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Prostate Cancer Microparticles as a Next Generation Screening Tool for Prostate Cancer

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Surgery

THE PROSTATE CANCER MICROPARTICLES AS A NEXT GENERATION SCREENING TOOL FOR PROSTATE CANCER»

(Thesis format: Integrated Article)

by

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Graduate Program in Surgery

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Abstract

Currently available screening tests for prostate cancer (PCa) are neither very sensitive nor specific. Microparticles (MP) are submicron tumor cell fragments released by PCa cells into the circulation and offer a possible means of sampling the tumor. We evaluated the utility of a MP blood test using nanoscale flow cytometry to distinguish patients with PCa from patients with benign prostatic hyperplasia (BPH). We used monoclonal antibodies against prostate specific membrane antigen, gastrin releasing peptide receptor and ghrelin peptide ligand.

We found higher but statistically insignificant, PSMA and Ghrelin dual positive MP counts in the BPH group. Our results show that although MP can be enumerated, better more exclusive surface antigens and different antibodies are required to test the utility of MP for this to be used as a "Next Generation Screening Tool" for PCa. In addition, comparison with a more suitable control group would improve accuracy of the experimental test.

Keywords

Prostate Cancer, Biomarkers, Microparticles, Nanoscale Flow Cytometer, Prostate Specific Membrane Antigen, Ghrelin Peptide Ligand, Growth Hormone Secretagogue Receptor, Gastrin Releasing Peptide Receptor

Co-Authorship Statement

Chapter 2: Pilot Study: Enumeration of Prostate Cancer Microparticles as a Tool to Identify Prostate Cancer

The experiment presented in this chapter was performed in the laboratory of Drs. Ann Chambers and Hon Leong. In this experiment samples were obtained from the patients of Dr. Nicholas Power and Ontario Cancer Institute of Research. The concept of this study was designed by Dr. Leong and funded through his grant (Prostate Cancer Canada). Dr. Leong and his laboratory technician, Colleen Biggs invented the microparticle assay and performed the initial optimization of this test. Both of them taught the technique and helped in analyzing the samples for this pilot study. Dr. Michele Billia also helped in analyzing the samples. Drs. Joseph Chin and Nicholas Power provided clinical insights and helped in writing of abstract and presentations of this pilot study in various conferences.

Chapter 3: Prostate Cancer Microparticles as a Next Generation Screening Tool for Prostate Cancer

The experiment presented in this chapter was performed in the laboratory of Drs. Ann Chambers and Hon Leong. The samples used in this study were obtained from the patients of Dr. Nicholas Power, Ontario Cancer Institute of Research and Princess Margaret Hospital-GU Bio-bank. The concept of this study was designed by Dr. Leong and funded through his grant (Prostate Cancer Canada. Drs. Michele Billia and Sohrab Ali helped in analyzing the samples. Through all experiments Dr. Hon Leong provided insights into the interpretation of the data collected and thesis preparation. Drs. Joseph Chin and Nicholas Power had an active involvement in the development of the experiment, interpretation and statistical analysis of the data and preparation of the thesis.

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The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

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Chapter 1

1 Introduction

1.1 General Overview

Prostate Cancer (PCa) is leading the list of all newly diagnosed visceral cancers in men, and each year it is responsible for approximately 9.3% of all cancer related deaths (Jemal *et al*. 2010). Currently, detection of PCa relies on a blood test known as Prostate Specific Antigen (PSA), digital rectal examination (DRE), and transrectal ultrasound guided biopsy (TRUS) of prostate. PCa is among the few solid organ malignancies which have a surrogate tumor marker to detect the disease and monitor its course. Although PSA is a highly sensitive and specific marker in the post treatment setting, especially post radical prostatectomy, it has a very low sensitivity and specificity as a screening tool. In 2011, the U.S. Preventive Services Task Force (USPSTF) drafted a recommendation against the routine use of PSA screening at any age and in October 2012 the USPSTF website posted that *"Prostate cancer is a serious health problem that affects thousands of men and their families. But before getting a PSA test, all men deserve to know what the science tells us about PSA screening: there is a very small potential benefit and significant potential harms. We encourage clinicians to consider this evidence and not screen their patients with a PSA test unless the individual being screened understands what is known about PSA screening and makes the personal decision that even a small possibility of benefit outweighs the known risk of harms."* (USPSTF Co-Chair Michael LeFevre, M.D., M.S.P.H. May 22, 2012).

A large prospective study from Europe Organization for Research and Treatment of Cancer (EORTC) looking at the role of PSA based screening program, ERSPC trial (European Randomised Study of Screening for Prostate Cancer) in reduction of mortality reported that a very large number of men need to be screened (1410 men) in order to save one life (Schroder *et al.* 2009). The Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial, also investigating the usefulness of PSA as a screening tool, showed that the incidence of death per 10,000 person-years was 2.0 (50 deaths) in the PSA based screening group and 1.7 (44 deaths) in the control group (Andriole *et al.* 2009). Both of these studies demonstrated only a small benefit with PSA based screening, thus underlining the point that PSA is not an ideal screening test. There is no absolute cutoff level defined for PSA based screening, with the majority of clinicians using the classic threshold of >4 mg/ml (Catalona *et al.* 1991). However, a large study looking at 2950 men with PSA of < 4mg/ml found that 15.2% of men with PSA's in this range were later found to have PCa (Thompson *et al.* 2004). Currently, based on "suspicious PSA", almost one million men in the USA undergo prostate biopsy each year and are exposed to significant complications (Welsh *et al.* 2007). Prostate biopsy carries a risk of potential complications such as hematuria and urinary tract infections and is reported to have a 6.9% hospitalization rate (Loeb *et al.* 2011). The current standard of performing a prostate biopsy is for systematic sampling of the prostate using TRUS guidance. Previously, only six cores (sextant) of prostate were obtained. However, this was found to be inadequate and today, most centers perform a 10-12 core biopsy. This scheme of biopsy has improved the detection rate to around 50% (Martinez *et al.* 2013). Therefore,

the majorities of men with suspicious PSA levels actually do not have PCa and are unnecessarily exposed to the risks of biopsy.

To meet the challenge of developing a screening test superior to PSA, we propose a blood test based on prostate cancer microparticles (PCMP). Microparticles (MP) are submicron (< 1μm) particles released from cells during their growth, malignant transformation or apoptosis (Rak, 2013). MP are released in the circulation and express surface receptors inherited from their cells of origin (Andreu *et al.* 2014). We enumerated PCMP using a combination of monoclonal antibodies (mAb) namely prostate specific membrane antigen, gastrin releasing peptide receptor and ghrelin peptide ligand which have been shown to bind to the extracellular portion of the receptors inherited by the MP derived from the PCa cells. We used these PCMP counts to distinguish between plasmas from patients with localized PCa and benign prostatic hyperplasia (BPH). The MP based test has the potential of functioning as a "fluid biopsy" which can continually sample the primary tumor to gain insight regarding the biology of these tissues.

1.2 Prostate Cancer

Prostate cancer (PCa) accounts for 27% of all newly diagnosed visceral malignancies among men in the USA (Siegel *et al.* 2014). In 2014, it is estimated to affect 233,000 men in the USA and will account for 10% of all male cancer related deaths in men (DeSantis *et al.* 2014). The Canadian Cancer Society estimates that 23,600 men will be diagnosed with PCa in 2014, representing 24% of all new cancer cases in men and nearly 4,000 men will die from PCa in Canada. This translates into 65 Canadian men being

diagnosed and approximately 11 men dying of PCa every day (www.cancer.ca). These statistics place prostate cancer as one of the leading cancers affecting males in North America. Prostate cancer typically has a long course, thus making it a major consumer of the health care budget. Overall, the lifetime risk of developing PCa is about 16.7%; therefore one in six men will be diagnosed with prostate cancer in their life time. The incidence of harboring a focus of clinically insignificant PCa is even higher as autopsy studies performed on prostate glands obtained from men dying of all causes has shown that 20% of men aged 50 to 60 years and 50% of men, aged 70 to 80 years, have histologic evidence of carcinoma prostate (Carter *et al.* 1990). This disparity between clinically significant cancer and incidental or indolent cancer has led to numerous efforts to risk stratify this disease. None are perfect and study in this area is the major focus of prostate cancer research. Naturally, this uncertainty provokes a genuine anxiety among men at the risk of developing prostate cancer (Kotwal *et al.* 2012).

The definitive reason for development of PCa is not yet known. However, there are several risk factors which increase the life-time risk of developing PCa. Risk factors include family history, ethnicity, diet, and environmental factors (Crawford, 2003). The risk of prostate cancer doubles among men having a first-degree relative with PCa and rises up to eightfold or greater if both a first and second degree relative previously had prostate cancer. Ethnicity is also a risk factor, as a wide variation in incidence has been reported between different ethnic groups. **Table 1** shows the incidence and mortality of PCa among men in different ethnic populations (Campbell-Walsh Urology, tenth edition, 2010). Asian men have the lowest mortality rates and highest incidence and mortality rates are observed in African–American men. Studies looking at the incidence of PCa

among immigrants, moving from an area of low incidence to higher incidence locations have reported significant increase in incidence of PCa in the immigrants compared to the natives in their country of origin. The increase in the incidence of PCa is more if immigration happened earlier in life of an individual. The higher incidence is also seen in the second generation of immigrants originating from areas of lower incidence.. This signifies the influence of environmental factors in development of cancers (Shimizu *et al.* 1991).

Prostate Cancer Incidence and Mortality by Race/Ethnicity, United States, 2000–2004

	INCIDENCE*	MORTALITY*
White	161.4	25.6
African-American	255.5	62.3
Hispanic/Latino	140.8	21.2
Asian-American and Pacific Islander	96.5	11.3
American Indian and Alaska Native	68.2	21.5

Table 1. Incidence and mortality rate of prostate cancer in different ethnic groups (Adopted from Wein AJ, Kavoussi LR, Novick AC, Partin AW & Peters CA, eds. Campbell-Walsh Urology, 10th Edition. Philadelphia: Saunders, 2010.

A high dietary intake of red meat, animal and polyunsaturated fats and milk appear to increase the risk of prostate cancer whereas, fruit and vegetables and polyphenols may be of preventive value for development of PCa. A review of dietary factors influencing the risk of PCa does not suggest any conclusive evidence to elucidate the role of the above agents in the development or prevention of PCa (Mandare *et al.* 2014). Anecdotal experiences and small nonrandomized studies have long promoted Selenium, Lycopene and Vitamin E as dietary supplements to prevent the development of PCa. To provide conclusive answer to this query a large prospective, multicenter study was designed. This Selenium, lycopene and Vitamin E Cancer Prevention Trial (SELECT) found no preventive effects of any of these substances in the development of prostate cancer (Klein *et al.* 2011). On the contrary, it provided some evidence that Vitamin E in high dose actually increased the risk of development of PCa.

The prostate gland is a walnut-sized structure located at the bladder neck, and the urethra passes through the center of this gland. **Figure 1** illustrates the anatomic location and relationship of prostate gland in a male pelvis (Campbell-Walsh Urology, tenth edition, 2010). The function of the prostate gland relates to its secretory role that nourishes and protects sperm during insemination. As this gland is located just anterior to the rectum, it can be easily palpated during digital rectal examination.

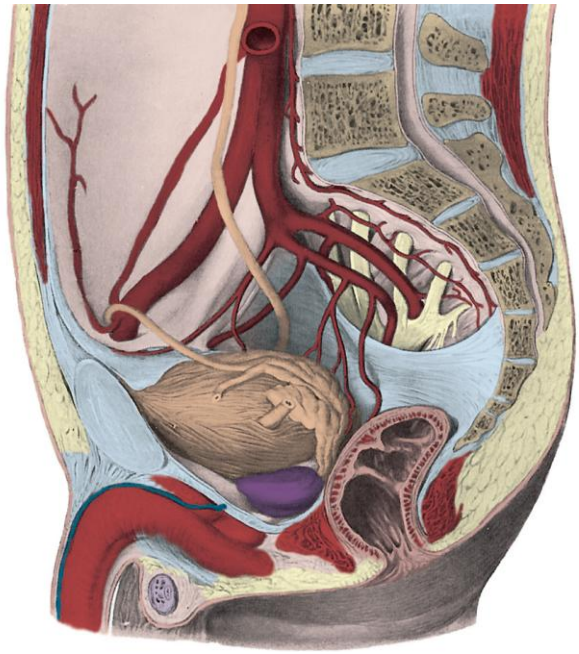

Figure 1. Anatomical location of the prostate gland. The gland is shown in purple color lying at the base of the urinary bladder and just anterior to the rectum. Adopted from Wein AJ, Kavoussi LR, Novick AC, Partin AW & Peters CA, eds. Campbell-Walsh Urology, 10th Edition. Philadelphia: Saunders, 2010.

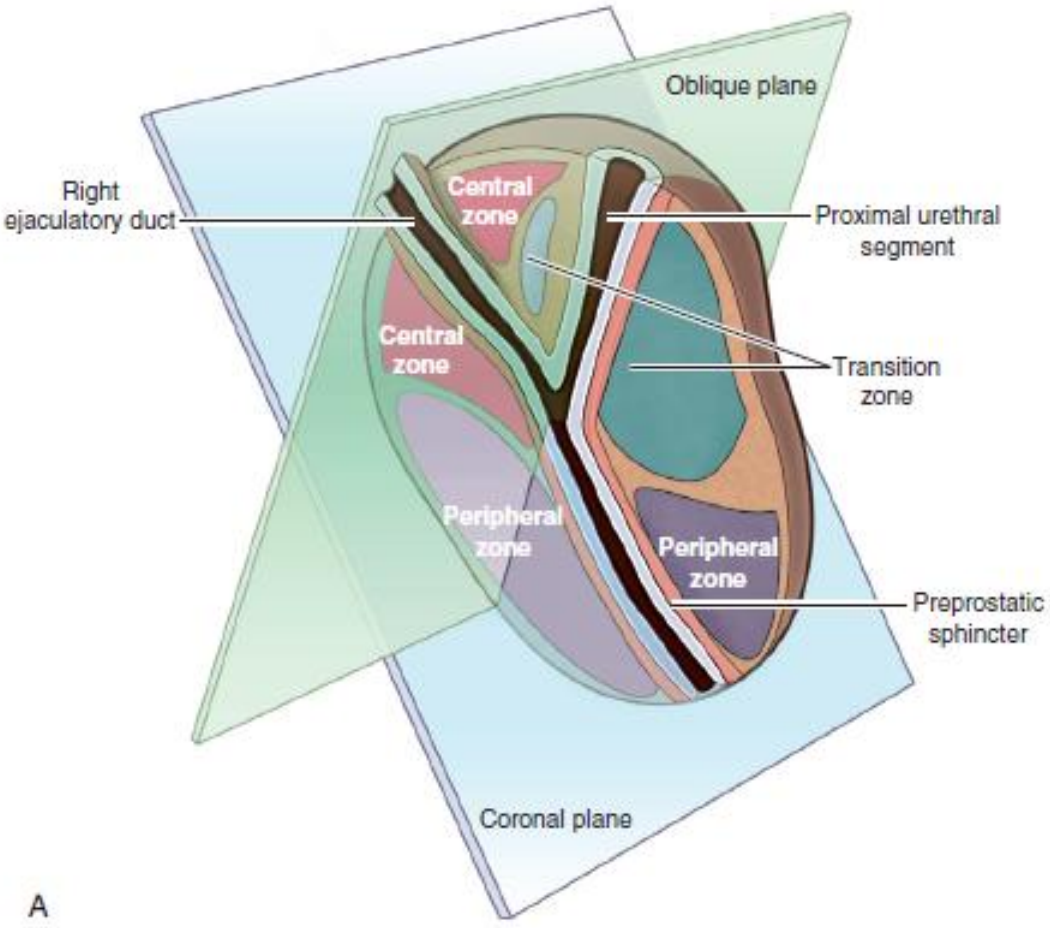

Figure 2. Zonal anatomy of prostate showing arrangement of zones of prostate. Adopted from Wein AJ, Kavoussi LR, Novick AC, Partin AW & Peters CA, eds. Campbell-Walsh Urology, 10th Edition. Philadelphia: Saunders, 2010.

McNeal, in his original description of the zonal anatomy of prostate gland, described four basic anatomic zones (McNeal, 1980). This description was further modified later and **Figure 2** shows the current understanding of the zonal anatomy of the prostate gland (Campbell-Walsh Urology, tenth edition, 2010).

Prostate carcinoma typically arises from the peripheral zone and cancer cells originate mainly from the secretory cells. Thus, prostate cancer is classified as 'Acinar adenocarcinoma'. Histologically, prostate carcinoma can range from well-differentiated tumors which simulate normal prostatic glands, to poorly differentiated lesions which have completely lost the glandular architecture and cannot be easily recognized as descending from prostatic origin. A numeric grading system was introduced by Dr. Donald Gleason to grade the degree of loss of differentiation (Gleason, 1974). PCa exhibits a variety of histological patterns within a prostate cancer specimen. The Gleason grading system incorporates this unique characteristic, in which the most predominant pattern is called 'Gleason Major' and the second most common is called 'Gleason Minor'. Gleason score, a sum of Gleason Major and Minor is assigned to pathological specimen incorporating both the patterns. This numeric score ranging from 2 to 10, out of a maximum of 10, is meant to reflect the histological pattern accounting for biological aggressiveness. The International Society of Urological Pathology made some modifications to this system in 2005 and this system is still prevalent in grading of PCa (Epstein *et al.* 2005). **Table 2** illustrates the patterns with assignment of grades according to the 2005 Modification of International Society of Urological Pathologist (Campbell-Walsh Urology, tenth edition, 2010).

2005 International Society of Urological Pathology Modified Gleason System

Pattern 1

Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini (larger glands than pattern 3)

Pattern 2

Like pattern 1, fairly circumscribed, yet at the edge of the tumor nodule there may be minimal infiltration

Glands are more loosely arranged and not quite as uniform as Gleason pattern 1

Pattern 3

Discrete glandular units

Typically smaller glands than seen in Gleason pattern 1 or 2

Infiltrates in and amongst non-neoplastic prostate acini

Marked variation in size and shape

Pattern 4

Fused microacinar glands

Ill-defined glands with poorly formed glandular lumina

Large cribriform glands

Cribriform glands

Hypernephromatoid

Pattern 5

Essentially no glandular differentiation, composed of solid sheets, cords, or single cells

Comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses

Table 2. Modified Gleason's histological grading system Adopted from Wein AJ, Kavoussi LR, Novick AC, Partin AW & Peters CA, eds. Campbell-Walsh Urology, 10th Edition. Philadelphia: Saunders, 2010.

Prostatic carcinoma can metastasize through lymphatic or hematogenous dissemination. Bone metastases are the most common site of hematogenous spread. Lymphatic metastases occur frequently to the obturator lymph nodes (Campbell-Walsh Urology, tenth edition, 2010).

Prostate cancer is staged according to the guidelines of the 7^{th} edition of American Joint Committee on Cancer (AJCC). This staging system is commonly referred to as TNM system. T stage refers to the volume of disease or the local extend of tumor, N reflects the lymph node status and M categorize the metastasis, if present. Clinical TNM stage based on local examination and imaging results is commonly referred to as cTNM. The pathological TNM or pTNM, however, is the final stage assigned after histological evaluation of the surgically removed prostate gland, along with regional lymph nodes. **Table 3** illustrates the details of TNM staging system (Campbell-Walsh Urology, tenth edition, 2010).

Tx	**Primary Tumor could not be assessed**
T0	**No evidence of Tumor**
T1	**Tumor not palpable**
T2a	**Tumor palpable in less than half of one lobe**
T2b	**Tumor palpable in more than half of one lobe**
T2c	**Tumor palpable in both lobes**
Nx	**Nodal metastasis cannot be assessed**
N0	**No nodal metastasis**
N1	**Nodal metastasis in single node less than 2 cm**
N2	**Nodal metastasis in single/multiple nodes less than 5 cm**
N3	**Nodal metastasis in multiple nodes more than 5 cm**
Mx	**Distant metastasis cannot be assessed**
M0	**No distant metastasis**
M1a	**Involvement of non regional lymph nodes**
M1b	**Involvement of bones**
M1c	**Involvement of other sites**

Table 3. Tumor staging (TNM) according AJCC 7th edition

In the last three decades there has been a migration in the stage at which PCa presents to the health care provider (Scosyrev *et al.* 2012). Most of the patients now present with asymptomatic and early stage cancer. Previously, a majority of patients were diagnosed with symptomatic bony metastasis or urinary obstruction. In the nineties, a blood test (PSA) was introduced in clinical practice that led to larger numbers of patients being diagnosed at an earlier and potentially curable stage. Prostate biopsy is now mostly recommended due to an abnormal PSA test. PSA is not a true tumor maker as such a marker should only be raised in a malignant condition but PSA may be elevated in a variety of other noncancerous prostate conditions. Interestingly, this PSA induced stage migration has had a very little impact on reducing the overall mortality of PCa. This observation led to the effort to critically appraise the role of PSA in reducing the mortality of PCa. In 2011, the U.S. Preventive Services Task Force (USPSTF) recommended against the routine use of PSA screening at any age. This recommendation was based mainly on the results of two large prospective studies demonstrating that a large number of men need to be screened (NNS) with PSA to detect PCa and prevent cancer related death. The EORTC study showed that 1410 men need to be screened and 48 men needed to be treated (NNT) in order to prevent one death related to PCa (Schroder *et al.* 2009). Although, the recent publication of extended follow-up (13 years) of this cohort has shown improvement in the NNS (Schroder *et al*. 2014), PSA still does not fulfill any currently acceptable standards for an ideal screening test. Urgent efforts are required to develop a new screening test for detection of PCa.

1.3 Prostate Specific Antigen (PSA)

PSA is a glycoprotein enzyme from the kallikrein-related peptidase family and is produced by prostatic epithelial cells. It is also known as gamma-seminoprotein or kallikrein-3 (KLK3). The physiological role is in the liquefaction of semen. It helps in dissolving the coagulum and allows the sperm to swim freely for insemination.

Prostate specific antigen was first purified in humans in 1979 (Wang *et al.* 1979). Prior to this, prostate acid phosphatase (PAP) was used as a marker for PCa. PAP was non-specific for prostate cancer and was elevated in a variety of other conditions like Paget's disease. Prior to the establishment of PSA as a blood-based tumor marker, it was shown to be an exclusive immunohistologic marker for prostatic cancer. In an experiment using histological sections, it was observed that all sections from primary and metastatic prostatic cancer reacted positively with PSA, whereas non-prostatic neoplasms did not stain with PSA (Nadji *et al.* 1981). Later, PSA was established as a blood test for monitoring the response of treatment for PCa (Stamey *et al.* 1987). Soon thereafter, PSA was also introduced as a screening tool for early detection of PCa (Catalona *et al.*1991). Using a cutoff of 4mg/ml, it was shown that this test was able to detect asymptomatic PCA in 22 % cases. Other studies looking at the performance of PSA as a screening tool have shown variable results and depend on the PSA cutoff used for screening. For example, increasing the cutoff of PSA to higher levels improves the predictive value. However, if a higher cutoff is used then the number of cancers detected at early stage decrease and more cancers are diagnosed at an advanced stage. The lack of specificity at lower cutoffs subject many patients with elevated PSA, often secondary to noncancerous causes, to a prostate biopsy. This underscores the fact that PSA is able to identify patients

with prostate disease and is not specific for PCa. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve is between 0.56 and 0.70 for the ability of PSA to identify patients with cancer, where a score of 1.0 is perfect discrimination and 0.5 is a coin toss (Brawer *et al.* 1999). Common non-cancerous causes of elevated PSA levels are prostatic infection, trauma, and BPH. Studies have also shown that using the classic cutoff of 4mg/ml will result in missing significant number of prostate cancer cases (Schroder *et al.* 2008). If the cutoff of < 4mg/ml is considered as a negative test, a large study found 15.2% men in this group were actually later diagnosed to have prostate cancer, and according to AUA risk classification, 14.9% of these patients exhibited high risk disease (Thompson *et al.* 2004).

To improve the predictive value of the PSA test, a number of dynamics of PSA have been tested. PSA density (PSAD) is one such tool. The calculations for PSAD are based on the concept that the normal prostate gland produces PSA but at lower concentrations. Each gram of normal prostate gland contributes to the total PSA but if there is a focus of cancer in the gland it produces significantly higher PSA. The total weight of the prostate is calculated by transrectal ultrasound and a predicted PSA is calculated by multiplying it by 0.12. Any value above the predicted value is suggestive of PCa. In one study, no cases of BPH were found in men with PSAD of > 0.1 mg per each gram of prostate (Benson *et al.* 1992). However, 2 out of 41 patients with prostate cancer had a PSAD of 0.05mg/gm or less. PSAD is a great tool to rule out BPH in men with high PSAD but fails to reliably rule out PCa, when PSAD is low. Recently, the role of PSAD is also being highlighted in the follow up of patients with low risk PCa managed with active

surveillance. A higher PSAD is found to be positively associated with the risk of progression and need for active treatment (Welty *et al.* 2014).

Age related PSA is another method to improve diagnostic efficiency. These age-specific reference ranges are designed to enhance the predictive value of PSA as a more discriminating tumor marker for detecting clinically significant cancers in older men (increasing specificity) and to find more potentially curable cancers in younger men (increasing sensitivity) (Oesterling *et al.* 1993).

PSA in circulation exists in both free form and complex form, bound with alpha 1-antichymotrypsin (ACT). Complex PSA is raised in PCa (Leinonen *et al.* 1993). A free to total PSA ratio of < 15 to 20% is generally considered to increase the risk of cancer (Oesterling *et al.* 1995). However, there is no exact watershed level. PSA velocity, the rate at which the PSA rises, is also used to improve the predictive value, where if the PSA increases by >0.75mg/ml per year it is considered to be an indication for prostate biopsy (Carter *et al.* 1993).

Extensive research in finding ways to promote the utility of PSA as a screening tool has failed to develop a perfect model capable of confidently selecting men suitable for aggressive and life-saving treatment.

1.4 Trans-rectal ultrasound (TRUS) guided Biopsy

This is the main modality of obtaining prostate tissue for histological diagnosis. Transrectal ultrasound is used as a guide to obtain systematic samples from the prostate. A probe is introduced into the rectum and a needle biopsy gun is used to obtain core

biopsies. Specific templates are used to systematically sample the prostate gland. Additional samples may be obtained from sonographic or clinically suspicious areas.

The chances of finding a focus of prostate cancer on TRUS biopsy increases with the number of cores taken but this may also increase the risk for complications which include hematuria, rectal bleeding and urinary tract infection. In North America, more than 1 million prostate biopsy procedures are performed each year, with approximately 25% being positive for cancer and approximately 75% negative for cancer (Thompson *et al.* 2004). The risk of complications after a prostate biopsy is not trivial, as hematuria occurs in 66% patients and rectal bleeding in 9% patients. Urinary tract infections occur in 0.5 to 3% patients and in rare cases these can lead to mortality. SEER- Medicare data reveals that the 30-day hospitalization rate after TRUS biopsy was 6.9%, which was significantly greater than the 2.7% risk of hospitalization in the control population (Loeb *et al.* 2011). Even after adjusting for age, ethnicity and comorbidities, prostate biopsy is associated with a 2.65-fold (95% CI 2.47-2.84) increased risk of hospitalization within 30 days, $p < 0.0001$ (Loeb *et al.* 2011). Interestingly, the risk of infectious complications requiring hospitalization after biopsy was significantly greater in more recent years, probably a reflection of a trend to acquire more biopsy cores. Increasing the number of cores of the biopsy increases the sensitivity but also increases the risk of complications. It is now recommended to have at least 10 cores for prostates <50 gms and up to 18 cores for larger glands (Chun *et al.* 2010). But even increasing the number of cores to >20, does do not eliminate the possibility of missing a focus of carcinoma and this may often require a repeat biopsy. If we develop a better marker of the diagnosis of PCa, we may be able to prevent a large majority of men undergoing a prostate biopsy. This test is critically

needed for screening PCa. In theory, every additional 5% increase in accuracy rates for such a prostate screening test would eliminate approximately 165,000 unnecessary biopsies and 6,930 hospitalizations each year in North America. This would potentially result in significant health care savings and improve the quality of life of our patients.

To meet the challenge of a screening test superior to PSA, we propose a prostate microparticle-based "fluid biopsy" which continually samples the prostate and its primary tumor. The microparticles may contain biological information from the parent cells and may help to gain insight regarding the biology of these tissues without the need of obtaining a tissue sample.

1.5 Microparticles

Microparticles (MP) are defined as plasma membrane derived structures of a diameter of less than 1000nm. They are released from the cell upon activation, death, apoptosis or malignant transformation (Rak, 2013). The release of microvesicles was first described in 1967 by Wolf while studying the particulate dust formed by active platelets (Wolf, 1967). MP are released directly from the cell membranes whereas, exosomes are enveloped by a inverted cell membrane that was first internalized and then released. Cell cultures from various normal and neoplastic cell lines have shown that exfoliated vesicles are present in the cell culture medium (Trams *et al.* 1981). These microparticles were found to contain significantly higher amounts of sphingomyelin (SM) and total polyunsaturated fatty acids. This study concluded that the shed MP constitute a select portion of the plasma membrane. Examination by electron microscopy showed the vesicles had an average diameter of 500 to 1000 nm. These particles were previously

labeled as “cell dust” or “cell garbage”, but advancements in imaging technology has enabled us to understand them better and this has opened up new avenues of research (Leong *et al.* 2011).

Living cells, through outward blebbing of the plasma membrane, generate MP from the region of membrane lipid rafts. They exhibit high levels of exposed phosphatidylserine (PS), integrins and metalloproteinases (Rak, 2013).

The process of the generation of a MP starts with vesiculation, initiated by focal and short-lived alterations in the plasma membrane phospholipids brought about by enzymatic changes. These changes produce a focal asymmetry, which is seen as a tentacle or a bleb. Some of these tentacles are shed off and enter the circulation. This is an energy-dependent and active mechanism, which maintains an architectural uniformity of the cell. This process guarantees that phosphatidylcholine and SM remain on the outer surface of the plasma membrane and phosphatidylserine, and phosphatidylethanolamine are present on the inner side. This action is brought about by lipid-translocating enzymes (flippases). Calcium is an integral part of this process because cytosolic calcium initiates significant changes in the state of the enzymes responsible for generation of MP. Aminophospholipid translocase, scramblase, gelsolin, lipid, floppase, and calpain are the main enzymes identified to play an important role in this process (Rak, 2013). This ultimately results in externalization of PS and changes in the membrane geometry. These cytoskeletal interactions are critical for the formation of the tentacles or blebs, which ultimately leads to the formation and release of a MP.

MP can be visualized using electron microscopy for morphological characterization. Gold-labeled immune electron microscopy was first used to assess the urinary MP (Mitchell *et al.* 2009). With the microparticles fluorescently labeled, MP can also be visualized indirectly using confocal microscopy (CM). However, MP are too small for direct visualization with standard CM. The lipophilic fluorescent dyes conjugated with antibodies against the antigens expressed on the plasma membranes of the MP can help detect them with relative ease. Dr. Leong, at our laboratory has validated the presence of MP analyzed using flow cytometry (Leong *et al.* 2011) and by atomic force microscopy (Leong *et al.* 2010). **Figure 3** shows atomic force microscopy images of prostate cancer microparticles.

The exact role of these MP is still elusive but there is great enthusiasm in the scientific community to explore the role of these structures in the vesicular transportation system both for intracellular and extracellular communication. In 2013, James Rothman, Randy Schekman and Thomas Sudhof were presented with the Nobel Prize for their work on the role of micro vesicles for intracellular transportation. Similarly, plasma membrane derived microparticles may also have a role in the extracellular transportation/communication (Camussi *et al.* 2010).

As with other newly discovered structures, these < 1 μm structures have been described in the literature using a variety of terminologies; microparticles, microvesicles, exosomes, oncosomes, secretory vesicles, ectosomes are a few examples. The terminologies have mostly originated from either the disciplines in where they have been studied or more recently based on the size. The most widely studied structures in this

category are the Exosomes that have a size up to 500nm (Pan, 1983) and have an established role in cellular communication.

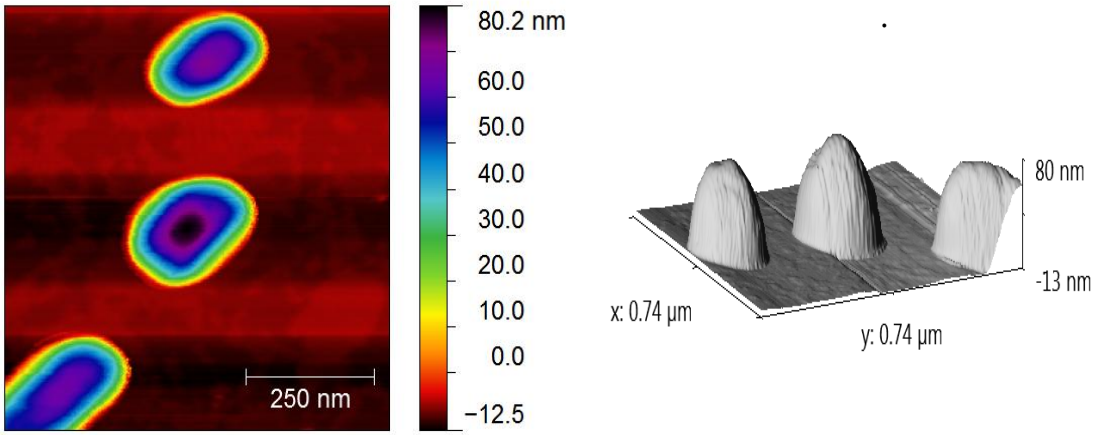

Figure 3 Atomic force microscopy images depicting the structure of individual prostate specific membrane antigen (PSMA) positive microparticles

However, the mechanism of release of exosomes is very different as they are first internalized and then reprocessed for release (Duijvesz *et al.* 2011). Ectosomes are plasma cell derived structures but are mainly released as a result of apoptosis (Diamant *et al.* 2004). To avoid use of confusing terminologies we will refer to microvesicles, exosomes, oncosomes, secretory vesicles and ectosomes as 'Microparticle (MP)' in our discussion. There is growing consensus that MP is the best-suited terminology to describe these biologically diverse structures.

The exact physiological importance of these structures in the development of cancer is not known. MP are enriched with specific antigens (Clayton *et al.* 2009). Elegant experiments in immunology have shown that MP affects the immune system by expressing and processing antigens (Raposo *et al.* 1996). As MP retains the surface characteristics of parent cells they may have a role in antigen presentation and immunomodulation. They may play a role in either promotion or prevention of metastasis. Assuming this is the mechanism of their release into the circulation, this feature may be exploited to develop diagnostic markers, which can function as a "fluid biopsy" of the entire gland.

Currently, prostate cancer microparticles is an emerging topic in oncology research, but many researchers lack the instrumentation needed to translate this work into clinical applications. Enumeration of MP in a sample remains a challenge. The number of MP is generally estimated by measuring the amount of protein (Nilsson *et al.* 2009).

Fluorescence-activated cell sorting (FACS) technology i.e. flow cytometer is capable of enumerating MP but identification of organ specific MP, which are relatively tiny compared to a cell, is difficult using a regular flow cytometry system due to the limitations of laser detectors. Other visualization techniques such as electron microscopy (EM) and confocal microscopy (CM) described previously are good for morphological characterization but cannot perform quantitative assays in a high throughput manner. A combination of enzyme-linked immunosorbent assay (ELISA) for exosome quantification has been used (Logozzi *et al.* 2009). In this experiment they used two different transmembrane proteins that are present on all exosomes. They postulated that by using one general transmembrane protein or so called 'capture protein', it is possible to identify exosomes and by using 'tissue- or cancer-specific' transmembrane protein, the number of exosomes derived from a specific tissue can be measured.

We used a similar hypothesis and selected prostate specific membrane antigen (PSMA) as the capture marker for identification of prostate specific MP. For cancer specific marker we selected ghrelin peptide and gastrin releasing peptide receptor (GRPR). We postulated that a combination of more than two markers would improve our ability to select the population of prostate cancer MPs. We proposed to do this using flow cytometry. Improvement in this technology with development of Agogee, A-50 nanoscale flow cytometer (® Apogee Flow systems, Hertfordshire, UK) has provided us the capacity to enumerate particles of less than one micron.

1.6 Instruments

We used a specialized instrument that analyzes cell fragments in a high-throughput, multi-parametric manner. This nanoscale flow cytometer is manufactured by Apogee Flow systems Incorporation, Hertfordshire, UK. The "Apogee A-50 micro nanoscale flow cytometer" shown in **Figure 4**, is specifically designed to analyze and enumerate cancer microparticles. This machine has three lasers installed; the Laser wavelengths are 375nm, 405nm, 488nm and 635nm. The multiple light scattering and fluorescence detectors help increase the detection limit to <100nm and increase the resolution to <10nm. **Figure 5** shows the basic design of the machine, the florescence channels and the laser with exposure to the column of cells. This machine is equipped with Peripheral Component Interconnect Express (PCIe), high-speed computer software used for data acquisition. It employs the latest Altera™ technology for data acquisition at speed of up to 100k events per second. Conventional flow cytometers rely on fluorescent probes to measure biological particles smaller than 500nm, this may produce dim signals and data may be inconsistent. The A50-Micro's light scatter performance allows small particles to be better discriminated.

Since our previous study demonstrated that prostate cancer MP are abundant in plasmas, we performed multi-parametric analyses with prostate-specific and cancer-specific biomarkers (Siddiqui *et al.* 2014). We used a combination of antibodies that bind prostate-specific and cancer-specific markers present in just 20 µl of patient plasma. Our initial experience of enumeration of MPs in the plasma samples from a PCa patient before and after radical prostatectomy and our previous pilot study encouraged us to use

this machine to develop a test to distinguish patients with benign prostatic hyperplasia (BPH) from patients with prostate cancer.

Figure 4. Apogee A50 Nanoscale Flow cytometer

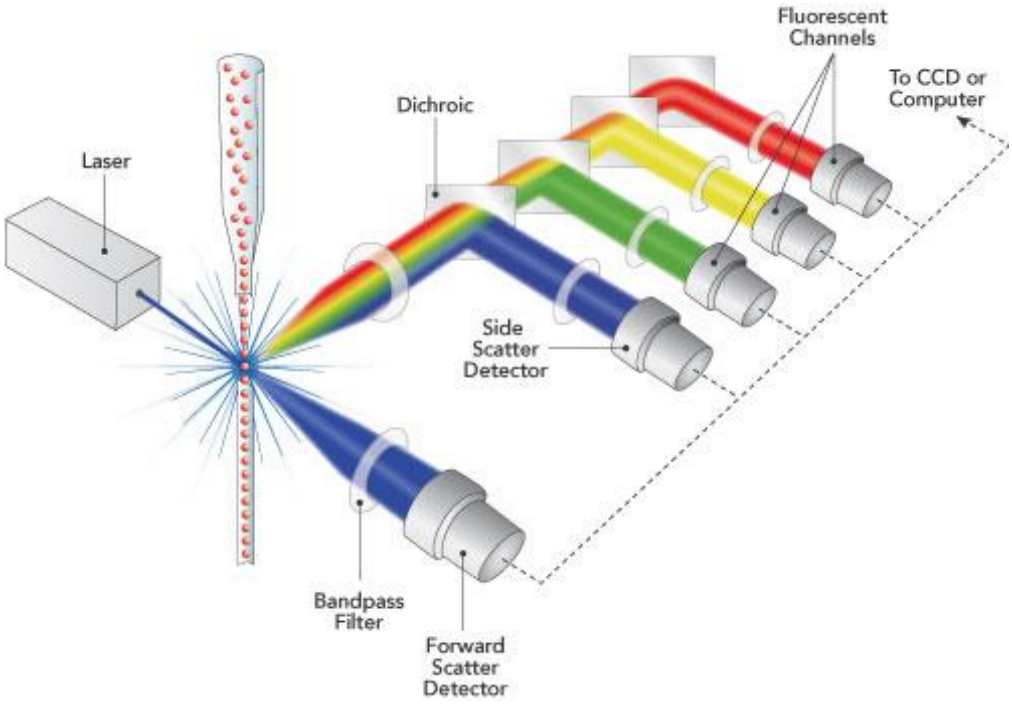

Figure 5. Fundamental framework of a flow cytometer with florescence channels and the laser detectors

1.7 Prostate Cancer Surface Receptors

Cells originating from a specific organ typically manifest some receptors exclusive to that organ. This usually helps in classifying the cell to the organ of origin. Histological interpretation of specimens from metastatic sites with unknown primary lesion have long relied on using monoclonal antibodies against cell specific receptors to identifying the origin of these metastatic lesions. Recently, they have attracted attention and are increasingly used to develop therapeutic and diagnostic agents for treatment of cancers (Deckert *et al.* 2009). These antibodies are conjugated with florescence dyes and used in imaging modalities. For example, the Prostascint scan uses PSMA to look for metastatic lesions from PCa (Rosenthal *et al.* 2001). Ghrelin has been used as a PET imaging agent to identify foci of carcinomas in the prostate gland (Fowkes, 2014). We hypothesize that these antibodies may be used to identify microparticles, as it is postulated that a MP retains the surface receptors from the parent cells. With the use of appropriate antibodies it may be possible to establish the lineage of the MP. Monoclonal antibodies (mAbs) are highly specific and adaptable for targeting cells. We used two antibodies against two surface receptors expressed on PCa cells namely, prostate specific membrane antigen (PMSA) and gastrin releasing peptide receptor (GRPR). We also used a peptide-ligand called Ghrelin. The antibodies and the peptide-ligand were conjugated with fluorescent agents and used to enumerate the MP using flow cytometry.

1.7.1 Prostate specific Membrane Antigen (PSMA)

PSMA is a type II transmembrane protein. It has a helical structure with an N-terminal cytoplasmic tail and was first cloned in 1993 (Israeli *et al.* 1993). This transmembrane

protein consists of a small intracellular domain of 19 amino acids, a transmembrane domain of 24 amino acids, and a large extracellular domain of 707 amino acids. The extracellular portion consists of a binding motif, including two zinc ions. The presence of PSMA is not actually unique to the prostate (Sacha *et al.* 2007). Its structure is almost identical to folate hydrolase and it is confirmed to be present in four sites in the body: prostate (secretory acinar epithelium), kidney (proximal tubules), nervous system glia (astrocytes and schwann cells), and the small bowel (Mhawech *et al* 2007). The physiological significance of PSMA in not completely understood in the prostate gland but it may be linked to the presence of folates in the seminal fluid. As intracellular folate is more abundant in rapidly dividing cells it is postulated that it may be more expressed in the higher grade prostate cancers.

PSMA was first identified in the prostate using IgG1 monoclonal antibody called 7E11-C5.3. This antibody was developed using the prostate cancer cell line known as LNCaP and was also used in development of Prostascint scan (Horoszewicz *et al.* 1987). This scan was approved by U.S. Food and Drug Administration (FDA) to be used as ^{111}In-labeled form (ProstaScint, Cytogen, Philadelphia, PA) but gained limited success (Rosenthal *et al* 2001). The main flaw in this antibody was that it had affinity for the intracellular portion and hence was not useful to identify living cells. Wolf et al developed three other mAbs (3/A12, 3/E7, 3/F11), which show a strong and specific extracellular binding to PSMA (Wolf *et al.* 2010). Wolf also demonstrated that 3/E7 was compatible with flow cytometric analysis and showed high affinity to human prostate tissue. This antibody could be obtained with >95% purity from the hybridoma. Progressively increasing expression of PSMA has been demonstrated in BPH, high grade

prostatic intraepithelial neoplasia (PIN) and prostate cancer (Bostwick *et al.* 1998). Similar association of PSMA expression in high grade prostate cancer has been shown by other investigators (Wright *et al.* 1995). This data support potential clinical use of PSMA in the diagnosis of PCa.

1.7.2 Ghrelin

Ghrelin peptide is a ligand for Growth Hormone Secretagogue Receptor (GHSR). Growth hormone (GH) is mainly released from the pituitary gland in response to growth hormone releasing hormone (GHRH) but there is also another pathway related to G-protein coupled receptor called growth hormone secretagogue receptor (GHSR). This receptor was first cloned in 1996 and unlocked the door for future research in this direction (Howard *et al.* 1996). The ligand for GHSR was purified in 1999 and named as Ghrelin, 'ghre' is the Proto-Indo-European root of the word 'grow' (Kojima *et al.* 1999). Ghrelin is composed of 28 amino acids, in which the 3-serine residues are n-octanoylated. The acylated peptide specifically releases GH both *in vivo* and *in vitro*, and O-n-octanoylation at 3-serine is essential for the activity. GHSR is also differentially expressed in human breast cancer cell lines (Casoni *et al.* 2001). Other studies have also documented that differential expression of GHSR in PCa and showed that Ghrelin has a role in growth of prostate cancer cell lines (Jeffery *at al.* 2002). He observed a 33% increased growth in the PC-3 cell lines when stimulated by ghrelin. Our collaborator, Dr. Luyt and colleagues at Western University modified the structure of ghrelin and created a novel fluorescent ghrelin analogue. They demonstrated the binding and uptake of this fluorescent ghrelin analogue in human PCa cells and showed the ability of Ghrelin to specifically bind PCa over normal adjacent tissue (Rosita *et al.* 2009). In our previous work using *ex vivo*

tissue, we were able to demonstrate that this novel fluorescein-ghrelin probe was able to distinguish between benign and cancerous cell lines and therefore has potential to be explored as a marker for diagnosis of PCa (Lu *et al.* 2012). In our pilot study, we have shown the usefulness of Ghrelin as our cancer-specific biomarker to define the population of prostate cancer microparticles in plasma. In the pilot study, we enumerated PSMA+ and GHSR+ microparticles in plasmas from three patient cohorts and found higher counts of PSMA+ GHSR+ dual positive MP in the metastatic and localized prostate cancer patient cohorts compared to the low counts observed in healthy volunteers. Based on this experience we elected to investigate Ghrelin as a biomarker in this study.

1.7.3 Gastrin Releasing Peptide Receptor (GRPR)

GRPR is a G-protein coupled receptor belonging to the family of Bombesin receptors. Bombesin is a 14-amino acid peptide, originally isolated from the skin of the European fire-bellied toad (Bombina bombina) (Nakajima *et al.* 1980). Erspamer, further characterized and isolated them (Erspamer, 1988). Gastrin Releasing Peptide (GRP) mediates its action through the membrane-bound GRPR. It activates several signaling pathways including those involved in cell cycle regulation (Jensen *et al.* 2008). GRPR expression has been identified on PC-3 prostate cancer cells (Bologna *et al.* 1989). Their differential expression was also documented in the breast cancer cell line (Giacchetti *et al.* 1990). These studies lead the way for further exploration of GRPR in the process of carcinogenesis. It has also been shown that antagonists of GRP can inhibit the growth of prostate cancer cells. (Milovanovic *et al.* 1992). GRPR also has been shown to be over expressed in PCa (Ananias *et al.* 2009). In this study they found GRPR staining in lymph node metastases in 85.7% of cases and PSMA staining in 100%. GRPR expression was

found to be present in 52% of the bone metastases. Another study also looked at GRPR expression in 299 primary prostate carcinomas and found 77% cases positive for GRPR (Beer *et al.* 2012). They also found inverse relation with the grade and volume of disease. In this study our hypothesis is that PCa cell release MP in the circulation. These MP express surface receptors which are inherited from their cell of origin. Our literature review suggests that PSMA, GRPR and GHSR are abundantly expressed on PCa cells.. Monoclonal antibodies and ligands specific for these receptors can be used as biomarkers for PCa. A combination of these biomarkers including PSMA mAb and Ghrelin-peptide and/or GRPR mAb can help us isolate PCMP in plasma samples to discriminate patients with PCa from patients with BPH.

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Chapter 2

2 Pilot Study: Enumeration of Prostate Cancer Microparticles as a Tool to Identify Prostate Cancer

2.1 Introduction

The search for development of an ideal tumor marker for prostate cancer has been on going for the better part of the last half century. There is a long list of emerging biomarkers for diagnosis and prognosis of prostate cancer (PCa) (Velonas *et al.* 2013). Only a few biomarkers have been able to survive the stringent tests of large clinical trials and prostate specific antigen (PSA) continues to be the most widely used marker for PCa. The current guidelines by the American Urology Association (AUA) only permit PSA testing for individuals between age 55 and 64 who understand the risks associated with PSA based screening (www.auanet.org). The AUC analysis for ability of PSA to confidently diagnose PCa is in the range of 0.56- 0.70 (Brawer et al. 1999). A recent report of the ESRPC trial report that 781 men are needed to be screened with PSA to prevent one PCa related death (Shroder *et al.* 2014). To meet the challenge of developing a screening test superior to PSA, we proposed a microparticle (MP) based test that enumerated prostate cancer MPs in minute volumes of patient blood in a high-throughput and multi-parametric manner. This pilot study is aimed to validate the clinical utility of this test to successfully distinguish patients with PCa from those who did not have cancer.

2.2 Material and Methods

We used A-50 Apogee, nanoscale flow cytometer which is specifically designed to analyze and enumerate cancer microparticles, to study prostate cancer microparticles present in four cohorts of patients:

a. Healthy volunteers (HV) (n=24); Included young men and women aged <35 with no known cancers.
b. Benign Prostatic Hypertrophy (BPH) (n=10); Included men who had a normal digital rectal examination and had a transurethral resection of prostate with a pathologic diagnosis of BPH.
c. Localized Prostate Cancer (n=112): Samples obtained from Ontario Institute of Cancer (OICR) from patients undergoing radical prostatectomy for localized prostate cancer.
d. Metastatic Prostate Cancer (n=23): Included samples from patients with metastatic castrate resistant prostate cancer.

We used a monoclonal antibody specific to the extracellular portion of PSMA (PSMA-RPE mAb) and ghrelin peptide, a growth hormone secretagogue receptor (GHSR) ligand (Ghrelin-FITC ligand) to identify and enumerate dual positive prostate cancer microparticles (PSMA + Ghrelin dual positive).

For the HV and BPH group we collected 1 vacutainer from each patient (10 mL sodium-heparin Green top tubes) of whole blood. The vacutainer was centrifuged at 2000 RCF for 20 min. This resulted in the blood separating into two distinct layers, a red layer (erythrocytes) and a yellow upper layer (platelet poor plasma-PPP). Using a plastic disposable pasteur pipet, the PPP was transferred into 3ml tubes and stored at -80ºC.

Before the experiment, we thawed PPP in the tube and transferred 20 uL to a sterile 1.5 mL eppendorf tube. This was designated as tube A. Using a 20 uL pipetman, another 20 uL of PPP was transferred to another sterile 1.5 mL eppendorf tube and labeled as tube B. 2uL each of IgG-FITC antibody and IgG-RPE antibody was then added to tube A. 2uL of Ghrelin-FITC ligand and 2uL of PSMA-RPE antibody was also added to tube B. Both the tubes A and B were mixed and immediately incubate in the dark for 30 min. 600uL of sheath fluid (1X PBS, pH 7.4) was then added to each tube and samples were vortexed. Samples were then analyzed using flow cytometry. Samples were analyzed in triplicates and the average calculated.

2.3 Results

We analyzed 169 plasmas and found significantly higher counts ($p<0.01$, ANOVA, bon ferroni test) of PSMA + Ghrelin, dual positive prostate cancer microparticle (PCMP) in patients with prostate cancer as compared to BPH and healthy volunteers. The numbers of PCMP in each group are shown in **Figure 6**.

However we did not find any significant correlation between the number of PCMP and Gleason score (one-way ANOVA test). The number of PCMP in each category of Gleason score are shown in **Figure 7**.

We did not find any correlation between number of PCMP and Tumor Stage (one-way ANOVA test). **Figure 8** shows the number of PCMP in each 'T' category. Similarly no statistical difference was observed between the numbers of PCMP in the localized group and metastatic group. **Figure 9** shows the distribution of PCMP counts in various patient cohorts. The scatter plots with means and respective 95% confidence intervals (red lines) reveal a cutoff (green dashed line) for distinguishing BPH patients from patients with

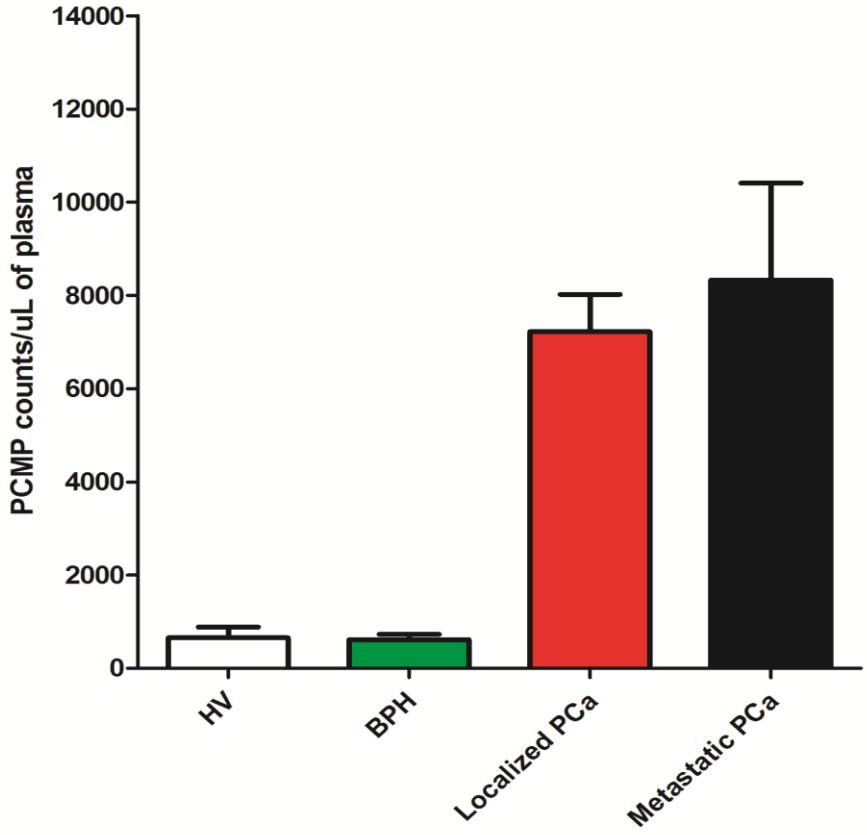

Figure 6. Comparison of Number of Prostate Cancer Microparticles (PCMP) Expressing PSMA+ Ghrelin, Dual Positive Events in Plasmas from Four Groups, Including Healthy Volunteers (HV) and Benign Prostatic Hypertrophy (BPH), Representing A Population with No Cancer and Other Two Groups; Localized Prostate Cancer and Metastatic Prostate Cancer, Representing A Population with Cancer..

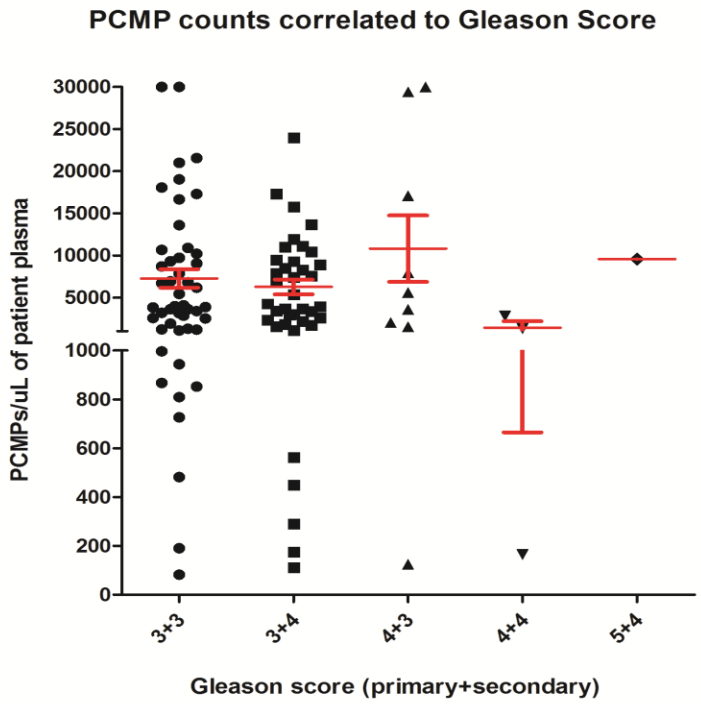

Figure 7. Comparison of Number of Prostate Cancer Microparticles (PCMP) Expressing PSMA+ Ghrelin, Dual Positive Events in Plasmas from Prostate Cancer Patients with Gleason Score 3+3, 3+4, 4+3, 4+4 and 5+4.

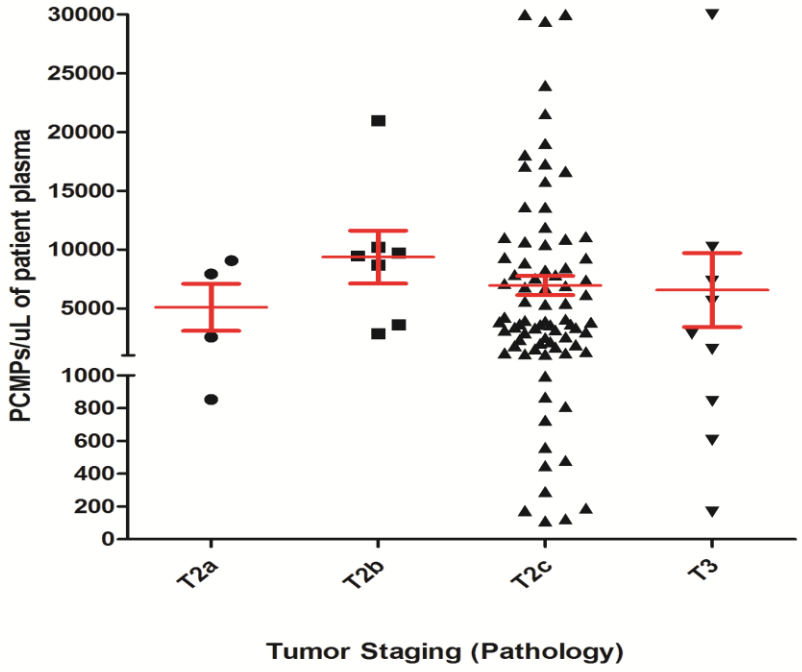

Figure 8. Comparison of Number of Prostate Cancer Microparticles (PCMP) Expressing PSMA+ Ghrelin, Dual Positive Events in Plasmas from Prostate Cancer Patients with Tumor Stages; T2a, T2b, T2c and T3.

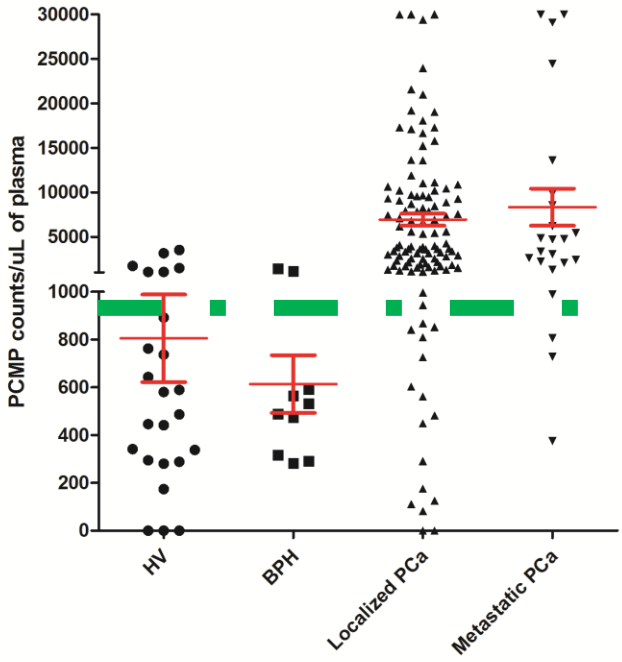

Figure 9. Distribution of Number of Prostate Cancer Microparticles (PCMP) Expressing PSMA+ Ghrelin, Dual Positive Events in Plasmas from Four Groups, Including Healthy Volunteers (HV) and Benign Prostatic Hypertrophy (BPH), Representing A Population with No Cancer and Other Two Groups; Localized Prostate Cancer and Metastatic Prostate Cancer, Representing A Population with Cancer.. The Green Dashed Line Distinguishes Patients with PCa from patients with No Cancer.

PCa. With this cutoff, the blood test is 89% accurate in identifying patients with PCa (localized PCa cohort) with 20% of patients being mistakenly identified as having PCa.

2.4 Discussion

Prostate cancer (PCa) is a leading cause of cancer-related death of men in the western world (Siegel *et al.* 2014). Majority of men are diagnosed with PCa based on a raised prostate specific antigen (PSA). Since its introduction, PSA has generated intense debate as an effective screening tool for PCa (Catalona et al. 1991). The recent recommendations by United States Preventive Task Force (USPSTF) has concluded that PSA based screening produces unacceptably high rates of false positive results and causes more harm than benefit.

The National Cancer Institute defines a biomarker as "a biological molecule found in the blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease". An ideal biomarker for PCa should not only be able to screen with high sensitivity and specificity but also predict the course of disease and help select high risk individuals for aggressive treatment. The ideal tumor marker should be economical, reproducible, non-invasive, not time consuming to perform and easily accessible for majority of population (Velonas *et al.* 2013).

Microparticles (MP) are plasma membrane derived structures of a diameter of less than 1000nm. The introduction of MP as a diagnostic test could enable more sensitive detection than current methods due to their origin and specificity (Rak 2013). A recent review of MPs, highlighted the importance of these sub micron structures as a biomaker for cancer (Gyorgy B *et al.* 2011). MP are secreted into the circulation, urine and semen,

thus if reliably identified, they can be exploited as biomarker treasure chests for prostate cancer (Dujivesz D *et al*. 2011). The main function of MP is proposed to be cell-to-cell communication but they are likely to play a significant role in oncogenesis (Yang et al. 2011). Exosomes (very similar to MP) have also been shown to promote metastasis by avoiding detection by immune system (Yang et al. 2007).

We developed a MP based blood test to identify patients with PCa using a combination of monoclonal antibody for prostate specific membrane antigen (PSMA) and ghrelin peptide, a ligand for growth hormone scretagogue receptor (GHSR). PSMA has been shown to be highly expressed on prostate cells and differential expression has been documented on high grade prostate cancer compared to BPH and normal prostate gland (Wright *et al.* 1995). Similarly, ghrelin has been shown to bind more strongly with PCa in both *in vitro* and *ex vivo* experiments (Lu *et al.* 2012).

Our results showed that PSMA + Ghrelin, dual positive MP are more abundant in PCa patient plasmas compared to plasma samples from healthy volunteers and patients with BPH. Compared with other established makers like PSA and prostate cancer antigen (PCA) 3 score, which have a diagnostic accuracy of 56% to 72% (Crawford *et al.* 2012). This blood test is able to identify PCa with a diagnostic accuracy of 89% in only a minute volumes of patient blood in a high-throughput and multi-parametric manner.

2.5 Conclusion

Our initial results show that the MP based blood test using PSMA+ Ghrelin as biomarker for PCa is capable of fulfilling the requirements of an ideal tumor marker and has potential to be the "Next Generation Screening Tool" for Prostate Cancer.

2.6 Limitations

The major limitation of this study was the disproportionately higher number of patients with prostate cancer. The total numbers of patients with PCa were 135 (112+23) compared to only 10 patients with BPH. Even if we combined BPH and healthy volunteers, the number was only 34 (24+10). In this pilot study the sample were neither randomized nor was the observer blinded.

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Chapter 3

3 Prostate Cancer Microparticles as a Next Generation Screening Tool for Prostate Cancer

3.1 Introduction

Prostate cancer (PCa) is the leading cancer among adult males and is one of the few solid organ malignancies, which can be diagnosed and monitored using a tumor marker i.e. prostate specific antigen (PSA). However PSA is not a true tumor marker and is also produced by normal prostate gland and may be raised in a number of benign conditions. Large studies have shown that >50% of men with raised PSA do not have PCa and are unnecessarily subjected to transrectal ultrasound (TRUS) guided systematic biopsy of prostate gland (Martinez *et al.* 2013). In 2012, the U.S. Preventive Services Task Force (USPSTF) recommended against the routine use of PSA screening at any age. Clearly, PSA does not fulfill any currently acceptable standard of a screening test and a new screening test is needed. To meet this challenge, we propose a prostate cancer microparticle based test.

Microparticles (MPs) are fragments of cells that are released by the prostate gland as the cells undergo division, necrosis, apoptosis, and/or exocytosis (Rak, 2013). Using a monoclonal antibody specific to the receptors expressed on prostate cancer we can identify prostate cancer microparticles (PCMP) and these novel markers may be used for diagnosis and monitoring of PCa.

3.2 Objective

The objectives of this study were;

1. To differentiate patients with prostate cancer from those with BPH or 'no cancer'.
2. To determine the difference in PCMP levels in the two groups namely,
    a. Men with localized PCa
    b. Men with biopsy proven BPH and no evidence of PCa.

3.3 Materials and Methods

After obtaining the necessary ethical approval was obtained from Western University (Appendix 1), we analysed 405 plasma samples. 249 PCa plasma samples were obtained from Ontario Institute of Cancer Research (OICR) and 147 samples of patients with prostate biopsy proven BPH were obtained from Princess Margaret Hospital GU Tissue Bank (PMH). We also recruited 9 patients with BPH at London Heath Sciences (LHSC), these patients had a TRUS biopsy to rule out PCa and also underwent transurethral resection with histologically confirmed BPH.

Collection of Samples

Sample from Tumor Bank: The samples were obtained from the tumour banks in aliquots of 3-5 ml of frozen plasma. Services of an appropriate transportation company were engaged to ensure that the samples were transported frozen at -80 degrees Celsius. These samples were obtained from patients prior to definitive treatment for PCa. Each patient donated around 10-15ml of blood which was obtained in a 4.5 mL EDTA vacutainer

(Purple top tubes). The vacutainer was centrifuged at 2000 RCF for 20 min. This resulted in the blood separating into two distinct layers, a red layer (erythrocytes) and a yellow upper layer (platelet poor plasma-PPP). Using a plastic disposable Pasteur pipet, the PPP was transferred into tube for storage at -80 degree Celsius.

Samples from London Health Sciences: Nine samples were obtained from patients undergoing transurethral resection of prostate (TURP). These patients were previously worked up for lower urinary tract symptoms and presence of PCa was ruled out by transrectal ultrasound guided biopsy. The final pathology report from the TURP specimen was consistent with histological diagnosis of BPH. Prior to surgery 10-15 ml of blood was drawn in a purple top tube and centrifuged at 2000 RCF for 20 min. This resulted in the blood separating into two distinct layers, a red layer (erythrocytes) and a yellow upper layer (platelet poor plasma-PPP). Using a plastic disposable Pasteur pipet, the PPP was transferred into tube for storage at -80 degree Celsius.

Randomization and blinding

All the samples were thawed and aliquoted again. The samples were randomized and numbered to blind the observer during analysis of samples. These randomized and relabelled samples were stored at -80 degree Celsius until they were used during the experiment. The master list was kept separately in the office of Dr. Hon Leong, PhD.

Preparation of samples

We prepared two mixtures of antibodies in two different tubes to be used in this experiment. We labeled one tube as 'positive antibody' and the second as 'isotype

antibody'. Isotype antibodies were used as control for each sample as the florescence-conjugated antibodies known to bind with trace amounts of nonspecific proteins and produce auto florescence. The use of isotype antibodies helped us in identifying this background noise. The numbers of events in the isotype sample were then subtracted from the counts observed in the sample, labeled as 'positive antibody'. This methodology helped in eliminating the effect of this non specific binding. The positive antibody tube was used to prepare a mixture of antibodies namely PSMA-PE antibody (3/E7), Ghrelin peptide, GRPR antibody. Similarly we prepared a mixture of isotype controls for using mouse IgG-RPE antibody for PSMA, Ghrelin/LCE antibody for ghrelin and rabbit IgG antibody for GRPR for the tube labeled as isotype antibody. The concentration of PSMA-PE antibody (3/E7) used was 408.42 µg/ml, the concentration of Ghrelin peptide used was 62.5mM and the concentration GRPR used was 0.5 µg/ml. The concentrations of isotype antibodies were matched.

To simplify the process of conjugation of the antibodies we prepared two cocktails. The tube labeled positive contained; 24µL of GRPR, 24 µL of 2° antibody, 12 µL each of PSMA mAb and Ghrelin peptide. The total volume of this mixture was 72 µL. Similarly we used 24 µL of rabbit IgG antibody for GRPR, 24 µL of 2° antibody and 12 µL each of mouse IgG-RPE antibody for PSMA, Ghrelin/LCE antibody for Ghrelin. The total volume of this mixture was also 72 µL. This volume was enough to conduct the experiment on a batch of 10 samples at one time.

We transferred 20 µL of platelet poor plasma (PPP) from a sample to two sterile 1.5 mL eppendorf tubes. The tubes were labeled as tube '+ve' and '–ve' and given a numbers xxx

according to previous randomization. We added 6 µL from tube labeled 'positive antibody' to the tube labeled '+ve' xxx and 6 µL from tube labeled 'isotype antibody' to the tube labeled '-ve'xxx.

Both the tubes were mixed well and immediately incubate in the dark for 30 min. After incubation we added 600uL of sheath fluid (1X PBS, pH 7.4) using 1000 uL pipetman to each tube and vortexed.

Analysis of samples on Flow cytometer

The samples were analysed on A−50 Micro nanoscale flow cytometer (Apogee Flow Systems Inc.) to enumerate events. The optimization was performed using samples from healthy volunteers and patients with high volume/grade PCa. We used varying concentrations for antibodies during the optimization stage and chose the concentration which revealed the maximum discrimination in the two selected populations. We then selected our gates for calculation of the events. Each event seen on the histogram is a reflection of a single MP captured as a result of binding to a specific antibody. The location of each event on the histogram is a reflection of the characteristic of its florescence and size. Apogee A-50 nanoscale flow cytometer has the capacity to discriminate events generated by submicron structures. We selected specific gates to capture a selected population of MP. These gates were created by drawing boundaries around the population of interest, which was identified during the optimization as most discriminatory between the two groups. **Figure 10** illustrates the settings for the histograms.

We then analyzed our study samples and used the same settings (identified during optimization) throughout the study. The numbers of events per microliter were recorded for each sample. The isotype controls were used to calculate the background activity of each sample. We subtracted the number of events in the isotype from the sample to calculate the 'compensated' number of microparticles events for each specimen.

3.4 Statistical Analysis

We used SPSS version 21.0 for statistical analysis. One-way ANOVA test (Ordinary) was used to compare the mean number of events in the two groups using. The confidence interval was set at 95% and the p-value of < 0.05 was considered significant.

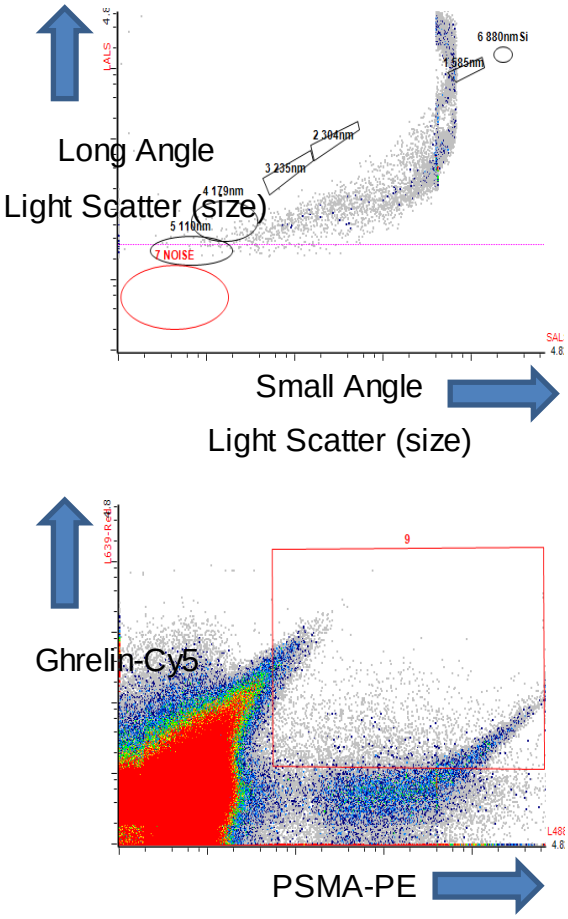

Figure 10. Histogram from Apogee A-50 nanoscale flow cytometer obtained from a patient plasma from group 1 representing patients with prostate cancer The top panel (A) represents all microparticle events analyzed according to size (long angle vs. small angle light scatter, Y vs. X axis respectively). The bottom panel (B) represents those same events but analyzed for PSMA + Ghrelin, dual positive MP, On Y axis is Ghrelin-Cy5 and on X axis is PSMA-PE.

3.5 Results

Four hundred and five randomized samples were analyzed. The patients were divided in two groups. Group 1 representing 249 patients with prostate cancer and Group 2 included 156 patients with BPH.

In Group 1 (PCa), 65% patients were aged between 40-64 years (range 49-79 years). The mean PSA was 13.88 $\pm$ 62.22 ng/mL (range 1.5 ng/mL to 541 ng/mL) and the median PSA was 7 ng/ml. The pathologic T stage of the tumors (Group 1) is shown in **Table 4** and the distribution of Gleason Score is shown in **Table 5.** In Group 2 (BPH), the mean age of patients was 64.5 $\pm$ 6.19 years (range 49-79 years). All patients in Group 2 had biopsy proven BPH, the median size of the prostate gland was 67.5 grams calculated on trans-rectal ultrasound. The mean PSA was 10.07 $\pm$ 5.82ng/mL and the median PSA was 8.7 ng/ml.

Group 1 contained 249 patients diagnosed with PCa. The mean number of PSMA positive events in this group was 160412 $\pm$ 11480 events/µL. The mean number of PSMA and Ghrelin (dual positive events), PSMA and GRPR (dual positive events) and PSMA/Ghrelin/GRPR (triple positive events) is shown in **Table 6**.

pT Stage (n=164)	**Number of Patients** (%)
pT2a	38 (23.1)
pT2b	16 (9.7)
pT2c	39 (23.7)
pT3a	46 (28)
pT3b	23 (14)
pTx	2 (1.2)

Table 4. Distribution of Pathologic T stage in Group 1

Gleason Score (n=185)	**Number of Patients** (%)
6	53 (28.6)
7	114 (61.6)
8	5 (1.0)
9	13 (7.0)

Table 5. Distribution of Gleason Score in Group 1

Group 2 was comprised of 156 samples from patients with BPH. The mean number of PSMA positive events in this group was 186119 $\pm$ 20231 events/mL. The mean number of PSMA and Ghrelin, PSMA and GRPR (dual positive events) and PSMA and Ghrelin and GRPR (triple positive events) is shown in **Table 7**. A comparison of the mean number of MP events for each type of MP in the two groups is shown in **Figure 12.**

We compared the number of events in the two groups using the one-way ANOVA (Ordinary) test to compare the means. The confidence interval was set at 95% and a p-value of < 0.05 was considered significant. **Table 8** shows the comparison of the number of microparticle events expressing various biomarkers in the two groups. This comparison is also shown in **Figure 13**.

We did not find any significant differences in mean number of PSMA positive microparticle events, PSMA+GRPR or PSMA+Ghrelin dual positive microparticle events, or PSMA+Ghrelin+GRPR triple positive microparticle events.

We also looked at the distribution of the number of MP subtypes in different subgroups of PSA and Gleason Score. **Figure 16** and **Figure 17** show the number of MP expressing various biomarkers in the three tiers of PSA levels i.e. < 4ng/ml, 4-10 ng/ml and >10ng/ml in Group 1 and Group 2. **Figure 18** shows comparison of number of MP events expressing various biomarkers in the two groups stratified by levels of PSA.

	PSMA +ve events/µL	PSMA+GRPR Dual +ve events/µL	PSMA+Ghrelin Dual +ve events/µL	PSMA+GRPR+ Ghrelin Triple +ve events/µL
Mean	160412	55177	56658	7909
Median	99585	21144	38594	3163
SEM	11480	5413	5376	696

Table 6**.** Microparticle Events Expressing Various Biomarkers (Prostate Specific Membrane Antigen/PSMA, Gastrin-Releasing Peptide Receptor/GRPR, Ghrelin peptide ligand/Ghrelin) in Plasmas from Patients with Prostate Cancer (PCa, Group 1). N=249. The Mean, Median and Standard Error were calculated using SPSS version 21.0.

	PSMA +ve events/µL	PSMA+GRPR Dual +ve events/µL	PSMA+Ghrelin Dual +ve events/µL	PSMA+GRPR+ Ghrelin Triple +ve events/µL
Mean	186119	45885	67284	8946
Median	86155	17407	49973	2483
SEM	20231	5549	5795	1142

Table 7. Microparticles Events Expressing Various Biomarkers (Prostate Specific Membrane Antigen/PSMA, Gastrin-Releasing Peptide Receptor/GRPR, Ghrelin peptide ligand/Ghrelin) in Plasmas from Patients with Benign Prostatic Hyperplasia (BPH, Group 2). N=156. The Mean, Median and Standard Error were calculated using SPSS version 21.0.

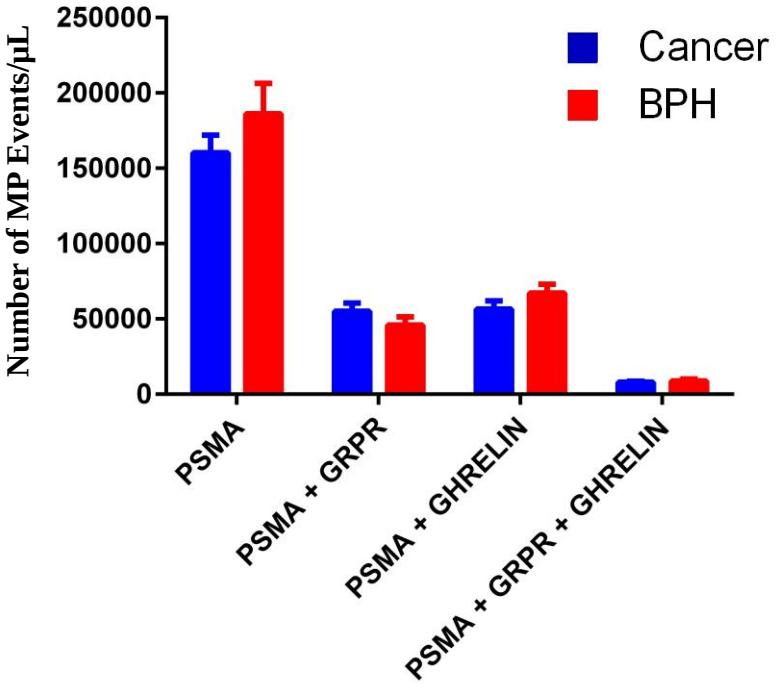

Figure 11. Mean Concentration of MPs Expressing Various Biomarkers; PSMA, PSMA + GRPR, PSMA + Ghrelin and PSMA + GRPR + Ghrelin in Prostate Cancer Patient Plasmas (Blue, N=249) and BPH Patient Plasmas (Red, N=156). One-way ANOVA (Ordinary) test was used to compare the means of the two groups.

Mean # events/µL	**Group 1** (n=249)	**Group 2** (n=156)	**p-value**
PSMA +ve MPs	160412 ± 11480	186119 ± 20231	0.235
PSMA+GRPR Dual +ve MPs	55177 ± 5413	45885 ± 5549	0.254
PSMA+Ghrelin Dual +ve MPs	56658± 5376	67284 ± 5795	0.196
PSMA+GRPR+Ghrelin Triple +ve MPs	7990 ± 696	8946 ± 1142	0.411

Table 8. Concentration of MPs Expressing Various Biomarkers in Prostate Cancer Patient Plasmas (Group 1) and BPH Patient Plasmas (Group 2). One-way ANOVA (Ordinary) test was used for comparison using SPSS version 21.0.

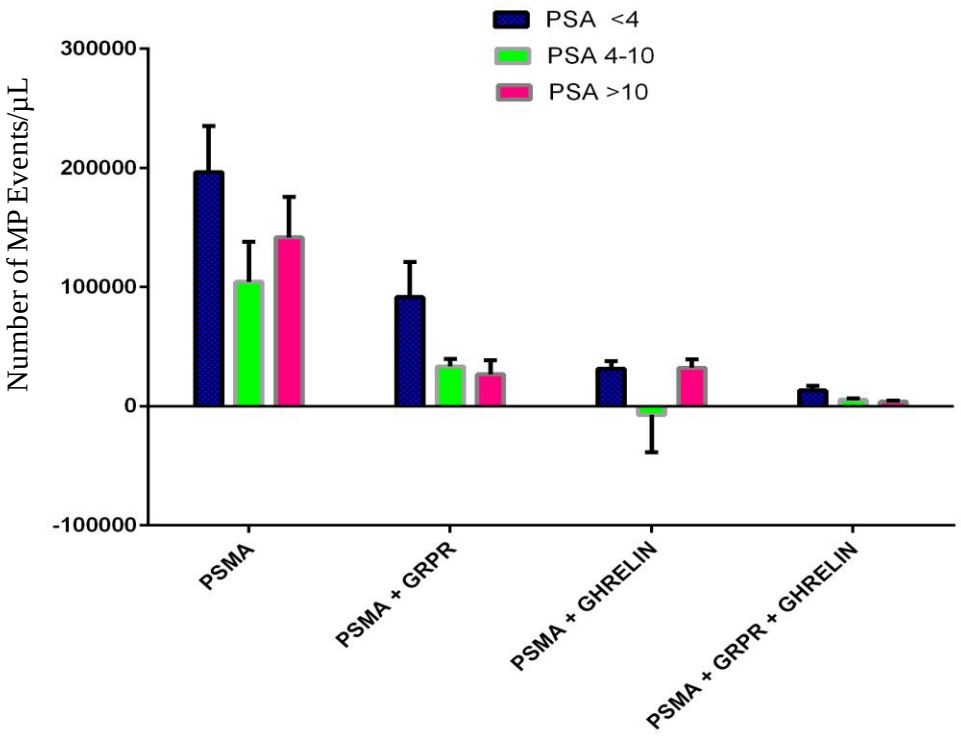

Figure 12. Mean Concentration of MPs Expressing Various Combinations of Biomarkers in Prostate Cancer Patient Plasmas (N=72) Stratified into Three PSA Groups (PSA < 4 ng/mL, 4-10 ng/mL and >10 ng/mL).

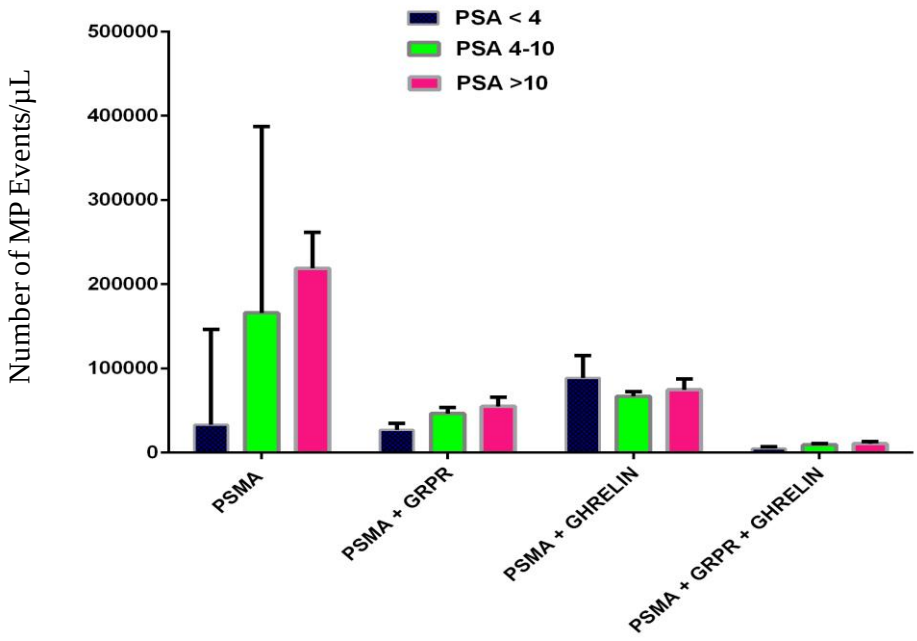

Figure 13. Mean Concentration of MPs Expressing Various Combinations of Biomarkers in BPH Patient Plasmas (N=143) stratified in Three PSA Groups (PSA < 4 ng/mL, 4-10 ng/mL and >10 ng/mL).

We compared PSMA positive microparticle events, PSMA+GRPR or PSMA+Ghrelin dual positive microparticle events, and PSMA+Ghrelin+GRPR triple positive microparticle events to determine if this test was able to differentiate between the patients with cancer and BPH in any subgroup, stratified on the basis of PSA cutoff, but we did not find any statistically significant difference. Table **9, 10** and **11** shows the comparison in different in the different PSA tiers with BPH group.

We also compared PSMA positive, PSMA+GRPR or PSMA+Ghrelin, dual positive, and PSMA+Ghrelin+GRPR triple positive microparticle events in patients with different Gleason score and BPH. We divided the patients with PCa in three groups comprising of patient plasma from Gleason score $\leq$ 6, 7 and $\geq$ 8. The main limitation of this comparison was the small number of patients in the subgroups. We found significantly higher number of PSMA+ Ghrelin dual positive in BPH group in comparison with all the subgroups of patients with PCa. This Gleason score wise comparison using various biomarkers with patient plasmas from BPH is shown in **Figure 15.** We also found a statistically significant difference in PSMA only positive MP events in the Gleason score 7 and $\geq$8 compared with BPH but the association was opposite in these groups. PSMA only positive MP were significantly higher in BPH compared to Gleason score 7 ($p=0.003$) and significantly lower in BPH compared to Gleason score $\geq$8 ($p=0.01$).

Events/µL	Group 1 (n=9)	Group 2 (n=8)	p-value and Confidence Interval
PSMA +ve	196346 ± 38709	331700 ± 113016	0.821 (-157910 to 198622)
PSMA+GRPR Dual +ve	91438 ± 29595	26933 ± 7931	0.057 (-252 to 98562)
PSMA+Ghrelin Dual +ve	31414 ± 6241	88785 ± 26352	0.998 (-53758 to 53660)
PSMA+GRPR+ Ghrelin Triple +ve	13226 ± 3707	4754 ± 2229	0.233 (-3909 to 15949)

Table 9. Comparison of Concentration of MPs Expressing Various Biomarkers in Prostate Cancer Patient Plasmas (Group 1) and BPH Patient Plasmas (Group 2) for Patients with PSA < 4ng/mL. One-way ANOVA (Ordinary) test was used for comparison.

Events/μL	Group 1 (n=49)	Group 2 (n=79)	p-value and Confidence Interval
PSMA +ve	104401 ± 33473	165908 ± 21516	0.832 (-152654 to 9476)
PSMA+GRPR Dual +ve	33435 ± 6349	46502 ± 7102	0.1033 (-37699 to 3503)
PSMA+Ghrelin Dual +ve	-7339 ± 31171	66945 ± 5675	0.1037 (-85615 to 8011)
PSMA+GRPR+ Ghrelin Triple +ve	5224 ± 1163	9227± 1422	0.3516 (-6168 to 2204)

Table 10. Comparison of Concentration of MPs Expressing Various Biomarkers in Prostate Cancer Patient Plasmas (Group 1) and BPH Patient Plasmas (Group 2) for Patients with between PSA 4 to 10 ng/mL. One-way ANOVA (Ordinary) test was used for comparison.

Events/μL	Group 1 (n=22)	Group 2 (n=56)	p-value and Confidence Interval
PSMA +ve	141613 ± 34053	218981 ± 42493	0.537 (-144092 to 75338)
PSMA+GRPR Dual +ve	26810 ± 11593	54918 ± 11170	0.3085 (-45366 to 14422)
PSMA+Ghrelin Dual +ve	32318 ± 6917	74733 ± 12894	0.9589 (-31844 to 33554)
PSMA+GRPR+ Ghrelin Triple +ve	3724 ± 1053	10646 ± 2338	0.2494 (-9429 to 2465)

Table 11. Comparison of Concentration of MPs Expressing Various Biomarkers in Prostate Cancer Patient Plasmas (Group 1) and BPH Patient Plasmas (Group 2) for Patients with between PSA >10 ng/mL. One-way ANOVA (Ordinary) test was used for comparison.

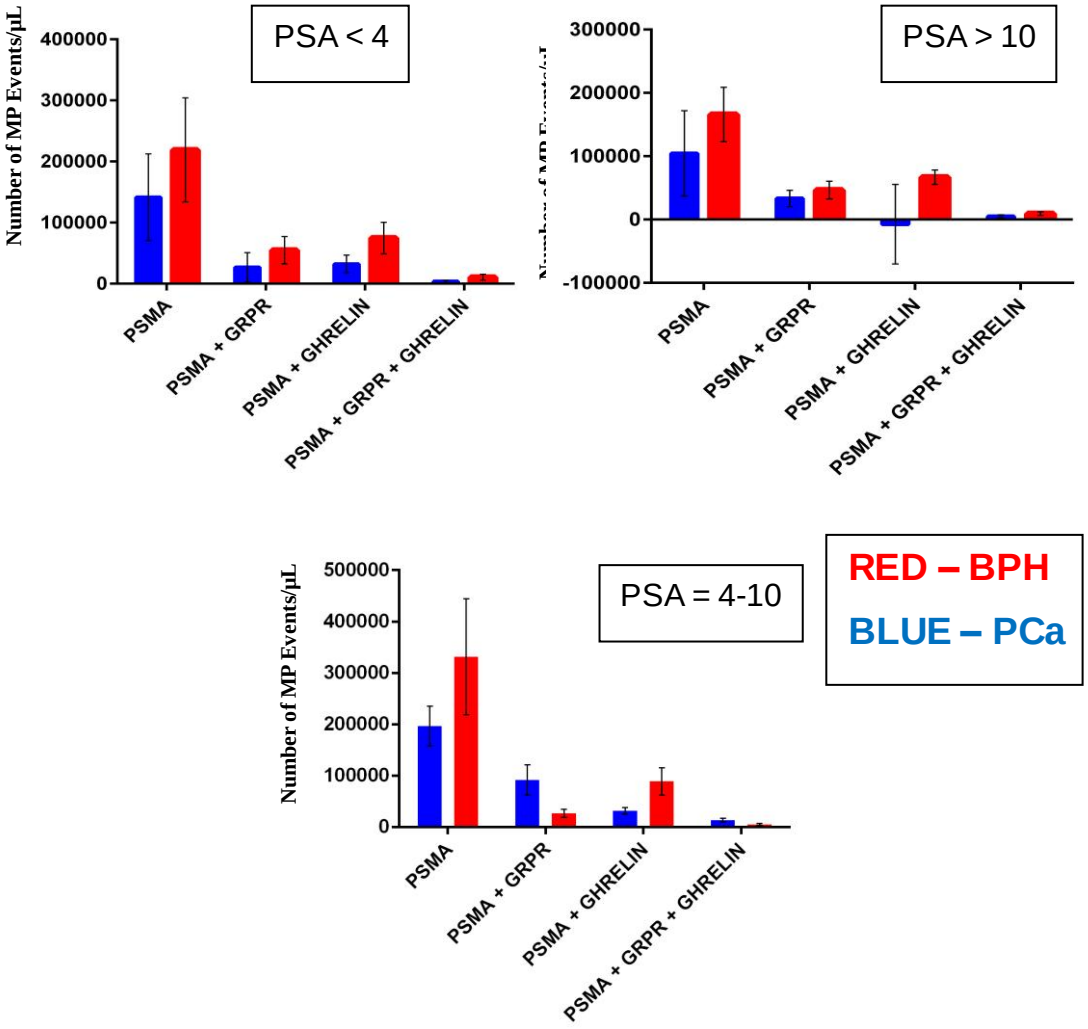

Figure 14.Comparison of Mean Concentration of MPs Expressing Various Combinations of Biomarkers in Prostate Cancer Patient Plasmas (Blue, N=74) and BPH Patient Plasmas (Red, N=143) stratified in Three PSA Groups (PSA < 4 ng/mL, 4-10 ng/mL and >10 ng/mL). One-way ANOVA (Ordinary) test was used to compare the means of the two groups.

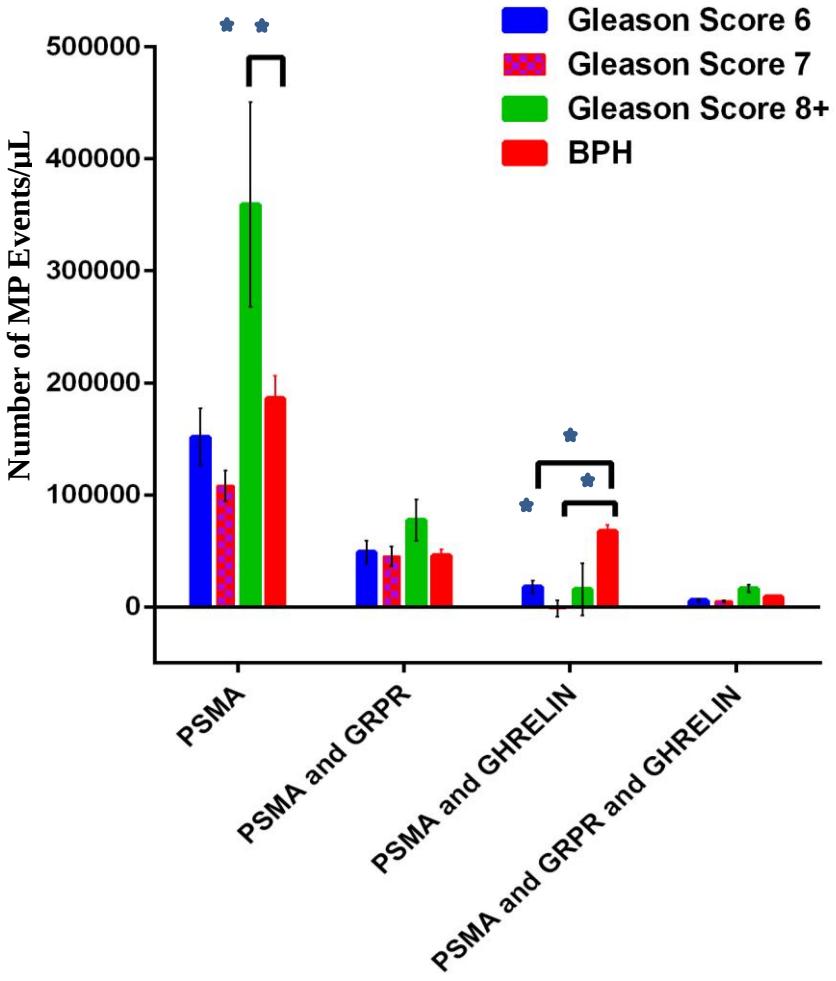

Figure 15. Comparison of Various Combinations of Biomarkers in Prostate Cancer Patient Plasmas with Gleason Score 6, 7 and $\geq$ 8 and BPH Patient Plasmas. One-way ANOVA (Ordinary) test was used to compare the means of the two groups. * denotes statistical significance.

3.6 Discussion

The concerted efforts to develop a biomarker for screening of prostate cancer (PCa) have been significantly accelerated after the publication of recent reports that have highlighted the potential harms of prostate specific antigen (PSA) based screening (Andriole *et al.* 2009). In this study we investigated a blood test based on enumeration of microparticle (MP) as a biomarker to identify PCa. MPs are defined as plasma membrane derived structures of a diameter of less than 1000nm. They are released from the cell upon activation, death, apoptosis or malignant transformation and inherit surface receptors from their cell of origin (Rak, 2013). Using monoclonal antibodies (mAb) specific for these antigens it may be possible to identify them. Surgical pathologists have used this technique to interpret specimens from metastatic sites with unknown primary lesion. mAb against cell specific receptors are used identify the primary site of origin of these metastatic lesions. Modified monoclonal antibodies have also been used as imaging agents and tagged with therapeutic arsenal to deliver targeted therapy (Deckert *et al.* 2009).

In this study we investigated four biomarkers to differentiate patients with PCa from benign prostatic hypertrophy (BPH). We selected mAb specific for prostate specific membrane antigen (PSMA) and gastrin releasing peptide receptor (GRPR). We also selected ghrelin peptide, a ligand for growth hormone secretagogue receptor (GHSR). The four biomarkers were PSMA alone and a combination of PSMA with GRPR and or Ghrelin.

Sokoloff *et al.* have demonstrated that PSMA is expressed more than 100 fold higher in prostate cancer cells in comparison to normal prostate cells (Sokoloff *et al.* 2000). PSMA based imaging (ProstaScint scan, Cytogen, Philadelphia, PA) for metastatic evaluation of PCa is US-FDA approved, however it has not been shown to be very effective (Rosenthal *et al.* 2001). The limited success of ProstaScint scan is mainly attributed to the long scanning time. The PSMA antibody used in this imaging modality was required to be injected several days before the planned imaging, thus making it less attractive for practical clinical use (Foss *et al.* 2012). A recent review analyzing published literature investigating PSMA in the last 20 years reveals robust research and an optimistic future for PSMA based treatment for castrate resistant PCa (Ritasu *et al.* 2013). In our study PSMA positive MP events were non-significantly elevated in the BPH group ($p=0.235$). The subgroup analysis according to the PSA tiers of <4 ng/ml, 4-10 ng/ml and >10 ng/ml also did not reveal any significant difference in the PSMA positive MP counts among the two groups. However, PSMA positive MP were significantly higher in BPH compared to Gleason score 7 ($p=0.003$) and significantly lower in BPH compared to Gleason score ≥ 8 ($p=0.01$). As the number of patients in Gleason >8 category was small (n=18) we are unable to draw any meaningful conclusion.

PSMA + GRPR, dual positive MP events were also investigated as a potential biomarker. Beer et al. in his study had demonstrated that two third of primary prostate cancer stain positively with GRPR (Beer *et al.* 2012). In another study looking at co-expression of PSMA and GRPR found the all cases of metastatic PCa stained positively for PSMA and 87% also co-stained with GRPR (Ananias *et al.* 2009). In our study we found statistically insignificant difference in PSMA+GRPR, dual positive MP in the PCa group ($p=0.254$).

In the sub group analysis we found this difference to approach statistical significance (p= 0.057) in the sub group of patient plasma with PSA of < 4ng/ml. This finding needs further investigation as the number of patients both the groups was very small (n=8 *vs.* n=9).

In our pilot study we found PSMA + Ghrelin, positive dual positive MP events to discriminate patients with PCa (Siddiqui *et al*. 2013). On contrary to our expectation in this study we found this biomarker to be elevated in patients with BPH. Overall the difference in PSMA + Ghrelin, dual positive MP events was not statistically significant (p=0.998). In subgroup analysis PSMA + Ghrelin, dual positive MP events were significantly higher in BPH group compared to all categories of Gleason score. This find may have been due to the fact that patients in BPH group had a higher median PSA compared to PCa group (7ng/ml *vs*. 8.7ng/ml). But when we controlled for PSA, the subgroup analysis did not show any significant difference. The other theoretical possibility may be that patients in the BPH group harbored an occult focus of PCa.

PSMA + GRPR + Ghrelin, triple positive MP was evaluated as the fourth biomarker in this study. We found this to be also non discriminatory (7990 $\pm$ 696 *vs*. 8946 $\pm$ 1142, p=0.411). Thus all four biomarkers studied in were not able to discriminate patients with PCa from BPH.

3.7 Conclusions

Prostate cancer MP can be enumerated by nanoscale flow cytometer proving the utility of Apogee A-50 flow cytometer to detect MP. This enhances our capacity of analyze MP in a high throughput and multi-parametric manner.

Although, all four biomarker were not able to discriminate patients with PCa from patient with BPH but in patients with PSA <4ng/ml, PSMA+GRPR dual positive MP showed a trend toward statistical significance in discrimination between the two groups. Further exploration of this biomarker with larger numbers may help in identifying a biomarker which can help discriminate patients with PSA in this range. The subgroup analysis comparing Gleason score and BPH showed that PSMA + Ghrelin, dual positive MP was higher in the BPH.

A blood test based on enumeration of MP may hold the promise as it has the potential to measure prostate cancer fragments continuously released from cancer cells. When perfected this test may be used as a fluid biopsy to continually sample the tumor.

3.8 Bibliography

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4 Chapter 4

4.1 General Discussion

Synchronous improvement in quality of care, dissemination of refined surgical technique, improvement in the radiation technology along with the early stage of PCa at presentation has contributed to better outcomes of prostate cancer. However, we now see a plateau in the mortality curves (Otis *et al.* 2012). The plateau reflects that we are not diagnosing all patients and that treatment is still not perfect. We know that PSA is not a true tumor marker for prostate cancer and this surrogate marker may be raised in a number of non-malignant conditions. Transrectal ultrasound (TRUS) guided prostate biopsy is the corner stone of management and is the most common modality to obtain a histological diagnosis which is a mandatory investigation for definitive diagnosis. A standard 8-12 core biopsy is reported to have a positive yield of around 50% (Lawrentschuk *et al.* 2009). Therefore, for the majority of men a biopsy recommended due to an elevated PSA fail to reveal the diagnosis of PCa and often they are undergo more than one biopsy procedure (Shinohara *et al.* 2014). This potentially exposes them to the complications of the procedure and is a cause of persistent anxiety. Ideally, we need a cost effective test that can confidently diagnose cancer and this test should be able to predict its clinical course. This test should preferably also avoid the need for biopsy or recommend biopsy for a very select population. To meet the challenge of a screening test superior to PSA, we attempted to explore a prostate microparticle-based "fluid biopsy" which could continually sample the prostate gland and its primary tumor to gain insight regarding the biology of these tissues.

MP released into blood, urine, and body fluids offers a novel opportunity to sample the biological information from the mutant cancer cell along with the normal stromal cells. This MP based sampling has the potential to be superior to conventional histopathological assessment as this fluid biopsy may be able to capture information not limited by the anatomical boundaries of the pathology slide and provide a holistic view regardless of the regional differences observed in a tumor effected prostate gland (Heppner *et al.* 1984).

Microparticles by our definition exhibit a diameter smaller than one micron and are manifest as fragments released from the cancer cell's surface. Our laboratory has validated the presence of MP by atomic force microscopy and have also analyzed them using flow cytometry (Leong *et al.* 2011). These MP retain the surface receptors present on the cells of origin. The significance of this in oncogenesis is still not determined but this property makes them potentially useful as disease biomarkers. We used the Apogee® A-50 nanoscale flow cytometer which has three lasers and is capable of recording dual and triple florescence positive events. Our laboratory was the first one to acquire this technology in North America and is still among the few in Canada to have the capacity to use the A-50 micro nanoscale flow cytometer.

We selected PSMA, GRPR and GHS receptor as these are surface receptors documented to be present on prostate cancer cells. It has been demonstrated that PSMA is expressed more than 100 fold higher in prostate cancer cells in comparison to normal prostate cells (Sokoloff *et al.* 2000). This led to development of PSMA antibodies which selectively target prostate cancer cells both at the primary site and at the site of metastasis. In a phase

1 trial, radio therapeutic agent Lutetium-177 was tagged to the PSMA antibody (J591) for treatment of metastatic lesions of castrate resistant prostate cancer with acceptable toxicity (Bunder *et al.* 2005). In a phase 2 trial, 60% PSA response was observed in patients treated with PSMA based antibody (Tagawa *et al.* 2008). A query for "PSMA" and "prostate cancer" reveals 14 current clinical trials (http://www.clinicaltrials.gov) exploring the role of PSMA in prostate cancer. The initial interest in PSMA antibody was for its potential role in imaging for prostate cancer however, the downside to antibody-based imaging is the long time required for the antibody to clear from non-target tissues. This means that the antibody should be injected several days before the planned imaging, making it less attractive for practical clinical use (Foss *et al.* 2012).

We explored the possibility of PSMA expression on MPs. In the pilot study we found that PSMA positive MPs can be enumerated using Apogee® A-50 nanoscale flow cytometer. In our pilot study we also found a stronger expression of PSMA positive MP in the patients with PCa in comparison with healthy volunteers and patients with BPH. In this study, the majority of samples in the control group were obtained from healthy volunteers and only 9 patients with BPH were included. The healthy volunteers in this study were aged younger than 35 years and did not have any history of cancer. All groups did express PSMA positive events and this showed that PSMA positivity is not exclusive for prostate cancer. In our study, we were able to enumerate PSMA positive MPs but found statistically insignificant differences in MPs enumerated in the two groups. In contrary to our expectation we found a trend towards higher number of events in patients with BPH compared to PCa. We postulated that looking for co-expression of more than one biomarker would increase the specificity of prostate cancer specific biomarkers. We used

PSMA as our prostate-specific biomarker, and GRPR antibody and Ghrelin peptide (ligand) as our cancer-specific biomarkers. Previous work in our laboratory had earlier confirmed that fluorescein labeled Ghrelin is over expressed in PCa compared to normal prostate (Lu *et al.* 2012). This study looked at PC3 and LNCaP cell lines along with specimens obtained from radical prostatectomy and concluded that this Ghrelin analog could be used as an imaging probe for PCa. In our pilot study with PSMA and Ghrelin dual positive markers we found this test to successfully discriminate PCa from the control but in our main study, we found that PSMA+Ghrelin dual positive MP events were higher in the BPH group compared to the cancer group (67284 $\pm$ 5795 vs. 56658 $\pm$ 5376, p=0.196).

This was an unexpected finding as based on our pilot study we anticipated this to be the contrary. The possible reasons for this difference in expected vs. observed maybe due to the fact that in our pilot study we compared patients with prostate cancer with mainly healthy controls and not BPH which is a benign tumor and may actually have over expression of PSMA. Due to the small number of patients with BPH in the pilot study this observation could have been masked. The patients in the BPH group also had a higher median PSA as compared to the cancer group, 8.7ng/ml vs.7 ng/ml. Although all the men in the BPH group had a TRUS biopsy to rule out prostate cancer, they were to begin with, at a high risk of having cancer which had initiated the biopsy. The risk of having a high volume PCa was ruled out with the prostate biopsy but we also understand that TRUS biopsy has its limitations and it is even possible that some of the patients in the BPH group actually had a focus of cancer which was missed at the biopsy. These are the inherent limitations of our control population. We designed this study to compare the

utility of these markers for distinguishing patients with prostate cancer from those with no cancer. This clinical equipoise is present in men who are recommended a biopsy hence we chose this control. This selection was made after we had documented in our pilot study that the MP are more abundant in the prostate cancer patients compared with healthy controls. We now desired to reproduce the results in a large number of samples from the patients who do not have prostate cancer and were able to have access to specimens from a bio bank which stored such samples. We presumed that this age and gender matched population would be an ideal control for the overall objective of the study. In our study we also noticed a large standard deviation in the number of events. This could mean that either the test fails to identify the MP or our group of patients in the control group is very heterogeneous mixture.

We also used an additional third marker, GRPR for this experiment and enumerated PSMA and GRPR positive MP. We also used a combination of all three makers to identify MP which express all three surface antigens. This strategy was designed to further enhance the specificity of the test for enumeration of PCMP. Enhanced GRPR expression has previously been reported on PCa cells *in vitro* (Bologna *et al.* 1989). GRPR expression was further confirmed by studies which demonstrated growth of prostate cancer cells stimulated by the gastrin releasing peptide and inhibited by antagonists of GRP (Milovanovic *et al.* 1992). Another study found co-expression of GRPR and PSMA in both nodal and bony metastatic lesions of PCa (Ananias *et al.* 2009). Similar expression was also demonstrated in primary prostate carcinomas (Beer *et al.* 2012).

In our study we enumerated PSMA and GRPR, dual positive MP and PSMA, Ghrelin and GRPR, triple positive MP and did not find any significant difference in the number of PSMA and GRPR dual positive (55177 $\pm$ 5413 *vs.*45885 $\pm$ 5549, p=0.254) and PSMA and GRPR and Ghrelin triple positive markers (7990 $\pm$ 696 *vs.* 8946 $\pm$ 1142, p=0.411) in the two groups. Thus this combination was also not able to discriminate patients with PCa from BPH.

When we controlled for PSA and divided our patients in three groups i.e. PSA <4 ng/mL, 4 to 10 ng/mL and >10 ng/mL and compared the number of MP events in the two groups, we did not find any statistically significant difference. However, when we compared the number of MP events for various biomarkers expressed in three different histological grades (Gleason scores 6, 7 and $\geq$ 8) with patient plasma from Group 2 (BPH), a statistically significant difference in MP expressing PSMA and Ghrelin dual positive events was seen in Gleason score 6 and 7 (p=0.001). Similarly a statistically significant difference was also observed in the number of MP expressing PSMA only, in the Gleason score $\geq$8 compared with BPH (p=0.01). The higher number of PSMA and Ghrelin, dual positive events in the BPH group was in contrary to our expectation. We anticipated a stronger expression of this biomarker in patient plasma with PCa. However the small sample size in this subgroup and the imbalanced groups used for comparison may have accounted for this statistical significance.

clusion

Our blood test based on PSMA, Ghrelin peptide ligand and GRPR has not been able to successfully differentiate patients with prostate cancer from those with BPH. However,

we have been able to enumerate microparticle expressing single, dual or triple positive events. This proves the utility of flow cytometer to detect MP in a high throughput and multiparametric manner. A blood test based on enumeration of MP holds the promise as it has the potential to measure prostate cancer fragments continuously released from cancer cells. When perfected this test may be used as a fluid biopsy to continually sample the tumor.

4.2 Future Directions

Our laboratory is currently investigating other biomarkers like prostate stem cell antigen (PSCA), TMPRSS2, AMACR etc. We plan to analyze PSMA + GRPR dual positive MP for patients with BPH and PCa having a PSA < 4 ng/ml. In this regard we have identified a source of patient plasmas with BPH and PSA of < 4ng/ml. Increasing the power of study to assess this biomarker may reveal interesting results. We are designing a study to explore PSMA + Ghrelin, dual positive MP as a biomarker for BPH. In collaboration with London bio-bank we are recruiting patients ahead of TRUS biopsy. These patients will also have serial blood samples. The longer follow-up will significantly strengthen the control group for future experiments.

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Appendices

Appendix 1. REB Approval Letter

Western Research

Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Hon Leong
File Number:103409
Review Level: Delegated
Approved Local Adult Participants:300
Approved Local Minor Participants:0
Protocol Title: The correlation of pre-biopsy prostate cancer micro particle and the yield of ultrasound guided prostate biopsy.
Department & Institution: Schulich School of Medicine and Dentistry\Oncology,London Regional Cancer Program
Sponsor:
Ethics Approval Date: April 02, 2013 **Expiry Date:**March 31, 2014
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Western University Protocol		2013/01/10
Instruments	Data collection form (revised, Feb 13 2013)	2013/02/13
Instruments	Master list (Feb 13, 2013)	2013/02/13
Letter of Information & Consent	Revised Letter of Information March 8th 2013	2013/03/08

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services

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Curriculum Vitae

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EDUCATION

FEBU,
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PROFESSIONAL Experience

July 2006 – July 2014 **Assistant Professor,** Department of Surgery,
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July 2003 – July 2006 **Senior Instructor,** Department of Surgery,
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July 2014 – present **Urologic Oncology Clinical Fellow (SUO)**
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Nov 2000-June 2003 **Instructor/Fellow** Urology, Department of Surgery,
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Nov1995-Oct 1999 **Resident,** Department of Surgery,
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RESEARCH GRANTS

2014 PI; Khurram Siddiqui, FRCS Co-investigators, Hon Sing Leong, PhD, Ann Chambers, PhD, Jonathan Izawa, FRCSC. Achieving the Oligometastatic State in End-Stage Prostate Cancer by Pharmacologic and Genetic Manipulation of Invadopodia Formed by Tumor Cells Canadian Urologic Association Scholarship Foundation (CUASF) grant CAD 50000

2010 PI; Khurram Siddiqui,Co PI: Khurshid Khawaja. Surgical Improvement Care Project (SCIP). Funded by AKU. Project to measure the SSI, Post-op VTE, cadiovasular morbidity and implement bundles for its prevention

2003 PI; Khurram Siddiqui. Co-investigators; J Talati, Q Huda, A Memon, K Khawaja and A Faroqui Comparison of Physician vs. Patient controlled analgesia for Pain Free Extra Corporeal Shock Wave Lithotripsy. (Funded by Seed money grant, The Aga Khan University, USD 6358)

2007 Co-PI. Khurram Siddiqui, PI Jamsheer Talati. Association of mitochondrial DNA haplotype M with sperm motility in Pakistani male visiting infertility clinic, Bill Gates foundation grant through population council $ 10,000.

TRAVEL GRANTS

2009 Asia Pacific Society of Sexual Medicine, USD 2000 to attend 12th Biennial Meeting of Asia Pacific Society of Sexual Medicine, 12-15th December 2009

2004 Society of Endourology, USD 24000

2003 SIU Fellowship, USD 5000

2003-4 Manpower Development Award, AKU USD 8000

Scholarships & Awards

2013 Western Graduate Scholarship
Masters in Surgery at University of Western Ontario, Canada

2013 Best Poster award at Surgery Research Day 2013
University of Western Ontario, Canada

PUBLICATIONS

1. Mazzola CR, Siddiqui KM, Billia M, Chin J. **Dovitinib : rationale, preclinical and early clinical data in urothelial carcinoma of the bladder**. Expert Opin Investig Drugs. 2014 Oct 4:1-10. PMID:25284004
2. Siddiqui KM, Billia M, Mazzola CR, Alzahrani A, Brock GB, Scilley C, Chin JL. **Three-Year Outcomes of Recovery of Erectile Function after Open Radical Prostatectomy with Sural Nerve Grafting**. J Sex Med. 2014 Jun 5
3. Zehri AA, Biyabani SR, Siddiqui KM, Memon A. **Triggers of blood transfusion in percutaneous nephrolithotomy**. J Coll Physicians Surg Pak. 2011 Mar;21(3):138-41.
4. Raheela Mohsin, Khurram M Siddiqui. **Recurrent urinary tract infection in female.** Journal Pak med assoc. Vol 60 No 1, page 55-59. Jan 2010
5. M Hammad Ather, Zaheer Alam, Anila Jamshaid, Khurram M Siddiqui, M Nasir Sulaiman. **Separate Submission of Standard Lymphadenectomy in 6 Packets Versus En Bloc Lymphadenectomy in Bladder Cancer**. Urology Journal,Volume 5, Number 2 , Spring 2008, Pages 94-98
6. Alam Z, Ather MH, Jamshaid A, Siddiqui KM, Sulaiman MN. **Predictors of lymph node involvement in bladder cancer treated with radical cystectomy**. J Pak Med Assoc. 2009 Aug;59(8):516-9
7. Ali A Zehri, M Hammad Ather, Khurram Siddiqui, M Nasir Sulaiman. **A randomized clinical trial of lidocaine jelly for prevention of inadvertent retrograde stone migration during pneumatic lithotripsy of ureteral stone.** J urol, vol 180, Sep 2008; 180: 966-968
8. M Hammad Ather, Zaheer Alam, Anila Jamshaid, Khurram M Siddiqui, M Nasir Sulaiman. **Practice of Lymphadenectomy during radical cystectomy for bladder cancer in a University Hospital.** Braz J Urol
9. Siddiqui K, Abbas F, Biyabani S R, Ather M H, Talati J. **Role of Estrogens in the secondary hormonal manipulation of hormone refractory Prostate Cancer**. J Pak Med Assoc Sep 2004; 54(9): 445-7
10. Abbas F, Siddiqui K, Biyabani R, Hasan SH, Talati J**: Early results with intent to treat by radical retropubic prostatectomy for clinically localized prostate cancer.** *J Pak Med Assoc* 2002; 52(5): 200-5.
11. Moazzam M, Siddiqui KM, Ather MH, Biyabani SR. **Surgical ligation of scrotal varicocele for male factor infertility is a valid option of treatment".** J Pak Med Assoc. 2006 Aug; 56(8) 363

12. Albala DM, Siddiqui KM, Fulmer B, Alioto J, Frankel J, Monga M. **Extracorporeal shock wave lithotripsy with a transportable electrohydraulic lithotripter: experience with >300 patients**. BJU Int. 2005 Sep;96(4):603-7.
13. Steven A. Terranova, M.D., Khurram Siddiqui, MD., David M. Albala, M.D. and Glenn M. Preminger, M.D. **Hand-Assist Laparoscopic Renal Surgery: Hand Port Incision Complication.**. *Journal of Endourology*. Oct 2004, Volume: 18 Number: 8 Page: 770-774.
14. Y.H. Tan, Khurram Siddiqui, D.M. Albala. **Hand Assisted Laparoscopic Nephrectomy for Inflammatory Renal Conditions.** *Journal of Endourology*. Oct 2004, Volume: 18 Number: 8 Page: 770-774.
15. Siddiqui K, Nazir Z and Ali SS. **Is routine histological evaluation of pediatric hernia sac necessary?** Pediatric surgery International. Pediatr Surg Int. 2004 Feb; 20(2): 133-5

Published Abstracts

1. Khurram M Siddiqui ; Biggs, Colleen; Billia, Michele; Mazzola, Clarisse R; Izawa, Jonathan; Power, Nicholas; Chin, Joseph; Leong, Hon L **Enumeration of Prostate Cancer Microparticles as a Tool to Identify Prostate Cancer**. London Health Sciences Centre, CUAJ Vol 8, No 5-6 (2014) June-SUPPL 3
2. Billia, Michele; Siddiqui, Khurram M; Yutkin, Vladimir; Al-Zharani, Ali; Williams, Andrew; Baumann, Glenn; Chin, Joseph **Salvage High Intensity Focused Ultrasound of Radio-recurrent Prostate Cancer: Clinical Outcomes of a Prospective Trial at Tertiary Referral Centre**. Department of Urology, Western University; London Health Sciences Centre; London Victoria Hospital, London, ON, Canada; CUAJ Vol 8, No 5-6 (2014) June-SUPPL 3
3. Khurram M Siddiqui, Billia, Michele; Mazzola, Clarisse R.; Al-Zahrani, Ali; Brock, Gerald; Chin, Joseph **Three Year Outcomes of Recovery of Erectile Function after Open Radical Prostatectomy with Sural Nerve Grafting by a Multidisciplinary Surgical Team** London Health Sciences Centre; London Victoria Hospital, London, ON, CUAJ Vol 8, No 5-6 (2014) June-SUPPL 3
4. Mazzola, Clarisse R.; Willie, Chantalle; Pardhan, Siddika; Siddiqui, Khurram M.; Billia, Michele; Izawa, Jonathan; Chin, Joseph; Chambers, Ann F.; Brugarolas, James; Tram, Ahn; Power, Nicholas; Leong, Hon. **Developing a Patient-derived Xenograft Model Using Chicken Embryos to Predict Targeted Therapy Tumour Resistance in Renal Cell Carcinomas.** Western University, London, ON, Canada; 2UT Southwestern, Dallas, TX, United States CUAJ Vol 8, No 5-6 (2014) June-SUPPL 3
5. Zehri AA, Ather MH, Siddiqui KM and Sulaiman MN **Efficacy of Lidocaine Jelly in prevention of proximal migration of ureteric calculi during intracorporeal lithotripsy (Best poster ward EUA-Milan 2008)**
6. Abbas F, Sanaullah R, Zaidi N, Talati J, Siddiqui A, Memon A, Bayabani R, Ather H, Siddiqui K. **A comparative assessment of the sensitivity of telomerase assay with other molecular markers for the diagnosis and of human bladder cancer**. British Journal of Urology, October 2004, Volume: 9, Supplement 2, pg 125.

7. Y.H. Tan, K. Siddiqui, D.M. Albala **Hand Assisted Laparoscopic Nephrectomy for Inflammatory Renal Conditions.** *Journal of Endourology*. Sep 2003, Volume: 17 Number: 7 Supplement: 1 Page: 155.
8. D.M. Albala, K. Siddiqui, J. Kabalin, J. Nuzzarello, B. Davis, G. Andriole, J. Lingeman, G. Eure. **Transurethral Microwave Thermotherapy (TUMT) Using The Thermatrx TMX-2000™: Durability Exhibited In A Study Comparing TUMT With A Sham Procedure In Patients With Benign Prostatic Hyperplasia (BPH)** *Journal of Endourology*. Sep 2003, Volume: 17 Number: 7 Supplement: 1 Page: 11.
9. Yeh Hong Tan, Jim L'Esperance, Khurram Siddiqui, Glenn Preminger, and David Albala **Hand Assisted Laparoscopic Heminephrectomy in Horseshoe Kidney.** *Journal of Endourology*. Sep 2003, Volume: 17 Number: 7 Supplement: 1 Page: 53.
10. W. Patrick Springhart, Khurram Siddiqui, Steven A. Terranova, Yeh Hong Tan, and David M. Albala**. Patient Positioning In Urologic Hand-Assisted Laparoscopic Renal Surgery: The Technique**. *Journal of Endourology*. Sep 2003, Volume: 17 Number: 7 Supplement: 1 Page: 344.

Book Chapters

1. **Urolithiasis; Basic Science and Clinical Application. Edited by J. Talat, D Albala, H Tesilius and Z Ye.** Springer 2012
   Contribution as**:** Associate Editor and Section Editor
   Chapter; Epidemiology of stone disease
   Chapter; Role of operating room nurse
   Chapter; Robotics for stone disease
   ISBN-13: 978-1447143833
2. **Mastery of Endoscopic and Laparoscopic Surgery**. Edited by Nathaniel J. Soper, MD, Lee L. Swanstrom, M.D., W Stephen Eubanks, M.D. 2nd Edition. Lippincott Williams and Wilkins. Philadelphia, P.A. 2005.
   ***Chapter;* Laparoscopy In Urology**. Steven A. Terranova, M.D., Khurram Siddiqui, MD., David M. Albala, M.D. and Glenn M. Preminger, M.D.
   ISBN-13: 978-0781744454
3. **Holmium Laser ; Endourological Applications**. Edited by Narmada P. Gupta; B I Publications. India 2004
   ***Chapter;* Ureteropelvic junction obstruction Khurram Siddiqui, Yeh Hong Tan, David M Albala The success in the endoscopic treatment of ureteropelvic junction.** ISBN-13: 978-8172252021 ISBN-10: 81-7225-202-1