



# Levels of plasma cytokine in patients undergoing neoadjuvant androgen deprivation therapy and external beam radiation therapy for adenocarcinoma of the prostate

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**Background:** Radiotherapy (RT) alone or in combination with androgen deprivation therapy (ADT) is most common non-operative treatments for localised prostate cancer (PC). Some circulatory cytokines are believed to play an important role in RT resistance and lead to tumour progression, invasion, and angiogenesis. The aim of this study is to assess the influence of ADT and RT on the expression of circulatory cytokines levels in plasma at different time points.

**Methods:** Between Nov 2015 and Aug 2016, 18 patients with localized PC were selected for this clinical study. All patients had received neoadjuvant ADT using a leuteinizing hormone-releasing hormone (LH-RH) analogs prior to RT. Peripheral blood samples were collected prior to ADT, before RT, at the end of RT and 3 months after the completion of RT. Blood plasma samples were monitored for the pro-inflammatory and profibrotic cytokines TNF- $\alpha$ , TGF- $\beta$ 1, IL-6, and IL-8, using enzyme-linked immunosorbent assay (ELISA) procedures.

**Results:** The concentration of TGF- $\beta$ 1 rose while IL-6 levels declined in post-ADT samples when compared pre-ADT. Levels of TGF- $\beta$ 1 increased in post-RT blood plasma compared to pre-RT blood plasma. Those changes were not statically significant. Three months post-RT completion, TGF- $\beta$ 1 levels decreased and IL-6 and IL-8 levels increased. Although levels of TGF- $\beta$ 1, IL-6 and IL-8 were found to be altered 3 months post-RT completion, only changes in IL-8 levels were found to be statistically significant (P=0.05).

**Conclusions:** In conclusion 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. Large clinical studies will be required to validate these findings and hopefully become useful biomarkers in the clinical setting to predict patient outcome and success of treatment received.

**Keywords:** Androgen deprivation therapy (ADT); biomarkers; cytokines; prostate cancer (PC); radiotherapy (RT)

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## Introduction

Prostate cancer (PC) is the second most commonly diagnosed malignancy (excluding non-melanoma skin cancer) and the fifth leading cause of cancer death in men worldwide (1,2).

In current medical practice, serum prostate-specific antigen (PSA) level, tumour Gleason score (GS) and clinical tumour stage are prognostic markers which predict clinical outcomes after radical therapy of clinically localised PC (3).

Radiotherapy (RT) is a non-surgical treatment modality which focusses megavoltage photon beams on the prostate gland with the aim of good local-regional control and prolonged disease-free survival (DFS) for PC patients (4). Normal tissue damage with RT leads to an increased cytokine production within the irradiated field, which then enters circulation (5). Cytokines are water soluble, low molecular weight proteins that transport signals between cells (6). Many previous studies reported that levels of inflammatory cytokines were increased during or after RT completion (7-10). Many pro-inflammatory cytokines can disturb microenvironment in radiated cell, thereby affecting PC progression, RT efficacy and toxicities (11).

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 high-risk PC (12-14). An understanding of the relationship between testosterone levels and inflammatory cytokine levels has been growing in recent times. Few clinical studies have evaluated the correlation between male hypogonadism and serum levels of pro- and/or anti-inflammatory cytokines (15,16). The immunological consequences of RT alone or in combination with ADT are not well explained in scientific literature. Therefore, there is clearly a need for more investigation into this area.

During this prospective study, we have measured plasma cytokine levels in PC patients treated with neoadjuvant ADT and definitive RT at various time points before and after treatment. The aim of this study is to assess the influence of ADT and RT on the expression of various circulatory cytokines in patient's plasma.

## Methods

### *Study population and patient information*

Eighteen patients were recruited who had agreed to undergo curative external beam RT (EBRT) +/- ADT for intermediate to high-risk PC using the D'Amico classification (3).

All patients were diagnosed between 2nd July 2015 and 21st April 2016 at Royal Darwin Hospital (RDH). Eligible patients were  $\geq 18$  years old, had histologically confirmed prostate adenocarcinoma, and had an ECOG (Eastern Cooperative Oncology Group) performance status of 0 to 1 and no history of prior surgery to the prostate. Exclusion criteria include prior prostate EBRT or brachytherapy, metastatic disease at presentation, prior history of malignancy (excluding non-melanoma skin cancer), and serious medical or psychiatric illness precluding safe administration of ADT or RT. Using the D'Amico classification, selected patients were classified into the following three groups: low risk,  $\leq$  cT2a, PSA  $< 10$  ng/mL and GS  $\leq 6$ ; intermediate-risk, cT2b, PSA 10–20 ng/mL, GS =7; and high risk,  $\geq$  cT2c, PSA  $> 20$  ng/mL, GS  $\geq 9-10$  (3).

This clinical study has been approved by the Human Research and Ethics Committee (HREC: 2015-2385) of the Northern Territory (NT) and the Department of Health and Menzies School of Health Research. The research team collected previous prostate tissue biopsies, blood samples at different time points during treatment. Medical and pathology records from RDH and Alan Walker Cancer Care Centre (AWCCC) were interrogated during this study.

### *Androgen deprivation therapy*

ADT included administration of subcutaneous or intramuscular long-acting gonadotrophin-releasing hormone (GnRH) agonists or antagonists and oral anti-androgens. All participants were treated with GnRH agonists/antagonists.

### *Radiation therapy*

All patients had fiducial markers inserted prior to RT and had daily cone beam imaging during treatment. Treatment planning was based on computed tomography (CT) performed with empty rectum, comfortably filled bladder, with patients in the prone position. 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.

### *Timing of patient blood sampling and plasma processing*

Blood was drawn from patients before ADT, immediately

**Table 1** Clinicopathological characteristics of patient population

Characteristics	N	Percentage (%)
Age (years)	66.83±7.93 (53–80)	
Initial serum (iPSA) levels (ng/mL)		
≤10	6	33
10–20	9	50
> 20	3	17
Risk stratification		
Low risk	0	0
Intermediate risk	5	28
High risk	13	72
cTNM-stage		
cT1a-c	4	23
cT2a-c	6	33
cT3a-c	8	44
Gleason score		
6	1	6
7	8	44
8–10	9	50
Radiation dose (Gy)		
78	4	23
80	14	77

Values for age are presented as the mean ± standard deviation (mean ± SD). TNF- $\alpha$ , tumour necrosis factor-alpha; TGF- $\beta$ 1, tumour growth factor beta1; IL-6, interleukin-6; PSA, prostate-specific antigen.

preceding the initiation of RT, at the end of RT and 3 months from the completion of RT. At each sampling, 5–10 mL of peripheral blood was drawn into blindly coded 5–7 mL vacutainer tubes containing powdered lithium heparin (14 Units/mL blood). Blood samples were immediately placed on ice for transport to the laboratory, aliquoted into conical 15 mL tubes and centrifuged (3,000 × 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 analysis was carried out under blind conditions and in accordance with the guidelines established by the Charles Darwin University committee for handling

biohazardous material.

### Assessment of plasma cytokines

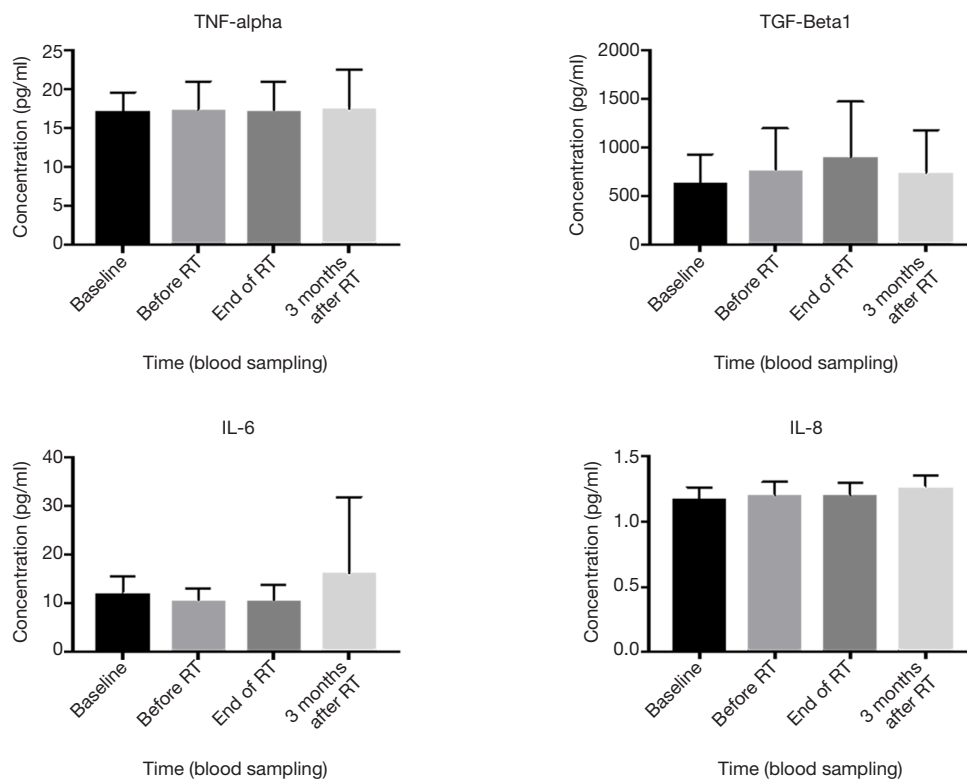
The concentration of cytokines TNF- $\alpha$ , TGF- $\beta$ 1, IL-6 and IL-8 were measured on plasma samples using sandwich ELISA kits including human TNF- $\alpha$  ELISA Kit (KAC1751), human TGF- $\beta$ 1 ELISA kits (EHTGFB1), human IL-6 ELISA kits (KAC1261) and human IL-8 ELISA kit (KAC1301) (Life 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 (17). These kits were designed to detect cytokines levels using a target-specific antibody on pre-coated 96 well microplates.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. The nonparametric Kruskal-Wallis test and Tukey's HSD (honestly significant difference) tests were used to assess pre-treatment and post-treatment plasma cytokine concentrations. Pearson's product moment correlation coefficients were used to determine the correlation between ADT and RT and cytokine levels in blood plasma. A P value of <0.05 was taken as significant.

### Results

The clinical characteristics of patients in this study are summarised in *Table 1*. The mean age of PC patients at the time of diagnosis was 66.8 years (range, 53–80 years). The mean initial serum PSA (iPSA) level was 16.03 ng/mL (range, 4–71 ng/mL) with 33% of patients at <10 ng/mL (6/18), 50% of patients with PSA of 10–20 ng/mL (9/18) and 17% with PSA concentration >20 ng/mL (3/18). The mean GS was 7.88 (range, 6–10). Six percent of patients had a GS of 6 (1/18), 44% had GS 7 (8/19) disease, and 50% GS 8–10 (9/18). Five (28%) of 18 patients had intermediate-risk PC and 13 (72%) had high-risk PC. Twenty-three percent (4/18) of patients were found to have T1a-c disease, 33% (6/18) with T2a-c and 44% (8/18) had T3a-b disease. Only one patient (6%) was found to have one pelvic node involved prior to therapy.



**Figure 1** Results demonstrating the influence of ADT and RT on circulatory cytokines levels in PC patients. Data represent the mean plasma levels (mean  $\pm$  SD) for the cytokines TNF- $\alpha$ , TGF- $\beta$ 1, IL-6 and IL-8 in PC patients before ADT (baseline), before RT, end of RT and 3 months after completion of RT. ADT, androgen deprivation therapy; RT, radiotherapy; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; TGF- $\beta$ 1, tumour growth factor- $\beta$ 1; IL-6, interleukin-6; IL-8, interleukin-8.

Moreover, patients were treated in combination with an oral anti-androgen. Neoadjuvant ADT commenced 3 to 6 months prior to RT. While all patients had neoadjuvant ADT, 12 patients ceased ADT after completion of RT and other 6 patients continued ADT as an adjuvant treatment up to 2 years based on their stage of cancer. Patients were planned and treated using IMRT. Definitive IMRT patients received a total dose of 78 Gy in 39 fractions (n=4) and 80 Gy in 40 fractions (n=14).

Effects of neoadjuvant ADT and RT of study cytokines levels are summarised in *Figure 1*. Plasma levels of TNF- $\alpha$  and IL-8 did not significantly alter (mean  $\pm$  SD, 17.2 $\pm$ 2.4 to 17.3 $\pm$ 3.7 and 1.2 $\pm$ 0.1 to 1.2 $\pm$ 0.1, respectively) in post-ADT samples when compared to pre-ADT values. The concentration of TGF- $\beta$ 1 was elevated (mean  $\pm$  SD, 643.7 $\pm$ 282.7 to 765.2 $\pm$ 426.3) and IL-6 was decreased (mean  $\pm$  SD, 12.2 $\pm$ 3.3 to 10.7 $\pm$ 2.4) in post-ADT samples. These changes were not statistically different.

The concentration of TNF- $\alpha$ , IL-6 and IL-8 did not alter

(mean  $\pm$  SD, 17.3 $\pm$ 3.7 to 17.2 $\pm$ 3.7, 10.7 $\pm$ 2.4 to 10.6 $\pm$ 3.2 and 1.2 $\pm$ 0.1 to 1.2 $\pm$ 0.1 respectively) in the post-RT blood plasma when compared with pre-RT (see *Figure 1*). In contrast, levels of TGF- $\beta$ 1 increased (mean  $\pm$  SD, 765.2 $\pm$ 426.3 to 915.9 $\pm$ 539.2) in post-RT blood plasma compared to pre-RT blood plasma. The changes in pre-RT and post-RT TGF- $\beta$ 1 levels were not statistically significantly different (see *Table 2*). The levels of IL-6, TNF- $\alpha$  and IL-8 did not alter, but expression levels of TGF- $\beta$ 1 were noticeably elevated after RT (not significantly significant).

Plasma cytokine levels at completion of RT were compared with those at the 3-month follow-up period. The concentration of TNF- $\alpha$  did not change (mean  $\pm$  SD, 17.2 $\pm$ 3.7 to 17.4 $\pm$ 5.1) during this time period (see *Figure 1*). However, TGF- $\beta$ 1 decreased (mean  $\pm$  SD, 915.9 $\pm$ 539.2 to 732.4 $\pm$ 441.7) and IL-6 and IL-8 increased (mean  $\pm$  SD, 10.6 $\pm$ 3.2 to 16.2 $\pm$ 15.5 and 1.2 $\pm$ 0.1 to 1.3 $\pm$ 0.1 respectively) over the same time period. Although levels of TGF- $\beta$ 1, IL-6 and IL-8 were found to be altered 3 months post-RT

**Table 2** Effects of ADT and RT on circulating cytokine levels

Cytokines (pg/mL)	Before ADT	Before RT	End of RT	3 months after RT
TNF- $\alpha$	17.2 $\pm$ 2.4	17.3 $\pm$ 3.7	17.2 $\pm$ 3.7	17.4 $\pm$ 5.1
TGF- $\beta$ 1	643.7 $\pm$ 282.7	765.2 $\pm$ 426.3	915.9 $\pm$ 539.2	732.4 $\pm$ 441.7
IL-6	12.2 $\pm$ 3.3	10.7 $\pm$ 2.4	10.6 $\pm$ 3.2	16.2 $\pm$ 15.5
IL-8	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1*

\*, statistically significant value. Values for age are presented as the mean  $\pm$  standard deviation (mean  $\pm$  SD); TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; TGF- $\beta$ 1, tumour growth factor- $\beta$ 1; IL-6, interleukin-6; IL-8, interleukin-8.

completion, only changes in IL-8 levels were found to be statistically significant ( $P=0.05$ ).

## Discussion

This prospective clinical study investigated the influence of neoadjuvant ADT and RT on plasma cytokine levels in patients with PC. Previous studies have reported changes in plasma cytokine levels before and after therapeutic treatments such as ADT and RT (15,16,18-22).

PC cells are androgen-dependent and ADT is a form of systemic therapy since ADT induces programmed cell death in normal, hyperplastic, pre-neoplastic, and malignant prostatic epithelial cells (23,24). Recently, there has been increasing scientific inquiry into the association between testosterone levels and inflammatory cytokines. Some previous studies have identified an association between male hypogonadism and serum levels of pro- and/or anti-inflammatory cytokines (15,16,25). Tanji *et al.* reported that six months of ADT significantly decreased serum fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) levels in serum of PC patients (15). Both FGF-2 and VEGF contribute to tumour angiogenesis in PC (26). Thus, ADT may control the concentrations of such cytokines, which suppress angiogenesis and may result in antitumor effects (27). Moreover, decreased levels of inflammatory cytokines such as IL-1, IL-6, IL-8, TNF- $\alpha$ , and stromal cell-derived factor 1 (SDF-1) were observed in blood samples after ADT treatment (19). One more study also identified that testosterone supplementation in hypogonadal men caused decreases in inflammatory biomarkers such as TNF- $\alpha$  and IL-1 $\beta$  (25). In this prospective study, we observed that plasma levels of TGF- $\beta$ 1 increased and inflammatory cytokine IL-6 decreased in post-ADT blood plasma when compared with pre-ADT plasma samples. However, these changes were not statistically different. Our findings agree with previous clinical studies that inflammatory cytokines

such as IL-6 decreased after administration of ADT.

Following RT, researchers believe that gene expression changes at the messenger RNA (mRNA) level leads to increased production of some cytokines (5,17). Cytokine levels may decrease or increase depending on tumour response to RT (5,17). Several previous clinical studies reported that levels of cytokines such as interleukin (IL)-1, transforming growth factor (TGF)- $\beta$ , and tumour necrosis factor (TNF)- $\alpha$ , Interferons- (IFN)- $\gamma$ , IL-6, IL-4 and chemokine (C-C motif) ligand-2 (CCL2) were increased during or after RT completion (7-10). Further clinical studies also identified that expression levels of cytokines such as TGF- $\beta$ 1 and TGF- $\beta$ 2, IFN- $\gamma$ , TNF- $\alpha$  and IL6 were elevated during intensity-modulated RT in the blood patients with PC (8,15,18,28). Our current study showed that expression levels of IL-6, TNF- $\alpha$  and IL-8 remained unchanged in post-RT plasma samples, when compared with pre-RT plasma. We did notice a trend to increasing levels of TGF- $\beta$ 1 post-RT, but this difference was not statistically significant.

A limited number of studies have evaluated the changes in cytokine levels after 3 months completion of therapeutic treatments. One study of head and neck cancer (HNC) reported elevated levels of serum IL-6 3 months after RT completion when compared to pre-treatment levels (29). Another study in lung cancer highlighted elevated IL-6 levels 3 months from treatment completion (17). We also have examined a time-based trend of cytokine concentrations 3 months from RT completion. Levels of TNF- $\alpha$  did not change after therapeutic treatments but we observed that TGF- $\beta$ 1 decreased, while inflammatory cytokines such as IL-6 and IL-8 increased 3 months following the administration of RT. A statically significant difference was only found between the changes in IL-8 concentration levels pre-RT and 3 months from completion of RT ( $P=0.05$ ). There is a dearth of PC studies evaluating a time-based trend of cytokine concentrations 3 months

from RT completion. Therefore, to evaluate the time-based trend of cytokines concentration is clearly needed in future studies and predicting the biochemical recurrence and RT-induced toxicity.

Elevated levels of the cytokines in patients with PC have been detected, suggesting that proteomic analyses of pre-treatment and post-treatment cytokine levels could be more broadly applicable for determining the response of ADT and RT. The limitations of this study included relatively small patient numbers which made interpretation of the significance of changes in cytokine levels very difficult.

## Conclusions

In conclusion 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. Large clinical studies will be required to validate these findings and hopefully become useful biomarkers in the clinical setting to predict patient outcome and success of treatment received.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-19-1913>). SSS serves as the unpaid editorial board member of *Annals of Translational Medicine* from Apr 2019 to Mar 2021. The other authors have no conflicts of interest to declare.

*Ethical Statement:* The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This clinical study has been approved by the Human Research and Ethics Committee (HREC: 2015-2385) of the Northern Territory

(NT) and the Department of Health and Menzies School of Health Research.

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