This is to certify that the
thesis entitled

DETERMINING THE EFFECTS OF CYTOKINE GENE POLYMORPHISMS AND THE HUMORAL RESPONSE ON THE PROGNOSIS OF HORMONE REFRACTORY PROSTATE CANCER PATIENTS

presented by

Kristin R. Landis

has been accepted towards fulfillment
of the requirements for the

Master of Science degree in Clinical Laboratory Sciences

Major Professor's Signature

4/6/04

Date

DETERMINING THE EFFECTS OF CYTOKINE GENE POLYMORPHISMS AND THE HUMORAL RESPONSE ON THE PROGNOSIS OF HORMONE REFRACTORY PROSTATE CANCER PATIENTS

By

Kristin R. Landis

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Medical Technology Program

2004

ABSTRACT

DETERMINING THE EFFECTS OF CYTOKINE GENE POLYMORPHISMS AND THE HUMORAL RESPONSE ON THE PROGNOSIS OF HORMONE REFRACTORY PROSTATE CANCER PATIENTS

By

Kristin R. Landis

Hormone therapy used as a treatment for prostate cancer (PC) may be indicated for individuals with tumor progression. This therapy may be effective initially, however, all patients will eventually become resistant. If hormone therapy is determined *a priori* to be less effective for certain patients, then an alternative treatment regimen that eliminates unnecessary and/or ineffective procedures could more efficiently benefit the patient. Over-expressed interleukin-6 and antibodies to PSA produced by the humoral response may indicate a poor prognosis in PC patients. This study attempted to demonstrate a novel approach to determine PC patient prognosis by testing patient serum for antibodies to PSA using flow cytometry and determining the predisposition of those patients to high interleukin-6 production by DNA analysis. A flow cytometry assay using micro-beads to test for anti-PSA was successfully developed and this reproducible assay could be used to test PC patients in the future.

ACKNOWLEDGEMENTS

Thank you to Dr. John Gerlach for all of his time spent guiding me through the research process and his uncanny ability to answer all of my endless questions. I am grateful for all that he has done for me. Furthermore, I would like to thank my committee members Dr. Aaron Scholnik and Dr. Kathleen Hoag for their attention to detail and support to the end. Also, thank you to my father, for inspiring me to pursue a career in clinical laboratory sciences and for being a wonderful educator. Finally, I thank my husband for his lasting encouragement and patience. These people made this degree possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Prostate cancer (PC) is the most commonly diagnosed cancer among men in the United States and claims the lives of nearly 40,000 individuals each year. Based on previous incidence rates, over 180,000 new cases will be diagnosed this year [1]. African-American men are 35-50 percent (%) more likely to be diagnosed with PC than Caucasian men and are twice as likely to die of the disease [2] due in part to socio-economic reasons. Prostate cancer varies in aggressiveness: some tumors progress to an invasive and potentially life-threatening disease, while others remain latent from the time of diagnosis throughout the remainder of an individual's lifetime [3].

Genetic factors influence the likelihood of developing PC as well as the degree to which the disease will be expressed in an individual. For example, a man with one close relative (father or brother) with PC has twice the risk of developing the disease. With two close relatives, a man's risk increases fivefold, and with three close relatives, a man's risk of PC is 97%. In addition to heredity, other factors may increase the risk of PC, such as a high fat diet, age of over 55 years, race, a sedentary lifestyle, and smoking, but the degree to which these factors contribute to PC is still inconclusive [2].

Prostate cancer is diagnosed by a combination of laboratory tests [prostate specific antigen (PSA) test], physical examinations (digital rectal exam), imaging technologies (transrectal ultrasonography), and tissue sample analysis (histology). Upon diagnosis of the disease, PC is graded and then staged to

assess its severity, and in combination with patient age and health, the treatment options for the patient are determined.

Treatment options for the PC patient include radical prostatectomy, radiation therapy, hormone ablation, and chemotherapy. If the cancer is discovered early, radical prostatectomy is often the best option; it results in the highest survival rates for healthy males (approximately 90% for organ-confined disease) [4, 5]. In contrast, if PC is not detected in its earliest stages, radical prostatectomy may prove ineffective. At this point, depending on the health of the individual, radiation therapy with or without hormone ablation may be indicated. Once PC becomes resistant to hormone ablation, patient treatment is limited to palliative chemotherapy and radiation therapy [6].

Hormone therapy is used to decrease the number of androgen-dependent cells by decreasing the production of androgens in the testes. Initially, this therapy can be effective. Approximately 75% of patients receiving hormone ablation therapy will show clinical improvement, although all patients will eventually become resistant to the treatment (median survival is 12-18 months) [7]. Because of the relatively short response or lack of any response to hormone ablation in some patients, a test that determines PC prognosis before the administration of hormone ablation could be beneficial. However, such a test does not currently exist. If hormone ablation therapy is determined *a priori* to be less effective for certain patients, then an alternative treatment regimen that eliminates unnecessary and/or ineffective procedures can be developed and utilized to the benefit of the patient.

The goal of this research was to develop a clinical model for predicting PC prognosis before commencing hormone therapy. This clinical model was to be founded on the analysis of the humoral response (antibodies against prostate specific antigen or anti-PSA) and the genetic predisposition for increased production of the cytokine interleukin-6 (IL-6) involved in the humoral response. The hypothesis was that up-regulated, naturally-occurring, humoral immunity, in combination with up-regulated IL-6 production may indicate a poor prognosis for the hormone-ablated PC patient [8-10]. Such a clinical model could provide a useful tool to help predict the life expectancy of PC patients.

The first component of the proposed clinical model is the detection of anti-PSA antibodies. Currently, anti-PSA is most often detected by the enzyme-linked immunosorbent assay (ELISA). An ELISA is a highly sensitive and specific technique that can detect the anti-PSA antibody [2, 11-13]. Simply described, an ELISA is used to detect the presence of antigens specifically recognized by an antibody, or to determine the presence of antibodies specific for an antigen; in this case, it is used to detect anti-PSA antibodies in the serum of men with PC [14]. Although ELISA methods are well-established and are a proven sensitive technique, particle-based flow cytometric assays are emerging as viable alternatives [15] and may prove to have higher sensitivity (up to 50%) than the standard ELISA [16-18].

Because flow cytometers are increasingly more commonplace in hospital and research laboratories, particle-based techniques, which are sensitive, specific, and technically no more difficult than ELISA methods, have been

developed. A flow cytometer can detect and quantify particles (cells or micro-beads) in the lower micrometer range and measure the physical properties of those particles.

Micro-beads with an attached antigen are regularly used as calibrators for flow cytometers. Additionally, the processes used to adsorb antigen onto micro-beads for the detection of soluble antibodies are well documented [16, 19-22] and relatively simple to perform. To detect specific free antibody in serum, plastic micro-beads could be coated with the complementary antigen, blocked to bind any residual protein binding sites, and incubated with the serum of interest [16]. If the specific serum antibody were present, it would bind to the micro-beads, which would be subsequently incubated with a fluorescent-labeled anti-human antibody. The fluorescent-label would be detected by flow cytometry according to certain parameters (forward vs. side scatter and forward scatter vs. fluorescence). Once this antibody detection method is perfected for anti-PSA (Figure 1), an estimate of the strength of the humoral immune response can be made based on the relative strength of fluorescent signal detected. This study will describe a particle-based assay that detects the anti-PSA antibody.

A second component of the proposed clinical model is the evaluation of specific cytokine genotypes that could be used to predict an individual's predisposition toward a pro-inflammatory (cell mediated) or anti-inflammatory (humoral) immune response profile. Variations in the DNA sequences (genotypes) determine the expression levels (low, intermediate, and high) of the cytokines. The genotypes can be determined by the use of the polymerase chain

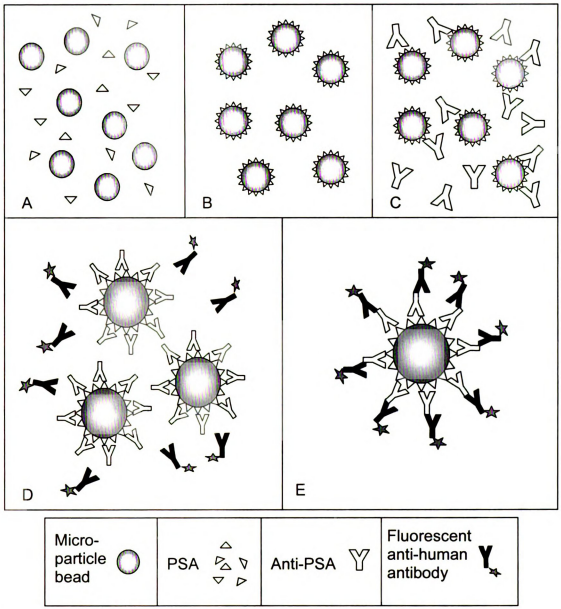


Figure 1: Micro-beads Coated with PSA, Anti-PSA, and Fluorescent Anti-human Antibody

(A) Micro-beads are incubated with PSA. (B) The PSA binds to the beads and the excess is washed away. The beads are then blocked with bovine serum albumin (not shown) to coat open protein binding sites on the beads that are not bound with PSA. (C) The PSA coated/blocked beads are incubated with human serum with antibodies to PSA. (D) The excess antibody is washed away and fluorescent-labeled anti-human antibody is added, which (E) binds to the anti-PSA. The excess fluorescent antibody is washed away and the beads are fixed in formaldehyde (not shown). The bead slurry is introduced to the flow cytometer and fluorescence is measured.

reaction and sequence specific primers (PCR-SSP) that are designed for variations in the DNA sequence. PCR-SSP is based on the principle that DNA synthesis is dependent upon correct base pairing at the 3 prime terminus of the primer. Amplification will not proceed if there is a mismatch. The DNA is amplified by PCR with multiple variant primers recognizing the polymorphisms affecting expression. The resulting products are separated by gel electrophoresis and visualized by staining with ethidium bromide and ultraviolet light exposure. Genotyping is determined by the presence of a band indicating amplification (positive reaction for that allele), the absence of a band (negative reaction for that allele), or the presence of a band in both alleles (heterozygosity). The use of sequence specific primers for the detection of genetic polymorphisms is available as a kit for cytokine genotyping (One Lambda, Inc).

Background and Significance

The Prostate Gland

The prostate is a walnut-sized gland located beneath the bladder and in front of the rectum. The prostate capsule consists of fibrous tissue and forms the outer covering of the gland. The prostate surrounds the urethra, producing much of the fluid component of semen and potentially regulating the flow of urine. Seminal fluid is delivered to the urethra by tubular structures within the gland. The prostate has a peripheral zone in which most cancers develop and a central zone where most benign prostatic hyperplasia take place.

Prostate Cancer

Like all cancers, PC is a disease of uncontrolled cell growth. Generally, as tumors grow, they crowd surrounding healthy tissue and may deprive healthy cells of oxygen. Tumors can also secrete angiogenic factors that initiate the formation of new blood vessels. Additionally, tumors release cells that metastasize into other organs, forming secondary tumors. Some prostate tumors are slow growing and cause few clinical symptoms, which allows individuals to live comfortably with the disease. However, aggressive tumors can spread rapidly to lymph nodes, other organs, and bone, inducing severe pain [23].

Prostate Cancer Diagnosis

Prostate cancer is diagnosed through a combination of laboratory tests, physical examinations, imaging technologies, and tissue sample analyses. The

age at which men should be screened for PC is somewhat controversial. One study suggests that PC screening should start with PSA tests at age 40 and again at age 45, with biennial testing after age 50. This testing strategy appears to lower prostate cancer deaths while simultaneously reducing the number of PSA tests and maintaining cost effectiveness [24]. The American Cancer Society currently recommends that annual PSA tests and digital rectal exams (DRE) should begin at age 50 for all males and at age 45 for African American males and individuals with a family history of the disease [23].

Prostate Specific Antigen

In the clinical setting, the most commonly tested serum protein to detect prostate gland abnormality is PSA, which is a very good indicator of abnormal glandular activity. PSA concentration is correlated with prostatic epithelial growth and men with PC show progressive increases in circulating PSA [25]. PSA is a serine protease (approximately 28.5 kilodalton (kD) [26]) encoded for by the Kallikrein gene hK3 [27]. When secreted into prostatic and seminal fluid, PSA hydrolyzes seminal vesicle proteins important in semen liquefaction. Furthermore, the enzymatic activity of PSA is partially responsible for PC tissue invasion and metastasis [28].

PSA levels in serum range from near 0 (undetectable or below reagent sensitivity) to greater than (>) 206 nanograms per milliliter (ng/ml) [29]. Normally PSA circulates in the bloodstream at concentrations below 2 ng/ml. A PSA concentration of 2-4 ng/ml may be considered high risk for the development of

PC in the future [30]. In addition, concentrations between 4-10 ng/ml are sometimes indicative of benign enlargement of the prostate, which is common with increasing age, yet 50% of men with concentrations >4 ng/ml have carcinoma [25]. Finally, a PSA concentration >10 ng/ml may indicate a malignant growth [31]. Hence, the higher the PSA level, the more likely the probability that PC is present. Blood levels of PSA are monitored often (testing occurs every 4-6 months) when a man has a prostate abnormality [23]. The PSA test can also monitor the effectiveness of treatments such as radical prostatectomy, radiation, or hormone ablation. A rise in PSA concentration can be indicative of disease recurrence, negative response to therapy, or disease progression [3].

In an attempt to extend the usefulness of the PSA blood test, PSA density and velocity testing have been developed. PSA density is determined by measuring the volume of the prostate by ultrasound in relationship to the level of serum PSA [density = serum PSA concentration (ng/ml) / prostate volume (cubic centimeter)]. A PSA density greater than or equal to ($\geq$) 0.15 ng/ml/cubic centimeter of prostatic tissue may be indicative of PC [32, 33]. Yet benign enlargement can also increase PSA levels and therefore the diagnostic value versus the cost-effectiveness of the PSA density test is questionable [25]. PSA velocity is determined by the elevation of PSA over time, assuming that PSA will increase more rapidly in men with PC than in normal individuals [34]. PSA concentrations are measured three times over the course of 18 months. A velocity $\geq$0.75 ng/ml/18 months is considered suspect and, although the PSA concentration may be normal, a biopsy should be performed for definitive

diagnosis [34, 35]. Because of daily fluctuations in PSA concentrations (up to 23% within the same patient) [36], and because of assay/reagent variability, interpretation of these tests can be slightly flawed [25, 37]. Therefore, the PSA density and velocity tests are not always useful.

Digital Rectal Exam (DRE)

An elevated PSA level warrants further testing, usually a physical examination. The most common physical diagnostic technique is the DRE, in which a physician inserts a gloved finger into the rectum to assess the texture and size of the rear portion of the gland. Irregular or abnormally firm areas may indicate cancer. Digital rectal exams in combination with PSA tests can lead to early diagnosis, potentially preventing 50-70% of PC deaths [38].

Transrectal Ultrasonography

Upon determination of an abnormality in the prostate gland by a DRE or an unexplained elevation in PSA, a transrectal ultrasonography (TRUS) may be requested. A visual image of the prostate is produced using sound waves and may indicate if the cancer has penetrated the prostate capsule. However, TRUS is most often used to visualize the organ for guidance of biopsies (usually in combination with PSA >4 ng/ml).

Histological Examination: Prostate Cancer Grading

Biopsied tissue is viewed under a microscope as a last effort for definitive diagnosis [39]. A grading system (specific to each type of cancer) is used to

classify cancer cells in terms of how abnormal they appear microscopically. The Gleason System (most widely accepted among physicians) is used to grade prostate cells based on their level of differentiation (on a scale of 1 to 5). Differentiated (mature) prostate cells secrete PSA at normal concentrations. Gleason Grades of 1 and 2 indicate well differentiated cells; Grade 3 indicates moderately differentiated cells; and Grades 4 and 5 indicate poorly differentiated cells. Malignant prostate cells produce elevated levels of PSA and form tubules that are distorted and that eventually form solid masses (Figure 2) [2, 39-41]. Because different sections of the prostate cancer may exhibit variation in cellular differentiation, Gleason Grades are determined for the two most prominent patterns of biopsy and added to obtain the Gleason Score (2-10). The probable growth rate of the tumor and its tendency to spread can be assessed based on the Gleason Score, which is an indicator of the aggressiveness of the prostate tumor. The higher the Gleason Score, the faster the cancer is likely to grow and spread beyond the prostate [23, 40, 41].

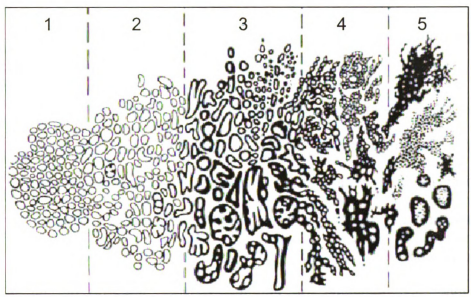


Figure 2. Gleason Grades 1-5 (adapted from Gleason and Mellinger, 1974)
The progression of cellular changes from Gleason Grade 1 (normal) to Gleason Grade 5 (cancer) is shown. Cells described as Gleason Grades 1 and 2 are well differentiated; Grade 3 is moderately differentiated; and Grades 4 and 5 are poorly differentiated. As cells are characterized as less differentiated, the cancer is likely to be more invasive.

Physical and Histological Examination: Prostate Cancer Staging

Repeatedly elevated PSA levels, an abnormal DRE and TRUS results, and the calculated Gleason Score are the basis for PC staging (Table 1). The stage of the cancer is determined by the extent of tumor spread within the body. The stages are termed T for tumor, N for lymph node, and M for distant metastasis [42]. Furthermore, each stage is assigned a number with degrees ranging from a to c.

Tumor status is categorized by the following schemes: If the tumor cannot be assessed or if no tumor is present, the stages are termed TX and T0 respectively. If the prostate cancer is thought to be contained within the prostate capsule and the tumor is neither palpable nor visible by imaging techniques, the disease is designated T1. If a lump is felt during DRE, but the cancer is visualized within the prostate capsule, patients are classified T2. When a palpable lump of cancer extends through the prostate capsule and may involve the seminal vesicles, patients are classified T3. Finally, when the cancer has metastasized to tissues adjacent to the prostate, such as the bladder's sphincter, the rectum, and/or the wall of the pelvis, men are classified T4. Additionally, stages N0 (lack of any lymph node involvement), N1 (metastasis to one or more regional lymph nodes), M0 (no metastasis beyond regional nodes), and M1 (metastasis to distant lymph nodes, bones, or other organs such as lungs, liver, or brain) are used to stage PC [35, 41-43] (Table 1).

To better distinguish the extent of PC, stage groupings (Table 2) are assessed upon determination of the T, N, and M categories and in combination

with the Gleason Score. Prostate cancers in stage groupings 1 and 2 are localized to the prostate gland. Stage 3 PC is locally advanced outside the gland and Stage 4 PC has spread to other organs or tissues. Prostate cancer grading, staging, and grouping help determine treatment decisions [35].

Table 1. Prostate Cancer Stages by T, N, and M Categories: Adapted from the American Joint Committee on Cancer: AJCC Cancer Staging Manual.

T categories:

- **TX: Primary tumor cannot be assessed**
- **T0: No evidence of primary tumor**
- **T1: Tumor not palpable nor visible by imaging**

  T1a: histologic finding 5% or less of tissue resected
  T1b: histologic finding in more than 5% of tissue resected
  T1c: Tumor identified by biopsy (e.g., because of elevated PSA)

- **T2: Tumor confined within prostate**

  T2a: Tumor involves 1 lobe
  T2b: Tumor involves more than half of one lobe but not both lobes
  T2c: Tumor involves both lobes

- **T3: Tumor extends through the prostatic capsule**

  T3a: Unilateral extracapsular extension
  T3b: Bilateral extracapsular extension
  T3c: Tumor invades seminal vesicle(s)

- **T4: Tumor is fixed or invades adjacent structures other than seminal vesicles:**

  T4a: Tumor invades bladder neck and/or external sphincter and/or rectum
  T4b: Tumor invades levator muscles, and/or pelvic wall

N categories:

- **N0: No regional lymph node metastasis**
- **N1: Metastasis in single lymph node <2 cm in dimension**
- **N2: Metastasis in single lymph node >2 cm, <5 cm in dimension**
- **N3: Metastasis in lymph node >5 cm**

M categories:

- **M0: No distant metastasis**
- **M1: Distant metastasis**

  M1a: Nonregional lymph node(s)
  M1b: Bone(s)
  M1c: Other site(s)

Table 2. Stage Groupings of Prostate Cancer: Adapted from the American Cancer Society Inc. Prostate Cancer Treatment Guidelines 2001.

Stage I	T1a, N0, M0, low Gleason score (2 to 4) **- Localized to prostate and less than 5% of tissue is cancerous**
Stage II	T1a, N0, M0, intermediate or high Gleason score (5 to 10) T1b, N0, M0, any Gleason score (2 to 10) T1c, N0, M0, any Gleason score (2 to 10) T2, N0, M0, any Gleason score (2 to 10) **- Localized to the prostate and includes one of the three characteristics below:** - more than 5% of the tissue contained cancer. - high PSA, cancer not detected by DRE, not detected by TRUS. - diagnosed by biopsy, can be detected by DRE, can be detected by TRUS.
Stage III	T3, N0, M0, any Gleason score (2 to 10) **- Cancer has metastasized to seminal vesicles, no lymph node involvement**
Stage IV	T4, N0, M0, any Gleason score (2 to 10) Any T, N1, M0, any Gleason score (2 to 10) Any T, any N, M1, any Gleason score (2 to 10) **- Cancer has metastasized to tissues other than the seminal vesicles, such as the bladder's external sphincter, the rectum, and/or the wall of the pelvis.** **- Cancer spread to the lymph nodes.** **- Cancer has spread to other, more distant sites in the body.**

Prostate Cancer Treatment Options

Observation

As a result of improved testing methods, approximately 83% of all PC cases are diagnosed while the disease is still confined to the prostate gland [2]. Patients are monitored by a PSA test performed every 4-6 months and palliative treatment may be employed. A PSA concentration above 10 ng/ml may warrant further treatment depending on the health status of the individual [39]. In some cases, observation alone is reserved for older patients (more than 70 years old) and/or for patients whose life expectancy is less than 10 years. The rationale for withholding treatment is that these patients will probably die of other causes and not from PC [39].

Radical Prostatectomy

When PC is confined to the prostate capsule, it is treatable with surgery. The most common treatment of localized PC is radical prostatectomy, which can be very effective, especially for patients in good health and who are younger than 70 years with a remaining life expectancy of more than 10 years [39]. Patients should have a negative bone scan and tumors confined to the prostate gland (stages I and II). Prostatectomy can be performed from perineal or retropubic approaches but surgery is not usually performed if frozen section evaluation of pelvic nodes reveals metastases. Patients with metastases may receive radiation therapy to control local symptoms [44]. Mortality rates are low and

incidences of impotence and urinary incontinence are decreasing as surgical techniques are improved. The 10-year survival rate for individuals with localized disease who have undergone radical prostatectomy is nearly 100% [45].

Radiation Therapy

Radiation therapy (external beam source) and brachytherapy (implanted radioactive seeds) are treatment options for patients with localized (stages T1-T2) and also more advanced PC (stages T3-T4). External beam radiation is painless, lasts for only minutes, and is administered in brief sessions, usually one session each weekday for several weeks. Brachytherapy employs radioactive seeds inserted directly into the prostate. This allows a higher dose of radiation than external beam therapy in close proximity to the cancer cells. Radiation therapy is a more appropriate treatment for individuals more than 70 years of age because it is less invasive than surgery [2]. Side effects of radiation therapy are manageable and survival rates are nearly as favorable as those after prostatectomy [39].

Hormone Therapy

Once the cancer penetrates the prostate capsule to involve adjacent organs, or metastasizes, treatment options are more limited. Advanced local-regional stage and metastatic PC are incurable at this time, although techniques such as hormone therapy can extend the lives of individuals suffering from the disease [39, 46]. The goal of hormone therapy is to decrease the amount of

androgens, specifically testosterone, produced by the testes. Some prostate cells depend on testosterone for proliferation and inhibition of apoptosis, therefore, removal of testosterone inhibits cell growth [46, 47]. One form of hormone therapy is bilateral orchiectomy, which is used to remove androgen because testes are major sources of testosterone. Although most men opt for other treatment, orchiectomy is effective because 90-95% of testosterone is eliminated immediately [48]. Another form of hormone therapy (also termed medical castration or hormone ablation) is a technique that inhibits the proliferation of prostate cells by the administration of luteinizing hormone-releasing hormone (LHRH) agonist. The LHRH agonist causes a primary increase in the release of luteinizing hormone (LH), which potentially worsens the symptoms of the disease. However, after approximately 5-7 days, the concentration of LH declines and consequently decreases the production of testosterone by the testes. The lack of testosterone halts the growth of testosterone-dependent cells in the prostate and elsewhere [39]. Although almost all patients initially respond to treatment, hormone therapy eventually becomes ineffective in metastatic cancer with patient survival decreasing from 50% at 2 years to 20% at 5 years [45, 47]. Tumor cell clones whose growth was temporarily inhibited by the removal of testosterone become testosterone-independent and proliferate. Once a patient stops responding to hormone ablation therapy, the patient's disease state is termed hormone refractory prostate cancer (HRPC).

Chemotherapy

Treatment for individuals with HRPC is limited, although chemotherapy can provide palliative benefit to some men with hormone-refractory disease [6]. Chemotherapeutics reported to improve symptoms and reduce PSA concentration include mitoxantrone/docetaxel, paclitaxel, estramustine, etoposide, and vinblastine, used separately and in varying combinations. Currently, no chemotherapy regimen can be considered standard [49] and survival rarely extends longer than one year [45].

Immunotherapy

Immunotherapy is being developed as a potential treatment for HRPC. This treatment attempts to induce tumor-specific cell-mediated immunity to target antigens to effectively bolster the immune system. PSA, which is the ideal target antigen, is tumor specific and expressed on all tumor cells. Immunotherapeutic methods include cytokine-based, tumor-associated antigen-based, tumor vaccines, and dendritic cell-based techniques. All of these techniques artificially induce the immune system to contain the tumor, yet none of them have been proven universally effective [50, 51].

Although clinical trials for immunotherapeutics are well documented, there are no immunology based trial groups that focus on determining the prognosis of PC [35]. In the absence of a curative therapy for HRPC, an estimate of life expectancy is valuable to the patient.

Prostate Cancer and the Immune System

Prior to the derivation of immunotherapy, it was well known that neoplastic tumors, including prostate tumors, are immunogenic. This implies that PC patients can mount a cell-mediated and/or humoral immune response to the tumor [11]. The immune system is composed of two equally important arms: a cell-based system (cell-mediated immunity) and an antibody-based system (humoral immunity). The elimination of tumors by cell-mediated immunity primarily involves cytotoxic T lymphocytes (CTLs), which have the ability to discriminate and lyse cells expressing tumor-associated antigenic peptides associated with Major Histocompatability Complex (MHC) class I molecules [52-54]. However, an effective CTL response requires the support of T helper (Th) cells, specifically the Th1 cells [55, 56] which secrete pro-inflammatory cytokines (Interleukin (IL-) IL-1, IL-2, IL-3, Interferon-γ (IFN-γ), and Tumor Necrosis Factor-α (TNF- α)) [55]. In contrast, the humoral immune response requires B cell involvement and the aid of Th2 cells, which secrete anti-inflammatory cytokines (IL-4, IL-5, IL-6, and IL-10) and aid antibody production [57].

Cytokines function as regulators of the immune response by mediating cell-to-cell communication, and thus the make-up of the cytokine network is of great importance. The generation of cytokines by Th cells and other cells of the immune response is induced by an immune challenge and the process is complex. The cytokines produced by Th1 cells suppress the Th2 response. Furthermore, Th2 cytokine production suppresses the Th1 response. Therefore,

the net effect of the interactions between the pro-inflammatory and anti-inflammatory cytokine profiles determine whether the immune response will be cellular or humoral in nature [57]. In general, a cell-mediated or a humoral-mediated response predominates in response to any given immune challenge.

The immune responsiveness toward the prostate tumor cells may determine the prognosis of the PC patient. Promoter regions of a few crucial pro- and anti-inflammatory cytokine genes contain polymorphisms that directly influence cytokine production leading to either low-, intermediate-, or high-level production of a given cytokine. These polymorphisms may cause differences in the immune response profile, which may ultimately influence the anti-tumor immune response in PC [8-10]. Together, the cell-mediated and humoral systems can contain tumor growth [51, 58]. However, a shift in immune function, such as cytokine elevation, can disrupt tumor containment [59].

This study attempts to connect the presence of cytokine gene polymorphisms with the clinical outcome of patients and correlate increased or decreased expression of a particular cytokine profile (pro- or anti-inflammatory) with the presence or absence of a humoral response. The cytokine gene polymorphisms determined in this study included transforming growth factor-β1, IL-10, and IL-6 (associated with the anti-inflammatory or humoral response) and TNF-α and IFN-γ (associated with the pro-inflammatory or cell-mediated response). However, this study places emphasis on IL-6 and the humoral response because of various reports indicating that increased production of the

cytokine and anti-PSA antibodies are associated with a poor prognosis in PC patients [11, 12, 60-62].

Interleukin-6

Normal Function

IL-6 is key to host defense mechanisms by regulating immune responses, hematopoiesis, and induction of the acute phase response [63]. IL-6 exerts both pro- and anti-inflammatory activities, such as the stimulation of T cell proliferation and their differentiation into CTLs and the stimulation of antibody production by B cells [64]. Production of IL-6 occurs in a number of cell types, especially macrophages, fibroblasts, and endothelial cells and IL-6 induces the differentiation of monocytes to macrophages [65]. Th2 cells also secrete IL-6, controlling the proliferation and differentiation of B cells into antibody-producing B cells. IL-6 stimulates IL-4 production, leading to a Th2 response and thus down-regulating a Th1 response [57, 66, 67]. Normal healthy males maintain serum IL-6 levels at approximately 0.7 picograms (pg)/ml [68].

Association with Prostate Cancer

Cytokines, especially IL-1, IL-6, and TNF-α, that are over-expressed in patients with cancer can cause symptoms such as anemia, malaise, fever, and cachexia [69-72] as seen in cancers such as renal cell carcinoma, ovarian carcinoma, plasmacytoma, myeloma, and in PC [10, 60]. These wasting syndromes can be harbingers of severe clinical deterioration and death [10].

Furthermore, IL-6 directly correlates with patient response to hormone therapy in PC [8, 68, 73]; hormone-dependent prostate cells can transfom into hormone-independent cells when IL-6 is over-expressed [60, 61, 74-76] and the secretion of IL-6 protects tumor cells from certain chemotherapeutic agents [77]. Over-expression of IL-6 can be caused by a polymorphism (G to C substitution) at position –174 in the promoter region upstream of the multiresponsive element (position -173 to -151) [66, 78-80]. This mutation causes a two-fold increase in transcription of IL-6 and occurs in approximately 40% of the population [78] and in 50% of PC patients [9].

Humoral Response

In addition to increased IL-6 production, an up-regulated humoral response (naturally-occurring anti-PSA antibodies) can be detected in PC patients [11, 12, 62], although high-titer antibody production is probably apparent exclusively in late-stage disease [11, 12]. Initiation of the humoral response may be indicated by antibodies to PSA (anti-PSA), which circulate in the bodies of men with PC [11, 12, 62]. In one study, 200 PC patients were tested [11] and 22 (11%) displayed detectable anti-PSA in serum at dilutions 1:50 and greater. In addition, 6 of 56 HRPC patients tested (11%) displayed high titers (1:100 dilution or greater) of circulating anti-PSA, yet it is possible that the antibody was masked *in vivo* by the high concentration of circulating PSA.

Project Goals

The evidence for increased IL-6 production and the potential for a humoral response to PSA in PC directed the goals of this project. These factors may be indicators of poor prognosis for individuals who become refractory to hormone therapy used to treat PC. This project set out to detect the humoral response (anti-PSA) by a novel flow cytometry technique and to correlate those findings to the genetic predisposition for increased anti-inflammatory cytokine profile, specifically, elevated IL-6 production by PCR-SSP detection.

Materials and Methods

All reagents described, unless otherwise stated, were supplied by various manufacturers and were purchased from Michigan State University Stores (East Lansing, Michigan).

Blood Collection

All human samples were handled in accordance with the University Committee on Research Involving Human Subjects. Ten patient samples were received in two tubes, 10 ml of whole blood and 10 ml of acid citrate dextrose (ACD) anti-coagulated blood. Whole blood samples were centrifuged for 10 min at 1,200 x gravity (g). The serum was removed and 200 microliter (μl) aliquots were frozen at -20 °C. The ACD samples were centrifuged for 10 min at 1,200 x g; the nucleated leukocytes contained in the buffy coat were removed and frozen at -20 °C for DNA isolation and purification.

Flow Cytometry

All flow cytometry methods were adapted from One Lambda, Inc. (Canoga Park, CA) and McHugh et al. (1997) [16] and all experiments were conducted in 1.5-1.9 ml polypropylene microcentrifuge tubes unless otherwise stated.

Micro-bead Washing

Polystyrene micro-beads, (Polysciences Inc., Warrington, PA) 3.0 micrometer (μm) in diameter, were used at a concentration of 10^7 micro-beads

per ml for all flow cytometry bead assays. All wash steps included a stock 10X flow cytometry wash buffer [1% volume per volume (v/v) Tween 20, 0.1% weight per volume (w/v) sodium azide, and 10X phosphate buffered saline, PBS (1.368 molar (M) sodium chloride, 0.027 M potassium chloride, 0.10 M sodium phosphate, 0.01 M potassium phosphate)], which was diluted to 1X with double distilled water before use. Micro-beads were washed by adding 1 ml of flow cytometry wash buffer (flow wash buffer), vortexing for 30 seconds (sec), and centrifuging for 6 minutes (min) at 11,000 x g. The supernatant was aspirated with a vacuum aspirator and was discarded after each wash.

Batched PSA Coating of Micro-beads

Lyophilized PSA (Biodesign International, Saco, ME) was reconstituted to a concentration of 1 mg/ml in sterile water using sterile techniques. Working aliquots of 20 μl were stored at -20°C to avoid protein denaturation. Micro-beads were coated with PSA by mixing 19.5 ml of binding buffer (0.1 M Na_2CO_3, pH 9.6) with 500 μl of micro-beads and 80 μl of PSA and incubating at 37°C for 3 hours (hr) and at 4°C for 18 hr with gentle rocking in a 50 ml conical polypropylene tube. After incubation, the micro-beads were washed twice using approximately 30 ml of flow wash buffer.

Batched Blocking of Micro-beads

A solution of 1X PBS containing 0.5% w/v bovine serum albumin, (Fraction V; Sigma-Aldrich, St. Louis, MO) and 0.1% w/v sodium azide (BSA-

PBS) was used for micro-bead blocking (block solution). The PSA-coated beads were incubated at 4°C with 20 ml block solution for 1 hr with gentle rocking. After incubation, the beads were washed twice using approximately 30 ml of flow wash buffer. After the last wash, 20 ml of flow wash buffer was added to the tube and 1 ml aliquots were made in 1.5-1.9 ml Eppendorf tubes. PSA-coated and blocked micro-beads were stored at 4°C with gentle rocking for up to 3 months.

Micro-bead Method

All controls and patient samples were tested using PSA-coated, blocked micro-beads prepared as above and all dilutions were made using flow wash buffer unless otherwise stated. Micro-beads were incubated at 37°C with 200 μl of a 1:100 dilution of the primary antibody (patient serum or controls) for 30 min. After incubation, the beads were centrifuged and washed twice using the wash method previously mentioned. Two hundred μl of 1:100 diluted secondary antibody (Fluorescein Isothiocyanate (FITC) conjugate) was added to the bead mixture and incubated at 37°C for 30 min. After incubation, the beads were centrifuged, washed twice, and 1 ml of fixing solution (0.5% v/v formaldehyde in 1X PBS) was added.

Positive Control

Mouse monoclonal anti-human PSA, subclass IgG_1, at a concentration of 0.5 mg/ml (Abcam Ltd, Cambridge, United Kingdom) was used as the positive

control (primary antibody). Goat anti-mouse IgG FITC (Sigma-Aldrich, St. Louis, MO) was used as the secondary antibody for detection on the flow cytometer.

Negative Control

Human female serum was used for the negative control. Goat anti-human IgG FITC (Abcam Ltd) was used for detection on the flow cytometer. All patient samples were treated in the same manner as the negative control.

Preliminary Test: Lower Limit of Reactivity

Five females were tested as potential negative controls. Triplicate testing of the females was performed to determine the range of the negative control and each repeat test included a positive control. One female sample was tested with an additional 10 replicates and was chosen as the negative control for all subsequent experiments.

Preliminary Test: Positive Control Dilution

Serial dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600) of the positive control were made using the flow wash buffer. Each dilution of mouse anti-PSA was added to PSA-coated, blocked micro-beads and incubated at 37°C for 30 min. After incubation, the beads were centrifuged and washed twice. Two hundred μl of 1:100 diluted secondary antibody was added to the bead mixture and incubated at 37°C for 30 min. After incubation, the beads were centrifuged,

washed twice, and 1 ml fixing solution was added. All dilutions were tested in three replicates.

Preliminary Test: Testing of Non-specific Binding

Positive and negative controls were used in four assays that tested non-specific binding (Table 3). Micro-beads were blocked and washed twice but were not coated with PSA prior to blocking. The negative control non-specific binding assay included a 200 μl of 1:100 diluted negative control, which was added to the blocked micro-beads, incubated at 37°C for 30 min, centrifuged, and washed twice. Two hundred μl of 1:100 diluted anti-human IgG FITC conjugate was added to the bead mixture, incubated at 37°C for 30 min, centrifuged, washed twice, and 1 ml fixing solution was added. The positive control non-specific binding assay included 200 μl of 1:100 diluted positive control, which was added to the blocked micro-beads, incubated at 37°C for 30 min, centrifuged, and washed twice. Two hundred μl of 1:100 dilution anti-mouse IgG FITC conjugate was added to the bead mixture, incubated at 37°C for 30 min, centrifuged, washed twice, and 1 ml fixing solution was added.

The anti-human IgG FITC conjugate non-specific binding assay included micro-beads coated with PSA, which were washed and blocked, incubated with 200 μl of 1:100 diluted anti-human IgG FITC conjugate at 37°C for 30 min. The micro-bead mixture was washed twice, and 1 ml fixing solution was added. Finally, the anti-mouse IgG FITC conjugate non-specific binding assay included micro-beads coated with PSA, which were washed and blocked, incubated with

200 μl of 1:100 dilution of anti-mouse IgG FITC conjugate at 37°C for 30 min. The micro-bead mixture was washed twice, and 1ml of fixing solution was added.

Table 3. Testing Non-specific Binding
The controls testing non-specific binding are listed below. The addition of each reagent is indicated by a plus symbol (+) and no reagent added is indicated by a negative symbol (-).

Control	Blocked micro-beads	Female serum 1:100	Mouse anti-PSA	Anti-human IgG-FITC	Anti-mouse IgG-FITC
1	+	+	-	+	-
2	+	-	+	-	+
3	+	-	-	+	-
4	+	-	-	-	+

Preliminary Test: Antigen/Antibody Binding

In the absence of micro-beads, anti-PSA was incubated with varying concentrations of PSA (ranging from undiluted to a 1:20,000 dilution) and the antigen/antibody complex was tested under experimental temperature conditions (room temperature and 37°C) for 18 hr. The anti-PSA was diluted 1:100 and 150 μl of that dilution was incubated with the varying PSA concentrations (1, 2, 5, 10, 15, 20, 25, 35, and 50 μg). The appropriate amount of PBS was added to each test to bring the final volume to 200 μl. Each antigen/antibody combination was incubated at room temperature and 37°C for 18 hr. Antigen/antibody binding was detected by flow cytometry using the secondary antibody as previously described.

Preliminary Test: Antigen/Antibody Acid Elution

The cold-acid elution technique was adapted from *Technical Manual*, American Association of Blood Banks (AABB) [82]. Glycine-HCl (0.1 M, pH 3.0)

and phosphate buffer (0.8 M, pH 8.2) were prepared according to the cold-acid elution protocol.

Anti-PSA and PSA were incubated at 37°C for 18 hr to obtain the optimal antigen/antibody complex. After incubation, 200 μl of the antigen/antibody complex was added to 200 μl of ice-cold glycine-HCl and mixed on ice for 1, 2, or 5 min. Phosphate buffer was added (20 μl) to restore neutrality. The antigen/antibody mixture was added to PSA-coated, blocked micro-beads and incubated at 37°C for 30 min. After incubation, the beads were centrifuged and washed twice. A 1:100 dilution of secondary antibody was added to the bead mixture and incubated at 37°C for 30 min. After incubation, the beads were centrifuged, washed twice, and 1 ml of fixing solution was added. The antigen/antibody complex was also tested as an untreated sample. Positive and negative controls were included as acid-treated or untreated.

Preliminary Test: Antigen/Antibody Heat Elution

Anti-PSA and PSA were incubated at 37°C for 18 hr to obtain the optimal antigen/antibody complex. The complex was heat eluted at 56°C for 15, 30, or 45 min or at 65°C for 15, 30, 45, 60, 90, or 120 min. After the heat elution, the antigen/antibody mixture was immediately added to PSA coated and blocked micro-beads for incubation at 37°C for 30 min. After incubation, the beads were centrifuged and washed twice. A 1:100 dilution of secondary antibody was added to the bead mixture and incubated at 37°C for 30 min. After incubation, the beads were centrifuged, washed twice, and fixing solution was added. The

antigen/antibody complex was also tested as an untreated sample, and positive and negative controls were tested as heat-treated samples as well as untreated samples.

Flow Cytometry

The BD FACSCalibur™ system (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for flow cytometric analysis. Two sets of parameters, forward scatter vs. side scatter and forward scatter vs. fluorescence were used to evaluate the samples. Median scores (fluorescence intensity) of the FITC intensity on a log scale were used as the raw data and were analyzed using the program Cellquest™ (Becton, Dickinson and Company). All results are reported as the median fluorescence.

DNA Isolation

Frozen buffy coat samples were isolated using the QIAmp® DNA Blood Mini Kit (QIAGEN Inc, Valencia, CA) according to the manufacturer's instructions. The GeneQuant RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd, Science Park, Cambridge, England) was used to measure the optical density of a 1:60 dilution of each sample. The DNA samples were stored in sterile water at concentrations between 0.025 and 0.200 μg/μl. A Cytokine Genotyping Tray (One Lambda, Inc) was used according to the manufacturer's instructions as well as the PCR amplification parameters. Ten μl of each PCR product was loaded into wells of a 2.5% agarose gel and was electrophoresed at 100 volts for 36 min

in 0.5X Tris borate buffer [0.445 M Trizma Base (Sigma-Aldrich), 0.444 M Boric Acid, 2% (v/v) 0.5 M EDTA]. The gels were stained in 95% ethidium bromide (Sigma-Aldrich) diluted 1:1,000 with double distilled water for 10 min and destained in double distilled water. DNA bands were visualized using the Chromato-Vue transilluminator model 75-36 (UVP Incorporated, San Gabriel, CA). The gels were photographed with type 667 black and white Polaroid® film (Polaroid Corporation, Cambridge, MA). The resulting photographs (Figure 3) were manually analyzed based on the presence or absence of allele specific bands and the presence of an internal control band (specific for the human β-globin gene). In accordance with the manufacturer's instructions, any visible band between the internal control band and the unincorporated primer band indicated a positive result.

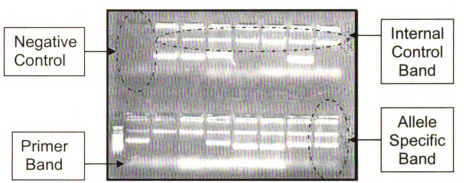


Figure 3. Photograph of Gel Electrophoresis
The One Lambda Cytokine Genotyping kit was used to determine allele specificity as shown above.

Results

Negative Control Study

The range of the negative control was determined using five human female sera (Table 4) following the negative control method described previously. Female 3 was repeated with 10 additional replicates to set the negative control range and was used as the negative control for all subsequent tests. After receiving a new lot of PSA, the chosen negative control was tested in 10 replicates to set the new negative control range. Two lots of PSA were used and the range for the negative control is depicted in Table 5.

Table 4. Negative Control Study.
The median fluorescence for three separate tests of five females is listed.

	Female 1	**Female 2**	**Female 3**	**Female 4**	**Female 5**	**MEAN**
Test 1	1.55	1.58	2.29	1.81	2.33	1.91
Test 2	1.00	1.00	1.21	1.35	1.00	1.11
Test 3	1.53	1.66	1.70	1.00	1.22	1.42
						1.48

Table 5. Negative Control Ranges
The mean for the two lots (1 and 2) of PSA used to test the negative control plus or minus (±) the standard deviations (SD) are listed.

	Mean ± SD PSA lot 1	**Mean ± SD PSA lot 2**
Female 3	1.96 ± 0.69	3.37 ± 1.5
Range ± 2 SD	1.0 - 3.34	1.0 - 6.37

Positive Control Serial Dilutions

The positive control serial dilutions (tested by the positive control method) were used to determine the optimal concentration for a statistically significant

($p<0.05$) control (Table 6). After analysis on the flow cytometer, the 1:100 dilution of the positive control was chosen. The 1:50 and 1:100 dilutions were compared and were determined to be not statistically different (two-tailed p-value = 0.9092). The 1:100 and 1:200 dilutions were also compared and were determined to be statistically different (two-tailed p-value = 0.0185). The 1:200 dilution compared to the 1:400 dilution was statistically different (two-tailed p-value = 0.0291), but the 1:400 and 1:800 were not statistically different (two-tailed p-value = 0.1326). The 1:100 dilution was used for all subsequent positive controls for its strength of reactivity.

Table 6. Positive Control Serial Dilutions
The median fluorescence for three separate tests of each positive control serial dilution is listed (ND = not detectable above background).

	1:50	**1:100**	**1:200**	**1:400**	**1:800**	**1:1,600**
Test 1	82.05	65.52	26.66	18.60	13.46	ND
Test 2	50.71	70.41	34.60	22.27	12.52	4.07
Test 3	106.5	96.47	39.95	12.63	3.37	1.57
MEAN	**79.75**	**77.47**	**37.74**	**17.83**	**9.78**	**2.82**

Testing Non-specific Binding

The results of the assays used to test non-specific binding are described in Table 7. After analysis on the flow cytometer and additional statistical analysis, the negative control non-specific binding assay and the positive control non-specific binding assay were determined negative as compared to the negative control (two-tailed p-value = 0.41 and 0.12 respectively). In addition, the anti-human IgG FITC conjugate non-specific binding assay and the anti-mouse IgG FITC conjugate non-specific binding assay were also determined negative (two-tailed p-value = 0.30 and 0.52 respectively).

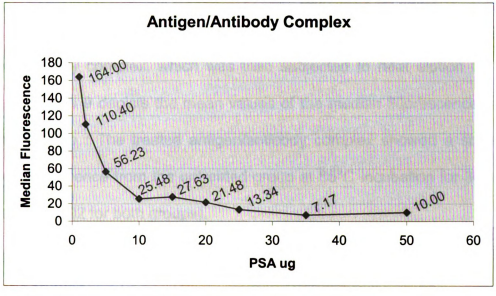
Table 7. Blocking of Non-specific Binding
The median fluorescence for four separate tests of each non-specific binding assay is listed.

	Test 1	**Test 2**	**Test 3**	**Test 4**	**MEAN**
Positive Control (mouse anti-human PSA)	26.18	49.14	61.53	67.93	**51.20**
Negative Control (Female serum)	2.25	3.85	2.46	1.63	**2.55**
Positive control non-specific binding assay	1.42	1.93	1.54	1.79	**1.67**
Negative control non-specific binding assay	1.83	2.94	1.76	1.67	**2.05**
Anti-mouse IgG FITC conjugate non specific binding assay	1.65	1.81	1.79	3.28	**2.13**
Anti-human IgG FITC conjugate non-specific binding assay	1.38	2.40	1.46	2.44	**1.92**

Antigen/Antibody Binding

After testing various combinations of the PSA concentration against the constant anti-PSA dilution (1:100), the most saturated antigen/antibody complex was achieved with 35 μg of PSA, incubated with 200 μl of 1:100 diluted anti-PSA, for 18 hours, at 37°C (Figure 4). There was no statistical significance between the room temperature incubations and the 37°C (data not shown; two-tailed p-value = 0.12).

Figure 4. Antigen/antibody Complex
The median fluorescence for 1, 2, 5, 10, 15, 20, 25, 35, and 50 μg of PSA incubated with 200 μl of diluted anti-PSA is shown.



Antigen/Antibody Acid Elution

Upon determining the appropriate concentrations of PSA and anti-PSA and the appropriate conditions for their maximum binding, the antigen/antibody complexes were subjected to acid in an attempt to elute them. Three replicates

of the acid elution test were performed. The flow cytometry results of the antigen/antibody elution are described in Table 8.

Table 8. Antigen/Antibody Acid Elution
The means of three replicates of the acid elution test on the antigen/antibody complex (A/A complex) are listed as the median fluorescence. Acid remained on the antigen/antibody complex for 1, 2, or 5 min before neutralization. The two-tailed p-values for the antigen/antibody complex are also shown.

	Untreated	1 min (p-value)	2 min (p-value)	5 min (p-value)
A/A Complex	6.58	6.27 (0.75)	7.17 (0.55)	5.98 (0.54)
Negative Control	3.71	3.38	3.22	3.53
Positive Control	210.66	109.98	54.57	35.84

Antigen/Antibody Heat Elution

Anti-PSA (1:100) and PSA (35 μg) were combined to form the antigen/antibody complex, which was then subjected to heat elution testing in triplicate. Table 9 depicts the mean values of the median fluorescence of each treatment group. The treated antigen/antibody complex showed a statistically significant difference from the untreated group at 65°C incubation for 30 and 45 minutes ($p = 0.02$ for both groups).

Patient Samples

All patient samples were tested for anti-PSA in triplicate using the micro-bead method and all were considered negative for anti-PSA as detected by flow cytometry (Table 10). Table 11 displays the cytokine production profile determined by PCR-SSP. Because of the varying levels (low, medium, and high) within the cytokine groupings, the presence of an up regulated pro- or anti-

inflammatory response was not determined, although, IL-6 production was high in 90% of patients.

Table 9. Antigen/Antibody Heat Elution

The means of three replicates of the heat elution test on the antigen/antibody complex are listed as the median fluorescence. The SD of the untreated antigen/antibody complex was 1.59 and the range ± 2 SD was 3.2 to 9.56.

	56°C			
	Untreated	**15**	**30**	**45**
Antigen/Antibody Complex	6.38	8.12	9.03	8.52
Negative Control	2.16	5.13	6.06	5.70
Positive Control	72.45	101.33	55.34	87.57

	65°C					
	15	**30**	**45**	**60**	**90**	**120**
Antigen/Antibody Complex	9.96	10.01	11.34	7.10	6.86	5.44
Negative Control	15.11	14.25	15.27	16.50	13.01	13.48
Positive Control	71.05	84.29	74.99	68.32	72.57	59.08

Table 10. Patient Results: Flow Cytometry
The patient status (hormone refractory or responsive) is listed along with the median fluorescence. All patient samples were determined negative for anti-PSA.

Patient status	Median Fluorescence
1. Refractory	1.33
2. Refractory	3.48
3. Refractory	2.04
4. Refractory	2.86
5. Responsive	3.59
6. Responsive	1.30
7. Responsive	1.87
8. Responsive	2.53
9. Responsive	2.21
10. Responsive	4.65

Table 11. Patient Results: Cytokine Production Profile
The patient status (hormone refractory or responsive) is listed along with the cytokine production profile (Low, High, or Intermediate).

	Anti-inflammatory			Pro-inflammatory	
Patient status	**TGF-β1**	**IL-10**	**IL-6**	**IFN-γ**	**TNF-α**
1. Refractory	Inter	Inter	Low	Low	Inter
2. Refractory	High	High	High	Inter	High
3. Refractory	High	High	High	Inter	High
4. Refractory	High	Inter	High	Low	High
5. Responsive	High	High	High	Inter	High
6. Responsive	High	High	High	High	Low
7. Responsive	High	Inter	High	Low	Low
8. Responsive	Inter	Inter	High	Inter	Low
9. Responsive	High	Inter	High	Low	Low
10. Responsive	High	Low	High	Inter	Low

Discussion

A clinical model for predicting PC prognosis before commencing hormone therapy was the goal of this research. This clinical model was to be founded on the analysis of anti-PSA, tested by flow cytometry using micro-bead technology, and the analysis of the genetic predisposition for increased production of IL-6.

Negative Control

To define the range of the negative control, five human female sera were tested and one female was chosen for all subsequent testing (Table 4). The negative control tested with PSA lot 2 ranged from 1.0 to 6.37 (Table 5) and this range included that of PSA lot 1. The negative control used in the flow cytometry studies provided reliable and reproducible results.

Positive Control

The positive control was able to successfully bind to the PSA-coated micro-beads. Furthermore, the positive control serial dilutions effectively determined the optimal concentration (1:100 dilution) for a statistically significant control (Table 6). Accepted positive control results were within 2 standard deviations (33.28) of the mean (77.47). However, the use of a human positive control would greatly enhance the value of this testing protocol for anti-PSA.

Blocking of Non-specific Binding

The non-specific binding potential of the positive control, the anti-mouse IgG FITC conjugate, the negative control, and the anti-human IgG FITC conjugate (Table 7) was tested. Incubating uncoated, blocked beads with the positive and negative controls, and the appropriate FITC conjugate, showed that the controls would not bind without the presence of an antigen (median fluorescence = 1.67 and 2.05, respectively). Furthermore, incubating the anti-human and anti-mouse FITC conjugates with coated and blocked beads, showed that the FITC conjugates could not bind without the presence of a primary antibody (median fluorescence = 2.13 and 1.92 respectively). Blocking of non-specific binding was successful and each component (positive control, negative control, anti-human FITC, and anti-mouse FITC) was specific to its counterpart.

Antigen/Antibody Binding

The antigen/antibody binding study was conducted after careful consideration of the PSA concentrations circulating in PC patients. It was proposed that high levels of circulating PSA, common in PC patients, could bind circulating anti-PSA and thus block capture of the antibody by the micro-beads. The antigen/antibody complex binding test was developed to simulate the possible *in vivo* conditions of the PC patient. After testing various concentrations of the PSA against a constant anti-PSA dilution a nearly saturated antigen/antibody complex was effectively obtained which nearly blocked all

binding of anti-PSA to the PSA-coated micro-beads (Figure 4). For all elution testing, 35 μg of PSA was incubated with 200 μl of 1:100 diluted anti-PSA at 37°C for 18 hr. These concentrations of the antigen and antibody produced a median fluorescence of 6.58. The antigen/antibody complex was > 2 SD from the negative control, yet the results were reproducible.

Antigen/Antibody Acid Elution

The acid elution of the antigen/antibody complex was conducted in order to test the hypothesis that the PSA and anti-PSA were bound *in vivo* and that anti-PSA could be detected once the complex was broken. The antigen/antibody complex was successfully formed, was subjected to acid treatment, and was neutralized before reintroducing the mixture to the PSA-coated micro-beads. The three replicates of the acid elution test showed no successful release of the antibody from the antigen (Table 8). Although the acid treatment had no effect on the negative control, the positive control lost considerable reactivity between the treatment groups (210.66 untreated to 35.84 with the 5 min treatment).

Antigen/Antibody Heat Elution

Further elution testing was required to determine if anti-PSA and PSA were bound *in vivo*. The heat elution test was employed (Table 9) and was somewhat successful. The antigen/antibody complexes treated at 65°C incubation for 30 and 45 minutes showed a statistically significant difference from the untreated group. However, the antigen/antibody complex treated at 56°C

was not successfully eluted. An upward shift was observed in the negative control after the 56°C incubation and an even greater shift was observed after the 65°C incubation, which remained constant for all negative controls in the heat elution testing. It is possible that heat elution could have some effect, although not likely significant.

Patient Samples

Although the micro-bead method was not able to detect anti-PSA in any of the patient samples tested (Table 10), it is possible that the antibody was present and the assay could prove to be successful if a larger population were studied. Furthermore, ELISA should be used in parallel with the micro-bead method as a known method to detect anti-PSA.

Cytokine production profiles were not determined in this study because of the varying levels within each grouping. However, 9 of the 10 patients had a genetic predisposition to produce high levels of IL-6 (Table 11), which may suggest that a humoral response to the tumor could have been initiated. Because IL-6 is produced at high levels in approximately 50% of PC patients, this study had great potential to link the humoral response with cytokine production and their effects on prognosis for HRPC patients. With greater sample numbers and a fully functional anti-PSA detection system by flow cytometry, these methods could be beneficial to predicting the outcome of PC patients.

Obstacles

Patient samples were very difficult to acquire. Most sources from outside the Michigan State University campus were unwilling to cooperate despite numerous efforts. A brief seminar, which included a pamphlet explaining the research project goals, was presented to area physicians to inform them of this study and to stimulate interest in this project. Mailers, which included an explanatory pamphlet, patient consent forms, blood collection tubes, and postage, were delivered to many area physicians and even though interest was expressed, few samples were returned. In addition, one area hospital seemed willing to provide serum samples with high PSA concentrations to be used for the elution studies and micro-bead testing. Unfortunately, those samples were never produced. Finally, one journal article author who was able to show antibody detection in PC patients was contacted. Again, interest was expressed, but the samples were not sent because of a conflict with the Institutional Review Board and the terms of the signed patient consent forms. With more time and more resources, additional patient samples could have been obtained for analysis in this study.

Conclusions

A flow cytometry assay using micro-beads to test for anti-PSA was successfully developed and this reproducible assay could be used to test PC patients. If a large number of patient samples were available for testing using the micro-bead method, it is possible that antibodies to PSA could be detected. The genetic predisposition encoding for high IL-6 production has been reported to occur in approximately 50% of PC patients, yet 90% of the patients in this study carried this genetic trait. Unfortunately, time constraints and few patient samples have limited further testing and correlation studies of IL-6 phenotype and anti-PSA to disease outcome.

BIBLIOGRAPHY

1. Garnick MB and Fair WR, *Combating Prostate Cancer.* Science America, 1998. **279**: p. 74-83.

2. American Cancer Society, *American Cancer Society, Inc. Cancer Facts & Figures 2002.* 2002. p. 3-15.

3. Barry MJ, *Prostate-Specific-Antigen Testing for Early Diagnosis of Prostate Cancer.* New England Journal of Medicine, 2001. **344**: p. 1373-1377.

4. Zincke H, Bergstralh EJ, Blute ML, Myers RP, Barrett DM, Lieber MM, Martin SK, Oesterling JE, *Radical prostatectomy for clinically localized prostate cancer: long-term results of 1,143 patients from a single institution.* Journal of Clinical Oncology, 1994. **12**(11): p. 2254-2263.

5. Zincke H, Oesterling JE, Blute ML, Bergstralh EJ, Myers RP, Barrett DM, *Long-term (15 years) results after radical prostatectomy for clinically localized (stage T2c or lower) prostate cancer.* Journal of Urology, 1994. **152**: p. 1850-1857.

6. Oh WK, *Chemotherapy for patients with advanced prostate carcinoma.* Cancer, 2000. **88**(Suppl 12): p. 3015-3021.

7. Knox JJ and Moore MJ, *Treatment of Hormone Refractory Prostate Cancer.* Seminars in Urologic Oncology, 2001. **19**(3): p. 202-211.

8. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC, *Elevated levels of circulating interleukin-6 and transforming growth factor-ß1 in patients with metastatic prostatic carcinoma.* Journal of Urology, 1999. **161**: p. 182-187.

9. Hoosein NM, Abdul M, McCabe R, Gero E, Deftos L, Banks M, Hodges S, Finn L, Logothetis CJ, C, *Clinical Significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostate cancer.* Urologic Oncology, 1995. **1**: p. 246-251.

10. Twillie DA, Eisenberger MA, Carducci MA, Hseih WS, Kim WY, Simons JW, *Interleukin-6; a Candidate Mediator of Human Prostate Cancer Morbidity.* Urology, 1995. **45**(3): p. 542-549.

11. McNeel, DG, Nguyen, LD, Storer, BE, Vessella, R, Lange, PH, Disis, ML, *Antibody Immunity to Prostate Cancer Associated Antigens can be Detected in the Serum of Patients with Prostate Cancer.* Journal of Urology, 2000. **164**: p. 1825-1829.

12. Chu TM, Kuriyama M, Johnson E, Papsidero LD, Killian CS, Murphy GP, Wang MC, *Circulating Antibody to Prostate Antigen in Patients with Prostatic Cancer.* Annals New York Academy of Sciences, 1983. **417**: p. 383-389.

13. Zisman A, Zisman E, Lindner A, Velikanov S, Siegel YI, Mozes E, *Autoantibodies to prostate specific antigen in patients with benign prostatic hyperplasia.* Journal of Urology, 1995. **154**(1052-5).

14. Stowell LI, Sharman LE, Hamel K, *An enzyme-linked immunosorbent assay (ELISA) for prostate-specific antigen.* Forensic Science International, 1991. **50**(1): p. 125-38.

15. Vignali DAA, *Multiplexed Particle-base Flow Cytometric Assays.* Journal Immunological Methods, 2000. **243**: p. 243-255.

16. McHugh TM, Viele MK, Chase ES, Recktenwald DJ, *The sensitive detection and quantitation of antibody to HVC by using a microsphere-based immunoassay and flow cytometry.* Cytometry, 1997. **29**(2): p. 106-12.

17. Chen R, Lowe L, Wilson JD, Crowther E, Tzeggai K, Bishop JE, Varro R, *Simultaneous Quantification of Six Human Cytokines in a Single Sample Using Microparticle-based Flow Cytometric Technology.* Clinical Chemistry, 1999. **45**(9): p. 1693-1694.

18. Carson RT and Vignali DAA, *Simultaneous quantitation of fifteen cytokines using a multiplexed flow cytometric assay.* Journal of Immunological Methods., 1999. **227**(1-2): p. 41-52.

19. Best LM, Veldhuyzen van Zanten SJ, Bezanson GS, Haldane DJ, Malatjalian DA, *Serological detection of Helicobacter pylori by a flow microsphere immunofluorescence assay.* Journal of Clinical Microbiology, 1992. **30**(9): p. 2311-7.

20. Syrjala MT, Tolo H, Koistinen J, Krusius T, *Determination of anti-IgA antibodies with a flow cytometer-based microbead immunoassay (MIA).* Journal of Immunological Methods, 1991. **139**(2): p. 265-70.

21. Wilson MR and Wotherspoon JS, *A new microsphere-based immunofluorescence assay using flow cytometry.* Journal of Immunological Methods, 1988. **107**(2): p. 225-30.

22. Drouvalakis KA, Neeson PJ, Buchanan RR, *Detection of anti-phosphatidylethanolamine antibodies using flow cytometry.* Cytometry, 1999. **36**(1): p. 46-51.

23. American Cancer Society, *American Cancer Society, Inc. Cancer Prevention & Early Detection Facts & Figures 2002.* 2002. p. 1-28.

24. Ross KS, Carter HB, Pearson JD, Guess HA, *Comparative Efficiency of Prostate-Specific Antigen Screening Strategies for Prostate Cancer Detection.* The Journal of the American Medical Association, 2000. **284**(11): p. 1399-1405.

25. Brawer MK, *Prostate-Specific Antigen.* Seminars in Surgical Oncology, 2000. **18**(1): p. 3-9.

26. Belanger A, van Halbeek H, Graves HC, Grandbois K, Stamey TA, Huang L, Poppe I, Labrie F, *Molecular mass and carbohydrate structure of prostate specific antigen: studies for establishment of an international PSA standard.* Prostate, 1995. **27**(4): p. 187-197.

27. Yousef GM and Diamandis EP, *The New Human Tissue Kallikrein Gene Family: Structure, Function, and Association to Disease.* Endocrine Reviews, 2001. **22**(2): p. 184-204.

28. Webber MM, Waghray A, Bello D, *Prostate-specific antigen, a serine protease, facilitates human prostate cancer cell invasion.* Clinical Cancer Research, 1995. **1**(10): p. 1089-94.

29. Murphy GP, Barren RJ, Erickson SJ, Bowes VA, Wolfert RL, Bartsch G, Klocker H, Pointner J, Reissigl A, McLeod DG, Douglas T, Morgan T, Kenny GM, Ragde H, Boynton AL, Holmes EH, *Evaluation and comparison of two new prostate carcinoma markers: Free-prostate specific antigen and prostate specific membrane antigen.* Cancer, 1996. **78**: p. 809-818.

30. Ito K, Yamamoto T, Ohi M, Takechi H, Kurokawa K, Suzuki K, Yamanaka H, *Long term follow-up of mass screening for prostate carcinoma in men with initial prostate specific Antigen levels of 4.0 ng/mL or less.* Cancer, 2001. **91**(4): p. 744-751.

31. Maattanen L, Auvinen A, Stenman UH, Tammela T, Rannikko S, Aro J, Juusela H, Hakama M, *Three-year results of the Finnish prostate cancer screening trial.* Journal of National Cancer Institute, 2001. **93**(7): p. 552-3.

32. Benson MC, Whang IS, Pantuck A, Ring K, Kaplan SA, Olsson CA, Cooner WH, *Prostate Specific Antigen Density: A means of Distinguishing*

Benign Prostatic Hypertrophy and Prostate Cancer. Journal of Urology, 1992. **147**: p. 815-6.

33. Bazinet M, Meshref AW, Trudel C, Aronson S, Peloquin F, Nachabe M, Begin LR, Elhilali MM, *Prospective evaluation of prostate specific antigen density and systematic biopsies for early detection of prostatic carcinoma.* Urology, 1994. **43**: p. 44-51.

34. Smith DS and Catalona WJ, *Rate of change in serum prostate specific antigen levels as a method for prostate cancer detection.* Journal of Urology, 1994. **152**: p. 1163–1167.

35. American Cancer Society, *American Cancer Society, Inc. Prostate Cancer Treatment Guidelines for Patients 2001*. 2001. p. 2-9.

36. Prestigiacomo AF and Stamey TA, *Physiological variation of prostate specific antigen in the 4.0 to 10.0 ng/mL range in male volunteers.* Journal of Urology, 1996. **155**: p. 1977-1980.

37. Nixon RG, Wener MH, Smith KM, Parson RE, Strobel SA, Brawer MK, *Biological variation of prostate specific antigen levels in serum: an evaluation of day-to-day physiological fluctuations in a well-defined cohort of 24 patients.* Journal of Urology, 1997. **157**(6): p. 2183-2190.

38. Jacobsen SJ, Bergstralh EJ, Katusic SK, Guess HA, Darby CH, Silverstein MD, Oesterling JE, Lieber MM, *Screening digital rectal examination and prostate cancer mortality: a population-based case-control study.* Urology, 1998. **52**(2): p. 173-179.

39. Cohen SP and Jaskulsky SR, *Prostate cancer Tx: Therapeutic options based on tumor grade, life expectancy, and patient preferences.* Geriatrics, 2001. **56**(2): p. 39-52.

40. Gleason DF, *Histologic grading and clinical staging of prostate carcinoma*. 1977, In: Tannenbaum M, ed. Urologic Pathology: the Prostate. Philadelphia, PA: Lea and Febiger: p. 171-198.

41. Gleason DF and Mellinger GT, *Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging.* Journal of Urology, 1974. **111**(1): p. 58-64.

42. Beahrs OH, Hensen DE, Hutter RVP, Kennedy BJ, *Prostate*. 1997, In: American Joint Committee on Cancer: AJCC Cancer Staging Manual. Philadelphia, PA: Lippincott-Raven Publishers, 5th ed.: p. 219-224.

43. Chodak GW, Thisted RA, Gerber GS, Johansson JE, Adolfsson J, Jones GW, Chisholm GD, Moskovitz B, Livne PM, Warner J, *Results of conservative management of clinically localized prostate cancer.* New England Journal of Medicine, 1994. **330**(4): p. 242-8.

44. Fournier GR Jr, Narayan P, *Re-evaluation of the need for pelvic lymphadenectomy in low grade prostate cancer.* British Journal of Urology, 1993. **72**(4): p. 484-488.

45. Pirtskhalaishvili G, Hrebinko, RL, Nelson JB, *The Treatment of Prostate Cancer An Overview of Current Options.* Cancer Practice, 2001. **9**(6): p. 295-306.

46. Feldman BJ and Feldman D, *The Development of Androgen-Independent Prostate Cancer.* Nature Reviews Cancer, 2001. **1**(1): p. 34-45.

47. Isaacs JT, *Role of androgens in prostatic cancer.* Vitamines and Hormones, 1994. **49**: p. 433-502.

48. Kish JA, Bukkapatnam R, Palazzo F, *The Treatment Challenge of Hormone-Refractory Prostate Cancer.* Cancer Control, 2001. **8**(6): p. 487-495.

49. Pienta KJ, Redman B, Hussain M, Cummings G, Esper PS, Appel C, Flaherty LE, *Phase II Evaluation of Oral Estramustine and Oral Etoposide in Hormone-Refractory Adenocarcinoma of the Prostate.* Journal of Clinical Oncology, 1994. **12**(10): p. 2005-2012.

50. Freedland SJ, Pantuck AJ, Weider JS, Zisman AS, Belldegrun, AS, *Immunotherapy of prostate cancer.* Current Urology Reports, 2001. **2**(3): p. 242-7.

51. Smyth MJ, Godfrey DI, Trapani JA, *A fresh look at tumor immunosurveillance and immunotherapy.* Nature Immunology, 2001. **2**(4): p. 293-299.

52. Markiewicz MA, Gajewski TF, *The immune system as anti-tumor sentinel: molecular requirements for an anti-tumor immune response.* Critical Review of Oncology, 1999. **10**(3): p. 247-60.

53. Kawakami Y and Rosenberg SA, *Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy.* International Review of Immunology, 1997. **14**(2-3): p. 173-92.

54. Klarnet JP, Matis LA, Kern DE, Mizuno MT, Peace DJ, Thompson JA, Greenberg PD, Cheever MA, *Antigen-driven T cell clones can proliferate in vivo, eradicate disseminated leukemia, and provide specific immunologic memory.* Journal of Immunology, 1987. **138**(11): p. 4012-7.

55. Baxevanis CN, Voutsas IF, Tsitsilonis OE, Gritzapis AD, Sotiriadou R, Papamichail M, *Tumor-specific CD4+ T lymphocytes from cancer patients are required for optimal induction of cytotoxic T cells against the autologous tumor.* Journal of Immunology, 2000. **164**(7): p. 3902-3912.

56. Keene JA and Forman J, *Helper activity is required for the in vivo generation of cytotoxic T lymphocytes.* Journal of Experimental Medicine, 1982. **155**(3): p. 768-82.

57. Janeway CA, Traver P, Walport M, Capra JD, *Immunobiology The Immune System in Health and Disease.* 1999, New York, NY: Garland Publishing: p. 8-20, 272-273, 392-393, 636-637, 641.

58. Pardoll DM, *Cancer vaccines.* Nature Medicine, 1998. **4**: p. 525-531.

59. McCarron SL, Edwards S, Evans PR, Gibbs R, Dearnaley DP, Dowe A, Southgate C, Easton DF, Eeles RA, Howell WM, *Influence of cytokine gene polymorphisms on the development of prostate cancer.* Cancer Research, 2002. **62**(12): p. 3369-72.

60. Smith PC, Hobisch A, Lin DL, Culig Z, Keller ET, *Interleukin-6 and Prostate Cancer Progression.* Cytokine and Growth Factor Reviews, 2001. **12**: p. 33-40.

61. Lee SO, Lou W, Hou M, de Miguel F, Gerber L, Gao AC, *Interleukin-6 Promotes Androgen-independent Growth in LNCaP Human Prostate Cancer Cells.* Clinical Cancer Research, 2003. **9**: p. 370-376.

62. Eder JP, Kantoff PW, Roper K, Xu GX, Bubley GJ, Boyden J, Gritz L, Mazzara G, Oh WK, Arlen P, Tsang KY, Panicali D, Schlom J, Kufe DW, *A Phase I Trial of a Recombinant Vaccinia Virus Expressing Prostate-specific Antigen in Advanced Prostate Cancer.* Clinical Cancer Research, 2000. **6**: p. 1632-1638.

63. Akira S, Taga T, Kishimoto T, *Interleukin-6 in biology and medicine.* Advances in Immunolology, 1993. **54**: p. 1-78.

64. Taga T and Kishimoto T, *Gp130 and the interleukin-6 family of cytokines.* Annual Review of Immunology, 1997. **15**: p. 797-819.

65. Chomarat P, Banchereau J, Davoust J, Palucka AK, *IL-6 switches the differentiation of monocytes from dendritic cells to macrophages.* Nature Immunology, 2000. **1**(6): p. 510-514.

66. May LT, Ghrayeb J, Santhanam U, Tatter SB, Sthoeger Z, Helfgott DC, Chiorazzi N, Grieninger G, Sehgal PB, *Synthesis and Secretion of Multiple Forms of ß2-Interferon/B cell Differentiation Factor 2/Hepatocyte-stimulating Factor by Human Fibroblasts and Monocytes.* Journal of Biological Chemistry, 1988. **263**: p. 7760-7766.

67. Mosmann TR and Coffman RL, *Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annual Review of Immunology, 1989. **7**: p. 145-173.

68. Drachenberg DE, Elgamal AA, Rowbotham R, Peterson M, Murphy GP, *Circulating Levels of Interleukin-6 in Patients With Hormone Refractory Prostate Cancer.* The Prostate, 1999. **41**(2): p. 127-133.

69. Bienvenu J, Monneret G, Fabien N, Revillard JP, *The Clinical Usefulness of the Measurment of Cytokines.* Clinical Chemistry Laboratory Medicine, 2000. **38**(4): p. 267-285.

70. Kurzrock R, *The Role of Cytokines in Cancer-Related Fatigue.* Cancer, 2001. **92**(Suppl 6): p. 1684-8.

71. Van Snick J, *Interleukin-6: an overview.* Annual Review of Immunology, 1990. **8**: p. 253-257.

72. Heinrich PC, Castell JV, Andus T, *Interleukin-6 and the acute phase response.* Biochemical Journal, 1990. **265**: p. 621-636.

73. Hobisch A, Eder IE, Putz T, Horninger W, Bartsch G, Klocker H, Culig Z, *Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor.* Cancer Research, 1998. **58**(20): p. 4640-5.

74. Culig Z, Bartsch G, Hobisch A, *Interleukin-6 regulates androgen receptor activity and prostate cancer cell growth.* Molecular and Cellular Endocrinology, 2002. **197**(1-2): p. 231-8.

75. Chen, T., L.H. Wang, and W.L. Farrar, *Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells.* Cancer Research 2000. **60**(8): p. 2132-5.

76. Borsellino N, Belldegrun A, Bonavida B, *Endogenous interleukin 6 is a resistance factor for cis-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines.* Cancer Research, 1995. **55**(20): p. 4633-4639.

77. Morse HR, Olomolaiye OO, Wood NA, Keen LJ, Bidwell JL, *Induced Heteroduplex Genotyping of TNF-α, IL-1ß, IL-6 and IL-10 Polymorphisms Associated with Transcriptional Regulation. Cytokine, 1999. **11**(10)*: p. 789-795.

78. Olomolaiye OO, Wood NA, Bidwell JL, *Identification of a novel polymorphism in the human IL-6 promoter region.* Immunology, 1997. **92**(Suppl 1): p. 66.

79. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P, *The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis.* Journal of Clinical Investigation, 1998. **102**(7): p. 1369-76.

80. Brecher, M.E., ed. *Technical Manual.* 14 ed. 2002, American Association of Blood Banks: Bethseda, MD.