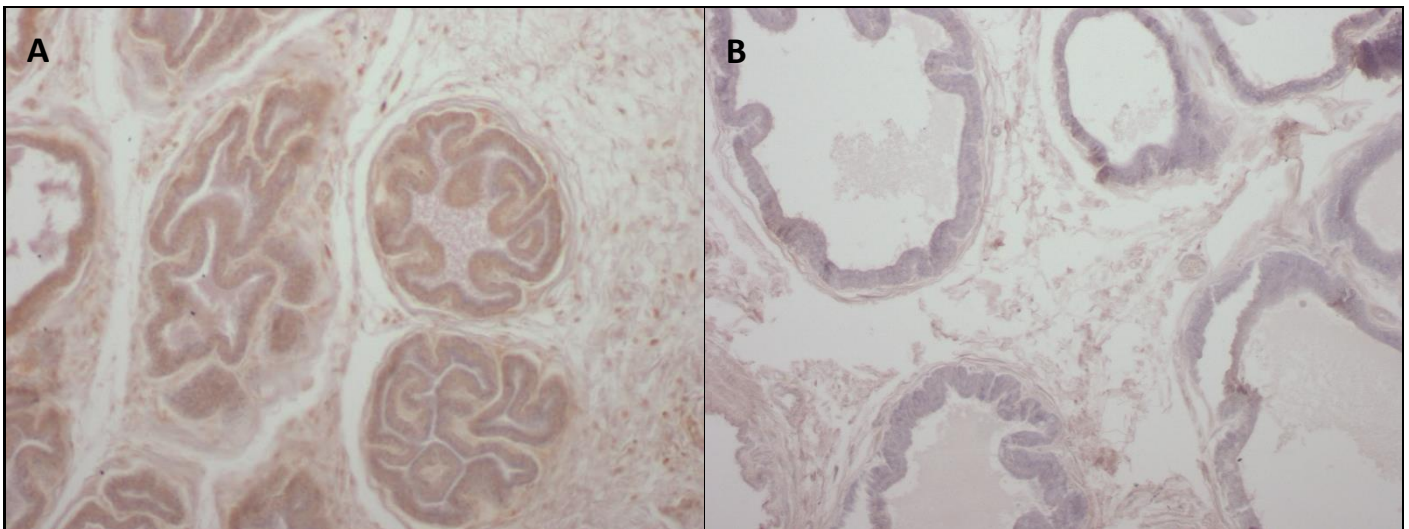
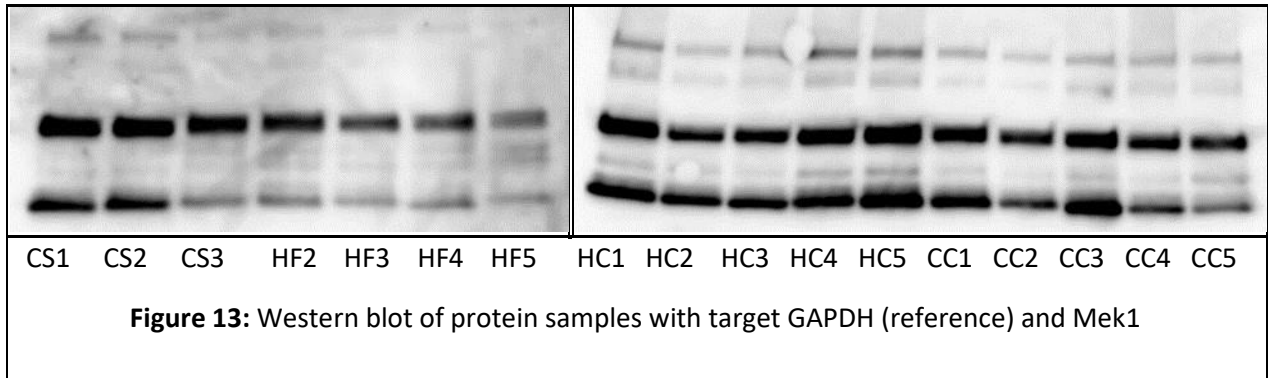


Figure 12: Effect of diet on the prostate gland **A:** Control CS rat (100X), **B:** Western diet HF rat (100X)

3.4 Western Blot

In the Western blot (figure 13) the reference GAPDH showed diffuse banding, while no new bands were observed after incubation with MEK1, consequently the protocol requires further optimisation to provide results.



Chapter 4: Discussion

The main findings of the investigation were the significant increase in TRPV6 gene expression in the prostate of rats fed a Western diet high in refined carbohydrates, and saturated fats compared to rats fed a normal diet and that the addition of capsaicin to this Western diet resulted in a statistically significant decrease in the gene expression of TRPV6. As TRPV6 is involved in prostate cancer tumourigenesis, the gene expression changes provide preliminary evidence that the addition of capsaicin to the Western diet can prevent diet-induced tumourigenesis in the prostate gland.

TRPV6, previously known as calcium transport protein or calcium transport protein-like, is not typically expressed, or expressed at low levels, in normal prostatic tissue, whereas overexpression is observed in cancerous prostate cells (Wissenbach et al. 2001). Overexpression of TRPV6 has also been shown to increase cell survival in both cancerous and non-cancerous prostate cell lines via apoptotic resistance and promoting cell proliferation (Raphaël et al. 2014). TRPV6 over expression is also observed in cancerous prostate cells and is associated with high Gleason scores (Peng et al. 2001). The reduced expression of TRPV6 in the capsaicin treated rat receiving the Western diet rats compared to the rats receiving only the Western diet suggest there may be a preventative effect, considering the association of increased TRPV6 expression with prostate cancer.

The increased expression of TRPV6 observed in this study suggests that the Western diet may be contributing to increased cellular proliferation via this receptor. As no significant effects were observed in other tumourigenic or proliferative markers, c-Myc, Ki-67, NF-kB1 and NF-kB2, the modification in expression of TRPV6 may be one of the earliest molecular changes in the rat prostate in response to a Western diet. Previously, the effect of diet on TRPV6 expression has only been reported in the intestine of rats, where a normal diet with 10% of calories from fat was compared to a high fat diet (45% of calories from fat) predominantly containing either monounsaturated fatty acids

or saturated fatty acids. No significant changes were observed in the intestinal expression of TRPV6 in response to diet (Wang et al. 2016). This study appears to be the first to examine TRPV6 expression in response to diet in the prostate and it would appear that unlike intestinal expression, TRPV6 expression increases in response to a Western, high-carbohydrate, high-fat diet.

When the rats were fed the Western diet supplemented with capsaicin for the last eight weeks of the protocol, the increase in TRPV6 expression stimulated by the Western diet was prevented. This may be due to capsaicin having an apoptotic effect on the proliferation-stimulated prostate cells.

In lung cancer cell lines, capsaicin has been shown to induce apoptosis via TRPV6 whereas very little apoptotic activity was observed in normal lung cell lines. Additionally, it was also observed that lung cancer cell lines highly expressed TRPV6 and normal cells demonstrated considerably lower expression (Lau et al. 2014), similarly to the finding in prostate cancer (Raphaël et al. 2014). In gastric cancer cells, capsaicin causes apoptosis primarily via TRPV6, as silencing of the gene significantly reduces the apoptotic effect (Chow et al. 2007).

Recently, a Phase I human trial in advanced solid cancers has been performed investigating the compound SOR-C13, a selective antagonist of TRPV6, with a high affinity for the receptor (Fu et al. 2017). The compound was originally isolated from a peptide located in the salivary glands of the northern short-tailed shrew and shown to inhibit proliferation in 12 cancer cell lines by preventing TRPV6 calcium uptake, resulting in apoptosis. In some cell lines COR-C13 was as effective as the chemotherapy drug cisplatin, resulting in the progression of research to the phase I trial (Bowen et al. 2013). In the trial, 22 patients with various types of advanced solid tumours received at least one cycle (20 patients completed at least two) of SOR-C13 treatment. The safety profile was acceptable, with the main adverse reactions consisting of hypocalcaemia and hypersensitivity, treatable with calcium and vitamin D supplementation and antihistamines, and could be combined with other chemotherapy agents with a minimal risk of cumulative toxicity. After two cycles of SOR-13, half of the patients were observed to have stable disease, including in two out of the three patients receiving the lowest dose.

Based on the promising results of the trial, further research was recommended (Fu et al. 2017). Given the anti-tumour effect in the trial, targeting TRPV6 shows excellent potential. In this study, the reduced expression of TRPV6 in the capsaicin treated HC rats suggests a possible chemopreventive mechanism of action for capsaicin.

The effect of diet and capsaicin on NKX3.1 expression haven't been previously studied. In a mutant mouse model of NKX3.1 with loss of both alleles of NKX3.1, dysregulation of genes coding for prooxidant and antioxidant enzymes were observed. This dysregulation could be seen as early as four months of age, eventually culminating in DNA damage, and the onset of pre-cancerous PIN lesions in mice over 12 months of age (Ouyang et al. 2005). The increased expression in the control corn-starch diet with the addition of capsaicin for most individuals would therefore suggest a potential beneficial effect. The increased expression of TRPV6 in the rats fed a Western diet and decreased expression in the rat fed the Western diet with the addition of capsaicin suggests the rats fed the Western diet would be more predisposed to forming pre-cancerous lesion with age, whereas the reduced TRPV6 expression in the rats fed the Western diet and treated with capsaicin suggested a lower risk.

In other tumourigenic or inflammatory genes, no statistically significant effects on expression were observed, with large biological variation occurring between individual animals of the same group.

The AR signalling pathway is complex, with the effects of expression on the prostate dependent on downstream targets, such as c-Myc (Vander Griend, Litvinov & Isaacs 2014). In the context of high-fat diet induced obesity, the expression of the AR has been shown to decrease along with circulating concentrations of testosterone in the prostate of rats. The prostates of obese rats also showed fat accumulation, increased proliferation in the epithelial and stromal tissue and higher rates of precancerous PIN lesions. The increased proliferation induced by the high-fat diet was attributed to an increase in oestrogen receptor signalling and insulin resistance (Ribeiro et al. 2012). The effects of the hormone oestrogen and its receptors haven't been explored in this study, but in future studies

would be important in elucidating the hormonal signalling effects of a Western diet on prostate tumourigenesis.

PMEPA1 expression is induced by the AR, then forming a negative feedback loop inhibiting expression of AR downstream targets and inhibiting its activity, as shown by Xu et al. (2003). In prostate cancer, PMEPA1 is silenced by methylation and also exhibits down-regulation (Akinsete et al. 2011). There was no significant change in expression of PMEPA in this study, however methylation of gene was not studied and potentially changes may not be observed in the prostate until later stages of tumourigenesis.

There was no significant change in c-Myc expression which is not entirely unexpected even though the AR promotes prostate cancer cell survival through c-Myc upregulation (Gao et al. 2013). In normal cells the highly regulated androgen dependent AR signalling causes a decrease in c-Myc expression, suppressing growth and an upregulation of c-Myc is observed early in tumorigenesis, as demonstrated by Vander Griend, Litvinov and Isaacs (2014). Tumourigenesis is not expected to have occurred in the rats used in this study at their age (approximately 28 weeks), therefore no significant change in c-Myc expression is consistent with the literature of non-cancerous prostate cells.

It would be expected that the expression of Ki-67 would increase in the Western diet fed rats, as previous research shows a high fat diet promotes proliferation of the prostate and therefore increases the expression of the proliferative marker Ki-67 (Kwon et al. 2016). The large decrease in some animals in the rats fed the high-carbohydrate, high-fat diet are unlikely to represent true results as the prostate in the HF were visually determined to be larger in size, therefore potentially errors in RT-qPCR procedure may account for this result, such as evaporation during plate loading or variation in cDNA conversion.

The high level of variation observed in animals in regards to Nf-kB1 expression also prevents the observation of any trends. NF-kB1 encodes the p50 subunit which is involved in the classical signalling pathway of NF-kB. The alternate NF-kB pathway includes p52, produced from the NF-kB2 precursor

protein (Jin et al. 2015). The expression of NF- κ B in the CC group is consistently decreasing in all individuals, suggesting capsaicin may reduce the expression compared to the CS group. It is difficult to determine the response in the HF and HC group due to the variation between the animals. It was expected that NF- κ B signalling would increase in the HF group, as previous research has shown increased activation of the NF- κ B p65 unit in response to a high fat diet (Shankar et al. 2012).

Histomorphological changes in rats fed a Western diet high in mammalian fat and simple carbohydrates suggested a negative impact on the prostate gland. A reduction in the presence of glandular infolding has been observed in a study of mice engineered to lack the expression of the epithelial AR. The mice developed various abnormal morphological features, including dedifferentiation, increased epithelial proliferation and sloughing off of epithelial luminal cells into the lumen of the gland, related to the loss of epithelial AR expression (Wu et al. 2007). This study has observed a minor decrease in AR expression in the Western diet fed rats where loss of glandular infolding was also observed. It is not known, however whether the expression of the AR could have been reduced differently in epithelial or stromal cells, and may be an interesting area for future research. Another study compared the morphology of the prostate gland in rats fed a control diet or various forms of high-fat diet, high in either saturated fatty acids, polyunsaturated fatty acids, or both. After approximately 16 weeks, the rats fed the high fat diets displayed abnormal cellular features, with the diet high in both saturated and unsaturated fatty acids showing the most significant increases in epithelial cell height, area density of stromal tissue and epithelial proliferation. The increase in epithelial cell height was attributed to a possible increase in the size of organelles, including the endoplasmic reticulum and the Golgi apparatus, therefore increasing their secretory abilities (Furriel et al. 2014). It would be beneficial to measure the PSA production in the prostate and serum concentrations to confirm if the changes in epithelial size correlate with PSA secretion.

There were several limitations to this study. Predominantly, the small number of animals (three for CS and five for the other three groups) made determining statistical significance difficult due to the

variation between the animals. Using a larger number of animals will likely improve the ability to determine trends and statistical significance. Another difficulty was the sampling of the prostate, as determining the boundary of the organ was problematic, particularly with a large peri-prostatic adipose tissue layer, as observed in the rats fed the Western diet. As rats have multi-lobed prostates, a representative sample may not have been obtained for all animals, which may have interfered with expression of gene and account for some variation. Additionally, the integrity of RNA extracted for RTqPCR analysis can affect the results, and therefore stringent conditions during tissue collection and RNA extraction were required. The integrity of the RNA was determined using the Agilent Bioanalyser however, and all samples were suitable for further analysis. Variation in technical replicates was most likely due to variations in cDNA conversion prior to RT-qPCR. Due to limited availability of iScript required for the conversion, multiple batches of cDNA were produced, whereas the ideal situation for reproducible results would be to use one batch of stock cDNA for all for RT-qPCR replicates.

From the results of this study, TRPV6 appears to be a promising target for capsaicin chemoprevention of prostate cancer. Supporting this is a case study of a prostate cancer patient who after receiving radiotherapy had an increase in PSA, indicating a biochemical failure, and due to side effects couldn't tolerate other forms of treatment including ADT. The patient began taking capsaicin, in habaneros chili sauce, and during this time the PSA concentrations stabilised and resumed increasing after cessation of capsaicin ingestion (Jankovic, Loblaw & Nam 2010).

It would be beneficial to thoroughly investigate the effect of TRPV6 overexpression and inhibition in cell culture on the expression of genes related to proliferation and tumorigenesis. Additionally, the apoptotic signalling pathways should be studied as TRPV6 has been shown to induce apoptosis in cancer cell lines and these pathways could potentially be involved in the preventative effect (Chow et al. 2007).

Furthermore, the use of older rats, particularly Lobund Wistar with the higher rate of testosterone induced prostate cancer, in a study could provide more clinically relevant information on the effects

of capsaicin on pre-cancerous prostate. This could determine if capsaicin may be used as a dietary intervention in men with pre-cancerous lesions to reduce the risk of progression to cancer.

Chapter 5: Conclusion

This study investigated the effect of a high-carbohydrate, high-fat Western diet on gene expression and cell morphology in the prostate. Using RT-qPCR, it was shown that the expression of TRPV6, a capsaicin receptor which is also overexpressed in prostate cancer, was significantly increased in response to the Western diet. Notably, the increase in expression was not observed in rats receiving the same diet when capsaicin was included in the diet, suggesting a potential chemopreventive effect. The expression of other genes related to proliferation, tumorigenesis and inflammation showed no significant change. The morphology of the tissue in the prostate gland showed the occurrence of a number of abnormal changes in the Western diet fed rats compared to the control diet. The increased epithelial cell height and reduction of glandular infolding are associated with pre-cancerous changes in the prostate, suggesting the Western diet is inducing abnormal cellular alterations.

As it appears capsaicin may have a chemopreventive effect in Western diet induced abnormalities in the prostate, future research should further investigate the tumourigenic, apoptotic and hormonal pathways involved in prostate tumourigenesis. Potentially, capsaicin could be an inexpensive and easily sourced dietary supplement to reduce the risk of prostate cancer.

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