Serum cytokine profiles in patients with prostate cancer

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Abstract

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the third leading cause of cancer death among men in western countries. The high incidence of prostate cancer underscores urgent need for clinical indicators (biomarkers) for early detection of the presence and progression of the disease. Emerging evidence suggest that cytokine, chemokine and growth factor are key mediators of prostate inflammation that may play an important role in prostate cancer initiation and progression and may facilitate the exploration of new markers of prostatic neoplasia and inflammation.

Cytokine profiling in serum and plasma has become an important biomarker discovery tool in the study of disease mechanism, pathogenesis and treatment. The purpose of this Master thesis was to implement multiplex immunoassay that is capable of measuring multiple cytokines simultaneously within a single sample in order to study the differences of serum cytokine profile between patients with prostate PCa and non-cancer individuals to explore new biomarker of prostatic neoplasia.

Concentrations of 27 cytokines (cytokines/chemokine's and growth factor) were measured in sera of 29 patients with PCa and 21 non-cancer individuals using multiplex ELISA-based immunoassay. Relationships of these cytokine levels to serum PSA, Gleason score and urinePCA3 was also assessed. There was no significant difference in cytokine level between PCa and non-cancer controls. No correlation of cytokine and serum PSA level among non-cancer and whole cohort was observed. However, a significant positive correlation was found between serum levels of twenty different cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, PDGF-BB, TNF-α, and VEGF) and PSA levels among PCa patients. Interferon gamma-induced Protein 10 (IP-10) was only found to be associated with relationship with age, and no correlation of serum cytokine with Gleason score was found. Negative correlations between the expression levels of IL-2, GM-CSF, MIP1α and urine PCA3 was found before Benjamini and Hochberg multiple corrections. Except IP-10, MCP1, MIP1α, MIP1β, PDGF, the majority of cytokine shows strong correlations between each other.

In conclusion, though there was no significant difference in cytokine level between prostate and non-cancer individuals, however the work presented in this thesis shows strong correlation of PSA level with cytokine among PCa and no correlation among non-cancer individuals. This could lead to a better description of disease state and better understanding of the pathophysiology of PCa and may improve clinical management in patients with PCa.

Symbols and abbreviations

ADT	Androgen Deprivation Therapy
AP-1	Activating Protein 1
AR	Androgen Receptor
BMI	Body Mass Index
BPH	Benign Prostatic Hyperplasia
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CT	Computed Tomography
CTLS	Cytotoxic T Lymphocytes
CZ	Central Zone
DCs	Dendritic Cells
DRE	Digital Rectal Examination
EFGR	Epidermal Growth Factor Receptor
FGF	Fibroblast Growth Factor
FI	Fluorescent Intensity
FZ	Fibromuscular Zone
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HK3	Human Kallikrein 3
IFN-γ	Interferon gamma
IL	Interleukin
IP-10	Interferon gamma-induced Protein 10
LUTS	Lower Urinary Tract Symptoms
MAP	Multianalyte Profiling
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Complex
MIG	Monokine induced by gamma interferon
MIP	Macrophage Inflammatory Protein
MRIs	Magnetic Resonance Imaging
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKs	Natural Killer Cells
PCa	Prostate Cancer

PCA3	Prostate Cancer Antigen 3
PDGF	Platelet-derived growth factor
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasm
PSA	Prostate Specific Antigen
PZ	Peripheral Zone
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
SA-PE	Streptavidin-Phycoerythrin
SPSS	Statistical Package for the Social Sciences
STAT	Signal Transducers and Activators of Transcription
TAMS	Tumor Associated Macrophages
TH	Helper T Cells
TNF-α	Tumor necrosis factor alpha
TNM	Tumor, Nodes and Metastasis
TRAIL	TNF-related apoptosis-inducing ligand
TRUS	Trans Rectal Ultrasonography
VEGF	Vascular Endothelial Growth Factor A
WW	Watchful Waiting

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Introduction

1.1 Prostate cancer

1.1.1 Anatomy

The prostate is a male secretory gland with the size and shape of a walnut located in the sub peritoneal compartment between the pelvic diaphragm and the peritoneal cavity (Figure 1). The normal prostate in adult measures 3 cm in length, 4 cm in width, and 2 cm in depth and weighs about 18 g [1]. As demonstrated in figure 2, the adult prostate gland can be subdivided into four anatomical zones: the peripheral zone (PZ), the transition zone (TZ), central zone (CZ), and fibromuscular zone (FZ). The zonal anatomy of prostate cancer is based on their histology, anatomic landmarks, biological functions, and susceptibility to pathological disorders. Approximately seventy percent (70%) of all prostate cancers arises in the peripheral zone, a 20 % rise in the transition zone and only 10% rise in the central zone [1, 2].

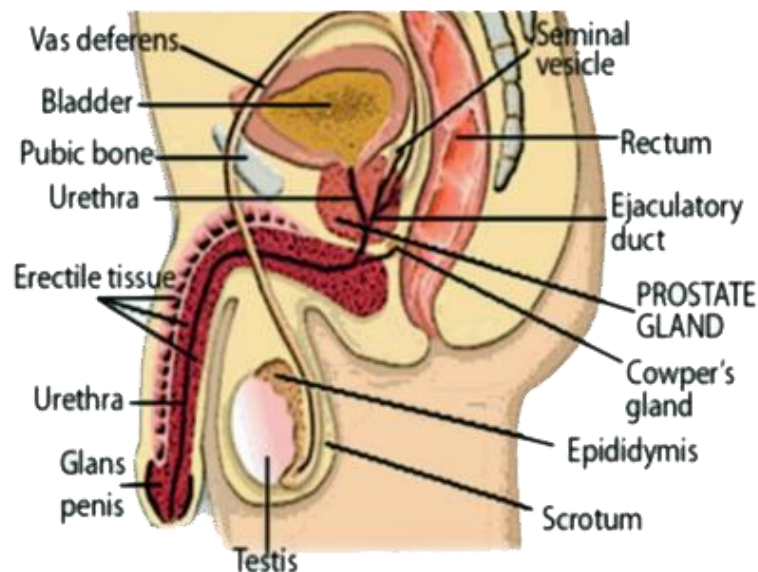


Figure 1: Male reproductive organ [3].

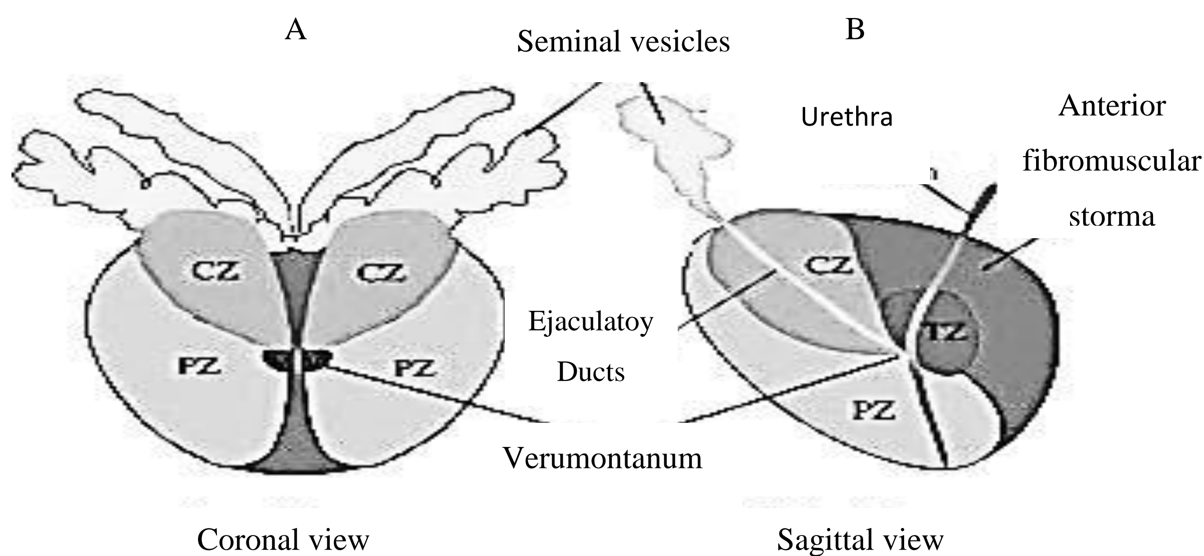


Figure 2: Zonal anatomy of the human prostate three glandular zones: central zone, peripheral zone and transition zone, indicated by CZ, PZ and TZ and one nonglandular zone (the anterior fibromuscular stroma). (A) Coronal section of the prostate. (B) Sagittal section of the prostate [4].

1.1.2 Epidemiology

Prostate cancer (PCa) is the most commonly diagnosed non skin cancer and is the third leading cause of cancer death among men in developed countries [5]. In the United States alone, an estimated 238,590 men were diagnosed with prostate cancer in 2013 and 29,720 died from these disease [6]. The incidence of PCa increases with age, and is very uncommon among the men under the age of 50. An early observation reports that the mean age of the patients with this disorder is 72-74 years, and about 85% of all prostate cancer is diagnosed at the age of 65 years [7]. Incidence of PCa varies greatly worldwide between ethnic population and countries. It is approximately 60% higher in black men compared to white while lowest rates are usually in Asia, especially among native Japanese and Chinese populations [7, 8]. The increased incidence rates of PCa in the western world are mainly due to growing awareness about prostate cancer and wide spread screening with prostate specific antigen (PSA) in men having no symptoms [7, 9]. The main risk factors for development of PCa are genetics, social and environmental factors (especially diet and lifestyle) which explains why some individuals are at high risk for developing prostate cancer than others [10].

1.1.3 Diagnosis

The current main tools for a clinical prostate examination are digital rectal exam (DRE), PSA measurements and trans rectal ultrasonography (TRUS) guided prostate biopsies [11]. The result from these tests can reveal the probability of the incidence of prostate cancer.

1.1.3.1 Digital Rectal Examination – DRE

DRE is considered as a basic tool for screening and early detection of prostate cancer. Despite its poor sensitivity (59%), DRE is routinely used for prostate cancer screening because it often detects cancer missed by other tests [12]. The advantage of DRE is that it is a relatively inexpensive procedure and may detect cancer in some men with normal PSA level and it can be handy to investigate other abnormal conditions of the prostate, such as benign prostatic hyperplasia (BPH) [8, 13].

1.1.3.2 Prostate specific antigen-PSA

PSA is a member of the human tissue kallikrein family of serine protease proteins with a molecular mass of approximately 30 kDa [8]. Prostate specific antigen (also known as human kallikrein 3 (hK3)) is a widely used biomarker for detecting , and monitoring prostate cancer in its early stage [14]. The expression of PSA is dependent on signaling by the androgen receptor (AR). Testosterone after conversion to dihydrotestosterone acts on prostate epithelium which binds to the androgen receptor (AR) which stimulates production of PSA in the nucleus. The main biological function of PSA is to break down coagulated semen and aid liquefaction and fertilization [14, 15]. However, in case of PCa or other pathology of prostate, disruption of the basal-cell layer allows PSA to leak in to the circulation resulting in increased serum levels of PSA [16]. The main advantage of PSA testing is its superior sensitivity (≥ 80% at > 4.0 ng/ml), and like DRE, PSA testing is a relatively inexpensive procedure. However, the main disadvantage of the PSA test is that it is not very specific (~50% at > 4.0 ng/ml) because levels of serum PSA may be raised by non-cancer related BPH (benign prostatic hyperplasia), prostatitis, diet alterations, medication and environment [16-18]. To improve specificity of PSA test several variations on the basic PSA test have been proposed based on different factors for example age, race, free vs. bound PSA, PSA velocity (when monitoring yearly) and volume of prostate (especially the transition zone where BPH arises) [19]. The value of prostate cancer screening using the PSA test is unclear however PSA levels of < 4ng/ml conferred a low cancer risk, that PSA levels of >4 ng/ml but

of <10ng/ml suggested an intermediate risk and that PSA levels of >10 ng/ml conferred a high cancer risk [20, 21].

1.1.3.3 Prostate Cancer Gene 3-PCA3

PCA3, originally named differential display clone 3 (DD3), was first described by Bussmarkers and colleagues in 1999. It is a prostate-specific noncoding mRNA located on chromosome 9q 21-22, which is highly overexpressed in PCa tissue compared to benign prostatic tissue [22, 23]. Initially PCA3 gene was described as consisting of four (4) exons with alternative polyadenylation at 3 different positions in exon 4. The most frequent transcript exon 1, 3, 4a and 4b was found in 60% of the cDNA (complementary deoxyribonucleic acid) clones whereas exon 2 was found to be absent due to alternative splicing. Recently PCA3 gene has been described with 4 new transcriptions starts sites, 2 new differentially spliced exons, and 4 new polyadenylation sites. Important observation within the PCA3 cDNA sequence was the presence of large sequence of stop codons and its nuclear localization support the hypothesis that PCA3 is an untranslated, noncoding RNA (ncRNA) [24]. The observations that strong association between PCA3 mRNA levels and prostate cancer lead to development of urinary assay to measure PCA3 to aid in PCa detection. Following DRE the first voided urine sample is used to detect PCA3 in urine sediment which increases the PCA3 mRNA signal in the urine specimen. Studies suggest that PCA3 measurement can be used to select the patients in which repeated biopsies should be done when the first biopsies were negative or in improving decision making about treatment choices (e.g., active surveillance vs. curative therapy) in patients with prostate cancer positive biopsies. PCA3 score also gives information about the aggressiveness about the cancer [25, 26].

1.1.3.4 Trans Rectal Ultra Sonography – TRUS

TRUS provides an ultrasound (harmless sound waves) to image the entire prostate gland and surrounding tissue and allows the physician to access prostate volume and examine the gland for abnormalities. It is basically used to locate the prostate to guide needle biopsies of the prostate gland for diagnostic purpose [8, 27]. Nowadays, various novel ultrasound techniques have been developed to increase the sensitivity of TRUS-guided prostate biopsy [1]. The use of PSA for prostate cancer screening has led to a great increase in the number of men undergoing TRUS [28].

1.1.3.5 Histopathological examination and grading-Gleason

Any positive cases from the common tests like DRE, PSA, TRUS are usually followed by biopsy and histological examination for verification. Various other test such as X-rays, MRIs (Magnetic Resonance Imaging), CT (Computed Tomography) scans, and scintigraphic bone scans can be used to detect any localized cancer outside the prostate [8]. TRUS guided biopsy sampling is most commonly used where the needle is inserted via a thin rectal membrane into the prostate. Several biopsy samples are harvested, from different areas of the prostate with half of the biopsies from left lobe and remaining from right lobe [29, 30]. The biopsies obtained are examined by pathologist to determine the Gleason score and tumor invasion [31].

Grading of prostate carcinoma is based on histopathological classification by examining the glandular architecture of the prostate gland. This grading system was described by Donald Gleason and according to the Gleason grading system, tumor growth pattern is scored on a scale from 1 to5, where 5 are the score of the most aggressive tumors (Figure 3),. Due to heterogeneous nature of prostate carcinoma, the Gleason score is given as the sum of the score of primary Gleason grade (most common pattern) and secondary grade (second most common pattern) [32]. The secondary grade should comprise of greater than 5% and less than 50% of the total tumor tissue. For example when only one pattern is identified the primary grade is doubled i.e. 3+3=6. The Gleason score as a result ranges from 2-10 where grade 2 is least aggressive and 10 is the most aggressive. The Gleason score system provides information regarding the type of cancer cells and in the decision making of optimal treatment in the individual patient [32-34].

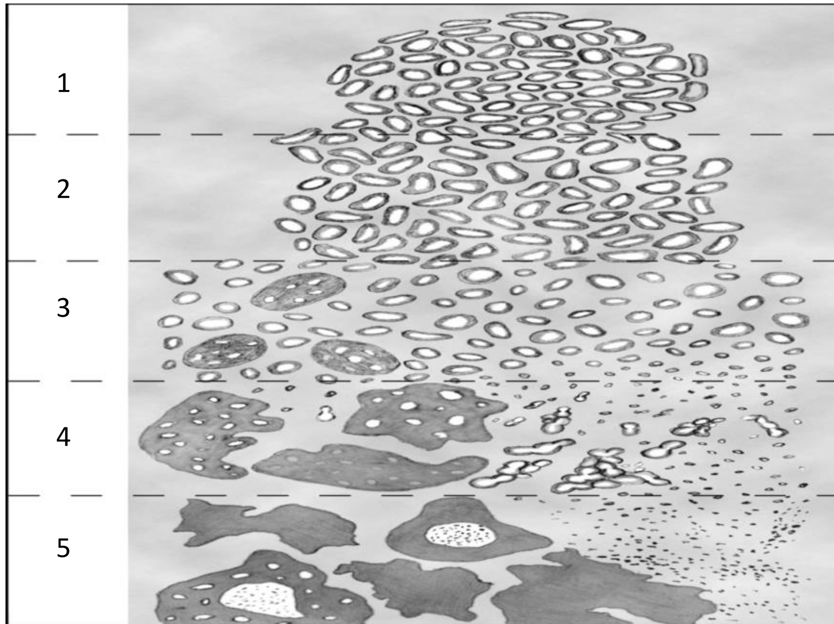


Figure 3: Schematic diagram of histological appearance of the growth patterns in the Gleason grading of prostate cancer [35]:

- Grade 1 (well differentiated), circumscribed mass of closely packed but separated, uniform shaped glands, with no evidence of stromal infiltration
- Grade 2 (well differentiated), fairly circumscribed, limited variation in gland size and spacing and some infiltration into the surrounding stroma
- Grade 3 (moderately differentiated), marked variation in size and shape, smaller glands than are seen in Gleason pattern 1 and 2, and less intervening stroma
- Grade 4 (poorly differentiated), fused glands, cribriform glands with an irregular border almost without any intervening stroma
- Grade 5 (undifferentiated), characterized by complete absence of glandular differentiation, tumor cells grow in sheets lacking of pattern, single infiltrating cells or cords

1.1.3.6 Clinical staging-TNM

TNM, which stands for Tumor, Nodes (lymph nodes), and Metastasis is a staging system to characterize cancer as described in Table 1. This widely used staging system is based on the extent of the tumor size and grade (T stage), the absence or presence of spread to nearby lymph node (N stage) and any other possible metastasis [31]. The TNM staging of disease is used as standard for predicting survival, to select best treatment options, and categorize patients. Besides PSA, DRE, and TRUS several other tests such as X-rays. MRIs, CT scans can be used to determine the stage of cancer [8, 36, 37]. These systems stratify the patients, according to the method of tumor detection, separating nonpalpable prostate cancers detected during transurethral resection and palpable cancers detected by digital rectal examination. These staging systems also categorize nonpalpable tumors detected by an increased serum prostate-specific antigen (PSA) level or an abnormal trans rectal ultrasound image [38].

Table 1: The TNM staging system

Stage	Description
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
T1	The primary tumor is evident- not palpable or visible by imaging.
T2	Palpable tumor limited within the prostate, but has not spread outside the prostate.
T3	Palpable tumor extends through prostate capsule.
T4	The tumor has spread into other body organs nearby.
NX	Metastasis in lymph nodes cannot be assessed.
N0	No lymph nodes metastasis.
N1	Metastasis in a single node, ≤ 2cm.
N2	Metastasis in a single node, >2cm, <5cm or multiple nodes, ≤5cm.
MX	No distant metastasis assessed.
M0	No distant metastasis.
M1	Distant metastasis.

1.1.4 Treatment

The therapeutic goals and treatment options for PCa patients is based on factors such as tumor characteristics and the patient's life expectancy [10]. Patients with very low risk of PCa can be subjects to watchful waiting (WW) treatment which is delayed symptomatic non-curative treatment. For men with low-grade PCa (T1or T2 disease), serum PSA 10 ng/ml, and a Gleason score of 6 or less who accept the slightly increased risk of late metastasis or death, active surveillance is an appealing option to minimize the harms of over diagnosis and over treatment [11, 39]. For patients with organ confined or localized PCa active treatment, including radical prostatectomy and radiotherapy is mostly recommended as a curative intent. Radical prostatectomy is very effective in the treatment of early stage cancer. It is associated with a reduction of the death rate from prostate cancer, as well as a reduced risk of metastases compared to the watchful waiting or active surveillance group [40]. Radiation therapy is intended for treatment of low risk, intermediate risk/high risk PCa. Patients with locally advanced or metastatic prostate cancer are candidates for 3 years of androgen deprivation therapy (ADT). ADT does not have curative intent, but prolongs the survival of PCa patients. A close follow-up of PCa patients are important either to detect recurrent disease or to identify disease progression in need of active therapy [10, 11].

1.2 Cytokine

Cytokines are 8-40kDa low-molecular weight soluble proteins produced mainly by immune cells as well as non-immune cells. They are a diverse group of protein comprised of hematopoietic growth factors, interferon's, lymphokines and chemokine's that regulate diverse physiological processes, such as growth, development, differentiation, wound healing, new blood vessel formation and immune response, including acute phase reactions and septic shocks [41-43]. Cytokines exert pleiotropic, i.e., they have ability to interact with a variety of cellular targets via specific receptors expressed on the surface of a target cell and redundant effects i.e., the same biologic function can be executed by several distinct cytokines. Cytokine interact with cells via high affinity glycoprotein receptors, located in cell membranes and are linked to intracellular signaling pathways. Their mechanism of action can be autocrine, juxtacrine, or paracrine, thereby initiating a signaling cascade by binding to their cognate receptors, consequently altering gene transcription and translation [44, 45]. Cytokines are key mediators of immunity and inflammation, including innate immunity,

antigen presentation, cell recruitment and activation, bone marrow differentiation, and adhesion molecule expression. Which cytokine produced is one of the signature functions of immune response whether that response is cytotoxic, humoral, cell-mediated, or allergic [46]. Cytokine may exert either pro- or anti-inflammatory activity or immunosuppressive activity, depending on the microenvironment of their production [41]. Some cytokines are pro-inflammatory which stimulate cell-mediated, humoral and/or allergic immunity whereas some have predominantly anti-inflammatory and immunosuppressive effects (for example, IL-10 and TGFβ) or both pro- and anti-inflammatory effects (for example, IL-6) (Table 2) [47, 48].

Table 2: Summary of pro-inflammatory and anti-inflammatory cytokine.

Activity	**Cytokine repertoire**
Cell-mediated immunity (pro-inflammatory)	IL1, IL2, IL4, IL6, IL7, IL10, IL11, IL12 IL15, IL16, IL17, IL18, IL21, IL23, TNFα, TNFβ, IFNα, IFNβ, IFNγ
Humoral immunity (pro-inflammatory)	IL1, IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL15, IL21, IL25, TGFβ
Allergic immunity (pro-inflammatory)	IL3, IL4, IL5, IL9, IL13, IL25, IFNγ, GM-CSF, SCF
Anti-inflammatory	IL4, IL5, IL6, IL10, IL13, IL19, IL20, IL22, IL24, IL26, TGFβ, IL1RA, signaling by IL1RII

1.2.1 The role of immune cells and cytokine in cancer

Cytokines are implicated in many aspects of tumor growth, tumor progression and immunosuppression rather than to the host's anti-tumor response. The tumor microenvironment contains macrophages, neutrophils, mast cells, myeloid derived suppressor cells, dendritic cells (DCs), natural killer cells (NKs), and cells of adaptive immunity (T and B lymphocytes) [49]. These cells communicate with each other by means of direct contact or cytokine production. Immune cells shows antitumorigenic and protumorigenic effect against tumor if the tumor is not rejected, the protumorigenic effect dominates. Tumor microenvironment is rich in tumor associated macrophages (TAMs), mature T cells, $CD8^+$ cytotoxic T cells (CTLs) and $CD4^+$ helper T (Th) cells, which include Th1, Th2, Th17, and T

regulatory (Treg) cells, as well as natural killer T (NKT) cells [50]. There is evidence that immune cells found within the tumor microenvironment can exert both tumors suppressive and promoting effects as determined by their effector functions. Cytokines are intricately involved in all immune reactions hence cytokine profiling of the tumor microenvironment determine what systems are activated, which are suppressed and the duration of activation or suppression which may be more relevant than its specific immune cell's content. Cytokine milieu either favors antitumor immunity (IL-12, TRAIL, IFNγ) or enhances tumor progression (IL-6, IL-17, IL-23) and also has direct effects on cancer cell growth and survival (TRAIL, FasL, TNFα, EFGR ligands, TGF-β, IL-6) through activation of various downstream effectors, such as NF-κB, Ap-1, STAT, and SMAD transcription factors, as well as caspases [49-51]. Like Th1 and Th2 cells, macrophages can be classified into M1 and M2 types which are an important source of cytokines. M1 macrophages express high levels of inflammatory cytokines (TNFα, IL-1, IL-6, IL-12, or IL-23), major histocompability molecules (MHC), and inducible nitric oxide synthase which are responsible for antitumor activity whereas M2 macrophage express high levels of anti-inflammatory cytokines (IL-4, IL-10, and IL-13) which promote immunosuppression, growth of cancer cells, tumor angiogenesis and tissue remodeling [50, 52, 53]. Besides macrophage and T cell other immune cells such as neutrophils, B- lymphocytes, mast cell dendritic cell also effect tumor genesis by production of cytokine or their effector functions [50].

1.2.2 Role of cytokine in prostate cancer

Cytokines play an important role in promoting the growth and metastatic spread of cancer. Elevated levels of a wide range of cytokines are found in serum and tumor microenvironment in different cancer patients. Local increase in tumor cytokine levels, which are clinically undetectable can have systemic effects. Besides the role of cytokine in inflammation and the development of prostate cancer miser effort has been carried out to relate relationship among serum cytokine and its role in prostate cancer [41, 54].

Cytokines act as a key component of cancer-related inflammation and an increased risk for developing prostate cancer. They can modulate tumor growth by mediating interactions between cancer cells and infiltrating inflammatory cytokine. However, the immune response in the prostate and the impact of an inflammatory environment on the prostate has yet to be conclusively demonstrated [55, 56]. The mechanism by which inflammation contributes to

carcinogenesis includes shifting cellular redox balance toward oxidative stress; induction of genomic instability; stimulation of cell proliferation; increased DNA damage; metastasis, and angiogenesis; deregulation of cellular epigenetic control of gene expression; and inappropriate epithelial-to-mesenchymal transition. Immune and tumor cells mediated production of pro-inflammatory cytokines, prostaglandins, nitric oxide, and matricellular proteins are closely involved in premalignant and malignant conversion of cells in a background of inflammation (Figure 4) [42, 57-59]. The term proliferative inflammatory atrophy (PIA) is characterized by discrete foci of proliferative glandular epithelium with the morphological appearance of simple atrophy or prostatrophic hyperplasia and is usually associated with inflammatory infiltrates [60-62]. Many studies suggest that PIA may be early precursors to prostatic intraepithelial neoplasm (PIN). The cause of PIA may include response to infection, cell trauma due to oxidant damage, autoimmunity or hypoxia related changes. However, there are both experimental and clinical evidences supporting the hypothesis that inflammation may be one of the many causes of prostate cancer [42, 50, 56, 62].

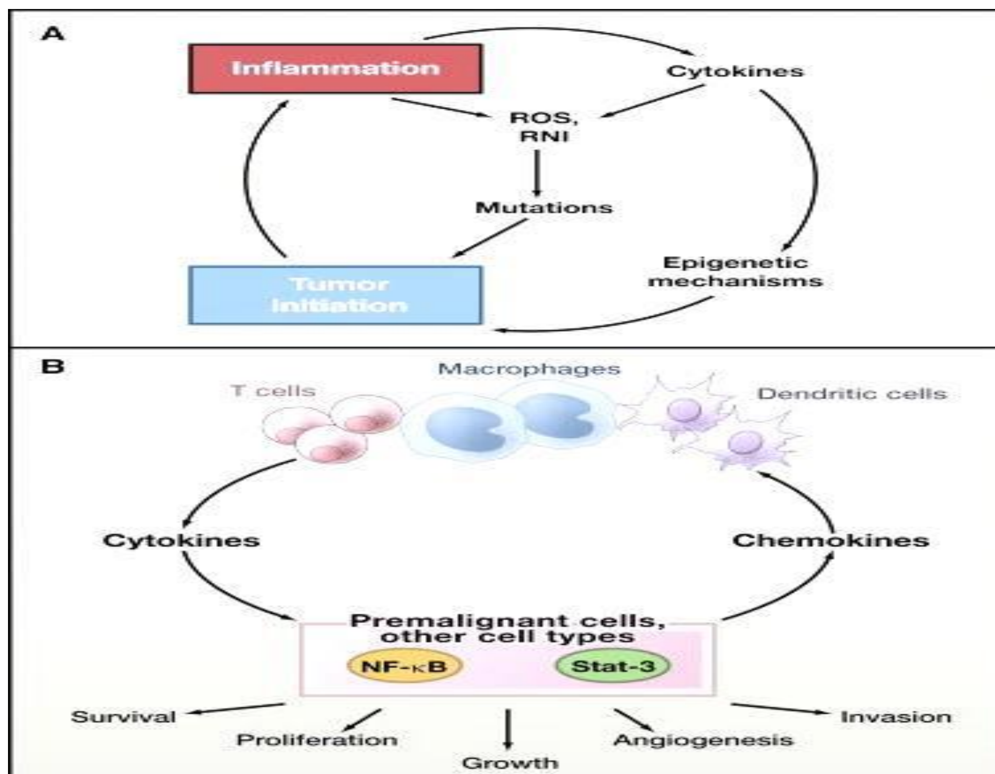


Figure 4: Role of inflammation in initiation and development of tumor [50].

Currently, cytokines have shown to play important roles in prostatic inflammation, carcinogenesis, and cancer progression [63]. IL-1 pro-inflammatory cytokine has been shown to promote tumor growth and metastasis in prostate cancer however but not sufficient alone [55, 64, 65]. IL-6 is another pro-inflammatory cytokine involved in prostate regulation and in prostate cancer development/progression. It acts as an autocrine growth factor in human prostate cancer cells via activation of Stat3 signaling. Serum concentrations of IL-6 are increased during prostate carcinogenesis and tumor progression, however elevated serum IL-6 is associated with aggressiveness of the disease, associated with poor prognosis in prostate cancer patients [55, 66, 67]. IL-8 is a pro-inflammatory CXC chemokine that recruits neutrophils and mononuclear cells into sites of inflammation. It has shown to have potent pro-angiogenic action and also regulate the expression of MMPs (matrix metalloproteinases), and thus it can promote angiogenesis and metastasis of tumors. Experimental evidence has shown that increased levels of IL-8 are associated with higher Gleason scores and metastatic disease [61, 68, 69].

IL-17 is a T-lymphocyte derived pro-inflammatory cytokine. This cytokine involves in induction of increased mRNA and protein expression of IL-6, IL-8 and IL-1α and β protein by prostate epithelial and stromal cells. IL-17 may be directly (acting on the prostate cells) or indirectly (increasing the level of inflammatory cytokine and growth factor) involved in increasing prostate tumor cell growth and metastasis [55, 61, 70].

TNF-α is another pro-inflammatory cytokine with both local effects in the tumor microenvironment and potential systemic effects. It has been found that elevated serum levels of TNF-α and IL-6 correlate in advanced disease and decreased survival in prostate cancer patients [49, 71].

Transforming growth factor (TGF-β) is a multifunctional cytokine which acts differently on transformed and non-transformed cells. TGF-β reduces the growth and proliferation of non-transformed epithelial cells, endothelial cells, as well as hematopoietic cells where as it increases the proliferation of transformed cells, including prostate epithelial cells, and the elevated levels in the serum of prostate cancer patients with metastatic disease [55, 72, 73]. TGF-β in metastatic prostate cancer serves as tumor promoter loss or mutation of TGF-beta transmembrane receptors during prostate tumorigenesis enable tumor cells to evade normal growth regulation by this cytokine. Increased production of TGF-β activate NF-κB pathway a major transcription factor promoting tumor cell growth and production of pro-inflammatory cytokines. Increased secretion of TGF-β by prostate epithelial cells causes extracellular

matrix degradation, epithelial to mesenchymal transition, immunosuppression, and angiogenesis [73-75].

Besides these important cytokines many others cytokines and chemokine may have direct or indirect effector function in tumoriogenesis in both BPH and PCa. In the invasiveness, antiapoptosis, and angiogenesis of cancer also IL-1, 4, 13, 15, 18, 23, IFN-γ, FGF-2 (fibroblast growth factor) are involved, but the full impact of these inflammatory mediators on the prostate has yet to be determined [55, 61]. Tumor cells express and produce various angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), IL-6, IL-8, and other cytokine, such as MCP-1, granulocyte CSF (G-CSF), M-CSF, tumor necrosis factor-α (TNFα), IL-1α, and IL-1β. Tumor-produced cytokine acts on cancer cells in autocrine manner or binds to their cognate receptors expressed by endothelial and hematopoietic/lymphoid cells and trigger production of additional types of cytokines. This leads to the increase of cytokine concentrations locally (ascites) as well as systemically (in the blood) [76-78].

In addition to their role in inflammation, cytokine deregulation (pro-inflammatory and anti-inflammatory cytokines) plays an important role in induction of various signs and symptoms including anorexia, cachexia, fever, and metabolic abnormalities. Studies in animal models and humans suggest that some cytokines might also contribute to other features of advanced cancer, including asthenia, drowsiness, pain, agitated delirium, cognitive failure, and autonomic dysfunction. It is also important in understanding the pathophysiology of advanced cancer. This deregulation of cytokines in plasma might serve as molecular markers of cancer related symptoms and measuring their concentration is helpful in finding treatments that modify or block cytokine production [41, 79, 80].

Along with inflammation, cytokine play important roles in the initiation and progression of prostate cancer [55]. Comprehensive cataloguing of cytokine profiles may provide further insight into the mechanisms of prostate cancer initiation and progression, and may identify cytokine as possible molecular markers for prostatic neoplasia and inflammation [55]. The widespread use of the prostate specific antigen (PSA) test has led to increased detection prostate cancer at earlier stages and a reduction in the number of patients where the metastatic disease is found at diagnosis. There are significant limitations of the PSA test such as its lack of specificity. Therefore, now PSA is commonly regarded as an indicator of prostate volume and is not independently diagnostic or prognostic in prostate cancer. Due to

its limitations, there is an urgent need for new prognostic biomarkers to enhance the clinical management of prostate cancer [81]. However, there is very little research carried out on plasma concentration of cytokines in patients with prostate carcinoma. This avenue of research holds tremendous effort for identifying cytokine markers as applicable diagnostic tools for detection of prostate cancer [82].

1.2.3 Cytokine analysis

Cytokine can be measured by a variety of assay formats among which Enzyme-linked immunosorbent assay (ELISA) is considered the 'gold standard'; however, their use is limited due to the large sample volume required for multiple analyte (cytokine) testing [83, 84]. A recent advancement in enzyme-linked immunosorbent assay is the multiplex immunoassay that is capable of measuring multiple cytokines simultaneously within a single sample. There are several reasons to adopt multiplexed immunoassay for cancer biomarker analysis. It allows reduction in sample volume, time, labor and material cost. Furthermore, there is evidence that biomarker sensitivity and specificity are improved by multiplexed measurements. Cytokine are low molecular weight proteins which serve as regulators of inflammation and immune response. These proteins are of particular interest in cancer biomarker studies because they play a key role in tumor initiation, promotion and progression [85]. Multiplex immunoassays are available in several different formats based on utilization of flow cytometry, chemiluminescence, or electochemiluminescence technology. One example of a multiplexed immunoassay platform is the flow-cytometric microbead assay, the most commonly used, which uses beads of different size or fluorescent intensity combined with analysis by flow cytometry. Multi-analyte profiling (xMAP) by Luminex Corporation is an example of commercially available microbead assays [86, 87].

1.2.4 Cytokine measurement by Luminex multiplex cytometric bead array assay

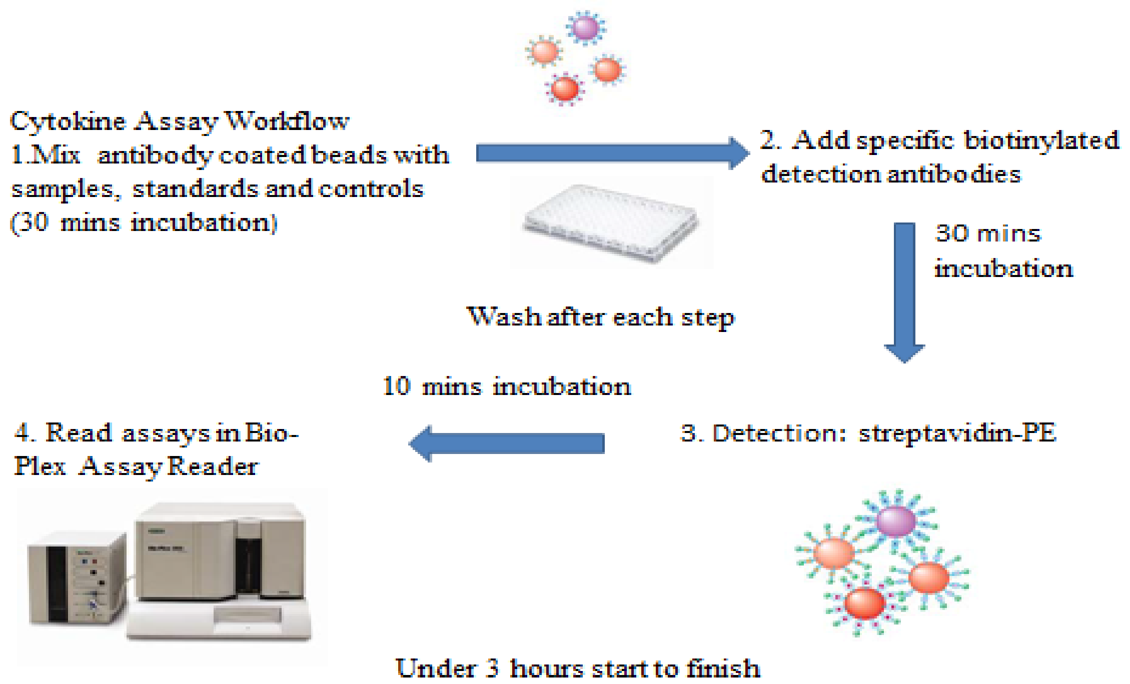
The assay principle is similar to that of sandwich ELISA; however, capture antibodies directed against the specific cytokine are attached to beads differing in color for each single type of cytokine. The principle of the method is described below and illustrated in Figure 5

- Beads of defined spectral properties which are attached to analyte specific capture antibodies are allowed to react with the sample of interest.

- Following the sample incubation, analyte bind to the capture antibodies. Any unbound protein is removed after a series of washings. After washing the beads, analyte specific biotinylated detection antibodies are added to the reaction. The analyte-specific biotinylated antibodies recognize their epitopes and bind to immobilized analyte, creating an antibody-cytokine-biotinylated antibody sandwich.
- After removal (washing) of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, phycoerythrin (SA-PE) reporter complex is added and then binds to biotinylated detection antibodies. Unbound SA-PE is washed away.
- The beads are analyzed using a Luminex based reader (dual-laser flow based readar system). By monitoring the spectral properties of the beads together with the reporter signal (SA-PE), the concentration of one or more analyte can be determined.

The bead detector identifies conjugated antibodies targeted against particular analyte by registering the bead color or fluorescence intensity (FI), while the analyte detector measures concentration of analyte bound to each bead by measuring the FI of the reporter signal (SA-PE). A standard curve generated for each cytokine is used to convert the FI measurements into the concentration of a given cytokine, e.g. in picograms per milliliter (pg/ml) [84, 87-89].

A.



B.

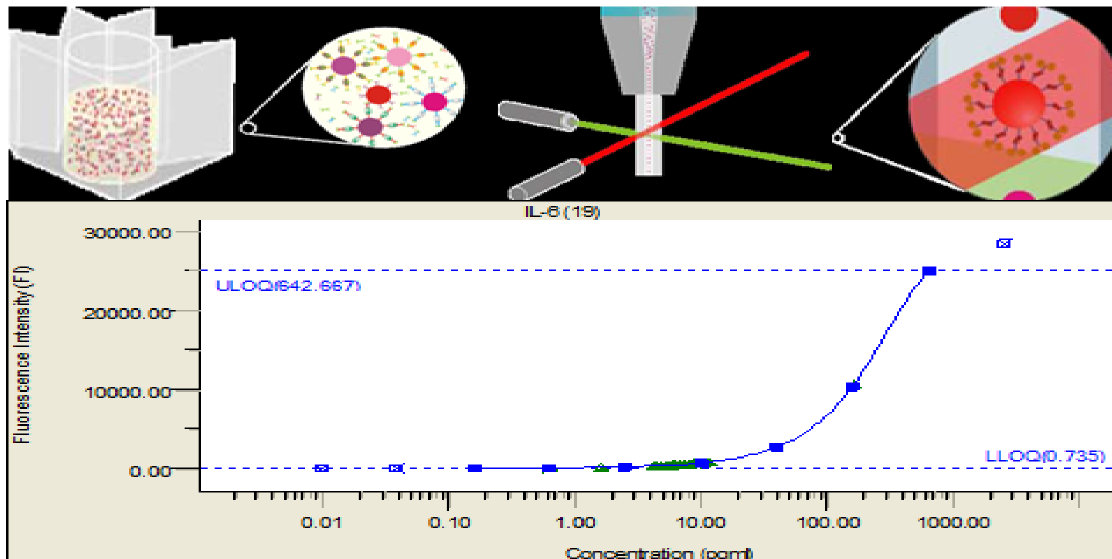


Figure 5: A. A stepwise description of multiplex assay procedure. B. A schematic illustration of luminex based reader (dual-laser-flow based reader system) [89, 90].

2 Aim of study

Cytokines are not detectable in body fluids or tissue under normal conditions; however an elevated level of cytokine expression is associated with inflammation or disease progression [44]. Emerging evidence suggests that cytokines, chemokines and growth factors play an important role in the initiation and progression of prostate cancer. Cytokine profiling from patients with prostate cancer has been reported, but a comprehensive cataloguing of serum cytokine has yet to be described [55]. This may provide further insight into the mechanisms of prostate cancer initiation and progression and may identify cytokine as possible molecular markers for PCa, as well as cancer-related symptoms that could become new targets for treatment.

The aim of this study was to profile serum cytokine levels in PCa patients and non-cancer individuals, to identify their potential as new diagnostic markers of prostatic neoplasia. The primary aim was to compare cytokine concentration in samples from PCa patient's and non-cancer individuals to identify cytokine as possible molecular markers for early cancer detection and prognosis. The secondary aim of this study was to explore the association of cytokine levels with serum PSA, age, Gleason score and urine PCA3 for increased understanding of the disease state and progression.

3 Materials and Methods

3.1 Patient population

The study population was recruited from the outpatient clinic to the research department of St. Olavs Hospital, 2011. Fifty men referred to the department of urology due to lower urinary tract symptoms (LUTS) were enrolled in the study. Written informed consent was obtained from all individuals included. They had the possibility to withdraw their consent along with the collected material throughout the course of the study. The study was approved by the Regional Committee for Medical Research Ethics, and all included subjects signed informed consents.

Spot urine, blood, and post prostatic-massage urine was collected from recruited population. The blood samples were collected twice a day from each patient (cancer and non-cancer) during two different days within the same week (Monday and Thursday) before and after fasting. Fasting blood samples were used for the analysis of serum cytokine. The following list of criteria was for inclusion of patients in the study cohort:

- Age 50-75 years
- Prostate cancer detected by trans rectal ultrasound (TRUS) guided biopsies.
- No prior tumor specific treatment (radiation, operation, hormones).
- Non-cancer group consists of individuals proven not to have PCa based on normal serum PSA (<4.0ng/ml), benign findings of digital rectal examination (DRE), trans rectal ultrasound and additionally prostate-biopsy (if this was done).

The clinical characteristics of the included individuals, patients with PCa and non-cancer are summarized in the results section, Table 4.

3.2 Multiplex analysis

Cytokine measurements in serum samples were performed using a reagent kit (#500034724) (Bio-Rad, Hercules, CA, USA) together with coupled magnetic beads and detection of antibodies targeted against 27 different cytokines. The quantitative 27-plex (Human Group I) assay was accomplished under the supervision of Liv Ryan, Chief Engineer, at the Institute of Cancer Research and Molecular Medicine, NTNU. The Multiplex Bead Immunoassay was performed according to [91] the instruction manual from suppliers with very few

modifications to the procedure. Data from the reaction was analyzed by Bio-Plex 200 system (Bio-Rad Laboratories) developed by Luminex xMAP® Technology.

In this study the levels of 27 different cytokines were measured. The cytokine panel included**:** IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, tumor necrosis factor alpha (TNF-α) , IFN-*γ*-induced protein 10 kDa (IP-10, CXCL10), monocyte chemotactic protein-1 (MCP-1, CCL2), macrophage inflammatory protein-1*α* (MIP-1α, CCL3), MIP-1β (CCL4), rantes(CCL5), eotaxin (CCL11), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor basic (FGF), platelet-derived growth factor PDGF, subtype BB (PDGF-BB), and vascular endothelial growth factor A (VEGF). Cytokine exerts pleiotropic and redundant effects, thus classification of cytokine as pro-inflammatory and anti-inflammatory is ambiguous. Cytokine may exert either pro- or anti-inflammatory activity or immunosuppressive activity, depending on the microenvironment of their production as shown above in Table 2. Cytokine produced by Th1 cells, macrophage and dendritic cells are mostly pro-inflammatory which promotes cell mediated immune-responses while cytokine produced by Th2 cells can act as both pro- or anti-inflammatory which promotes humoral immune responses. Based on cytokine produced by Th1 and Th2 cells and literature review [47, 48, 92, 93] cytokine in our measurement panel was categorized as pro-inflammatory (Th1 cytokine or Th2 cytokine), anti-inflammatory (Th2 cytokine), chemokine and growth factors for analysis as listed in Table 3.

Table 3: Categorization of 27 different cytokines:

Pro-inflammatory	Anti-inflammatory	Chemokine	Growth factors
IL-1β	IL-1ra	Eotaxin/CCL11	Basic FGF
IL-2 (Th1)	IL-4(Th2)	IP-10/CXCL10	PDGF-BB
IL-4(Th2)	IL-5 (Th2)	MCP-1/CCL2	G-CSF
IL-5 (Th2)	IL-6 (Th2)	MIP-1α/CCL3	GM-CSF
IL-6 (Th2)	IL-10(Th2)	MIP-1β/CCL4	VEGF
IL-7	IL-13(Th2)	RANTES/CCL5	
IL-8			
IL-9			
IL-12 (Th1)			
IL-13(Th2)			
IL-15 (Th1)			
IL-17			
IFN-γ (Th1)			
TNF-α			

3.2.1 Serum preparation

Peripheral blood was drawn from all subjects. Samples were allowed to clot for 30 minutes by leaving them undisturbed at room temperature. The serum was extracted by removing the clot by centrifugation at 1800xg for 10 minutes. The resulting serum supernatant was transferred into a clean propylene tube and stored at -80˚C until further analysis. The frozen serum samples were brought to room temperature just before they were assayed for cytokines. The serum samples were diluted in 1:4 concentrations by adding 1 volume of sample to 3 volumes of sample diluent supplied by manufacturer (50µl serum sample in 150 µl sample diluent).

3.2.2 Standard preparation

Lyophilized standards for 27-plex (Human group I) cytokines panel for the preparation of standard curves were included in the reagent kit for cytokine analysis described before. The standards are provided as a premixed set of related markers. Standard were prepared according to [91] in which 500µl of standard diluent is added to the lyophilized vial containing the standard. The reconstituted standard was vortexed for a few seconds, and incubated on ice for 30 minutes. Next, a fourfold dilution series with single reconstituted standards were prepared in wells (S1-S8). 25µl of each of the standard dilutions (S1-S8) were run in duplicates on each plate.

3.2.3 Preparation of couple beads

The 27 different sets of couple magnetic beads provided in the reagent kit (conjugated to specific primary capture antibodies) were vortexed at mid speed for 30 seconds to ensure mono dispersion and maximum bead recovery. Beads were then diluted to 1x working solution using assay buffer before being added to the assay plate.

3.2.4 Preparation of detection antibodies and SA-PE

Secondary reporter antibody, i.e. detection antibody included in the reagent kit were diluted to a working solution using detection antibody diluent. A vial containing 27-plex detection antibodies were vortexed at medium speed for 15-20 seconds and spun for 30 seconds to collect the entire volume at the bottom of the vial. The detection antibody was diluted using

antibody diluent to 0.5x working solution instead of 1x as described in the protocol just before 10-15 minutes were added to the assay plate. Similarly, the fluorophore containing streptavidin-PE was prepared by diluting 100x stock solution to 1x working solution before being added to the assay plate.

3.2.5 Assay procedure

The assay was set in a 96-well Bio-Plex Pro flat bottom plate (5033989, Bio-Rad, Hercules, CA, USA), and subsequent wash steps were performed using a magnetic bead based setting of the Bio-Plex wash station (Bio-Rad, Hercules, CA, USA). Buffers and kit reagents were left to stabilize at room temperature prior to the assay. The preparation of the sample comprised the following steps:

- Diluted magnetic beads solution (25µl) was added to each well in the 96-wells plate using a multichannel pipette. The diluted couple beads were vortexed for 30s at minimum speed immediately prior to use in the assay plate. The assay plate with beads was then washed twice using a magnetic based washing Pro wash station.
- Next, 50µl of all serum samples diluted in sample diluent were added to the 96- well plate. Similarly, 25µl of standard and blank (standard diluent) was added to designated wells on the assay plate changing the pipette tips of multichannel pipette after every volume transfer. The samples, standards, and blank were gently vortexed for 1-3 seconds prior to use in assay plate.
- The assay plate was then covered with sealing tape and aluminum foil and placed on a micro plate shaker, with moderate shaking speed for 30 seconds, followed by 30 minutes incubation at room temperature with orbital shaking at 400 rpm. Shaking should be done to keep the beads suspended during incubation.
- After 30 minutes of capture bead incubation, the assay plate was washed three times in the magnetic wash station. Next, 25µl of diluted detection antibody was added to each well and the plate was covered with a new sheet of sealing tape and aluminum foil and incubated for 30 minutes on an orbital shaker as described above.
- Similarly, ten to fifteen minutes prior to end of the detector incubation step the streptavidin-PE working solution was prepared from stock solution. After detector incubation step the assay plate was washed three times in the magnetic wash station. Twenty five (µl) of the diluted streptavidin-PE was then added to each well. The plate was covered and incubated for 10 minutes as described in [91] with 400rpm orbital

shaking to keep the beads suspended during incubation. After incubation the assay plate was washed three times.

- Finally, (125µl) of assay buffer was added to each well and the plate was covered with a new sheet of sealing tape. The plate was shaken at 1100 rpm for 30 seconds to resuspend the beads. Following shaking the assay plate was uncovered and the plate was inserted into the XY platforms of the Bio-Plex-200 system, which analyzed the samples.

To reduce the cost of the study, samples from both the PCa and non-cancer individuals were run in a singlet. The cytokine data were analyzed using the Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). Standard curves were obtained from the 8 point standard dilution series supplied with the kit by manufacturers. The cytokine concentrations in the samples were determined from the standard curve using the curve fitting software. Five parameter logistic regression models (5PL) were used to create standard curves due to give the greatest dynamic range for each standard curve. Using the standard curve obtained from the 8-point standard dilution series, the concentration of the unknown analyte (sample) was measured and expressed as pg/ml [91]. The cytokines IL-15 and Rantes were not detected in any of the samples, and were thus excluded from the further analysis.

3.3 Statistical analysis

Principal component analysis (PCA) was carried out to determine the variance structure of the cytokine profiles, and to investigate potential clusters of samples in which PCa patients were discriminated from the from the non-cancer individuals. Multivariate analyses (PCA) were performed in MATLAB 7.8.0 (The Mathworks, Inc., USA). Univariate analysis were performed in the software package SPSS (IBM* SPSS* STATISTIC, USA) version 21.0. Data were tested for normality distribution using Q-Q plots. Generalized extreme studentized deviate test (ESD) was performed to remove outliers (MedCalc version 13.0.0, Belgium). For normal distributed data, the statistical differences between two groups were determined using independent t-test. The data which are not normally distributed; Mann-Whitney U-test was used to analyze the difference of two categories. Spearman's correlation coefficients were computed to assess any correlations among age, PSA, urine PCA3 and Gleason score with 25 of the 27 different cytokines. Statistical significance was defined as a p-value <0.05 and correction for multiple testing was calculated using Benjamini and Hochberg [94] multiple correction (Matlab 2013a).

4 Results

The clinical characteristics of the included individuals, patients with PCa and non-cancer individuals are summarized in Table 4. The PSA and PCA3 levels were significantly higher in the patients with PCa compared to the non-cancer individuals. Independent t-test difference showed that there was a significant difference between the PSA value of the patients with prostate cancer and non-cancer individuals (P-value=0.00012). Since PCA3 score did not show normality distribution, Mann-Whitney U-test was used to compare differences in PCA3 score between PCa and non-cancer individuals. The test showed that there was a significant difference in PCA3 score between the group of PCa and non-cancer individuals (P-value= 0.003).

Table 4: Clinical characteristics and statistical analysis of study population.

		Prostate (n=29)	Non-cancer (n=21)	P-value
Age	Mean	65	62	-
	Range	58-76	52-69	
PSA (ng/ml)	Mean	11.5	1.9	0.00012*
	Range	4.3-50.4	0.3-13.0	
Gleason scores	Mean	7	-	-
	Range	5-9	-	
Urine PCA3	Mean	67	63	0.003**
	Range	2-166	6-75	

* Independent t- test ($p<0.05$)

** Mann-Whitney U-test ($p<0.05$)

4.1 xMAP-Based Analysis

Concentrations of 27 different cytokines were evaluated in a multiplexed assay using xMAP technology, in serum samples of patients with PCa and non-cancer individuals. Of the 27 cytokines, two (Rantes and IL-15) were undetectable in more than half of the serum samples in either PCa or non-cancer sera, and were thus excluded during the subsequent data analysis.

4.2 PCA analysis

Before PCA analysis the data was autoscaled so that cytokine in low concentrations is given the same influence on the model as a cytokine present in high concentrations. The PCA score plot (Figure 6) based on PC1 and PC2 shows that there is no clear discrimination between PCa patients and non-cancer individuals. No separation between PCa patients and non-cancer individuals were also found in PC2, PC3, etc. However, as shown in (Figure 6) six samples from PCa patients, characterized with higher score values for PC1 are associated with higher levels of IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-13, IL-17, MCP-1, TNF-α, and IFNγ compared to the rest of the patients. But no any distinct clinical characteristics were found among these six PCa patient samples compared to rest of the patients population confirms that it is difficult to discriminate PCa patients and non-cancer individuals on the basis of serum cytokine profiles.

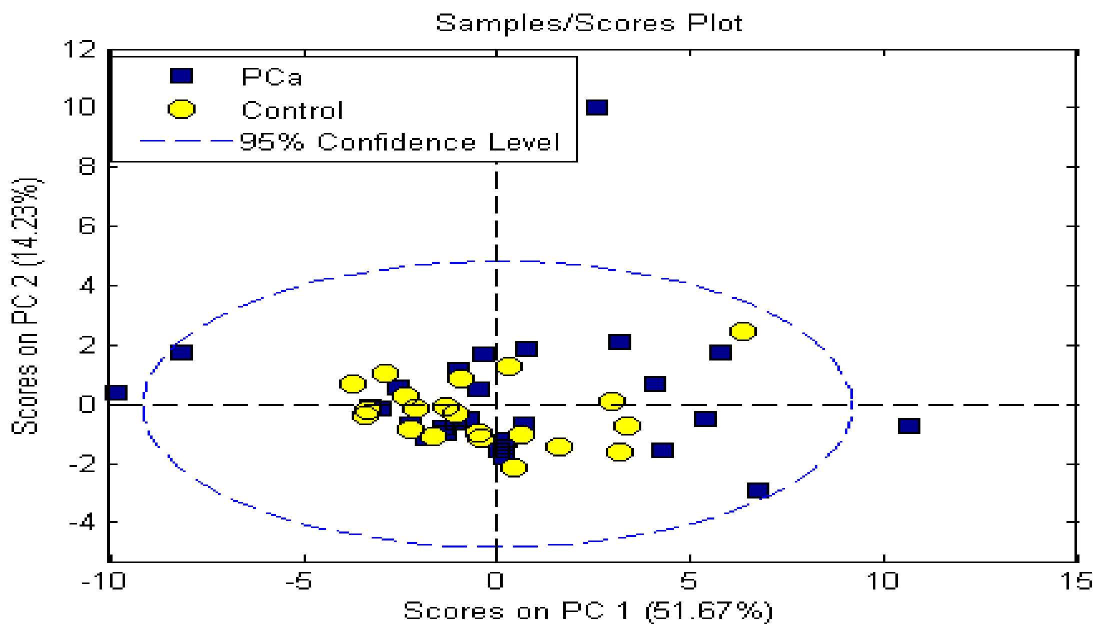


Figure 6: PCA score plot of serum cytokines (n=25) from study population (n=50).

4.3 Comparison of cytokine levels in PCa patients and non-cancer individuals

The Mann-Whitney U-test (Table 5) showed a significantly higher level of the IL-12 cytokine in PCa compared to non-cancer individuals. However, after multiple testing for P-values using the Benjamini and Hochberg test the difference was no longer significant.

Table 5: Comparison of cytokine levels among PCa patients compared with non-cancer individuals. P-values from Mann-Whitney U-test and adjusted P-value from Benjamini and Hochberg test.

Cytokine	Group	N	Mean Rank	P-value	Adjusted P-value
IL6	Patient	29	26.72	0.485	0.865
	Non-cancer	21	23.81		
IL1β	Patient	29	26.43	0.596	0.865
	Non-cancer	21	24.21		
IL1ra	Patient	29	26.34	0.63	0.865
	Non-cancer	21	24.33		
IL2	Patient	29	28.09	0.14	0.798
	Non-cancer	21	21.93		
IL4	Patient	29	27.41	0.275	0.798
	Non-cancer	21	22.86		
IL5	Patient	29	26.93	0.415	0.798
	Non-cancer	21	23.52		
IL7	Patient	29	26.98	0.398	0.798
	Non-cancer	21	23.45		
IL8	Patient	29	26.03	0.761	0.864
	Non-cancer	21	24.76		
IL9	Patient	29	24.62	0.616	0.864
	Non-cancer	21	26.71		
IL10	Patient	29	27.97	0.16	0.798
	Non-cancer	21	22.1		
IL12	Patient	29	28.97	0.048	0.798
	Non-cancer	21	20.71		
IL13	Patient	29	26.62	0.523	0.864
	Non-cancer	21	23.95		
IL17	Patient	29	27.41	0.275	0.798
	Non-cancer	21	22.86		
Eotaxin	Patient	29	25.41	0.961	0.961
	Non-cancer	21	25.62		
BasicFGF	Patient	29	27.29	0.307	0.798
	Non-cancer	21	23.02		

Cytokine	Group	N	Mean Rank	P-value	Adjusted P-value
GCSF	Patient	29	26.97	0.403	0.798
	Non-cancer	21	23.48		
GMCSF	Patient	29	27.1	0.361	0.798
	Non-cancer	21	23.29		
IFNγ	Patient	29	25.83	0.852	0.887
	Non-cancer	21	25.05		
IP10	Patient	29	28.17	0.128	0.798
	Non-cancer	21	21.81		
MCP1	Patient	29	24.81	0.694	0.864
	Non-cancer	21	26.45		
MIP1α	Patient	29	26.98	0.398	0.798
	Non-cancer	21	23.45		
MIP1β	Patient	29	25.83	0.852	0.887
	Non-cancer	21	25.05		
PDGFBB	Patient	29	24.76	0.673	0.864
	Non-cancer	21	26.52		
TNFα	Patient	29	24.95	0.753	0.864
	Non-cancer	21	26.26		
VEGF	Patient	29	27.9	0.172	0.798
	Non-cancer	21	22.19		

4.4 Correlation of cytokine levels with age, PSA, urine PCA3, and Gleason score

Table 6 shows the correlation of cytokine levels with age, PSA, urine PCA3, and Gleason score in PCa patients (Spearman's rank correlation). Among PCa patients significant positive correlation was found between PSA levels and serum levels of 20 out of the 25 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-17A, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, PDGF-BB, TNF-α, and VEGF). However, the serum cytokine levels had no significant correlation with the stage of cancer (Gleason score). Only one cytokine, the IP-10, showed positive correlation with age. Serum levels of IL-2, GM-CSF, and MIP1α were negatively correlated with urine PCA3 score among PCa patients before Benjamini and Hochberg correction for multiple testing.

Among non-cancer individuals and the whole cohort study population no correlation was observed among urine PCA3, age, and serum PSA and cytokine levels. Table for correlation among non-cancer (Table 7) and a whole cohort (Table 8) is depicted in Appendix A.

Table 6: Spearman's correlation test of cytokine level with Age, PSA, urine PCA3 and Gleason score in PCa patients. P-values from Spearman's test and adjusted P-value from Benjamini and Hochberg test.

Cytokine	Gleason score			Serum PSA			Age			UrinePca3		
	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value
IL6	.052	.796	.983	.475**	.009	.028	.130	.500	.991	-.261	.199	0.340
IL1β	.004	.983	.983	.423*	.022	.034	.069	.724	.991	-.250	.218	0.340
IL1ra	.077	.704	.983	.450*	.014	.029	.099	.609	.991	-.286	.156	0.340
IL2	-.095	.638	.983	.574**	.001	.025	.054	.780	.991	-.525*	.006	0.15
IL4	.012	.951	.983	.411*	.027	.036	.125	.519	.991	-.269	.184	0.340
IL5	.012	.953	.983	.280	.142	.155	.014	.941	.991	-.202	.323	0.459
IL7	.019	.926	.983	.507**	.005	.028	.014	.942	.991	-.277	.171	0.340
IL8	.006	.976	.983	.336	.075	.089	.096	.621	.991	-.252	.214	0.340
IL9	.014	.945	.983	.468*	.010	.028	.026	.895	.991	-.196	.338	0.459
IL10	.036	.859	.983	.532**	.003	.025	.026	.895	.991	-.335	.094	0.340
IL12	.039	.848	.983	.491**	.007	.028	.171	.374	.991	-.192	.349	0.459
IL13	.020	.920	.983	.533**	.003	.025	.073	.707	.991	-.142	.489	0.555
IL17	-.235	.237	.983	.450*	.014	.029	.083	.667	.991	-.365	.067	0.335
BasicFGF	-.165	.410	.983	.412*	.026	.038	-.082	.671	.991	-.300	.137	0.335
Eotaxin	-.225	.259	.983	.478**	.009	.028	.119	.539	.991	-.371	.062	0.335
GCSF	.096	.632	.983	.437*	.018	.034	.003	.986	.991	-.276	.173	0.340
GMCSF	-.109	.589	.983	.425*	.022	.034	.068	.725	.991	-.429*	.029	0.335
IFNγ	.009	.964	.983	.389*	.037	.046	.060	.757	.991	-.287	.154	0.340
IP10	.007	.974	.983	.273	.151	.157	.550*	.002	.050	.073	.725	0.725
MCP1	-.011	.956	.983	.414*	.026	.036	.066	.732	.991	-.099	.631	0.657
MIP1α	-.045	.823	.983	.279	.143	.155	-002	.991	.991	-.400*	.043	0.335
MIP1β	-.345	.078	.983	.163	.398	.398	.135	.486	.991	-.158	.441	0.525
PDGF	-.102	.614	.983	.452*	.014	.029	.090	.644	.991	-.164	.424	0.525
TNFα	.067	.741	.983	.430*	.020	.034	.101	.603	.991	-.310	.124	0.340
VEGF	-.033	.871	.983	.483**	.008	.028	.087	.655	.991	-.122	.554	0.602

r_s: Spearman rank correlation coefficient

****:** Correlation is significant at the 0.01 level (2-tailed)

***:** Correlation is significant at the 0.05 level (2-tailed)

4.5 Correlation among cytokines

Table 9 (Appendix B) shows the internal correlations among the 25 cytokines in PCa patients (Spearman rank correlation). The analysis of the correlations between the individual cytokine revealed that the most of the cytokine shows strong correlations. Among 25 cytokines IL-2, Eotaxin, GMCSF shows complete (strong positive) correlation with all other cytokine. IL-4, IL-8, IL-13, TNFα (except with MIP1β), IL-7 (except with IP-10) shows a strong positive correlation among the rest of the cytokine. Similarly, IL-9, IL-10, GCSF (except with IP-10, MIP1β), IL-12, IL-17 (except with IP-10, MCP1), IL-5 (except with MIP1β, VEGF) shows strong correlation among other cytokine. MCP1, MIP1α, MIP1β, PDGF are relatively uncorrelated to the majority of the cytokine, and IP-10 was found to be uncorrelated with all cytokine after Benjamini and Hochberg correction for multiple testing.

5 Discussion

Cytokine and chemokine are essential immune effector molecules that form a complex network of regulatory proteins, and often many cytokines are essential to synergize to bring about an optimal effect [95]. To our knowledge, this work is the first preliminary screening study for 27 different cytokines among prostate and non-cancer individuals. The method used in our study offers an opportunity to study relative differences in cytokine levels between prostate cancer patients and non-cancer individuals. Multiplex enzyme immunoassay technology was used for analyzing 27 cytokines in the sera of men with prostate cancer and non-cancer subjects. The hypothesis that the cytokine profile, including pro-inflammatory and anti-inflammatory cytokine would be useful not only for appraisal of prostatic inflammation, but also for early cancer detection and prognosis [96-99] was not completely supported by our findings.

5.1 Clinical finding

The main objective of this thesis was to evaluate the potential of serum cytokine profile in discriminating the PCa patients from non-cancer individuals. The results of the PCA analysis in the present study confirmed the complexity of discriminating PCa and non-cancer controls based on levels of serum cytokine concentration. The Mann-Whitney U-test showed differences in serum level of IL-12 among prostate and non-cancer controls before Benjamini and Hochberg correction for multiple testing. Research has found that IL-12 may be applicable for detecting PCa in early stage [99]. Interleukin 12 is a Th1 cytokine which enhances the activity of cytotoxic T lymphocytes ($CD8^+$) and has an essential role in cell-mediated immunity. Release of IL-12 from macrophages and DCs, shows remarkable antitumor properties that are mainly mediated by interferon (IFN) -γ secretion by CD4+, CD8+ T cells, natural killer (NK). IL-12 through IFN- γ -dependent induction of the anti-angiogenic factor interferon-inducible protein (IP) 10 and monokine induced by gamma interferon (MIG) adds to tumor suppression [49, 100]. Studies have shown that IL-12 gene therapy is effective for orthotopic tumor control and suppression of pre-established metastases in a preclinical prostate cancer model and improved survival [101, 102].

Previous studies concerning cytokine profiling in PCa patients and non-cancer controls were carried out using few different cytokine which shows significant difference of cytokine levels among prostate and non-cancer controls [79, 96, 97, 99, 103]. However, in our study, we found no significant difference in the cytokine levels between prostate and non-cancer

individuals. This may be due to the fact that the matched controls in our cohort consist of patients who were suspected to have prostate cancer. Studies suggest that prostate cancer progress through proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) precursor lesions prior to malignant transformation [104]. PIA is a hyperproliferative epithelial atrophy that is frequently associated with inflammation, which acts as a precursor of prostate cancer development and is found in patients with prostate disorders. The presence of inflammatory cells and their secreted products, i.e. cytokines, chemokines, and growth factors in prostate indicates a causative role for these mediators in development of PCa. Studies also suggest that inflammatory cells and their secreted products are found in PCa and patients with prostate disorders such as BPH [98]. Inflammatory cytokines, chemokines and growth factors are present as the result of the cancer or as the result of transformation events which precede the cancer are ambiguous. This may explain why our control group which consists of patients with prostate disorder might be difficult to discriminate from the subjects with confirmed prostate cancer on the basis of cytokine profiling. Second, lack of information about co-morbidity in both PCa patients and non-cancer individuals, may have resulted in the lack of significant association between cytokine levels among prostate and non- cancer individuals. Third, the small sample size (n=29 prostate cancer), and fourth inconsistencies between approaches and assay manufacturers can result in markedly different results which is further discussed in section 5.4 and 5.5.

In this study, we also determined the correlation of cytokine levels with age, serum PSA, urine PCA3 and Gleason score. We found a significant relationship between increasing PSA and serum cytokine in prostate cancer patients, but not in non-cancer individuals. A significant positive correlation was found between PSA and serum levels of 20 out of 25 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-17A, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, PDGF, TNF-α, and VEGF). Prior research has found an increased serum concentration of pro-inflammatory cytokines IL-1α, IL-6, IL-4, IL-8, IL-12, IL-17, IFN-γ, TNF-α, Eotaxin and anti-inflammatory cytokines such as IL-10 and serum PSA level in patients with prostate cancer [71, 103, 105]. It would be interesting to understand the expression of cytokine with PSA levels in prostate cancer. Several studies in prostate cancer cells have demonstrated that cytokine activates and up-regulates PSA gene expression and enhance endogenous PSA expression in an androgen-dependent and androgen-independent manner [106, 107]. High expression of cytokines with increased PSA levels in PCa patients may be indirectly involved in the PSA leakage into the circulation due

to increased loss of epithelial cells, polarity and basal cell number. However, the exact underlying mechanisms for the increase in diverse members of cytokines with an increase in PSA level are unknown. Our finding holds future effort for better understanding of the biologic mechanism and the role played by elevation of circulating PSA related to elevation of serum cytokine to understand the diverse roles of various cytokines in order to further address the pathophysiology of PCa and may improve clinical management in patients with PCa.

We observed no association between the expression level of cytokine and age, except IP-10 which shows a positive correlation with age. This finding was similar to observations in patients with colorectal cancer which showed increased serum concentration of IP-10 with increase in age [108]. We have no specific biological explanation for this finding at present, but it could offer a new facet for studying age-related differences of cytokine in patients with PCa.

We found negative correlations between the expression levels of certain cytokines: IL-2, GM-CSF, MIP1α and urine PCA3 before Benjamini and Hochberg correction for multiple testing. The findings from this study are interesting as there are no previous published data showing relationships among cytokine and urine PCA3. PCA3 seems to be a regulator of the expression of AR target genes. Studies have shown that increased in PCA3 and cytokine expression increases expression of AR target genes among which PSA is one [106, 109]. But in our study, we found no correlation between urine PCA3 and PSA level (Appendix C) and positive correlation of cytokine with PSA levels. This finding could offer a new aspect of studying the biological mechanism and the relationship between these markers in prostate cancer. Decrease in urine PCA3 expression with increase in serum cytokine level holds tremendous future effort for better understanding of mechanisms of prostate cancer initiation and progression and may identify cytokine as possible molecular markers for disease progression and decision making about treatment choices.

In our study there was no relationship between cytokine levels with stage of cancer (Gleason score). Investigators have linked increased expression of cytokine levels with tumor progression, however, no association between Gleason score and cytokine levels was reported in prior study [96]. Our findings are consistent with this, suggesting that Gleason score is not the major factor affecting the level of cytokine.

5.2 Correlation between pro-and anti-inflammatory cytokine

Strong correlation between cytokine were shown in this study. Among 25 cytokines IL-2, Eotaxin43, GMCSF shows significant (strong) correlation with all other cytokines. IL-4, IL-8, IL-13, TNF alpha (except with MIP beta), IL-7 (except with IP-10) shows strong correlation among the rest of the cytokines. Similarly, IL-9, IL-10, GCSF (except with IP-10, MIP beta), IL-12, IL-17 (except with IP-10, MCP1), IL-5 (except with MIP-beta, VEGF) shows strong correlation among other cytokine. IP-10 which was only cytokine found to be correlated with age shows no correlation among all cytokine after Benjamini Hochberg correction for multiple testing. Except MCP1, MIP1alfa, MIP1beta, PDGF, which are relatively uncorrelated the majority of marker shows strong correlations among each other. The alterations in the serum levels of major pro-inflammatory cytokine (IL-1β, IL-2, IL-6, IL-7, IL-8, IL-9, IL-12, IL-15, IL-17, IFN-γ, TNF-α, IFN-γ) and anti-inflammatory cytokine (IL-1ra, IL-4, IL-5, IL-10, IL-13) was one of our interests, as, an imbalance of pro- and anti-inflammatory cytokine has been proposed to occur in the development of cancer. We could not demonstrate a clear polarization of the systemic pro-and anti-inflammatory cytokine levels in prostate cancer patient, as our PCa patients showed a strong association between both pro-and anti-inflammatory cytokine. Multiple pieces of evidence have confirmed that inflammatory cells have powerful effects on tumor development, but recruitment of inflammatory cells may also be inhibitory to tumor development. Inflammatory cells secrete a wide array of both pro-and anti-inflammatory cytokine such as TNF-α, IL-1 are usually classed as a key pro-inflammatory cytokine whereas IL-1ra, IL-10 are key anti-inflammatory cytokine. The balance between pro-and anti-inflammatory cytokine determines the outcome of inflammation. Several studies have linked both the pro-and anti-inflammatory cytokine in the development and progression of prostate cancer [110, 111]. Correlation between cytokine in our study shows that, an imbalance of pro- and anti-inflammatory cytokine, may prevent the normal self-limiting nature of the immune response, leading to prolonged inflammation with chronic exposure to cytotoxic mediators such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability which aids in PCa progression [112]. Elucidating the reason for the strong correlation among most of these cytokine pairs will need further studies to establish their role in prostate cancer.

5.3 Methodological discussions

We found that out of 29 PCa patients, two of the patient serum samples showed three to four fold high serum cytokine concentration compared to rest of PCa and non- cancer individuals. This may be due to a technical error. For example, the inappropriate addition of sample diluent to serum during the performance of the assay could result in unexpected high concentration of cytokines in these 2 patients. The inflammatory response is complex, nonlinear, and redundant, influenced by both epigenetic and environmental factors. This results in a high degree of intraindividual variability. High fluctuations of these cytokines levels among two patients serum may likely signify sub-clinical, innate responses to slight immunologic or physiologic insults that are necessary to maintain homeostasis [113].

In our study observed values for two cytokines IL-15 and Rantes were missing. The concentration of IL-15 was below the range of detection (<0.116) and concentration for Rantes was above the range of detection (>847) so these values were excluded during data analysis. These findings are similar to other studies carried out to assess physiological cytokine variations in healthy volunteers that have shown low serum levels of IL-15 and high serum levels of Rantes [114, 115]. We have no specific explanation for this, but it may be due to the lack of sample duplicates. To reduce costs of analysis each sample from prostate patients and non-cancer controls were run in singlets. So our data lack the average of duplicates to measure serum cytokine concentration. Similarly, IL-15 concentration is low or undetectable in serum. Its expression is post-transcriptionally regulated and soluble protein is not detected in physiological conditions. Rantes concentration is high in serum and is above the limit of detection of the standards using the multiplex immunoassay. Rantes assays need to be run separately or at appropriate dilution of samples.

5.4 Limitation of the study

The findings of this study provide information related to cytokine networks, prostate inflammation and oncogenessis in PCa. However, discrepancies between the present study results and previous finding should be evaluated in the context of the following limitations. The main limitation of our study was lack of clinical information about control group as we do not know the comparison group in our study consists of patients with LUTS, BPH, prostatitis or other inflammatory disease condition. Our study also lacks information about the co-morbidity among PCa and non-cancer individuals. Therefore, our results and approach

needs to be confirmed by subsequent studies with independent study populations, and in studies including healthy individuals and in individuals with inflammatory conditions and LUTS. The limitation of the study may result, in part, from the small sample size. However, the most important factors responsible for variability in cytokine levels between studies is the method of measurement.

Cytokine can be measured by a variety of assay formats among which ELISA is considered the 'gold standard', However, their use is limited by the large sample volumes that are required for multiple analyte (cytokine) testing. A recent advancement in enzyme-linked immunosorbent assay is the multiplex immunoassay, capable of measuring multiple cytokine simultaneously within a single sample. Multiplex assay allows reduction in sample volume, time, labor, and material cost [116]. However, as with all immunoassays, performance of the multiplex assay also depend on antibody quality, manufacturer, and the experience and skill of the operator. The results from a growing number of research studies indicate that there is significant variation between the absolute cytokine concentration determined by ELISA and either multiplex kit, but that trends in cytokine levels are comparable [117, 118]. Despite of the many advantages of using multiplex technology, comparison of bead based assays utilizing kits from different manufacturers, researchers found comparable trends, but difference in absolute values among the kits and cytokine [119, 120]. Considering the important role of multiplex in the detection of cytokine, inconsistencies between approaches and assay manufacturers can thus result in markedly different results.

Although the relation between cytokine and inflammation in prostate cancer is well documented, most of the studies confined their focus in examining cytokine levels in PCa patients undergoing radiation treatment, associated with symptoms, and most of them are looking only for pro-inflammatory and anti-inflammatory cytokine among which IL-6, IL-8, TGF-β, TNF-α, and IFN-γ are of importance. This exploratory study, in which we sought to quantify difference in cytokine levels among PCa and non-cancer is the first preliminary screening study for 27 different cytokines among prostate and non-cancer controls. The negative findings in the present study confirmed the complexity of discerning (discriminating) cytokine levels and changes in those levels among prostate and non-cancer individuals.

5.5 Future perspectives

Future research should explore inherent interindividual variability in the inflammatory response and the methodological inconsistencies between studies measuring cytokine, additional information related to the role of cytokines, chemokines and growth factors in prostate inflammation and its effects on prostate and matched control into their study designs. Future studies are warranted to validate these findings using high number of patients (with more clinical variables) and validation of these results using the same and/ or different experimental methods. As we for the first time describe the serum cytokine profile in PCa patients, future research also should focus on the release of these markers and its consistency and reliability in serum. Future studies should be carried out to evaluate the individual inflammatory cell response to reveal immune reaction patterns in prostate cancer patients, and as well as to measure the specificity of the alterations of the cytokine profile in PCa patients in relation to other inflammatory disorders and neoplasms. Future studies should also be focused on establishing these and other cytokine detection not only in serum, but also in prostatic fluid obtained from prostate biopsy and in post-DRE urine specimen which may perhaps be useful in early detection and prognosis if they are present in prostatic secretions or post-DRE urine specimen. This may provide further insight into the mechanisms of prostate cancer initiation and progression and may identify cytokine as possible molecular markers for PCa, as well as cancer-related symptoms that could become new targets for treatment. Future research should also focus on establishing relation of cytokine levels with both prostate biomarker serum PSA and urine PCA3.

6 Conclusion

Evidence from pre-clinical and clinical literature suggest that cytokines, chemokines and growth factors may serve as indicators of tumor progression. These factors may have potential as novel biomarkers in predictive assays. In conclusion, although there was no significant difference in serum cytokine levels among PCa patients and non-cancer individuals, we found a strong positive correlation between cytokine levels and PSA in PCa patients. A better understanding of the biological mechanism and the role played by the elevation of circulating PSA related to elevation of serum cytokine level among PCa patient could lead to a better description of the disease state that might improve therapeutic strategies against the disease. Cytokine may perhaps be useful for early detection and prognosis efforts and mechanisms associated with the process leading from inflammation to cancer, and clinical management of PCa patients if they are expressed in both prostatic secretions and post-DRE urine samples in addition to serum.

7 References

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Appendix A

Table 7: Spearman's correlation coefficient analysis of age, urinepca3 and serum cytokines in non-cancer individuals.

Cytokine	Serum PSA			Age			UrinePca3		
	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value
IL6	-.063	.793	.947	-.309	.172	.663	-.204	.403	0.906
IL1β	-.169	.475	.947	-.347	.124	.660	-.090	.713	0.906
IL1ra	-.048	.839	.947	-.291	.200	.663	-.190	.435	0.906
IL2	.101	.673	.947	-.068	.770	.925	-.121	.621	0.906
IL4	-.266	.258	.947	-.373	.096	.660	-.182	.456	0.906
IL5	.048	.839	.947	-.279	.221	.663	-.061	.803	0.945
IL7	-.105	.659	.947	-.242	.292	.690	-.118	.629	0.906
IL8	-.167	.481	.947	-.181	.433	.728	-.134	.584	0.906
IL9	.073	.761	.947	-.260	.255	.663	-.104	.670	0.906
IL10	-.091	.704	.947	-.344	.127	.660	-.102	.678	0.906
IL12	-.086	.718	.947	.049	.833	.942	-.165	.500	0.906
IL13	.155	.514	.947	.118	.610	.835	.224	.357	0.906
IL15	-.020	.933	.947	-.260	.255	.663	-.172	.481	0.906
IL17	.036	.879	.947	.022	.926	.963	-.218	.371	0.906
Eotaxin	.183	.440	.947	-.066	.776	.925	.108	.660	0.906
BasicFGF	.020	.932	.947	.002	.993	.993	-.158	.518	0.906
GCSF	-.092	.701	.947	-.202	.381	.728	-.084	.732	0.906
GMCSF	-.058	.807	.947	-.189	.413	.728	-.051	.836	0.945
IFNγ	-.131	.581	.947	-.410	.065	.660	-.023	.926	0.952
IP10	.294	.209	.947	.175	.448	.728	.183	.452	0.906
MCP1	-.104	.661	.947	-.495*	.023	.598	-.020	.935	0.952
MIP1α	.042	.862	.947	.024	.917	.963	-.176	.472	0.906
MIP1β	-.059	.805	.947	-.189	.413	.728	.516*	.024	0.624
PDGFBB	.016	.947	.947	-.153	.509	.778	-.368	.121	0.906
TNFα	-.124	.602	.947	-.129	.579	.835	-.127	.604	0.906
VEGF	-.234	.321	.947	-.064	.783	.925	.015	.952	0.952

Table 8: Spearman's correlation coefficient analysis of age, urinepca3 and serum cytokines in whole cohort.

Cytokine	Serum PSA			Age			UrinePca3		
	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value	rho 'r_s'	P-value	Adj P-value
IL6	.219	.131	.252	-.037	.798	.990	-.187	.218	0.552
IL1β	.185	.202	.306	-.092	.524	.990	-.125	.412	0.582
IL1ra	.205	.158	.263	-.052	.722	.990	-.205	.177	0.552
IL2	.411**	.003	.063	.033	.820	.990	-.220	.147	0.552
IL4	.178	.221	.307	-.057	.692	.990	-.178	.243	0.552
IL5	.221	.126	.252	-.108	.456	.990	-.070	.649	0.763
IL7	.286*	.046	.188	-.046	.749	.990	-.114	.456	0.582
IL8	.122	.405	.463	.002	.990	.990	-.157	.304	0.567
IL9	.126	.388	.463	-.089	.538	.990	-.148	.333	0.567
IL10	.314*	.028	.180	-.079	.586	.990	-.139	.362	0.567
IL12	.398**	.005	.063	.137	.342	.990	-.007	.963	0.963
IL13	.301*	.036	.180	.112	.439	.990	.012	.938	0.963
IL17	.277	.054	.188	-.006	.965	.990	-.194	.202	0.552
Eotaxin	.207	.154	.263	.076	.598	.990	-.209	.168	0.552
BasicFGF	.244	.091	.207	-.050	.731	.990	-.159	.296	0.567
GCSF	.257	.075	.188	-.053	.714	.990	-.123	.422	0.582
GMCSF	.260	.071	.188	-.008	.958	.990	-.200	.188	0.552
IFNγ	.140	.337	.443	-.127	.379	.990	-.139	.363	0.567
IP10	.309*	.031	.180	.381**	.006	.150	.185	.223	0.552
MCP1	.120	.410	.463	-.158	.273	.990	-.111	.466	0.582
MIP1α	.183	.208	.306	.012	.933	.990	-.224	.138	0.552
MIP1β	.089	.544	.566	-.005	.972	.990	.065	.672	0.763
PDGFBB	.084	.566	.566	-.007	.961	.990	-.294*	.050	0.552
TNFα	.116	.426	.463	-.009	.948	.990	-.247	.102	0.552
VEGF	.265	.065	.188	.023	.872	.990	.012	.939	0.963

Appendix B

Table 9: Correlation among cytokines.

Cytokine	IL6			IL1β			IL1ra			IL2		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL6	1			.932**	0	0	.967**	0	0	.616**	0	0
IL1β	.932**	0	0	1			.926**	0	0	.591**	0.001	0.001
IL1ra	.967**	0	0	.926**	0	0	1			.621**	0	0
IL2	.616**	0	0	.591**	0.001	0.001	.621**	0	0	1		
IL4	.854**	0	0	.897**	0	0	.844**	0	0	.697**	0	0
IL5	.687**	0	0	.738**	0	0	.669**	0	0	.657**	0	0
IL7	.794**	0	0	.837**	0	0	.823**	0	0	.712**	0	0
IL8	.835**	0	0	.910**	0	0	.819**	0	0	.619**	0	0
IL9	.781**	0	0	.813**	0	0	.765**	0	0	.651**	0	0
IL10	.827**	0	0	.805**	0	0	.863**	0	0	.765**	0	0
IL12	.504**	0.005	0.0075	.459*	0.012	0.016	.576**	0.001	0.001	.746**	0	0
IL13	.783**	0	0	.888**	0	0	.792**	0	0	.664**	0	0
IL17	.419*	0.024	0.030	.464*	0.011	0.016	.402*	0.031	0.041	.833**	0	0
Eotaxin	.450*	0.014	0.019	.424*	0.022	0.027	.394*	0.034	0.042	.733**	0	0
BFGF	.401*	0.031	0.037	.456*	0.013	0.017	.407*	0.028	0.039	.832**	0	0
GCSF	.686**	0	0	.766**	0	0	.679**	0	0	.797**	0	0
GMCSF	.678**	0	0	.694**	0	0	.683**	0	0	.930**	0	0
IFNγ	.944**	0	0	.971**	0	0	.939**	0	0	.585**	0.001	0.001
IP10	.438*	0.018	0.024	.399*	0.032	0.036	0.353	0.061	0.069	.378*	0.043	0.043
MCP1	.854**	0	0	.889**	0	0	.789**	0	0	.416*	0.025	0.026
MIP1α	0.254	0.18	0.19	0.316	0.095	0.099	0.285	0.134	0.146	.753**	0	0
PDGFBB	.365	.052	.056	.329	.082	.091	.270	.157	.167	.656**	.000	0
MIP1β	0.222	0.248	0.248	0.224	0.243	0.243	0.209	0.277	0.277	.463*	0.011	0.012
TNFα	.893**	0	0	.902**	0	0	.895**	0	0	.712**	0	0
VEGF	0.356	0.058	0.063	.405*	0.029	0.034	0.36	0.055	0.066	.582**	0.001	0.001

Cytokine	IL4			IL5			IL7			IL8		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL6	.854**	0	0	.687**	0	0	.794**	0	0	.835**	0	0
IL1β	.897**	0	0	.738**	0	0	.837**	0	0	.910**	0	0
IL1ra	.844**	0	0	.669**	0	0	.823**	0	0	.819**	0	0
IL2	.697**	0	0	.657**	0	0	.712**	0	0	.619**	0	0
IL4	1			.798**	0	0	.868**	0	0	.943**	0	0
IL5	.798**	0	0	1			.725**	0	0	.760**	0	0
IL7	.868**	0	0	.725**	0	0	1			.808**	0	0
IL8	.943**	0	0	.760**	0	0	.808**	0	0	1		
IL9	.873**	0	0	.664**	0	0	.759**	0	0	.853**	0	0
IL10	.845**	0	0	.699**	0	0	.826**	0	0	.764**	0	0
IL12	.585**	0.001	0.0013	.476**	0.009	0.011	.641**	0	0	.476**	0.009	0.012
IL13	.883**	0	0	.737**	0	0	.895**	0	0	.841**	0	0
IL17	.625**	0	0	.539**	0.003	0.004	.670**	0	0	.566**	0.001	0.001
Eotaxin	.502**	0.005	0.006	.457*	0.013	0.015	.503**	0.005	0.006	.465*	0.011	0.013
BFGF	.612**	0	0	.608**	0	0	.667**	0	0	.556**	0.002	0.002
GCSF	.876**	0	0	.829**	0	0	.803**	0	0	.855**	0	0
GMCSF	.753**	0	0	.741**	0	0	.768**	0	0	.707**	0	0
IFNγ	.890**	0	0	.761**	0	0	.816**	0	0	.897**	0	0
IP10	.408*	0.028	0.029	.386*	0.038	0.041	0.335	0.076	0.076	.418*	0.024	0.025
MCP1	.749**	0	0	.644**	0	0	.672**	0	0	.824**	0	0
MIP1α	.483**	0.008	0.009	.503**	0.005	0.006	.474**	0.009	0.010	.449*	0.015	0.017
MIP1β	0.365	0.052	0.052	0.194	0.312	0.312	.402*	0.031	0.032	0.306	0.107	0.107
PDGFB	.472**	0.01	0.011	.391*	0.036	0.041	.422*	0.023	0.025	.419*	0.024	0.025
TNFα	.886**	0	0	.860**	0	0	.860**	0	0	.858**	0	0
VEGF	.445*	0.016	0.017	0.274	0.15	0.156	.639**	0	0	.455*	0.013	0.015

Cytokine	IL9			IL10			IL12			IL13		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL6	.781**	0	0	.827**	0	0	.504**	0.005	0.008	.783**	0	0
IL1β	.813**	0	0	.805**	0	0	.459*	0.012	0.014	.888**	0	0
IL1ra	.765**	0	0	.863**	0	0	.576**	0.001	0.002	.792**	0	0
IL2	.651**	0	0	.765**	0	0	.746**	0	0	.664**	0	0
IL4	.873**	0	0	.845**	0	0	.585**	0.001	0.002	.883**	0	0
IL5	.664**	0	0	.699**	0	0	.476**	0.009	0.001	.737**	0	0
IL7	.759**	0	0	.826**	0	0	.641**	0	0	.895**	0	0
IL8	.853**	0	0	.764**	0	0	.476**	0.009	0.012	.841**	0	0
IL9	1			.724**	0	0	.432*	0.019	0.021	.834**	0	0
IL10	.724**	0	0	1			.681**	0	0	.791**	0	0
IL12	.432*	0.019	0.021	.681**	0	0	1			.541**	0.002	0.002
IL13	.834**	0	0	.791**	0	0	.541**	0.002	0.003	1		0
IL17	.644**	0	0	.568**	0.001	0.001	.675**	0	0	.627**	0	0
Eotaxin	.513**	0.004	0.005	.462*	0.012	0.014	.609**	0	0	.557**	0.002	0.002
BFGF	.620**	0	0	.587**	0.001	0.001	.663**	0	0	.620**	0	0
GCSF	.823**	0	0	.775**	0	0	.606**	0	0	.809**	0	0
GMCSF	.641**	0	0	.757**	0	0	.723**	0	0	.743**	0	0
IFNγ	.790**	0	0	.801**	0	0	.431*	0.02	0.021	.808**	0	0
IP1048	0.293	0.123	0.123	0.341	0.07	0.073	0.36	0.055	0.057	.419*	0.024	0.027
MCP1	.698**	0	0	.607**	0	0	0.284	0.136	0.136	.726**	0	0
MIP1α	.504**	0.005	0.006	.489**	0.007	0.008	.544**	0.002	0.003	.407*	0.028	0.029
MIP1β	0.302	0.111	0.115	0.196	0.308	0.308	.459*	0.012	0.014	0.348	0.065	0.065
PDGFB	.530**	0.003	0.004	.406*	0.029	0.031	.479**	0.009	0.012	.408*	0.028	0.029
TNFα	.737**	0	0	.889**	0	0	.564**	0.001	0.002	.834**	0	0
VEGF	.399*	0.032	0.034	.437*	0.018	0.020	.684**	0	0	.549**	0.002	0.002

Cytokine	IL17			Eotaxin			BasicFGF			GCSF		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL6	.419*	0.024	0.027	.450*	0.014	0.017	.401*	0.031	0.033	.686**	0	0
IL1β	.464*	0.011	0.013	.424*	0.022	0.026	.456*	0.013	0.017	.766**	0	0
IL1ra	.402*	0.031	0.033	.394*	0.034	0.035	.407*	0.028	0.032	.679**	0	0
IL2	.833**	0	0	.733**	0	0	.832**	0	0	.797**	0	0
IL4	.625**	0	0	.502**	0.005	0.008	.612**	0	0	.876**	0	0
IL5	.539**	0.003	0.004	.457*	0.013	0.017	.608**	0	0	.829**	0	0
IL7	.670**	0	0	.503**	0.005	0.008	.667**	0	0	.803**	0	0
IL8	.566**	0.001	0.001	.465*	0.011	0.016	.556**	0.002	0.003	.855**	0	0
IL9	.644**	0	0	.513**	0.004	0.008	.620**	0	0	.823**	0	0
IL10	.568**	0.001	0.001	.462*	0.012	0.016	.587**	0.001	0.001	.775**	0	0
IL12	.675**	0	0	.609**	0	0	.663**	0	0	.606**	0	0
IL13	.627**	0	0	.557**	0.002	0.004	.620**	0	0	.809**	0	0
IL17	1			.799**	0	0	.960**	0	0	.786**	0	0
Eotaxin	.799**	0	0	1			.744**	0	0	.598**	0.001	0.001
BasicFGF	.960**	0	0	.744**	0	0	1			.825**	0	0
GCSF	.786**	0	0	.598**	0.001	0.002	.825**	0	0	1		
GMCSF	.816**	0	0	.761**	0	0	.829**	0	0	.824**	0	0
IFNγ	.420*	0.023	0.027	.373*	0.046	0.046	.411*	0.027	0.032	.748**	0	0
IP10	0.274	0.15	0.15	.415*	0.025	0.028	0.265	0.166	0.166	0.35	0.063	0.065
MCP1	0.361	0.055	0.057	.411*	0.027	0.029	0.348	0.065	0.067	.651**	0	0
MIP1α	.827**	0	0	.589**	0.001	0.002	.867**	0	0	.744**	0	0
MIP1β	.583**	0.001	0.001	.525**	0.003	0.006	.448*	0.015	0.018	0.292	0.124	0.124
PDGF	.788**	0	0	.777**	0	0	.750**	0	0	.637**	0	0
TNFα	.515**	0.004	0.005	.460*	0.012	0.016	.536**	0.003	0.004	.807**	0	0
VEGF	.731**	0	0	.654**	0	0	.682**	0	0	.560**	0.002	0.0021

Cytokine	GMCSF			IFNγ			IP10			MCP1		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL6	.678**	0	0	.944**	0	0	.438*	0.018	0.076	.854**	0	0
IL1β	.694**	0	0	.971**	0	0	.399*	0.032	0.076	.889**	0	0
IL1ra	.683**	0	0	.939**	0	0	0.353	0.061	0.088	.789**	0	0
IL2	.930**	0	0	.585**	0.001	0.001	.378*	0.043	0.082	.416*	0.025	0.04
IL4	.753**	0	0	.890**	0	0	.408*	0.028	0.076	.749**	0	0
IL5	.741**	0	0	.761**	0	0	.386*	0.038	0.082	.644**	0	0
IL7	.768**	0	0	.816**	0	0	0.335	0.076	0.096	.672**	0	0
IL8	.707**	0	0	.897**	0	0	.418*	0.024	0.076	.824**	0	0
IL9	.641**	0	0	.790**	0	0	0.293	0.123	0.147	.698**	0	0
IL10	.757**	0	0	.801**	0	0	0.341	0.07	0.093	.607**	0	0
IL12	.723**	0	0	.431*	0.02	0.03	0.36	0.055	0.088	0.284	0.13	0.14
IL13	.743**	0	0	.808**	0	0	.419*	0.024	0.076	.726**	0	0
IL17	.816**	0	0	.420*	0.023	0.032	0.274	0.15	0.17	0.361	0.055	0.073
Eotaxin	.761**	0	0	.373*	0.046	0.057	.415*	0.025	0.076	.411*	0.027	0.040
BFGF	.829**	0	0	.411*	0.027	0.036	0.265	0.166	0.181	0.348	0.065	0.082
GCSF	.824**	0	0	.748**	0	0	0.35	0.063	0.088	.651**	0	0
GMCS	1			.680**	0	0	.502**	0.005	0.076	.543**	0.002	0.003
IFNγ	.680**	0	0	1		0	.370*	0.048	0.082	.887**	0	0
IP1048	.502**	0.005	0.005	.370*	0.048	0.057	1			.398*	0.032	0.045
MCP1	.543**	0.002	0.002	.887**	0	0	.398*	0.032	0.768	1		
MIP1α	.711**	0	0	0.31	0.102	0.111	0.116	0.551	0.551	0.181	0.349	0.364
MIP1β	.467*	0.011	0.011	0.196	0.309	0.309	0.253	0.185	0.193	0.039	0.839	0.839
PDGFB	.630**	0	0	0.301	0.112	0.11	.411*	0.027	0.076	0.317	0.094	0.107
TNFα	.779**	0	0	.914**	0	0	.459*	0.012	0.076	.761**	0	0
VEGF	.615**	0	0	0.312	0.1	0.11	.375*	0.045	0.082	0.319	0.091	0.107

Cytokine	MIP1α			MIP1β			PDGF		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL619	0.254	0.183	0.199	0.222	0.248	0.313	0.365	0.052	0.062
IL1β	0.316	0.095	0.12	0.224	0.243	0.313	0.329	0.082	0.093
IL1ra	0.285	0.134	0.153	0.209	0.277	0.325	0.27	0.157	0.157
IL238	.753**	0	0	.463*	0.011	0.041	.656**	0	0
IL452	.483**	0.008	0.014	0.365	0.052	0.124	.472**	0.01	0.021
IL533	.503**	0.005	0.012	0.194	0.312	0.325	.391*	0.036	0.045
IL774	.474**	0.009	0.015	.402*	0.031	0.082	.422*	0.023	0.040
IL854	.449*	0.015	0.024	0.306	0.107	0.190	.419*	0.024	0.040
IL977	.504**	0.005	0.012	0.302	0.111	0.190	.530**	0.003	0.008
IL10	.489**	0.007	0.014	0.196	0.308	0.325	.406*	0.029	0.040
IL12	.544**	0.002	0.006	.459*	0.012	0.041	.479**	0.009	0.021
IL13	.407*	0.028	0.039	0.348	0.065	0.13	.408*	0.028	0.040
IL17	.827**	0	0	.583**	0.001	0.012	.788**	0	0
Eotaxin	.589**	0.001	0.003	.525**	0.003	0.024	.777**	0	0
BFGF	.867**	0	0	.448*	0.015	0.045	.750**	0	0
GCSF	.744**	0	0	0.292	0.124	0.198	.637**	0	0
GMCS	.711**	0	0	.467*	0.011	0.041	.630**	0	0
IFNγ	0.31	0.102	0.122	0.196	0.309	0.325	0.301	0.112	0.116
IP10	0.116	0.551	0.551	0.253	0.185	0.277	.411*	0.027	0.040
MCP1	0.181	0.349	0.364	0.039	0.839	0.839	0.317	0.094	0.102
MIP1α	1			0.358	0.057	0.124	.677**	0	0
MIP1β	0.358	0.057	0.076	1			.465*	0.011	0.022
PDGF	.677**	0	0	.465*	0.011	0.041	1		
TNFα	.444*	0.016	0.024	0.243	0.204	0.288	.393*	0.035	0.045
VEGF	.502**	0.006	0.013	.568**	0.001	0.012	.617**	0	0

Cytokine	TNFalfa			VEGF		
	rho ‘r_s’	P-value	Adj P-value	rho ‘r_s’	P-value	Adj P-value
IL619	.893**	0	0	0.356	0.058	0.0662
IL1β	.902**	0	0	.405*	0.029	0.0435
IL1ra	.895**	0	0	0.36	0.055	0.066
IL238	.712**	0	0	.582**	0.001	0.0026
IL452	.886**	0	0	.445*	0.016	0.0274
IL533	.860**	0	0	0.274	0.15	0.15
IL774	.860**	0	0	.639**	0	0
IL854	.858**	0	0	.455*	0.013	0.024
IL977	.737**	0	0	.399*	0.032	0.0451
IL10	.889**	0	0	.437*	0.018	0.0288
IL12	.564**	0.001	0.0015	.684**	0	0
IL13	.834**	0	0	.549**	0.002	0.0043
IL17	.515**	0.004	0.00533	.731**	0	0
Eotaxin	.460*	0.012	0.0144	.654**	0	0
BFGF	.536**	0.003	0.00424	.682**	0	0
GCSF	.807**	0	0	.560**	0.002	0.0043
GMCS	.779**	0	0	.615**	0	0
IFNγ	.914**	0	0	0.312	0.1	0.104
IP1048	.459*	0.012	0.0144	.375*	0.045	0.0568
MCP1	.761**	0	0	0.319	0.091	0.099
MIP1α	.444*	0.016	0.018	.502**	0.006	0.012
MIP1β	0.243	0.204	0.204	.568**	0.001	0.0026
PDGFB	.393*	0.035	0.038	.617**	0	0
TNFα	1			.376*	0.044	0.056
VEGF	.376*	0.044	0.045	1		

Appendix C

Table 10: Correlation among urine PCA3 and serum PSA.

Spearman's rho		Urine PCA3	Serum PSA
Urine PCA3	Correlation coefficient	1	-0.078
	P-value		0.706
Serum PSA	Correlation coefficient	-0.078	1
	P-value	0.706	