MOI UNIVERSITY

SCHOOL OF MEDICINE

"*Evaluating Engrailed-2 and Cytokines in urine with serum PSA as potential biomarkers in patients with prostatism at MTRH Eldoret, Kenya*"

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science (MSc) in the Department of Immunology of Moi University

Department of Immunology
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DECLARATION

This thesis has been prepared as partial fulfillment of requirements for award of Master of Science (MSc) in Immunology by Moi University School of Medicine.

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DEDICATION

This work is dedicated to God.

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ABSTRACT

***Background*:** Majority of adult male patients in the developing world present in clinics with advanced stage prostate cancer (CaP) leading to high mortality and a heavy cancer burden. PSA is currently used for CaP screening and is produced by both malignant and non-malignant cells. PSA levels do not provide information to distinguish among benign prostatic hyperplasia (BPH), prostatitis or malignancy. PSA is therefore prostate-specific, but not prostate cancer–specific. Engrailed-2 (EN-2) is an important protein in the development of the human embryo and a candidate oncogene in prostate cancer; its production is switched off at birth. It is a homeodomain-containing transcription factor, expressed in CaP cell lines. The influx of inflammatory cells, macrophages and lymphocytes, produces pro-inflammatory cytokines like IL-6 and TNF-α that insult normal prostatic tissue propagating the inflammatory process.

Objective: To evaluate EN-2 and Cytokines in urine with serum PSA as potential biomarkers in patients with prostatism at Moi Teaching and Referral Hospital Eldoret-Kenya.

Methods and procedures: This cross-sectional study involved 39 male adult patients, 13 cases-CaP and 26 controls-(24 with BPH & 2 with prostatitis), matched by age. These patients were recruited from the Moi Teaching and Referral Hospital (MTRH) urology clinic and male surgical ward between October and December 2013. A questionnaire was used to record demographic and clinical information about the patients. Digital rectal examination (DRE) for each patient was conducted by a urologist and results were obtained from the patients chart. Participants were asked to provide an early morning urine specimen in a sterile container. The concentrations of EN-2, IL-6 and TNF-α were determined from the urine deposit using ELISA. Serum PSA results were obtained from the Immunology laboratory. Pelvic/abdominal ultrasound reports were retrieved from the patient's file.

Results

The mean age of cases was 73.1years and controls 71.1 years. Over 92.3% of the cases and 73.1% of controls had PSA values >4.0ng/ml. Both cases and controls had elevated levels of EN-2 in their urine. The difference was not statistically significant. There was no correlation between urinary EN-2 and serum PSA. IL-6 and TNF-α expressions were higher in cases compared to controls (p-values 0.0001 and 0.004 respectively), and these differences were statistically significant.

Conclusion and Recommendations:

This study shows that EN-2 expression in urine was not a predictive marker as it was elevated in both cases and controls. IL-6 and TNF-α levels were elevated in urine of CaP patients. The potential for using these cytokines as urinary biomarkers in CaP should be further explored.

We recommend a prospective study recruiting a larger number of participants for evaluating EN-2 and cytokines in urine, using histology of prostate biopsy as a gold standard test.

Limitations: Most of the patients sought medical attention late and presented at the clinic when the disease had already progressed. Biopsies were not available for these patients

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBEVIATIONS

AR	Androgen Receptor
BPH	Benign Prostatic hyperplasia
BRCA1&2	Breast cancer genes 1&2
CaP	Prostate cancer
DRE	Digital Rectal Examination
ELISA	Enzyme linked immuno-sorbent Assay
EN-2	Engrailed-2
HPC1	Hereditary Prostate Cancer gene 1
IL-6	Interleukin 6
IREC	Institutional Research Ethics Committee
LUTs	Lower Urinary Tract symptoms.
MTRH	Moi Teaching and Referral Hospital
NIH	National Institute of Health
OPD	Out Patient Department.
PAX	Paired genes
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
SPSS	Statistical Package for Social science
TNF-α	Tumor Necrosis Factor alpha

CHAPTER ONE

1.0 INTRODUCTION.

1.1 Background;

Prostatism is a clinical syndrome, occurring mostly in older men, usually caused by enlargement of the prostate gland and manifested by irritative (nocturia, frequency, sensory urgency, and urgent incontinence) and obstructive (hesitancy, decreased stream, terminal dribbling, double voiding, and urinary retention) symptoms (Farlex, 2012). Prostate cancer (CaP) is a disease in which cells in the prostate gland become abnormal and start to grow uncontrollably, forming tumors (Beers *et al*. 2004). CaP is the most common non-cutaneous and the second leading cause of cancer related death in men worldwide, especially in the developed world (Jemal *et al.* 2006). Approximately 913,000 new cases of CaP were diagnosed worldwide in 2008 (Ferlay *et al.* 2010). It is predicted that the number of cases will almost double (1.7m) by 2030. In Kenya, the estimated numbers of new cases of CaP stands at 1007 per year.

The prostate gland is about the size of a walnut and lies just behind the urinary bladder. A tumor in the prostate gland interferes with proper control of the bladder and normal sexual function. Often, the first symptom of CaP is difficulty in urinating. CaP is associated with poor prognosis globally and specifically in Africa. However, the molecular mechanisms underlying the disease remain largely unknown. Several factors, including age, race, family history of prostate cancer, hormone levels (high serum androgen levels), high saturated fat intake, low intake of carotenoids, vasectomy and other sexual factors, are suspected to play a role in the development of prostate cancer.

No gene is directly responsible for the initiation and progress of prostate cancer. Mutations in *BRCA1* and *BRCA2*, important risk factors for ovarian cancer and breast cancer in women, have been implicated in prostate cancer (Struewing *et al*. 1997). Other linked genes include the Hereditary Prostate cancer gene 1 (HPC1), the androgen receptor, and the vitamin D receptor (Gallagher & Fleshner, 1998). According to Reynard J. *et al* (2006), 10% of prostate cancers are believed to be inherited. The risk of a man developing prostate cancer is doubled if there is one affected first-degree relative, and is 4-fold if there are two (Reynard J. *et al*. 2006)

African-American males compared to Caucasian males have a greater number of prostatic intraepithelial neoplasia (PIN) lesions, which are precursors to cancer, and larger tumors, possibly related to the higher levels of testosterone seen in African-American males (Fauci *et al.* 2008). This implies that high testosterone concentrations could be a reliable indicator of CaP in adult men.

Among males there is a one in six lifetime probability of being diagnosed with prostate cancer (Robbins and Cotran, 2010). It is one of the most remarkable tumors, exhibiting a wide range of clinical behaviors from very aggressive lethal cancers to incidentally discovered, clinically insignificant cancers. While localized or organ-confined CaP can be cured in a large proportion of patients by surgery or radiotherapy, prostatectomy may be a treatment option in metastatic CaP, although, a majority of men with metastatic CaP, refuse the option of prostatectomy. Advanced and metastatic CaP continues to be associated with a poor prognosis (Di Blasio *et al*. 2009).

Early diagnosis and treatment of CaP remains a challenge to physicians, as there are a number of key limitations with diagnosis based on prostate specific antigen (PSA), the only

marker to diagnose the disease to date. Prostatic carcinoma can be suspected when elevated levels of PSA are found in the blood. However, PSA levels may also be elevated in benign prostatic hyperplasia (BPH) and in prostatitis. Furthermore, an elevated PSA level does not confirm that a patient has prostate cancer. In most laboratories a serum level of 4 ng/mL is used as the cutoff distinguishing between normal and abnormal. However, according to Robbins and Cotran (2010), this simplified approach to serum PSA tests is not appropriate, and has led to the delay in diagnosis of many prostate cancers. Catalona *et al*. 1997, found that 22% of men with a normal DRE and a serum total PSA level between 2.6 and 4.0 ng/ml have CaP, and 81% of them have organ-confined disease. Data from the Prostate Cancer Prevention Trial (PCPT) revealed that as many as 15% of men with normal DRE and a serum total PSA less than 4.0 ng/ml have CaP (Thompson *et al*, 2004). Thus, the PSA test suffers from both low sensitivity and low specificity, causing physicians to rely heavily on the histological examination of prostate biopsy as a differential diagnosis for CaP (Robbins *et al.* 2010). Prostate biopsy is a special procedure carried out by urologists who are very few in number compared to the population. Most healthcare facilities can't afford to pay for their services. In addition prostate biopsy is an invasive procedure which is not comfortable to patients.

Engrailed-2 (EN-2) belongs to a family of homeodomain-containing transcription factors, which determine the early identity of cells and tissues (Shah, 2010). Recent studies have shown that EN-2 is secreted into urine by prostate cancer cells; its expression, therefore, may distinguish among prostatitis, BPH, and CaP.

1.2 The problem statement

The prostate specific antigen (PSA) test result in an adult male patient identifies levels of PSA detected in blood. An individual is considered not to have CaP if he has a PSA reading of less than or equal to 4ng/mL. Virtually all prostate epithelial cells, whether normal, hyperplastic or cancerous, synthesize PSA. PSA levels are not only high in malignant carcinoma, but also in BPH and prostatitis. Thus, PSA levels do not provide information to distinguish among benign prostate conditions and cancer (Thompson et al. 2004). American College of Physician have stated that "PSA values may fluctuate for no apparent reason; thus, an isolated abnormal value should be confirmed before proceeding with further testing", concluding that PSA is prostate-specific, not prostate cancer–specific, and elevated serum PSA may accompany prostatitis, BPH, or prostate cancer. Moreover, some aggressive CaPs do not produce PSA. In addition, the large number of unnecessary biopsies due to false-positive PSA results places a large burden on the healthcare system and lead to patient discomfort (Damber JE, 2008). PSA testing increases cancer detection but has not led to a decrease in mortality. However, because of the low specificity of PSA (40%) and the high prevalence of latent prostate cancer, opponents argue that many men would suffer unnecessary anxiety, biopsies, over-diagnosis (50%), and over-treatment when PSA is the only diagnostic test (Reynard J.et al. 2006). Therefore, determination of the PSA value is not sufficient to assess cancer. Hence there is need to investigate and validate a novel biomarker with a high specificity and sensitivity that will distinguish CaP, BPH and prostatitis.

1.3 RESEARCH QUESTION

Can a urinary biomarker, Engrailed-2 (EN-2), distinguish the different forms of prostatism and be more reliable than PSA?

1.4 Objectives.

1.4.1 Broad Objective

To evaluate EN-2 and Cytokines in urine with serum PSA as potential biomarkers in patients with prostatism at the urology clinic and male surgical ward of Moi Teaching and Referral Hospital Eldoret, Kenya.

1.4.3 Specific Objectives

1.) To determine the concentration of EN-2 in urine in individuals with prostatitis, BPH and CaP

2.) To determine the serum concentration of PSA in individuals with prostatitis, BPH and CaP

3.) To determine the concentration of pro-inflammatory cytokines, that is, IL-6 and TNF-α in the urine of patients with prostatism.

1.5 Justification

EN-2 is an antigen expressed by CaP cells that is secreted into urine. Results from this study may assist in distinguishing the different forms of prostatism. It is anticipated that physicians might be able to distinguish among prostatitis, BPH, and CaP using urinary EN-2 and cytokines as potential biomarker. The use of urine as the sample for analysis instead of blood or tissue biopsy is advantageous, as it is a noninvasive approach which will be of benefit to the patient and medical personnel because of its availability ease of collection,

storage, processing and low risk of infection to the health worker. The risk of harmful side effects such as bleeding and infection will be avoided.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tumor Biology:

The division, differentiation and growth of body cells are processes which are tightly controlled. Sometimes, the cells of a tissue will undergo more specialized cell division or differentiation. Such normal but not routine tissue changes include *hyperplasia*- more cells are produced than lost, *metaplasia*- a different cell type is produced, and *dysplasia*- undifferentiated or partially differentiated cells appear with fully differentiated cells. On rare occasions the cells of a tissue undergo division that is unusual and serves no useful function for the host. This abnormal proliferation creates an abnormal tissue mass called either a *neoplasm* ("new growth") or *tumo*r ('swelling'). All tumors can be classified as either benign or malignant (Tak W. *et al*. 2005). A benign tumor is relatively slow growing because it contains cells that are well-differentiated and well organized so that the tumor is very much like the normal tissue from which it originated. In contrast to benign tumors, malignant tumors are directly lethal to the host unless they are completely removed or killed. The malignant transformation of a cell is due to a multi-step process called carcinogenesis that culminates in the deregulation of cellular growth. The four steps of carcinogenesis necessary to establish a primary tumor include: initiation, promotion, progression and malignant conversion. In the initiation step, the DNA in the nucleus of a cell experiences either a strand break or a nucleotide alteration. In some cases, however, the alteration or break introduces an error that is not repaired and alters the sequence of a protein involved in growth regulation. Such a cell is said to be the target cell of a tumor. Promotion involves the exposure of the initial target cell to a stimulus that allows the selective proliferation of this cell. This cell goes through pre-neoplastic stages and

eventually become a neoplastic clone with a significant growth advantage over normal cells.

2.2 Benign Prostatic Hyperplasia (BPH)

Benign prostatic hyperplasia also known as nodular hyperplasia is an extremely common disorder in men over age 50 (Umtergasser G. et al: 2005). It is characterized by hyperplasia of prostatic stromal and epithelial cells, resulting in the formation of large, fairly discrete nodules in the peri-urethral region of the prostate. When sufficiently large, the nodules compress and narrow the urethral canal to cause partial, or sometimes virtually complete, obstruction of the urethra (Robbins and Cotran, 2010). It is believed that the main component of the "-hyperplastic-" process is impaired cell death. It has been proposed that there is an overall reduction of the rate of cell death, resulting in the accumulation of senescent cells in the prostate (Umtergasser G, *et al*: 2005). The main androgen in the prostate, constituting 90% of total prostatic androgens, is dihydrotestosterone [DHT] (Marks LS, *et al:* 2005). It is formed in the prostate from the conversion of testosterone by the enzyme type 2 5α-reductase. DHT binds to the nuclear androgen receptor (AR) present in both stromal and epithelial prostate cells. Binding of DHT to AR activates the transcription of androgen-dependent genes and induces the growth factors which act by increasing the proliferation of stromal cells and decreasing the death of epithelial cells (Robbins and Cotran, 2010). Fibroblast growth factor-7(FBG-7), produced by stromal cells, is probably the most important factor mediating the paracrine regulation of androgen-stimulated prostatic growth in BPH.

2.3 Immunopathogenesis of prostate cancer

Although the prostate was first described by Venetian anatomist Niccolò Massa in 1536, and illustrated by Flemish anatomist Andreas Vesalius in 1538, prostate cancer was not identified until 1853 (Adams J., 1853). The prostate is a part of the male reproductive system that helps make and store seminal fluid. In adult men, a typical prostate is about 3 centimeters long and weighs about 20 grams (Aumüller, G,. 1999). It is located in the pelvis, under the urinary bladder and in front of the rectum. The prostate is composed of branching tubuloalveolar glands arranged in lobules and surrounded by a stroma. The acinal unit includes an epithelial compartment made up of epithelial, basal, and neuroendocrine cells and a stromal compartment that includes fibroblasts and smooth-muscle cells. The compartments are separated by a basement membrane. The prostate surrounds part of the urethra, the tube that carries urine from the bladder during urination and semen during ejaculation (Moore, K & Dalley, A. 1999). Because of its location, prostate diseases often affect urination, ejaculation, and rarely defecation. Prostate cancer is classified as an adenocarcinoma, or glandular cancer, that begins when normal semen-secreting prostate gland cells mutate into cancer cells. Prostate cancer was initially considered a rare disease, probably because of shorter life expectancies and poorer detection methods in the 19th century. The region of prostate gland where the adenocarcinoma is most common is the peripheral zone. The tumor spreads locally through the poorly formed prostatic capsule (this is absent at the apex and base of the gland) into surrounding tissue, hence, may involve the urethral sphincter, corpora of the penis, seminal vesicles, and trigone of the bladder including the distal ureters (Reynard *et al*. 2006). This invasion of other organs is called metastasis. The most frequent sites of metastasis are

lymph nodes and bone, although the lungs, liver, testis, and brain are not uncommon. Bone metastases are characteristically sclerotic, rarely lytic (Michael F. *et al*. *2004*). The axial skeleton (spine and pelvis) are most commonly affected, followed by the proximal long bones, ribs, clavicles, and the skull (Reynard *et al*. 2006).

The prostate is a zinc accumulating, citrate producing organ. According to Pamela E. *et al.* (2003), the reason for this is not clear, but appears to help in fighting off infections. The protein ZIP1 is responsible for the active transport of zinc into prostate cells. One of zinc's important roles is to change the metabolism of the cell in order to produce citrate, an important component of semen (Leav I. *et al.* 2010). The process of zinc accumulation, alteration of metabolism, and citrate production is energy inefficient, and prostate cells sacrifice enormous amounts of energy (ATP) in order to accomplish this task. Prostate cancer cells are generally devoid of zinc. This allows prostate cancer cells to save energy not making citrate, and utilize the new abundance of energy to grow and spread (Leav I. *et al.* 2010). The absence of zinc is thought to occur via a silencing of the gene that produces the transporter protein ZIP1 (Giles G. G. *et al.* 2003). ZIP1 is now considered a tumor suppressor gene product for the gene SLC39A1. The cause of the epigenetic silencing is unknown ((Narizhneva NV *et al.* 2009). Zinc inhibits NF-κB pathways, is anti-proliferative, and induces apoptosis in abnormal cells. Unfortunately, oral ingestion of zinc is ineffective since a high concentration of zinc into prostate cells is not possible without the active transporter, ZIP1 (Michael F. *et al*. 2004). RUNX2 is a transcription factor that prevents cancer cells from undergoing apoptosis thereby contributing to the development of prostate cancer (Leav I *et al*. 2010). Loss of cancer suppressor genes, early in the prostatic carcinogenesis, has been localized to chromosomes 8p, 10q, 13q, and 16q (Cooper C, S. *et*

al. 2008). p53 mutations in the primary prostate cancer are relatively low and are more frequently seen in metastatic settings, hence, p53 mutations are late events in the pathology of prostate cancer (Cooper C, S. *et al.* 2008). Other tumor suppressor genes that are thought to play a role in prostate cancer include PTEN (gene) and KAI1 (Reynard J.*et al*., 2006). Up to 70 percent of men with prostate cancer have lost one copy of the PTEN gene at the time of diagnosis (Beuzeboc P, *et al.* 2009). Relative frequencies of loss of E-cadherin and CD44 have also been observed, together with activation of c-myc and bcl-2 proto-oncogenes (Reynard J.*et al*. 2006). The growth and survival of prostate cancer cells depend on androgens, which bind to the androgen receptor (AR) and induce the expression of pro-growth and pro-survival genes. It's been found that prostate specific membrane antigen (PSMA) stimulates the development of prostate cancer by increasing folate levels for the cancer cells to use to survive and grow. Men with germline mutations of the tumor suppressor *BRCA*2 have a 20-fold increased risk of CaP, but the vast majority of familial prostate cancers are due to variation in other loci that confer a small increase in cancer risk (Struewing JP. *et al.* 1997). Family and genome-wide association studies have identified a number of risk-associated loci, including one at 8q24 that appears to selectively increase the risk among African American men (Wiklund *et al*. 2003). A number of genes are involved in innate immunity, leading to speculations that inflammation may set the stage for the development of prostate carcinoma, as has been shown with respect to other human cancers.

2.4 Prostate carcinogenesis and inflammation

Prostatitis is an inflammation of the prostate gland (British Medical Association- 2010). Bacterial prostatitis usually results from bacteria that cause urinary tract infection such as

Escherichia coli, staphylococci, enterococci and other gram negative rods (Robbins and Cotran, 2010). The organisms become implanted in the prostate usually by intra-prostatic reflux of urine from the posterior urethra or from the urinary bladder. When urine stays for long in the bladder due to retention, it becomes a medium for bacteria growth. Prostatitis sometimes follows surgical manipulation of the urethra or prostate gland itself, such as catheterization, cystoscopy, urethral dilation, or resection procedures on the prostate. This is associated with painful and frequent urination, fever and sometimes discharges from the penis. Most malignancies have an association with either a specific infectious agent and/or a defined environmental exposure. Inflammation, regardless of etiology is thought to incite carcinogenesis by (i) causing cell and genome damage, (ii) promoting cellular replacement and creating a tissue microenvironment rich in cytokines and growth factors that can enhance cell replication, angiogenesis and tissue repair (Federico A, *et al.* 2007). Salman H. *et al* (2012) in their recent publication provide evidence that inflammation may play a significant role in the pathogenesis of prostate cancer via increased activity of inflammatory cytokines, particularly IL-6. Chronic insult to normal prostatic epithelial cells, due to infection, ischemia or a toxin (exogenous or endogenous), can result in the influx of inflammatory cells (lymphocytes and macrophages) and increased expression of carcinogen-detoxification enzyme such as glutathione S-transferase (GSTP 1). The primary mediators of the non-specific host immune defense system are free radicals, predominantly oxygen and nitrogen species. Hydroxyl radical (OH), peroxynitrite (ONOO--) and nitric oxide (NO) are the reactive oxygen species (ROS) and reactive nitrogen oxide (RNOS) species most commonly linked to the deleterious oxidative effects of inflammation (Espey *et al*. 2002). These reactive species can alter protein structure and function, cause lipid

peroxidation and induce somatic gene changes (Ernst *et al*. 2000). Free radicals have been shown to cause post-translational modifications of several key proteins, including those involved in DNA repair, apoptosis, cell signaling and essential enzymatic pathways. Experimental non-prostatic models of chronic inflammation have revealed that NO is able to cause structural changes to p53 that can affect its function (Hofseth *et al*. 2003). It is, therefore, expected that substances like free radicals will promote carcinogenesis. Lipid peroxidation creates the reactive aldehyde species malondialdehyde and can trigger the prostaglandin synthesis pathway via activating cyclooxygenase COX-2 (Romero *et al.* 1998). With the influx of inflammatory cells, i.e., lymphocytes and macrophages, into normal prostatic tissue during an insult, pro-inflammatory cytokines (IL-1, IL-6, TNF-α) are secreted to propagate the inflammatory process. Cytokines are small proteins important for the orchestration of inflammatory processes. The most-potent pro-inflammatory cytokines are tumor necrosis factor alpha (TNF) and interleukin 6 (IL-6) (Dinarello C.A., 1996). Mar Royuela (2008), studying pro-inflammatory cytokines in CaP, found that there may be a link between high expression of pro-inflammatory cytokines, IL-6 and TNF-α and high serum levels of PSA with the progression of the cancer. Several endogenous mechanisms that can modulate the production and/or activity of TNF and/or IL-6 have been identified. It is possible that a better understanding of the biological mechanism of such an association could lead to a therapeutic target in patients suffering from a prostate pathology.

2.5Immune response to prostate cancer

Cancer immunosurveillance is a theory formulated in 1957 by Burnet and Thomas, who proposed that lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells (Dunn G.P *et al.* 2002). Cancer immunosurveillance

appears to be an important host protection process that inhibits carcinogenesis and maintains regular cellular homeostasis. It has also been suggested that immunosurveillance primarily functions as a component of a more general process of cancer immunoediting (Dunn G.P *et al.* 2004). Immunoediting is a process by which a person is protected from cancer growth and the development of tumor immunogenicity by their immune system.

2.5.1 Innate Immune responses

Cells of the innate immune system recognize the presence of a growing tumor that has undergone stromal remodeling, causing local tissue damage. This is followed by the induction of inflammatory signals that are essential for recruiting cells of the innate immune system (e.g. natural killer cells, natural killer T cells, macrophages and dendritic cells) to the tumor site (Tak W. *et al* 2005). During this phase, the infiltrating lymphocytes such as the natural killer cells and natural killer T cells are stimulated to produce IFN-gamma. Newly synthesized IFN-gamma induces tumor death (to a limited amount) as well as promoting the production of chemokines CXCL10, CXCL9 and CXCL11 (Edward E. Max., 2005). These chemokines play an important role in promoting tumor death, by blocking the formation of new blood vessels. Tumor cell debris produced as a result of tumor death is then ingested by dendritic cells, followed by the migration of these dendritic cells to the draining lymph nodes. The recruitment of more immune cells also occurs and is mediated by the chemokines produced during the inflammatory process (Tak W. *et al.* 2005). Natural killer cells and macrophages trans-activate one another via the reciprocal production of IFN-gamma and IL-12. This again enhances the killing of tumor cells via apoptosis and the production of reactive oxygen and nitrogen intermediates.

2.5.2 Adaptive immune responses.

T cells, and especially CD8+ CTL, play a major role in tumor immunity (George P., 2002). Tumor antigens are processed in the cytosol, and their peptides presented in the context of MHC class I molecules. In the draining lymph nodes, tumor-specific dendritic cells trigger the differentiation of Th1 cells, which in turn facilitates the development of CD8+ T cells.

2.6 Biomarkers for prostate cancer

According to the National Institutes of Health (NIH) in the USA, a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmaceutical responses to a therapeutic intervention (Ilyin *et al*. 2004). Cancer biomarkers are either produced by the tumor or by the body in response to the tumor.

2.6.1 Prostate Specific Antigen (PSA)

Prostate specific antigen was identified by Ming Chu and his co-workers as a single chain 34-kDa protein containing 7% carbohydrate, a product of prostatic epithelium, that is produced by normal prostate cells and helps cleave and liquefy seminal fluid after ejaculation (Edward E. Max., 2001). It is produced by both malignant and non-malignant epithelial cells. PSA has been the most important test used in the diagnosis and management of CaP (Gretzer *et al.* 2003). The routine implementation of this test in clinics in the early 1990's has had a profound impact on the early diagnosis of prostate CaP and has resulted in an increase in reported CaP incidence (McDavid *et al.* 2004). However, the use of this marker is currently being debated, since it is not clear if PSA screening has led to a decline in mortality due to CaP (Lin et al. 2008). In addition, the large number of unnecessary biopsies due to false-positive PSA results, places a large burden on the healthcare system (Damber *et al.* 2008). Thus, this simplified approach to serum PSA tests

is not appropriate, and has led to the delay in diagnosis of many prostate cancers. Elevated blood levels of PSA occur in association with localized as well as advanced cancer. Neoplastic cells produce somewhat lower and varying tissue levels of PSA compared to benign epithelial cells, although both conditions cause total PSA elevation in the blood (Shariat, *et al*, 2004). In most laboratories a serum level of 4 ng/mL is used to distinguish between normal and abnormal. An elevated PSA level can reflect the presence of cancer, but can also be caused by BPH, infection and/or chronic inflammation. Virtually all prostate epithelial cells, whether normal, hyperplastic or cancerous, synthesize PSA. The performance of a prostate biopsy can also increase PSA levels up to tenfold for 8–10 weeks (Fauci *et al*. 2011). PSA levels are inherently variable, thereby affecting the interpretation of any single result (Ankerst *et al.* 2009). Variation in total PSA includes analytical (i.e. pre-analytical sample handling, laboratory processing, assay performance, and standardization) and biological variation (i.e. metabolism, renal elimination, medication, physical and sexual activity, size and integrity of the prostate). Oscillations up to 20 –30% in the total PSA range 0.1 – 20 ng/ml may be due to biologic variation (Soletormos *et al.*, 2005). Bunting et al., in 2002 reported a critical difference, defined as the minimum percent change between two consecutive measurements that suggest a significant change beyond the normal variation, close to 60% over a time period of one year. No single total PSA level separates men at high risk of CaP from men at low risk, nor men affected with high-grade disease from those with low-grade disease. At a total PSA level of 4 ng/mL, a significant number of CaPs remain undetected (Thompson *et al*. 2004). Nortably, intervention at lower total PSA levels has been proposed to improve patient outcomes (Berger *et al*. 2002).

Relaying on PSA as a diagnostic marker has lead to false positive results, false negative results over-diagnosis and over treatment. For every 1000 men tested, 87men who do not have prostate cancer will have a false positive PSA test that will lead to a biopsy (Howard , 2009). From this number, 28 men will experience a side effect from the biopsy that they consider to be a moderate/major problem that may require healthcare and 1 will experience hospitalization (Rosario, 2012). For every 1000 men tested, 25 men will choose to undergo treatment (surgery or radiation) because of uncertainty about which cancers need to be treated, many of whom would do well without treatment (Moyer *et al,* 2012). Of these 25 men, 7–10 will develop persistent impotence and/or urinary incontinence, and some will develop persistent bowel problems, due to treatment (Moyer *et al*, 2012).

2.6.2 Engrailed-2 (EN-2)

Developmental genes that encode transcription factors have an important role in the regulation of specific genes and are necessary for normal growth. In addition, aberrant expression and structural alteration of transcription factors are often primary molecular mechanisms in tumorigenesis (Rabbitts, 1994). *PAX* (paired) genes, a family of developmental control genes and transcription factors regulate tissue development and cellular differentiation in embryos by promoting cell proliferation, migration and survival (Wallin *et al.*, 1998; Buttiglieri *et al*., 2004; Gnarra and Dressler, 1995). Gibson *et al*., 2007 demonstrated that PAX2 expression is an essential requirement for prostate cancer cell survival. Furthermore, *PAX* genes have been shown to be capable of acting as proto-oncogenes by transactivating promoters of target genes involved in the regulation of cell growth and apoptosis (Stuart *et al*., 1995). Therefore, these transcription factors can function both as activators and repressors of transcription. It has been observed that *PAX*

and *Engrailed* genes are part of genetic networks that control the development of the brain and occupy a prominent position in the developmental regulatory hierarchy (Joyner, 1996). EN-2 is a homeodomain transcription factor involved in many aspects of multicellular development (Brunet *et al.*, 2005). First known for its role in arthropod embryological development, working in consort with the Hox genes, EN-2 has been found to be important in other areas of development. It has been identified in many bilaterians, including the vertebrates, echinoderms, molluscs, nematodes, brachiopods, and polychaetes. It acts as a selector gene, conferring a specific identity to defined areas of the body, and co-ordinating the expression of downstream genes (Hidaldo, 1994).

EN-2 is a homeodomain-containing transcription factor protein, expressed in CaP cell lines and secreted into urine by CaP cells. Homologous Engrailed proteins are found in a diversity of organisms. EN-2 is a transcriptional repressor, and also has a role in translational regulation (Morgan, 2006). In addition to its developmental role, EN-2 has recently been shown to be a potential oncogene in breast cancer, as EN-2 over-expression in non-malignant mammary cells induces a malignant phenotype, including increased cell proliferation and a loss of contact dependence (Martin *et al.*, 2005). Richard *et al.,* (2011), demonstrated that EN-2 is expressed in, and secreted by, CaP cells but not normal prostatic tissue; notably, there was no correlation with PSA. It has also been shown that urinary EN-2 is a highly specific and sensitive candidate biomarker of CaP (Richard *et al*., (2011). It has recently been demonstrated that EN-2 is over-expressed in human prostate cancer cells as compared to normal prostate epithelial cells and that down-regulation of EN-2 expression causes a dramatic decrease in CaP cell proliferation (Emma k. *et al*., 2012).

2.7 Diagnosis of prostate cancer

2.7.1 Digital rectal examination (DRE)

In a normal adult, the prostate gland weighs approximately 20 gm. In an adult, prostatic parenchyma can be divided into four biologically and anatomically distinct zones or regions: the peripheral, central, transitional zones, and the region of the anterior fibromuscular stroma (McNeal 1981). The periurethral portion of the gland increases in size during puberty and after the age of 55 due to the growth of non-malignant cells in the transition zone of the prostate that surrounds the urethra. Localized prostate cancer is asymptomatic, and is usually discovered by the detection of a suspicious nodule on rectal examination (Robbins and Cotran, 2010). Because the prostate gland lies in front of the rectum, the back wall of the prostate gland can be felt by putting a gloved, lubricated finger into the rectum and feeling the prostate by pressing on the anterior wall of the rectum. The rectal examination allows one to feel only the back part of the prostate. The DRE focuses on prostate size, consistency and abnormalities within or beyond the gland (Fauci. *et al.*, 2008). Most (75%) of adenocarcinomas occur in the peripheral zone of the prostate and most (85%) are multifocal, 20% appear to arise from the transition zones and 5% from the embryologically distinct central zone (Reynard J *et al*., 2006). Over 75% of prostate cancer patients have a palpable nodule that can be felt by a DRE (Pamela E. *et al.*, 2003). Carcinomas are characteristically hard, nodular, and irregular, while induration may be due to BPH or to calculi or tumor. Overall, 20–25% of men with an abnormal DRE have cancer (Fauci *et al*., 2008). Currently, no blood tests or radiographic studies are superior to the combination of DRE and PSA in the screening for prostate cancer (Pamela E. *et al.*, 2003)

2.7.2 Transrectal Ultrasound

According to Reynard J.*et al*., (2006), the most common diagnostic modality for prostate cancer is currently transrectal ultrasonography (TRUS) with guided biopsies. Ultrasound looks at tissue by sound waves. The ultrasound probe, which is a little larger than the thumb, is gently placed into the rectum. The probe emits sound waves, and the waves hit the prostate and are bounced off the prostate and the surrounding tissue. The waves then return on the ultrasound probe and a picture is developed on the screen (Pamela E. *et al*., 2003). Even though, the sound waves do not cause discomfort, introduction of the probe into the rectum may cause some discomfort. Transrectal ultrasonography can image the outline of the prostate, cysts, abscesses, and calcifications within the prostate (Reynard J. *et al*., 2006). CaP tends to cause less reflection of the sound waves, a trait referred to as hypoechoic, so the area often looks different in an ultrasound image than the normal prostate tissue (Pamela E. *et al*., 2003). The transrectal ultrasound allows the urologist to visualize location for the biopsies and collects about six to eight from different sections of the gland for histological examination. According to a study carried out by Carter *et al* (1989), transrectal ultrasound detected 13 of 25 unsuspected cancers for a sensitivity of 52% and for the 34 patients with no contralateral lobe lesion transrectal ultrasound was correct in 23 for a specificity of 68%, however, there was no significant difference in the pathological size of the clinically suspected and clinically unsuspected cancers as measured by average largest dimension.

2.7.3 Histological examination of the tissue

According to Epstein J.I. (1995), histological diagnosis of CaP from biopsy specimens is one of the most difficult challenges for pathologists. Generally, examination of biopsy specimens under the microscope is done to differentiate normal prostate cells from prostatitis, BPH or cancerous cells. Histologically, most lesions are adenocarcinomas that produce well-defined, readily demonstrable gland patterns (Eble J.N. *et al*., 2004). The glands are typically smaller than benign glands and are lined by a single uniform layer of cuboidal or low columnar epithelium. In contrast to benign glands, prostate cancer glands are more crowded, and characteristically lack branching and papillary in-folding (Epstein J.I., 2008). The outer basal cell layer typical of benign glands is absent. The cytoplasm of the tumor cells ranges from pale-clear as seen in benign glands, to a distinctive amphophilic appearance. Nuclei are large and often contain one or more large nucleoli. There is some variation in nuclear size and shape, but in general, pleomorphism is not marked (Robbins and Cotran, 2010).

The grading schema used for prostate cancer is the Gleason system (Gleason D.F. *et al.*, 1974). According to this system, prostate cancers are stratified into five grades on the basis of glandular patterns of differentiation. Grade 1 represents the well-differentiated tumors, in which the neoplastic glands are uniform and round in appearance and are packed into well-circumscribed nodules. By contrast, grade 5 tumors show no glandular differentiation, and the tumor cells infiltrate the stroma in the form of cords, sheets, and nests. The other grades fall in between. Most tumors contain more than one pattern, where one assigns a primary grade to the dominant pattern and a secondary grade to the second most frequent pattern.

The two numeric grades are then added to obtain a combined Gleason grade or score (Robbins and Cotran, 2010).

Staging of prostatic cancer is also important in the selection of the appropriate form of therapy (Matzkin H. *et al.,* 1994). Stage T1 refers to incidentally found cancer, either on transurethral resection of the prostate (TURP) done for BPH symptoms (T1a and T1b depending on the extent and grade), or on needle biopsy typically performed for elevated serum PSA levels (stage T1c) (Epstein J.I. et al., 1994). Stage T2 is organ-confined cancer. Stage T3a and T3b tumors show extra-prostatic extension, with and without seminal vesicle invasion, respectively. Stage T4 reflects direct invasion of contiguous organs. Any spread of tumor to the lymph nodes regardless of extent is eventually associated with a fatal outcome, such that the staging system merely records the presence or absence of this finding (N0/N1) (Matzkin H. *et al*., 1994).

M0, M1, M1a, M1b and M1c represents no metastases, distant metastases present, metastases to distant lymph node, bone metastases and other distant sites respectively (Robbins and Cotran, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out in Moi Teaching and Referral Hospital (MTRH), in the urology clinic and surgical ward. MTRH is situated in Eldoret (the 5th largest town in Kenya) municipality, Uasin Gishu County, North Rift region of Kenya. It is the second teaching and referral hospital in Kenya. It lies at 2300 metres above sea level, 310km from Nairobi, Kenya's capital city. Being the only referral hospital in Western Kenya, it has a catchment population of 13 to 15 million, about 40 percent of Kenya's population.

3.2 Study population

The study population comprised adult male patients, age 50 and above, who presented with lower urinary tract symptoms due to prostate disorders. Digital rectal exams and ultrasound features deemed to clinically suggest malignancy or histologically examined samples indicating prostate cancer constituted cases, while those that indicated BPH and normal prostatic tissue were considered as controls.

3.2.1 Inclusion criteria

All adult male patients of age 50 and above who presented with lower urinary tract symptoms, ascribable to the prostate, with or without an enlarged prostate gland and consented to participate, were recruited to the study.

3.2.2 Exclusion criteria

Adult male patients of age 50 and above who presented with lower urinary tract symptoms not due to obstructive prostate or with another conditions besides enlargement of the

prostate were not recruited into the study. Any other disease in addition to lower urinary tract could have altered the concentrations of the biomarkers.

3.2.3 Sampling

A sample size of 39, including controls, was arrived at as follows: according to information from the Cancer Registry and average of 14 CaP cases/year had been reported in MTRH. Considering that data collection for this study was going to run for close to a year, the investigator assumed that all cases presenting with CaP within this period were to be recruited into the study. It was presumed that 13 cases may present within the period, and for every case, two controls were allocated, that is, 13+ [2x13] =39. Applying a formula to calculate sample size will require reference to a related study in the same region of which none has been conducted in this region. A large sample size would require a longer duration for the study which was suppose to last for only one year.

3.3 Study design and procedure

This was a cross-sectional, descriptive study. All eligible patients that were seen in the clinic and ward within the period were recruited into the study. Those who gave consent were interviewed (using the questionnaire) to collect demographic data. Clinical data and results of diagnostic tests done were obtained from the patient files and entered onto data sheets. These data included:

1) DRE
2) Pelvic and or abdominal ultrasound results
3) Histological report (if prostate biopsy was collected)
4) PSA test results

Ultrasound signals were read by a specialist who interpreted the wave motion for each patient and made the diagnosis accordingly.

The study subjects were also asked to produce an early morning urine sample in a standard, sterilized urine container. The well-mixed urine sample was centrifuged at 3000 rpm for 15 minutes. The sediments were obtained and stored at -80^{0}C. After all the samples had been collected, EN-2, IL-6 and TNF-α levels were measured using the ELISA sandwich method using the urine sediment.

3.3.1 EN-2 Assay

This assay employed the quantitative sandwich enzyme immunoassay technique. EN-2 monoclonal antibody was purchased from *Cloud Clone Corp.*

Early morning urine was collected from all participants, centrifuged and sediment stored at -80º C. Samples were analyzed in duplicate, according to instructions for use.

Standard and samples (100µl) were added into the appropriate wells, covered with a plate sealer and incubated for 2 hrs at 37º C. After incubation, the liquid was removed and 100µl of detection reagent A added to each well. The wells were incubated at 37º C for 1 hour. After incubation the liquid was aspirated and each well washed three times using an auto-washer. After the last wash, excess wash buffer was removed by blotting the plate against an absorbent paper. 100µl of detection reagent B solution was added to each well and incubated for 30 minutes at 37º C. After incubation, the liquid was removed and the wash process repeated for a total of five times. 90 µl of substrate solution was added to each well for 25 minutes, in the dark. This was followed by the addition of 50 µl of stop solution. Absorbance was read using an ELISA reader immediately following the addition of the stop solution. The averages of the optical densities of the standards were plotted on the X-

axis against the corresponding concentrations on the Y-axis. A straight line was drawn through the meeting points on the graph and the concentrations of the sample extrapolated from the graph.

3.3.2 IL-6 Assay

This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody (R&D Systems) specific for human IL-6 was pre-coated on a microplate.

Early morning urine was collected from all participants, centrifuged and sediment kept at -80º C. Samples were analyzed in duplicate, according to the manufacturer's instructions.

100µl of assay diluent was added to each well. This was followed by the addition of 100µl of standards and samples in the appropriate wells. The plate was covered with adhesive tape and incubated for 2 hours at room temperature. After incubation the liquid was aspirated and each well washed four times using an auto-washer. After the last wash excess wash buffer was removed by blotting the plate against an absorbent paper. Two hundred (200) µl of Human IL-6 conjugate was added to each well and incubated for 2 hours at room temperature. After incubation the liquid was removed and the wash process repeated for a total of four times. Two hundred (200) µl of substrate solution was added to each well for 20 minutes at room temperature in the dark. This was followed by the addition of 50 µl of stop solution. Absorbance was read using an ELISA reader immediately following the addition of the stop solution. The averages of the optical densities of the standards were plotted on the Y-axis against the corresponding concentrations on the X-axis. A straight line was drawn through the meeting points on the graph and the concentrations of the sample extrapolated from the graph.

3.3.3 TNF-α Assay

This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody (R&D Systems) specific for Human TNF-α was pre-coated on a microplate.

Early morning urine was collected from all participants, centrifuged and sediment stored at -80º C. Samples were analyzed in duplicate, according to the instructions for use.

Fifty (50) µl of assay diluents was added to each well. This was followed by the addition of 200µl of standards and samples to the appropriate wells. The plate was covered with adhesive tape and incubated for 2 hours at room temperature. After incubation the liquid was aspirated and each well washed four times using an auto-washer. After the last wash, excess wash buffer was removed by blotting the plate against an absorbent paper. Two hundred (200) µl of Human TNF-α conjugate was added to each well and incubated for 1 hour at room temperature. After incubation the liquid was removed and the wash process repeated for a total of four times. Two hundred (200) µl of substrate solution was added to each well for 20 minutes at room temperature in the dark. This was followed by the addition of 50 µl of stop solution. Absorbance was read using an ELISA reader immediately following the addition of the stop solution. The averages of the optical densities of the standards were plotted on the Y-axis against the corresponding concentrations on the X-axis. A straight line was drawn through the meeting points on the graph and the concentrations of the sample extrapolated from the graph.

3.4 Data management

Data was coded and stored in Microsoft excel and analyzed using the statistical package for social science (SPSS) version 20. Results were presented on bar charts, graphs, tables and figures.

3.5 Ethical Considerations

The project proposal was reviewed by Moi University School of Medicine/Moi Teaching and Referral Hospital (MUSOM/MTRH) Institutional Research and Ethics Committee (IREC) and approval (*appendix)* obtained before the study commenced. Consent was sought from all participants before recruitment into the study. It would have been best to select controls from individual without any prostate anomaly; however, it will be unethical to subject individuals without any problem through a DRE and ultrasound, given that both have side effects. No risk was reported in the study subjects.

3.6 Study limitation

In the study, prostate biopsy was not collected from the subjects and hence, histology was not performed. Therefore, prostate cancer was diagnosed based on DRE and pelvic/ultrasound report. Biopsies were not available for this cohort of patients.

CHAPTER FOUR

4.0 RESULTS

The study was carried out during the months of September to December 2013, with a total of 39 patients participating. Data describing demographic and clinical characteristics of the patients were collected. Results of serum PSA was retrieved from the patient's medical record and laboratory registers. EN-2, IL-6 and TNF-α concentrations were measured in urine samples obtained from both cases and controls. These results are indicated in the section below.

4.1 DEMOGRAPHIC DATA

A total of 39 (13 cases and 26 controls) patients, matched by age were included in the study. The mean age for the cases was 73.6years with an age range of 61 to 94years, while the mean age for the controls was 71.1yrs with an age range 62 to 89yrs. Majority of the cases were of the age group 60-69, same for the controls (see table 1 below). Cases and controls were matched by age to rule out age as a disease contributing factor.

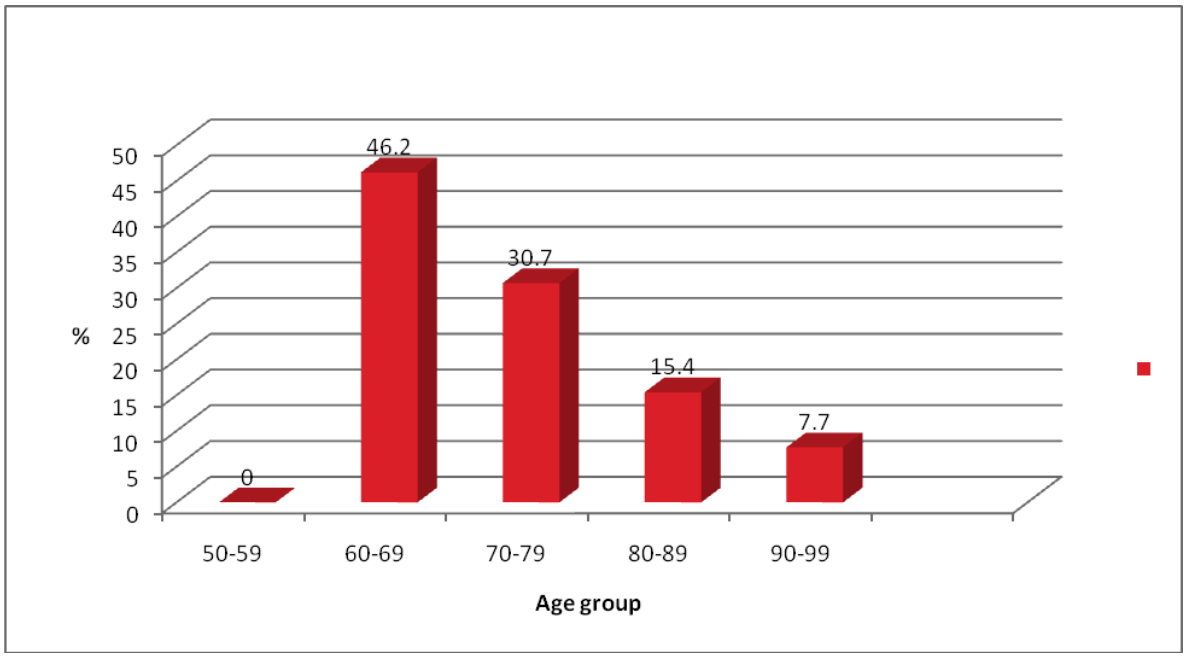

Fig1a: Patient distribution by age-Cases

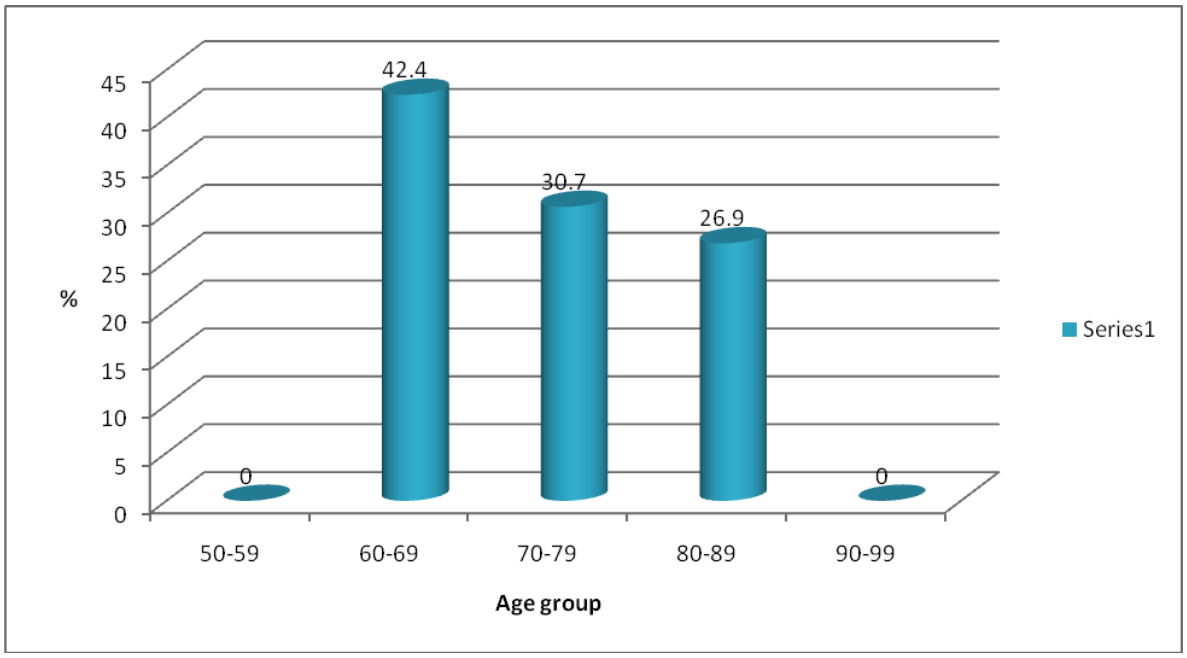

Fig 1b: Patient distribution by age-Controls

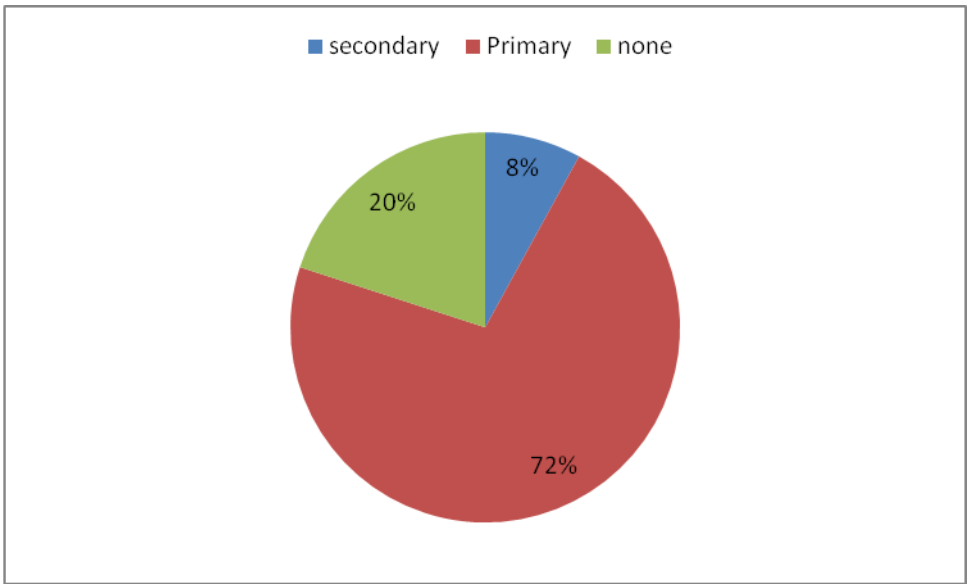

Fig 2: Level of education of the study population.

4.2CLINICAL CHARACTERISTICS

CASES						**CONTROLS**					
sample #	age	PSA	EN-2	IL-6	TNF-α	sample #	age	PSA	EN-2	IL-6	TNF-α
1	67	45.15	28.4	399.4	700.8	**7**	68	100	14.8	54.1	126.5
						11	67	1.42	18.2	12.2	75.3
8	65	44.65	28.8	367.9	150.8	**28**	64	0.399	7.5	15.2	72.3
						64	64	0.78	23.2	12.3	77.2
15	61	6.4	20.6	398.7	200.3	**52**	62	100	11.6	200.1	120.2
						35	63	0.604	12	14.1	69.5
16	66	7.82	10.5	306.9	475.7	**59**	62	22.49	2.5	24.3	71.2
						34	63	22.7	18	12.2	73.2
22	73	100	21.8	65.8	148.5	**60**	76	1.01	20.1	12.3	77.2
						13	73	15.72	44.1	25.1	148.4
23	79	0.622	17.6	89.5	70.8	**48**	81	14.96	26.5	15.2	73.2
						29	79	10.25	22.5	25.2	124.3
32	78	100	17.5	5	10.1	**26**	76	100	18.4	28.4	138.2
						54	77	4.36	16.5	18.2	71.2
40	94	179.43	7	300.5	180.6	**37**	89	45.95	14.2	17.1	80.3
						57	89	68.89	20	75.1	100.1
43	85	251	17.9	20.3	72.3	**21**	88	7.625	37.5	12.1	77.1
						58	88	49.39	28.5	12.1	72.6
44	67	100	17.5	20.5	77.1	**31**	69	5.72	>60	25.7	130.5
						55	68	1	28.2	9.2	74.5
38	65	10.47	18	30.7	10.2	**50**	68	5.1	38.1	18.1	145.6
						10	70	32.7	21.8	77.2	120.9
3	84	100	36	57.9	125.7	**18**	80	54.39	44.1	23	146.1
						4	85	2.4	40.1	25.1	130.7
61	73	100	2.56	210.5	122.1	**63**	73	21.14	1.5	18.1	20.2

Table 1: Results of the different biomarkers in cases and controls with their ages

A DRE was carried out on all patients. Eleven (11) of the 13 cases (84.6%) had an enlarged peripheral nodule on the prostate gland, indicating CaP, while the other 2 (15.4%) presented with asymmetrically enlarged prostate glands. They were a total of 26 controls, all with BPH.

There was a good correlation between DRE and US records with 84.6 % of the case suggested by DRE as having CaP getting similar diagnosis by ultrasound, while 76.9 % of the controls suggested by DRE as BPH, getting ultrasound concordance.

4.3 LABORATORY DATA

4.3.1 EXPRESSION OF URINARY EN-2 LEVELS.

Both cases and controls expressed EN-2 in urine with controls showing higher concentration than cases.

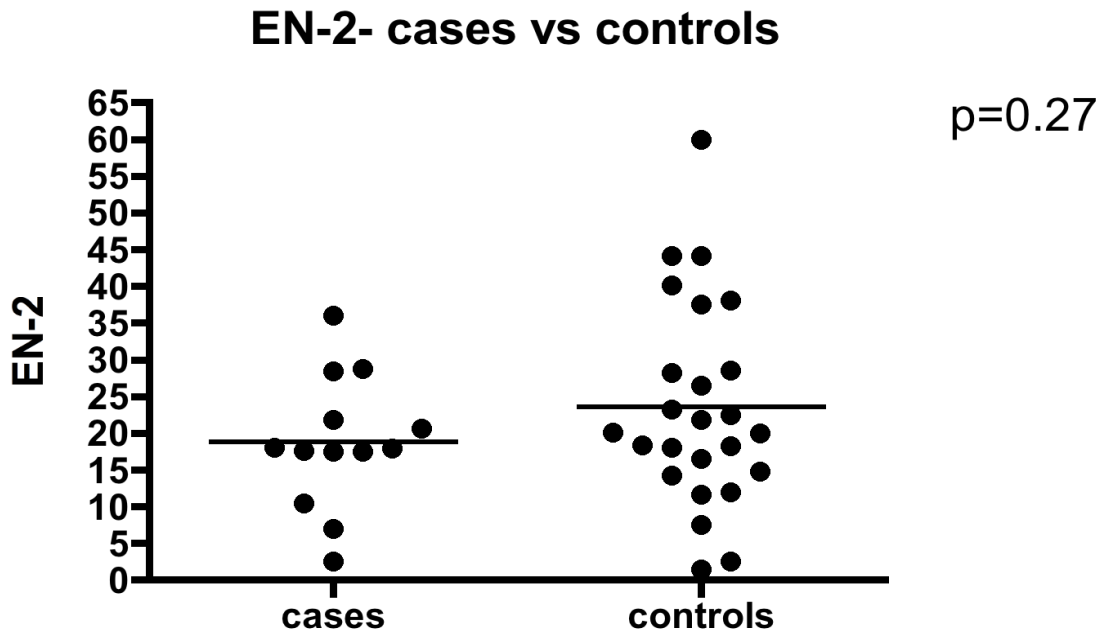

Figure 3: Urinary EN-2 concentrations among cases and controls

4.3.2 EXPRESSION OF SERUM PSA LEVELS.

About three quarters (73.1%) of the controls had PSA values greater than 4.0.

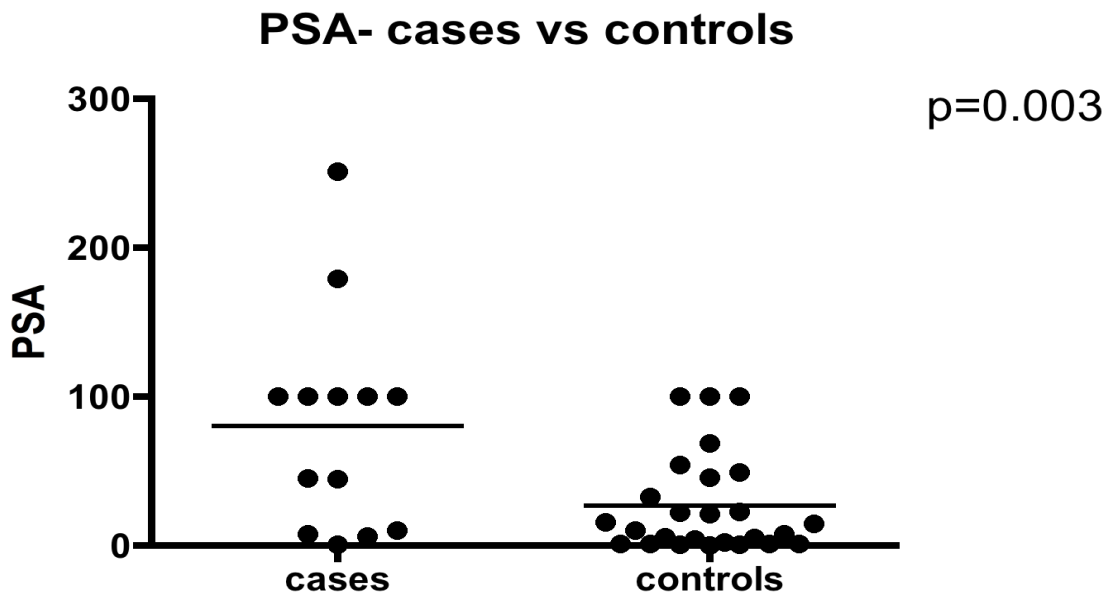

Figure 4: Serum PSA levels in cases and controls.

Table 2: Serum PSA levels in cases and controls with p-value.

Indicator	Cases		Controls	P-value
	Mean	80.43	25.54	
PSA (ng/ml)				0.003
EN-2		17.9	20.1	0.27

There were significant differences in serum PSA levels between cases and controls (p=0.003). Table 2: Urinary EN-2 levels between cases and controls indicating median values and p-value

As Indicated in table 2 and figure 2, there were no statistically significant difference in urinary EN-2 levels between cases and controls (p-value 0.27)

4.3.3 CORRELATION OF URINARY EN-2 LEVELS WITH SERUM PSA CONCENTRATIONS

Relationship between urinary EN-2 and serum PSA for both cases and controls indicated a no correlation. Higher values of EN-2 urinary concentrations were seen in both cases and controls and did not correspond to higher serum PSA levels (fig3). When cases and controls were plotted separately, there was still no correlation (see Fig 5a and 5b).

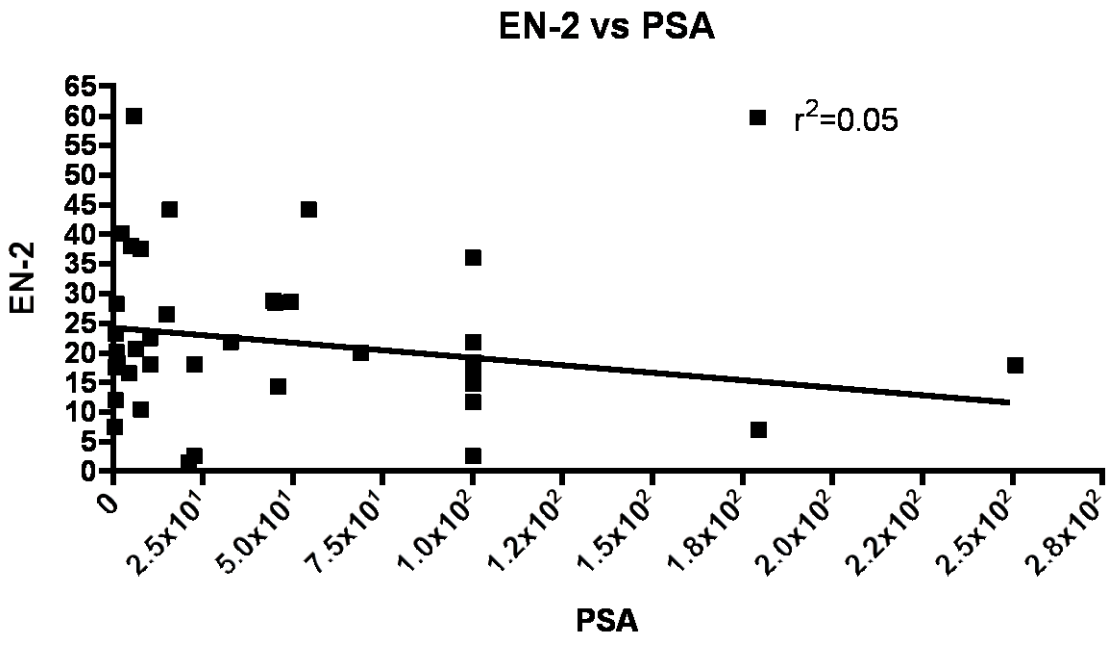

Fig 5: Correlating urinary EN-2 levels against serum PSA concentration

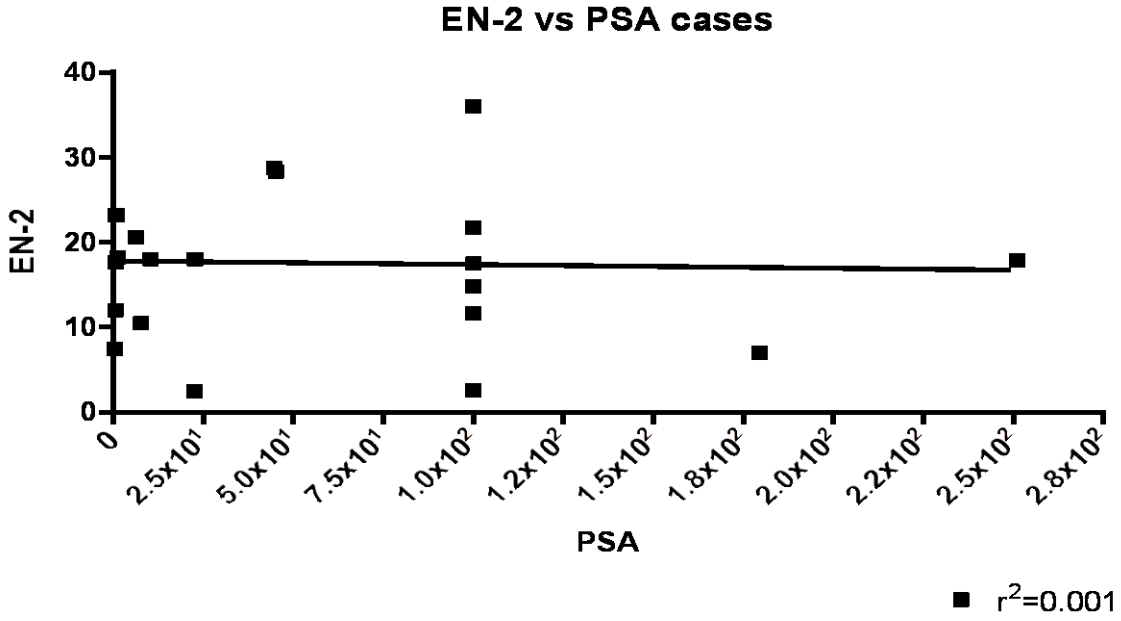

Fig 5a: Correlating urinary EN-2 levels against serum PSA concentration (cases).

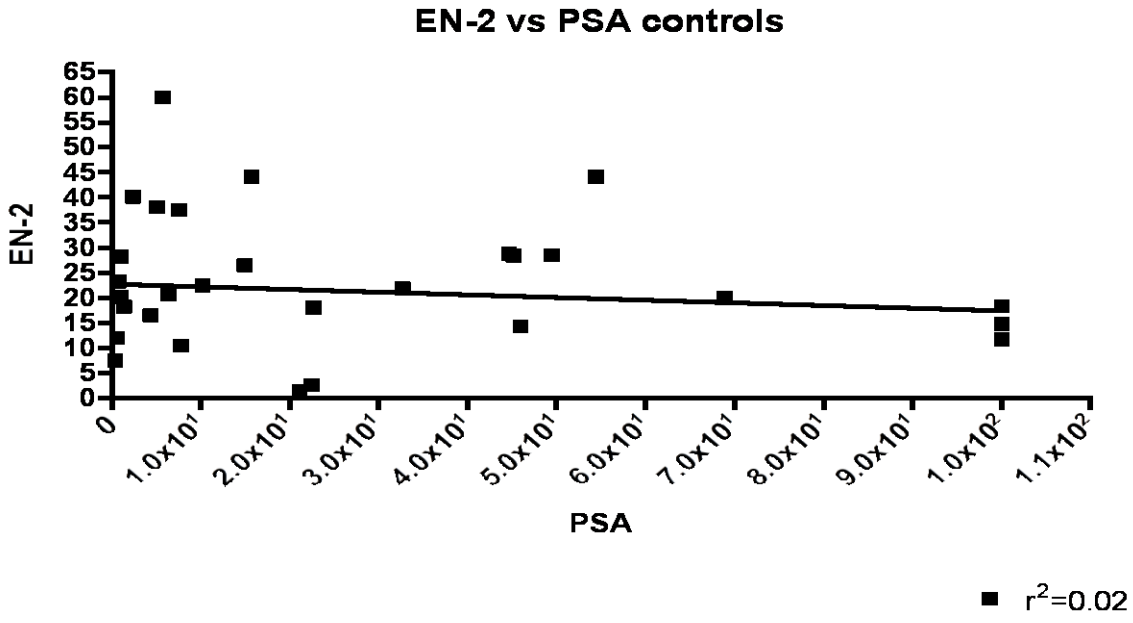

Fig 5b: Correlating urinary EN-2 levels against serum PSA concentration (controls).

4.3.4 EXPRESSION OF URINARY IL-6 LEVELS

The median value for urinary IL-6 levels in CaP was 89.5, while in controls it was 18.2. This difference was statistically significant (p=0.0001) (table 3 figure 4).

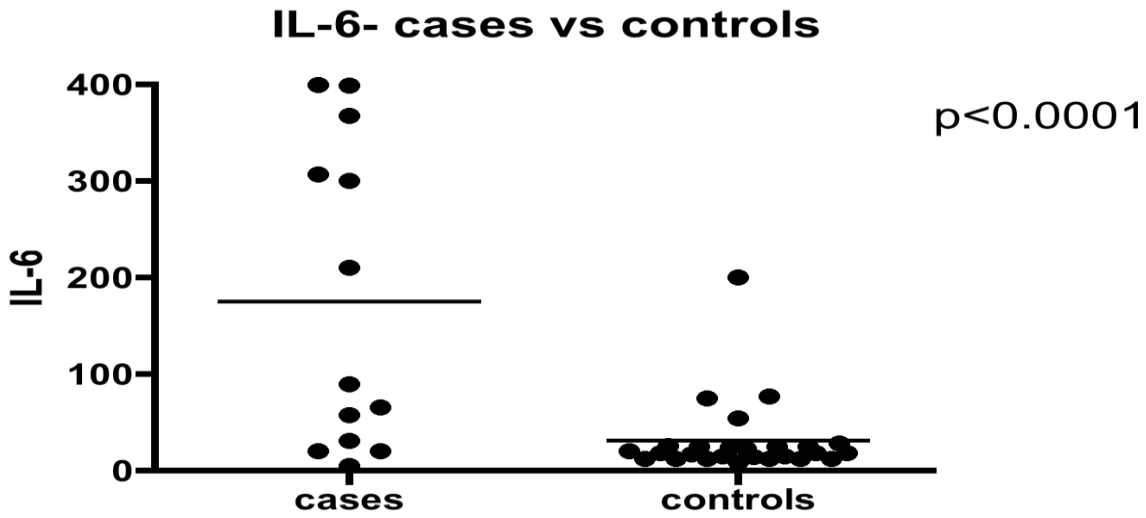

Figure 6: Urinary IL-6 levels between cases and controls.

Table 3: Urinary IL-6 levels between cases and controls indicating median and p values.

indicator	Measure	Cases	Controls	Z-value	P-value
IL-6 (pg/ml)	Median	89.5	18.2	3.278	0.0001

4.3.5 CORRELATION OF SERUM PSA CONCENTRATIONS WITH URINARY IL-6 LEVELS

When all the data points for PSA were plotted against IL-6 values, there was a positive correlation (figure 5). When cases and controls were plotted, the plots appeared differently and there was a trend towards correlation. Cases had a negative correlation while controls had a positive correlation. More than 50 % of the cases expressed higher IL-6 levels

alongside lower PSA concentration. It appears that for the CaP cases, higher IL-6 values correlate with lower PSA values (fig 7a)

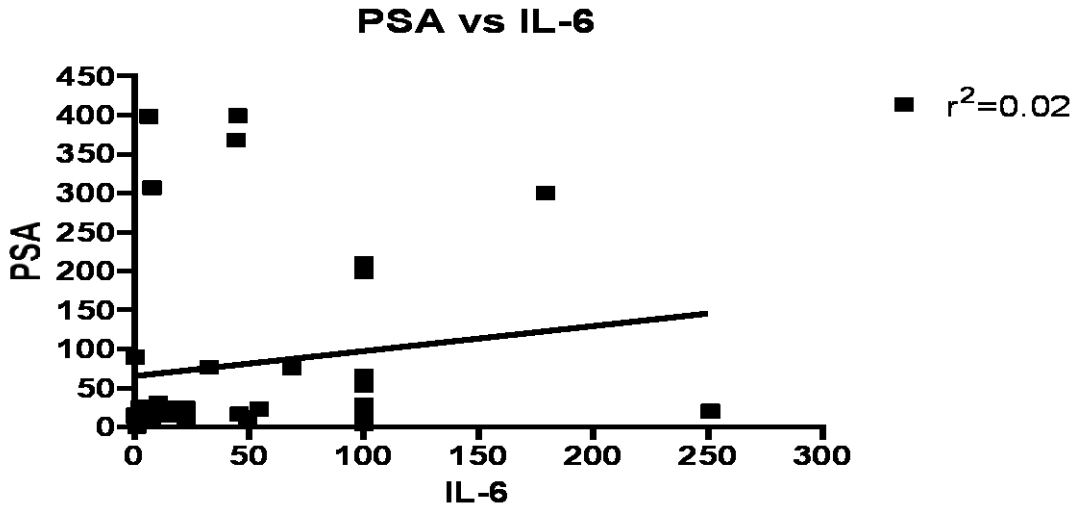

Figure 7: Correlating serum PSA on the Y-axis against urinary IL-6 on the X-axis.

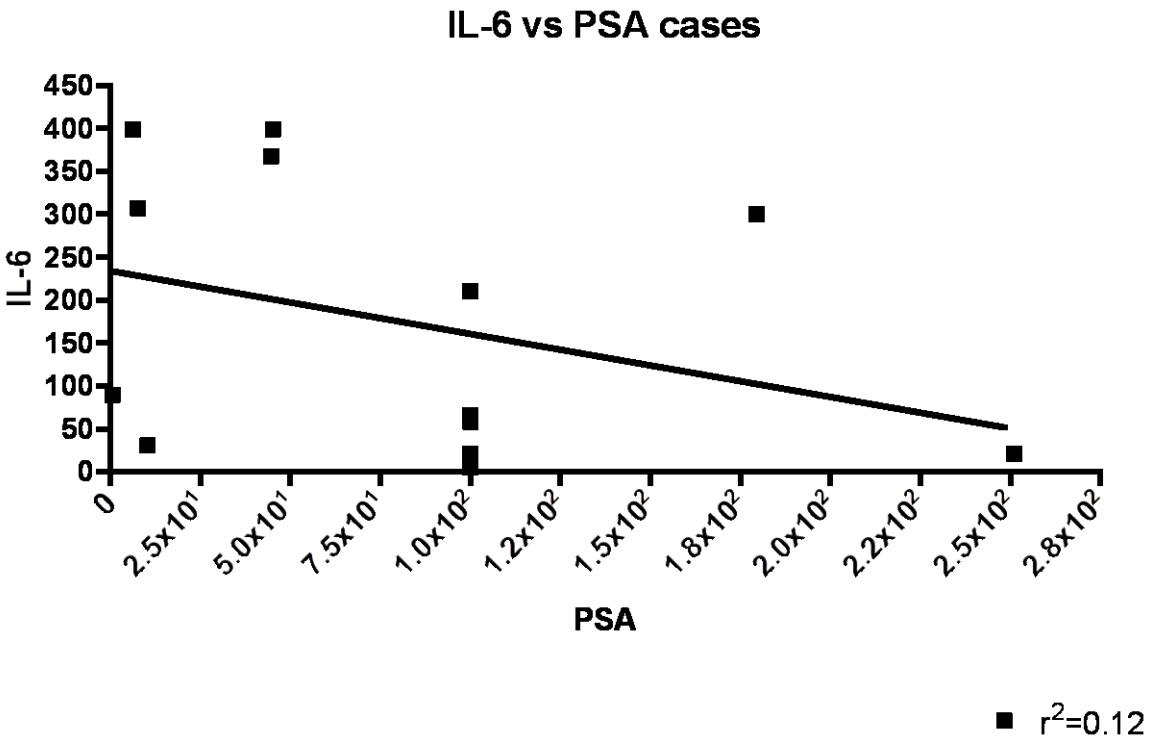

Figure 7a: A plot of IL-6 against PSA (cases)

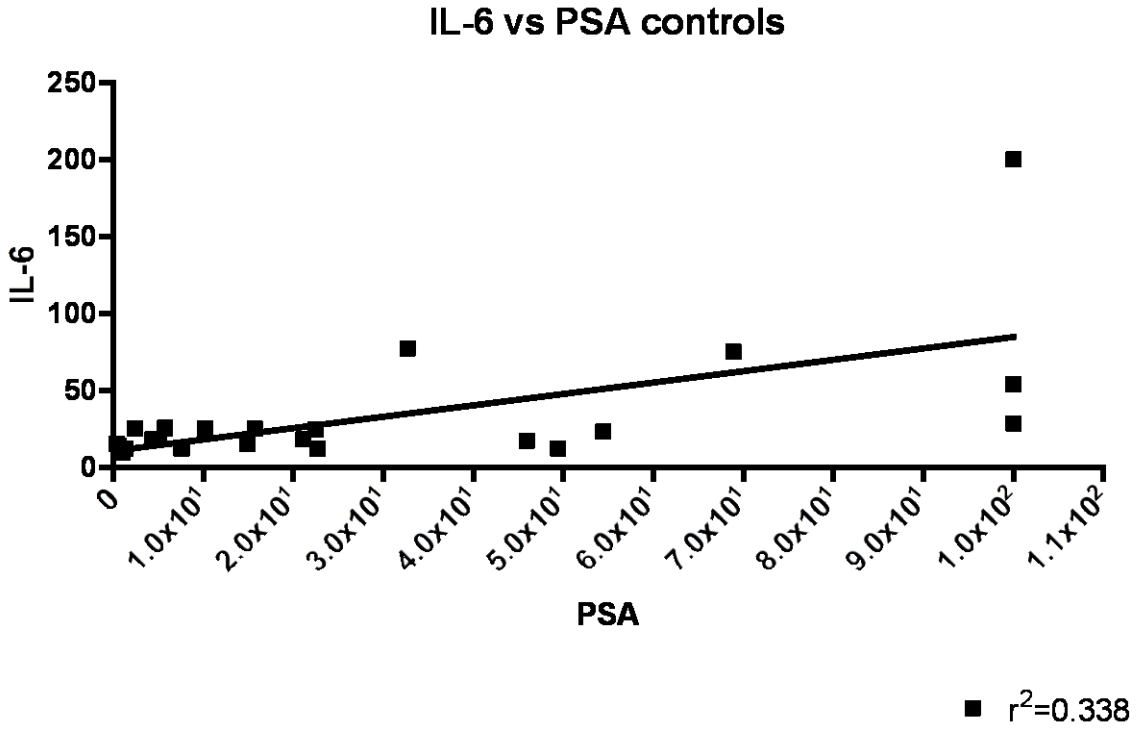

Figure 7b: Correlating IL-6 against PSA (controls).

4.3.6 CORRELATION OF URINARY EN-2 LEVELS WITH URINARY IL-6 LEVELS

The relationship between EN-2 and IL-6 for both cases and control indicated no correlation (r=0.02). When cases and controls were plotted separately, they were still no correlation (fig 8a & fig 8b).

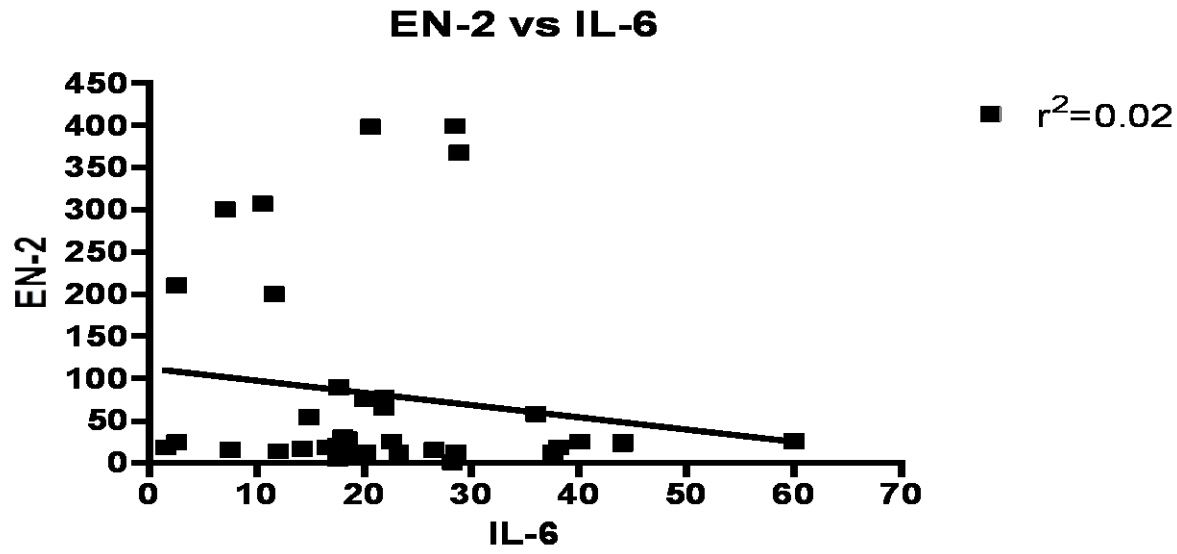

Figure 8: Correlating urinary EN-2 levels on the Y-axis against IL-6 levels on the x-axis.

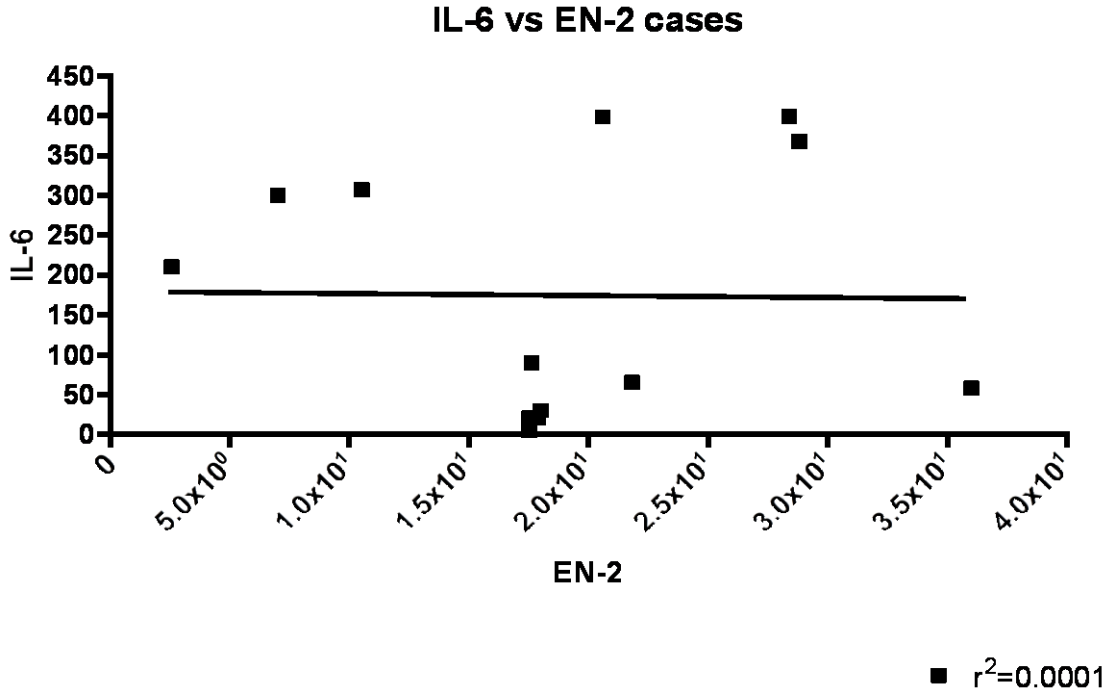

Figure 8a: Correlating urinary IL-6 against urinary EN-2 for cases.

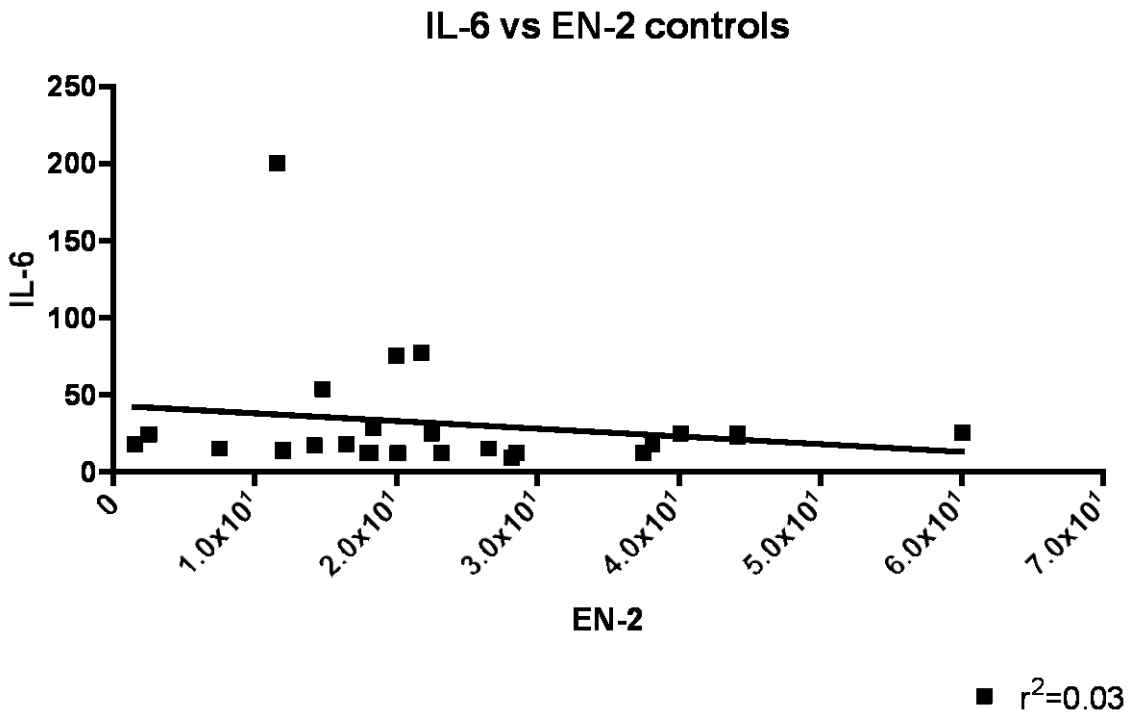

Figure 8b: Correlating IL-6 against urinary EN-2 for controls.

4.3.7 EXPRESSION OF URINARY TNF-α

There was a statistically significant difference in urinary TNF- α levels between case and controls (p=0.04)**.** This biomarker was expressed more in cases (median 125.7) than in controls (median 77.2)

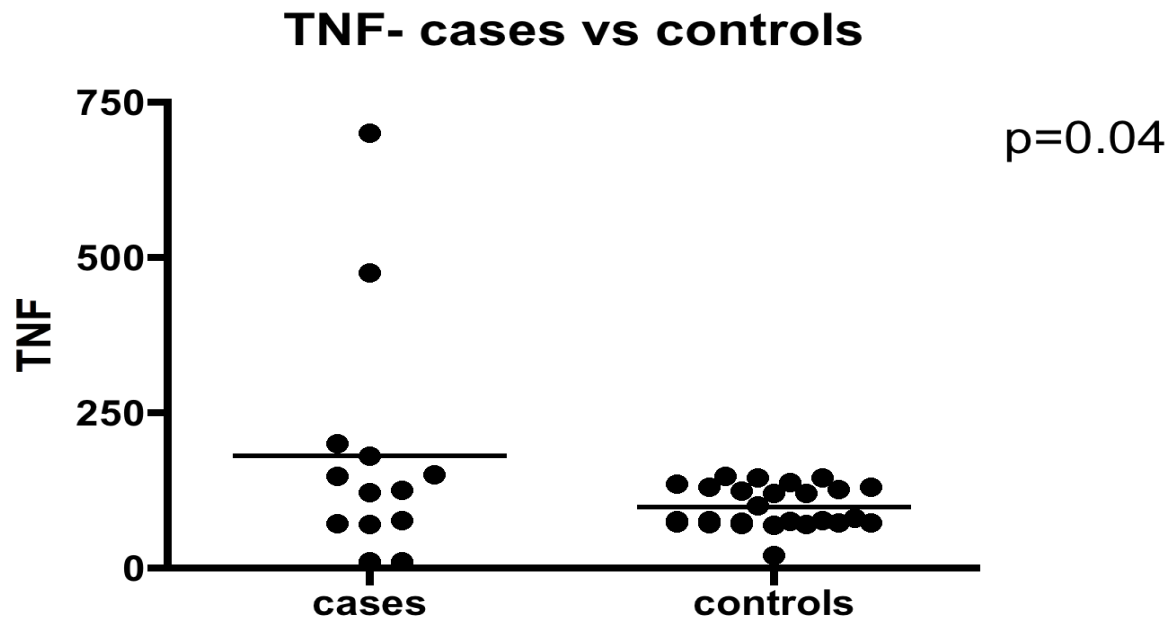

Figure 9 Urinary TNF-α levels between cases and controls

4.3.7 CORRELATION OF SERUM PSA LEVELS WITH URINARY TNF-α LEVELS.

When the data points for PSA were plotted against TNF-α, there was no correlation (figure 10). When the cases and controls were plotted separately there was a trend in correlation fig10a and 10b). Controls with PSA value <4.0ng/ml had lower values of TNF-α compared to those with PSA value > 4.0ng/ml.

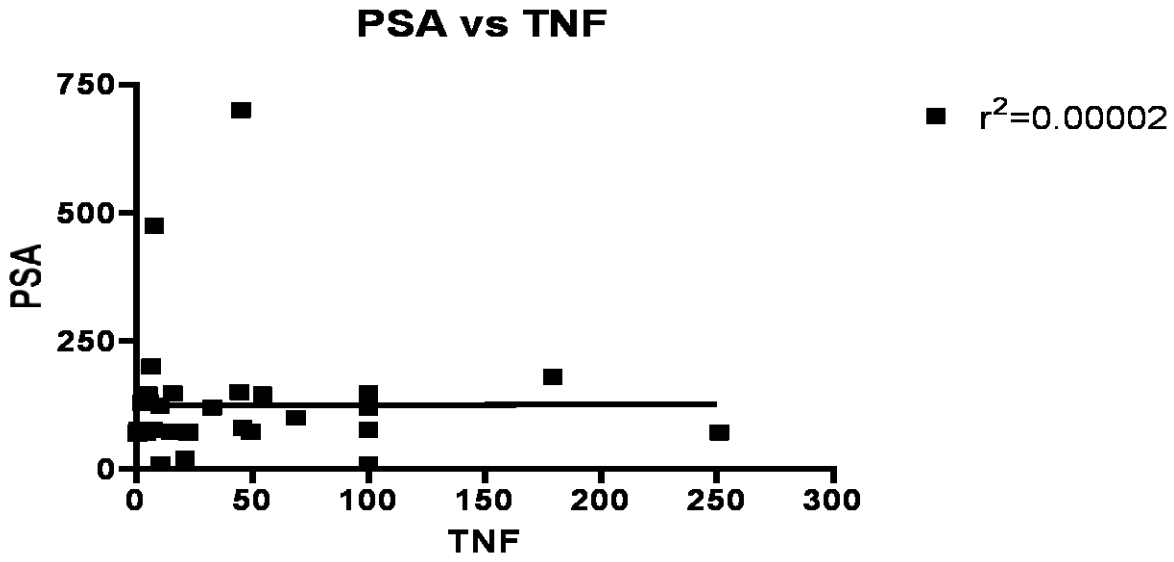

Figure 10: Correlating serum PSA on the Y-axis against urinary TNF-α on the X-axis.

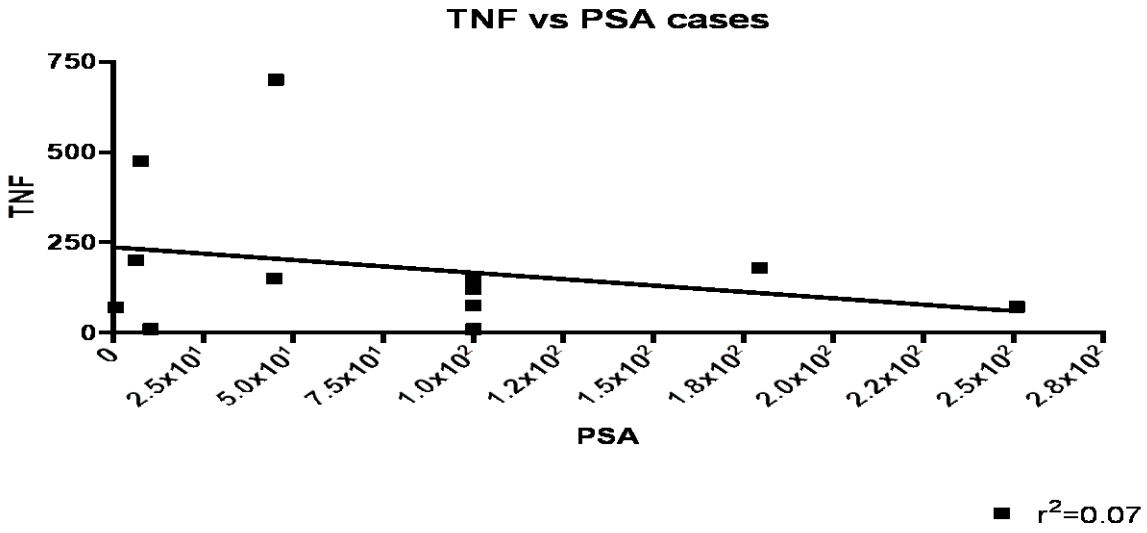

Figure 10a: Correlating urinary TNF-α against PSA (cases)

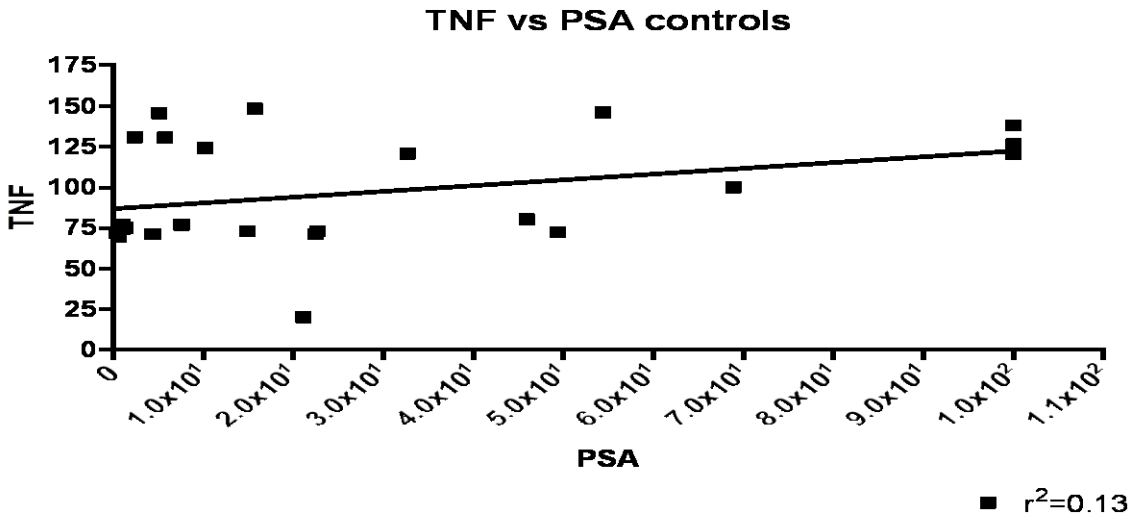

Figure 10b: Correlating TNF-α against serum PSA (cases).

4.3.8 CORRELATION OF URINARY EN-2 AND URINARY TNF-α

The relationship between urinary EN-2 and urinary TNF-α indicated no correlation (r=0.01) (fig 11). However, when EN-2 values for controls were plotted with the TNF values, there was a correlation: higher EN-2 values, higher TNF values (fig11a and 11b).

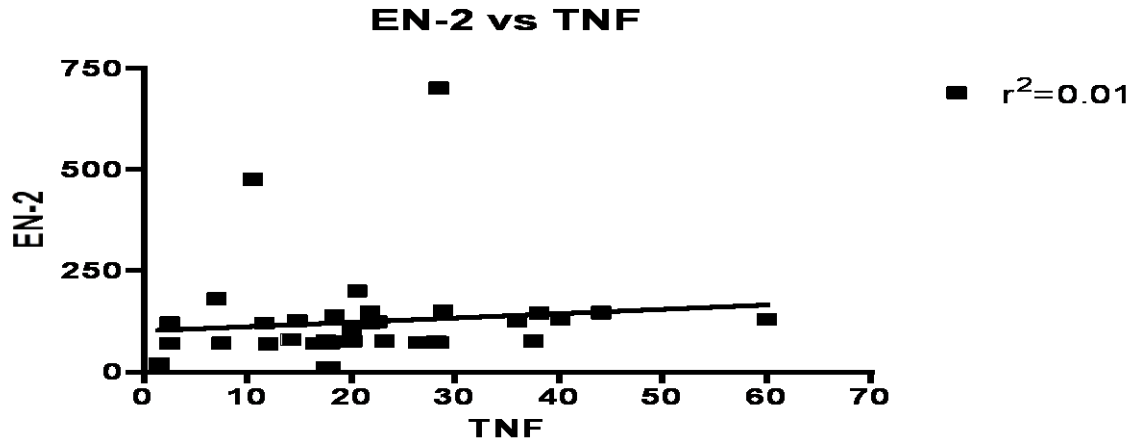

Figure 11: Correlating urinary EN-2 against urinary TNF-α.

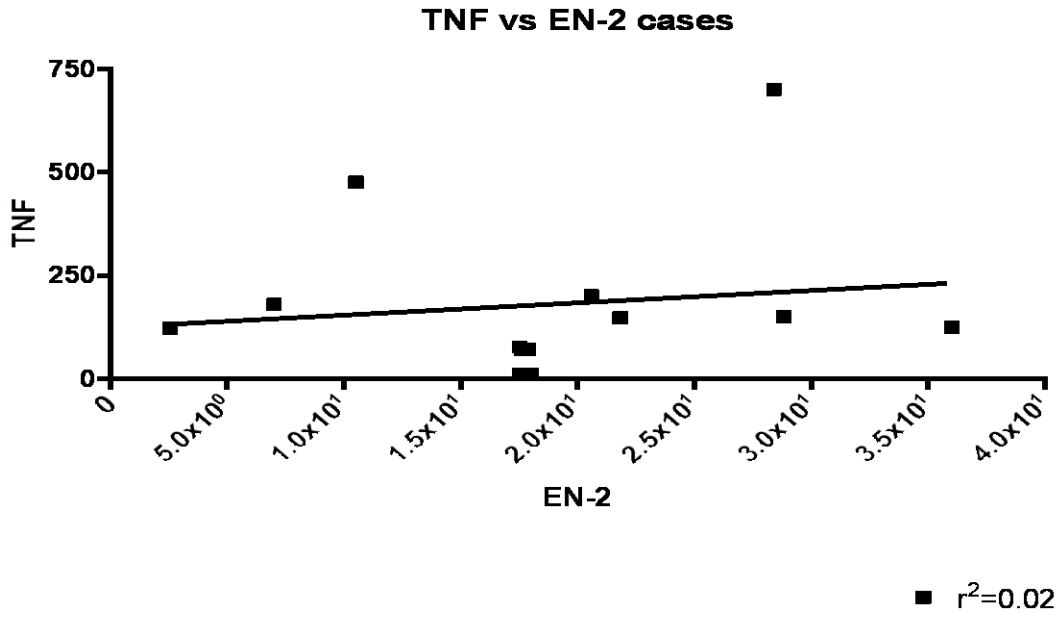

Figure11a. Correlating urinary TNF-α against urinary EN-2 (cases).

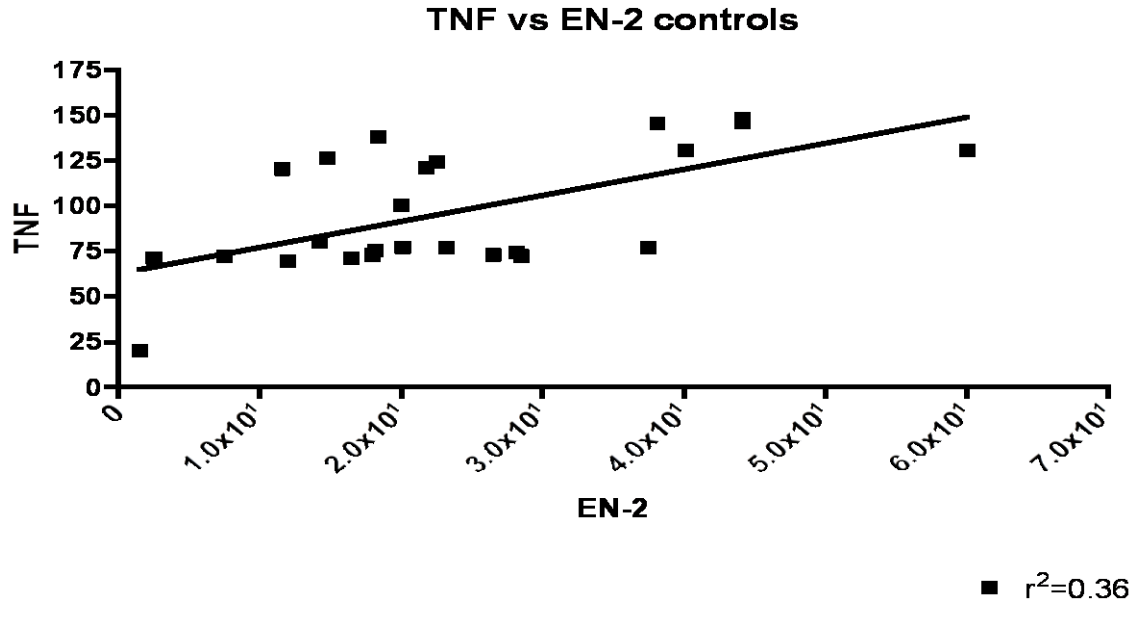

Figure 11b: Correlating urinary TNF-α against urinary EN-2 (controls).

4.3.9 CORRELATION OF URINARY IL-6 LEVELS WITH URINARY TNF-α LEVELS.

There was a good correlation between urinary IL-6 levels and urinary TNF-α levels (r=0.49) (fig 12). High IL-6 levels corresponded with high TNF-α level in both cases and controls.

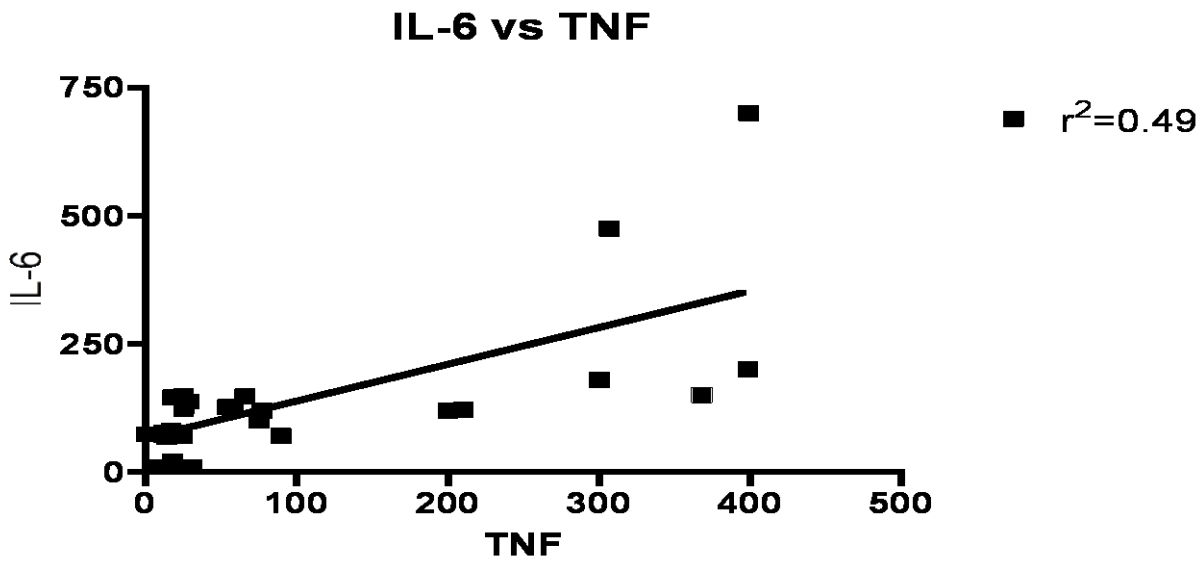

Figure 12: Correlating urinary IL-6 levels against TNF-α level.

CHAPTER FIVE

5.0 DISCUSSION

The study was aimed at evaluating EN-2 and Cytokines in urine with serum PSA in the diagnosis of prostate cancer. Although there is need for a CaP biomarker that has diagnostic and prognostic utility, and which can aid in treatment, DRE and ultrasound are still very useful in the diagnosis and follow-up of the disease. Old age is a contributing factor to both CaP and BPH. Although, older men are at a higher risk of CaP compared to younger men (cases mean age= 73.6, controls mean age=71.1). Data from this study revealed that majority of men are likely to develop prostate cancer from age 60. This agrees with Hans-Joachim et al. (2007) who found that about two-thirds of all prostate cancers are diagnosed in men age 65 and older. The older the patient, especially if they are over 70, the less aggressive the disease usually behaves.

Although serum PSA concentrations were significantly higher in cases than in controls, p=0.003, 73.1% of the controls had PSA values >4.0ng/ml. This confirms with Catalona et al (1997), who showed that 81 % of men with PSA >4 had no CaP. According to Catalona et al. (1997), the specificity of the PSA test is suboptimal, and as a result, about 75 percent of men who undergo a prostate biopsy because they have PSA levels of 4.0to 10.0ng per milliliter do not have cancer. This is an indication that PSA is secreted by both malignant and non-malignant cells, confirming that PSA is prostate-specific and not prostate cancer-specific.

There was no statistically significant difference between EN-2 in cases and controls (p-0.27). The presence of EN-2 in urine might not be predictive of CaP prostate or distinguish

between CaP and BPH. Data from this study suggest that EN-2 is secreted into urine by both malignant and BPH cells. This contradicts the findings of Richard et al (2012) in which urinary EN2 levels were measured by ELISA and had a sensitivity of 66.7% and specificity of 89.3% for cancer detection. The study by Richard et al had as controls men with no prostetism as opposed to this study in which controls were those with BPH. This could be a possible reason for the difference in findings. Data from this study suggest that EN-2 may not be a reliable diagnostic marker for CaP in Kenya.

The relationship between urinary EN-2 levels and serum PSA showed no correlation for both cases and controls combined (r=0.05). Richard et al, (2012) had a weak correlation between PSA and EN-2.When cases were separated from controls and each group plotted against the other, there was still no correlation. That is, the presence of EN-2 in urine those not predict the presence of PSA in serum. This could be an indication that these two parameters are produced by two different pathologic or physiologic mechanisms and if used together may not be reliable markers in CaP diagnosis.

Torre et al, (1996) from in vitro and in vivo found that proinflammatory cytokines, that is, IL-6 and TNF-α has been proposed as important mediators in the development of heart failure and can be measured in urine. It is from the study that the researcher thought it wise to measure the same cytokines in urine to investigate prostate cancer as urine flows through the prostate gland and can easily be obtained without any invasive procedure as opposed to blood. Analysis of urinary IL-6 levels in cases and controls revealed it is highly expressed in cases compared to controls. However, it was noted that cases with very high PSA values had lower IL-6 values and vice vice-versa.This observation shades more light on the

suggestion that inflammation may plays a key role in CaP especially at an early stage. Recent studies have shown that CaP patients express high levels of IL-6 with low levels of PSA and have shorter survival in late stage CaP (Hoosein, N. et al.1995). Inflammatory processes may be reduced in CaP metastasis. IL-6 could play a key role in the pathogenesis and progress of CaP. IL-6 may further serve as a diagnostic and prognostic marker suggesting therapeutic targets. For the controls, higher IL-6 values correspond to higher PSA values (fig 5b). Some studies have reported high levels of IL-6 and high PSA concentrations in BPH patients (Al-Maghrebi M. et al. 2012).

There was a negative correlation between IL-6 and EN-2 (r=0.02). When cases and controls were plotted separately there was still no correlation for cases but still a negative correlation for controls. This could be an indication that the presence of EN-2 in urine does not result from inflammatory processes.

TNF-α had a positive correlation with PSA in controls, ie, higher levels of TNF-α corresponding with higher levels of PSA. In cases, there was no correlation though the concentration of TNF-α was generally higher in cases than in controls.

TNF-α had no correlation with EN-2. When the controls were plotted separately from cases there was still no correlation, the same as in IL-6. This is an indication that EN-2 concentrations alongside pro-inflammatory markers may not be considered in CaP or BPH diagnosis.

There was a correlation (r=0.49) between IL-6 and TNF-α suggesting that both cytokines are expressed in both CaP and BPH. This expression is seen more in cases than in controls, although CaP metastasis may turn to show lower levels of IL-6.

CHAPTER SIX
6.0 CONCLUSIONS AND RECOMMENDATIONS
6.1 CONCLUSIONS

The study has demonstrated that inflammation may play a major role in the onset and progress of prostate cancer. This is confirmed by the high concentrations of IL-6 in CaP and its correlation with TNF-α. From the study that IL-6and TNF-α could be reliable diagnostic and prognostic markers to differentiate prostetism. Trends in IL-6 expression may also be useful in understanding of the pathogenesis of CaP. In addition, urine may be a good milieu in which to study these cytokines and may have diagnostic, prognostic and therapeutic implications. Changes in inflammation pro-inflammatory cytokines markers may be considered to be of value in discriminating BPH and CaP.

The study has also shown that the sensitivity of urinary EN-2 is not significantly higher than that of serum PSA. The EN-2 marker is therefore, not yet useful in application for CaP diagnosis in the study population. It has however, demonstrated that serum PSA is prostate-specific and not prostate cancer-specific. It should therefore be used in association with DRE, pelvic/abdominal ultrasound and/or prostate biopsy.

6.2 RECOMMENDATIONS

The result of this study suggests that more is needed to be done in terms of research to confirm EN-2 as a reliable marker for CaP especially in Africa. Further studies should embark on recruiting a large number of participants and should utilize histology as the standard for differentiating cases from controls. A prospective study is also recommended to establish a prognostic and therapeutic marker.

We also recommend studies that will determine the expression of pro-inflammatory cytokines in serum. This may add to the fact that IL-6 may be a reliable marker for Cap, and could suggest therapeutic targets.

REFERENCES.

Adams J (1853). The case of scirrhous of the prostate gland with corresponding affliction of the lymphatic glands in the lumbar region and in the pelvis".

Al-Maghrebi M, Kehinde EO, Al-Mulla F, Anim JT (2012). The effect of prostate tissue inflammation in benign prostatic hyperplasia on enhancer of zeste homolog 2 ribonucleic acid expression. Ann Saudi Med 2012;32(3):262-268.

Ankerst DP, Miyamoto R, Nair PV, Pollock BH, Thompson IM, Parekh DJ (2009) Yearly prostate specific antigen and digital rectal examination fluctuations in a screened population; Urology 181:2071 – 5;

Aumüller, G. (1999). Prostate Gland and Seminal Vesicles. Berlin-Heidelberg: Springer-Verlag.

Beckman,J.S. and Koppenol,W.H. (1996) Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. Am. J. Physiol., 271, C1424--C1437.

Beers, Mark H., MD, and Robert Berkow, MD. (2004). Prostate Cancer. In The Merck Manual of Diagnosis and Therapy. Whitehouse Station, NJ: Merck Research Laboratories, 2004.

Berger AP, Volgger H, Rogatsch H, Strohmeyer D, Steiner H,Klocker H, et al (2002) Screening with low PSA cutoff values results in low rates of positive surgical margins in radical prostatectomy specimens. Prostate; 53: 241 – 5.

Beuzeboc P, Soulié M, Richaud P, Salomon L, Staerman F, Peyromaure M, Mongiat-Artus P, Cornud F, Paparel P, Davin JL, Molinié V (2009). Fusion genes and prostate cancer. From discovery to prognosis and therapeutic perspectives Prog. Urol. **19** (11): 819–24.

Beuzeboc P, Soulié M, Richaud P, Salomon L, Staerman F, Peyromaure M, Mongiat-Artus P, Cornud F, Paparel P, Davin JL, Molinié V (December 2009). [Fusion genes and prostate cancer. From discovery to prognosis and therapeutic perspectives]. Prog. Urol. (in French) 19 (11): 819–24. doi:10.1016/j.purol.2009.06.002. PMID 19945666.

Bunting PS, DeBoer G, Choo R, Danjoux C, Klotz L,Fleshner N (2002) Intraindividual variation of PSA, free PSA and complexed PSA in a cohort of patients with prostate cancer managed with watchful observation. Clin Biochem; 35:471 – 5.

Burnet, F.M. (1957). "Cancer—A Biological Approach: I. The Processes Of Control. II. The Significance of Somatic Mutation. Brit. Med. Jour. 1 (5022): 779–786. doi:10.1136/bmj.1.3356.779. JSTOR 25382096. PMC 1973174. PMID 13404306.

Carter HB, Hamper UM, Sheth S, Sanders RC, Epstein JI, Walsh PC.(1989). J Urol. 142 (4):1008-10

Catalona WJ, Smith DS, Ornstein DK (1997) Prostate cancer detection in men with serum PSA concentrations of 2.6 to 4.0 ng/mL and benign prostate examination. Enhancementof specifi city with free PSA measurements. JAMA; 277:1452 – 5.

Cooper, CS; English, DR; Hopper, JL; Neal, DE; Easton, DF (2008 Mar). Multiple newly identified loci associated with prostate cancer susceptibility. Nature genetics 40 (3): 316–21. PMID 18264097.

Damber JE, Aus G (2008) Prostate cancer. Lancet; 371(9625):1710–21.

Di Blasio CJ, Malcolm JB, Hammett J, Wan JY, Aleman MA, Patterson AL, (2009) Survival outcomes in men receiving androgen-deprivationtherapy as primary or salvage treatment for localized or advanced prostate cancer: 20-year single-centre experience. BJU Int; 104:1208–14.

Dinarello C. A.(1996) Biologic basis for interleukin-1 in disease. Blood 87:2095–2147.

Dunn, G.P.; Bruce, A.T.; Ikeda, H.; Old, L.J.; Schreiber, R.D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. Nature Immunology 3 (11): 991–998. doi:10.1038/ni1102-991. PMID 12407406.

Eble JN, (2004): Pathology and Genetics: Tumors of the urinary system and male genital organs. WHO classification of tumors, Geneva, World Health Organization, 2004.

Eble JN, Epstein JI (1990). Stage A carcinoma of the prostate. In: Roth LM, ed. Pathology of the Prostate, Seminal Vesicles, and Male Urethra, New York: Churchill Livingstone; 1990:61-82

Epstein JI (1995). Diagnostic criteria of limited adenocarcinoma of the prostate on needle biopsy. Hum Pathol 1995; 26:223.

Epstein JI (2011). Prognostic significance of tumour volume in radical prostatectomy and needle biopsy specimens. *JUrol* ;186:790-7

Epstein JI, Walsh PC, Carmichael M, Brendler CB (1994). Pathologic and clinical findings to predict tumor extent of nonpalpable (stage T1c) prostate cancer. JAMA ; 271:368-74.

Ernst,P.B. and Gold,B.D. (2000). The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastricancer. Annu. Rev. Microbiol., 54, 615--640.

Espey,M.G., Miranda,K.M., Thomas,D.D., Xavier,S., Citrin,D., Vitek,M.P. and Wink,D.A. (2002). A chemical perspective on the interplay between NO, reactive oxygen species and reactive nitrogen oxide species. Ann. N. Y. Acad. Sci., 962, 195--206.

Fauci L, (2008) Harrison's Principle of internal medicine 17th edition. McGraw Hills

Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C.(2007): Chronic inflammation and oxidative stress in human carcinogenesis. Int J Cancer, 121:2381-2386.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN Int JCancer; 127:2893–917.

Gallagher RP, Fleshner N (1998). Prostate cancer: 3. Individual risk factors. CMAJ **159** (7): 807–13.

Gibson, W., Green, A., Bullard, R.S., Eaddy, A.C. and Donald, C.D. (2007) Inhibition of PAX2 expression results in alternate cell death pathways in prostate cancer cells differing in p53 status Cancer Lett, 248:251–61.

Gleason DF, Mellinger GT (1974). Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J Urol 1974; 111:58.

Gnarra, J.R. and Dressler, G.R. (1995) Expression of Pax-2 in human renal cell carcinoma and growth inhibition by antisense oligonucleotides. Cancer Res., 55:4092–8.

Gretzer MB, Partin AW (2003) PSA markers in prostate cancer detection. Urol Clin North Am ; 30:677.

Hidalgo.A, (1994). Three distinct roles for the engrailed gene in Drosophila wing development. Current Biology **4** (12): 1087–1098.

Holick MF, (2007) Vitamin D deficiency. N Engl J Med ; 357:266.

Hoosein N[1], **Abdul M**, **McCabe R**, **Gero E**, **Deftos L**, **Banks M**, **Hodges S**, **Finn L**, **Logothetis C (1995).**Clinical significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostate cancer. **Urol Oncol.** Nov-Dec;1(6):246-51.

Howard K, (2009). A Model of Prostate-Specific Antigen Screening Outcomes for Low- to High-Risk Men', Arch Intern Med, vol. 169, pp. 1603-1610.

Ilyin SE, Belkowski SM, Plata-Salaman CR (2004) Biomarker discovery and validation: Technologies and integrative approaches. Trends Biotechnol; 22:411 – 6.

Jemal A, (2007) Cancer statistics. CA Cancer J Clin ; 57:43.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ (2009) Cancer statistics, CA Cancer J Clin ;59:225 – 49.

Joyner, A.L, (1996) Engrailed, Wnt and Pax genes regulate midbrain—hindbrain development. Trends Genet, 12:15–20.

Jump up Steive, H. (2000). Männliche Genitalorgane. Handbuch der mikroskopischen Anatomie des Menschen. Vol. VII Part 2. Berlin: Springer. pp. 1–399.

Jump up Moore, K.; Dalley, A. (1999). Clinically Oriented Anatomy. Baltimore, Maryland: Lippincott Williams & Wilkins. ISBN 0-683-06132-1.

Leav I, Plescia J, Goel HL, Li J, Jiang Z, Cohen RJ, Languino LR, Altieri DC (January 2010). Cytoprotective Mitochondrial Chaperone TRAP-1 As a Novel Molecular Target in Localized and Metastatic Prostate Cancer. Am. J. Pathol. 176 (1): 393–401.

Lin K, Lipsitz R, Miller T, Janakiraman S. (2008) Benefits and harms of prostate specific antigen screening for prostate cancer: an evidence update for the U.S. Preventive Services Task Force.Ann Intern Med;149(3):192

Malcolm JB, Hammett J, Wan JY, Aleman MA, Patterson AL, (2009) Survival outcomes in men receiving androgen-deprivation therapy as primary or salvage treatment

for localized or advanced prostate cancer: 20-year single-centre experience BJU Int; 104: 1208–14.

Male Genitals - Prostate Neoplasms. Pathology study images. University of Virginia School of Medicine. Archived from the original on 2011-04-28. Retrieved 2011-04-28.

Marks LS, (2008): Prostate tissue androgens: history and current clinical relevance. Urology; 72:247.

Martin NL, Saba-El-Leil MK, Sadekova S, Meloche S, Sauvageau G. (2005) EN-2 is a candidate oncogene in human breast cancer. Oncogene; 24:6890–901.

Matzkin H, (1994): Stage T1A carcinoma of prostate. Urology; 43:11.

McDavid K, Lee J, Fulton JP, Tonita J, Thompson TD (2004) Prostate cancer incidence and mortality rates and trends in the United States and Canada. Public Health Rep; 119(2):174–86.

McNeal JE. (1981). Normal and pathologic anatomy of prostate. Urology; 17:11.

Morgan R. (2006) Engrailed: complexity and economy of a multi-functional transcription factor. FEBBS lett; 580:2531–3.

Moyer VA. (2012). On behalf of the U.S. Preventive Services Task Force, 'Screening for Prostate Cancer: U.S. Preventive Services Task Force Recommendation Statement',
Ann Intern Med, vol. 157, pp. 120–134

Narizhneva NV, Tararova ND, Ryabokon P, Shyshynova I, Prokvolit A, Komarov PG, Purmal AA, Gudkov AV, Gurova KV (2009) Small molecule screening reveals a transcription-independent pro-survival function of androgen receptor in castration-resistant prostate cancer. 15;8(24):4155-67. Epub Dec 13.

Ohtani, H.; Dunn, IF; Curry, WT (2007). Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma. Cancer Immunity 7: 4. PMC 2935751. PMID 17691714.

Pamela E, John H, and Cliff G (2003): 100 Questions and Answers about Prostate Cancer.

Pervin,S., Singh,R. and Chaudhuri,G. (2001). Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1. Proc. Natl Acad. Sci. USA, 98, 3583--3588.

Rabbitts, T.H. (1994). Chromosomal translocations in human cancer. Nature, 372:143–9.

Reynard, John; Brewster, Simon; Biers, Suzanne (2006). Oxford Handbook of Urology, 1st Edition.

Robbin and Cotran (2010). Pathologic Basis of Disease**.** 8th Ed. SAUNDERS ELSEVIER. William Schmitt

Romero,F.J., Bosch-Morell,F., Romero,M.J., Jareno,E.J., Romero,B., Marin,N. andRoma,J. (1998) .Lipid peroxidation products and antioxidants in human disease. Environ. Health Perspect., 106 (suppl. 5), 1229-1234.

Rosario DJ. (2012). 'Short Term Outcomes of Prostate Biopsy in Men Tested for Cancer by Prostate Specific Antigen: Prospective Evaluation within ProtecT Study', BMJ, vol. 344, pp. d7894.

Salman H[1], Ori Y, Bergman M, Djaldetti M, Bessler H (2012). Human prostate cancer cells induce inflammatory cytokine secretion by peripheral blood mononuclear cells. Jul;66(5):330-3. doi: 10.10

Shah N, Sukumar S. (2010) The Hox genes and their roles in oncogenesis. Nat Rev Cancer; 10:361–71.

Shariat SF, Canto EI, Kattan MW, Slawin KM (2004). Beyond prostate-specific antigen: New serologic biomarkers for improveddiagnosis and management of prostate cancer. Rev Urol; 6:58 – 72.

Soletormos G, Semjonow A, Sibley PE, Lamerz R, Petersen PH, Albrecht W, (2005). Biological variation of total prostatespecific antigen: A survey of published estimates and consequences for clinical practice. Clin Chem ; 51:1342 – 51.

Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA (May 1997). The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. N. Engl. J. Med. **336** (20): 1401–8.

Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA (May 1997). The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. N. Engl. J. Med. 336 (20): 1401–8. doi:10.1056/NEJM199705153362001. PMID 9145676.

Stuart, E.T., Haffner, R., Oren, M. and Gruss, P (1996). Loss of p53 function through PAX-mediated transcriptional repression. Embo J. Biol Chem14:5638–45.

Sudeep K. Bose, Rebecca S. Bullard and Carlton D. Donald (2008). Oncogenic Role of Engrailed-2 (EN-2) in Prostate Cancer Cell Growth and Survival:3 37-43.

Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Pames HL, (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level < or = 4.0 ng per milliliter. New England Journal of Medicine*; 350:2239–46.*

Umtergasser G ,(2005) : Benign prostatic hyperplasia: age related tissue-remodeling. Exp Gerontol ; 40:121.

Van der Poll T., Lowry S. F.(1995). Endogenous mechanisms regulating TNF and IL-1 during sepsis. in Year-book of intensive care and emergency medicine. ed Vincent J. L. (Springer-Verlag, New York), pp 385–397.

Wallin, J.J., Gackstetter, E.R. and Koshland, M.E. (1998). Dependence of BSAP repressor and activator functions on BSAP concentration. Science, 279:1961–4.

Wiklund F, Gillanders EM, Albertus JA, Bergh A, Damber JE, Emanuelsson M, (2003). Genome-wide scan of Swedish families with hereditary prostate cancer: suggestive evidence of linkage at 5q11.2 and 19p13.3. Prostate 57:290.

Wray, C. Wray, C.G.; Jacobs, D.K.; Kostriken, R.; Vogler, A.P.; Baker, R.; deSalle, R. (1995). Homologues of the engrailed gene from five molluscan classes. FEBS Letters 365: 71–00. Elsevier

Yao V, Berkman CE, Choi JK, O'Keefe DS, Bacich DJ (February 2010). Expression of prostate-specific membrane antigen (PSMA), increases cell folate uptake and proliferation and suggests a novel role for PSMA in the uptake of the non-polyglutamated folate, folic acid. Prostate 70 (3): 305–16. doi:10.1002/pros.21065.

APPENDIX1:CLINICAL/LABORATORY DATA

s/n	Sample	age	DRE rpt	US rpt	PSA	EN-2	IL-6	TNF-α	Remark
1	1	67	CaP	CaP	45.15	28.4	399.4	700.8	Case
2	7	68	BPH	BPH	100	14.8	54.1	126.5	Control
3	11	67	BPH	normal	1.42	18.2	12.2	75.3	Control
4	8	65	CaP	CaP	44.65	28.8	367.9	150.8	Case
5	28	64	BPH	BPH	0.399	7.5	15.2	72.2	Control
6	64	64	BPH	blada pro	0.738	23.2	12.3	77.2	Control
7	15	61	CaP	CaP	6.4	20.6	398.6	200.3	Case
8	52	62	BPH	BPH	100	11.6	200.1	120.2	Control
9	35	63	?	BPH	0.604	12	14.1	69.5	Control
10	16	66	CaP	CaP	7.82	10.5	306.9	475.7	Case
11	59	62	BPH	BPH	22.49	2.5	24.3	71.2	Control
12	34	63	?	BPH	22.7	18	12.2	73.2	Control
13	22	73	CaP	CaP	100	21.8	65.8	148.5	Case
14	60	76	BPH	PBH	1.01	20.1	12.3	77.2	Control
15	13	73	BPH	BPH	15.72	44.1	25.1	148.4	Control
16	23	79	CaP	CaP	0.622	17.6	89.5	70.8	Case
17	48	81	BPH	BPH	14.96	26.5	15.2	73.2	Control
18	29	79	?	BPH	10.25	22.5	25.2	124.3	Control
19	32	78	CaP	CaP meta	100	17.5	5	10.1	Case
20	26	76	BPH	BPH	100	18.4	28.4	138.2	Control
21	54	77	BPH	BPH	4.36	16.5	18.2	71.2	Control
22	40	94	CaP	CaP	179.43	7	300.5	180.6	Case
23	37	89	?	BPH	45.96	14.2	17.1	80.3	Control
24	57	89	BPH	prostatis	68.89	20	75.1	100.1	Control
25	43	85	CaP	CaP	251	17.9	20.3	72.3	Case
26	21	88	BPH	BPH	7.625	37.5	12.1	77.1	Control
27	58	88	BPH	BPH	49.39	28.5	12.1	72.6	Control
28	44	67	CaP	CaP	100	17.5	20.5	77.1	case
29	31	69	BPH	BPH	5.72	>60	25.7	130.5	control
30	35	68	BPH	BPH	1	28.2	0.92	74.5	control
31	38	65	?	CaP	10.47	18	30.7	10.2	case
32	50	68	BPH	BPH	5.1	38.1	18.1	145.6	control
33	10	70	BPH	BPH	32.7	21.8	77.2	120.9	control
34	3	84	CaP	CaP	100	36	57.9	125.7	case
35	18	80	BPH	BPH	54.4	44.1	23	146.1	control
36	4	85	BPH	BPH	2.4	40.1	25.1	130.7	control
37	61	73	CaP	CaP meta	100	2.56	210.5	122.1	case
38	63	73	BPH	BPH	21.14	1.5	18.1	20.2	control
39	17	73	BPH	BPH	1.22	501	20.1	135.5	control

CASES						**CONTROLS**					
sample #	age	PSA	EN-2	IL-6	TNF-α	sample #	age	PSA	EN-2	IL-6	TNF-α
1	67	45.15	28.4	399.4	700.8	7	68	100	14.8	54.1	126.5
						11	67	1.42	18.2	12.2	75.3
8	65	44.65	28.8	367.9	150.8	28	64	0.399	7.5	15.2	72.3
						64	64	0.78	23.2	12.3	77.2
15	61	6.4	20.6	398.7	200.3	52	62	100	11.6	200.1	120.2
						35	63	0.604	12	14.1	69.5
16	66	7.82	10.5	306.9	475.7	59	62	22.49	2.5	24.3	71.2
						34	63	22.7	18	12.2	73.2
22	73	100	21.8	65.8	148.5	60	76	1.01	20.1	12.3	77.2
						13	73	15.72	44.1	25.1	148.4
23	79	0.622	17.6	89.5	70.8	48	81	14.96	26.5	15.2	73.2
						29	79	10.25	22.5	25.2	124.3
32	78	100	17.5	5	10.1	26	76	100	18.4	28.4	138.2
						54	77	4.36	16.5	18.2	71.2
40	94	179.43	7	300.5	180.6	37	89	45.95	14.2	17.1	80.3
						57	89	68.89	20	75.1	100.1
43	85	251	17.9	20.3	72.3	21	88	7.625	37.5	12.1	77.1
						58	88	49.39	28.5	12.1	72.6
44	67	100	17.5	20.5	77.1	31	69	5.72	>60	25.7	130.5
						55	68	1	28.2	9.2	74.5
38	65	10.47	18	30.7	10.2	50	68	5.1	38.1	18.1	145.6
						10	70	32.7	21.8	77.2	120.9
3	84	100	36	57.9	125.7	18	80	54.39	44.1	23	146.1
						4	85	2.4	40.1	25.1	130.7
61	73	100	2.56	210.5	122.1	63	73	21.14	1.5	18.1	20.2

APPENDIX 2 :DATA COLLECTION FORM

1) Client's ID ……. age……. date
2) Marital status; (tick) Single married divorce widower
3) Educational status: 0 1^0 2^0 3^0
4) Occupation
5) Report on the DRE

   Prostate gland enlarged: not enlarged:

6) Laboratory results:

   EN-2 concentration: ----------ng/mL

   PSA concentration: ----------ng/mL

   IL-1 concentration:----------ng/mL

   IL-6 concentration:----------ng/mL

   TNF-α concentration:----------ng/mL

7) Histological examination (tick where appropriate)

   Benign Prostatic Hyperplasia

   Malignancy –grade 1 2 3 4 5 6 7

   Inflammation

   Normal prostatic tissue

Appendix 3: Research Participant Informed Consent Form (English)

Title of Study: Correlating Engrailed-2 in urine with serum PSA to diagnose Prostate cancer at Moi Teaching and Referral Hospital.

Investigator:
Donald BURI, Department of Immunology,
School of Medicine, Moi University
P.O Box 4606, Eldoret, Kenya.
Phone number 0701374937

Informed consent. We are asking you to volunteer for a research study. Cancer is a disease that affects any organ or part of the body. Most often the cause of this disease is unknown, but characterized by uncontrolled multiplication of cell in the area affected. Prostate cancer affects the prostate gland in Men, particularly those of 50years and above. This condition can affect the way a man urinates, cause the penis not to erect, hence sexual difficulties and even death. Before you decide whether to take part in the study or not, we would like to explain the purpose, benefits, risk and what is expected of you in the study.

Purpose of the study.

Up to date, prostate cancer has been diagnosed by testing PSA in serum and collecting prostate biopsy as a confirmatory test. In this study we intend to introduce a new method where urine instead of blood/prostate biopsy will be used as a specimen. The purpose of the study is to compare the reliability of urine as a sample to diagnose prostate cancer with blood/prostate biopsy.

Procedure:

Alongside the normal DRE, blood/prostate biopsy specimens that should and will be collected from the clients, an early morning urine sample will also be collected from the participants. These samples will be analyzed in the laboratory and the different results will be compared.

Benefit: They will be no direct benefits for the participants in this study, however, the participants and others may benefit in the future as information from the study and other studies may introduce a non-invasive, less costly and a more reliable method of diagnosing prostate cancer.

Risk: The participant may experience discomfort or pain when undergoing phlebotomy. They may also develop a bruise, swelling or infection where the needle is inserted. However, well trained phlebotomist will be in-charge of sample collection to minimize the risk involve.

Confidentiality: Every effort will be made to keep personal information confidential. All information obtained in this study will be treated with strict confidentiality and will not be revealed to unauthorized persons. The participants' name will not be used in any publication made from the study.

Right to refuse or withdraw: participation in this study is entirely voluntary. There freedom not to take part or withdraw at any time.

Statement of consent and signature

I have read this form or had it read to me. I have discussed the information with the study staff and my concerns have been answered. I understand that my decision where or not to partake in the study is voluntary.

Participant name……Signature ……date……

Study staff…….Signature……..date…… …..

Witness name……Signature……...date……

Appendix 4 :Research Participant Informed Consent Form (Kishwahili)

Kichwa cha masomo: kuunganisha EN-2 katika mkojo na serum PSA to kukagaua saratani ya korodani katika hospitali ya masomo ya Moi na Refferal.

Kibali kilicho arifiwa: Twawaomba kujitolea katika uchunguzi wa masomo. Saratani ni ugonjwa ambao hudhuru sehemu yoyote ya mwili, mara nyingi chanzo cha ugonjwa haijulikani, yatambuliwa kwa chembe chembe lilizozidishwa katika sehemu iliyoathiriwa. Saratani ya korodani hudhuru kibofo cha sehemu ya ndani ya uzazi wa mwanaume sana sana wale walio na umri wa miaka hamsini na zaidi. Hali hi yaweza dhuru jinsi mwanaume anavyokojoa, kusababisha kutosimamisha uume wake, kisha baadaye ugumu katika kufanya mapenzi, alafu kifo hufuatia. Kabla hauja amua kushiriki katika somo ama hapana tungependa kuwaelezea lengo, faida, hatari na yale yanayotarajiwa katika masomo.

Lengo la somo: Hadi leo, saratani ya korodani imechunguzwa kwa kupimwa. korodani mahsusi antigeni na katika kukusanya sampuli kidogo kidogo kutoka kwa kibofu cha mwanaume, kudhibitisha kama kuna saratani. Katika somo ili twakusudia kuanzisha mbinu mpya ambapo mkojo baadala ya damu itatumiwa kama sampuli. kusudi la somo ni kulinganisha kutegemewa kwa mkojo na damu kama sampuli ya kuchunguza kuwepo kwa saratani ya korodani .

Njia: Pamoja na uchunguzi kwa mwili, uchunguzi wa sampuli ya damu, mkojo ambayo itachukuliwa mapema asabuhi, sampuli hizi litachambuliwa katika karakana na matokeo tofauti yatalinganishwa.

Faida: Hapatakua na faida inayo walenga wahusika, hata hivyo wahusika na wengine wanaweza faidika siku zijazo kama wahusika katika usaidizi wa somo na somo lingine la weza anzishwa lisilo la garama na la kutegemewa kama njia ya kupima saratani ya korodani.

Hatari: Mhusika anaweza hisi hali isiyo ya kawaida ama uchungu anapopitia uchunguzi. Pia anaweza kua na chubuko, kufura ama ambukizo wakati amedungwa sindano. Hata hivyo, walio hitimu vizuri watasimamia katika ukusanyaji wa sampuli kupunguza hatari.

Yenye Siri: Juhudi zitaimarishwa katika kuweka mambo yote kuwa siri na wala haziwezi fichuliwa kwa mtu yeyote.
Wasio ruhusiwa: Majina ya wahusika hayatumika katika matangazo yote yatakayo patikana katika somo.
Hali ya kukata ama kujiondoa: Kuhusika katika masomo haya ni kwa kujitolea kabisa, kuna uhuru kuhusika ama kujiondoa wakati wowote.

Neno la kukubali na sahili: Nimesoma sura hii ama nimesomewa nimejadiliana somo na watendakazi wa somo hili na haja zangu zimejibiwa. Nimeelewa kwamba uamuzi wangu kuhusika katika somo hili ni kwa kujitolea.

Mhusika Jina……mhusika sahihi……..tarehe……

Somo
Mtenda
kazi…….Sahihi……Tarehe…….

Mashahidi……...……..Sahihi……Tarehe……
……

APPENDIX 5:IREC APPROVAL

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
COLLEGE OF HEALTH SCIENCES
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/202
Approval Number: 000774

9th March, 2012

Mr. Bongkiyung Donald Buri,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET- KENYA.

Dear Mr. Bongkiyung,

FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"Correlating Engrailed – 2 in Urine with Serum PSA to Diagnose Prostate Cancer at Moi Teaching and Referral Hospital, Eldoret".

Your proposal has been granted a Formal Approval Number: **FAN: IREC 000774** on 9th March, 2012. You are therefore permitted to start your study.

Note that this approval is for 1 year; it will thus expire on 8th March, 2013. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc:	Director	-	MTRH
	Principal	-	CHS
	Dean	-	SOM
	Dean	-	SPH
	Dean	-	SOD
	Dean	-	SON

Appendix 6: MTRH APPROVAL

MOI TEACHING AND REFERRAL HOSPITAL

Telephone: 2033471/2/3/4
Fax: 61749
Email: director@mtrh.or.ke

P. O. Box 3
ELDORET

Ref: ELD/MTRH/R.6/VOL.II/2008

9th March, 2012

Mr. Bongkiyung Donald Buri,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET – KENYA.

RE: APPROVAL TO CONDUCT RESEARCH AT MTRH

Upon obtaining approval from the Institutional Research and Ethics Committee (IREC) to conduct your research proposal titled:

"Correlating Engrailed – 2 in Urine with Serum PSA to Diagnose Prostate Cancer at Moi Teaching and Referral Hospital, Eldoret".

You are hereby permitted to commence your investigation at Moi Teaching and Referral Hospital.

DR. J. KIBOSIA
DIRECTOR
MOI TEACHING AND REFERRAL HOSPITAL

CC - Deputy Director (CS)
- Chief Nurse
- HOD, HRISM

Appendix 7: APPROVAL ADENDMENT

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/202
Approval Number: 000774

13th December, 2013

Mr. Bongkiyung Donald Buri,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS APPROVED 13 DEC 2013 P.O. Box 4606 ELDORET

Dear Mr. Bongkiyung,

RE: CONTINUING APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:

"Correlating Engrailed – 2 in Urine with Serum PSA to Diagnose Prostate Cancer at Moi Teaching and Referral Hospital, Eldoret."

Your proposal has been granted a Continuing Approval with effect from 13th December, 2013. You are therefore permitted to continue with your study.

Note that this approval is for 1 year, it will thus expire on 12th December, 2014. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE.

cc:

Director	-	MTRH
Principal	-	CHS
Dean	-	SOM
Dean	-	SPH
Dean	-	SOD
Dean	-	SON

Appendix 8: Approval of amendment

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/202
Approval Number: 000774

12th February, 2014

Mr. Bongkiyung Donald Buri,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
12 FEB 2014
APPROVED
P. O. Box 4606-30100 ELDORET

Dear Mr. Bongkiyung,

RE: APPROVAL OF AMENDMENT

The Institutional Research and Ethics Committee has reviewed the amendment made to your proposal titled:-

"Correlating Engrailed – 2 in Urine with Serum PSA to Diagnose Prostate Cancer at Moi Teaching and Referral Hospital, Eldoret".

We note that you are seeking to make an amendment as follows:-

1. Results from digital rectal examination (DRE), pelvic and abdominal ultrasound and/or histological report from prostate biopsy will be used to differentiate those with prostate cancer from those with only benign prostatic hyperplasia (BPH) and prostatitis as opposed to using only histological report from prostate biopsy.

The amendment has been approved on 12th February, 2014 according to SOP's of IREC. You are therefore permitted to continue with your research.

Note that this amendment approval will expire on the date of expiry of your Formal Approval. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change(s) or amendment(s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

DR. W. ARUASA
DEPUTY-CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc:	Director	-	MTRH	Dean	-	SPH
	Principal	-	CHS	Dean	-	SOD
	Dean	-	SOM	Dean	-	SON