Analysis of Cytokine Expression in Prostate Cancer Patients Undergoing Radiation Therapy: Correlation with Clinicopathological Outcomes

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<td>Allan Walker Cancer Care Centre</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer 2</td>
</tr>
<tr>
<td>BRIP1</td>
<td>BRCA1 interacting protein1</td>
</tr>
<tr>
<td>CHEK2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CSS</td>
<td>Cancer specific survival</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>GS</td>
<td>Gleason score</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPPC</td>
<td>Higher percentage of positive cores</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin - 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin - 8</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity-Modulated Radiotherapy</td>
</tr>
<tr>
<td>JAK1</td>
<td>Janus kinase 1</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Ng/ML</td>
<td>Nano gram/Millilitre</td>
</tr>
<tr>
<td>NIK</td>
<td>Transcription factor inducing kinase</td>
</tr>
<tr>
<td>NT</td>
<td>Northern Territory</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner and localizer of BRCA2</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PCAP</td>
<td>Predisposing for prostate cancer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>RP</td>
<td>Radical prostatectomy</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>T1c</td>
<td>Tumour 1c</td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour 2a</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor- β1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour Node Metastasis</td>
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THESIS DECLARATION

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of Charles Darwin University, is the result of my own investigations, all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not been accepted in substance for any degree and is not being currently submitted in candidature for any other degree.

Jagtar Singh

Date: 21-08-2019
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- Our laboratory technicians, who helped to locate specimen in the biobank, and were always happy to facilitate experiments with their invaluable technical assistance.
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The capacity to diagnose and stage prostate cancer (PC) is restricted and generally based on pre-treatment assessments such as the prostate-specific antigen (PSA), TNM stages, and the pathologic Gleason score. However, existing pre-treatment tools cannot be used to predict acute RT-induced toxicity. Therefore, new protein biomarkers are required in RT oncology to improve decision-making, treatment and therapy monitoring for PC patients.

This prospective study aimed to assess the magnitude and frequency (increase/decrease) of changes in cytokine expression in patients receiving androgen deprivation therapy (ADT) and intensity-modulated radiotherapy (IMRT) for PC and to link these changes to clinicopathological characteristics and acute RT-induced toxicity. Principal findings include the following:

Firstly, IHC expression levels of TNF-α, TGF-β1 and IL-6 were significantly reduced with increased GSs and a significant correlation was only found between TNF-α expression levels and GSs.

Secondly, a statistically significant correlation was found between the amount of pre-RT plasma levels and the staining intensity of the corresponding prostatic needle-biopsy specimens.

Thirdly, profibrotic cytokine TGF-β1 levels were elevated at the end of RT over baseline. In addition, IL-6 increased after 3 months completion of RT when compared with baseline and end of RT blood plasma samples.
Fourthly, elevated levels of TNF-α and IL-6 were associated with the higher probability of acute genitourinary and gastrointestinal toxicity.

Finally, the levels of profibrotic TGF-β1 decreased as the severity of genitourinary and gastrointestinal increased.

This dissertation provides the evidence of the overexpression of cytokines in prostatic needle-biopsy specimens and influence of androgen deprivation therapy (ADT) and radiotherapy (RT) on the changes in cytokine levels in blood plasma before and after curative treatments. In addition, our study also provides the evidence of the association of cytokines levels in blood and acute RT-induced toxicity.
Chapter 1 - Introduction

1.1 Background

Prostate cancer (PC) is the most commonly diagnosed malignancy (excluding non-melanoma skin cancer) and accounting for nearly 1 in 5 new diagnoses (Siegel, Rebecca L, Miller & Jemal 2019). In 2019, an estimated of 174,650 men in the United States will be diagnosed with PC (Siegel, Rebecca L, Miller & Jemal 2019). It accounts for 15% of cancers diagnosed in men, with almost 70% of cases occurring in developed countries including the United States of America (USA), Australia, New Zealand and Europe (International Agency for Research on Cancer 2014). Asian and African countries exhibit the lowest incidence rates for PC (Kvale et al. 2007; Marugame & Katanoda 2006; Shin et al. 2010). The common risk factors for PC are age, family history, ethnicity, diet, and genetic factor (Hosseini et al. 2010; Tao et al. 2015).

In Australia, PC is the third most common cancer diagnosed and the third most common cause of death (Feletto et al. 2015). It is more common in older men, with 63% of cases diagnosed in men over 65 years of age. In the Northern Territory (NT), the incidence rate of PC in non-Indigenous men was 19% lower than the total Australian rate; however, their mortality rate was similar (Xiaohua Zhang et al. 2014). The incidence and mortality for Indigenous males were much lower than total Australian rates; incidence was 74% lower and mortality 81% lower than non-Indigenous males (Xiaohua Zhang et al. 2014).

Treatment option for PC depends on a number of tumour and patient factors. Radical prostatectomy (RP) or radiotherapy (RT) are common treatment modalities utilized for localized PC. RT is a non-surgical treatment modality which focusses megavoltage photon
beams on the prostate gland and close surrounding tissue, with the aim of good local-regional control and prolonged disease-free survival (DFS) for PC patients (Wu et al. 2013). However, loco-regional post-RT relapse still occurs in some treated patients (Wu et al. 2013). RT is often administered with androgen deprivation therapy (ADT) for intermediate and high-risk PC (Basaria et al. 2002; Harle et al. 2006). The immunological consequences of ADT need to be studied as they may enhance the possible immunosuppressive actions of RT, resulting in serious clinical consequences (Basaria et al. 2002; Harle et al. 2006). Unfortunately, the immunological consequences of ADT, both alone and in combination with RT, are not well described and therefore, there is clearly a need for more investigation into this area.

The capacity to diagnose and stage PC is restricted and generally based on pre-treatment assessments such as the prostate-specific antigen (PSA), TNM stages, and the pathologic Gleason score (GS) (Nichol, Warde & Bristow 2005). However, existing pre-treatment factors cannot be used to predict acute RT-induced toxicity. Therefore, new protein biomarkers are required in RT oncology to improve decision-making, treatment and therapy monitoring for PC patients. Furthermore, many pro-inflammatory cytokines can influence the redox status in irradiated cells and the surrounding microenvironment, thereby affecting PC progression, RT efficacy and RT-induced toxicities (Miao et al. 2014). Still, an accurate understanding of cytokines behaviour remains elusive.

Cytokines are water soluble, low molecular weight proteins that transport signals between cells (George et al. 2005). Inflammatory cytokines have been shown to influence tumour progression, invasive potential, angiogenesis and RT resistance (Deorukhkar & Krishnan 2010). Rubin et al. (1995) were among the first to describe the role of cytokines in mediating RT-induced toxicity. They reported that levels of interleukin (IL)-1, transforming growth
factor (TGF)-β, and tumour necrosis factor (TNF)-α were increased immediately after radiation exposure and that elevated TGF-β levels were associated with increased risk of pulmonary fibrosis. Another study also reported that Interferon-γ (IFN-γ) and Interleukin-6 (IL-6) significantly increased during prostate RT with an associated increase in acute gastrointestinal and genitourinary toxicity (Christensen et al. 2009).

Over the last two decades, many studies have focused upon investigating the prognostic potential of circulating cytokines to act as biomarkers of RT-induced toxicities and complications (Chen, Y et al. 2001; Chen, Y et al. 2002; Fleckenstein et al. 2007; Kovacs et al. 2003; Muller & Meineke 2007). Generally, RT-induced toxicity is considered as acute or late toxicity, according to the time before the appearance of symptoms. RT-induced toxicity is classified as acute if it occurred during RT and in the first 3 months thereafter (Berkey 2010; D'Avino et al. 2015). Whereas it is classified as late if it occurred after 3 months from the completion of RT (D'Avino et al. 2015; Dorr & Hendry 2001). However, late effects could develop months to years after radiation exposure (Berkey 2010; D'Avino et al. 2015).

There are many pieces of evidence in scientific literature that several cytokines are systematically upregulated in response to cancer progression and radiation exposure. The assessment of cytokines that are involved in cancer progression and treatment response may lead to the identification of potential reliable biomarkers and aid in the development and validation of immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) assays. In addition, the immunological consequence of ADT and RT in PC patients is not well defined within the scientific literature. Therefore, there is clearly a need for research in this area. In our current research, prostatic tumour tissue and normal prostate tissue as a control were examined for expression of cytokines with IHC method. In addition to this,
blood plasma of same patients and healthy individual plasma as a control were also assessed with ELISA to investigate the cytokines levels before ADT, before and after RT. It is proposed that identifying cytokines as innovative biomarkers will contribute to the understanding of the effect of ADT and RT on cytokines expression and their side effects on the PC patients.

1.2 Knowledge gap

The scientific literature reported the association of cytokine expression or their levels with clinicopathological characteristics and acute RT-induced toxicity in PC patients who underwent ADT and RT (Christensen et al. 2009; Johnke et al. 2009). There was an important gap between previous findings and the proposed research. This clinical study will analyse the knowledge gap from the following angles:

First, this is not the first study to attempt to find any correlation between plasma cytokine levels and acute RT-induced toxicity and clinicopathological outcomes in PC patients receiving RT (Christensen et al. 2009; Johnke et al. 2009). Previous studies measured the cytokine levels before RT and designated time intervals during RT (Christensen et al. 2009; Johnke et al. 2009). However, in this clinical study, cytokines levels were assessed in blood before ADT, prior to RT, at the end of RT and after the completion of 3 months of RT. The assessment of 3 months blood is actually needed to fully address the cytokines levels and their association with acute RT-induced toxicity in PC patients. The above studies did not assess the patient’s blood before ADT and after 3 months completion of RT.
Second, this is foremost a clinical study in NT, prostatic needle-biopsy specimens and blood plasma obtained only from the PC patients of the NT and assessed by using IHC and ELISA methods respectively. This gap is also actually needed to be addressed.

1.3 Hypotheses

In this observational study, the expression of the cytokines TNF-α, TGF-β1, IL-6 and IL-8 were selected for investigation in PC patients. These four cytokines have been recognized as playing a potential role in PC invasion and metastasis. In this study, it was hypothesized that there is a potential association between cytokine expression and clinicopathological characteristics and acute RT-induced toxicity. This work aimed to analyse cytokines TNF-α, TGF-β1, IL-6 and IL-8 in the maximum number of plasma samples. In addition, prostatic needle-biopsy specimens were also investigated from a subset of the same PC patients from whom plasma samples were studied. It is important to note that levels of selected cytokines in blood plasma of PC patients were analysed prior to ADT, prior to RT, at the completion of RT, and 3 months after RT. Other parameters for the post-RT cohort were rectal, bladder and bowel function.

1.4 Objectives

1. To assess cytokines expression in prostatic needle-biopsy specimens;
2. To assess cytokines levels in blood plasma before and after ADT and RT;
3. To assess the influence of ADT and RT on changes in cytokine levels in blood plasma;
4. To assess any correlation between cytokines expression/levels and clinical outcomes including RT-induced toxicity (acute toxicity).
1.5 **Expected study output**

The discovery of potential cytokines as molecular biomarkers may aid in the treatment of PC and could help predict which patients undergoing RT will suffer symptoms and side effects. However, several assessments for validated biomarkers may be needed with the highest degree of accuracy and specificity of RT. This clinical prospective study will significantly contribute to monitoring prostatic needle-biopsy specimens and blood plasma for the concentration of cytokines and allow for predictive risk assessment of patients irradiated for RT. Hopefully, this clinical study will identify reliable biomarkers of acute RT-induced toxicity and therefore provide the opportunity to rectify the treatment modality to improve the therapeutic outcome. Moreover, findings from this study will highlight the rate of complications in the management of PC in the NT and this will allow for comparisons with other centres.

1.6 **Purpose of study**

In the current research, prostatic needle-biopsy specimens and blood plasma were assessed with IHC and ELISA methods to investigate the pattern changes or variation in cytokines levels. The research will raise awareness of the impact of ADT and RT on cytokine expression in PC in the NT population and the association with clinicopathological characteristics and acute RT-induced toxicity and data will be comparable to other Australian data sets.

1.7 **The rationale for the study**

The NT population experiences several aggressive cancers and a higher mortality rate than the other States and Territories of Australia. No clinical research has been done on PC in the NT population. However, some epidemiological studies have reported on PC incidence and
mortality rates among the NT population. Survival after diagnosis is poorer for the Aboriginal population and that may be due to the difference in the timing of diagnosis and access to PC treatment between Aboriginal and non-Aboriginal men (Cunningham et al. 2008; Morrell, You & Baker 2012).

In addition, an immunological consequence of ADT and RT in PC patients from the NT is not defined within the scientific literature. Thus, there is clearly a need for research in this area. In our current research, prostate needle-biopsy specimens and blood plasma were assessed to investigate the expression and variation in cytokine levels before and after RT. In doing so, we hoped to find a relationship between changes or variations in expression levels of selected cytokines TNF-α, TGF-β1, IL-6 and IL-8 with clinicopathological characteristics and acute RT-induced toxicity. It is estimated that identifying cytokines as novel biomarkers will contribute to the understanding of the effect of ADT and RT and their side effects in the NT population and will inform future service delivery of therapeutic treatments.
1.8 Thesis Outline

**Chapter 1: Introduction**  
Provides the context and an overview of the aims

**Chapter 2: Expression of circulatory cytokines in prostate cancer patients undergoing androgen deprivation therapy and radiotherapy and the association with clinical outcomes: a systematic review**  
Introduction the relevant background research

**Chapter 3: Methods and Materials**  
Outline the research methodology

**Chapter 4: Immunohistochemical investigation of cytokine expression in prostatic needle-biopsy specimens and correlation with clinicopathological outcomes**  
Describe the cytokines expression and their correlation with pre-operative serum PSA levels, Gleason scores and pre-RT plasma cytokines levels

**Chapter 5: Levels of plasma cytokines in patients undergoing androgen deprivation therapy and radiation therapy for adenocarcinoma of the prostate cancer and correlation with clinical outcomes**  
Describe the effect of Androgen (ADT) deprivation therapy and radiotherapy (RT) on plasma cytokines levels

**Chapter 6: The predictive role of circulatory cytokines in RT oncology for prostate cancer and RT-induced acute toxicity**  
Describe the cytokines levels before and after RT and correlation with RT-induced toxicity such as genitourinary and gastrointestinal acute RT-induced toxicity

**Chapter 7: Summary of Chapters, Recommendations and Conclusion**  
Summarise the finding of thesis and future research directions

*Figure 1-1* A schematic research framework and thesis outline
Chapter 2- Expression of circulatory cytokines in prostate cancer patients undergoing androgen deprivation therapy and radiotherapy and the association with clinical outcomes: a systematic review

2.1 Anatomy of the prostate gland

The prostate gland is a male sex organ situated at the floor of the pelvis. It sits inferior to the bladder and anterior to the rectum (Figure 2-1). The urethra passes through the centre of the prostate from the bladder to the penis and drains urine from the body. The normal prostate is composed of glands and stroma. The glands are coated by two cell layers; the outer low cuboidal layer and an inner columnar mucin-secreting epithelium. The fibromuscular stroma between the glands cover-up is about half of the volume of the prostate. The seminal vesicles are glands found on each side of the prostate. These store the sperm for many days until ejaculation. During ejaculation, the prostate muscles contract and eject the sperm into the prostatic urethra towards the tip of the penis.
Figure 2-1: The prostate gland, adapted from (Theodorescu 2001).
2.2 Function of the prostate gland

The main function of the prostate is the production of seminal fluid that helps nourish and protect the sperm during and after intercourse. This slightly alkaline seminal fluid helps sperm motility and viability. The core components of prostatic secretion are prostate-specific antigen (PSA), citrate (18.7 mg/ml), zinc (488 μg/ml), spermine (243 mg/ml) and cholesterol (78 mg/ml) (Blandy JP & Lytton B 1986).

2.3 Prostate cancer

Prostate tissues are predominantly glandular and malignant transformation from these glandular structures make adenocarcinoma the most common form of PC. PC is mostly adenocarcinoma; however, there is approx. 1% of other type of PC such as sarcomas, small cell carcinoma, neuroendocrine tumour and transitional cell carcinoma (Cecen et al. 2014; Hicks et al. 2016; Parimi et al. 2014). However, other types of PC are very rare, and if someone has cancer it is almost certain to be an adenocarcinoma. The extent of cancer at the time of diagnosis is a key factor used to define treatment and to assess the chance of successful treatment outcome. Cancer staging systems codify the extent of cancer to provide clinicians and patients with the means to quantify prognosis for individual patients and to compare groups of patients in clinical trials and who receive standard care around the world.
2.4 Epidemiology of prostate cancer

2.4.1 Worldwide incidence and mortality rates

PC is the most common malignancy and the fifth leading cause of cancer death in men worldwide (excluding non-melanoma skin cancer) (Siegel, Rebecca L, Miller & Jemal 2019). The incidence of PC differs worldwide, with the highest age-standardized rates in Western developed countries, such as the United States of America (USA) (Ferlay et al. 2013). In the USA, PC is the most common cancer among men, with 180,890 new cases diagnosed and 26,120 deaths recorded in 2016 (Siegel, R. L., Miller & Jemal 2017).

2.4.2 Australian incidence and mortality rates

In Australia, age-standardised incidence and mortality rates for PC were 167/100,000 and 23.4/100,000 respectively in 2010, making it the fourth leading cause of mortality, with 3,294 deaths in 2011 (Feletto et al. 2015). From 2001 to 2010, the age-standard incidence rate of PC in the NT was 119.4 per 100,000. This rate was slightly higher for non-Indigenous males (133.3 per 100,000 when compared to Indigenous males (43.0 per 100,000) (Xiaohua Zhang et al. 2014). Mortality figures are currently unavailable for Indigenous males with PC but the non-Indigenous mortality rate was 29.3 per 100,000 from 2001-2006 (Xiaohua Zhang et al. 2014).
2.5 Major risk factors

The cause of PC is not fully understood and may be multifactorial. Previous studies have suggested some major risk factors include age, family history, ethnicity, diet, and genetic factors (Hosseini et al. 2010; Tao et al. 2015).

2.5.1 Age

PC is almost exclusively a disease of elderly men, aged 50 years and above (Alam et al. 2009; Bashir, Ahmad & Malik 2014; Hosseini et al. 2010). Histological examination of tissue samples from autopsy studies worldwide have indicated that PC incidence increases with age and nearly 75% of men above 80 years show some evidence of latent disease (Billis 1986). It has been suggested that the higher risk of PC among older men is likely because these men were not screened intensely at their younger age (Bechis, Carroll & Cooperberg 2011).

2.5.2 Family history

Males with a first-degree relative, such as a parent or sibling with PC have a higher risk of developing PC compared to those with no such history (Alam et al. 2009; Johns & Houlston 2003). This is also supported by earlier studies, which reported that approximately 11.6% of PC cases had strong associations with the familial history of the disease (Gronberg et al. 1999; Zeegers, Jellema & Ostrer 2003). More recent work has also reported that first-degree relatives of PC patients might have double the risk for developing PC than those without such history (Tao et al. 2015).

2.5.3 Ethnicity

PC is recognised as a disease of the western world. The incidence of PC varies between different ethnic groups and countries, with the lowest incidence rates observed in Asian
populations as compared to the relatively high rates of the disease in the western world (Tao et al. 2015). Many epidemiology studies have reported that PC is much higher among black men who also have a higher stage of disease at presentation (Tao et al. 2015). Moreover, significant evidence for an increased PC incidence in African Americans in comparison to Asian Americans has been presented, which is indicative of both genetic as well as racial differences (Eeles et al. 2014; Ha et al. 2013; Virnig et al. 2009). In Australia, the non-Indigenous population is more likely to diagnosed with PC than Indigenous (Xiaohua Zhang et al. 2014).

2.5.4 Diet
Consumption of diets low in fat and high in vegetables and plant-based foods have a negative impact on the incidence of PC according to some epidemiological studies (Daniyal et al. 2014; Tao et al. 2015; Tyagi, Manoharan & Raina 2010). Less intake of high caloric foods, fatty foods such as red meat and dairy products, and lower calcium intake along with adequate intake of vitamin D and lycopene have been suggested to lower the risk of PC (Lawson et al. 2007). Some previous studies reported that obesity is linked to higher risk of some cancers, such as colon, breast, and hepatic cancer (Basen-Engquist & Chang 2011; Calle & Kaaks 2004). Similarly, obesity was also found to be associated with a higher risk of developing PC as well as cancer recurrence (Allott, Masko & Freedland 2013). Additionally, obesity is also associated with poorer post-treatment results and increased risk of PC death (Buschemeyer & Freedland 2007).

2.5.5 Genetic factors
Early gene mapping studies have identified several chromosomal regions that may be linked to a higher risk of developing PC (Datta et al. 2006). The International Consortium for PC
Genetics (ICPCG) study identified 12 PC risk regions on different chromosomes, including 1q23, 5q11, 5q35, 6p21, 8q12, 11q13, and 20p11-q11 (Xu et al. 2005). Genetic mutations in BRCA1 or BRCA2 appear to increase the risk for PC; however, mutations of BRCA1 or BRCA2 are relatively infrequent in PC with < 0.3% cases (Kote-Jarai et al. 2011; Thorne et al. 2011). In addition, there is high-risk of PC in men above 65 years of age, with germline mutations in the BRCA1 (risk increased by 3.5-fold) and BRCA2 (8.6-fold increase in risk) genes (Kote-Jarai et al. 2011). Other mutations in DNA repair-related genes such as PALB2, BRIP1, CHEK2, NBS1 genes and mutations in DNA mismatch repair genes MLH1, MSH2, MSH6, and PMS2 also have been found to relate to the higher risk of PC (Bauer et al. 2011; Raymond et al. 2013).

### 2.5.6 Other risk factors

In addition to the above-mentioned risk factors, occupation associated risk factor for PC such as the use of or exposure to herbicides and pesticides have been identified, although no specific carcinogenic agent has been recognized (Ragin et al. 2013). Lifestyle factors including smoking and excessive alcohol consumption have also been shown to increase the risk of developing PC (Gago-Dominguez et al. 2016; Roswall & Weiderpass 2015). Zu et al. (2009) believe that smoking encourages the development of more aggressive PC by affecting sex steroid hormone levels and mutations in p53 tumour suppressor genes.
2.6 Diagnosis and staging

PC is often an indolent and asymptomatic disease at an early stage. Initial screening tests employed by the medical community include a prostate-specific antigen (PSA) blood test and digital rectal examination (DRE) (Heidenreich et al. 2011).

2.6.1 Prostate-specific antigen (PSA) analysis

Prostate-specific antigen (PSA) is a glycoprotein produced by the luminal epithelial cells of the prostatic ducts, acini, and peri-urethral glands (Adhyam & Gupta 2012). Prostate epithelial cells secrete PSA and both normal and neoplastic prostate tissues express PSA (Chang, SG et al. 2006). PSA levels can be elevated in non-malignant conditions including benign prostatic hyperplasia (BPH) and prostatitis (Chang, SG et al. 2006).

Some individuals with normal PSA levels (which is ≤ 4ng/mL) and benign DRE had PC, including high-grade cancer; therefore, the uses of DRE and PSA as screening tools have become gradually uncertain (Thompson et al. 2005; Thompson et al. 2004). This particular study assessed 2,950 men, whose PSA levels were below 4ng/ml, and 23.9 % of men diagnosed with PC with PSA values of 2.1-3.0 ng/ml and 26.9 % of those with PSA levels of 3.1 to 4.0ng/ml and 25% of males in the 3.1-4.0 ng/mL had high-grade cancers (Thompson et al. 2004).

2.6.2 Digital rectal examination

Digital rectal examination (DRE) provides information regarding local disease extent and involves a clinician inserting a gloved finger into the rectum to feel the posterior aspect of the prostate through the anterior rectal wall. An experienced clinician can determine whether a patient has a benign feeling prostate or if there is a disease present. The extent of disease can
be separated into unilateral or bilateral lobe disease or evidence of extracapsular extension. The benefits of DRE are that it is a quick, low priced assessment tool and easy to perform, allowing access to the dimensions, shape and boundaries of the prostate (Mestrinho et al. 2011). However, DRE examination of patients with PSA level of up to 2ng/ml has a positive predictive value of only 5 – 30% (Loeb & Catalona 2009).

2.6.3 Histopathologic examination of prostatic needle-biopsy specimens

If PC is suspected based on initial screening tests or symptoms, more tests are required to confirm the diagnosis.

Histological assessment remains the gold standard for confirmation of a diagnosis of PC (Gardiner 2011 ). Prostate biopsies are commonly performed under transrectal ultrasound guidance (TRUS). Using TRUS, a thin, hollow needle is inserted through the wall of the rectum, into the prostate gland. When the needle is removed it yields a small core of prostate tissue (see Figure 2-2) (Hara et al. 2008; Takenaka et al. 2008). This process is repeated several times with multiple cores obtained from the base, midportion and apex of both lobes of the prostate gland. Furthermore, TRUS-gauge core biopsy has become the standard method to obtain tissue material for diagnosing and staging of malignancy. Current techniques for TRUS guided needle biopsy have low morbidity and provide precise information about the grade and extent of a tumour within the prostate gland (Gardiner 2011 ).

Magnetic resonance imaging (MRI) of the pelvis with trans-perineal biopsy is a more recent method for targeted prostate biopsy. MRI-guided prostate biopsies have significantly higher rates of cancer detection, being associated with a higher percentage of positive cores (HPPC)
and maximum cancer core length (MCCL) compared to systematic biopsies (Anastasiadis et al. 2006; Hambrock et al. 2010; Roethke et al. 2012).

Diagnosis of PC is based on histologic examination; therefore, ancillary staining techniques (e.g. basal cell staining) and extra (deeper) tissue may be analysed if a suspect glandular lesion is identified (van der Kwast, TH et al. 2003). During analysis of each prostate core, the proportion of prostatic adenocarcinoma and the GS are recorded (Epstein, J. I. et al. 2005; Gleason, Donald F. 1992; Gleason, Donald F. & Mellinger 1974; van der Kwast, TH et al. 2003). Furthermore, the presence of high-grade prostatic intraepithelial neoplasia (PIN) and perineural invasion are also usually reported. The clinical pathologist also reports the range of malignancy as this measurement could be an indication for a further diagnosis of the tissue or a re-biopsy before choosing treatment options (Herkommer et al. 2004; Trpkov et al. 2006).
Figure 2-2: Transrectal ultrasound (TRUS) plus biopsy, adopted from https://www.cancer.gov/types/prostate/patient/prostate-treatment-pdq).
2.6.4 Prostate imaging

Once the patient has been diagnosed histologically with PC, staging investigations are performed to accurately determine the extent or stage of the disease, to better inform treatment options. These investigations include computed tomography (CT) scan, MRI of the prostate, whole-body bone scan (WBBS) and more recently prostate-specific membrane antigen (PSMA) Positron emission tomography (PET)/CT scan.

MRI has been used to diagnose PC, with varying success, for over a decade. Multiparametric MRI (mpMRI) prostate is currently the most accurate imaging modality to detect, localize, and stage PC (Hoeks et al. 2011). The mpMRI of the prostate detects both high-grade and larger tumours accurately (Futterer 2017). In addition, mpMRI techniques may be used to differentiate between low- and intermediate/high-grade PC (Donati et al. 2014; Kobus et al. 2011).

If cancer metastasises, it often goes to the bone first. In patients with PC, WBBS is the most commonly used imaging technique for detecting or identifying bone metastases, for assessing tumour response to treatment and predicting the survival of PC patients (Zafeirakis 2014). Though WBBS is a highly sensitive technique for the detection of metastatic lesions, the interpretation of changes in the intensity and size of metastatic lesions on bone scans can be a difficult task causing inconsistency between different readers, with unacceptably high false-negative interpretations (Peters et al. 2004).

CT and WBBS were the standard staging investigations for the locoregional and distant disease for PC until recent times. PSMA PET/CT scanning is fast becoming the staging investigation of choice for intermediate and high-risk PC, given its superior specificity and
sensitivity as a staging investigation when compared to CT and WBBS (Rauscher et al. 2016).

PSMA is a transmembrane protein with significantly elevated expression in PC cells compared to benign prostatic tissue. These features of PSMA make it an optimal target for imaging in PC (Rauscher et al. 2016; Virgolini et al. 2018).
2.7 TNM staging

The most commonly used staging system amongst clinicians is the TNM system developed by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) (Edge & Compton 2010). This system codes the extent of a primary tumour (T), regional lymph nodes (N), and distant metastases (M) and provides a ‘‘stage grouping’’ based on T, N, and M. The TNM staging system for PC is briefly described in Table 2-1. This staging system is one of the most significant factors for predicting survival rates for PC patient (Tobisu 2005).
Table 2-1: TNM staging of prostate cancer

<table>
<thead>
<tr>
<th>T</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>A doctor cannot feel a tumour or see it with imaging like transrectal ultrasound</td>
</tr>
<tr>
<td>T2</td>
<td>Doctor can feel cancer during a digital rectal exam (DRE) or see it using the image</td>
</tr>
<tr>
<td>T3</td>
<td>Cancer has begun to grow and spread outside the prostate, potentially into seminal vesicles</td>
</tr>
<tr>
<td>T4</td>
<td>Cancer has spread to tissue next to the prostate, such as rectum, bladder or wall of the pelvis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx</td>
<td>Nearby lymph nodes were not assessed</td>
</tr>
<tr>
<td>N0</td>
<td>Cancer not spread to any nearby lymph nodes</td>
</tr>
<tr>
<td>N1</td>
<td>Cancer has spread to one or more nearby lymph nodes in the pelvis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Cancer has not spread past nearby lymph nodes</td>
</tr>
<tr>
<td>M1</td>
<td>Cancer has spread past nearby lymph nodes</td>
</tr>
<tr>
<td>M1a</td>
<td>Cancer has spread to lymph nodes outside of the pelvis</td>
</tr>
<tr>
<td>M1b</td>
<td>Cancer has spread to bones</td>
</tr>
<tr>
<td>M1c</td>
<td>Cancer has spread to organs including lung, liver or brain</td>
</tr>
</tbody>
</table>
2.8 Gleason grading system

The Gleason grading system was developed by Donald Gleason, a pathologist, specifically for PC (see Figure 2-3). The Gleason grading system is based on the histologic pattern of arrangement of carcinoma cells in hematoxylin and eosin (H&E) stained prostatic tissue sections (Gleason, Donald F. 1992; Gleason, Donald F. & Mellinger 1974). The histologic GS can range from 2 to 10. Gleason noted that more than one histological pattern was present in a tissue and he designated the predominant pattern as the primary grade, while the subordinate pattern designated as the secondary grade. If there was only one pattern present, then he considered both the primary and secondary pattern for analytical purposes (Gleason, Donald F. 1992; Gleason, Donald F. & Mellinger 1974).

The Gleason grading system highlights the pathologic aggressiveness of PC and is also one of the key factors in classifying PC into low, intermediate and high-risk categories, together with TNM staging and PSA (D'Amico et al. 2001). Clinical researchers believe that overall survival and prostate cancer-specific survival vary according to the GS (see Figure 2-4). As can be seen from the following two graphs, low GS shows a better survival rate than the high GS (Rusthoven et al. 2014).
Figure 2-3: Gleason grading of prostate cancer, adopted from (Gleason, Donald F 1988).

Gleason Pattern

1. Small, uniform glands
2. More stroma between glands
3. Distinctly infiltrative margins
4. Irregular masses of neoplastic glands
5. Only occasional gland formation

Well differentiated

Moderately differentiated

Poorly differentiated/Anaplastic
Figure 2-4: Overall survival and prostate cancer-specific survival according to the Gleason score, adopted from (Rusthoven et al. 2014).
2.8.1 Modifications to the Gleason grading system

The existing PC grading system was developed between 1966 and 1974 by Donald Gleason and the system assigns histologic patterns 1 through 5, adding the most and second most common patterns with GSs ranging from 2 to 10 (Gleason, Donald F. & Mellinger 1974). Over the following four decades, histologic and clinical diagnosis of PC along with its treatment has changed, leading to modifications of the Gleason system first codified in 2005 and more recently in 2014 (Epstein, J. I. et al. 2005; Epstein et al. 2016). The current Gleason grading system differs dramatically from the original system. The GSs 2 – 5 are currently no longer assigned and certain patterns that Gleason defined as a score of 6 are now graded as 7, therefore leading to contemporary GS 6 cancers having a better prognosis than historic score 6 cancers (Epstein et al. 2016)

The International Society of Urological Pathology (ISUP) also endorses grading needle-biopsy specimens with the most common Gleason pattern as the primary pattern and the highest Gleason pattern as the secondary pattern (Kır, Seneldir & Gumus 2016). This is a significant modification from the previous grading system (Epstein 2010; Epstein, J. I. et al. 2005; Kır, Seneldir & Gumus 2016). Under improved Gleason grading system, the GS 3 + 3 = 6 prostate carcinomas are scored as GS 3 + 4 = 7 (see Figure 2-5). This is reflected in the percentage of GS 6 cancers having reduced from 48% to 22%, while the percentage of GS 7 cancers has significantly increased from 26% to 68% (Billis et al. 2008; Huang et al. 2014).
Figure 2-5: Typical Gleason patterns of the modern Gleason grading system and the corresponding new Grade Group system (H&E: 100×–200×), adopted from (Chen, N & Zhou 2016).
2.9 Risk stratification

Risk stratification of PC patients in the absence of metastatic disease depends upon the PSA level, T stage, and GS at presentation. The original system divided men into 3 groups: low, intermediate, and high risk (D'Amico et al. 1998). In addition, treatment options and clinical outcomes of patients vary in the three different groups (see Table 2-2).
Table 2-2: Risk stratification of prostate cancer based on PSA level, T stage and Gleason scores

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleason Score</td>
<td>≤ 6</td>
<td>7</td>
<td>8, 9, 10</td>
</tr>
<tr>
<td>PSA level</td>
<td>&lt; 10 ng/ml</td>
<td>10 – 20 ng/ml</td>
<td>&gt; 20 ng/ml</td>
</tr>
<tr>
<td>T-Stage</td>
<td>T2 (Organ confined)</td>
<td>T2 (Organ Confined)</td>
<td>T3 or above</td>
</tr>
</tbody>
</table>
2.10 Treatments modalities

Management approaches are made after discussion with a multidisciplinary team regarding all options (including urologists, radiation oncologists, medical oncologists, pathologists, and radiologists), including benefits and side effects of each therapy modality together with the patient. It is generally impossible to state that one therapy is superior over another. However, there are some treatment options for patients with locoregional PC including watchful waiting or active surveillance, RP, hormone therapy and RT. Taking the important decisions considering management approach needs very careful assignment of patients to risk groups, which completely depends on the clinical T stage and radiologic results, GSs and degree of malignancy in the prostatic needle-biopsies, and serum PSA level (D'Amico et al. 1998).

2.10.1 Watchful waiting or active surveillance

Because the PC often grows very slowly, some men (with low risk and especially those who are older or have other health problems) may not require any treatment for their PC. As an alternative, their doctor may recommend watchful waiting or active surveillance.

Watchful waiting describes a less intensive type of follow-up that includes fewer tests and more reliance on changes in the symptoms to decide whether treatment is needed or not; whereas, active surveillance requires that the cancer is followed closely (Romero-Otero et al. 2016; Xu et al. 2012). This approach includes a doctor visit with PSA blood test and DRE at every 6 months. A prostate biopsy may be done every year. If test results show changes, the doctor would inform the patient of further treatment options (Romero-Otero et al. 2016; Xu et al. 2012).
2.10.2 Radical prostatectomy

Radical prostatectomy (RP) is the only treatment for localised PC that has revealed a cancer-specific survival benefit when compared with watchful waiting option (Bill-Axelson et al. 2008). In this method, the surgeon removes the entire prostate gland plus some surrounding tissue, including the seminal vesicles. Nerves paring RP represents the approach of choice in all men with a normal erectile function and organ-confined disease (Walsh 2007). High-risk of extracapsular disease, including any cT2c or cT3 or any GS >7, are usual contraindications (Steuber et al. 2006). A validated nomogram predicting side-specific extracapsular extension can help in decision making. Moreover, mpMRI could be useful for choosing a nerve-sparing method because it has good specificity but low sensitivity for detecting microscopic pT3a stages (de Rooij et al. 2016). Then the experience of the radiologist remains of supreme importance. It is increasingly evident that surgery is suitable in treating locally advanced PC with outstanding 5-, 10-, and 15-years overall and cancer-specific survival rates of 95%, 90% and 79%, respectively (Joniau et al. 2007).

2.10.3 Radiation therapy

Dose-escalated intensity-modulated RT (IMRT), with or without image-guided RT, is the gold standard for external-beam RT (EBRT) because it is associated with less RT-induced toxicity compared to three-dimensional conformal RT (3D-CRT) techniques (Zelefsky et al. 2008). Some previous studies reported that escalating the dose into the range 74–80 Gy leads to a significant improvement in 5-years biochemical-free survival (Beckendorf et al. 2011; Heemsbergen et al. 2014; Kuban et al. 2011). Using IMRT in mainly intermediate-risk localised PC reveals 60 Gy in 20 fractions (3 Gy/fraction) is non-inferior to 74 Gy in 37 fractions with 5-years recurrence-free rates of 90% (Dearnaley et al. 2016).
For low-risk disease, the standard care is dose-escalated IMRT at the dose 74–78 Gy without androgen deprivation therapy (ADT) (Mottet et al. 2017). In addition, for intermediate-risk, the patients appropriate for ADT should be given combined dose-escalated IMRT (76–78 Gy) with short-term ADT (4–6 months) (Jones et al. 2011). However, for the patients unsuitable for ADT (e.g. due to comorbidities) or unwilling to accept ADT, the recommended treatment is IMRT at a dose of 76–80 Gy or a combination of IMRT and brachytherapy (Mottet et al. 2017). The patients with localised high-risk are recommended for dose-escalated IMRT, perhaps together with the pelvic lymphatics and long-term ADT, usually for 2 to 3 years (Mottet et al. 2017). The standard of care for patients T3–4 N0, M0 locally advanced PC is IMRT combined with long-term ADT for at least 2 to 3 years as it results in better overall survival (OS) (Bolla et al. 2010). In both high-risk localised and locally advanced PC, an upfront combination with docetaxel only improves recurrence-free survival (Fizazi et al. 2015).

2.10.4 Brachytherapy (internal radiation therapy)

Low-dose rate (LDR) brachytherapy uses radioactive permanent seeds implanted into the prostate and it is recommended for those patients with low-risk disease and selected cases with intermediate-risk disease (GS 3 + 4), prostate volume <50 cm³, and an IPSS ≤12 (Ash et al. 2000). LDR as a boost with EBRT can be used to dose escalate radiation in patients with intermediate- and high-risk (Ash et al. 2000). Although it is a low-impact treatment modality, some patients experience significant urinary complications following implantation, such as urinary retention (1.5–22%), post-implantation TURP (8.7% of cases), and incontinence (0–19%) (Budaus et al. 2012).
High-dose rate (HDR) brachytherapy uses a radioactive source temporarily implanted into the prostate to deliver radiation. HDR brachytherapy can be delivered in single or multiple fractions and it is frequently combined with EBRT as a boost at least 45 Gy to dose escalation radiation in patients with intermediate or high-risk (Vordermark et al. 2006). HDR brachytherapy as monotherapy has been established in a few centres with low toxicity and high biochemical control rates (Hoskin et al. 2013).

2.10.5 Hormone therapy

For locally confined PC patients, RP and/or RT therapy have been the main treatment options; however, for patients with recurrent or metastatic PC, ADT is frequently used (Maggio et al. 2006). ADT is designed to avoid the physical and psychological discomforts of orchiectomy (Lepor & Shore 2012). ADT is standard of care for men with intermediate or high-risk disease patients undergoing RT, or men with locally obstructive or metastatic disease (Lepor & Shore 2012). In 1941, Charles Huggins recognized the dependence of the prostate gland on androgens, providing a pathway for ADT in the treatment of PC (Rashid & Chaudhary 2004). Generally, ADT is achieved through 3 mechanisms in PC patients including gonadotrophin-releasing hormone (GnRH) agonists, antiandrogens, and GnRH antagonists (Currie et al. 2013). GnHR is thought to interrupt the supply of testosterone to the PC cell, interfering with its growth. Though, because androgens also are important hormones of body composition, glucose and insulin levels, and insulin resistance, this medically-induced hypogonadism can cause significant side effects on the development of metabolic syndrome (Basaria et al. 2006; Smith 2007).
2.11 Molecular biomarkers

2.11.1 Cytokines as molecular biomarkers

Pre- and post-treatment PSA could predict DFS and long-standing response to RT (McLean et al. 1997; Ray et al. 2006). However, analysis of post-RT, PSA profile may be complex because there is a possibility of PSA “bounces” following RT, which can be incorrect for cancer relapse. Therefore, new biomarkers are needed in radiation oncology to improve PC decision-making, treatment and therapy monitoring for individual patients.

The following four cytokines were selected as targets for investigation within this clinical study as there is strong evidence in the scientific literature linking them to aggressive tumour growth and possible resistance to RT.

2.11.1.1 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine which is produced by macrophages, neutrophils, fibroblasts, keratinocytes, NK cells, T and B cells, and tumour cells (Anderson, Nakada & DeWitte 2004). TNF-α is a soluble 17-kDa protein containing 157 amino acids and it was first isolated from the serum of Bacillus-Calmette-Guerin-infected mice treated with endotoxin (Carswell et al. 1975). TNF-α mediates host responses in acute and chronic inflammatory conditions and it also helps in protection from infection and malignancy (Anderson, Nakada & DeWitte 2004). Elevated levels of TNF-α are identified in many cancers such as oesophageal (Hubel et al. 2000), prostate (de Miguel et al. 2000; Dirksen, Kirschner & Belyea 2014; Sharma et al. 2014), ovarian (Rzymski et al. 2005) and breast cancer (Garcia-Tunon et al. 2006). Yoshida et al. (2002) have observed that serum levels of TNF-α are associated with advanced stages of cancer and it could be a useful biomarker in the early diagnosis of PC.
2.11.1.2 Transforming growth factor-β1 (TGF-β1)

Transforming growth factor-β1 (TGF-β1) is a ubiquitous profibrotic cytokine regulating cellular functions such as cell proliferation, apoptosis, differentiation, migration, immune response and angiogenesis (Elliott & Blobe 2005; Massague 1990). TGF-β1 is a homodimer with a mass 25 kDa and is produced by almost all human cells (Kajdaniuk et al. 2013). Basically, TGF-β1 is produced by platelets, macrophages, monocytes, lymphocytes, fibroblasts and epithelial cells (Kato, Inoue & Yoshioka 1999). The association between TGF-β1 overexpression and tumour progression is well recognized in the scientific literature and this protein has become the target of an investigation on therapeutic options for some malignancies (Yang, Pang & Moses 2010). TGF-β1 has been shown to promote cancer cell progression and its overexpression is linked with aggressive PC (Katsuno, Lamouille & Derynck 2013; Reis et al. 2011). Earlier clinical investigations described that elevated levels of TGF-β1 are also connected with metastasis stage, and biochemical recurrence in PC patients (Reis et al. 2011; Shariat et al. 2004).

2.11.1.3 Interleukin-6 (IL-6)

Interleukin-6 (IL-6), a 26-kDa protein, is a cytokine which plays an important role in immunoglobulin synthesis, enhances B cell growth and controls the acute phase protein synthesis by hepatocytes (Zhang, Lin & Vilcek 1988). IL-6 is released not only by inflammatory cells but also by PC cells and prostate stromal cells (Nguyen, Li & Tewari 2014). Giri et al. (2001) have also confirmed that IL-6 is secreted by normal and neoplastic prostatic epithelial cells and its concentration was higher in localized PC than normal prostate tissue.
Furthermore, elevated levels of IL-6 in the serum were found to be associated with poor clinical outcomes (Hong, Angelo & Kurzrock 2007; Trikha et al. 2003). With regard to treatment response, patients with high IL-6 concentration showed a poor response to therapeutic treatments including chemo/RT and ADT (Chen, MF et al. 2013). Additionally, elevated level of IL-6 has been identified in many cancers such as oral squamous cell carcinoma (OSCC) (Shinriki et al. 2009), lung cancer (Songur et al. 2004), colorectal cancer (Belluco et al. 2000), renal cell carcinoma (Altundag, Altundag & Gunduz 2005), cervical cancer (Wei, L-H et al. 2003), breast cancer (Alokail et al. 2014; Salgado et al. 2003), and ovarian carcinoma (Penson et al. 2000; Zakrzewska & Poznański 2001) and PC (Culig et al. 2005; Johnke et al. 2009; Lippitz & Harris 2016).

2.11.1.4 Interleukin-8 (IL-8)

Interleukin-8 (IL-8), also known as CXCL8, is a pro-inflammatory CXC chemokine and its gene encodes for a protein of 99 amino acids (Waugh & Wilson 2008). IL-8 is produced by a number of cells including lymphocytes, monocytes, endothelial cells, fibroblasts hepatocytes and keratinocytes (Waugh & Wilson 2008). The expression of IL-8 has been identified in various human cancers including breast cancer (Singh et al. 2013; Todorovic-Rakovic & Milovanovic 2013), colon cancer (Jin et al. 2014), cervical cancer (Markowska 2007), gastric cancer (Lee et al. 2013), lung cancer (Zhang et al. 2012) and PC (Caruso et al. 2008; Waugh & Wilson 2008).

Subsequently, elevated serum levels of IL-8 have been identified in patients with PC compared with healthy volunteers (Roumeguere et al. 2018). Moreover, the serum levels of IL-8 were found to be associated with pathological tumour stages of PC and high GS (Uehara
et al. 2005). Significantly higher levels of IL-8 expression in patients with aggressive PC or presenting with biochemical recurrence have been reported (Neveu et al. 2014).

2.12 Longitudinal studies with biomarker measurements

The flowchart for longitudinal studies is shown in Figure 2-6. The article search was conducted using the following databases: Cochrane library, PubMed, MEDLINE, and Google Scholar. Principal supervisor and I carried out a systematic search using keywords such as PC, cytokine expression, TNFα, TGF-β1, IL-6, IL-8, IHC, and ELISA, clinical outcomes and RT-induced toxicity.

Inclusion criteria were (I) studies investigating the association between cytokine expression and clinical outcomes such as DFS and OS; (II) studies investigating cytokine expression and association with RT-induced toxicity; (III) studies using blood plasma or serum and prostatic tissues for cytokines analysis. Publications such as editorials, commentaries and review articles were excluded. Studies not subject to peer-review were also excluded. If there were more than one study resulting from the same patient cohort, to prevent data duplication, these were also excluded. Animal studies were also excluded.

The literature search identified 431 unique citations following the removal of duplicates. Of these 431 citations, 405 were excluded after the first screening stage involving titles and abstracts review. Twenty-three citations were inspected during a full-text review in the second screening stage. Out of 23 studies, 6 articles were excluded for the following reasons: outcome not assessed = 1, review article = 1, duplication of study cohort = 1, outcome relation with cytokines not assessed = 1, studies on animal’s samples = 2. Rest of 17 records were finally included in our literature review.
**Figure 2-6:** Flow chart of prostate cancer studies investigating molecular biomarkers.
A few longitudinal studies have tracked cytokines of interest on blood samples over the whole course of RT from pre-treatment baseline through to follow-up (Christensen et al. 2009; Dirksen, Kirschner & Belyea 2014; Feng et al. 2016; Holliday et al. 2016; Johnke et al. 2009; Kovacs et al. 2003; Tanji et al. 2015). In addition, some studies have identified the cytokine expression levels by using tissue biopsies of PC patients (Bouraoui et al. 2008; Cansino Alcaide et al. 2009; Caruso et al. 2008; Hobisch et al. 2000; Ma et al. 2015; Milicevic et al. 2015; Murphy et al. 2005; Rodriguez-Berriguete et al. 2013; Royuela et al. 2004; Wikstrom et al. 1998). These studies are summarized in Table 2-3 and discussed briefly here.

Regarding clinicopathological characteristics, some included studies did not report PSA levels, GSs and TNM stages. In addition, the patient’s biopsies were observed with immunoreactivity of cytokines and most of them were identified using cytoplasmic staining. The included studies investigating the level of cytokines with clinicopathological characteristics of patients is summarised in Table 2-4.
Table 2-3: Studies investigating cancer molecular biomarkers for radiation oncology

<table>
<thead>
<tr>
<th>Years (References)</th>
<th>(n=)</th>
<th>Cancer</th>
<th>Sample collection</th>
<th>Cytokines investigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Johnke et al. 2009)</td>
<td>37</td>
<td>prostate cancer</td>
<td>Blood was taken immediately prior to TAS, prior to RT, after 24 hours to the RT, and weekly during</td>
<td>IL-1β, IL-6 and TGF-β</td>
</tr>
<tr>
<td>(Kovacs et al. 2003)</td>
<td>37</td>
<td>prostate cancer</td>
<td>Blood samples were taken prior to RT, intervals throughout the RT and follow-up period</td>
<td>IL-1-α - TGF-β</td>
</tr>
<tr>
<td>(Christensen et al. 2009)</td>
<td>42</td>
<td>prostate cancer</td>
<td>Blood was taken prior to RT education and computed tomography simulation, and at every 5th fraction during IMRT and end of treatment</td>
<td>IFN-γ, TNF-α, IL-1α, IL-2, IL6, IL-8, IL-10, and IL-12p70</td>
</tr>
<tr>
<td>(Tanji et al. 2015)</td>
<td>30</td>
<td>prostate cancer</td>
<td>Blood samples were taken before and during RT</td>
<td>FGF-2, VEGF, G-CSF, GRO, TGFβ-1 and TGFβ2</td>
</tr>
<tr>
<td>(Dirksen, Kirschner &amp; Belyea 2014)</td>
<td>35</td>
<td>prostate cancer</td>
<td>Blood was collected before and after RT</td>
<td>TNF-α, IL-1b, IL-1b, IL-6, IL-10, and IL-4</td>
</tr>
<tr>
<td>(Holliday et al. 2016)</td>
<td>28</td>
<td>prostate cancer</td>
<td>Peripheral blood was collected before RT, 1 h after, end of week 3, end of week 5, and 39th (final) or end of RT</td>
<td>IL-1a, IL-1b, TNF-α, IL-6, IL-8, IL-10</td>
</tr>
<tr>
<td>(Feng et al. 2016)</td>
<td>34</td>
<td>prostate cancer</td>
<td>Blood samples were taken at baseline, the midpoint of EBRT, and 1 year following EBRT</td>
<td>IL-2  IL-3, IL-8, IL-9, IL-10, IL-16, IFN- γ, IFN-α2, and stromal cell-derived factor 1α</td>
</tr>
<tr>
<td>(Milicevic et al. 2015)</td>
<td>148</td>
<td>prostate cancer</td>
<td>Formalin-fixed paraffin-embedded archival tissues were selected</td>
<td>IL-6</td>
</tr>
<tr>
<td>(Hobisch et al. 2000)</td>
<td>17</td>
<td>prostate cancer</td>
<td>Frozen prostatic adenocarcinomas were selected for IHC and supernatants of primary culture, epithelial prostatic cell and immortalized cell</td>
<td>IL-6</td>
</tr>
<tr>
<td>(Wikstrom et al. 1998)</td>
<td>73</td>
<td>prostate cancer</td>
<td>Formalin-fixed, paraffin-embedded specimens from 73 tumours were randomly selected</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>(Rodriguez-Berriqueta et al. 2013)</td>
<td>93</td>
<td>prostate cancer</td>
<td>Formalin-fixed, paraffin-embedded specimens from 93 tumours were selected</td>
<td>IL-1 and TNF-α</td>
</tr>
</tbody>
</table>
(Ma et al. 2015) 128 prostate cancer Formalin-fixed paraffin-embedded were selected for IHC staining T-2A, E-cadherin, IL-6, cyclin-E, PCNA and Bcl-2

(Murphy et al. 2005) 40 prostate cancer Prostate biopsy containing histologically normal and neoplastic tissues were taken for IHC IL-8 and IL-8 receptor (CXCR1 and CXCR2)

(Royuela et al. 2004) 58 prostate cancer Prostatic biopsies were taken from 28 men diagnosed with BPH; prostatic biopsies from 25 men diagnosed with prostate carcinoma and from 5 men histologically normal prostates IL-6

(Caruso et al. 2008) 103 prostate cancer Formalin-fixed paraffin-embedded tissues were selected for IHC staining OPN and IL-8

(Cansino Alcaide et al. 2009) 47 prostate cancer Tissue section was taken from 5 normal, 25 benign prostatic hyperplastic (BPH) and 17 PC human prostates IL-1, TNF-α and IL-6

(Bouraoui et al. 2008) 25 prostate cancer Each prostate tissue sample was divided into two portions; one portion was processed for IHC, and the other portion was frozen in liquid nitrogen and maintained at 80 ºC TNF-α, IL-6, IL-1

Abbreviations used: TAS = total androgen suppression; RT = radiation treatment; IMRT = intensity-modulated radiotherapy; EBRT = external beam radiation therapy; and other abbreviations as in text; IL-1α, IL-1β = Interleukin 1 (α and β); IL-1α, IL-1β = Interleukin 1 (α and β); TGF-β = tumour growth factors-β; IFN-γ = interferon-γ; TNF-α = tumour necrosis factor-α; IL-2, IL-3, IL-4, IL-6, IL-8, IL-9, IL-10, IL-16 = Interleukin (2, 3, 4, 6, 8, 9, 10 and 16); IL-12p70 = interleukin-12p70; FGF-2 = fibroblast growth factor-2; VEGF = Vascular endothelial growth factor; G-CSF = Granulocyte colony-stimulating factor; GRO = growth-related oncogene; IFN-α2 = interferon-α2; Stromal cell-derived factor 1α, OPN = Osteopontin
Table 2-4: Studies investigating cytokines expression with clinicopathological features of patients

<table>
<thead>
<tr>
<th>Authors</th>
<th>PSA level</th>
<th>Gleason Scores</th>
<th>TNM stages</th>
<th>Sample</th>
<th>Methodology</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Johnke et al. 2009)</td>
<td>&lt; 21 ng/ml</td>
<td>≤ 6</td>
<td>T1-T a grade T1-2, N0, M0</td>
<td>blood plasma</td>
<td>ELISA</td>
<td>An elevated level of IL-1β and IL-6 found during RT, but TGF-β decreased immediately following the initiation of RT.</td>
</tr>
<tr>
<td>(Kovacs et al. 2003)</td>
<td>&lt; 21 ng/ml</td>
<td>≤ 6.5</td>
<td>T1-T a grade T1-2, N0, M0</td>
<td>blood plasma</td>
<td>ELISA</td>
<td>Elevated plasma concentration of IL-1α - TGF-β identified in PC group following RT.</td>
</tr>
<tr>
<td>(Christensen et al. 2009)</td>
<td>≥ 0.05 ng/ml</td>
<td>6, 7, or 9</td>
<td>T1c-3c</td>
<td>blood plasma</td>
<td>Multiplex Immunoassay</td>
<td>IFN-γ and IL-6 increased during RT and association was found between increased IL-2 and IL-1 and acute gastrointestinal and genitourinary toxicity.</td>
</tr>
<tr>
<td>(Tanji et al. 2015)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>blood serum</td>
<td>Multiplex assay system</td>
<td>Levels of epidermal G-CSF, and IFN-γ, G-CSF, GRO, TGFβ-1 and TGFβ2 were significantly increased during RT compared to the levels observed before RT.</td>
</tr>
<tr>
<td>(Dirksen, Kirschner &amp; Belyea 2014)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>blood serum</td>
<td>Multiplex assay system</td>
<td>Elevated TNF-α was associated with depression, anxiety, urinary irritation, and bowel problems, and IL-4 was related to urinary irritation symptoms.</td>
</tr>
<tr>
<td>(Holliday et al. 2016)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>blood serum</td>
<td>Immunofluorescence assays</td>
<td>IL-6 increased during RT but not associated with fatigue scores or sleep disturbance.</td>
</tr>
<tr>
<td>(Feng et al. 2016)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>blood serum</td>
<td>Bio-Rad Bio-Plex Cytokines Assay kit</td>
<td>Significant correlations were observed between increased levels of IL-2, IL-3, IL-8, IL-9, IL-10, IL-16, IFN-γ, IFN-α2, and stromal cell-derived factor 1α at T2 with worsening of fatigue from T1 to T3.</td>
</tr>
<tr>
<td>(Milicevic et al. 2015)</td>
<td>N/A</td>
<td>3+4 = ≥ 7 4+3 = ≤ 7</td>
<td>N/A</td>
<td>tumour tissue</td>
<td>IHC</td>
<td>IL-6 immunoreactivity was observed in the cytoplasm of benign, premalignant and malignant tissue samples.</td>
</tr>
<tr>
<td>(Hobisch et al. 2000)</td>
<td>N/A</td>
<td>≥ 6</td>
<td>N/A</td>
<td>tumour tissue and tissue culture</td>
<td>ELISA and IHC</td>
<td>IL-6 IHC expression found in the cytoplasm of epithelial cells of benign, preneoplastic, and malignant prostatic tissue.</td>
</tr>
<tr>
<td>Study (Year)</td>
<td>Serum PSA cut-offs</td>
<td>Patients</td>
<td>Tumour tissue</td>
<td>Methodology</td>
<td>Findings</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------</td>
<td>----------</td>
<td>---------------</td>
<td>-------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Wikstrom et al. 1998</td>
<td>N/A</td>
<td>N/A</td>
<td>T&lt;sub&gt;0.4&lt;/sub&gt;M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>Elevated level of TGF-β1 in tumour tissue of patients who had shorter median cancer-specific survival.</td>
</tr>
<tr>
<td>Rodriguez-Berriguete et al. 2013</td>
<td>≥10 ng/ml, &lt; 10 ng/ml</td>
<td>6, 7, and 8</td>
<td>T&lt;sub&gt;2.4&lt;/sub&gt;</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>Significant associations between expression of IL-1 and TNF-α and high pre-operative serum PSA levels and advanced pathological T-stage.</td>
</tr>
<tr>
<td>Ma et al. 2015</td>
<td>&lt;10 ng/ml, &gt;20 ng/ml</td>
<td>≤6, 7 and ≥8</td>
<td>≤cT2a, cT2b and ≥cT2c</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>Decreased biochemical recurrence-free survival rate was noted in PC cases with positive MT-2A and cyclin E expression respectively.</td>
</tr>
<tr>
<td>Murphy et al. 2005</td>
<td>≤4.00 ng/mL, ≤10.00 ng/mL, ≥10.00 ng/mL</td>
<td>6 in 14 patients, 7 in 12 patients, 8 to 10 in 14 patients</td>
<td>T&lt;sub&gt;2.3&lt;/sub&gt;</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>IL-8 was localized to the cytoplasm of cancer cells and significant correlation found with advanced stage of the disease. Over expression of IL-8 found in cancer cells of Gleason pattern 3 and 4, whereas circumferential expression was present in Gleason pattern 5.</td>
</tr>
<tr>
<td>Royuela et al. 2004</td>
<td>N/A</td>
<td>low grade= 1-2, medium = 3, high = 4-5</td>
<td>N/A</td>
<td>Tumour tissue</td>
<td>IHC, Western blot and RT-PCR</td>
<td>Both medium and high Gleason grade specimens showed a pattern of immunoreactivity in both the epithelial and the stromal, the intensity of staining increasing with Gleason grade.</td>
</tr>
<tr>
<td>Caruso et al. 2008</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>Increased IL-8 staining is observed in specimens from patients who had a biochemical recurrence.</td>
</tr>
<tr>
<td>Cansino Alcaide et al. 2009</td>
<td>0-4 ng/ml, 4-20 ng/ml</td>
<td>3 to 5</td>
<td>N/A</td>
<td>Tumour tissue</td>
<td>IHC</td>
<td>High expression of pro-inflammatory cytokines (TNFα, IL-6, IL-1) associated with elevated PSA serum levels and tumour progression.</td>
</tr>
<tr>
<td>Bourouei et al. 2008</td>
<td>NP- &lt; 4 ng/ml, BPH- 4-20 ng/ml, PC- 4-20 ng/ml</td>
<td>3 to 8</td>
<td>N/A</td>
<td>Tumour tissue</td>
<td>Western blotting IHC</td>
<td>Significant association found between high expression of TNF-α, IL-6, IL-1 and elevated PSA serum levels and tumour progression in PC.</td>
</tr>
</tbody>
</table>

ELISA= enzyme-linked immunosorbent assay; IHC= immunohistochemistry; NP= normal prostate; BPH= benign prostatic hyperplasia; PSA= Prostate-specific antigen
2.13 Diagnostic approaches for cytokines expression analysis

The detection of cytokine patterns has become increasingly crucial in human cancer. In this chapter, the diagnostic methods which have been used for measuring cytokine expression/levels are described. Each of these methods has advantages as well as limitations.

2.13.1 Analysis of cytokine expression on prostatic needle-biopsy specimens

IHC analysis of paraffin-embedded prostatic needle-biopsy specimens for cytokine determination is a well-established procedure in PC studies (Milicevic et al. 2015; Rodriguez-Berriguete et al. 2013; Royuela et al. 2004; Wikstrom et al. 1998). This particular diagnostic method can detect small numbers of cytokine-producing cells in needle biopsy specimens; however, it might not be possible to detect these cells using other diagnostic methods such as Western Blotting, ELISA, or Real-time quantitative polymerase chain reaction (RT-PCR) on prostatic needle-biopsy specimens (Amsen, de Visser & Town 2009).

This particular diagnostic method reveals the type and localization of cytokine-producing cells. Many previous studies have identified cytokine expression in the cytoplasm of epithelial cells of benign, preneoplastic, and malignant prostate tumour tissue and significant association was found with elevated PSA serum levels and tumour progression (Bouraoui et al. 2008; Hobisch et al. 2000; Milicevic et al. 2015; Rodriguez-Berriguete et al. 2013; Wikstrom et al. 1998). However, this method is poorly quantitative and can suffer from low sensitivity for detection of small amounts of secreted proteins.
2.13.2 Analysis of cytokines expression in blood plasma

ELISA is a plate-based assay designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction (Laing et al. 2010). Moreover, ELISA is a monoclonal antibodies-based method that provides results more satisfactory than using polyclonal antibodies, because monoclonal antibodies are highly specific.

The ELISA method has been established as the most practical and powerful tool for the analysis of cytokine levels in blood plasma or serum because it produces sensitive and reliable results without depending on sophisticated equipment (Johnke et al. 2009; Michalaki et al. 2004). Predominantly, the sandwich ELISA method has been used for the detection of cytokines in the blood of many cancer patients including PC (Chadha et al. 2014; Chen, Y et al. 2002; Johnke et al. 2009; Michalaki et al. 2004), breast (Janselsins et al. 2012; Westbury et al. 2014), lung (Siva et al. 2014; Zhao, L. et al. 2008), and head and neck (Chen, MF et al. 2013; Diakowska 2013). In our clinical study, a highly sensitive and specific monoclonal antibodies-based sandwich ELISA was used to detect cytokine expression levels in blood plasma of PC patients.

2.14 Association of cytokines expression with clinicopathological characteristics

Many previous clinical studies have focused on molecular assessment of the tumour tissue and blood plasma for cytokines expression and association with clinicopathologic characteristics of PC patients. Rodriguez-Berriguete et al. (2013) reported that elevated
expression of TNF-α on tumour tissue was significantly associated with high pre-operative serum PSA levels and pathological T (pT) stage of malignancy. Furthermore, high expression of TNF-α on tumour presented a strong association with cancer recurrence in those who had lymph node metastases (Grimm et al. 2011). Regarding blood analysis, elevated levels of TNF-α in blood serum revealed an association with stage of disease and the presence of metastatic disease (Michalaki et al. 2004).

In addition, elevated levels of TGF-β1 were significantly associated with cancer progression, metastasis and biochemical progression in PC patients (Reis et al. 2011; Shariat et al. 2004). Analysis of tumour tissues found that high expression of TGF-β1 was significantly associated with clinicopathological characteristics of PC patients including high GS and clinical T (cT) stage of cancer (Reis et al. 2011; Wu et al. 2015). Regarding survival rate, patients with overexpression of TGF-β1 were found to have short cancer-specific survival (Wikstrom et al. 1998).

Moreover, the elevated level of IL-6 in prostate tumour tissue and serum confirmed its influence on the tumour development and the microenvironment (Milicevic et al. 2015). Ishiguro et al. (2011) reported a significant association of IL-6 expression with pT stage and risk stratification but there was no significant correlation with biochemical recurrence. Furthermore, expression levels of IL-6 were higher in patients with GS ≥7 than the patients with GS ≤7 and with metastases stage (Michalaki et al. 2004; Shariat et al. 2004). The elevated serum levels of IL-6 were also significantly associated with pre-operative serum PSA levels (Hobisch et al. 2000).
As a possible prognostic biomarker, IL-8 expression has been investigated in many clinical studies using PC patient tumour specimens and animal models using established cell lines. Caruso et al. (2008) reported that PC cells stained positively for IL-8 more than for benign prostatic hyperplasia or normal prostate cells. They also identified low-grade IL-8 staining in tumour tissues from patients with GS 6, 7, or 8 and who had a biochemical recurrence. A previous clinical study involving blood analysis found elevated levels of IL-8 in blood serum and a significant association was found with GS and pT stage of PC patients and bone metastasis (Lehrer et al. 2004; Uehara et al. 2005).
2.15 Association of cytokines expression with RT-induced toxicity

Generally, RT-induced toxicity is considered as acute or late toxicity, according to the time before the appearance of symptoms. RT-induced toxicity is classified as acute if it occurred during RT and in the first 3 months thereafter (D'Avino et al. 2015). Whereas it is classified as late if it occurred after 3 months from the completion of RT (D'Avino et al. 2015; Dorr & Hendry 2001). However, late effects could develop months to years after radiation exposure (Dorr & Hendry 2001).

Use of molecular biomarkers of RT-induced toxicity could provide useful information to clinicians or radiation oncologist and help in the treatment plan for PC patients. Christensen et al. (2009) determined that there was an association between cytokine expression and patient-reported gastrointestinal and genitourinary acute RT-induced toxicity. They found that increases in IL-2 and IL-1 levels over baseline were significantly associated with increased gastrointestinal and genitourinary toxicity respectively (Christensen et al. 2009). In addition, an elevated level of IL-6 was also correlated with the higher probability for gastrointestinal toxicity; however, it did not show any statistical significance (Christensen et al. 2009).

Cytokines release in response to ionizing radiation is a standard phenomenon and may play a major role in subsequent RT-induced lung toxicity (Zhao, L. et al. 2008). They found that elevated level of circulating TGF-β1 during RT was significantly associated with RT-induced lung toxicity after completion of RT. However, Rube et al. (2008) reported no positive association between elevated levels of IL-6 or TGF-β1 and lung toxicity. Interestingly, in some previous studies, a decreased level of IL-8 was significantly associated with RT-
induced lung toxicity and was identified as a biomarker for developing RT-induced lung toxicity (Hart, Justin P et al. 2005; Stenmark et al. 2012).

2.16 Summary and conclusions

Although the above studies provide some evidence that cytokine changes modulate RT response, new promising biomarkers are needed for clinical use in predicting acute RT-induced toxicity. IHC and ELISA analyses of samples from PC patients receiving RT may therefore provide an early warning of future toxicity arising from the treatment. Many previous clinical studies, in the above scientific literature, have focused on molecular assessment of the tumour tissue and blood plasma for cytokine expression and association with clinicopathologic features and acute RT-induced toxicity of prostate and lung cancer patients.
Chapter 3- Methods and materials

3.1 General procedure
The routine and specific methods for cytokine analysis on prostatic needle-biopsies and in blood plasma of PC patients are described in this chapter. All the reagents, buffers, antibodies and solutions were obtained from the pharmaceutical companies Sapphire Bioscience, In Vitro Technologies Pty Ltd, Thermo Fisher Scientific Pty Ltd, Life Technologies Pty Ltd and Dako Pty Ltd. Antibodies and all solutions were validated in the clinical laboratory based at Charles Darwin University (CDU).

3.2 Reagents for IHC and ELISA procedures

3.2.1 Reagents for IHC staining method
The reagents and chemicals for IHC staining were used as described in the protocol (See section 3.3.8.2) on prostatic needle-biopsies of PC patients.

3.2.1.1 Dako REAL Detection kit
The Dako REAL detection kit contained the single ready-to-use Streptavidin-peroxidase, 3-diaminobenzidine (DAB) + chromogen (X50), horseradish peroxidase (HRP) substrate buffer and Link-biotinylated secondary antibodies.

3.2.1.2 Other reagents and chemicals for IHC staining

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein block serum-free</td>
<td>Dako Pty Ltd, Australia</td>
</tr>
<tr>
<td>Antibody diluent</td>
<td>Dako Pty Ltd, Australia</td>
</tr>
<tr>
<td>Target retrieval solution, pH 9 (10x)</td>
<td>Dako Pty Ltd, Australia</td>
</tr>
<tr>
<td>Wash buffer (10x)</td>
<td>Dako Pty Ltd, Australia</td>
</tr>
</tbody>
</table>
Hematoxylin solution Mayer's  
Dako Pty Ltd, Australia
Mounting medium  
Dako Pty Ltd, Australia
Xylene  
Thermo Fisher Pty Ltd
Ethanol (100%)  
Thermo Fisher Pty Ltd
Ethanol (90%)  
Thermo Fisher Pty Ltd
Ethanol (70%)  
Thermo Fisher Pty Ltd

**Primary antibodies for IHC staining**

- Monoclonal TGF-β 1 antibody  
Novus Biologicals
- Monoclonal TNF-α antibody  
Novus Biologicals
- Polyclonal rabbit IL-6 antibody  
Novus Biologicals
- Polyclonal CXCL8/IL-8 antibody  
Novus Biologicals

### 3.2.2 Reagents for ELISA

The ELISA kits were obtained from Life Technologies Australia Pty Ltd. These kits contained single ready-to-use solutions and were prepared according to the instructions of the manufacturer (*See section 3.3.9.3*).

#### 3.2.2.1 Reagents provided in TNF-α ELISA kit

- Microtiter plate with 96 well anti-TNF-α coated wells  
Life Technologies
- Standard 0 in human plasma with preservative (diluent)  
Life Technologies
- Standards 1 to 5 in human plasma with preservatives  
Life Technologies
- Incubation buffer with preservatives  
Life Technologies
- Anti-TNF-α -HRP conjugate  
Life Technologies
- Conjugate buffer  
Life Technologies
Controls 1 and 2 in human plasma with preservatives
Washing solution concentrated
Concentrated chromogen: TMB (Tetramethylbenzidine)
Substrate buffer: H$_2$O$_2$ in acetate/citrate buffer
Stop solution: H$_2$SO$_4$ 1.8 N

Reagents preparation

1. **Standards and controls** were reconstituted to the volume specified on the vial label with distilled water and then allowed to remain undisturbed until completely dissolved, and then mixed well by gentle inversion.

2. **Concentrated conjugate solution** was diluted by adding 600ul (concentrated conjugate) into 6000ul (conjugates buffer).

3. **Wash solution** was diluted by adding 2 ml (washing solution concentrate) into 400 ml (distilled water) or all the contents of the washing solution concentrate vial in 2000 ml of distilled water.

4. **Chromogenic solution** was diluted by adding 0.2 ml of the concentrated Chromogen (TMB) into one of the vials of Substrate Buffer (H$_2$O$_2$ in acetate/citrate buffer).

3.2.2.2 Reagents provided in TGF-β1 ELISA kit

- Anti-human TGF-β1 pre-coated 96-well strip plates
- Lyophilized recombinant human TGF-β1 standard 2 vials
- 20X Wash buffer 25mL
- Biotinylated antibody reagent 2 vials
- Streptavidin-HRP reagent 200uL
- TMB substrate 12mL
Sample preparation

The plasma samples were used in ELISA methods and 100μL per well of diluted sample was added. Assay Diluent A was used for dilution of plasma samples. The suggested dilution for plasma sample was 1:2000 for TGF-β1 analysis.

Reagents preparation

1. **Assay diluent B** was diluted 5-fold with deionized or distilled water before use.

2. **Preparation of standard**: 400μL Assay diluent A was added into the lyophilized standard vial to prepare a 50 ng/mL standard. The powder was dissolved thoroughly in a gentle mix. Then, 60μL betaIG-H3 standard (50 ng/mL) from the vial of the lyophilized standard was added into a tube with 440μL assay diluent A to prepare a 6,000 pg/mL standard solution. Following, 300μL assay diluent A was added into each tube. 6,000 pg/mL standard solutions were used to produce a dilution series.

3. **Wash solution** was diluted by adding 2 ml (washing solution concentrate) into 400ml (distilled water) or all the contents of the washing solution concentrate vial in 2000ml distilled water.

4. **Biotinylated antibody reagent**: 100μL Assay diluent B was added into the biotinylated antibody reagent vial to prepare a biotinylated antibody concentrate. Then biotinylated antibody concentrate was diluted 80-fold with assay diluent B.
5. **Streptavidin-HRP Reagent**: 50μL of HRP-Streptavidin concentrate was added into a tube with 15mL assay diluent B to prepare a 300-fold diluted HRP- Streptavidin solution.

### 3.2.2.3 Reagents provided in IL-6 ELISA kit

- Microtiter plate with 96 well anti-IL-6 coated wells: Life Technologies
- Standards 0 to 5 in human plasma with preservatives: Life Technologies
- Solution A (human plasma with preservative): Life Technologies
- Solution B (buffer with preservative): Life Technologies
- Anti-IL-6 conjugate: Life Technologies
- Control 1 and 2 in human plasma with preservative: Life Technologies
- Washing solution: Life Technologies
- Chromogen: TMB: Life Technologies
- Stop solution: Life Technologies

### Reagents preparation

1. **Standards and controls and solution A** were reconstituted to the volume specified on the vial label with distilled water and then allowed to remain undisturbed until completely dissolved, and then mixed well by gentle inversion.

2. **Wash solution** was diluted by adding 2 ml (washing solution concentrate) into 400 ml (distilled water) or all the contents of the washing solution concentrate vial in 2000 ml of distilled water.

### 3.2.2.4 Reagents provided in IL-8 ELISA kit

- Microtiter plate with 96 well anti-IL-6 coated wells: Life Technologies
- Standards 0 to 5 in human plasma with preservatives: Life Technologies
Solution A (human plasma with preservative) Life Technologies
Solution B (buffer with preservative) Life Technologies
Anti-IL-8 HRP conjugates Life Technologies
Control 1 and 2 in human plasma with preservative Life Technologies
Washing solution Life Technologies
Chromogen: TMB (Tetramethylbenzidine) Life Technologies
Substrate buffer: \( \text{H}_2\text{O}_2 \) in acetate/citrate buffer Life Technologies
Stop solution Life Technologies

**Reagent Preparation**

1. **Standards and controls and solution A** were reconstituted to the volume specified on the vial label with distilled water and then allowed to remain undisturbed until completely dissolved, and then mixed well by gentle inversion.

2. **Wash solution** was diluted by adding 2 ml (washing solution concentrate) into 400 ml (distilled water) or all the contents of the washing solution concentrate vial in 2000 ml of distilled water.

3. **Chromogenic solution** was prepared by adding 0.2ml of the concentrated chromogen (TMB) into 21ml of substrate buffer vial.
3.3 Methods

3.3.1 Study design

In this prospective observational study, patients were recruited from those referred to the Alan AWCCC diagnosed with PC, who agreed to undergo curative external beam RT (EBRT). Participants were interviewed prior to any treatment including ADT and RT and signed consent was obtained before using their samples and clinical information. This study was conducted on the paraffin-embedded tissue sections and blood plasma of PC patients. The patient’s clinicopathological characteristics and related demographic, clinical and pathological details were collected from AWCCC and securely stored during this study (Table 3-1). The study schema of cytokines such as TNF-α, TGF-β1, IL-6 and IL-8 is mentioned in (Figure 3-1). Every sample of the patient was allocated with an alphanumerical code and was stored according to the storage location assigned by the laboratory information management system.

3.3.2 Ethics

This study was approved by the Human Research Ethics Committee (HREC) of the NT and Department of Health and Menzies School of Health Research (HREC: 2015-2385). The participant information sheet (PIS) was provided to each participant prior to consent being obtained. The signed consent form from patients permitted the research team to access their tissue biopsies, blood samples, medical and pathology records from Royal Darwin Hospital (RDH) and AWCCC during this study. Results from deceased PC patients after treatments were included for statistical analysis. The total number of deceased patients was recorded, along with the causes of death.
Table 3-1: Clinicopathological characteristics collected from prostate cancer patients

<table>
<thead>
<tr>
<th>AGE</th>
<th>≥ 50</th>
<th>≤ 50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIAGNOSIS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA (pre-operative PSA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsy core results</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical TNM assessment</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>CLINICAL MANAGEMENT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgen Deprivation Therapy (ADT)</td>
<td>Agent start date</td>
<td>Agent stop date</td>
</tr>
<tr>
<td>RELAPSE/RECURRENTCE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical relapse</td>
<td>Occurrence</td>
<td>Region</td>
</tr>
<tr>
<td>Biochemical relapse</td>
<td>Occurrence</td>
<td>Date of initial event</td>
</tr>
<tr>
<td>FOLLOW-UP</td>
<td>3 months after radiation</td>
<td></td>
</tr>
</tbody>
</table>

TNM- Tumour, Node and Metastasis; Gy- Gray; PSA- prostate-specific antigen.
Figure 3-1: Study schema of the prospective observational study.
3.3.3 Eligibility criteria for PC patient recruitment

3.3.3.1 Inclusion criteria

Eligible patients were aged ≥18 years, had histologically confirmed prostate adenocarcinoma of the prostate, had a ECOG (Eastern Cooperative Oncology Group) performance status of 0 or 1 or a Karnofsky performance score of 80-100 at the time of diagnosis and had no history of prior surgery or RT to the prostate.

3.3.3.2 Exclusion criteria

Exclusion criteria include prior external beam RT or brachytherapy to the prostate, metastatic stage disease at presentation, prior history of malignancy (excluding basal cell or squamous cell skin cancer), and serious medical or psychiatric illness, precluding safe administration of RT +/- ADT. This clinical study was carried out in cooperation with Charles Darwin University (CDU) and the AWCCC based in Darwin, NT, Australia.

3.3.4 Selection of cytokines

In this study, the cytokines TNF-α, TGF-β1, IL-6 and IL-8 were selected as targets for investigation as there is strong evidence in the scientific literature linking them to aggressive tumour growth and possible resistance to RT.

3.3.5 Patient recruitment

Eighteen PC patients referred to the AWCCC and diagnosed with PC were recruited for the study. Using the D’Amico classification, selected patients were identified into the following three groups: low, intermediate and high-risk disease. Low-risk, GS ≤6, PSA< 10 ng/ml, T2 (Organ confined) and Treatments RT alone; intermediate-risk, GS =7, PSA 10-20 ng/ml and cT2b (Organ confined); and high-risk, GS ≥8, PSA >20 ng/ml and ≥cT2c (Organ confined)
(D'Amico et al. 1998). Clinicopathological data including patient age, pre-operative PSA, risk stratification, pTNM-stages, cTNM-stages, MRI TNM-stage, node involvement, GS, RT Dose, RT fraction and 3 months serum PSA levels were also recorded.

3.3.6 Samples size consideration
A total of twenty-seven newly diagnosed PC patients were interviewed between July 2015 and April 2016 at AWCCC. Out of the 27, eighteen patients were recruited for this clinical observational study according to our eligibility criteria. From these 18 patients, 200 prostatic needle-biopsy specimens were collected from RDH for IHC analysis. In addition to this, 61 blood samples were also collected from selected patients for plasma analysis.

3.3.7 Confidentiality
Identity and privacy of the participants were protected as follows:

- Identifiable patient data was de-identified with a means of re-identifying if necessary, which was known only to the research team.

- Clinical data was stored in the electronic database at CDU accessible only to the research team. In addition, the patient’s data was also stored on the hard drive which was password protected and the computer was in a locked room.

- Recruited patients were not identified in any publication with strict adherence to patient confidentiality guidelines.
3.3.8 IHC analysis of prostatic needle-biopsy specimens for cytokine expression levels

3.3.8.1 Collection of prostatic needle-biopsy specimens

During this study, prostatic needle-biopsy specimens were taken prior to any treatment (including RT and ADT), once consent has been obtained from the study participant. The collected tumour tissues were evaluated for the expression levels of TNF-α, TGF-β1 and IL-6 with IHC technique. Importantly, the pathology reports of recruited patients were reviewed by two clinical pathologists for the selection of positive tumour tissues.

3.3.8.2 Immunohistochemical (IHC) staining analysis of needle-biopsy specimens

Paraffin-embedded, formalin-fixed tumour tissue was sectioned to 4µm and mounted on poly-lysine-coated slides (DAKO, Australia). Sections on microscope slides were kept in 50°C water bath. Microscope glasses were dried for 30 min in a thermostat at 60°C. In preparation for IHC staining, all sections were de-paraffinised using xylene and subsequently rehydrated with a series of graded ethanol dilutions. Then, antigen retrieval was performed by placing slides in a coplin jar of target retrieval solution (Dako Pty Ltd) with pH 9.0 for 20 minutes at 90 – 95 °C hot water bath. All the sections were circled with DAKO pen.

Furthermore, sections were incubated in methanol containing 3% hydrogen peroxide for 30 minutes and then washed twice (3 minutes per wash) with Tris Buffer Saline (TBS) washing buffer. Following protein blocking, serum was applied to all the sections and incubated for 10 minutes. In addition, primary antibodies (Novus Biologicals) were used to identify the expression of TNF-α, TGF-β1 and IL-6 on tumour biopsies from PC patients. Sections were incubated with primary antibodies for 1hr and washed twice (3 minutes each wash) with TBS. The primary antibodies were used with dilutions: mouse monoclonal TNF-α (1:50), mouse monoclonal TGF-β1 (1:100) and rabbit polyclonal IL-6 (1:100) as mention in Table
3-2. Antibody diluent was substituted with a primary antibody for negative control sections (DAKO, Australia). All sections were rinsed in TBS as previously described. Following 3-4 drops of secondary antibody (Dako REAL Link-biotinylated secondary Ab) were applied to all sections and incubated for 10 minutes at room temperature. Again, all the sections were rinsed twice with TBS. Then tissue sections were incubated with STREP peroxidase-HPR for 10 minutes by adding 3-4 drops to the slides (DAKO, Australia).

All sections were developed with 3’-diaminobenzidine (DAB) for 5 minutes at room temperature and counter-stained with hematoxylin. In addition, all sections were dehydrated through a graded series of ethanol dilutions followed by xylene. After staining, coverslips were applied and sealed using permanent mounting medium.
Table 3-2: Dilution factors of primary antibodies for IHC analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibody</th>
<th>Cat.</th>
<th>Dilutions</th>
<th>Company Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TNF-alpha Antibody</td>
<td>NB600-1422</td>
<td>1:50</td>
<td>Novus Biologicals</td>
</tr>
<tr>
<td>2</td>
<td>TGF-beta 1 Antibody</td>
<td>NBP2-22114</td>
<td>1:100</td>
<td>Novus Biologicals</td>
</tr>
<tr>
<td>3</td>
<td>IL-6 Antibody</td>
<td>NB600-1131</td>
<td>1:100</td>
<td>Novus Biologicals</td>
</tr>
</tbody>
</table>
3.3.8.3 Microscopic examination for cytokine expression levels

Following the IHC staining, every IHC stained slide was evaluated for the expression or variation of TNF-α, TGF-β1 and IL-6 on tumour biopsies with light microscopy in a blinded manner by two clinical pathology consultants. The range of score scale was classified from “negative” (mostly marked as “-“) to “positive”, which was assigned different numbers of “+”, depending on staining strength (Ida et al. 2013; Sand et al. 2014; Soares et al. 2010). Expression of TNF-α, TGF-β1 and IL-6 were evaluated according to a semi-quantitative scale: –, less than 10% staining; +, 10 - 50% of the cells stained positive; + +, 51 - 80% positive cells; + + +, more than 80% of cells positive (Kapoor & Deshmukh 2012; Rodriguez-Berrigüete et al. 2013; Zimmermann et al. 2014). Semiquantitative scoring systems are generally used to convert subjective perception of IHC-biomarker expression levels into quantitative data for statistical analysis and establishing conclusions (Klopfleisch 2013).

In this study, the Immunoreactive (IRS) scoring method was used for IHC expression analysis on PC core biopsies and IRS gives a range of 0 – 12 by multiplication between positive cells proportion score (0 – 4) and staining intensity score (0 – 3) (Table 3-3) (Fedchenko & Reifenrath 2014; Remmele & Stegner 1987).
Table 3-3: Immunoreactive score system of IHC expression analysis

<table>
<thead>
<tr>
<th>A (percentage of positive cells)</th>
<th>B (intensity of staining)</th>
<th>IRS score (multiplication of A and B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = no positive cells</td>
<td>0 = no colour reaction</td>
<td>0-1 = negative</td>
</tr>
<tr>
<td>1 = &lt;10% of positive cells</td>
<td>1 = mild reaction</td>
<td>2-3 = mild</td>
</tr>
<tr>
<td>2 = 10-50% positive cells</td>
<td>2 = moderate reaction</td>
<td>4-8 = moderate</td>
</tr>
<tr>
<td>3 = 51-80% positive cells</td>
<td>3 = intense reaction</td>
<td>9-12 = strongly positive</td>
</tr>
<tr>
<td>4 = &gt;80% positive cells</td>
<td>Final IRS score (A × B): 0-12</td>
<td></td>
</tr>
</tbody>
</table>
3.3.8.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Established clinical variables included in the study were age, pre-operative PSA, risk stratification, cTNM-stages, pTNM-stages, and GS. Spearman’s correlation tests were performed to assess the correlation between TNF-α, TGF-β1 and IL-6 expression levels and clinicopathological variables. Fisher’s exact test or $\chi^2$-test was used to compare expression or variation of cytokines between two groups including diseased and healthy individuals. Cox proportional hazard regression analysis was utilized to estimate hazard ratios (HR), with 95% confidence interval (95% CI).

3.3.9 Plasma analysis for cytokine expression levels

3.3.9.1 Patient blood sampling

To examine blood biomarkers, early consideration is very important as whether to use plasma (anti-coagulated blood) or serum (coagulated blood, clot removed) samples. Moreover, the time from blood collection to primary processing (i.e. centrifuge and aliquoting) can have a significant impact on protein stability. In this study, blood plasma samples were used to examine the cytokines expression levels before and after RT with ELISA methods.

Blood samples were obtained before and after RT treatment from the patient. The blood samples were taken prior to the commencement of RT, last week of RT and 3 months from completion of RT as mentioned in Table 3-4. Patients requiring hormone therapy were required to give one extra blood sample prior to the commencement of ADT. At each sampling time, 5-10 ml of peripheral blood was drawn into blindly coded 5-7 ml vacutainer tubes containing powdered lithium heparin (14 Units/ml blood). The blood samples were immediately placed on ice for transport to the laboratory, aliquoted into conical 15 ml tubes
and centrifuged (3000 × g × 20 min) to separate out the plasma. The platelet-free plasma layer was separated from the blood, transferred into coded cryotubes and frozen at −80°C until they were analysed (see Figure 3-2). The analysis was carried out under blind conditions and in accordance with the guidelines established by the CDU committee for handling biohazardous material.
<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gleason score</strong></td>
<td>$\leq 6$</td>
<td>7</td>
<td>8, 9, 10</td>
</tr>
<tr>
<td><strong>PSA level</strong></td>
<td>$&lt; 10 \text{ ng/ml}$</td>
<td>$10 – 20 \text{ ng/ml}$</td>
<td>$&gt; 20 \text{ ng/ml}$</td>
</tr>
<tr>
<td><strong>T stages</strong></td>
<td>T2 (Organ confined)</td>
<td>T2 (Organ Confined)</td>
<td>T3 or above</td>
</tr>
<tr>
<td><strong>Treatments</strong></td>
<td>RT alone</td>
<td>3 months neoadjuvant ADT– RT / 3 months concurrent ADT</td>
<td>3-6 months neoadjuvant ADT followed by RT and adjuvant ADT for total 2-3 years</td>
</tr>
<tr>
<td><strong>Sample Collection</strong></td>
<td>-Prior to RT</td>
<td>-Before ADT</td>
<td>-Before ADT</td>
</tr>
<tr>
<td></td>
<td>-Last week of RT</td>
<td>-Prior to RT</td>
<td>-Prior to RT</td>
</tr>
<tr>
<td></td>
<td>-3 months to RT</td>
<td>-Last week of RT</td>
<td>-Last week of RT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-3 months to RT</td>
<td>-3 months to RT</td>
</tr>
</tbody>
</table>
Figure 3-2: Systematic movements of blood sampling and procedure for further analysis.
3.3.9.2 Enzyme-linked Immunosorbent Assay

Measurement of the cytokines TNF-α, TGF-β1, IL-6 and IL-8 in the blood plasma was conducted by using sandwich ELISA. In this method, the target protein is bound in between two antibodies such as the capture (primary) antibody and the detection (secondary) antibody (see Figure 3-3).

There are four elementary steps involved in sandwich ELISA method. These steps are as below:

1. Capturing target cytokines from the sample with the capture antibody;
2. Detecting captured antibody with detection antibody (also specific for captured antibody) that is labelled with biotin;
3. Detection amplification with streptavidin that has been conjugated with an enzyme, in most instances horseradish peroxidase (HRP); and
4. Substrate addition and signal measurement via optical density (OD) with a microplate reader.
**Figure 3-3:** Schematic of sandwich ELISA using HRP-linked detection antibody and cytokines specific capture antibodies.
3.3.9.3 Sandwich ELISA protocol for plasma analysis

Plasma concentration of cytokines TNF-α, TGF-β1 and IL-6 and IL-8 were measured using sandwich ELISAs methods which are commonly available in kits including human TNF-α ELISA (KAC1751), human TGF-β1 ELISA (EHTGFBI), human IL-6 ELISA (KAC1261) and human IL-8 ELISA (KAC1301) (Life Technologies Australia Pty Ltd). For ELISA detection, plasma samples were removed from a storage location in the –80°C freezer and thawed on crushed ice. In order to detect pro-inflammatory cytokines TNF-α, IL-6 and IL-8, undiluted plasma samples were used respectively. However, in the case of profibrotic TGF-β1, the plasma samples were diluted using standard solution or Calibrator diluent which was provided in the kit. The ELISA assays targeting TNF-α, TGF-β1, IL-6 and IL-8 were carried out following the manufacturer’s protocol as shown in Table 3-5.

To analyse TGF-β1 in plasma samples, assay diluent A was used for dilution of plasma samples. The suggested dilution for plasma was 1:2000 by the manufacturer as mention in Table 3-6. For the preparation of standard, 400μL assay diluent A was added into the lyophilized standard vial to prepare a 50 ng/mL standard. The powder was dissolved thoroughly in a gentle mix. Then, 60μL standard (50ng/mL) was added from the vial of the lyophilized standard; into a tube with 440μL assay diluent A to prepare a 6,000 pg/mL standard solution. Next 300μL assay diluent A was added to each tube. 6,000 pg/mL standard solutions were used to produce a dilution series. Then each tube was mixed thoroughly before the next transfer. Assay diluent A serves as the zero standards (0pg/mL). The serial dilutions of plasma samples for profibrotic TGF-β1 detection using sandwich ELISAs are shown in Figure 3-4.
Table 3-5: ELISA protocols for measurement of cytokines levels in blood plasma

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagents</th>
<th>TNF-α</th>
<th>TGF-β1</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Incubation Buffer or Solution B</td>
<td>Incubation buffer 50μl</td>
<td></td>
<td>Solution B 50 μl</td>
<td>Solution B 100 μl</td>
</tr>
<tr>
<td>2</td>
<td>Standard, Control and Plasma sample</td>
<td>200 μl for 2 hrs incubation</td>
<td>100 μl for overnight at 4 °C</td>
<td>100 μl for 1 hr incubation</td>
<td>100 μl and following by step 5</td>
</tr>
<tr>
<td>3</td>
<td>Wash</td>
<td>400 μl for 3 times</td>
<td>400 μl for 3 times</td>
<td>400 μl for 3 times</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Standard 0</td>
<td>100 μl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Anti- TNF-α conjugate Or Biotinylated antibody</td>
<td>Anti- TNF-α conjugate 50 μl for 2 hrs</td>
<td>100 μl biotinylated Ab. for 1 hr incubation</td>
<td>Anti-IL-6 conjugates 100 μl and 50 μl solution A for 1 hr incubation</td>
<td>Anti-IL-8 100 μl conjugate for 2 hrs incubation</td>
</tr>
<tr>
<td>6</td>
<td>Wash</td>
<td>400 μl for 3 times</td>
<td>400 μl for 3 times</td>
<td>400 μl for 3 times</td>
<td>400 μl for 3 times</td>
</tr>
<tr>
<td>7</td>
<td>Chromogenic Solution</td>
<td>200 μl for 30 mins and following step 11</td>
<td></td>
<td>200 μl for 30 mins and following step 11</td>
<td>200 μl for 30 mins following step 11</td>
</tr>
<tr>
<td>8</td>
<td>Streptavidin-HRP solution</td>
<td>100 μl for 45 mins incubation</td>
<td>400 μl for 3 times incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Wash</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>TMB Substrate</td>
<td>100 μl for 30 mins incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Stop Solution</td>
<td>50 μl</td>
<td>50 μl</td>
<td>100 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Dilution factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>undiluted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1:2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>undiluted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>undiluted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-4: Serial dilutions of recombinant protein standards for sandwich ELISA.
During the assessment of plasma samples, 100μL of each standard, control and plasma sample was added to the appropriate wells. The 96 wells microplate was incubated overnight at 4°C with gentle shaking to bind the target to the primary (capture) antibody. Following the overnight incubation, the solution of each well was discarded, and wells were washed 3 times with wash buffer provided in each kit. Washing was done by filling each well with 300μL wash buffer using a multi-channel pipette. After the last wash, remaining wash buffer was removed by aspirating or decanting. Then the plate was inverted and blotted against clean paper towels.

Next, 100μL of prepared biotinylated antibody was added to each well and incubated for 1 hour at room temperature. As before, the solution was discarded, and the wells washed three times with wash buffer. Further, 100μL of the prepared streptavidin-HRP solution was added to each well and was incubated for 45 minutes at room temperature with gentle shaking. Following this step, again the solution was discarded, and the plate washed three times with wash buffer.

Finally, 100μL of TMB substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Then 50 μl stop solution was added to each well. The microplate was evaluated within 30 minutes of stopping the reaction. The measured absorbance on Titertek Multiskan MCC/340 plate reader was set at 450nm and 550nm. The values 450nm were subtracted from 550nm to correct for optical imperfections in the microplate.
3.3.9.4 Statistical analysis for ELISA

Statistical analysis was performed using GraphPad Prism 7. The nonparametric Kruskal-Wallis test and Tukey’s HSD test were used to assess pre-treatment and post-treatment blood cytokine concentrations. Spearman’s correlation tests were used to determine if any linear relationships existed among clinicopathological variables such as age, risk stratification, cTNM-stages, pTNM-stages, MRI TNM-stage, pre-operative PSA, GS 3 months PSA levels and acute RT-induced toxicity. A p-value of <0.05 was taken as significant. Acute RT-induced toxicity was modelled as International Index Prostate Symptom Scoresheet (IIPS) and Expanded Prostate Cancer Index Composite (EPIC) 0/2 (defined as no toxicity) vs. Grade 3/5 = toxicity (defined as toxicity). Spearman’s correlation test was used determine the correlation between cytokine expression and patient-reported gastrointestinal and genitourinary acute RT-induced toxicity graded prospectively.

3.3.10 Grading RT-induced toxicity

The frequency and severity of RT-induced toxicity were self-rated by patients using criteria based on the Common Terminology Criteria for Adverse Events (CTC-AE) (van der Laan et al. 2008). Patients were asked to complete questionnaires concerning symptoms of bladder or rectal injury prior to RT, last week of RT and after 3, 9 and 12 months of RT completion as part of their standard care. The monitoring of patients for RT-toxicity was part of the clinical routine, follow-up visits were planned every 3 months for the first year, then every 6 months for the next 3 years and yearly thereafter.

The grading of RT-related toxicity is as follows: Grades 0/2 are minimal and asymptomatic. Grade 3 effects are considered moderate, usually symptomatic, and sometimes require interventions. Grades 3-5 effects are considered severe and undesirable, usually with multiple
and disruptive symptoms. For our analysis, we considered Grade 0/2 = no toxicity vs. Grade 3/5 = toxicity. International Index Prostate Symptom Scoresheet (IIPS) Table 3-7 (Li et al. 2014; Rodrigues et al. 2004) and Expanded Prostate Cancer Index Composite (EPIC) Table 3-8 (Chang, P et al. 2011) were used which were based on the answers to questions concerning urinary and bowel symptoms. Each question concerning urinary and bowel symptoms allowed the patient to choose one answer indicating increasing severity of the particular symptom. The answers were assigned points from 0 to 5. The total score, therefore, ranged from 0 to 35 (asymptomatic to very symptomatic) for IPSS and 0 to 28 (asymptomatic to very symptomatic) for EPIC (Li et al. 2014; Wei, JT et al. 2000). The RT-induced toxicity was considered as mild if the total score was equal to or less than 7, while it was considered as moderate if total score range was 8-19, and it was considered as severe if the total score range was 20-35 (Li et al. 2014; Wei, JT et al. 2000).
Table 3-7: International Index Prostate Symptom Scoresheet (IIPS)

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not at all</th>
<th>Less than 1 time in 5</th>
<th>Less than half the time</th>
<th>About half the time</th>
<th>More than half the time</th>
<th>Almost always</th>
</tr>
</thead>
<tbody>
<tr>
<td>INCOMPLETE EMPTYING</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>FREQUENCY</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>INTERMITTENCY</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>URGENCY</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>WEAK STREAM</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>STRAINING</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>NOCTURIA</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Total =…./35
Table 3-8: Expanded Prostate Cancer Index Composite (EPIC)

1. How many bowel moments have you had on a typical day during the last 4 weeks

   - Two or less  1
   - Three to four  2  (Circle one number)
   - Five or more  3

2. How big of a problem, if any, have each of the following been for you?
   (Circle one number on each line)

<table>
<thead>
<tr>
<th></th>
<th>Not at all</th>
<th>Less than 1 time in 5</th>
<th>Less than half the time</th>
<th>About half the time</th>
<th>More than half the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Urgency to have a bowel movement</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>b. Increased frequency of bowel movements</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>c. Losing control of your stools</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>d. Bloody stools</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>e. Abdominal/Pelvic/Rectal pain</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

3. Overall, how big a problem have your bowel habits been for you during the last 4 weeks?

   - No Problem  1
   - Very small problem  2
   - Small problem  3  (Circle one number)
   - Moderate problem  4
   - Big problem  5

   **Total =……/28**
4.1 Introduction

In current medical practice, the standard clinicopathological features such as the serum prostate-specific antigen (PSA) level, GS and clinical tumour stage can be used to select treatment possibilities for PC; though, the prognostic accuracy with respect to disease recurrence remains an important issue (Dabbs 2013; Schroder et al. 2009; Swanson & Basler 2010). Clinical researchers believe that the accuracy of prediction could be improved by including new prognostic biomarkers into clinical practice. Therefore, many molecular biomarkers have been evaluated for their potential role in the better prediction of disease progression, response to therapy and survival in PC patients (Epstein, J. I. et al. 2005; Ma et al. 2015; Milicevic et al. 2015; Rodriguez-Berriguete et al. 2013).

Some pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α are believed to play an important role in RT resistance and lead to tumour progression, invasion, and angiogenesis (Deorukhkar & Krishnan 2010; Poutahidis et al. 2009; Steiner et al. 2003). Thus, identification of new biomarkers with the use of diagnostic techniques to examine them becomes crucial. Several clinical researchers have used ELISA, western blot (WB), IHC and proteomic or flight-mass spectrometry approaches for the detection of cytokine expression in serum or plasma and on tumour biopsies of PC patients.

The IHC expression level of TNF-α has been identified in prostatic biopsies specimens of PC (Rodriguez-Berriguete et al. 2013). Moreover, overexpression of TGF-β1 in tumour biopsies was related with aggressive PC initiated tumour progression (Katsuno, Lamouille & Derynck
2013; Reis et al. 2011). Some previous studies have also reported that elevated expression of TGF-β1 was associated with metastasis condition and biochemical recurrence in PC patients (Reis et al. 2011; Shariat et al. 2004). Regarding IHC expression of IL-6, the overexpression of IL-6 was also reported in tumour biopsies in patients with PC (Hobisch et al. 2000; Ma et al. 2015; Milicevic et al. 2015; Royuela et al. 2004).

Generally, PC is diagnosed on needle-biopsy specimens prompted by higher levels of serum PSA, DRE test or transrectal ultrasonography results and symptoms of urinary tract obstruction. In addition to higher serum PSA levels in blood and other preliminary assessments, complete histopathological investigation of PC in needle-biopsy specimens predicts the tumour behaviour and helps in therapeutic decision-making. In systematic clinical practice, the pathology report of PC includes the grade of tissue differentiation according to the Gleason grading system, and a quantitative assessment of the tumour volume per biopsy in either length in millimetre (mm) or percentage (%) of a tumour (Epstein, Jonathan I et al. 2005; Van der Kwast, T et al. 2013).

Histological examinations of prostatic needle-biopsies specimens reveal several significant changes in tissue biopsies and allow monitoring of the disease progression (Arlen et al. 2008; Billis et al. 2008; Freedland & Moul 2007). However, qualitative IHC examination of prostatic needle-biopsies specimens has shown a significant difference and inconsistency of results among different viewers (Doganaevsargil et al. 2006; Ercole et al. 2008). The extensive use of antibodies has also generated big controversy, published data often from false-positive or false-negative results produced with antibodies that have not been appropriately validated (Pradidarcheep et al. 2008). It is important to add-on histopathological examination of prostatic needle-biopsies specimens with other approaches for diagnosis and staging of PC.
because it has some limitations. These few important limitations comprise the presence of morphological mimickers of carcinoma prostate, for example, adenosis (a non-cancerous condition) and atypical adenomatous hyperplasia and a very low-grade or very high-grade carcinoma, all which cause trouble in the interpretation of tumour biopsies (Epstein et al. 2012; Hoogland, Kweldam & van Leenders 2014).

The aim of this clinical study was to evaluate the expression levels of TNF-α, TGF-β1, and IL-6 in prostatic needle-biopsy specimens using IHC staining approach. In addition, we aimed to analyse the possible association of TNF-α, TGF-β1, and IL-6 expression levels on needle-biopsy specimens with pre-operative serum PSA levels, GS and pre-RT cytokines plasma levels.

4.2 Methods and materials

4.2.1 Collection of tissue specimens and clinical data

Between July 2015 and April 2016, a total of 18 PC patients who underwent TRUS biopsies at Royal Darwin Hospital (Darwin, Northern Territory, Australia) were included in this prospective observational study. These patients did not receive RT or ADT prior to TRUS biopsies and clinical follow up data was also collected. All these PC cases were classified into the following three groups: low–risk, ≤ cT2a, PSA <10 ng/ml and GS ≤ 6; intermediate–risk, cT2b, PSA 10–20 ng/ml, GS = 7; and high–risk, ≥ cT2c, PSA > 20 ng/ml, GS ≥ 9 – 10 (D'Amico et al. 1998).

This clinical study was approved by the Human Research Ethics Committee (HREC) of the Northern Territory (NT) and Department of Health and Menzies School of Health Research (HREC:2015-2385). The participant information sheet (PIS) was provided to the individuals.
to read and to understand our clinical study. The signed consent from patients allowed the research team to access their prostatic tissue biopsies, blood samples, medical and pathology records from Royal Darwin Hospital (RDH) and AWCCC during this study.

4.2.2 Immunohistochemical staining protocol for cytokine expression
Paraffin-embedded tumour biopsies from PC patients were cut for TNF-α, TGF-β1, and IL-6 IHC expression analysis. These sections were subsequently analysed with mouse monoclonal TNF-α, mouse monoclonal TGF-β1, and rabbit polyclonal IL-6 antibodies (Novus Biologicals Pty Ltd). The procedure of IHC staining for TNF-α, TGF-β1, and IL-6 antibodies are briefly described in chapter 3 (See section 3.3.8.2). These antibodies were used at TNF-α (1: 50), TGF-β1 (1: 100), and IL-6 (1: 100) dilutions after validation of these antibodies.

4.2.3 Microscopic analysis
IHC stained slides were evaluated for the expression or variation of cytokines on tumour biopsies with light microscopy in a blinded manner by two clinical pathology consultants. The microscopic analysis method is described in chapter 3 (See section 3.3.8.3).

4.2.4 Statistical analysis
The statistical analysis was performed using GraphPad Prism 7 software and methods are described in chapter 3 (See section 3.3.8.4).
4.3 Results

4.3.1 Clinical characteristics of PC patients

Clinicopathological data of PC cases including age, pre-operative PSA, risk stratification, clinical TNM-stages, pathological TNM-stages, and GS was also recorded, as reported in Table 4-1.

The mean age of PC patients at the time of diagnosis was 66.83 ± 7.93 years (range, 53 - 80 years). In the initial assessment, they were assessed for pre-operative serum PSA level, clinical TNM assessment and biopsies core results. The mean pre-operative serum PSA levels were 16.03 ± 15.81 ng/ml (range, 4 – 71 ng/ml) with 33% patients at <10 ng/ml (6/18), 50% of patients with PSA at 10-20ng/ml (9/18) and 17% at PSA concentration > 20ng/ml (3/18). In addition, the mean GS was 7.88 ± 1.14 (range, 6 – 10) with 6% patients at GS 6 (1/18), 44% of patients with GS 7 (8/19) and 50% at GS 8-10 (9/18).

In this study, 5 out of 18 PC patients were identified with intermediate-risk of PC and 13 patients were identified with high-risk of cancer. In clinical TNM-stage group, 23% of patients were found with T1a-cN0M0 stage (4/18), 33% with T2a-cN0M0 (6/18) and 44% of patients with T3a-cN0M0 (8/18). In pathological TNM-stage group, no patient was identified with T1a-cN0M0 stage (0/18), 89% with T2a-cN0M0 (16/18) and 11% with T3a-cN0M0 clinical T-stage (2/18). Out of 18 patients, only one patient i.e. 6% was found to have node involvement (involvement of a movable homolateral regional lymph node smaller than 3 cm) whereas rest of them had no metastatic condition.
### Table 4-1: Clinicopathological characteristics of prostate cancer patients

<table>
<thead>
<tr>
<th>Clinicopathological Characteristics</th>
<th>N</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.83 ± 7.93 (53 – 80)</td>
<td></td>
</tr>
<tr>
<td>Pre-operative PSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &lt; 10 ng/ml</td>
<td>6</td>
<td>(33%)</td>
</tr>
<tr>
<td>• 10 -20 ng/ml</td>
<td>9</td>
<td>(50%)</td>
</tr>
<tr>
<td>• &gt; 20 ng/ml</td>
<td>3</td>
<td>(17%)</td>
</tr>
<tr>
<td>Risk stratification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Low-risk</td>
<td>0</td>
<td>(0%)</td>
</tr>
<tr>
<td>• Intermediate-risk</td>
<td>5</td>
<td>(28%)</td>
</tr>
<tr>
<td>• High-risk</td>
<td>13</td>
<td>(72)</td>
</tr>
<tr>
<td>cTNM-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• cT1a-cN0M0</td>
<td>4</td>
<td>(23%)</td>
</tr>
<tr>
<td>• cT2a-cN0M0</td>
<td>6</td>
<td>(33%)</td>
</tr>
<tr>
<td>• cT3a-cN0M0</td>
<td>8</td>
<td>(44%)</td>
</tr>
<tr>
<td>pTNM-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• pT1a-cN0M0</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>• pT2a-cN0M0</td>
<td>16</td>
<td>(89%)</td>
</tr>
<tr>
<td>• pT3a-cN0M0</td>
<td>2</td>
<td>(11%)</td>
</tr>
<tr>
<td>Gleason scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 6</td>
<td>1</td>
<td>(6%)</td>
</tr>
<tr>
<td>• 7</td>
<td>8</td>
<td>(44%)</td>
</tr>
<tr>
<td>• 8-10</td>
<td>9</td>
<td>(50%)</td>
</tr>
</tbody>
</table>

Values for age are presented as the mean ± standard deviation; ng/ml, nanogram/millilitre; cTNM, clinical Tumour Node and Metastatic stage; pTNM-stage, pathological Tumour Node and Metastatic stage and PSA, prostate-specific antigen.
4.3.2 IHC expression of cytokine levels in prostatic needle-biopsy specimens

Figure 4-1 displays the intensity of IHC staining with negative, mild, moderate and strong for the immunoreactivity of cytokines. To examine the distribution of cytokines, prostatic needle-biopsies specimens from 18 patients were tested using the IHC method. The results indicate that cytokines were expressed in the tumour tissues of the majority of the patients. Cytokine expression was evident in the cytoplasm of malignant epithelial prostate cells of the prostate tissue samples. For those cytokines showing less than 10% of patients with negative immunoreactions, patients were assigned with an immunostaining score. Expression rates of TNF-α, TGF-β1 and IL-6 in prostatic needle-biopsies specimens were 82.3, 71.6, and 73.3 respectively.

Tumour epithelial cells were classified according to a semi-quantitative scale: –, negative staining or ≤ 10% of the cell stained positive; +, less than 10% of the cells stained positive; ++, 10±50% cells positive; and ++++, more than 50% of cells positive. In this thesis, IRS scoring method was used for IHC expression analysis on needle-biopsy specimens and IRS method gives a range of 0 – 12 by multiplication between positive cells proportion score (0 – 4) and staining intensity score (0 – 3). IRS method is described in chapter 2 (See section 3.3.9.3)
**Figure 4-1**: Immunohistochemical IL-6 staining in prostatic needle-biopsy specimens with PC. The figure displays the intensity of IHC staining with negative, mild, moderate and strong for the immunoreactivity of cytokines.
4.3.3 Correlation between IHC cytokines expression levels and pre-operative serum PSA levels

Immunostaining for TNF-α, TGF-β1 and IL-6 was seen in 33% of samples with PSA levels between ≤ 10 ng/ml and 50% of patients with PSA levels between 10 – 20 ng/ml and 17% of patients with PSA ≥ 20 ng/ml. When the results were evaluated according to pre-operative PSA values, the TNF-α expression levels were not correlated with pre-operative PSA levels. Also, we found no significant differences between TNF-α expression levels and pre-operative serum PSA levels (p = 0.1833). Similarly, TGF-β1 expression levels also were not correlated with pre-operative serum PSA levels and there was no significant difference between TGF-β1 expression and pre-operative serum PSA levels (p = 0.3564). Moreover, IL-6 was also not correlated with serum pre-operative PSA levels. The results showed no significant association between IL-6 expression levels and pre-operative serum PSA levels of PC patients (p = 0.2323). In Figure 4-2, IHC expression levels of TNF-α, TGF-β1 and IL-6 respectively, were plotted against the pre-operative serum PSA levels of PC patients.
Figure 4-2: Correlation between TNF-α, TGF-β1 and IL-6 IHC levels and pre-operative serum PSA levels.
4.3.4 Correlation between IHC expression of cytokines levels and Gleason scores

To explore the correlation between expression levels of the TNF-α, TGF-β1 and IL-6 and GSs, Spearman’s correlation test was performed. Of note, TNF-α, TGF-β1 and IL-6 expression levels were correlated in a negative fashion with GSs. Figures 4-3 and 4-4 shows the staining with standard H&E and IHC staining techniques respectively in prostatic needle-biopsy specimens with GS 3 + 3 = 6, GS 3 + 4 = 7, GS 4 + 3 = 7, GS 4 + 4 = 8, GS 4 + 5 = 9, GS 5 + 4 = 9 and GS 5 + 5 = 10.

The IHC expression levels of TNF-α, TGF-β1 and IL-6 in prostatic needle-biopsy specimens according to GS are summarised in Table 4-2, 4-3 and 4-4 respectively. The median TNF-α expression levels were reduced with increases in the GSs and we have found no significant correlation with TNF-α expression levels and GSs (p = 0.06). Similarly, TGF-β1 and IL-6 expression levels were also reduced with an increase in the GS. However, there were also no significant differences in TGF-β1 and IL-6 expression levels and GSs (p = 0.31 and p = 0.10) respectively. In Figures 4-5 the IHC TNF-α, TGF-β1 and IL-6 expression levels respectively, were plotted against GSs.
Table 4-2: Expression of TNF-α in prostatic needle-biopsy specimens according to Gleason scores

<table>
<thead>
<tr>
<th>Gleason scores</th>
<th>Samples (n)</th>
<th>Negative (−)</th>
<th>Mild (+)</th>
<th>Moderate (++)</th>
<th>Strong (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 + 3 = 6</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3 + 4 = 7</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>4 + 3 = 7</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4 + 4 = 8</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4 + 5 = 9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5 + 4 = 9</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>5 + 5 = 10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total 47</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemical staining: − = less than 10% staining; + = 10-50% of the cells stained positive; ++ = 51-80% positive cells; +++ = more than 80% of cells positive.
Table 4-3: Expression of TGF-β1 in prostatic needle-biopsy specimens according to Gleason scores

<table>
<thead>
<tr>
<th>Gleason scores</th>
<th>Samples (n)</th>
<th>Negative (-)</th>
<th>Mild (+)</th>
<th>Moderate (++)</th>
<th>Strong (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 + 3 = 6</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3 + 4 = 7</td>
<td>15</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4 + 3 = 7</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>4 + 4 = 8</td>
<td>8</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>4 + 5 = 9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>5 + 4 = 9</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5 + 5 = 10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Total 47

Immunohistochemical staining: – = less than 10% staining; + = 10-50% of the cells stained positive; ++ = 51-80% positive cells; +++ = more than 80% of cells positive.
Table 4-4: Expression of IL-6 in prostatic needle-biopsy specimens according to Gleason scores

<table>
<thead>
<tr>
<th>Gleason scores</th>
<th>Samples (n)</th>
<th>Negative (−)</th>
<th>Mild (+)</th>
<th>Moderate (++)</th>
<th>Strong (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 + 3 = 6</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3 + 4 = 7</td>
<td>15</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>4 + 3 = 7</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4 + 4 = 8</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>4 + 5 = 9</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>5 + 4 = 9</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5 + 5 = 10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Total 47

Immunohistochemical staining: − = less than 10% staining; + = 10-50% of the cells stained positive; ++ = 51-80% positive cells; +++ = more than 80% of cells positive.
Figure 4-3 Immunohistochemical staining with standard H&E method and immunohistochemical staining with mouse monoclonal TNF-α (1:50), mouse monoclonal TGF-β1 (1:100) and rabbit polyclonal IL-6 (1:100) antibodies in prostatic needle-biopsy specimens with PC with GS 3 + 3 = 6, GS 3 + 4 = 7, GS 4 + 3 = 7, and GS 4 + 4 = 8. Original magnifications 20X.
Figure 4-4 Immunohistochemical staining with standard H&E method and immunohistochemical staining with mouse monoclonal TNF-α (1:50), mouse monoclonal TGF-β1 (1:100) and rabbit polyclonal IL-6 (1:100) antibodies in prostatic needle-biopsy specimens with PC with GS 4 + 5 = 9, GS 5 + 4 = 9 and GS 5 + 5 = 10. Original magnifications 20X.
Figure 4-5: Correlation between TNF-α, TGF-β1 and IL-6 IHC expression levels with Gleason scores.
4.3.5 Correlation between pre-RT cytokine plasma levels and IHC expression in corresponding prostatic needle-biopsies specimens

To evaluate the possible impact of tumour-derived cytokine production on circulating plasma levels, the prostatic needle-biopsy specimens of the PC patients were IHC stained for TNF-α, TGF-β1 and IL-6. The 141 prostatic needle-biopsies specimens of 18 patients revealed a heterogeneous IHC staining pattern for TNF-α, TGF-β1 and IL-6 with variably intense cytoplasmic epithelium staining of the malignant prostate cells.

In Figure 4-6, the pre-RT plasma levels for TNF-α, TGF-β1 and IL-6, respectively, were plotted against the staining intensity of the corresponding prostatic needle-biopsy specimens for every patient. For all cytokines, a statistically significant correlation was found between the amount of pre-RT plasma levels and the staining intensity of the corresponding prostatic needle-biopsy specimens. Our results suggest that irrespective of the subsequent RT the TNF-α, TGF-β1 and IL-6 plasma levels in PC patients were influenced by the cytokine release of their tumours (TNF-α: $r = 0.76$; $p < 0.01$; TGF-β1: $r = 0.57$; $p = 0.05$ and IL-6: $r = 0.5294$; $p < 0.05$).
Figure 4-6: Correlation between the pre-RT TNF-α, TGF-β1 and IL-6 plasma levels, respectively and IHC staining expression levels of the corresponding prostatic needle-biopsy specimens.
4.4 Discussion

The use of IHC biomarkers for the detection of PC in small morphologically atypical foci of prostate tissue is a modern clinical practice (Billis et al. 2008; Liska et al. 2007). The examination of some potential prognostic biomarkers in PC and the patient’s decision about the type of treatment option has been one of the great challenges of clinical practice. Concato et al. (2007) believe that when the biological variables can be identified, it is probable that cancer recurrence rates will decline. Many clinical studies have reported the relevance of IHC biomarkers that will possibly be used in the future as predictors of prognosis and tumour development (Doganavsargil et al. 2006; Freedland & Moul 2007). Tissue biomarkers such as p63 and 34betaE12 for IHC staining in PC are well-established biomarkers and currently being used in clinical practice (Googe, McGinley & Fitzgibbon 1997; Rice & Stoyanova 2018).

In the last 20 years, serum PSA has been the most effective and extensively used prognostic biomarker, predominantly for PC patients. Though, as a functional product of normal prostate epithelial tissue, serum PSA levels can change due to inflammation, trauma or benign proliferation. Pre-operative serum PSA levels are normally used as prognostic biomarkers for biochemical recurrence after RT. Rodriguez-Berriguete et al. (2013) demonstrated that there were some significant associations between elevated stromal expression of interleukin-1 receptor-associated kinase 1 (IRAK-1) and high pre-operative serum PSA levels. Remarkably, IL-1b expression in a tumour and IL-1RII and IRAK-1 expression in stromal cells had prognostic value after adjusting for the effects of pathological T stage, GS and total pre-operative serum PSA (Rodriguez-Berriguete et al. 2013). However, when assessing the P27 biomarker, there was a significant association between the patients with positive expression (P27 Positive) with lower mean serum PSA level (p = 0.091) (Nassif & Tambara...
Filho 2010). Shariat et al. (2004) reported that serum levels of IL-6 before any treatment were significantly associated with pre-operative PSA levels. In addition, the patients with elevated expressions of IL-1α also had higher serum PSA levels (PSA>20ng/ml) (Cansino Alcaide et al. 2009). In our study, when the results were evaluated according to pre-operative PSA values, the TNF-α, TGF-β1 and IL-6 IHC high expression levels were not associated with high production of PSA. We also found no significant differences between TNF-α, TGF-β1 and IL-6 IHC expression levels and different groups of preoperative PSA level (p = 0.1833, p = 0.3564 and p = 0.2323) respectively.

The Gleason scoring system for grading PC has been shown to be a highly significant predictor for localised PC. GS of 8 – 10 represents a more aggressive form of the disease and is used to classify patients as high-risk (Stock et al. 2009). High-grade cancer can develop an increased risk for both biochemical and distant failure (Stock et al. 2009). Michalaki et al. (2004) demonstrated that serum levels of IL-6 were significantly higher in patients with metastatic disease and GS > 6. Another clinical study also reported that an elevated level of IL-6 was associated with GS ≥ 7 and in patients with metastases in regional lymph nodes (Shariat et al. 2004). In addition, the percentage of immunoreactivity of six-transmembrane epithelial antigens of the prostate 1 (STEAP1) was also associated with GS. Patients with higher GS (7–9) expressed higher STEAP1 immunoreactivity, whereas patients with lower GS (5–6) indicated moderate immunoreactivity (Gomes et al. 2014). However, in our study, our results were opposite to the above clinical studies. The immunoreactivity of TNF-α, TGF-β1 and IL-6 were lower in prostatic needle-biopsy specimens among the patients with higher GS. Our results showed that only TNF-α IHC expression levels were significantly associated with GS (p = 0.0207). In addition, the expression levels of TGF-β1 and IL-6 were not significantly associated with GS (p = 0.1090 and 0.1000) respectively.
Previous research suggests that normal tissue damage and gene expression changes occur at the messenger RNA (mRNA) level that lead to increased cytokine production within the irradiated area, which then the cytokines enters the blood circulation (Rube et al. 2008; Siva et al. 2014). The half-life of cytokines and their potential degradation become important factors for the stability and storage of biological samples (Zhou, X et al. 2010). Therefore, cytokine half-life and their degradation nature may impact on circulating cytokine measurement. In this study, an effort has been made to assess the potential impact of tumour-derived cytokine production on circulating plasma levels which may impair the identification of patients at risk for RT-induced toxicity. A statistically significant correlation was found between the amount of pre-RT plasma IL-6 and TGF-β1 cytokines levels in patients irradiated for PC and the staining intensity of the corresponding tumour biopsies (Rube et al. 2008). In our study, we found a correlation between the pre-RT plasma levels of TNF-α, TGF-β1 and IL-6 and the respective IHC expression of TNF-α, TGF-β1 and IL-6 in the corresponding prostatic needle-biopsies specimens. Our results suggested that the elevated plasma levels TNF-α, TGF-β1 and IL-6 in PC patients result from the overproduction of TNF-α, TGF-β1 and IL-6 in their prostatic needle-biopsies specimens. Furthermore, our data indicate a significant association between the TNF-α, TGF-β1 and IL-6 plasma levels and the individual tumour responses (p = 0.01, p = 0.05 and p = 0.05) respectively.

By evaluating prostatic needle-biopsy specimens from patients with PC, we observed the same TNF-α, TGF-β1 and IL-6 expression pattern in all malignant tissue. Our important finding demonstrated a significant association of IHC expression levels of TNF-α, TGF-β1 and IL-6 with GSs and pre-RT plasma levels. Therefore, IHC expression levels of TNF-α, TGF-β1 and IL-6 might be predictive biomarkers in combination with GSs and pre-RT plasma cytokine levels in a population of PC patients. However, we did not find any
significant association between TNF-α, TGF-β1 and IL-6 expression levels in prostatic needle-biopsy specimens and pre-operative PSA levels; this may be due to the small number of PC patients included in this study. However, further studies are needed to confirm this hypothesis with a large sample size to demonstrate the prognostic value of TNF-α, TGF-β1 and IL-6 in PC patients.

4.5 Conclusion

In conclusion, this is the first report focusing on the immuno-expression of TNF-α, TGF-β1 and IL-6 in patients with PC and their association with clinical variables such as pre-operative serum PSA levels, GSs and pre-RT blood plasma. We conclude that in PC there is no association between TNF-α, TGF-β1 and IL-6 IHC expression levels and pre-operative serum PSA levels. In addition, the presence of TNF-α, TGF-β1 and IL-6 IHC expression in the epithelium was inversely associated with Gleason grades, suggesting a role for TNF-α, TGF-β1 and IL-6 in the proliferation of epithelial cells of low Gleason grade carcinomas. The pre-RT plasma levels of TNF-α, TGF-β1 and IL-6 in patients with PC depends on the cytokine production in their tumours. Further release of TNF-α, TGF-β1 and IL-6 into the blood circulation because of RT-induced toxicity is often superimposed by the variable cytokine production of a tumour and therefore will be difficult to detect. Hopefully, future studies will identify reliable predictors of adverse RT side effects, and thus provide the opportunity to modify the treatment to improve the therapeutic outcome.
Chapter 5- Levels of plasma cytokines in patients undergoing androgen deprivation therapy and radiation therapy for adenocarcinoma of the prostate cancer and correlation with clinical outcomes

5.1 Introduction

Many pre- and postoperative prostate cancer (PC) nomograms have been developed to predict PC stage, progression and treatment response after attempted curative therapy (Shariat et al. 2004). Clinical stage, tumour GS and prostate-specific antigen (PSA) are established pre-operative prognostic markers (Ferro et al. 2017). Some previous clinical studies have cast doubt as to the prognostic potential of PSA levels in the plasma, as there was difficulty distinguishing between the presence of benign prostatic hyperplasia (BPH) and low risk PC (Shariat, Karam & Roehrborn 2007; Shariat et al. 2004). Previous clinical studies reported that sensitivity and specificity of PSA were 90.0% and 71.9% respectively when normal PSA is defined less than or 4.0 ng/ml (Mettlin et al. 1994; Wolf et al. 2010). However, approximately 15% of men with PSA levels below 4ng/ml have PC as diagnosed by prostatic tissue biopsy (Thompson et al. 2004). Therefore, there is a clear need for new molecular biomarkers that are specifically associated with biologically aggressive PC for improved prediction for presence of clinically significant disease and clinical outcomes after treatments in patients diagnosed with clinically localized PC.

Radiotherapy (RT) is the most common non-operative treatment option for PC. Delivery of RT to the prostate is limited by the acute and late side effects of RT on the integrity of surrounding normal tissue (Tanji et al. 2015). Some earlier clinical studies have focussed upon the prognostic potential of circulating cytokines to act as biomarkers of RT-induced toxicity and complications (Chen, Y et al. 2002; Christensen et al. 2009; Johnke et al. 2009;
Rube et al. 2008). Most biomarker studies in RT have concentrated on the degree of tumour response and survival after RT (Okunieff et al. 2008). Some pro-inflammatory and profibrotic cytokines can change the redox status in radiation-exposed cells, by this affecting PC progression, RT effectiveness and RT-induced toxicities (Miao et al. 2014). Researchers believe that during the RT inflammatory cytokines are released from malignant cells and normal tissue, thus enhancing the RT effect. During this response, signals are produced by irradiated malignant cells to neighbouring normal cells (bystander effect) or to distant tumour cells (abscopal effect) and support an immunomodulatory response (Miao et al. 2014). These events frequently are associated with the release of proinflammatory and profibrotic cytokines such as IL-6, IL-8, TGF-β1 and TNF-α (Prise & O'Sullivan 2009).

RT is often administered in combination with androgen deprivation therapy (ADT). The use of ADT in combination with RT has become a popular treatment modality for localised intermediate and high-risk PC (Basaria et al. 2002; DeWeese & Song 2000; Harle et al. 2006). An understanding of the relationship between testosterone levels and inflammatory cytokine levels has been growing in recent times. Tanji et al. (2015) evaluated the association between male hypogonadism and serum levels of pro- and/or anti-inflammatory cytokines. In this particular study, 17 out of 42 cytokines were measured at detectable levels before and during RT. They reported that ADT for six months significantly reduced the serum levels of both FGF2 and VEGF; however, ADT did not affect the levels of the other cytokines. In contrast, testosterone replacement in hypogonadal men results in a significant reduction in inflammatory cytokines TNF-α and IL-1β and an increase in anti-inflammatory cytokine IL-10 (Malkin et al. 2004). Maggio et al. (2006) have further confirmed the inverse relationship between testosterone and inflammatory cytokine levels.
During this prospective study, we have measured cytokine levels for PC patients treated with ADT and definitive RT at various time points before, during and after treatment, to investigate the association between these treatments and induction of the inflammatory response. We then hope to investigate any association between cytokine levels as predictors of disease aggressiveness, response to treatment, and association with acute/late treatment effects.

5.2 Patients and Methods

5.2.1 Study population and patient information

The study cohort consisted of 18 patients who agreed to undergo ADT and curative external beam RT (EBRT). They were included in this observation study according to eligibility criteria as described in chapter 3 (See section 3.3.3). Patients were classified into three groups; low, intermediate and high-risk disease using D’Amico classification. The signed consent form from patients allowed the research team to access their prostatic tissue biopsies, blood samples, medical and pathology records from Royal Darwin Hospital (RDH) and AWCCC during this study.

5.2.2 Androgen Deprivation Therapy

ADT included subcutaneous or intramuscular administration of long acting gonadotrophin-releasing hormone (GnRH) agonists or antagonists and oral anti-androgens. All 18 participants were treated with GnRH agonists/antagonists; however, three out of 18 patients were treated in combination with an oral anti-androgen. Neoadjuvant ADT commenced 3 to 6 months prior to RT and continued until RT was completed or an overall treatment time of two to three years was achieved. In this study, all included patients had neoadjuvant ADT; however, 12 patients had ceased ADT after completion of RT. Adjuvant ADT has yielded
significant improvement in DFS for men with high-risk features treated with definitive RT. Out of the 18, six patients in this clinical study had 2-3 years adjuvant ADT.

Gasoerelin, a Luteinizing hormone-releasing hormone (LHRH) analog is used for locally advanced or metastatic carcinoma of the prostate. Gasoerelin 10.8mg comes in a special syringe, called the SafeSystem™ and a siliconised needle. The drug was injected into a pocket of subcutaneous fat every 3 months beginning before RT for an overall time of two to three years. In addition, Degarelix is used to treat locally advanced or metastatic PC and may slow or stop the growth of cancer. Degarelix is given as an injection under the skin (subcutaneously), usually near the stomach every month. Bicalutamide (Cosudex) is a pill, usually taken once per day in the morning or evening. Bicalutamide is given as part of a combination PC treatment with LHRH. These medicines prevent the testicles from producing testosterone.

5.2.3 Radiation therapy
All PC patients were treated with intensity modulated radiation therapy (IMRT), with four (23%) patients receiving 78Gy in 39 fractions and fourteen (77%) patients received 80Gy in 40 fractions. All patients had 3 gold seeds inserted as fiducial markers prior to RT and had daily cone beam imaging during their treatment. Treatment planning was based on CT performed with empty rectum, comfortably filled bladder, and with patients in prone position using vacuum-locked mattress. Clinical target volume (CTV) included the prostate gland or the prostate gland plus seminal vesicles. A 1cm margin was added around the CTV to define the planning target volume (PTV), except the boundary between the anterior rectal wall and the prostate, where a 0.7 cm margin was used.
5.2.4 Patient blood sampling and plasma processing

For plasma cytokine analysis, 5-10 ml of peripheral blood was drawn into blindly coded 5-7 ml vacutainer tubes containing powdered lithium heparin (14 Units/ml blood). Following, the blood samples were immediately transported to the laboratory and centrifuged (3000 × g × 20 min) to separate out the plasma. The platelet-free plasma layer was separated from the blood, transferred into coded cryotubes and frozen at –80°C until they were analysed. The procedure of blood sampling and processing for cytokines TNF-α, TGF-β1, IL-6 and IL-8 analysis is briefly described in chapter 3 (See section 3.3.8.2).

5.2.5 Assessment of plasma cytokines

Concentration of cytokines TNF-α, TGF-β1, IL-6 and IL-8 was carried out on plasma samples (61 PC samples) using sandwich ELISA kits including human TNF-α ELISA Kit (KAC1751), human TGF-β1 ELISA kits (EHTGFB1), human IL-6 ELISA kits (KAC1261) and human IL-8 ELISA kit (KAC1301) (Life Technologies Australia Pty Ltd). Assay kits were chromogen-based and cytokine concentration (colour) was quantified using a Titertek Multiskan MCC/340 plate reader at the appropriate wavelength dictated by the particular kit utilized. Each assay was run against a standard curve with a full range predetermined for each cytokine and sample source (Rube et al. 2008). These kits were designed to detect cytokines levels using a target-specific antibody on pre-coated 96 well microplates. The procedure of sandwich ELISA method for plasma analysis for cytokines TNF-α, TGF-β1, IL-6 and IL-8 expression levels is completely described in chapter 3 (See section 3.3.9.3).

5.2.6 Statistical methods

Statistical analysis was performed using GraphPad Prism 7. Pre- and post-treatment blood plasma samples were evaluated for cytokine expression levels. In addition, their expression
levels were also assessed for any linear relationships which might exist among clinicopathological characteristics of patient such as age, risk stratification, cTNM-stages, pTNM-stages, pre-operative PSA, GS, RT Dose (Gy), and RT Fractions. A p-value of <0.05 was taken as significant. All the tests are briefly described in chapter 3 (See section 3.3.9.4)

5.3 Results

5.3.1 Clinical characteristics of the patient population

The clinical characteristics of patients in this study are included in Table 5-1. The mean age of PC patients at the time of PC diagnosis was 66.83 ± 7.93 years (range, 53 - 80 years). All patients were diagnosed between 2nd July 2015 and 21st April 2016 at RDH. The mean initial serum PSA (iPSA) levels were 16.03 ± 15.81 ng/ml (range, 4 – 71 ng/ml) with 33% patients at <10 ng/ml (6/18), 50% of patients with PSA at 10-20ng.ml (9/18) and 17% at PSA concentration > 20ng/ml (3/18). In addition, the mean GS was 7.88 ± 1.14 (range, 6 – 10) with 6% patients at GS 6 (1/18), 44% of patients with GS 7 (8/19) and 50% at GS 8-10 (9/18).

Five (28%) of 18 PC patients had intermediate-risk PC and 13 (72%) patients had high-risk PC. The tumour staging was divided into subgroups such as clinical T- stage, pathological T-stage and Magnetic Resonance Imaging T-stage. In clinical T-stage group, 23% of patients were found with T1a-cN0M0 stage (4/18), 33% with T2a-cN0M0 (6/18) and 44% of patients with T3a-cN0M0 (8/18). In pathological T- stage group, 0% of patient was identified with T1a-cN0M0 stage (0/18), 89% with T2a-cN0M0 (16/18) and 11% with T3a-cN0M0 clinical T-stage (2/18). Only one patient (6%) was found to have one ipsilateral pelvic node involved prior to therapy.
Table 5-1: Clinicopathological characteristics of prostate cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.83 ± 7.93 (53 – 80)</td>
<td></td>
</tr>
<tr>
<td>Pre-operative PSA</td>
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<td></td>
</tr>
<tr>
<td>≤ 10 ng/ml</td>
<td>6</td>
<td>33%</td>
</tr>
<tr>
<td>10 -20 ng/ml</td>
<td>9</td>
<td>50%</td>
</tr>
<tr>
<td>&gt; 20 ng/ml</td>
<td>3</td>
<td>17%</td>
</tr>
<tr>
<td>Risk stratification</td>
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</tr>
<tr>
<td>Low-risk</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Intermediate-risk</td>
<td>5</td>
<td>28%</td>
</tr>
<tr>
<td>High-risk</td>
<td>13</td>
<td>72%</td>
</tr>
<tr>
<td>cTNM-stage</td>
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<td></td>
</tr>
<tr>
<td>T1a-cN0M0</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>T2a-cN0M0</td>
<td>6</td>
<td>33%</td>
</tr>
<tr>
<td>T3a-cN0M0</td>
<td>8</td>
<td>44%</td>
</tr>
<tr>
<td>Node involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>6%</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>44%</td>
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<tr>
<td>8-10</td>
<td>9</td>
<td>50%</td>
</tr>
<tr>
<td>Radiation dose</td>
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</tr>
<tr>
<td>78 Gy</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>80 Gy</td>
<td>14</td>
<td>77%</td>
</tr>
</tbody>
</table>

Values for age are presented as the mean ± standard deviation; PSA, prostate-specific antigen.
5.3.2 Effect of ADT on circulating cytokine levels

Because androgens are known to be potent immune modulators, it was of interest to determine what effect, if any, that ADT may have upon the levels of circulating cytokines. To accomplish this, plasma cytokine concentrations in PC patients prior to treatment (pre-ADT) were compared to those following ADT (post-ADT) therapy (post-ADT levels were assessed from blood drawn from patients immediately prior to initiation of their RT). The results displayed in Figure 5-1 demonstrate that two months of ADT do not significantly change the plasma levels of the four cytokines monitored. Plasma levels of the proinflammatory cytokines TNF-α and IL-8 did not significantly alter (mean ±SD, 17.2 ± 2.4 to 17.3 ± 3.7 and 1.2 ± 0.1 to 1.2 ± 0.1, respectively) in the post-ADT samples when compared to pre-ADT values; therefore, ADT had no significant effect on these cytokines. Whereas the concentration of the profibrotic cytokine TGF-β1 was noticed to be elevated (mean ±SD, 643.7 ± 282.7 to 765.2 ± 426.3) and proinflammatory cytokines IL-6 was decreased (mean ±SD, 12.2 ± 3.3 to 10.7 ± 2.4) in the post-ADT samples. However, the expression levels of TNF-α, TGF-β1, IL-6 and IL-8 cytokines were not statistically different when compared pre-ADT and post-ADT levels.
Figure 5.1 Influence of androgen deprivation therapy (ADT) and radiation therapy (RT) on circulatory cytokines levels before ADT (baseline), before RT, End of RT and 3 months after RT for prostate cancer patients.
Table 5-2: Effects of ADT and RT on circulating cytokine levels in patients with PC

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>Before ADT</th>
<th>Before RT</th>
<th>End of RT</th>
<th>3 months after RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>17.2 ± 2.4</td>
<td>17.3 ± 3.7</td>
<td>17.2 ± 3.7</td>
<td>17.4 ± 5.1</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>643.7 ± 282.7</td>
<td>765.2 ± 426.3</td>
<td>915.9 ± 539.2</td>
<td>732.4 ± 441.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.2 ± 3.3</td>
<td>10.7 ± 2.4</td>
<td>10.6 ± 3.2</td>
<td>16.2 ± 15.5</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1*</td>
</tr>
</tbody>
</table>

TNF-α, Tumour necrosis factor-alpha; TGF-β1, Tumour growth factor-beta1; IL-6, Interleukin-6; IL-8, Interleukin-8.
5.3.3 Effect of RT on circulating cytokine levels

Further, we examined the effect of RT on the levels of circulating cytokine. The proinflammatory cytokines TNF-α, IL-6 and IL-8 did not change (mean ±SD, 17.3 ± 3.7 to 17.2 ± 3.7, 10.7 ± 2.4 to 10.6 ± 3.2 and 1.2 ± 0.1 to 1.2 ± 0.1 respectively) in post-RT blood plasma when compared with pre-RT blood plasma (see Figure 5-1); thus, RT had no effect on the levels of these cytokines. In contrast, the level of profibrotic cytokine TGF-β1 increased (mean ±SD, 765.2 ± 426.3 to 915.9 ± 539.2) more in post-RT blood plasma than pre-RT blood plasma of PC patients. However, we found no significant differences in the pre-RT and post-RT cytokine levels (see Table 5-2). While the temporal pattern of blood cytokine IL-6, TNF-α and IL-8 were remained the same after RT, but expression levels of TGF-β1 were noticeably elevated after RT (not significantly significant).

5.3.4 Levels of circulating cytokine after 3 months of RT

Patients were carefully evaluated at 3 months follow-up period after ADT and RT treatments. In addition, diagnostic tests included at least serum PSA measurement, DRE, and careful evaluation of symptoms to assess the treatment response and the side effects of treatments given. In order to examine the cytokines levels in the 3 months follow-up period after ADT and RT, the plasma cytokine levels at the end of RT were compared with 3 months follow-up period of treated patients. Again, proinflammatory cytokines TNF-α and IL-8 did not change (mean ±SD, 17.2 ± 3.7 to 17.4 ± 5.1 and 1.2 ± 0.1 to 1.3 ± 0.1 respectively) in blood plasma when compared with the values at the end of RT (see Figure 5-1). On the other hand, profibrotic cytokine TGF-β1 decreased (mean ±SD, 915.9 ± 539.2 to 732.4 ± 441.7) and proinflammatory cytokine IL-6 significantly increased (mean ±SD, 10.6 ± 3.2 to 16.2 ± 15.5) after 3 months of therapeutic treatments. However, after 3 months follow-up the changes of TGF-β1 and IL-6 cytokines levels were not significant.
5.4 Discussion

In the last few years, many clinical studies have focused on the elucidation of clinically useful molecular biomarkers of RT-induced toxicity. Researchers believe that the ability to identify a patient’s radiation sensitivity profile could lead to more suitable treatment options, improved local control and survival. Regarding this, the possible association between altered circulating cytokines levels with RT and the cause of RT-induced toxicity has resulted in much discussion in the clinical literature (Chen, Y et al. 2002; Christensen et al. 2009; Fleckenstein et al. 2007; Johnke et al. 2009). A large section of the discussion has concentrated on determining the potential of circulating cytokines to predict the development of RT-induced toxicity following thoracic RT for lung cancer (Fleckenstein et al. 2007). The aim of this study was to evaluate the expression of the proinflammatory and profibrotic cytokines in the plasma of PC patients receiving ADT and RT and to determine the effect of neoadjuvant ADT and RT on cytokines expression as well. In this study, cytokines TNF-α, TGFβ1, IL-6 and IL-8 were carefully chosen on the basis of their well-documented role in the humoral response to RT (Christensen et al. 2009; Fleckenstein et al. 2007; Johnke et al. 2009; Muller & Meineke 2007; Wu et al. 2013).

Our clinical study has revealed several features regarding circulating cytokine levels in patients diagnosed with adenocarcinoma of the prostate. As well as the alterations in circulating cytokine profiles based on tumour, our study also clearly demonstrated the influence of RT on the changes in plasma cytokine levels. Cytokine release in response to ionizing radiation is recognized and might play a major role in RT-induced lung toxicity (Ding, Li & Sun 2013; Provatopoulou, Athanasiou & Gounaris 2008). Previous studies have reported that TGF-β1, IL-6 and IL-10 during RT may be useful as predictive biomarkers for RT-induced toxicity (Arpin et al. 2005; Citrin et al. 2012; Zhao, Lujun et al. 2009). Certainly,
the administration of RT resulted in noticeable variations of circulating cytokine levels. Johnke et al. (2009) have reported elevated levels of TGFβ, IL-1β, and IL-6 at the end of the RT regimen of PC patients. In addition, TGF-β1 and TGF-β2 were significantly increased during RT compared to those before RT (Tanji et al. 2015). Christensen et al. (2009) have also reported that the expression of IFN-γ and IL6 were significantly elevated during intensity-modulated RT for PC. Moreover, the circulating serum levels of IL6 and TNFα significantly increased following RT (Hurwitz et al. 2010). In our study, the pro-inflammatory cytokines IL-6, TNF-α and IL-8 remained unchanged in the post-RT when compared with pre-RT plasma phase of PC patients. We did notice an increased level of profibrotic TGF-β1 in post-RT phase compared to pre-RT, but this difference was not statistically significant, and may be due to limited sample size.

In the last few years, there has been increasing interest in understanding the association between testosterone levels and inflammatory biomarkers. Some previous clinical studies have estimated the association between male hypogonadism and serum levels of pro- and/or anti-inflammatory cytokines (Maggio et al. 2006; Malkin et al. 2004; Tanji et al. 2015). Maggio et al. (2006) found that testosterone levels were inversely associated with soluble IL-6 receptor levels but not with levels of the other inflammatory markers such as IL-6, TNF-α, IL-1β, and CRP. In addition to this, testosterone supplementation in hypogonadal men caused significant decreases in TNF-α and IL-1β as well as an increase in IL-10 (Malkin et al. 2004). Another study also showed elevated levels of several inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, and SDF-1) at baseline or before ADT treatment (Saylor et al. 2012). To gain additional insight into the systemic changes after ADT for PC patients, we measured circulating proinflammatory and profibrotic cytokines in plasma. We found that plasma levels of the profibrotic cytokine TGF-β1 increased in post-ADT blood; however, IL-6 decreased
when compared with pre-ADT blood plasma. The results were not statistically different from that seen in pre-ADT.

However, it is interesting to note that the addition of ADT to conventional RT for treatment of PC may have the potential to attenuate the increases in TGF-β1 after RT that have been correlated with RT-induced toxicity in other types of cancers (Chen, Y et al. 2002; Fleckenstein et al. 2007). Johnke et al. (2009) have also explored the immunological influence of combining neoadjuvant hormonal therapy with RT for PC by monitoring the serum cytokine levels. They detected the progressive alteration of cytokine levels, which seemed to be significantly affected by the addition of hormonal therapy. Unfortunately, the exact biological mechanisms underlying the hormone-induced shift toward a more pro-inflammatory state remain unclear.

5.5 Conclusion

The result of the current study is the observation that the administration of neoadjuvant ADT altered the levels of various RT-induced cytokines. No significant differences were observed before and after RT in the patients treated with neoadjuvant ADT and RT. Tanji et al. (2015) also investigated the immunological impact of combining neoadjuvant ADT with RT for PC by monitoring the serum cytokine levels. They observed the temporal change of cytokine levels, which appeared to be significantly affected by the addition of ADT. The results of this study suggest that the profibrotic TGF-β1 and proinflammatory IL-6 cytokine expression is influenced by ADT and RT in patients with PC. However, we found no significant results in our observational study and the reason could be small sample sizes, thus limiting the statistical power to detect significant differences. Further study should be done to better define important biomarkers and their clinical implications among PC patients. In particular,
the roles of the profibrotic TGF-β1 and proinflammatory IL-6 cytokines in men receiving ADT and RT warrant further clarification.
Chapter 6- The predictive role of circulatory cytokines in RT oncology for prostate cancer and RT-induced acute toxicity

6.1 Introduction

Prostate cancer (PC) is one of the most frequent tumours diagnosed in men worldwide (Zhou, CK et al. 2016). External beam radiotherapy (EBRT) is an effective and common treatment modality for locally advanced PC (De Langhe et al. 2013). Refinement of EBRT technologies, reliable predictive biomarkers of response to a given treatment, possible new targets for biological therapy and advanced knowledge of radiobiology have changed the approach to PC radiotherapy (Boccellino et al. 2015; De Cobelli et al. 2015).

Moding et al. (2013) reported that RT is a significant treatment modality for numerous malignancies as 60% of cancer patients receive ionizing radiation as a part of their therapeutic regimen. Although RT has variable success depending on the cancer being treated, the RT-induced toxicity or side effects associated with its use can affect the quality of life of the patients (Furst 1996). RT-induced toxicity can be classified as acute or late toxicity. Acute toxicities occur during RT and up to 3 months from the completion of treatment, while late effects are observed from 3 months to years after completion of RT (Berkey 2010). RT-induced toxicity can have an adverse effect on a patient’s quality of life (Furst 1996). During the treatment of PC, the most common acute toxicities of RT manifest as gastrointestinal and genitourinary symptoms based on validated scoring criteria [Common Terminology Criteria for Adverse Events (CTC-AE) (Christensen et al. 2009)]. The escalation of RT dose using intensity-modulated RT (IMRT) results in less toxicity and better biochemical tumour control (Christensen et al. 2009).
Molecular biomarkers have gained much attention in recent times, in order to monitor and inform the degree and severity of normal tissue toxicity from RT. This has led to an interest in the development of molecular biomarkers with adequate sensitivity and specificity to become clinically useful. Cytokines are a group of novel biomarkers involved in tissue damage response and tissue remodelling, and have been examined in numerous clinical studies (Christensen et al. 2009; Holliday et al. 2016; McDonald et al. 2016; Rube et al. 2008). Rubin et al. (1995) were among the first to define the role of cytokines in mediating RT-induced toxicity. They reported that levels of interleukin (IL)-1, transforming growth factor (TGF)-β, and tumour necrosis factor (TNF)-α were elevated immediately after RT exposure and that chronically increased TGF-β levels were correlated with an increased risk of pulmonary fibrosis in clinical lung studies. The association between acute RT-induced toxicity and cytokine expression is supported by other studies showing that elevated cytokine expression post-RT is associated with RT-induced lung toxicity (Fleckenstein et al. 2007; Hart, Justin P et al. 2005; Okunieff et al. 2008; Zhao, L. et al. 2008).

Most clinical studies investigating associations between cytokine expression and clinical outcomes after RT treatment have concentrated on measurements prior to and at the end of RT treatment (Christensen et al. 2009). Elevated levels of IL-6, IL-8, vascular endothelial growth factor (VEGF), HGF, and GRO-1 during RT were significantly associated with decreased cancer cause-specific survival in Head and Neck Squamous Cell Carcinoma (HNSCC) (Allen et al. 2007). Similarly, another study reported that elevated expression of IL-1α, M-CSF, and TGF-β in patients during RT for PC was associated with accumulated dose of RT (Kovacs et al. 2003). Although cytokine levels are elevated in response to RT, the temporal pattern of these changes during and after RT has not been closely addressed in clinical studies.
The goals of our clinical study were to assess the levels of proinflammatory and profibrotic cytokines in blood plasma before ADT, prior to RT, at the end of RT and 3 months after the completion of RT and interrogate any association between cytokines levels with acute RT-induced toxicity.

6.2 Methods and material

6.2.1 Study population

Eighteen patients were recruited from November 2015 to December 2016 and informed consent was obtained from all patients prior to study enrolment. The patients with age ≥ 18 years, histologically confirmed adenocarcinoma of the prostate and no history of prior surgery to the prostate were enrolled for this study. Exclusion criteria included metastatic disease at presentation, prior history of malignancy (excluding basal cell or squamous cell skin cancer), and serious medical or psychiatric illness, precluding safe administration of RT +/- ADT. All patients were planned and treated using image-guided IMRT. Definitive IMRT patients received a total dose of 78 Gy in 39 fractions (n = 4) and 80 Gy in 40 fractions (n = 14). Quality assurance for image-guided RT was attained by daily fiducial marker matching for definitive IMRT treatments and by bony anatomic matching for postoperative IMRT treatments.

6.2.2 Patient blood collection and plasma processing

To study blood biomarkers, early consideration is important whether to use plasma (anti-coagulated blood) or serum (coagulated blood, clot removed). The time from blood collection to primary processing (i.e. centrifuge and aliquoting) can have a significant impact on protein stability. In this clinical study, blood samples were obtained before and after RT treatment from the patient. The blood samples were taken prior to the commencement of RT, last week
of RT and 3 months from completion of RT. The process of blood collection and plasma processing is briefly described in chapter 3 (See section 3.3.8.2)

6.2.3 Grading acute RT-induced toxicity

In this study, all recruited patients were asked to complete questionnaires concerning symptoms of rectal or bladder dysfunction prior to RT, on the last week of RT and 3 months after the completion of RT as part of their standard care. International Index Prostate Symptom Scoresheet (IIPS) (Li et al. 2014; Rodrigues et al. 2004) and Expanded Prostate Cancer Index Composite (EPIC) (Chang, P et al. 2011) were the tools used to interrogate the acute bladder and rectal toxicities respectively. The total score can range from 0 to 35 for IIPS and 0 to 28 for EPIC respectively (asymptomatic to very symptomatic). The RT-induced toxicity is considered as mild if the total score is equal or less than 7, while it is considered as moderate if total score range is 8-19, and it is considered as severe if the total score range is 20-35 (Li et al. 2014; Wei, JT et al. 2000). The grading of acute RT-induced toxicity is briefly described in chapter 3 (See section 3.3.10)

6.2.4 Assessment of plasma cytokines

All analysis tests were carried out blinded to patient and therapy factors. The expression levels of the cytokines TNF-α, TGF-β1, IL-6 and IL-8 were measured on plasma samples. All assay kits were chromogen-based and cytokine concentration (colour) was quantified at the appropriate wavelength recommended by the particular kit utilised. The completed procedure of plasma analysis for cytokine expression levels is described in chapter 3 (See section 3.3.9.3).
6.2.5 Statistical analysis

All analysis tests were completed using GraphPad Prism 7 software program and \( p \leq 0.05 \) was taken as significant. Acute RT-induced toxicity was modelled as grade 0/2 (defined as no toxicity) versus grade 3/5 (defined as toxicity). The plasma expression levels of cytokines TNF-\( \alpha \), TGF-\( \beta 1 \), IL-6 and IL-8 were tested for their association with gastrointestinal and genitourinary acute RT-induced toxicity using Spearman’s correlation tests which are briefly described in chapter 3 (See section 3.3.9.4)
6.3 Results

6.3.1 Clinical characteristics of the patient

The mean age of PC patients at the time of diagnosis was 66.83 ± 7.93 years (range, 53 - 80 years). The mean pre-treatment serum PSA level was 16.03 ± 15.81 ng/ml (range, 4 – 71 ng/ml) and post-RT serum PSA were 0.34 ± 0.77 ng/ml (range, 0.05 – 3.48 ng/ml). The mean GS was 7.94 ± 1.01 (range, 6 – 10). Four (23%) patients had a GS of 3 + 4 = 7, five (28 %) patients had GS of 4 + 3 = 7, three (16.5 %) patients had GS of 4 + 4 = 8, three (16.5 %) patients had GS of 4 + 5 = 9, one (5 %) patient had GS of 5 + 4 = 9 and two (11 %) patients had GS of 5 + 5 = 10. All PC patients were treated with external beam RT, 23% (4/18) of patients were treated with 78Gy RT dose and 77% (14/18) of patients with 80Gy RT dose. Table 6-1 summarises the clinicopathological characteristics of the patients.
Table 6-1: Clinicopathological characteristics of prostate cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.83 ± 7.93 (53 – 80)</td>
<td></td>
</tr>
<tr>
<td>cT-stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• T1c</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>• T2a</td>
<td>3</td>
<td>16.5%</td>
</tr>
<tr>
<td>• T2b</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>• T2c</td>
<td>2</td>
<td>11%</td>
</tr>
<tr>
<td>• T3a</td>
<td>3</td>
<td>16.5%</td>
</tr>
<tr>
<td>• T3b</td>
<td>5</td>
<td>28%</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 3 + 3 = 6</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>• 3 + 4 = 7</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>• 4 + 3 = 7</td>
<td>5</td>
<td>28%</td>
</tr>
<tr>
<td>• 4 + 4 = 8</td>
<td>3</td>
<td>16.5%</td>
</tr>
<tr>
<td>• 4 + 5 = 9</td>
<td>3</td>
<td>16.5%</td>
</tr>
<tr>
<td>• 5 + 4 = 9</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>• 5 + 5 = 10</td>
<td>2</td>
<td>11%</td>
</tr>
<tr>
<td>Pre-RT PSA (ng/ml)</td>
<td>16.03 ± 15.81 (range, 4 – 71)</td>
<td></td>
</tr>
<tr>
<td>Post RT PSA (ng/ml)</td>
<td>0.35 ± 0.77 (range, 0.05 – 3.48)</td>
<td></td>
</tr>
<tr>
<td>Radiation dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 78 Gy in 39 fractions</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>• 80 Gy in 40 fractions</td>
<td>14</td>
<td>77%</td>
</tr>
</tbody>
</table>

Values for age are presented as the mean ± standard deviation; cT-stage, clinical tumour stage; RT, radiotherapy; PSA, prostate-specific antigen and Gy, gray, unit of radiation dose.
6.3.2 Baseline (pre-RT) cytokines expression versus post-RT

Blood samples were collected by a Consultant Radiation Oncologist and analysed at the PC2 laboratory. We conclude that the risk of artifact due to handling and processing is low. Patient compliance was excellent with all patients going on to complete treatment. Regarding sample collection, 61 of 72 blood collections were successfully completed, which translates to an 85% success rate.

There was no significant difference between the baseline (pre-RT), end of RT and 3 months after RT cytokines expression levels as summarised in Table 6-2. We conclude that the time of blood sampling before and after RT is not a significant factor for cytokine expression in this treatment cohort. When the average cytokine levels from baseline, end of RT and after 3 months of RT were compared, we found that profibrotic cytokine TGF-β1 levels were elevated (mean ±SD, 765.2 ± 426.3 to 915.9 ± 539.2) at the end of RT over baseline. Although there were higher IL-6 (mean ±SD, 10.6 ± 3.2 to 16.2 ± 15.5) at 3 months post RT, the results were not statistically significant. In addition, in Figure 6-1, the TNF-α, TGF-β1 and IL-6 expression levels, respectively, were plotted against the time of blood sampling.
Table 6-2: Effects of RT on circulating cytokine levels in patients with PC

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>Before RT</th>
<th>End of RT</th>
<th>3 months after RT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td>Mean</td>
<td>17.3 ± 3.7</td>
<td>17.2 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>median</td>
<td>16.6 (14.4 to 19.0)</td>
<td>16.0 (14.6 to 20.3)</td>
</tr>
<tr>
<td><strong>TGF-β1</strong></td>
<td>Mean</td>
<td>765.2 ± 426.3</td>
<td>915.9 ± 539.2</td>
</tr>
<tr>
<td></td>
<td>median</td>
<td>681 (515.7 to 782.7)</td>
<td>894.5 (572.9 to 995.2)</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>Mean</td>
<td>10.7 ± 2.4</td>
<td>10.6 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>median</td>
<td>10.5 (8.8 to 12.3)</td>
<td>9.7 (8.8 to 12.1)</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>Mean</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>median</td>
<td>1.1 (1.1 to 1.3)</td>
<td>1.2 (1.1 to 1.2)</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± standard deviation and median and interquartile range;

TNF-α, Tumour necrosis factor-alpha; TGF-β1, Tumour growth factor-beta1; IL-6, Interleukin-6; IL-8, Interleukin-8; RT, radiotherapy
Figure 6-1: Expression of individual mean plasma levels (± SEM) for cytokines TNF-α, TGF-β1, IL-6 and IL-8 for PC patients, before RT, end of RT and after 3 months of RT.
6.3.3 Association of cytokine expression with patient-scored RT-induced toxicity

In our study, we sought to determine if there was an association between cytokine expression and patient-reported genitourinary and gastrointestinal acute RT-induced toxicity graded prospectively (summarized in Table 6-3 and 6-4).

With regards to genitourinary toxicity, the level of cytokines measured before and after therapy showed increased TNF-α (mean ±SD, 13.8 ± 1.9 to 17.7 ± 3.7) and IL-6 increased (mean ±SD, 9.8 ± 3.6 to 12.1 ± 4.5) at post-RT. In addition, TNF-α and IL-6 levels were elevated (mean ±SD, 15.4 ± 4.2 to 23.9) and 18.7 ± 20.3 to 44.6) respectively after 3 months completion of RT. The analysis of TNF-α and IL-6 suggested that the increase of TNF-α and IL-6 is associated with the higher probability of genitourinary toxicity but it did not reach statistical significance.

In contrast, the levels of profibrotic TGF-β1 decreased (mean ±SD, 1201.7 ± 892.9 to 726.5 ± 657.2) as the severity of genitourinary increased at the end of RT. Furthermore, TGF-β1 decreased (mean ±SD, 936.0 ± 580.3 to 343.1) as the genitourinary increased after 3 months completion of RT. The analysis of TGF-β1 suggested that the decrease of TGF-β1 is associated with a higher probability of genitourinary toxicity but it did not reach statistical significance.
Table 6-3: Expression of cytokines and the severity of acute genitourinary RT-induced toxicity

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Phase of Blood</th>
<th>Genitourinary toxicity = 0</th>
<th>Genitourinary toxicity = 1</th>
<th>Genitourinary toxicity = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>End of RT</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3 months after RT</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>End of RT</td>
<td>13.8 ± 1.9</td>
<td>18.6 ± 3.6</td>
<td>17.7 ± 3.7</td>
</tr>
<tr>
<td>3 months after RT</td>
<td>15.4 ± 4.2</td>
<td>17.1 ± 3.9</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>End of RT</td>
<td>1201.7 ± 892.9</td>
<td>858.3 ± 256.4</td>
<td>726.5 ± 657.2</td>
</tr>
<tr>
<td>3 months after RT</td>
<td>936.0 ± 580.3</td>
<td>610.1 ± 254.9</td>
<td>343.1</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>End of RT</td>
<td>9.8 ± 3.6</td>
<td>10.1 ± 1.9</td>
<td>12.1 ± 4.5</td>
</tr>
<tr>
<td>3 months after RT</td>
<td>18.7 ± 20.3</td>
<td>10.2 ± 2.6</td>
<td>44.6</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>End of RT</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>3 months after RT</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

Values for cytokine concentration are presented as the mean ± standard deviation; TNF-α, Tumour necrosis factor-alpha; TGF-β1, Tumour growth factor-beta1; IL-6, Interleukin-6; IL-8, Interleukin-8; genitourinary toxicity (0 = mild, 1 = moderate and 2 = severe toxicity).
Regarding gastrointestinal toxicity, the level of the proinflammatory cytokine TNF-α increased (mean ±SD, 16.9 ± 3.8 to 19.3 ± 3.6) as the severity of gastrointestinal toxicity increased at post-RT. In addition to this, TNF-α was elevated (mean ±SD, 14.9 ± 4.0 to 23.9) as the gastrointestinal toxicity increased after 3 months completion of RT. The analysis of TNF-α suggested that the increase of TNF-α is associated with a higher probability of gastrointestinal toxicity but it did not reach statistical significance. Furthermore, the level of the proinflammatory cytokine IL-6 increased (mean ±SD, 9.9 ± 2.5 to 14.3 ± 4.6) as the severity of gastrointestinal increased at post-RT. In addition to this, IL-6 elevated (mean ±SD, 17.7 ± 18.9 to 44.6) as the gastrointestinal increased after 3 months completion of RT. The analysis of IL-6 suggested that the increase of IL-6 is associated with the higher probability of gastrointestinal toxicity but it did not reach statistical significance.

However, the levels of profibrotic TGF-β1 decreased (mean ±SD, 972.7 ± 536.5 to 269.3 ± 1.4) as the severity of gastrointestinal increased at the end of RT. Furthermore, TGF-β1 decreased (mean ±SD, 882.1 ± 558.5 to 343.1) as the gastrointestinal increased after 3 months completion of RT. The analysis of TGF-β1 suggested that the decrease of TGF-β1 is associated with a higher probability of gastrointestinal toxicity but it did not reach statistical significance.
**Table 6-4: Expression of cytokines and the severity of acute gastrointestinal RT-induced toxicity**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Phase</th>
<th>Gastrointestinal toxicity = 0</th>
<th>Gastrointestinal toxicity = 1</th>
<th>Gastrointestinal toxicity = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>End of RT</td>
<td>13</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3 months after RT</td>
<td>9</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>End of RT</td>
<td>$16.9 \pm 3.8$</td>
<td>$15.4 \pm 3.3$</td>
<td>$19.3 \pm 3.6$</td>
</tr>
<tr>
<td></td>
<td>3 months after RT</td>
<td>$14.9 \pm 4.0$</td>
<td>$17.9 \pm 3.7$</td>
<td>$23.9$</td>
</tr>
<tr>
<td><strong>TGF-β1</strong></td>
<td>End of RT</td>
<td>$972.7 \pm 536.5$</td>
<td>$362.5 \pm 40.3$</td>
<td>$269.3 \pm 1.4$</td>
</tr>
<tr>
<td></td>
<td>3 months after RT</td>
<td>$882.1 \pm 558.5$</td>
<td>$627.6 \pm 250.5$</td>
<td>$343.1$</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>End of RT</td>
<td>$9.9 \pm 2.5$</td>
<td>$8.7 \pm 1.7$</td>
<td>$14.3 \pm 4.6$</td>
</tr>
<tr>
<td></td>
<td>3 months after RT</td>
<td>$17.7 \pm 18.9$</td>
<td>$10.2 \pm 2.8$</td>
<td>$44.6$</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>End of RT</td>
<td>$1.2 \pm 0.1$</td>
<td>$1.3 \pm 0.1$</td>
<td>$1.2 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td>3 months after RT</td>
<td>$1.3 \pm 0.1$</td>
<td>$1.2 \pm 0.1$</td>
<td>$1.2$</td>
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</tbody>
</table>

Values for cytokine concentration are presented as the mean ± standard deviation; TNF-α, Tumour necrosis factor-alpha; TGF-β1, Tumour growth factor-beta1; IL-6, Interleukin-6; IL-8, Interleukin-8; gastrointestinal toxicity (0 = mild, 1 = moderate and 2 = severe toxicity).
Figure 6-2: Plasma cytokines levels against acute RT-induced toxicity at the end of RT (plots A, C, E and G) and after 3 months of RT completion (plots B, D, F and H). Maximum expression of (TNF-α and IL-6) as a function of maximum toxicity. However, TGF-β1 is decreasing while increasing toxicity. Closed circles, GU toxicity; open circles, GI toxicity.
6.4 Discussion

When planning RT for the treatment of PC, the Radiation Oncologist considers numerous tumour and treatment related factors. These factors include possible patterns of regional tumour spread, to ensure coverage of local tumour extension, uncertainties in positioning the patient for each treatment and tumour and organ movement during and between treatments. To accomplish these goals, normal tissues surrounding the tumour are irradiated, which may result in symptomatic injury. These RT-induced side effects are acute or chronic and have an influence on the quality of life of the PC patients. In general, as the RT dose increased, the probability of genitourinary and gastrointestinal toxicity also increased.

Preclinical and clinical studies have revealed that RT induces inflammatory cytokine (IL-1α, IL-6, IFN-α, IL-β1 and TNF-α) responses and that could play a major role in mediating acute RT-induced toxicity (Bentzen et al. 2010; Christensen et al. 2009; McDonald et al. 2016; Meirovitz et al. 2010; Stankovic et al. 2016). Inflammatory cytokines such as IL-1, IL-6 and TNF-α are identified to stimulate C-reactive protein (CRP) production and elevated plasma CRP has been recognized as a prognostic marker for poor cancer specific survival (CSS), OS and DFS in PC patients undergoing RT (Thurner et al. 2015). The administration of RT resulted in noticeable changes of circulating cytokine levels as shown by Johnke et al. (2009) who reported elevated levels of TGFβ, IL-1β, and IL-6 towards the end of a fractionated course of RT for PC. In addition, TGF-β1 and TGF-β2 were significantly increased during RT compared to those before RT (Tanji et al. 2015). Christensen et al. (2009) have also reported that the expression of IFN-γ and IL6 were significantly elevated during RT for PC when compared with Pre-RT. Moreover, the circulating serum levels of IL6 and TNFα were significantly increased following RT (Hurwitz et al. 2010). In our clinical study, when the average cytokine levels from baseline (before RT), end of RT and after 3 months of RT were
compared, we found that elevated levels of profibrotic cytokine TGF-β1 were observed at the end of RT and proinflammatory cytokines IL-6 at 3 months post-RT over baseline. However, the differences were not significant.

The levels of proinflammatory cytokines IFN-γ and IL-6 were found to increase during IMRT and were significantly associated with increased genitourinary toxicity (Christensen et al. 2009). Another study also presented a statistically significant association between the level of TGF-β1 and subsequent development of RT-induced fibrosis, therefore demonstrating that this is a potential predictive marker for this RT-induced toxicity (Boothe et al. 2013). During RT, the absolute levels of TGF-β1 increased in patients who developed RT-induced lung toxicity (RILT), resulting in an elevation of the TGF-β1 ratio at weeks 2 (2.6 ± 0.7 vs. 1.0 ± 0.01) and 4 (2.0 ± 0.5 vs. 0.8 ± 0.1) (Stenmark et al. 2012). This RT-induced elevation of TGF-β1 was significantly associated with RILT (P=.02 and P=.01 for TGF-β1 ratios at 2- and 4-weeks during RT, respectively). In our clinical study, the levels of proinflammatory cytokines TNF-α and IL-6 increased and were associated with the higher probability for genitourinary toxicity. Our results are in agreement with the above studies but did not reach statistical significance. However, in contrast to previous lung studies, profibrotic cytokine TGF-β1 decreased in our clinical study with the higher probability for genitourinary toxicity but once more was not statistically significant.

Regardless of the treatment technique used, RT for PC exposes a portion of the lower gastrointestinal (GI) tract to ionizing radiation and consequently carries a risk of gastrointestinal toxicity. It is extensively documented that irradiation at large volumes is associated with a high risk of normal tissue toxicity. For example, in protocols applying to irradiation of the bowel, moderate to severe acute gastrointestinal toxicity, primarily diarrhea
is observed in a significant fraction of patients. Christensen et al. (2009) reported that the increased level of IL-6 was associated with the higher probability for gastrointestinal toxicity; however, it did not reach statistical significance. Moreover, elevated level of TNF-α has also been associated with gastrointestinal symptoms (Dirksen, Kirschner & Belyea 2014). Another study also reported that IL-1 expression was associated with increased probability of gastrointestinal toxicity, although we were not able to establish a dose-response relationship (Kovacs et al. 2003). In our clinical study, the levels of TNF-α and IL-6 increased and were associated with the higher probability for gastrointestinal toxicity. However, in contrast to previous studies, profibrotic cytokine TGF-β1 decreased in our clinical study with the higher probability for gastrointestinal toxicity. However, there was no statistically significant correlation with gastrointestinal toxicity.

In our clinical study, no significant correlation was observed between acute gastrointestinal and genitourinary RT-induced toxicity and individual levels of TNF-α, TGF-β1, IL-6 and IL-8. As part of their plasma cytokine analysis, Hart et al. (2005) examined plasma levels of IL-1β, IL-6, and TNF-α prior to RT, while Rube and co-workers also measured the levels of these 3 cytokines before, during, and after RT (Rube et al. 2008). In agreement with our findings, those studies also found no correlation between plasma concentrations of IL-1β, IL-6, and TNF-α and RT-induced lung toxicity (RILT) in patients irradiated for advanced non-small-cell lung cancer (NSCLC). In contrast, the expression levels of IL-6 were significantly higher before and during RT, respectively, in patients who developed RILT (Arpin et al. 2005; Chen, Y et al. 2001).

The clinical study reported here was proposed to be investigative and to guide future larger trials. Future studies in larger cohorts of patients are necessary to control the time course of
plasma cytokine changes after RT exposure and how this correlate to normal tissue RT-induced toxicity with a better conclusion. We observed that TNF-α and IL-6 significantly increased after prostate IMRT, and we found a promising association between increase levels of proinflammatory cytokines TNF-α and IL-6 and acute gastrointestinal and genitourinary toxicity. Although our results are not statistically significant, this early data should be interpreted carefully given the relatively small sample size, but they deserve further study in a larger cohort.

6.5 Conclusion

This study confirmed the positive relationship between cytokine levels and acute gastrointestinal and genitourinary toxicity. This clinical study, due to its small sample size, cannot provide a statistically significant association between levels of TNF-α, TGF-β1 and IL-6 and acute gastrointestinal and genitourinary toxicity during and after RT. In addition, it should be highlighted that because of the small sample size this clinical observation study can only give an indication for further studies with a larger sample size, especially in relation to the proinflammatory cytokines TNF-α and IL-6.
Chapter 7- Summary of Chapters, Recommendations and Conclusion

7.1 Summary of Chapters

This section of the final chapter presents a summary of the work presented in chapters 2 to 6 and discusses the clinical implications of this work. The outcome of the research presented from chapters 2 to 6 is based on a prospective observational study of PC population based in Darwin from Nov 2015 to Dec 2016. Chapters included in this thesis are literature review (Chapter 2), methods and materials (Chapters 3), and a consolidated analysis of cytokine expression in prostatic tumour biopsies (Chapters 4), an analysis of cytokines expression in blood plasma and association with clinicopathological characteristics (Chapter 5) and analysis of plasma-based cytokines and their associations with RT-induced toxicity (Chapter 6). Figure 7-1 summarises overall finding of the thesis.
Research Questions  | Finding  | Conclusions  | Future Directions
--- | --- | --- | ---
**RQ1**- To assess cytokine expression in prostatic needle biopsy specimens  | Expression rates of TNF-α, TGF-β1 and IL-6 were 82.3, 71.6, and 73.3 respectively.  | Significant correlation found between TNF-α, TGF-β1 and IL-6 expression and pre-RT plasma cytokines level. No significant association found between cytokines expression levels in tissue biopsy specimens and pre-operative PSA levels and GS; this is most likely explained by the small patient numbers included in this study.  | Based on the results of this study, future studies should investigate panel of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) and profibrotic cytokine TGF-β1 in a larger cohort of PC patients undergoing ADT and RT and with longer follow-up to investigate late RT-induced toxicity and overall survival. Interestingly, I have found higher plasma cytokines levels in patients who developed metastatic cancer from high-risk disease and future studies can also identify early biomarkers for metastatic PC.  

**RQ2**- To assess the influence of ADT and RT on changes in cytokines levels in blood plasma  | TNF-α, TGF-β1 and IL-6 expression levels were reduced with increases GSs  | The changes in cytokines levels have been found after ADT and RT, which strengthen the finding of other clinical studies. Accept that small numbers of samples made difficult to attain significant results.  |  

**RQ1**- To assess any correction between cytokine expression/levels and clinical outcomes including acute RT-induced toxicity  | Pre-RT plasma TNF-α, TGF-β1 and IL-6 were increased with staining intensity increases of the prostatic needle-biopsy specimens.  | Study confirmed positive relationship between cytokines levels and acute GU and GI toxicity, though the difference was not statistically significant.  |  

TNF-α and IL-6 increased the severity of GU and GI toxicity increased, TGF-β1 decreased as severity of GU and GI increased.  

TGF-β1 was elevated after ADT, IL-6 was decreased after ADT.  

TGF-β1 was elevated after RT,  

TGF-β1 was decreased and IL-6 increased 3 months post-RT completion.  

TNF-α and IL-6 increased the severity of GU and GI toxicity increased, TGF-β1 decreased as severity of GU and GI increased.  

Based on the results of this study, future studies should investigate panel of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) and profibrotic cytokine TGF-β1 in a larger cohort of PC patients undergoing ADT and RT and with longer follow-up to investigate late RT-induced toxicity and overall survival. Interestingly, I have found higher plasma cytokines levels in patients who developed metastatic cancer from high-risk disease and future studies can also identify early biomarkers for metastatic PC.

Figure 7-1 A figure summarises overall finding of the thesis
The clinical observational study presented in this thesis was an exclusive opportunity to study the impact of ADT and RT at the molecular level with collaborators from a range of disciplines including ADT, RT, radiation oncology, laboratory medicine, and bioinformatics. This work represents a significant first step towards describing protein radiation responses before and after RT to identify potential biomarkers of RT exposure in tumour and normal tissues. The future of radiation medicine lies not only in improving treatment delivery technologies but in modifying treatment at a molecular level as well. Identifying potential biomarkers of a tumour and normal tissue response will improve our understanding of radiation biology.

7.1.1 Chapter two

In chapter two, we presented a comprehensive report of cytokine expression in PC, and an extensive search was carried out on PubMed, MEDLINE, and Google Scholar and Science Direct using terms. RT is one of the key modalities used to treat localized PC. Currently, PSA is the gold standard biomarker for assessing tumour radiation response. Though patients show a heterogeneous range of responses to RT, potential biomarkers would be beneficial to screen a tumour and normal tissue response to RT. As cancer is a proteomic disease and that multitudes of proteins are affected by RT exposure; single biomarker studies are simply unrealistic for describing tumour and host responses to RT. Therefore, we wanted to assess more than one including inflammatory and profibrotic cytokines before and after RT for PC. The key endpoint of this study was to determine the possibility of obtaining blood samples from patients undergoing ADT and RT. As a second endpoint, the influence of ADT and RT in patterns of protein levels before and after RT were analysed to determine the variability of individual protein response signatures. This clinical study has significant implications for future PC prospective trials at our university which will compare plasma cytokines radiation-
responses to clinical outcome based on PSA-related relapse rates and RT-associated toxicity.

7.1.2 Chapter three

In chapter three, entitled “Methods and materials” we have reported our protocols such as IHC and sandwich ELISA methods for examining cytokines expression using tissue and plasma samples from patients undergoing ADT and RT. In this study, we observed that clinical research is possible at our university based on our study design. When blood collection was expedited and standardized, the risk of handling artefacts was negligible. This study was successful in patient enrolment, tissue and blood sampling, plasma storage and particularly in view of all the challenges and limitations imposed by the clinical research environment. We carried out an investigative statistical analysis using the limited data set to answer the following questions:

- Does cytokine expression change according to the clinicopathological characteristics of patients
- Do baseline plasma samples fluctuate in cytokine expression?
- Are cytokines more likely to change with the influence of ADT and RT treatments?
- Do changes in cytokines expression correlate with acute RT-induced toxicity?

7.1.3 Chapter four

In the fourth chapter, entitled "Immunohistochemical analysis of cytokine expression in benign epithelium and malignant human prostate biopsies and correlation with clinicopathological characteristics and clinical outcomes" which focused on cytokine measurements on prostatic needle-biopsy specimens and their association with clinicopathological characteristics. This clinical study reported that the expression levels of TNF-α, TGF-β1 and IL-6 cytokines on prostatic needle-biopsy specimens were not
significantly associated with pre-operative PSA values. Moreover, TNF-α, TGF-β1 and IL-6 expression levels were correlated in a negative fashion with GSs. Only TNF-α expression levels were found to be significantly correlation with GSs. To evaluate the possible impact of tumour-derived cytokine production on circulating plasma levels, the prostatic needle-biopsy specimens of the PC patients were IHC stained for TNF-α, TGF-β1 and IL-6. We found a statistically significant correlation between the amount of pre-RT plasma levels and the staining intensity of the corresponding prostatic needle-biopsy specimens.

7.1.4 Chapter five

In the fifth chapter, entitled “Levels of plasma cytokines in patients undergoing androgen deprivation therapy and radiation therapy for adenocarcinoma of the PC and correlation with clinical outcomes” we focused on cytokine measurements in blood plasma at different intervals of blood collection, the influence of ADT and RT on cytokine expression and possible associations with clinicopathological characteristics and clinical outcomes. The present clinical study revealed the effect of ADT and RT on the proinflammatory IL-6 and profibrotic TGF-β1 cytokine levels in the blood plasma of patients with PC. Thus, the different interval of blood plasma testing was associated with changes of IL-6 and TGF-β1 cytokines levels, but these changes were not significant. Further study should be done to better define important molecular biomarkers and their clinical implications among PC patients. In particular, the roles of the proinflammatory IL-6 and profibrotic TGF-β1 cytokines in men receiving ADT and RT warrant further clarification.

7.1.5 Chapter six

In the sixth chapter, entitled “The predictive role of circulatory cytokines in RT oncology for PC and RT-induced acute toxicity”, we reported the levels of proinflammatory and
profibrotic cytokines in blood plasma before ADT, prior to RT, end of RT and after 3 months completion of RT and any association of cytokine levels with acute RT-induced toxicity. This study confirmed the positive relationship between cytokine levels and acute gastrointestinal and genitourinary toxicity. However, due to its small sample size, this study cannot provide a statistically significant association between levels of TNF-α, TGF-β1, IL-6 and IL-8 and acute gastrointestinal and genitourinary toxicity. Thus, this clinical observation study can only give an indication for further studies with larger sample size, especially in consideration of the proinflammatory TNF-α and IL-6 whose altered levels were associated with severe acute gastrointestinal and genitourinary toxicity during and after RT in PC patients.

The statistical analysis reported here was proposed to be investigative and to guide future larger trials. Future studies in larger cohorts of patients are necessary to control the time course of plasma cytokine changes after RT exposure and how these correlates to normal tissue RT-induced toxicity with a better conclusion. We observed that TNF-α and IL-6 significantly increased after prostate IMRT, and we found a promising association between increased levels of proinflammatory cytokines TNF-α and IL-6 and acute gastrointestinal and genitourinary toxicity. As our results are not statistically significant, this early data should be interpreted carefully given the relatively small sample size, but they deserve further study in a larger cohort.

7.2 Recommendations

Based on the results of our experiments, future studies should investigate our panel of proinflammatory and profibrotic cytokines in a larger cohort of PC patients undergoing ADT and RT. In carrying out this study, we have created a tissue bank at Charles Darwin University with multiple aliquots of plasma samples for every enrolled patient. Additionally, we have
also established a prostate tissue bank for each patient who enrolled in this clinical observation study. Considering that only two of eight plasma aliquots were required for the sandwich ELISA experiments presented in this thesis, there remains a wealth of biological material available for future biomarker studies.

7.3 Conclusion

In radiation oncology, cytokines have potential as substitution indicators of RT sensitivity and exposure. Although results indicate that cytokines reflect RT response, we still require potential biomarkers for clinical use in monitoring tumour kill and normal tissue toxicity. This cytokine study was carried out with uniform study populations, demanding standards for sample collection and processing, and the latest sandwich ELISA protein analysis technology, which have significant potential to recognise clinically useful cytokines and to generate future, hypothesis-driven experiments.

In future studies, by planning the study carefully and controlling for RT dose volume as a prejudice, dose-specific biomarker profiles can be inter-compared within similar cohorts to afford more adapted treatment planning. It is hoped that the work presented in this thesis will contribute to the identification of significant biomarkers that contribute not only to the development of radiation biology in the long-term but also allow us to make a difference in the lives of cancer patients in the future.

7.4 Future directions

Based on the results of this study, future studies should investigate panel of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) and profibrotic cytokine TGF-β1 in a larger cohort of PC patients undergoing ADT and RT and with longer follow-up to investigate late RT-induced
toxicity and overall survival. Interestingly, I have found higher plasma cytokines levels in patients who developed metastatic cancer from high-risk disease and future studies can also identify early biomarkers for metastatic prostate cancer.
09 June 2015

Dr Paolo De Iesso
Alan Walker Cancer Care Centre
Royal Darwin Hospital
Rocklands Drive
Tiwi NT 0810

Dear Dr De Iesso,

HREC Reference Number: 2015-2385
Project Title: Analysis of cytokine expression in prostate cancer patients undergoing radiation therapy: Correlation with clinicopathological outcomes.

Thank you for letter sent on 05/06/15 and taking the time to respond to the issues of concern identified by the Fast Track sub-committee of the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC)

I am pleased to advise that the Chair of the HREC has granted full ethical approval of this research project. Please note that HREC approval applies only to research conducted after the date of this letter.

This approval will be ratified at the next meeting of the Human Research Ethics Committee.

The nominated participating sites in this project is/are:

- Royal Darwin Hospital
- Charles Darwin University

The documents listed below are approved:

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<thead>
<tr>
<th>Document</th>
<th>Version</th>
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<tr>
<td>Participant Sheet Information</td>
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Approved Project Timeline: 09/06/2015 – 31/12/2020

Approval is granted for a maximum period of twelve months. An annual progress report or final report is required on or before the 09/06/2016.

APPROVAL IS SUBJECT TO the following conditions being met:
1. The Coordinating Principal Investigator will **immediately report anything that might warrant review** of ethical approval of the project.

2. The Coordinating Principal Investigator will notify the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) of **any event that requires a modification or amendment to the protocol or other project documents** and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found on the Menzies’ website, or by **clicking here**.

3. The Coordinating Principal Investigator will submit any necessary reports related to the **safety of research participants (e.g. protocol deviations, protocol violations)** in accordance with the HREC’s policy and procedures. These guidelines can be found on the Menzies’ website, or by **clicking here**.

4. The Coordinating Principal Investigator will **report to the HREC annually and notify the HREC when the project is completed at all sites using the specified forms. Forms and instructions may be found on the Menzies’ website, or by clicking here**.

5. The Coordinating Principal Investigator will notify the HREC if the project is **discontinued at a participating site before the expected completion date, and provide the reason/s for discontinuance**.

6. The Coordinating Principal Investigator will notify the HREC of any plan to **extend the duration of the project past the approval period listed above** and will submit any associated required documentation. The preferred time and method of requesting an extension of ethical approval is during the **annual progress report**. However, an extension may be requested at any time.

7. The Coordinating Principal Investigator will notify the HREC of his or her **inability to continue as Coordinating Principal Investigator**, including the name of and contact information for a replacement.

8. The safe and ethical conduct of this project is entirely the responsibility of the investigators and their institution(s).

9. Researchers should immediately report anything which might affect continuing ethical acceptance of the project, including:
   - Adverse effects of the project on subjects and the steps taken to deal with these;
   - Other unforeseen events;
   - New information that may invalidate the ethical integrity of the study; and
   - Proposed changes in the project.

10. Approval for a further twelve months, within the original proposed timeframe, will be granted upon receipt of an annual progress report if the HREC is satisfied that the conduct of the project has been consistent with the original protocol.
11. Confidentiality of research participants should be maintained at all times as required by law.

12. The Patient Information Sheet and the Consent Form shall be printed on the relevant site letterhead with full contact details.

13. The Patient Information Sheet must provide a brief outline of the research activity including: risks and benefits, withdrawal options, contact details of the researchers and must also state that the Human Research Ethics Administrators can be contacted (telephone and email) for information concerning policies, rights of participants, concerns or complaints regarding the ethical conduct of the study.

14. You must forward a copy of this letter to all Investigators and to your institution (if applicable).

**This letter constitutes ethical approval only.** This project cannot proceed at any site until separate research governance authorisation has been obtained from the CEO or Delegate of the institution under whose auspices the research will be conducted at that site.

Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: ethics@menzies.edu.au or telephone: (08) 8946 8687 or (08) 8946 8692.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research wishes you every continued success in your research.

Yours sincerely,

Associate Professor Phil Giffard
Chair
Human Research Ethics Committee
of Northern Territory Department of Health
and Menzies School of Health Research
NHMRC Registration No. EC00153
http://www.menzies.edu.au/page/Research/Ethics_approval/

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This HREC is constituted and operates in accordance with the National Health and Medical Research Council’s (NHMRC) National Statement on Ethical Conduct in Human Research (2007). The processes used by this HREC to review multi-centre research proposals have been certified by the National Health and Medical Research Council.
22 June 2017

Dr. Paolo De Ieso
Alan Walker Cancer Care Centre
Royal Darwin Hospital
Rocklands Drive
Tiwil NT 0810

Dear Dr. De Ieso,

HREC Reference Number: 2015-2385
Project Title: Analysis of cytokine expression in prostate cancer patients undergoing radiation therapy: Correlation with clinicopathological outcomes

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) thanks you for taking the time to complete and return your annual progress report for the above project.

The report has been reviewed and noted. Continued ethical approval is granted for the above research project. This approval will be ratified at the next meeting of the HREC.

Please note that this approval applies only to research conducted after the date of this letter.

Approved Timeline: 09/06/2015 – 31/12/2020
Annual progress report due: 09/06/2018

APPROVAL IS SUBJECT TO the following conditions being met:

1. The Coordinating Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.

2. The Coordinating Principal Investigator will notify the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) of any event that requires a modification or amendment to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found on the Menzies’ website.

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5. The Coordinating Principal Investigator will notify the HREC if the project is discontinued at a participating site before the expected completion date, and provide the reason/s for discontinuance.

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Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: ethics@menzies.edu.au or telephone: (08) 8946 9887 or (08) 8946 8892.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research wishes you every continued success in your research.

Yours sincerely,

[Signature]

Dr. Gabrielle McCallum
Report Review Representative
Human Research Ethics Committee
of the Northern Territory Department of Health
and Menzies School of Health Research
http://www.menzies.edu.au/ethics

This HREC is registered with the Australian National Health and Medical Research Council (NHMRC) and operates in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007). NHMRC Reg no. EC00153

menzies.edu.au

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4 July 2018

Dr Paolo De Ieso
Peter MacCallum Cancer Centre
305 Grattan St
Melbourne VIC 3000

CC: Mr Jagtar Singh

Dear Dr De Ieso,

HREC Reference Number: 2015-2385
Project Title: Analysis of cytokine expression in prostate cancer patients undergoing radiation therapy: Correlation with clinicopathological outcomes.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) thanks you for taking the time to complete and return your annual progress report for the above project.

The report has been reviewed and noted.

The HREC notes that:
- Principal Investigator Dr Paolo De Ieso has left Alan Walker Cancer Care Centre and is now with the Peter MacCallum Cancer Centre in Melbourne. Dr De Ieso remains as PI.
- Dr Thanuji Thachil has left Alan Walker Cancer Care Centre but remains as an investigator on the project.
- Dr Sukhwinder Singh Sohal replaces Dr Rama Jayaraj as student supervisor.

Continued ethical approval is granted for the above research project.

Please note that this approval applies only to research conducted after the date of this letter.

Approved Timeline: 09/06/2015 – 31/12/2020
Annual progress report due: 09/06/2019

APPROVAL IS SUBJECT TO the following conditions being met:

1. The Coordinating Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.

2. The Coordinating Principal Investigator will notify the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) of any event that requires a modification or amendment to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found on the Menzies website.

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14. You must forward a copy of this letter to all Investigators and to your institution (if applicable).

Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: ethics@menzies.edu.au or telephone: (08) 8946 8687 or (08) 8946 8692.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research wishes you every continued success in your research.

Yours sincerely,

Dr Lewis Campbell
Chair
Human Research Ethics Committee
of the Northern Territory Department of Health
and Menzies School of Health Research
http://www.menzies.edu.au/ethics

This HREC is registered with the Australian National Health and Medical Research Council (NHMRC) and operates in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007). NHMRC Reg. no. EC00153
15 August 2019

Dr. Paolo De Leo
Peter MacCallum Cancer Centre
305 Grattan St
Melbourne VIC 3000

CC: Jagtar Singh

Dear Dr. De Leo,

HREC Reference Number: 2015-2385
Project Title: Analysis of cytokine expression in prostate cancer patients undergoing radiation therapy: Correlation with clinicopathological outcomes.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) thanks you for the amendment request and taking the time to complete and return the final progress report for the above projects.

This letter acknowledges the finalisation of this study. The Committee notes that this project has now been completed. It would be appreciated if you could notify the HREC of any further publications or other outputs that may result from the research undertaken with this ethics approval.

Congratulations on the successful completion of this research study. I take this opportunity to wish you well in your future endeavors and research activities.

Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: ethics@menzies.edu.au or telephone: (08) 8946 8587 or (38) 8946 8602.

Yours sincerely,

Dr. Lewis Campbell
Chair
Human Research Ethics Committee
of the Northern Territory Department of Health
and Menzies School of Health Research
http://www.menzies.edu.au/ethics

This HREC is registered with the Australian National Health and Medical Research Council (NHMRC) and operates in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007), NHMRC Reg no. EC06153
29 September 2016

Dr. Rama Jayaraj and Mr. Jagtar Singh
Menzies School of Health Research
Via email rama.jayaraj@cdu.edu.au

Dear Rama and Jagtar,

RE: H15103 – Analysis of Cytokine expression in Prostate Cancer patients undergoing Radiation Therapy: Correlation with clinicopathological outcomes

Human Research Ethics Committee – Annual Report Approval

Thank you for submitting an annual report for the above approved protocol. The annual report has been considered under the auspices of the Charles Darwin University Human Research Ethics Committee (CDU-HREC) and has been approved.

EXPIRY DATE: 02/11/2017

Further annual progress reports must be provided to the Ethics Office before each anniversary of the commencement date. This approval is contingent on submission of a satisfactory annual progress report.

APPROVAL IS SUBJECT TO the following:

1. The safe and ethical conduct of this project is entirely the responsibility of the investigators and their institution(s).

2. The Principal Investigator must report immediately any event or circumstance that might affect the ethical acceptability of the project, including:
   - Adverse effects of the project on participants and the steps taken to deal with these;
   - All other unforeseen events that influence the protocol or participants; and
   - New information that may invalidate the ethical integrity of the study.

3. The Principal Investigator must obtain approval for any variation to the protocol (including the addition of new investigators) prior to implementation of the proposed variations. Requests for approval of variations must be submitted in accordance with the procedures of the Ethics Office.

4. The Principal Investigator must advise the University immediately of unapproved protocol deviations or protocol violations.

5. The Principal Investigator may request an extension of the project past the expiry date listed above. An extension may be requested at any time, however, the preferred time and method of requesting an extension of ethical approval is in the annual progress report.

6. The Principal Investigator must notify the Ethics Office of his or her inability to continue as Principal Investigator, including the name of and contact information for their replacement. The
research may not proceed without an approved Principal Investigator.

7. Confidentiality of personal information of research participants should be maintained at all times as required by law.

8. You must forward a copy of this letter to all investigators and to any associated organisations.

This letter constitutes ethical approval from the CDU Human Research Ethics Committee only.

Should you wish to discuss the above research project further, please contact the Executive Officer of the Ethics Office via email: ethics@cdu.edu.au or telephone: (08) 8946 6923.

Best wishes for the success of your project.

Yours sincerely

Dr Bev Turnbull
Chair, Human Research Ethics Committee
Charles Darwin University, NHMRC Registration No. EC00154

This HREC is constituted and operates in accordance with the National Health and Medical Research Council’s (NHMRC) National Statement on Ethical Conduct In Human Research (2007).
Participant Information Sheet

Analysis of Cytokine expression in Prostate Cancer patients undergoing Radiation Therapy: Correlation with clinicopathological outcomes

“This is for you to keep”

This participant information sheet will describe the research project and explains what your involvement will be. Please read the following information and feel free to ask questions about anything you don’t understand or want to know more about.

We are inviting you to take part in a research project called the “Analysis of Cytokine expression in Prostate Cancer patients undergoing Radiation Therapy: Correlation with clinicopathological outcomes”.

What are Chemo/cytokines?

Chemo/cytokines are proteins which are expressed in cancer tissue and in the blood and are known to have specific functions in the healthy immune system. However, they have also been shown by some to affect the outcome of a patient’s cancer and the severity of their side effects from radiotherapy (RT).

Particular proteins such as TNFα, TGFβ, IL-6 and IL-8 can be studied for their levels in both cancer tissue and blood with sophisticated laboratory techniques.
There is some evidence that RT affects the levels of Chemo/cytokines during and after the treatment for prostate cancer, so we are looking to investigate the link between prostate cancer biopsy expression of Chemo/cytokines and the initial levels of these proteins in the blood. We then want to assess the effect of hormone therapy and RT on the levels of these proteins in blood at various time intervals.

**Study design**

If you agree to participate in this study, samples of prostate cancer tissue taken during the prostate biopsy (no repeat biopsy required) will be examined for cytokine levels. This project will also test the blood levels of cytokines in prostate cancer patients at various stages during and after radiation therapy. We are hoping to link the changes in cytokine levels with differences in patient outcomes, like cure rates and side effects from RT.

Blood samples will be taken prior to ADT, prior to the commencement of RT, in the last week of therapy, 3 months from the end of RT and 6 months from completion of RT. Only 5-10 ml of peripheral blood will be drawn per test.

**Participation**

Your participation in this project is voluntary. If you don’t wish to take part, you don’t have to (this means you can say NO). You will receive the best possible care whether or not you take part in this study. Your decision to participate or not will not impact upon your current or future relationship with your doctors at the Royal Darwin Hospital and Alan Walker Cancer Care Centre.

If you agree to participate you will be contacted by one of the research team to tell you more about this project. If you are happy to take part, we will need your written consent and plans will be made for a convenient time and place to meet you for sample collection.

If you agree to participate in this project you will need to provide:

1. Cancer tissue (Prostate biopsy tissue at diagnosis-NO extra biopsy required)
2. Blood samples prior to commencement of RT, in the last week of therapy, 3 months from end of RT and 6 months from completion of RT.
3. Access to your medical and pathological records from Royal Darwin Hospital and Alan Walker Cancer Care Centre for the duration of this study

**Expected Benefits**

There will be no clear benefit to you from your participation in this research. However, possible benefits include:
1. Providing evidence for future blood tests which could help determine how well or poorly a particular patient with prostate cancer will do after RT. This information could change management decisions and provide future patients with more specific information about their particular prostate cancer or treatment side effects.

2. Patients may benefit from other treatments in future involving targeting of these chemo/cytokines specific to their prostate cancer.

Risks
There are no obvious risks associated with your participation in this project. However, if you become distressed as a result of your participation in this research, the study research staff will identify the issues to be dealt with and treat accordingly. You are free to withdraw consent at any time during the study, for any reason without prejudice.

Confidentiality
Any information we gather for this project will remain confidential and will be held securely, and only accessible to the research team.

Questions/ Concerns/ Complaints Regarding the conduct of this project
Please contact one of the study members listed if you have any questions or if you require more information about the project.

Alan Walker Cancer Care Centre and Charles Darwin University are committed to research integrity and the ethical conduct of research projects. If you do have any concerns or complaints about the conduct of the project you can inform the researchers.

OR
You are invited to contact the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research on (08) 89227922 or (08) 89228705 or email-ethics@menzies.edu.au”

For further information please contact to research team members:

Dr Paolo De Ieso (Chief Investigator)
Consultant Radiation Oncologist (AWCCC)
Ph. +61 8 89448220
Email. paolo.deieso@nt.gov.au

Dr Sukhwinder Singh Sohal
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Dr Thanuja Thachil
Consultant Radiation Oncologist (AWCCC)
Ph. +61 441116199
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Jagtar Singh
PhD student (Charles Darwin University)
Ph. +61 08 8946 7538
Email. jagtar.singh@cdu.edu.au
Analysis of Cytokine expression in Prostate Cancer patients undergoing Radiation Therapy: Correlation with clinicopathological outcomes

Consent Form

This Means You Can Say NO

1. I confirm that I have read the Participant Information Sheet for the “Analysis of Cytokine expression in Prostate Cancer patients undergoing Radiation Therapy: Correlation with clinicopathological outcomes” or someone has read it to me in a language that I understand.

2. I allow the research team to access tissue biopsies and blood samples, medical and pathology records from Royal Darwin Hospital and Alan Walker Cancer Care Centre for the duration of the study. **I understand that such information will remain confidential.**

3. I give permission for my tissue and blood samples to be stored and used for future research:
   a) Without contact  Y □  N □
   b) But contact me before use  Y □  N □

4. I have had an opportunity to ask questions about participation in the study and am satisfied with the answers I have received.
DEPARTMENT OF HEALTH

5. I understand the purposes, procedures and risks associated with participation in this research.

6. I freely agree to participate in this research project as described and understand that I am free to withdraw (I CAN SAY NO) at any time during the project without affecting the quality of my health care.

7. I understand that the researcher will not reveal my identity and personal details if results from this research is published or presented in any public forum.

(Please tick ✓ the appropriate box below for the part/s of the study in which you are participating)

☐ I agree to participate in this study.

☐ I do not agree to participate in this study.

Name of Participant (please print) __________________________________________________________
Signature ___________________________ Date ______________________

Name of Witness to Participant’s Signature (please print) ____________________________
Signature ___________________________ Date ______________________

Declaration by the Interpreter
I hereby declare that I was present and interpreted for the informed consent form with the patients

Name of Interpreter (if required) (please print) __________________________________________
Signature ___________________________ Date ______________________
DEPARTMENT OF HEALTH

Declaration by the Investigator

I hereby declare that I was present and interpreted for the informed consent form with the patients

Name of Principal Investigator/ Delegate ________________________________
Signature ________________________________ Date ________________

You will be given a signed copy of the Consent Form and Participant Information Sheet
References


Alokail, MS, Al-Daghri, NM, Mohammed, AK, Vanhoutte, P & Alenad, A 2014, 'Increased TNF alpha, IL-6 and ErbB2 mRNA expression in peripheral blood leukocytes from breast cancer patients', *Med Oncol*, vol. 31, no. 8, p. 38.

Altundag, O, Altundag, K & Gunduz, E 2005, 'Interleukin-6 and C-reactive protein in metastatic renal cell carcinoma', *Journal of clinical oncology*, vol. 23, no. 5, pp. 1044-.


Berkey, FJ 2010, 'Managing the adverse effects of radiation therapy', Am Fam Physician, vol. 82, no. 4, pp. 381-8, 94.


Dabbs, DJ 2013, *Diagnostic immunohistochemistry*, Elsevier Health Sciences.


Fedchenko, N & Reifenrath, J 2014, 'Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review', *Diagn Pathol*, vol. 9, p. 221.


Gardiner, R 2011 'Prostate Cancer Diagnosis. Encyclopedia of Cancer 310.1007/978-3-642-16483-5_6825© Springer-Verlag Berlin Heidelberg.'.


Kato, Y, Inoue, H & Yoshioka, U 1999, 'Effects of transforming growth factor β1, interleukin-1b, tumor necrosis factor a and platelet-derived growth factor on the collagen


Kır, G, Seneldir, H & Gumus, E 2016, 'Outcomes of Gleason score 3+4≤ 7 prostate cancer with minimal amounts (< 6%) vs≥ 6% of Gleason pattern 4 tissue in needle biopsy specimens', *Annals of diagnostic pathology*, vol. 20, pp. 48-51.

Klopfleisch, R 2013, 'Multiparametric and semiquantitative scoring systems for the evaluation of mouse model histopathology--a systematic review', *BMC Vet Res*, vol. 9, p. 123.


McDonald, TL, Hung, AY, Thomas, CR & Wood, LJ 2016, 'Localized External Beam Radiation Therapy (EBRT) to the Pelvis Induces Systemic IL-1Beta and TNF-Alpha Production: Role of the TNF-Alpha Signaling in EBRT-Induced Fatigue', Radiat Res, vol. 185, no. 1, pp. 4-12.


CXCR1, and CXCR2 correlates with cell proliferation and microvessel density in prostate cancer', *Clin Cancer Res*, vol. 11, no. 11, pp. 4117-27.

Nassif, AE & Tambara Filho, R 2010, 'Immunohistochemistry expression of tumor markers CD34 and P27 as a prognostic factor of clinically localized prostate adenocarcinoma after radical prostatectomy', *Rev Col Bras Cir*, vol. 37, no. 5, pp. 338-44.


Rice, M & Stoyanova, T 2018, 'Biomarkers for diagnosis and prognosis of prostate cancer'.


Thurner, EM, Krenn-Pilko, S, Langsenlehner, U, Stojakovic, T, Pichler, M, Gerger, A, Kapp, KS & Langsenlehner, T 2015, 'The elevated C-reactive protein level is associated with poor


Wei, JT, Dunn, RL, Litwin, MS, Sandler, HM & Sandler, MG 2000, 'Development and validation of the expanded prostate cancer index composite (EPIC) for comprehensive assessment of health-related quality of life in men with prostate cancer', *Urology*, vol. 56, no. 6, pp. 899-905.


Wu, CT, Chen, MF, Chen, WC & Hsieh, CC 2013, 'The role of IL-6 in the radiation response of prostate cancer', *Radiat Oncol*, vol. 8, p. 159.


Albright, LA & Seminara, D 2005, 'A combined genomewide linkage scan of 1,233 families for prostate cancer-susceptibility genes conducted by the international consortium for prostate cancer genetics', *Am J Hum Genet*, vol. 77, no. 2, pp. 219-29.


Yang, L, Pang, Y & Moses, HL 2010, 'TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression', *Trends in Immunology*, vol. 31, no. 6, pp. 220-7.


combined analysis from Beijing and Michigan', *International Journal of Radiation Oncology• Biology• Physics*, vol. 74, no. 5, pp. 1385-90.


