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Prostate Cancer Microparticles in Men Undergoing Radical Prostatectomy

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Abstract

Objectives: To determine changes in prostate microparticle (PMP) concentrations in men with prostate cancer (PCa) after digital rectal examination (DRE), after radical prostatectomy (RP), and at follow-up.

Materials and Methods: 22 men were recruited before RP. Four blood specimens were collected – baseline (specimen 1), post-DRE (specimen 2), immediately post-RP (specimen 3), and follow-up (specimen 4). Pre- and post-DRE urine was collected (Specimen A and B respectively). Flow cytometric analysis of biofluids was performed with fluorescent-labeled antibodies against prostate-specific membrane antigen (PSMA) and polysialic acid. Total MP (TMP) and dual positive (PMP) events per µl of plasma or urine were recorded.

Results: Median TMPs from specimen 1 to 4 were 3 005 500, 1 600 600, 976 353, and 3 951 400 events/µl, respectively ($p<0.001$). Median proportional PMPs increased from 9.6% to 17.3% after DRE ($p<0.001$), to 25.7% ($p<0.001$) post-RP, and decreased to 4.1% at follow-up ($p=0.170$). Urinary PMPs were unchanged after DRE.

Conclusions: Compared to baseline levels, proportional PMP events were significantly increased following DRE, and further following RP.

Keywords: Prostate Cancer, Biomarker, Liquid Biopsy, Diagnosis, Risk Stratification, Microparticle, Extracellular Vesicle, Exosome, Ectosome, Digital Rectal Examination

Dedication

To my wife Lucille, who has selflessly and lovingly supported me through this fellowship, this degree, and the writing of this thesis.

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AS	Active Surveillance
BPH	Benign Prostatic Hyperplasia
BT	Brachytherapy
DRE	Digital Rectal Examination
EBRT	External Beam Radiotherapy
EM	Electron Microscopy
EV	Extracellular Vesicle
FITC	Fluorescein Isothiocyanate
IHC	Immunohistochemical
LHRI	Lawson Health Research Institute
MP	Microparticle
mpMRI	Multiparametric Magnetic Resonance Imaging
MVB	Multivesicular Body
NPV	Negative Predictive Value
PAP	Prostatic Acid Phosphatase
PBS	Phosphate-buffered Saline
PE	Phycoerythrin
PCa	Prostate Cancer
phi	Prostate Health Index
PMP	Prostate Microparticle
PPV	Positive Predictive Value
PSA	Prostate-Specific Antigen
PSMA	Prostate-Specific Membrane Antigen
ROI	Region of Interest
RP	Radical Prostatectomy
RT	Radiotherapy
STEAP1	Six-Transmembrane Epithelial Antigen of the Prostate 1
TRUS	Transrectal Ultrasound
TURP	Transurethral Resection of the Prostate

Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer among men worldwide and accounts for approximately 6.6% of male cancer deaths (1). The large majority of prostate cancers are indolent and remain organ-confined throughout their natural history. A small proportion, however, are either aggressive *ab initio* or become so at a later stage. Of men who have prostate cancer diagnosed, few will have life-threatening disease. This is known from epidemiological data, but discerning at an early stage which particular men will die from prostate cancer from those that will not is very difficult.

Prostate-specific antigen (PSA), also called Human Kallikrein-3, was discovered and characterised in the early 1980's (2). Initial studies showed a positive correlation between serum PSA levels and the presence of prostate cancer. It rapidly became, and has remained, the standard biomarker for prostate cancer. Its clinical use extends from screening to diagnosis, risk stratification, and disease monitoring. The advent of the anatomic radical retropubic prostatectomy by Walsh in the 1980's, the improvement in technology for delivering radiation therapy, and then widespread screening for asymptomatic disease with prostate-specific antigen (PSA) testing in the early 1990's all led to a massive increase in the number of men being treated aggressively for localised prostate cancer. The widespread screening and treatment that occurred from the early 1990's onward resulted in a minor reduction in prostate cancer mortality, at approximately 30-40% lower than in the pre-PSA era (3). The reduced mortality rate, however came at a significant cost. There was substantial over-investigation of asymptomatic men, since many would undergo unnecessary prostate biopsy and other investigations. It also led to aggressive treatment of men with latent prostate cancer who were at low risk of progressive or fatal disease. This marginal benefit of PCa screening suggested by epidemiological studies was confirmed in several randomised controlled trials, most notably the European Randomised Study of Screening for Prostate Cancer (ERSPC). As a result of these trials, in 2012 the United States Preventative Services Taskforce (USPTF) recommended against routine screening for PCa (4). They cited a high risk of men being "overdiagnosed" and "overtreated". Overdiagnosis refers to the diagnosis of

cancer that would otherwise never have been symptomatic, and overtreatment is when men undergo radical therapy for disease that would never have been life threatening. The most recent analysis of the data from the ERSPC suggests that to save one man from death due to prostate cancer, 781 men need to be offered screening, and 27 additional cancers need to be diagnosed (5). After the recommendation by the USPSTF, many other healthcare organisations followed suit, and these recommendations were subsequently taken to heart among primary care providers. There is much evidence that fewer men were indeed screened in the years subsequent to the 2012 recommendations, and an upward stage migration in PCa has occurred since that time (6). This presumably indicates delayed diagnosis.

There is therefore an apparent paradox when it comes to screening for PCa. On the one hand, it is a common disease and a common cause of death among men. Early detection is possible, and several effective curative treatments are available. On the other hand, however, evidence from observational data and from randomised trials has shown limited benefit to screening of asymptomatic men, with a high rate of overinvestigation, overdiagnosis, and overtreatment.

There are several underlying factors that explain this apparent paradox. The first is that although the mortality from prostate cancer is high, the prevalence of PCa in middle aged and elderly men in the population is *extremely* high. While men have a 3-6% chance of dying of prostate cancer, men over 60 years old have a greater than 50% chance of harbouring at least a small focus of prostate cancer (7). So for any one man with prostate cancer, the chance of dying of the disease is low. There has recently emerged the concept of “clinically significant” PCa, which excludes the small volume, low grade cancers that are unlikely to be immediately dangerous. Secondly, screening for prostate cancer currently utilises a combination of two poor tests. These are serum PSA and transrectal ultrasound-guided (TRUS) biopsy. In most series, only 25-40% of men with abnormal PSA levels will have a positive biopsy. Many of the cancers diagnosed are clinically insignificant, while up to 20% of clinically significant cancers are missed due to the random nature of the biopsy. The histological grade of PCa - the Gleason score - is currently the most accurate predictor of disease behaviour, but the transrectal biopsy necessary to obtain the prostate tissue is invasive and carries the risk of complications. Common side effects

of transrectal biopsy include pain, rectal and urethral bleeding, worsening of lower urinary tract symptoms, and a temporary worsening of erectile function (8). Less common, but potentially more serious is post-biopsy sepsis. Up to 1.7% of men are hospitalised for infectious complications following prostate biopsy (8). In the ERSPC trial, there were 28 biopsies performed for every 100 men screened (5).This means that many men who opt for PSA screening will undergo a lengthy, costly, and potentially morbid process of investigation, and then turn out not to have clinically significant PCa. The third reason that screening has not worked well is that a much higher proportion of men undergo curative treatment for prostate cancer than would be expected to die of the disease. Another estimate from the ERSPC data is that an additional 17 cases of prostate cancer need to be diagnosed in order to save one life. The majority of those 17 patients would undergo radical treatment, indicating the high degree of overtreatment. This treatment is usually radical prostatectomy (RP), external beam radiotherapy (EBRT), or interstitial brachytherapy (BT).

These treatments all have significant side effects, including urinary incontinence, urinary obstruction, erectile dysfunction, and bowel dysfunction. They also place a burden of time, anxiety, pain, and discomfort. The financial impact of these investigations and treatment is significant. An analysis from 2006 estimated the total cost per patient diagnosed with prostate cancer was in excess of US$18,000 from diagnosis to four years of follow-up (9). The number of men undergoing these treatments has decreased progressively over the last 15 years as it has been recognised that men with low risk prostate cancer benefit less from curative treatment, leading to the increased uptake of Active Surveillance (AS) in that population. This strategy has now been universally accepted as a way to reduce unnecessary treatment of PCa. However, only 20-30% of men with newly diagnosed PCa will meet strict criteria for AS, and up to 50% of those men will proceed to curative treatment within a few years. The follow-up regimens for AS are also relatively onerous on the patient. This leaves the majority of men diagnosed with PCa as non-candidates for AS, the majority of whom will also not die of PCa even if left untreated. They would thus still be both overdiagnosed and overtreated for PCa. The obvious problem is the imperfection of current methods of risk stratification.

Because of a large degree of uncertainty, healthcare providers err quite heavily on the side of overtreatment.

These clinical challenges highlight the current need for a better biomarker for prostate cancer. Currently, PSA is used for screening, risk stratification, prognostication, and follow-up. Many novel biomarkers have been brought to market since the advent of PSA, but all of them have only been able to augment the role of PSA, and not replace it entirely.

Extracellular vesicles, also known as “microparticles”, represent one of the most promising new frontiers for biomarker research. They are subcellular cell-derived vesicles that carry membrane antigens of their parent cells and carry specific protein and genomic cargo. While their existence has been recognised for several decades, the technology to accurately quantify and to characterise microparticles has been a limiting factor up until relatively recently. Publications citing the term “extracellular vesicle” have increased 10 fold over the last 15 years, which illustrates the rapidly increasing interest in the field. It is now recognised that almost all cells in the body release extracellular vesicles. Many of which find their way into the circulation, and this is also true of the prostate (10). Circulating concentrations of microparticles have been shown to be increased in patients with cancer (11). Microparticles or extracellular vesicles originating from the prostate that are detectable in the blood are a promising non-invasive biomarker for prostate cancer.

The study that forms the basis of this dissertation is an analysis of prostate microparticles in the blood and urine of a cohort of men undergoing radical prostatectomy for localised prostate cancer. The aim of the study was to determine whether a digital rectal examination would increase the levels of microparticles in the blood or urine. The first chapter is a literature review that reviews the currently available biomarkers for prostate cancer, as well as extracellular vesicles, with a focus on prostate microparticles. The second chapter deals with technical details related to specimen collection, sample preparation, and flow cytometry. The third chapter reports the findings of our study examining PCMPs in the serum of men before and after DRE, as well as immediately after prostatectomy and at short term follow-up. The fourth chapter reports the findings of the study on PCMPs in the

urine of the same cohort of men before and after DRE prior to radical prostatectomy. The fifth chapter is a general discussion around the issues raised and hypotheses generated.

Chapter 1: Literature Review

This dissertation is focused on the role that blood borne microparticles may play clinically as a novel biomarker for prostate cancer. The literature review therefore has two parts. The first covers the currently available biomarkers for prostate cancer, with special emphasis on their test performance characteristics for diagnosis and risk stratification. The second part aims to give a general overview of the literature on microparticles, as well as a more detailed account of what is known about microparticles and prostate cancer.

Methods

A literature review was performed using the Medline database and Google Scholar. For the section on prostate cancer biomarkers, the search terms used were "prostate cancer", "biomarkers", "diagnosis", "risk stratification", "imaging", and "active surveillance" in varying combinations. For the microparticle section, the terms "microparticle", "extracellular vesicle", "endosome", "exosome", "prostasome", and "oncosome" were used. The search was limited to original peer-reviewed articles published in English since 2000. Articles were screened on title and abstract. Selected references from applicable articles were also looked up where appropriate. Articles on microparticles that dealt with the prostate and prostate cancer were all reviewed fully, while articles specific to other organ systems, general reviews, or technical aspects were reviewed on a more selective basis.

Current Biomarkers for Prostate Cancer

The term "biomarker" is a portmanteau of the term "biological marker". There are myriad and sometimes conflicting definitions. The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (12). In the context of prostate cancer, this broad definition would incorporate blood tests, urine tests, imaging investigations, and histological investigations. Since the prostate cancer microparticle test would seek to fill the role of a diagnostic and risk

stratification test, this literature review has been limited to currentcommercially available blood and tissue biomarkers, and to a more limited extent, currently available imaging modalities.

Prostate-Specific Antigen

The prototypical biomarker for prostate cancer is prostate-specific antigen (PSA). PSA is a normal exocrine secretory product of the prostate gland. It is a 33 kilodalton serine protease, and is a member of the human kallikrein family. Its physiological function it to liquefy the seminal coagulum (13). PSA was identified in human semen in the 1970's by various investigators, most notably by Richard Ablin in Buffalo, NY (2). At that time, the only available biomarker for prostate cancer was prostate acid phosphatase (PAP) which was only a reliable marker for metastatic disease (14). The scientific work that would bring PSA to the fore was done by Wang and Chu at the Roswell Park Memorial Institute, with their first of a series of important papers being published in 1979. They purified the protein, characterised it, and suggested it as a biomarker for prostate cancer. Based on their initial studies and those of Stamey et al., PSA was initially patented and approved by the FDA as a tool to monitor disease progression and response to treatment in men undergoing radical prostatectomy, radical radiotherapy, androgen deprivation therapy, and treatment of castrate-resistant disease (15). In the early 1990's it was also found to be useful for screening asymptomatic men for PCa (16). This new clinical indication led to the massive increase in prostate cancer screening seen in most developed countries, and the subsequent increase in treatments for localised disease (17).

In addition to disease monitoring and screening, PSA was also incorporated into methods of risk stratifying men with apparent localised disease. The system proposed by D'Amico et al. in 1998 used pre-treatment PSA together with Gleason sum on prostate biopsy and findings on digital rectal examination to stratify men into low, intermediate, and high risk groups to predict their chance of biochemical recurrence after local treatment (18). This tool was subsequently validated in multiple external cohorts and quickly became the standard method of risk stratification, as well as the basis for newer risk classification systems (19). As it became more widely recognised that localised prostate cancer was being

overtreated, the strategy of active surveillance was developed in the late 1990's and early 2000's. Again, PSA was used in all large series as one of the primary indicators of disease progression, with a rise either prompting further investigation, or triggering treatment (20,21). This newer use for PSA as a biomarker is a direct extension of its role in risk stratification.

PSA testing has proven to be very accurate in detecting recurrent prostate cancer after surgery (22). It is also fairly reliable for recurrence of cancer after radiotherapy and disease progression in metastatic PCa. However, PSA performs poorly for other indications, most notably screening and risk stratification. For screening purposes, there is not a cut-point below which PCa is certain to be absent, or a reasonable cut-point above which cancer is certainly present. For instance, at all PSA cut-points, there will be patients with prostate cancer that will be missed (Type 2 error) and there will be a significant number of patients that have been incorrectly "flagged" as having prostate cancer (Type 1 error). The most widely used value for "normal PSA" is ≤4.0 ng/ml. The positive predictive value at this level (≥4.0 ng/mL) is only 25-40% (23). Also, more than 20% of the men thus diagnosed will have clinically insignificant cancer. Data from the Prostate Cancer Prevention Trial (PCPT) suggested that, in order to achieve a sensitivity of 80%, the PSA threshold would need to be reduced to 1.0 ng/ml, but at that cut-point, the false-positive rate would be above 60% (24). Using data from the Prostate Cancer Prevention Trial, Thompson et al. reported an area under the receiver operating curve (AUC) of 67.8% for PSA alone (25). 20% of men meeting very low risk criteria have "significant disease" at RP (26).

Two of the refinements made in PSA testing since its introduction are the calculation of PSA density and the measurement of free PSA. PSA density is a measure of the serum PSA level as a proportion of the prostate volume. This corrects somewhat for the effect of prostate size and makes the test more sensitive for the detection of cancer (27). While the PSA density improves prediction of cancer better than PSA alone, the shortcoming is that it generally requires TRUS to accurately assess prostate volume, which is impractical to do for all patients in a screening situation. The role of PSA density in risk stratification is less clear.

Free PSA is a measure of the concentration of serum PSA that is not bound to plasma proteins. It is absorbed from the glandular lumen after inactivation of mature

active PSA, and is therefore underrepresented when a high serum PSA is driven by a malignant process with disordered glands (28). A high proportion of free PSA is indicative of a lower likelihood of malignancy, and vice versa. Free PSA is recommended in the “grey zone” of PSA 2.5-10 where it can sometimes assist with decision-making during screening. Unfortunately, it also represents a spectrum of risk, with the majority of patients remaining in a new grey zone somewhere in the middle. A large meta-analysis of studies on free PSA calculated an AUC of 0.70 for the detection of cancer, which is only marginally better than total PSA alone (29). Based on extrapolation from external data, percent free PSA was included in an updated version of the PCPT risk calculator, but it was not found to be a particularly strong discriminator between high grade, low grade, and no prostate cancer (30). A similar study using a subset of pre-screened men in the ERSPC found that %fPSA had no predictive power (31). As with PSA density, there is no clearly established role for free PSA measurements in risk stratification.

Other attempts at improving PSA testing in clinical practice include differing age and race cutoffs, PSA changes over time (PSA doubling time or PSA velocity), and baseline PSA at a younger age, but these modifications have not found a firm place in clinical practice. To diagnose prostate cancer, a histological diagnosis is typically made by means of a random multiple core TRUS biopsy. By its random nature, transrectal biopsy misses up to 25% of cancers (32), and can underestimate tumour extent and grade by 30%, thus further reducing the usefulness of PSA as a screening test (33,34). These same shortcomings of the random TRUS biopsy approach also reduce the accuracy of risk stratification in prostate cancer.

These issues underscore the major deficiencies of PSA as a clinical tool, and the need for better methods of screening asymptomatic men and risk stratifying those with an established diagnosis of prostate cancer.

Non-invasive biomarkers

PCA3

Prostate Cancer Antigen 3 (PCA3) was first described by Bussemakers et al. in 1999 (35), where it was initially referred to as DD3. It is a gene that is highly overexpressed in prostate cancer tissue – up to 60 times higher than in benign prostate tissue (36). Hessels et al. tested the concentration of PCA3 mRNA in first voided urine after a vigorous prostate massage in 108 men with raised PSA values prior to prostate biopsy. This was expressed as a ratio of PCA3 to PSA mRNA concentrations. The test performed with an AUC of 0.717 (0.58–0.85), which was better than that of serum PSA. The Progensa^{TM} PCA3 assay (Gen-Probe Inc, San Diego, Calif) was granted FDA approval in 2006, becoming the first prostate biomarker to be approved since the approval of PSA 20 years earlier.

The initial results were validated in numerous other cohorts, generally producing AUC values of above 0.690 (37,38). At a cut off value of 50 x 10^{-3}, the sensitivity of the test was found to be 0.69, while maintaining a relatively modest specificity of 0.79. Hansen et al. incorporated the PCA3 score into a pre-biopsy nomogram that also utilised age, PSA, prostate volume, and DRE findings. On univariate analysis, they found that PCA3 was the strongest single predictor of a positive biopsy. By using this nomogram, the authors concluded that 55% of unnecessary biopsies could be avoided at the cost of missing less than 2% of high grade cancers (39). A study performed at the National Cancer Institute prospectively enrolled 859 men prior to scheduled prostate biopsy and performed a PCA3 test (40). For biopsy naïve men, a PCA3 score of >60 gave a PPV of 80% for detection of any cancer, while for men undergoing repeat biopsy, a PCA3 score of <20 gave a NPV of 88%. While the latter indication might be clinically useful, the sensitivity of PCA3 >60 for first time biopsies was only 0.42. When used in combination with the PCPT risk calculator, the AUCs for detecting high grade disease were 0.78 and 0.79 for initial and repeat biopsies, respectively.

PCA3 has also been evaluated in the context of risk stratification. Ploussard et al. studied 106 men with low risk PCa who underwent RP with the intention of using the PCA3 test to determine eligibility for AS (41). PCA3 score >25 was significantly

associated with a more than threefold higher risk of having a tumour volume of >0.5 cm^3. However, it did not accurately which patients would have extraprostatic disease or an overall unfavourable pathology (Gleason pattern ≥4, T3+, N+, or tumour volume >0.5 cm^3). Furthermore, the PCA3 did not seem to add much more value than the so-called Epstein biopsy criteria which are aimed at identifying low grade, low volume disease. The authors concluded that while PCA3 might be incorporated into an AS selection protocol, it could not be used as a sole prognostic variable among low risk men. The implication of their statement is that AS might be advised against in the setting of a high PCA3, even if other factors suggest very low risk disease, although this conjecture was not clear from their reported analysis. Two studies have shown that PCA3 score is an independent predictor of multifocal disease among men undergoing radical prostatectomy (42,43).

In the 13 years since this test was first described, usage of the PCA3 test has remained fairly limited in most centres, possibly due to increased cost and/or the need for a prostate massage prior to testing.

TMPRSS2:ERG

Transmembrane protease, serine 2 (TMPRSS2) is a serine protease that contains a type II transmembrane domain and is expressed in prostatic basal cells and in prostate carcinoma (44). It is encoded by the TMPRSS2 gene.

In 2005, Tomlins et al. described abnormal fusion of the TMPRSS2 gene to various transcription factors of the ETS family, most commonly ERG and ETV1, in prostate cancer cells (45). This was postulated to be an oncogenic mechanism, whereby a cell's attempted expression of TMPRSS2 would lead to overexpression of these transcription factors. It was subsequently found that approximately 50% of screen-detected prostate cancers had TMPRSS2:ETS fusions, and that ERG accounted for approximately 90% of such chromosomal rearrangements (46). Detection of either the gene fusion itself or the resultant truncated ERG protein is almost 100% specific for PCa. The protein product is not detectable in the blood or urine, but TMPRSS2:ERG mRNA can be detected in the urine, which has led to its use as a non-invasive biomarker. In an independent cohort, Esgueva et al. identified TMPRSS2:ERG fusions in 37% of 540 cases of PCa (47).

The group that first developed this urine PCR test then developed a urine test that utilised the same technology as the commercially available PCA3 test. The collection also involved a first catch urine sample after a vigorous DRE. This test is sometimes referred to as the T2 score, and is also reported as a ratio of the mRNA to that of PSA mRNA. It was quickly realised that the very high specificity, but low sensitivity of the TMPRSS2:ERG test could be coupled to the PCA3 test which had a higher sensitivity at a lower specificity. A prospective multicentre Dutch study performed PCA3 and T2 tests on 497 men prior to prostate biopsy (48). In that study, addition of PCA3 to the ERSPC risk calculator improved the AUC for positive biopsy from 0.799 to 0.833. With the addition of T2, the AUC improved further to 0.842. T2 score was also a predictor of Gleason score, with OR 7.16 (2.54-20.15). Among the 61 patients in the cohort who underwent RP, the T2 score correlated with extraprostatic extension – with OR 4.91 (1.13-21.98) – but not with Gleason upgrading or seminal vesicle invasion.

Investigating the combination of PCA3 and T2 scores as a risk stratification tool, Lin et al. tested the urine of 387 men in the Canary Prostate Active Surveillance Study (49). The aim was to determine whether the tests could predict higher volume or pathological upgrading on repeat prostate biopsy. While the markers did correlate with biopsy findings on repeat biopsy, neither marker alone or a combination of the two added any statistically significant improvement in prediction over PSA alone. In a similar study, Cornu et al. performed TMPRSS2:ERG and PCA3 on men on active surveillance prior to a second biopsy, but with additional genotyping tests (50). They too found that the results of the urine assays did no better than PSA or PSA density at predicting pathological upgrading or increased tumour volume.

The combination of PCA3 and T2 scores is now commercially available as the Mi-Prostate Score, or MiPS. This test combination was investigated as a tool to reduce unnecessary repeat biopsy by Merdan et al. (51). Among men with raised PSAs and an initial negative biopsy, at thresholds for PCA3 and T2 of ≥25 and ≥10, respectively, biopsies could be reduced by 54.4% and 63.2%, respectively. The calculated cost of this reduction in biopsies was a reduction in 10-year cancer-specific survival of only 0.9% and 1.4%, respectively.

[-2]proPSA and PHI

[-2]proPSA was first described by Mikolajczyk et al. in 2001 (52). PSA is produced as a proenzyme that has a 17 amino acid leading sequence. Cleavage of this protein gives rise to proPSA, which has an additional 7 amino acids at the N terminal compared to normal PSA ([-7]proPSA). Mature PSA is then generated in the glandular lumen by cleavage of those 7 amino acids by Human Kallikren 2 (hK2) and to a lesser extent hK4 (28). Other proteolytic enzymes can cleave the N terminal amino acids in other positions, leading to generation of inactive [-2] and [-5] proPSA. These isoforms of the PSA enzyme occur at higher concentrations in prostate cancer, presumably because of less exposure to hK2 in the glandular lumen. As mentioned in the section on PSA above, active PSA becomes inactive after it undergoes "nicking" in any of three different locations. It then also circulates as free PSA. This process is more likely to occur in benign tissue. The ratio of free PSA to the [-2]proPSA has turned out to be more useful than [-2]proPSA concentrations alone in distinguishing benign from malignant causes of a raised PSA.

The "prostate health index" (phi) is a formula that utilises the [-2]proPSA level in combination with the free and total PSA values (phi = p2PSA/fPSA x $tPSA^{½}$) (53). In a prospective study by Catalona et al., a cohort of men with PSA values in the range of 2-10 ng/ml had phi determined prior to first biopsy (54). The test achieved an AUC of 0.703 for the detection of cancer overall, and an AUC of 0.724 for distinguishing Gleason 7 and above from benign disease or Gleason 6. The clinical usefulness might be limited due to the fact that 26.1% of men with a phi score of 0-24.9 had Gleason ≥7 disease. In a study on samples from two sites of the ERSPC, Jansen et al., 2010 (53) did not find the levels of p2PSA or phi before biopsy to be significantly different between men with Gleason ≤6 vs ≥7 disease. Guazonni et al., 2011 also found that %p2PSA and phi improved the diagnostic accuracy prior to biopsy, with AUC's of 0.756 and 0.757, respectively (55). However, they did not improve prediction of Gleason score. The same group analysed p2PSA and its derivatives in 350 men undergoing RP. They found that %p2PSA and phi were both significantly higher in the presence of pT3 disease, Gleason ≥7, and Gleason upgrading (56). Levels were lower in men with tumour volume <0.5 ml. The main advantage of this study was that it

was correlated with operative histology rather than with the results of TRUS biopsy. However, this was not in a screening population, since most men were intermediate or high risk. The group developed multivariate predictive models for T3, Gleason ≥7, Gleason upgrading, and low volume disease that included other clinical and pathological variables (such as age, clinical stage, PSA, biopsy results). The addition of %p2PSA and phi improved AUCs by up to 6%, with phi being slightly superior to %p2PSA. The best AUC was for predicting low volume disease (0.875). Of particular pertinence for an AS setting is the Gleason upgrading from 6 to ≥7. AUCs for predicting upgrading were 0.696, 0.747, and 0.753 for the base model, the model including %p2PSA, and the model including phi, respectively. Again, the other characteristics of this population (prostate volume, volume of cancer on biopsy, PSA, clinical stage) might limit the applicability. Although the addition of %p2PSA and phi significantly improved risk assessment, the benefit was marginal and there seems to be a large tradeoff between sensitivity and specificity at all cutpoints.

Makarov et al. (2009) performed a retrospective analysis of sera of 71 men on AS (57). 39 men in that cohort subsequently progressed to radical treatment, either because of higher Gleason grade or higher tumour volume on prostate biopsy. 32 of those men retained favourable biopsy criteria. Retrospective measurement of %p2PSA showed higher values at diagnosis in men who subsequently needed treatment for PCa. Hazard ratio for progression was 2.53 (1.18-5.41) p = 0.02.Using the same cohort of 71 men, Isharwal et al. also measured the prostate health index (phi) as well as the DNA content of their biopsy specimens using the AcuCyte imaging system. In that study, the phi performed equally as well as the %p2PSA, with AUCs of 0.625 and 0.616, respectively. Ferro et al. obtained %p2PSA, phi, and PCA3 on a cohort of 251 men undergoing first prostate biopsy. Both the phi and the PCA3 scores offered significant improvement over PSA alone, free PSA, %free PSA, and p2PSA in predicting positive biopsy. With specificity fixed at 0.9, the sensitivities of phi and PCA3 were similar at 0.36 (0.13-0.62) and 0.32 (0.04-0.49), respectively.

4Kscore

The 4Kscore® (OPKO Health, NJ) is a test named for the four kallikreins that are measured, namely total PSA, free PSA, intact PSA, and hK2. Using a proprietary

algorithm, the values are combined with clinical factors such as age and previous biopsy history to estimate the risk of a high grade disease at biopsy (58).

In a cohort of men with PSA 3-15 undergoing first time biopsy, Nordstrom et al. compared 4Kscore and phi to a base model of age and PSA alone in predicting positive biopsy and high grade (Gleason ≥7) tumour (59). Both were superior to the base model by very similar margins. For predicting a positive biopsy, AUCs for 4Kscore and phi were 0.690 and 0.704, respectively. For high grade cancer, AUCs were 0.718 and 0.711, respectively. Both are simple blood tests that do not require a vigorous DRE. The authors concluded that 29% of biopsies could be avoided at a cost of initially missing 10% of high grade cancers.

A similar study in a larger cohort, reported by Parek et al., aimed to assess the 4Kscore in predicting Gleason ≥7 PCa (60). The 4Kscore showed an AUC of 0.820 which was significantly better than the PCPT risk calculator. The authors calculated that 30-58% of biopsies could be avoided, while missing 1.3-4.7% of Gleason ≥7 cancers. Vickers et al. examined the use of the 4Kscore among men "outside of the diagnostic gray zone" – with PSAs of 10-25 ng/ml or positive digital rectal examination in a large cohort (61). Their findings suggested that its use in this population could still reduce biopsy rates by 20% while only missing 3% of high grade cancers. Vedder et al. investigated the added value of PCA3 and the 4Kscores in men undergoing repeat screening in the fourth round of the Dutch ERSPC screening arm (31). While the 4k score was slightly better than PCA3 at predicting a positive biopsy among men with raised PSAs, the PCA3 performed better overall in a multivariable model. All differences were small, indicating that the tests performed similarly but also that the added benefit of these ancillary tests in that setting was relatively minimal. A recent meta-analysis by Voigt et al. confirmed a net improvement in diagnostic ability above PSA of 8-10%, and their cost analysis suggested that its widespread use could potentially result in annual savings of $1 billion in the USA (62).

While the majority of research on the 4Kscore has been performed in the context of pre-biopsy prediction of positive biopsy and high Gleason tumours, there is some data on the ability of the 4Kscore to risk stratify men known to have cancer. Carlsson et al. studied a cohort of 392 men with PCa from the Rotterdam arm of the ERSPC

who underwent RP between 1994 and 2004. They found that 4Kscore accurately predicted significant disease (Gleason ≥7 or volume >0.5 cm^3) on final pathology, most markedly in the low and very low risk groups (63). The AUC was calculated at 0.81. Punnen et al. analysed pathological findings at RP among 141 men who had had a 4Kscore prior to their initial prostate biopsy (64). While the score correlated with the histological grade, tumour volume, and extraprostatic disease at RP, its use did not improve upon established clinical risk stratification such as D'Amico classification. Part of the discrepancy between the results of this study and that of Carlsson et al. is possibly due to lower numbers studied, but is also likely to be affected by differences in clinical characteristics. The former study included a much larger proportion of men with low risk disease, while the latter was composed mainly of men known to have at least intermediate disease. This highlights the potential clinical use of the 4Kscore test in selecting out low risk men for radical treatment rather than selecting intermediate risk men for surveillance.

It is known that PSA level at a young age is predictive of likelihood of increased PCa-specific mortality later in life (65). Stattin et al. investigated the use of the 4Kscore to sub stratify men in at age 50-60 yrs with high baseline PSA values (66). They found that half of men with PSA values >2 ng/ml could be regarded as low risk based on their 4Kscore, with a risk of metastatic PCa at 15 yrs of less than 1%.

The 4Kscore has yet to receive approval from the FDA.

Newer Non-Invasive Biomarkers

ExoDx Prostate Intelliscore

The ExoDx™ Prostate (IntelliScore) (Exosome Diagnostics, Waltham, MA), also called the EPI score, is an assay measuring three different exosomal proteins in a first void urine sample. Unlike the PCA3 test, there is no need for a DRE prior to taking the sample (67). The test is intended to predict Gleason ≥7 cancer in a pre-biopsy setting. Development was on a training cohort of 499 patients with PSA values of 2 to 20 ng/ml, and validation then performed in a multi-institutional cohort of 1064 patients. For tumours of Gleason score ≥7, the negative predictive value of the test

was 91.3%, with a sensitivity of 91.9% (67). Using the test, 37% of biopsies could be avoided at the cost of missing 13% of Gleason ≥7 tumours. A poster presentation at the AUA 2017 meeting reported on a study analysing EPI scores in a pre-RP cohort. The EPI scores correlated with pathological stage and Gleason/ISUP grade. Among men who had pathological upgrading from a pre-RP biopsy grade of ISUP 1 (Gleason 6), the EPI score was significantly associated with upgrading at RP ($p<0.001$) (68). This suggests that the test may have some utility in selecting men for AS, or for subsequent monitoring on AS. However, the test has not yet been studied in an active surveillance population.

Circulating RNA

There has been much recent interest in circulating genetic material as biomarkers for PCa. Non-coding RNA (ncRNA) are segments of RNA that are transcribed from non-coding DNA and are not transcribed into proteins, but are nevertheless important for normal cellular functions (69). Alterations in ncRNA regulation also have a role in tumour biology. ncRNA can be subdivided based on size into long non-coding RNA (lncRNA) and small non-coding RNA (sncRNA). There is evidence that certain lncRNAs regulate certain cellular processes, including proliferation, differentiation, or apoptosis (69). The most well-known lncRNA PCa biomarker is PCA3, which has been discussed above. Another promising lncRNA marker is SchLAP1 which was found to predict 10 year metastasis with an odds ratio(OR) of 2.45 (1.70–3.53) when identified in PCa tissue after RP (70). Although this OR was slightly better than that of the Gleason score at final pathology, it is not clear what the additive benefit of this marker might be. There are several other lncRNA sequences that correlate to the presence of prostate cancer.

MicroRNAs (miRNAs) are a subtype of sncRNA, and have recently received more research attention in the biomarker space, possibly due to their increased accessibility in the blood and urine. miRNAs are small (17-27 nt) single stranded RNA molecules that bind to non-coding regions of messenger RNA, thereby down regulating gene expression. Not only are miRNAs integral to normal cellular functioning, they can also be phenotypic markers of malignant cells (71). The majority of circulating miRNA is found within extracellular vesicles, but miRNA is also

present free in the circulation, bound to ribonucleoprotein complexes, or within liposomes (72). Multiple studies have shown that at least 61 different miRNAs have altered expression in PCa cells, and at least 19 of those show some correlation to prognostic indicators such as Gleason score or metastasis (71). The studies on miRNA in PCa are thus far only preliminary in nature (73). There is much promise that large panels of miRNA will be commercially available in the future, but significantly more clinical study is still required.

DNA hypermethylation

DNA hypermethylation of various PCa-promoting genes is an important epigenetic mechanism in carcinogenesis and cancer progression. Hypermethylated GSTP1, T1G1, EDNRBRASSF2, HIST1H4K, TFAP2E, and others have been shown to correlate with the presence of PCa (74,75). The presence of hypermethylated genes in benign prostate tissue has been used as an indicator of occult malignancy elsewhere in the prostate. This forms the basis of the ConfirmMDx test that is performed on FFPE specimens from prostate biopsy to predict the likelihood of a positive repeat biopsy (see below).

These same hypermethylated genes can also be detected by PCR in the blood or urine. Zhao et al. analysed 8 DNA methylation biomarkers in the urine of 153 men on AS (76). APC, CRIP3, GSTP1 and HOXD8 were identified as predictors of disease reclassification on repeat biopsy, with OR of 2. 559 (1.257-5.212). They found that these markers were superior to clinical criteria such as PSA values or findings on initial biopsy. There is no commercially available test at this time.

Proteomics, Metabolomics, Autoantibodies

Proteomics and metabolomics are fields in which proteins in the serum and urine are detected and quantified, generally using mass spectrometry. Protein signatures have been used make predictions on biochemical recurrence after treatment or response to chemotherapy (77). Prostarix (Metabolon Inc, Morrisville, NC) is a commercially available test that uses tandem liquid chromatography and mass spectroscopy to quantify 4 different metabolites in post-DRE urine (78). It is designed to predict chance of a positive first or repeat prostate biopsy. In the initial study, the

AUC for detection of cancer was a modest 0.64. When combined in a logistic regression with other clinical parameters, such as age, PSA, and TRUS-measured prostate volume, the AUC increases to 0.78.

Another new avenue of biomarker research is autoantibodies to prostate cancer specific antigens. One team has shown that antibody responses to PRDX6 and ANXA11 are found in greater concentrations in PCa patients (79).

Circulating Tumour Cells

Detection of malignant cells in the circulation is possible with specialised commercially available flow cytometry systems, most commonly the CellSearch® system (Janssen Diagnostics, Raritan, NJ). Among men with metastatic prostate cancer, the number of circulating tumour cells (CTCs) correlates with PSA values, metastatic burden, time to progression, response to treatment, and overall survival (80–82).

More recently, molecular analysis of genetic material within CTCs has been the subject of research. The most notable is the identification of mRNA for a mutated version of the androgen receptor, named AR-V7. Positivity for this variant predicts poor response to newer generation androgen inhibitors such as Abiraterone and Enzalutamide (83). This biomarker could prove useful in treatment selection for men with metastatic disease. Other studies have analysed CTCs for the presence of abnormalities such as PTEN loss and TMPRSS2:ERG fusion, with promising results (84).

Tissue biomarkers

Many genetic abnormalities can be identified using immunohistochemical techniques. Examples in PCa include p53, Bcl-2, MDM2, Ki-67, and AMACR and loss of PTEN, and these have all been shown to correlate with worse outcomes, such as higher biochemical recurrence rates after RP (85). Unfortunately, measurement of the degree of staining of these markers is somewhat subjective and there is significant variation between laboratories and between different commercially

available antibodies. This makes these methods difficult to investigate as viable biomarkers.

On June 13, 2013, the US Supreme Court ruled that naturally occurring genes could not be patented, thus invalidating over 4,300 existing gene patents. This has been a major factor leading to the proliferation of genetic panels used to risk stratify patients for various types of cancer. Additionally, improvements in technology and reductions in cost have made genetic testing more accessible and affordable.

Currently commercially available genetic tissue biomarkers for prostate cancer test for somatic mutations in patient prostate tissue. Notable tests on the market currently are the Genomic Prostate Score (Oncotype DX® GPS), quantification of DNA methylation (ConfirmMDx®), the Cell Cycle Progression Score (Prolaris®), and the Genomic Classifier Score (GenomeDX Decipher®).

The former two (Oncotype DX GPSa and ConfirmMDx), are performed on prostate tissue from a prostate biopsy, while the latter two (Prolaris and Decipher) are performed on tissue from the radical prostatectomy specimens.

Genomic Prostate Score (Oncotype DX® GPS)

The Oncotype DX Genomic Prostate Score Assay (GPS) is a multi-gene panel designed to be performed on small volumes of formalin-fixed paraffin-embedded (FFPE) prostate tissue samples taken at prostate needle biopsy. The initial study was reported by Klein et al. (86). Gene expression was quantified in a set of contemporary prostatectomy and biopsy specimens as a development cohort. Of the 732 candidate genes identified, 12 genes that represented a spectrum of biological pathways involved in PCa were found to correlate well with stage and grade of PCa at on final pathology at RP. These were combined with 5 reference genes to form a 17 gene panel. The GPS is a score from 0 to 100, with a higher score indicating higher relative risk. The test was then validated in a low- to intermediate risk PCa population deemed eligible for active surveillance but who had radical prostatectomy, to determine whether it could accurately predict adverse pathology at RP. In the validation component of the study, 31% of the patients were deemed to have adverse pathological features. These were Gleason score of ≥4+3 or pathological extraprostatic disease. Significant positive correlation was found

between GPS and adverse pathology. For every 20 unit increase in GPS, the RR for extraprosatic disease increased by 1.9 and the RR for high grade disease increased by 2.3.

These findings were further validated in an external cohort of 402 cases (87). In a secondary analysis of those two cohorts, Brand et al. showed that NCCN very low risk men had an 80% likelihood of favourable pathology, which comprised 11% of the combined cohort (87). When using the GPS in combination with the NCCN risk group, the proportion of men with an 80% likelihood of favourable pathology was increased to 23%. When GPS was utilised in conjunction with the CAPRA score, the proportion increased to 31%. This demonstrates the risk stratification potential of the test, and potential use in decision-making in an active surveillance setting. Prospective validation of these findings would lend strength to the evidence.

In a study to determine the clinical usefulness of the Oncotype DX, Badani et al. interviewed urologists on their hypothetical treatment recommendations using a cohort of 157 men who had undergone both the test and subsequent radical prostatectomy (88). They found that there was discordance between the NCCN risk group and the Oncotype DX risk score in 37% of men. The test led to a 24% higher relative rate of recommending active surveillance (absolute difference of 41% vs 51%) and the clinicians were of the opinion that the test was clinically useful in 79% of patients. Although the authors proved that the test might have changed the clinical recommendation, there was no evidence presented that the altered recommendations were any better.

DNA hypermethylation (ConfirmMDx®)

Standard TRUS biopsy of the prostate is randomly directed, and as such can miss foci of prostate cancer (89). However, there are epigenetic changes that can be present in adjacent benign cells. Epigenetic changes are alterations to the DNA that affect gene expression but are reversible and do not affect the primary DNA sequence. DNA methylation is one such change. Methylation of certain dinucleotides can silence expression of tumour promoters.

The ConfirmMDx EpiScore is calculated from the analysis of the DNA methylation status of GSTP1, APC, and RASSF1 in benign prostate biopsy tissue (90). The purpose

of the score is to estimate the risk of a subsequent prostate biopsy being positive. The score has been designed to have a strong negative predictive value to reduce the number of unnecessary repeat biopsies being performed for men with continued high suspicion of prostate cancer after an initial negative biopsy. Absent DNA methylation of all three of the genes has a negative predictive value for high-grade (Gleason ≥7) PCa of 96%.

Van Neste et al. developed a risk score that incorporated the EpiScore along with other clinical parameters such as PSA, age, DRE findings, and pathological findings. For predicting men with Gleason ≥7 on repeat biopsy, this score achieved an AUC of 0.762, compared to an AUC of 0.742 for the EpiScore alone. The EpiScore has been validated in several external cohorts (91,92). The conclusion is that this test could reduce the number of unnecessary biopsies by up to two thirds without missing a significant number of significant cancers.

Cell Cycle Progression Score (Prolaris®)

The Cell Cycle Progression (CCP) score is a 46 gene panel comprised of 31 genes responsible for cell cycle progression, in addition to 15 housekeeper genes that are included for control and standardization. It is available commercially as the Prolaris test from Myriad Genetics, Salt Lake City, Utah.

The test was first reported by Cuzik et al. in 2011 (93). The genes were identified in prostate cancer tissue, and were chosen based on their individual concordance with overall increased expression of known cell cycle promoting genes. The aim was to measure the proliferative activity of a tumour in preference to other characteristics, such as invasive potential, etc. The predefined score was then tested on a cohort of men who had undergone radical prostatectomy and one who had had PCa diagnosed incidentally on TURP, to determine the accuracy for predicting final outcome. The CCP score was highly correlated to the risk of biochemical progression after RP (HR 1.89 for each 1 unit change in CCP score, $p < 0.001$), and also highly correlated to risk of PCa-specific death after incidental diagnosis following TURP (HR 2.92, $p < 0.001$).

CCP score has been externally validated in two RP cohorts, where it again was predictive of biochemical recurrence and cancer specific death, increasing the concordance index from 0.71 for a clinical model (CAPRA-S) to 0.77 for a model

combining clinical factors with the CCP (94). While a non-randomised study has shown that CCP might alter the recommendations that a physician might make to a patient (95), such studies do not really reflect the performance of the test, and importantly do not demonstrate improved outcomes as a result of the altered recommendations.

These studies have shown that the CCP score might be a good predictor of disease outcome after treatment for prostate cancer. How this would affect clinical decision-making should be directed by future studies.

Genomic Classifier Score (GenomeDX Decipher®)

A test that is similar to the CCP score is the Genomic Classifier Score, available commercially as the Decifer test from GenomeDX Biosciences, Vancouver, Canada. An RNA microarray is performed on FFPE prostate tissue taken from an area of the highest Gleason score in the RP specimen.

The score was first reported by Erho et al. in 2013 (96). A cohort of 639 men who underwent RP was used for development, 213 of whom developed early metastatic disease recurrence. Using computer learning algorithms processing data on 1.4 million markers from 545 individual tumours, the investigators identified 22 markers that best predicted early metastasis after RP. In validation, the test did indeed correlate well with diagnosis of early metastatic disease, with an AUC of 0.75 (0.67-0.83). In addition, higher score correlated with reduced time to PCa-specific death and reduced overall survival within Gleason grade groups.

An external validation of the Decipher genomic classifier test was performed by Karnes et al. in a cohort with more low risk men and a lower incidence of disease recurrence (96). They found that the test increased the predictive power of a clinical model and achieved an AUC of 0.79 (0.68-0.87) for predicting metastatic disease at 5 years.

In another validation of the test, Cooperberg et al. retrospectively studied a random sample of 185 men from larger cohort treated with RP who were all at a high risk of recurrence (97). GCS was strongly predictive of cancer specific mortality. 24% of the men had a GC score of >0.6, 64% of whom died of PCa. Conversely, men with scores lower than 0.6 had a 7% 10 year CSM. The GC score performed better at predicting

outcome when combined with the post-surgical Cancer of the Prostate Risk Assessment (CAPRA-S) Score. The authors conclude that Genomic Classifier Score could be useful in selecting men for early secondary therapies or clinical trials.

Imaging

Potential tools for improved screening and risk stratification are not limited to biomarkers. Recent improvements in imaging have recently made these modalities viable options to fulfil these roles, with some advantages and disadvantages relative to biomarkers.

Multiparametric Magnetic Resonance Imaging

Despite magnetic resonance imaging being available since the early 1980's, the utility of prostate MRI has until recently been limited. Initial investigations for assessing prostate cancer showed MRI to have poor sensitivity and specificity for distinguishing prostate cancer from benign disease (REF).

Recent improvements in technology as well as the combination of different imaging techniques, namely diffusion weighted imaging, dynamic contrast enhancement, and MRI spectroscopy, have dramatically improved the test characteristics of MRI. ultiparametric magnetic resonance imaging (mpMRI) is now finding its place in clinical practice. The Prostate Imaging Reporting and Data System version II (PI-RADS II) is the standard reporting scheme for prostate mpMRI, which defines anatomical regions of the prostate and classifies lesions on likelihood that they are malignant (98). This system has somewhat reduced the inter-observer variation in prostate MRI (99).

The main use for mpMRI of the prostate has been its use in conjunction with MRI-guided biopsy. While various studies have used in-bore biopsy in the MRI suite or "cognitive" guidance of regular TRUS biopsy, the majority of contemporary publications report on MRI-ultrasound fusion technology which allows MRI images from a recent MRI to be fused with real-time ultrasound images during TRUS biopsy (100). Some have investigated the use of mpMRI and subsequent MRI-targeted biopsy in biopsy naïve men with suspicion of PCa (101,102). mpMRI was found to

improve overall cancer detection to a small extent, to increase the detection of clinically significant PCa, and to reduce the diagnosis of clinically insignificant cancers. This presumably makes the TRUS biopsy more representative of the true pathological picture. However, the PPV and NPV of MRI-US fusion biopsy in the biopsy setting is still relatively modest. So, because of the low NPV, template biopsies are recommended in addition to targeted ones, and even in the case of a negative mpMRI, a biopsy still needs to be done to avoid missing a clinically significant cancer. Furthermore, because of the low PPV, many men with suspicious lesions on mpMRI will turn out to have negative biopsies (101). mpMRI can improve positive biopsy rates in the repeat biopsy setting and improve risk stratification for men with apparent low risk PCa (103). However, for these indications too, the MRI does not replace either a PSA measurement or the TRUS biopsy. In fact, without the use of a concurrent MRI-guided biopsy, the utility of mpMRI of the prostate is limited.

While mpMRI is has been widely incorporated into prostate cancer management and is likely to become more widely practiced, it falls far short of being a perfect assessment tool.

Positron Emission Tomography

Another imaging modality that has been used to evaluate men with prostate cancer is PET scanning. Traditional FDG-PET had limited usefulness in prostate cancer due to the relatively low metabolic rate of PCa cells compared to other cancers. Improvements in PET scanners as well as newer technologies, such as choline, N-[N-[(S)-1,3-dicar- boxypropyl]carbamoyl]-4-(18)Ffluorobenzyl-L-cysteine (18F-DCFBC), hyperpolarised C^{13}, and gallium-PSMA radiotracers, have improved the utility of PET scanning (104). ^{68}Ga-PSMA has shown highest promise in identifying otherwise occult nodal and distant metastatic disease in men with high risk prostate cancer. There is also some evidence that 68Ga-PSMA PET-MRI can improve disease localisation within the prostate compared to mpMRI alone (105). While these scanning technologies add clinical utility in a small proportion of men, PET would also not reduce the burden of investigation.

A comprehensive review of imaging for PCa is beyond the scope of this literature review.

Summary

PSA is the prototypical biomarker for prostate cancer that was brought into clinical use in 1986. It is used for screening, risk stratification, monitoring of disease progress, and response to treatment. However its accuracy in all of these contexts is limited because of individual variation, potential underproduction by malignant tissue, PSA production by normal and inflamed prostate tissue, and various other factors.

	Biomarker	**Basis**	**Role**	**Comment**
Blood	PSA	Serum protein	Scr, RS, DM, TR	Derivatives: PSAd, PSA-dt, %fPSA
	phi	p2PSA, tPSA, fPSA	Scr, RS	Uses p2PSA, tPSA, fPSA
	4k score	tPSA, fPSA, iPSA, hK2	Scr, RS	Uses tPSA, fPSA, intact PSA, and hK2.
	CTCs	Flow cytometry	RS and TR (in metastatic disease)	Not useful in localised PCa.
Urine	PCA3	Non-coding RNA	Scr, RS	Both require DRE before urine sample.
	TMPRSS2:ERG	Non-coding RNA	Scr, RS	
	ExoDx	Exosomal proteins	Scr	No DRE required Predicts GS≥7
	Prostarix	Protein metabolites	Scr	
Tissue – Biopsy specimen	Oncotype DX GPS	17 genes, multiple pathways	Predicts aggressive PCa in low risk	Tested on FFPE tissue of highest Gleason.
	ConfirmMDx	Hypermethylation of 3 genes	Predicts Positive repeat biopsy	Tested in benign FFPE tissue.
Tissue – RP specimen	Prolaris	46 genes, cell cycle progression	Prognosis after RP	Both could be used to select men for adjuvant or investigational therapy.
	Decipher	22 genes, predict early mets	Prognosis after RP	

Scr = Screening; RS = Risk Stratification; DM = Disease Monitoring; TR = Treatment Response

Table 1: Biomarkers for Prostate Cancer

From the time of its introduction, PSA remained the only useful biomarker for PCa until the advent of PCA3 almost 20 years later. PCA3 and a host of other non-invasive biomarkers that were introduced subsequently have not been able to replace PSA, but only to potentially augment its clinical role. Most have been evaluated in the context of selecting men with raised PSA for prostate biopsy, in risk stratifying men prior to treatment, and in predicting response following definitive treatment. Only marginal improvements have been shown for all of these functions.

Like most cancers, histological diagnosis remains the definitive diagnosis of PCa. While genomics performed on the biopsy specimen can improve on prognostication of the disease, little more is gained over the standard Gleason histological grading.

Newer imaging modalities, including mpMRI and PET scanning, occupy the same clinical space as many of these described biomarkers, but these too have not replaced PSA and prostate biopsy.

This review has highlighted the need for newer and better biomarkers for prostate cancer to reduce overinvestigation, overdiagnosis, and overtreatment of prostate cancer, while reducing deaths from the disease.

Extracellular Vesicles and Prostate Cancer

Extracellular vesicles (EVs) are membrane-bound cell fragments that range in size from 30-100 nm, and are derived from various cell populations (106). They are found in almost all biological fluids (107). EV membranes can be derived from the outer cell membrane of their cell of origin, and because of this often share the same surface proteins and other antigens. EV's contain proteins, RNA, DNA, and other cytoplasmic substances common to the cell of origin.

History

Circulating cell fragments were first described by Wolf in 1967 (108). He identified these subcellular particles by ultracentrifugation of platelet poor plasma and electron microscopy, and correctly recognised their platelet origin. The term he coined at the time was "platelet dust". He found that these particles were lipid-rich,

and that they were responsible for the thrombogenic properties of plasma that had previously contained activated platelets.

The role of these particles in thrombosis became more clearly elucidated over time, but it was some time before it was realised that other cells also released similar microvesicles.

The first description of extracellular vesicles being released from neoplastic cells was from Trams et al. in 1981 (109). Electron microscopy was used to examine conditioned culture medium from various mouse, rat, and human cells – among which were neuroblastomas, gliomas, astrocytomas, and melanomas.

Classification and Origins of EVs

Extracellular vesicles are nano sized bodies that consist of cytoplasm and a surrounding cell membrane derived from the cell of origin. The multidisciplinary nature of this field of research, as well as the many poorly understood aspects of microvesicle physiology, has led to a diverse and often conflicting nomenclature Some names give clues to the cell of origin (e.g. prostasome, oncosome), while others refer to their size or functional origin (e.g. exosome, ectosome) (110).

Based on a recent consensus conference, the international society for extracellular vesicles has recommended that the term “extracellular vesicle” be used for all such membrane-bound subcellular structures. Allowance, however, is made for individual scientists to use their discretion in choosing their preferred term, as long as their reports contain accurate descriptions of the methods used to isolate and study the particles concerned (110).

Subdivision of EVs is usually then based on size, into “exosomes”, “microparticles” (interchangeable with “ectosomes”), and “apoptotic bodies”, which correlates to some degree with the origin of the particles (111). The usual upper limit of size for EV’s is 1 µm, since normal platelets can be as small as 1-3 µm (112). Exosomes are extracellular vesicles that are produced via the endocytic pathway (111). They are formed inside cells as intraluminal vesicles (ILV) within multivesicular bodies (MVB). The ILVs within the MVBs are derived from the outer membrane of the cell, but endocytosed into the cell. Enrichment of the ILVs with various lipids and proteins can occur. Exocytosis of the MVB releases the ILVs into the extracellular environment,

which are then referred to as “exosomes” (113). Exosomes are typically 30-100 nm in size, although the upper limit can be considerably higher than that. Exosomes have a classically cup-shaped appearance under transmission electron microscopy, although this may be an artefact related to sample preparation (110). Microparticles (MPs) are extracellular vesicles that arise directly from budding of the outer cell membrane into the extracellular environment (113). They are typically defined as being between 100-1000 nm in diameter. Microparticles are referred to interchangeably as ectosomes or microvesicles (114). Apoptotic bodies are membrane-bound cell fragments, generally larger than 750 nm that are released during apoptosis. Apart from the size discrepancy between exosomes and MPs, where there is most likely an overlap, there is no reliable way to distinguish one from the other when found freely (111). Among healthy people, approximately 70-90% of EVs in the blood are derived from platelets, with the remainder being produced by leukocytes, endothelial cells, and red blood cells (115). There is a small proportion of EVs present in the circulation that is derived from cells outside the circulatory system, which can vary substantially in various disease states.

Qualitative and Quantitative Measurement

There is no standard qualitative or quantitative technique for microparticle analysis, despite the increasing volume of research in the field (115). In the original paper by Wolf, EVs were observed in ultracentrifuged platelet-poor plasma using electron microscopy (108). Since then, many other techniques for identifying, counting, and characterising exosomes and MPs have been described.

Analysis consists of determining concentration, measuring size, and determining qualitative aspects such as surface antigens and cytoplasmic contents such as RNA, DNA, and protein constituents.

Flow cytometry is perhaps the most commonly used technique for quantifying microparticles and it has been suggested that it should be the reference method of choice (116). When performing flow cytometry, techniques for discriminating microparticle populations from one another include: staining with either fluorescent dyes or with antibodies labelled with fluorescent molecules, or by their various light-scattering properties (112). These properties include their degree of forward scatter

and side scatter (Figure 1), which correlate with their size and internal complexity, respectively. The flow cytometer can be calibrated with artificial microspheres of known size. These can be composed of latex or silica (117). Fluorophore-labelled monoclonal antibodies specific to a relevant cell surface antigen can then be used to identify EVs of interest. Isotype controls that are derived from the same species should be used to control for non-specific antibody binding (118). An appropriate isotype control is an antibody of the same class as the test antibody and derived from the same animal, but without a specific target. The marker used for detection of MPs using flow cytometry in many of the earlier studies was Annexin V, although now there are Annexin V-negative MPs that have been described (119). Targeting a surface antigen that is more specific to the EV population of interest can be more useful when analysing complex fluids. With the use of flow cytometry, there is some evidence that some large protein complexes can be confused with MPs due to similar properties such as size, light scatter, and sedimentation (120). Flow cytometry is the quickest and easiest method of quantifying EVs and determining the cell of origin (121). The disadvantage of flow cytometry is the inability to analyse the composition of the vesicles.

To analyse the composition or other properties of EVs, a process of isolation needs to be performed. As with nomenclature, there is no standardised method of isolating EVs for analysis (119). An international survey of researchers with respect to methods used for EV isolation and characterisation revealed that ultracentrifugation was most commonly used (122). This technique isolates EV populations in terms of size by serial centrifugations at progressively higher spin rates and longer time. However, slight differences in centrifugation protocol risk selective depletion of MP population (119). To remove platelets from a suspension, an 800 nm filter is required, and this should ideally be gravity-driven rather than forced, to prevent platelet fragmentation. Very often, ultracentrifugation is used as the initial step in a multi-step process. Further isolation can then be performed using techniques such as microfiltration, magnetic bead sorting or fluorescence activated cell sorting (FACS) (123). Once isolated, EVs or their constituents can be analysed using techniques such as transmission EM, atomic force microscopy, Western blotting, or PCR (124).

Structure and Function

The production of both exosomes and MPs are cellular functions that are highly evolutionarily preserved, occurring in all eukaryotic cells, with very similar processes present in prokaryotes (125).

MPs seem to be produced via an active process of budding from the outer plasma membrane. Phospholipid composition differs between the inner and outer leaflets of the cell membrane. Phosphatidylserine and phosphatidylethanolamine are maintained at higher concentration on the inner leaflet, and are both actively transported there by an enzyme called “flippase” (126). It has been suggested that a “floppase” enzyme can mediate ATP dependent transport of these molecules to the outer leaflet of the cell membrane. Flippase activity is inhibited by a concurrent influx of intracellular calcium as would occur during cell activation. The resultant membrane asymmetry seems to be a common feature activated of apoptotic cells releasing MPs. “Scramblase” is another enzyme that plays a role in MP release in platelets. It allows the membrane polarity to be neutralised by allowing substances to follow concentration gradients.

Exosomes and MPs most likely have differing functions based on their membrane structure and protein content. Exosomes have membranes that are lipid-enriched and have higher concentrations of signalling proteins. MPs on the other hand are enriched with proteins associated with gene expression and translation, as well as various enzymes.

The multiple physiological roles played by MPs are now emerging. They seem to serve various functions related to cell homeostasis, such as regulating the surface area and composition of the plasma membrane – part of a process called membrane trafficking (113). MP generation could also be an important function in cytoskeleton remodelling (126) and management of cellular waste products (107).

Many exosomes and MPs have effects external to their cells of origin. From the time of their first discovery, it was known that MPs were released by platelets during activation during the course of normal coagulation and fibrinolysis (112). It subsequently appeared that these platelet MPs were most likely also key mediators of inflammation and immunity (116).

Intercellular communication is effected by various signalling molecules, as well as a variety of other molecules, including RNA, proteins, and lipids (127). These molecules act either by direct stimulation of external cellular receptors, or via delivery of a specific cargo into a cell. There is evidence that some MPs can transfer surface proteins or RNA horizontally between cells (127). This transfer occurs in several different ways. It can happen via direct fusion of the EV to the plasma membrane, with release of the constituents into the cytoplasm. It can also occur via phagocytosis or micropinocytosis. Subsequent fusion of the vesicles with the cells can then occur within the cells endocompartments. Clarithrin-mediated endocytosis is a process that allows an EV to be transported directly, and intact, into the cytoplasm (113). There is also increasing awareness that substances that were previously thought of as free constituents of the plasma, are actually contained within microparticles (116).

Microparticles derived from endothelial cells, platelets, and sometimes leukocytes have been found to be increased in various disease states, such as coronary artery disease, deep venous thrombosis, pulmonary embolism, sickle cell disease, chronic renal failure, and others (123). Activation of various cell types, induction of apoptosis, and promotion of cytokine secretion have all been reported in response to certain MPs (123).

There is much literature examining potential functions of exosomes and MPs. Apart from their previously well-established role in thrombosis, there is evidence that some EVs have immune suppressing abilities, can present antigens to immune cells, transfer signalling proteins, promote inflammation, transfer genetic information, and promote tumour growth (107). Viral particles and prions have been found within EVs, suggesting that they might be involved in pathophysiological processes or disease transmission.

Microparticles in Cancer

The first report of tumour-derived MPs was by Trams et al. in 1981 (109). Other investigators in the early 1980s reported finding similar microvesicles in other malignant cell lines (114). Interestingly, Trams et al. observed a bimodal population of EVs, with one set at approximately 50 nm and another population at 500-1000 nm

in diameter. This might correspond to populations of exosomes and MPs, respectively. However, early investigators only reported observing vesicles arising from the outer plasma membrane. It was not until 2001 that Wolfers et al. showed that tumour-derived EVs were also produced in MVBs and released as exosomes (128).

Overall circulating EV concentrations have been found by some to be significantly higher in cancer patients, compared to healthy controls (129). A large part of this increase may be due to higher concentrations of platelet MPs, but that increase may also be due to MPs originating from tumour cells themselves. Microparticle tissue factor activity is increased in several different cancers, including prostate cancer (130). Whether this increased tissue factor activity is from platelet-derived microparticles or those directly from cancer cells is not clear. Increased MP tissue factor activity plays a role in the link between malignancy and increased risk of thrombosis. Circulating tumour-derived MPs carry tumour-specific antigens on their surface, such as HER-2, CEA, and EGFRvIII [19, 157, 159].

It was initially thought that EV release from malignant cells was an aberrant process taking place in dysfunctional cells, but there is abundant evidence that this process serves various physiological functions. Tumour-derived MPs have been shown to not only carry protein and genetic cargo, but to be actively enriched in these substances (131). This might be part of a waste-clearing process, by carrying away excess cell membrane and cytosolic products, or as a way to influence the micro- or macro-environment.

It has been postulated that MP generation by tumours could play roles in defensive or offensive responses to the host immune response (132). Valenti et al. showed that melanoma and colorectal carcinoma MPs could inhibit the differentiation of monocytes, or promote their development into myeloid-derived suppressor cells (133). They therefore have an immunosuppressive effect that would serve to promote tumour survival. Surface proteins shed by MP release could also divert drugs as well as antibody and cellular immune responses. They could also be used to actively export substances such as cytokines or intracellular drug (132). Tumour-derived MPs may also act in a paracrine and juxtacrine fashion to transfer chemotherapy and radiation resistance between nearby cells (134).

There is also evidence that tumour-derived EVs have effects that could promote oncogenesis in other cells, facilitate metastasis by promoting cell invasion, or promote angiogenesis within the tumour microenvironment.

Keerthikumar et al. performed a proteogenomic analysis of exosomes and endosomes derived from cancer cells (111). They found that certain proteins known to be associated with various types of cancers, including specific mutant proteins associated with oncogenesis or drug resistance, were enriched within exosomes and MPs. This occurred to a greater extent in exosomes compared to MPs, although some proteins were exclusively found in one or the other. This suggests that these extracellular vesicles could play a role in cell proliferation or cell migration. The enrichment of these tumour-specific proteins was also highlighted as a promising potential source for biomarkers for these cancers. There is some evidence that these tumour-derived EVs are capable of microRNA (miRNA) biogenesis, which is a feature not present in EVs derived from normal cells (129). Cells incubated with cancer EVs have also been shown to exhibit increased rates of malignant transformation, suggesting that the transfer of miRNAs could confer a field effect that causes surrounding benign cells to also undergo malignant transformation (129).

Janowska-Wieczorek et al. observed that platelet-derived MPs could transfer various matrix metalloproteins (MMPs) to breast cancer cells and thereby increase in vitro measures of invasiveness (135). Skog et al. showed that MPs derived from glioblastoma cells contained angiogenic mRNA, miRNA, and proteins. These MPs are taken up by endothelial cells, and the authors demonstrated a resultant increase in angiogenesis (136).

Microparticles and the Prostate

Both seminal fluid and expressed prostatic secretions have been shown to have high concentrations of EVs (137). Submicron vesicles were detected in the seminal fluid by a group from Uppsala University in Sweden in the late 1970s. Using electron microscopy, they found evidence that these vesicles were produced by the prostatic glandular epithelial cells, where they were observed to be present within larger vacuoles (138). The same group proposed the name “prostasome” for this new microvesicle that was actively secreted into the glandular lumen by various

mechanisms. Much work has subsequently been done on investigating the structure and function of prostasomes.

The group initially used purified EVs isolated from semen to generate their own anti-prostasome antibody (139). The primary antigen was subsequently found to be dipeptidyl peptidase IV (DPP IV), also known as CD26 (140). Prostatic exosomes are composed of a high proportion of cholesterol, sphingomyelin, certain enzymes, and calcium (141,142). They have a heterogeneous size, varying from 40-500 nm, with a mean diameter of 120-150 nm (11). Using EM, the group observed prostasome generation occurring within MVBs in prostatic luminal cells. Their contention is that, despite their size of up to 500 nm, these EVs are exosomes rather than MPs. There are, however, currently no reliable markers available for distinguishing exosomes from MPs (111).

The proposed normal functions of prostatic EVs in the ejaculate are to support certain spermatozoal functions as well to interact with the microenvironment within the female genital tract (141). They protect spermatozoa from acidic pH changes (which occurs via fusion of these vesicles with the spermatozoa in low pH conditions), delay the acrosome reaction, enhance sperm motility, transfer enzymatic activity (such as CD26) to spermatozoa, and suppress the local female immune response (141). Prostasomes have been shown to have the capacity to generate ATP via the glycolytic pathway (143). This process might be important for supporting spermatozoa within the female genital tract. Ronquist et al. has proposed a theory that prostasomes from benign cells might play a role in malignant transformation of prostate glandular cells via transfer of matrix metalloproteinases (139).

Malignant prostatic epithelial cells also produce EVs. EM studies in malignant prostate tissue have shown that PCa cells have golgi-associated storage vesicles containing various prostasome precursors (139). These early MVBs are also present, although to a lesser degree, in metastatic PCa cells, suggesting that even very malignant prostate cells retain the ability to produce exosomes. The same group did indeed identify prostasomes surrounding surgically resected vertebral PCa metastases (144). Not only do they retain the ability to produce exosomes, but they may produce more plasma membrane-derived MPs. The cytoskeletal regulator

Diaphanous-related formin-3 (DIAPH3) is often lost in PCa, and this loss leads to an amoeboid cells that shed large quantities of large EVs (145).

As with other malignancies, it seems that tumour-derived MPs from PCa cells have functional roles that promote neoplastic transformation and malignant behaviour.

In an intriguing and very complex study, Abd Elmageed et al. isolated adipose stem cells derived from prostate cancer patients, selectively cultured those with a particular tropism for conditioned media of PCa cells, and then exposed these cells to PCa-derived MPs (versus control), and injected the cells into athymic nude mice (146). The stem cells that had been exposed to the PCMPs developed into large aggressive tumours that showed histological and biochemical mimicry of prostate cancer cells. This study further illuminates the role that MPs might have in influencing their host environment, and might explain the observed clinical correlation between disease aggressiveness and raised BMI.

Ramteke et al. demonstrated that LNCaP and PC3 prostate cancer cells grown under hypoxic conditions (1% O_2) produce exosomes that were structurally and constitutively different to those produced under normoxic conditions (147). Not only were they smaller, they had higher concentrations of various signalling molecules such as TGF-b2, TNF1a, IL6, b-catenin, and others. They also had higher levels of tetraspanins (CD63 and CD81), heat shock proteins (HSP90 and HSP70), and Annexin II. Naïve cells grown in the presence of these exosomes then showed greater motility and invasiveness. These findings could suggest that hypoxic prostate cancer tissue in vivo promotes tumour aggression via MP release.

Role as a Prostate Cancer Biomarker

Platelet EV levels have been investigated in PCa. Men with advanced PCa have been found to have increased levels of platelet MPs, and these levels correlate with disease-specific and overall survival (148). Another group has shown increased levels of MP-derived tissue factor in men with localised PCa (149). In these men, the values returned to normal within one week after RP.

Tumour-derived MPs carry tumour-specific cell surface antigens, proteins and genomic cargo (150). These can be used to identify the origin of the MP populations and can also indicate specific phenotypic properties of the tumour. These features

have potential as biomarkers for diagnosis, staging, risk stratification, or for personalised treatment selection.

Recently, several investigators have analysed prostate-derived EVs in plasma or urine in search of new biomarkers (137). These have varied from known proteins (such as PTEN and survivin) to miRNAs and N-linked glycans.

An investigational proteomics study by Hosseini-Beheshti et al. identified over 220 proteins in a panel of PCa cell lines, some of which were previously thought to be only present in cell nuclei (151). Khan et al. found that exosomal survivin levels were higher in men with PCa compared to men with BPH and healthy volunteers – showing promise for potential biomarker application (152).

Mizutani et al. studied PCMPs derived from conditioned media from LNCaP and PC3 cells (153). The media was ultracentrifuged, and the resuspended pellets then incubated with magnetic beads coated with anti-CD9 and anti-PSMA antibodies, with subsequent Western blotting. Using this technique, isolated MP populations from both CD9 and PSMA groups had identifiable androgen receptors (AR) present. The group then used the same technique to isolate prostate MPs from 5 men with PCa and 2 controls. All 5 had high grade PCa, but PSMA MPs were only different to controls in 3 of the men, all of whom had high volume metastatic or castrate-resistant disease. This study showed that PCMPs carry AR, and also that PSMA is a useful marker of these MPs. The method of using magnetic beads might limit the detection of lower quantities.

Prostate microparticles have also been identified in the urine. Duijvesz et al. used a time-resolved fluorescence immunoassay (TR-FIA) to identify MPs in ultracentrifuged urine of men with PCa and in controls (154). Samples were incubated in biotinylated CD9 and CD63 antibodies, then transferred to streptavidin-coated plates, after which Europium-labelled CD9 and CD63 antibodies were used for detection. They found significantly higher levels of prostate MPs in the urine of men with PCa compared to men without PCa, women, and men post RP. The team also investigated the influence of DRE prior to specimen collection. Interestingly, they also found that a DRE prior to specimen collection significantly increased the concentration of prostate MPs in all men. The CD9 and CD63 assays were separate, and they did not

test for dual positives. ROCs generated for each of the assays showed AUCs of 0.59 and 0.68 for CD9 and CD63, respectively, compared to an AUC of 0.61 for serum PSA.

Mitchell et al. analysed urinary EVs in PCa patients using ultracentrifugation and immune-blotting (155). They found that men with PCa had a 1.2x higher urinary concentration of prostate-derived EVs, and this dropped rapidly with the commencement of ADT. This is an early exploration of the use of PCMPs for disease monitoring. Another interesting finding from this paper was the finding that urinary EVs were stable in urine for longer than 18 hours at 37°C and that they were resistant to both osmotic and proteolytic damage.

There has been little work done on simple enumeration of prostate-derived MPs using flow cytometry. Our laboratory at Lawson Health Research Institute (LHRI) has recently published our experience of using nano-scale flow cytometry in conjunction with fluorophore-labelled antibodies to prostatic surface antigens (156). Using a monoclonal antibody to prostate-specific membrane antigen clone 3E7 (PSMA-3E7) conjugated to phycoerythrin (PE), our team tested plasma samples from men with localised and metastatic prostate cancer, men with BPH, men after radical prostatectomy, and male and female healthy volunteers. While the quantities of PSMA positive MPs were significantly higher with Gleason ≥8 tumours, they did not reliably distinguish between Gleason 6, Gleason 7, and BPH. The finding that there was a degree of positivity among men post RP as well as male and female healthy volunteers led our team to conclude that there was a degree of non-specificity of the PSMA clone used. Unpublished data from our laboratory shows greater specificity using multiple antibodies, which will be discussed below.

Cell surface antigens

Prostate cancer cells are known to express many different surface antigens, including CD9 and CD63 (153). Of interest to the study are three different cell surface antigens on benign and malignant prostate cells, namely PSMA, STEAP1, and polysialic acid. Also notable to this study are cell surface antigens present on microparticles derived from platelets and endothelial cells. CD41a and CD31 are expressed on MPs derived from platelets and endothelial cells respectively.(157).

A recent study on proteomic analysis of prostatic extracellular vesicles identified over 300 different proteins (158). They found high concentrations of PSA-antichymotrypsin complex, as well as many others. There are several limitations to this and similar studies. The proteomic assay used, although able to detect over 1,200 different proteins, is not able to detect proteins specific to the prostate, other than PSA. Differing technologies and difficulties purifying the extracellular vesicles also make for large inter-study variations.

It has been shown that tumour-derived MPs carry the cell surface proteins of their parent cells (159), which rationalises the approach of using known cell surface antigens of a particular cell type to identify MPs from those cells.

Prostate-Specific Membrane Antigen (PSMA)

Prostate-specific membrane antigen (PSMA) is the protein product of the gene officially known as FOLH1, which is a glutamate carboxypeptidase. It is a type II transmembrane glycoprotein that has folate hydrolase activity, and is produced mainly by prostatic epithelial cells (160). PSMA is also detectable in cells of the duodenal mucosa, some proximal renal tubules, and neuroendocrine cells of colonic crypts. Immunohistochemical (IHC) studies have shown that benign and malignant prostate epithelial cells strongly express PSMA, and this expression is maintained in metastatic cells in the majority of cases (161).

An initial IHC study by Sweat et al. showed PSMA immunoreactivity of 46.2% ± 27.5% for benign tissue versus 79.3% ± 18.5% for prostate cancer tissue (161). Staining was similarly high in 98% of metastatic lymph nodes examined. Although not commonly expressed by other tumour cells, PSMA expression has been demonstrated in neovascular endothelium of a wide variety of other neoplasms, such as renal cell, urothelial, and colonic carcinoma (162). Multiple authors have reported that increased tissue expression of PSMA in PCa measured with immunohistochemistry correlates with tumour grade, PSA, and biochemical recurrence (163–165).

Levels of free PSMA in the blood have been tested as a biomarker for PCa. In 1996, Murphy et al. examined multiple cohorts of patients at initial biopsy, repeat biopsy, at radical prostatectomy, and at diagnosis of metastatic disease (166). Serum PSMA

level was found not to be predictive of positive biopsy, with an AUC of 0.53, but it did correlate with disease progression among men with metastatic disease. Interestingly, PSMA levels were higher in men up to 4 years after prostatectomy compared to their pre-surgery levels. This study showed that free serum PSMA was not a feasible biomarker.

PSMA has shown utility for several clinical applications. The first widespread application was in nuclear scintigraphic imaging. Indium-labelled capromab pendetide anti-PSMA antibody was approved by the FDA for imaging purposes – available commercially as "Prostascint". Unfortunately the imaging used lacked sensitivity or specificity most likely due to the fact that the antibody targeted the intracellular epitope of PSMA (167). Although still commercially available, Prostascint is not recommended by any major guideline due to its limited usefulness.

More recently, second generation antibodies to the external domain of the protein have produced improved results, also notably in the imaging sphere. ^{68}Gallium-labelled PSMA antibodies used in PET scanning have showed substantially better performance over older PET tracers, and usage is becoming widespread in many places (168).

Six-Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1)

Six-transmembrane epithelial antigen of the prostate 1 (STEAP1) is a membrane-bound enzyme of the metalloreductase family (169). It is more correctly referred to as "metalloreductase STEAP1". It was first described by Hubert et al. in 1999 (170). Initially, because of its configuration and location at intercellular junctions, it was thought to be a channel or transport protein. STEAP1 and its relatives (STEAP 2,3, and 4) have subsequently been shown to be metalloreductases, responsible for reducing iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state, as well as reducing copper from the cupric (Cu^{2+}) to the cuprous (Cu^{1+}) state (169). This function is most likely necessary for transportation of these metals into the glandular lumen. Both iron and copper are present in the normal ejaculate, and are most likely physiologically important (171,172).

STEAP1 can be demonstrated on cell surfaces by immunohistochemistry. It has been shown to be overexpressed in prostate cancer cells compared to benign

prostate cells. The degree of STEAP1 overexpression has also been shown to correlate positively with Gleason score and with biochemical recurrence following treatment (173,174). Ihlaseh-Catalano et al. studied STEAP1 and STEAP2 RNA transcript levels using RT-PCR in normal, malignant, and hyperplastic prostate tissue (173). A non-significantly higher level of STEAP1 transcripts was found in higher Gleason tumours and also in patients who subsequently presented with recurrent disease. Multivariate analysis showed that STEAP1 expression (but not STEAP2) was an independent predictor of worse prognosis in PCa.

STEAP1 has also been found to be overexpressed in 10 other cancers, and overexpression specifically correlates with worse prognosis in colorectal cancer, acute myeloid leukaemia, multiple myeloma, and diffuse large B cell lymphoma (175).

Polysialic Acid

Polysialiation is a unique post-translational modification that occurs on neural cell adhesion molecules (NCAMs). Polysialic acid is an unusual oligosaccharide that is found almost exclusively on the NCAM molecule. As the name suggests, NCAMs are (along with the cadherins) responsible for cell-cell adhesion. Although particularly abundant in neural tissue, but they are also prevalent on many different cell types throughout the body, particularly in epithelial and endothelial membranes.

The adhesion properties of NCAM can be modulated by a specific process of polysialation (176). Polysialic acid is an α2,8-polymer of the carbohydrate molecule N-acetylneuraminic acid (177). Polysialation of NCAM results primarily in reduced cell-cell adhesion. And this function is thought to be important in growth and development of tissues (178). Areas of the brain that exhibit high polysialic acid expression have high neural plasticity (179). It is particularly highly expressed in the hippocampus, and has been shown to play a role in spatial memory (180). Two polysialyl-transferases in the golgi apparatus, ST8SiaII and ST8SiaIV, are responsible for polysialation (178). Inhibition of these enzymes in mice leads to fatal maldevelopment with major defects in brain morphology.

Increased polysialation in malignant cells was first noted in 1973 by van Beek et al. (181). Polysialic acid interferes with cell adhesion because of a physical inhibition of

the NCAM molecule to which it is attached, as well as nearby cadherin molecules (179). The strongly anionic charge of polysialic acid also produces repulsive forces between cells. This process is upregulated in certain neoplastic cell types, with the resultant reduced cell-cell adhesion facilitating metastatic behaviour. The degree of polysialation can be dramatic, with the polysialic acid component sometimes being more massive than that of the NCAM molecule to which it is adherent (>100 kDa). Because of the abundance of NCAM on many cells, this could produce a complete outer carbohydrate coating to a cell (176).

Increased polysialation has been documented to occur in non-small cell lung cancer, Wilms' tumour, neuroblastoma, pancreatic carcinoma, and others (182). In a murine model of pancreatic carcinoma, experimental loss of NCAM induces metastatic spread, mainly to lymph nodes (183).

Polysialation in PCa has to date not been described. Unpublished work from our laboratory at LHRI has shown that polysialic acid is consistently expressed on cultured PCa cells and is detectable by flow cytometry on MPs in conditioned media.

Chapter 2: Study Methodology

Background

Previous work done in Dr Hon Leong's laboratory by Karla Williams, Colleen Biggs, Sabine Brett, Khurram Siddiqui, and others investigated prostate MPs using nano-scale flow cytometry (156,184). Their investigations focused on analysis of patient plasmas from men with no prostate pathology, benign prostatic hyperplasia, localised PCa, and metastatic PCa. Analysis was performed on patient plasma samples from healthy volunteers, as well as from men with benign prostatic hyperplasia (BPH), localised PCa, and metastatic PCa, in an effort to develop a biomarker to accurately identify their stage. The ultimate aim was to develop a non-invasive biomarker that could accurately discern high grade localised prostate cancer from low grade disease.

Using fluorescent-labelled antibodies targeting various cell surface antigens specific to benign and malignant prostate cancer, populations of microparticles in the plasma samples were isolated that were likely to be derived from prostate cells. Using two or three antibodies, with different fluorophores of unique peak emission wavelengths, improved the specificity of the test.

The most promising combination of markers was that of prostate-specific membrane antigen (PSMA), six-transmembrane epithelial antigen of the prostate 1 (STEAP1), and polysialic acid. PSMA and STEAP1 are both transmembrane proteins found on normal prostate cells. Their physiological functions involve folate metabolism and reduction of iron and copper, respectively. Both have been found to be overexpressed in prostate cancer. Polysialic acid is a post-translational modification of the NCAM molecule that has been found on cell surfaces of a variety of different tumours. Work done in our laboratory had shown that polysialic acid is expressed strongly on PCa cells. Unpublished data from the Leong laboratory showed that the triple test could distinguish Gleason 4+3 PCa from benign or low grade disease with an AUC of 0.86, which is substantially better than most biomarker tests.

It is known that digital rectal examination increases the level of serum PSA. DRE also increases the concentration of secretory products of the prostate (such as PSA),

as well as genetic biomarkers (such as PCA3 and TMPRSS2:ERG) in first void urine. It is unknown what the acute effects of surgery are on these markers.

Objectives of the DRE Study

The primary objective of the study was to determine whether a digital rectal examination in men known with prostate cancer would increase plasma PMP concentrations.

Secondary objectives were to determine whether microparticle levels were higher than at baseline after the surgery, and whether they returned to below the baseline at post-operative follow-up. Another secondary objective was to determine whether microparticles could be detected in the urine of these men, and if the levels are influenced by digital rectal examination.

Recruitment

Ethics approval was obtained from the Western University institutional research ethics board (REB) prior to patient recruitment (REB #107043, see Appendix 2). The study was carried out according to the Declaration of Helsinki and established Good Clinical Practice guidelines.

All men booked for robotic radical prostatectomy at an academic referral centre in London, Ontario, were considered candidates for recruitment. Men were identified and approached either in the clinic when booked for surgery, or during the preadmission process on the day of surgery. Fully informed consent was taken and a letter of information, that included contact details for the investigators, was given for the participants to take home.

Specimen Collection and Processing

Each patient was given a study number to de-identify the case report form (CRF) and specimens. Data was collected on age, most recent PSA value, prostate volume, Gleason score at biopsy, number of cores taken, number of cores positive, percentage cores positive, and presence of atypical histological features (See Appendix 1). Participants were asked to give a voided sample of urine within an hour before entering the operating room. Men who were unable to produce this

sample had urine taken via an in-out catheterisation after induction of anaesthesia. The first urine specimen was labelled “Urine Specimen A”. After induction of anaesthesia, as part of routine anaesthetic practice at our institution, a radial arterial line was placed for invasive blood pressure monitoring. All intraoperative blood specimens were drawn from the arterial line after aspiration of 10 ml to purge saline from the line. A baseline blood sample was taken, labelled “Plasma Specimen 1”. Then a vigorous DRE was performed for at least 2 minutes, with firm strokes from lateral to medial on alternating sides of the prostate. At 5 minutes post-DRE, the second blood specimen was taken, labelled “Plasma Specimen 2”. The second urine sample, labelled “Urine Specimen B”, was taken after passage of the urethral catheter immediately prior to starting the surgery. Once the prostate had been completely freed from all attachments intraoperatively, the third blood sample was taken, labelled “Plasma Specimen 3”. Participants were seen again in the urology clinic during their routine follow-up between 3 weeks and 3 months following their surgery. A fourth blood sample was drawn at that time, labelled “Plasma Specimen 4”.

Blood and urine samples were processed as soon as possible following surgery, or after collection in the clinic. Each 10 ml vial of blood and 20 ml of each urine specimens were centrifuged at 3,000 G for 15 minutes. This was to generate platelet poor plasma and to pellet out urine sediment, respectively. Plasma was divided in 500 µl aliquots, and placed into cryopreservation vials. Each 10 ml blood sample generated ± 10 aliquots. Urine supernatant was similarly aliquoted into 10 cryopreservation vials per sample at 1.5 ml per vial.

Initial samples were stored at -20°C, but concerns about MP stability at that temperature led us to transfer to -80°C storage. This affected sample from the first 5 patients in the cohort. All samples collected subsequently, were flash-frozen to -80°C immediately following centrifugation. No cryopreservation agent was used.

Thawing of specimens for the purpose of analysis was done by a rapid thaw in a 37°C bath in an attempt to minimise MP loss. At each thaw, the cryo vial was marked in order to track the number of freeze-thaw cycles for each aliquot. Re-freezing of samples was also done via flash freezing to -80 °C. When repeated analyses were performed, our preference was to use samples that had previously not been thawed.

Although there is debate in the literature about the optimal method of freezing and thawing, ours is consistent with processes followed in many other laboratories (121).

Antibody Conjugation

The principles of antibody labelling in the context of this series of experiments are as follows. Antibodies directed against cell surface antibodies are selected, with a strategy of targeting multiple antigens to improve the specificity of the test. The antibodies are labelled with fluorescent dyes that absorb light maximally at a specific wavelength, and release light at a slightly longer wavelength with a predictable maximal emission frequency.

Fluorophores to be used in this study were selected to have as little overlap of emission spectra as possible, while still being compatible with the lasers equipped on the flow cytometer in the laboratory. Commercial fluorophore-protein conjugation kits were used to conjugate the antibodies according to their specified protocols. The three fluorophores used were: fluorescein isothiocyanate (FITC), phycoerythrin (PE), and Alexa Fluor 647. These molecules have emission maximums at 519 nm (green), 575 nm (orange), and 665 nm (yellow), respectively (Figure 1).

Antibody concentrations were measured before conjugation using both a NanoDrop microvolume spectrophotometer and a Bradford spectroscopic protein assay. Concentrations were optimised prior to conjugation and assumed to be equivalent to the final concentration after conjugation. Determinations of the protein concentrations after conjugation using spectroscopy were found to be unreliable due to the absorbance of the fluorophore. This was less of a problem with the Bradford assay generally, except for PSMA-PE, where Bradford overestimated protein concentrations by at least one order of magnitude. This was thought to be because PE is itself a large protein that could multiply bind to one antibody molecule. Its maximum absorption frequency being close to that measured in the protein assay, combined with a very high extinction coefficient, mean that it would absorb far more light at 595 nm than ordinary protein – thus giving rise to the error.. For each antibody, their respective isotype control was conjugated at the same concentration to the same fluorophore. When possible, conjugation of active antibody and corresponding isotype were performed at the same time.

Active antibodies, isotype controls, and their respective fluorophores used in the DRE study were:

- **Anti-Prostate Specific Membrane Antigen (Anti-PSMA)** – Clone 3E7 – Obtained from an affiliated laboratory (Dr. Phillipp Wolf, University of Freiburg, Germany); Isotype control: Mouse IgG2a.
  - Conjugated to Phycoerythrin (PE) – Lightning-Link® R-Phycoerythrin (R-PE) conjugation kit (Catalogue no: 703), Innova Biosciences, Cambridge, UK.
- **Anti-Six Transmembrane Epithelial Antigen of the Prostate (Anti-STEAP1)** – Clone J2D2 – Abcam, Cambridge, UK (Catalogue no: ab207914); Isotype: Mouse IgG2b.
  - Conjugated to Alexa Fluor 647 – Molecular Probes® Alexa Fluor® 647 antibody labelling kit (Catalogue no: A-20186), Invitrogen, ThermoFisher Scientific, Waltham, MA.
- **Anti-Polysialic Acid** – Clone 735 – Absolute Antibody; Cat: Ab00240-2.0. Isotype IgG2a.
  - Conjugated to Fluorescein Isothiocyanate (FITC) – FluoReporter™ FITC Protein Labeling Kit, ThermoFisher Scientific, Waltham, MA.

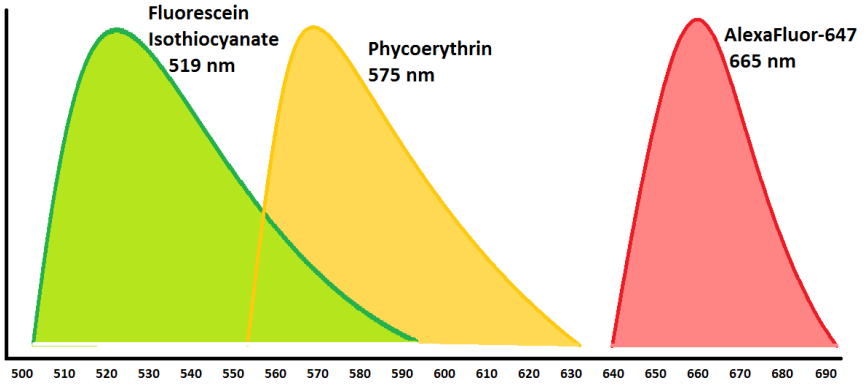


Figure 1: Fluorophores and their emission spectra. Image demonstrates the emission spectra of the three fluorophores used in the experiments.

Problems with Anti-STEAP1 [J2D2]

The original intention of the DRE study was to use all three antibodies to perform triple positive tests in all samples. While this approach produced initially promising results, quality control concern arose with the STEAP1 antibody during the study period that could not be resolved. Antibody received from the supplier showed a

lack of staining for MPs, whereby previous strong staining of distinct MP populations could not be reproduced. This problem persisted despite several re-orders of the same antibody and repeat conjugation with alternate fluorophore conjugation kits. The same antibody clone, but in a pre-conjugated form, was procured from a different company, and this antibody similarly did not produce the expected positive MP populations. In communication with both suppliers, it was not clear whether either sourced third party antibody clones.

It was therefore decided to complete the entire set of experiments using only the PSMA and polysialic acid antibodies, analysing both single positives and dual positives. The loss of specificity that might have resulted from such an approach could be justified because the study analysed sequential samples from the same patients. The non-specific component of each sample would therefore presumably be relatively consistent from one specimen to the next. The remainder of the discussion here deals with the analysis of dual positives of PSMA and polysialic acid, referred to as “Prostate Microparticles” (PMPs).

Nano-scale Flow Cytometry – Calibration, Thresholds, and Laser Settings

Flow cytometry is a process that passes a stream of fluid through a very narrow channel and analyses the components of the fluid passing through using either electrical impedance or laser light. The Apogee A50 nanoscale flow cytometer was used for all experiments in this study. The device analyses the fluid stream using an array of lasers, detecting light scatter as well as stimulated emission (Figure 2).

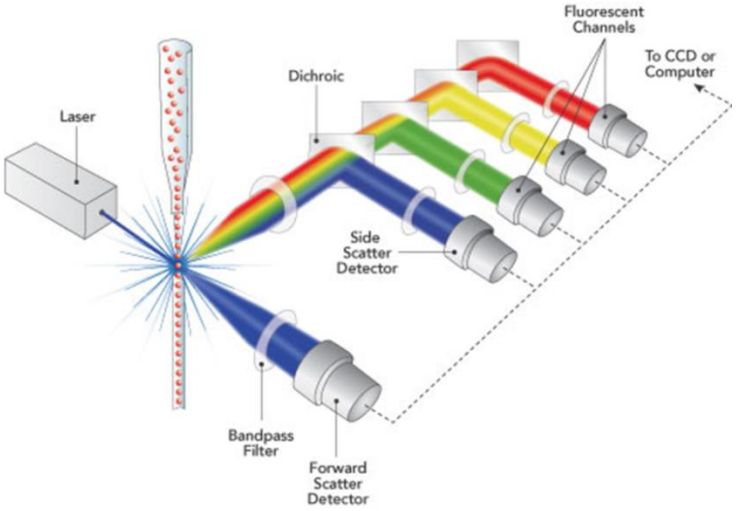


Figure 2: Principles of flow cytometry. A narrow column of fluid is passed through a laser beam. Multiple light detectors measure forward and side scatter of the laser light at the same wavelength as the primary light source, as well as scattered light of different wavelengths caused by fluorescence.

Laser-based flow cytometers can measure individual particles in three different ways. Forward scatter is measured by a detector located opposite the laser source, and measures light that is minimally scattered. When analysing whole cells, the forward (or long) angle light scatter (LALS) is a measure that correlates with the size of the cell. A photo detector perpendicular to the laser source detects side angle light scatter (SALS), which is a measure of the inner complexity of the cell. Structurally simple cells, such as lymphocytes, have little side scatter, while cells with many intracellular organelles, such as granulocytes, have more side scatter. It is not clear whether it can be assumed that long and short angle scatter represent the same properties when measuring subcellular particles, especially at the lower end of the detection limit. The third characteristic of particles that can be measured is fluorescence. Incident laser light can stimulate fluorescent molecules, which then emit light at a different (usually a longer) wavelength. Most flow cytometers have detectors for light at multiple wavelengths, as well as an array of different lasers to provide excitation at appropriate frequencies. It is this function that was exploited most in this series of experiments.

The flow cytometer that was utilised is able to report data in histographic format, which helps to visualise, characterise, and analyse the output more easily. Initial calibration of the machine was performed using suspensions of silica beads of standardised size. Although silica beads have somewhat different optical properties

to cells and EVs, the populations generated on the histograms allow for a fair estimation of EV size. Populations generated using silica beads were marked off for future reference on the flow template (Figure 3). The lower limit of detection for the Apogee A50 machine is ~100 nm, below which, the output on the histogram represents noise. This is roughly indicated by the red circle in the histogram below. A large Region of Interest (ROI), labelled “9 MPs” in the histogram shown, counted the total number of MPs in the sample, excluding the events smaller than 100 nm and the noise in the lower left corner of the histogram. This ROI served as a “gate” for MPs that performed two functions. The first was to use it to exclude all of the smaller particles and noise from the other histograms showing fluorescence. The second was to use the MP count as a denominator for calculating the proportion of fluorescent or “positive” MPs in each sample.

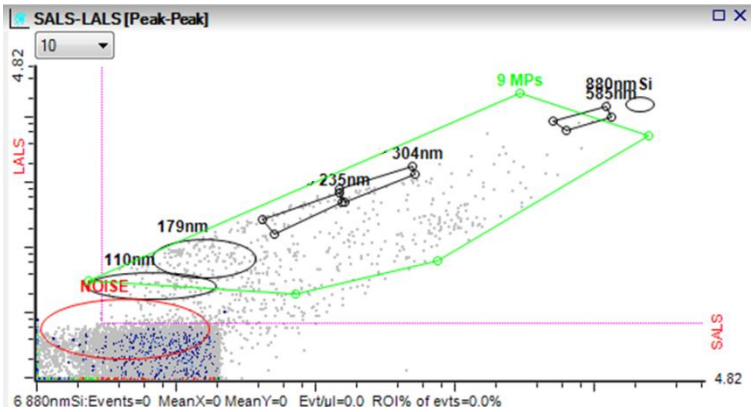


Figure 3: LALS-SALS Histogram of sterile PBS, illustrating gates marked from silica bead calibration. Regions of interest (ROIs) in black labelled the locations of populations of the variously sized silica beads that were determined on previous calibrations. The red ROI labelled “noise” in the lower left portion of the histogram indicates the location of smaller non-specific events. The green ROI labelled “MPs” was created to count the total number of events in the size range of 100-1000 nm.

The main area of interest when analysing our samples was enumeration of fluorescent microparticles. The software was set up with multiple histograms of LALS vs fluorescence on the X and Y axes, respectively (Figure 4). These diagrams showed particles of increasing size from left to right, and increasing fluorescence from bottom to top. The histograms were also gated for MPs, as described above, to exclude the smaller particles and noise. One histogram was created for each fluorophore: orange for PSMA-PE and green for Polysialic acid-FITC.

Samples analyzed without antibodies conjugated to fluorophores showed a flat population of events with autofluorescence across the bottom of the histogram. ROIs (red boxes 10 and 11 in Figure 4) were then drawn to exclude this non-fluorescent "noise" so that non-fluorescent samples would generate zero counts within the gates. A third histogram showed "dual positive" events (or "PMPs") by essentially combining the ROIs for each of the single positives. A ROI (red box 16 in Figure 4) drawn in this histogram counted the total number of dual positives. Figure 4 shows analysis of a single sample. The top row shows the isotype control for both single positive and the dual positive populations. The few positive events within the red gates likely represent non-specific binding. The bottom row of three histograms shows MPs stained for PSMA and polysialic acid, and the dual positives.

For all samples run, records were made of the total event count per sample, the MP count, the absolute number of positive events for PSMA and polysialic acid, and the absolute number of dual positives. Using the MP count as the denominator, the proportional numbers of PSMA, polysialic acid, and dual positive MPs were also recorded.

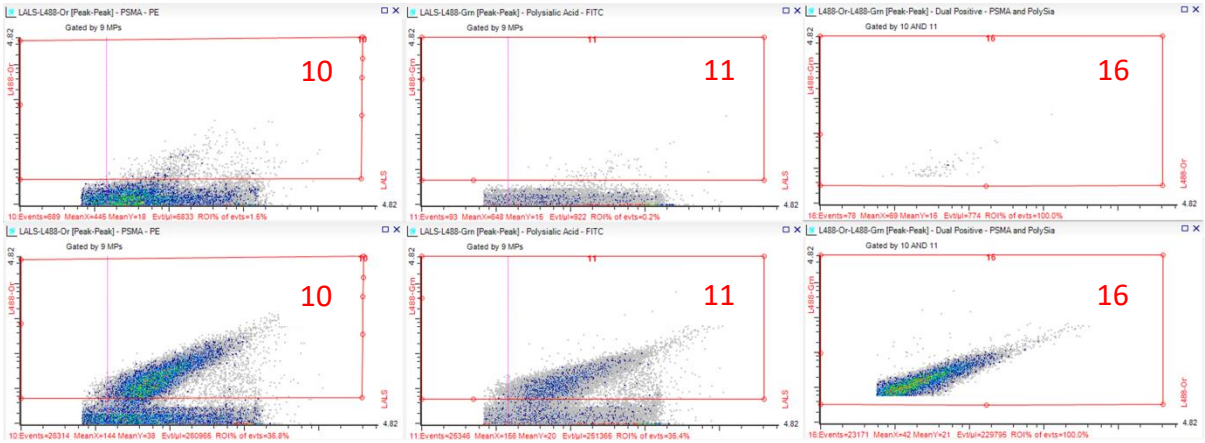


Figure 4: Isotype controls (top row) and antibodies (bottom row) for PSMA (left), Polysialic acid (middle), and Dual Positives (right), respectively. Active antibodies showed strong fluorescent signal outside of the baseline noise, while the isotype controls showed only a low level of background fluorescence.

Machine settings used were as follows:

- Laser thresholds: SALS at 5, LALS at 15.
- No lower thresholds for any of the fluorescent detectors.
- Sample size: 150 µl
- Sample rate: 1.5 µl/min

- Flush cycles: 2x at 150 mbar

Optimisation of Antibody Concentrations

Optimisation of antibody to sample proportions was performed prior to running study specimens. During the first phase of optimisation, conditioned media from PC-3M-LN4 and LNCaP cell cultures was used. Optimising using these samples was perhaps not ideal due to the wide variation in MP concentrations between different batches of conditioned media, and recognising that the ranges of concentration found in conditioned media might not be the same as would occur in human plasma. For that reason, final optimisation was performed on plasma from healthy volunteers as well as assorted plasma specimens from the DRE cohort. It was hoped that these would represent the spectrum of MP concentrations that would be expected.

For all optimisation experiments, the sample size was fixed at 10 µl, and adjustments were made to the amount of antibody used. Antibody concentrations were generally assessed with the NanoDrop spectrophotometer or Bradford protein assay prior to conjugation. For optimisation a range of antibody masses (per 10 µl sample) was tested. Antibodies were diluted accordingly to allow pipetted volumes of 1-3 µl. Each optimisation was also performed with the same volume and concentration of the corresponding fluorophore-labelled isotype control. MP counts for each fluorophore and each sample were recorded and plotted on histograms.

MP counts tended to increase with increasing antibody, up to a plateau. Optimum antibody mass per sample was then taken as the lowest antibody mass to give plateau values (Figure 5).

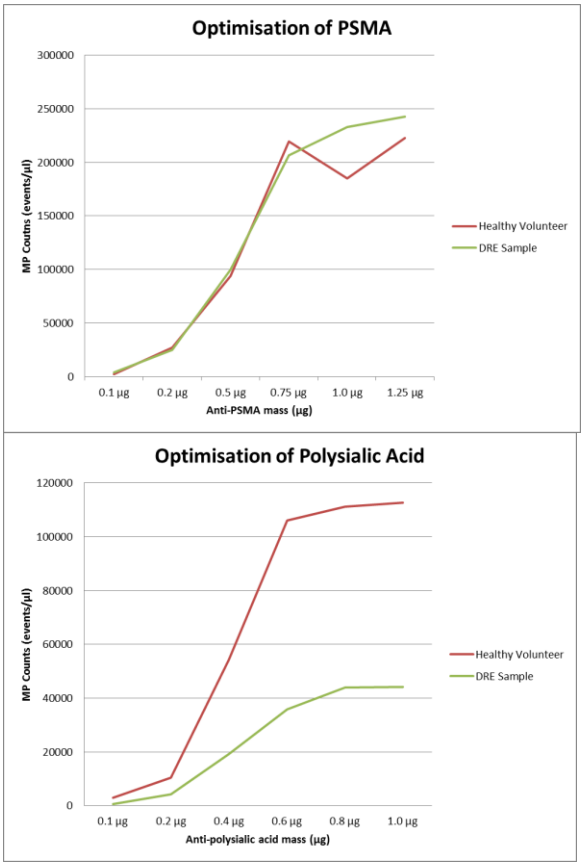


Figure 5: Optimisation of PSMA and polysialic acid antibodies. The same volume of plasma was incubated with varying amounts of antibody. Positive counts increased progressively with higher mass of antibody until a plateau was reached, indicating saturation of binding sites. Optimum antibody mass for each test was taken as the point of inflection of the respective curve.

Optimum antibody masses for PSMA and polysialic acid were found to be 0.75 µg and 0.8 µg, respectively. These were the amounts used for each 10 µl sample processed throughout the study. For the complete protocols used for plasma and urine samples, see Chapters 3 and 4, respectively.

Chapter 3: Influence of Digital Rectal Examination on Prostate Cancer Microparticles

Background and Rationale

Quantification of extracellular vesicles derived from the prostate is a promising candidate as a liquid biopsy for prostate cancer. There is currently no non-invasive test that can predict with a high degree of accuracy whether a prostate harbours cancer, or whether or not that cancer is clinically significant. Serum prostate-specific antigen (PSA), a secretory product of the prostate gland, is the primary biomarker used for prostate cancer in clinical practice. It is known that a vigorous DRE (or prostate massage) can increase the serum concentration of PSA.

It is therefore plausible that a vigorous DRE might increase the levels of prostate cancer microparticles (PCMP) in both the blood and in the urine. Furthermore, as is the case with PCA3, those levels might be disproportionately raised in the presence of prostate cancer, and possibly more so with high grade prostate cancer.

Aims and Objectives

The aim of this component of the study was to investigate the effect of digital rectal examination (DRE) on the levels of prostate cancer microparticles (PCMPs) in the blood.

The hypothesis was that digital rectal examination (DRE) would increase the levels of detectable plasma prostate cancer microparticles in men known to have prostate cancer. This formed the primary objective of the study.

Secondary objectives were to determine the changes in microparticle levels immediately after prostatectomy and at 3 to 12 weeks postoperatively, and to determine whether DRE could improve the accuracy of microparticle analysis in predicting histological features of prostate cancer.

Materials and Methods

Study Design: The study was prospectively planned and the protocol was approved by the institutional research ethics board of Western University (REB #107043). The

study was designed and carried out in accordance with local ethics regulations, as well as with the Declaration of Helsinki.

Participants: All patients who were booked for robotic prostatectomy at an academic referral centre (London, Ontario) were approached for participation in the study. There were no further inclusion or exclusion criteria. Twenty-two (22) potential participants were eligible for the study and were approached. All men agreed to participate in the study. To detect a 50% increase in microparticle levels after DRE with an alpha of 0.05 and a power of 80%, the required sample size was calculated at 31. Assuming a smaller difference of 10% with the same power, the required sample size was 83. However, since there had been no pilot study or guidance from the literature, the degree of increase or the standard deviation of such an increase could not be accurately estimated prior to the study. Sample collection and analysis was therefore planned to continue in parallel, with recruitment truncated as necessary once deemed adequate.

Sample Collection Protocol: Study ID numbers were assigned sequentially as DRE001 to DRE022, in order to immediately de-identify specimens and case report forms. An encrypted master list was kept behind the hospital firewall. Each participant had four blood samples (10 ml each) taken during the study. At the study institution, an arterial line is routinely placed for the purpose of intraoperative invasive blood pressure monitoring. All intraoperative blood samples in this study were taken directly from this arterial line, with 10 ml aspirated before sampling to clear the tubing of IV fluid. The anaesthetist would assist with this procedure when necessary. Post-operative blood samples taken in the clinics were venous blood.

After induction of anaesthesia a baseline blood sample was taken (“Plasma Specimen 1”). A DRE was then performed for 2 minutes, with firm pressure anteriorly, and sequential strokes from lateral to medial on alternating sides. At 5 minutes after completion of the DRE, a second 10 ml blood sample was taken (“Plasma Specimen 2”). The third blood sample was taken close to the end of the operation, once the prostate had been completely excised (“Plasma Specimen 3”). At completion of the surgery, the samples were centrifuged at 3000 g for 15 minutes

to generate platelet-depleted plasma. Each sample was aliquoted into ±10 labelled cryo tubes, each containing 0.5 ml plasma. Samples were then stored at -80°C.

At 3 to 12 weeks postoperatively, during routine follow-up, a fourth blood sample was drawn ("Plasma Specimen 4"). This sample was similarly centrifuged, aliquoted, and stored at -80°C.

Specimen Testing Protocol: See Chapter 2 for more complete details on sample handling, antibody preparation, flow cytometer settings, and antibody optimisation. For each sample, the following were added to a centrifuge tube:

Patient plasma – 10 µl

Anti-PSMA-3E7-PE – 0.75 µg (2 µl @ 0.375 mg/ml)

Anti-polysialic-acid-FITC – 0.8 µg (2 µl @ 0.4 mg/ml)

Each sample above was prepared in duplicate, with a third sample prepared with the corresponding isotype controls at the same volumes and concentrations.

Specimens were mixed with a vortex mixer and then spun down in a low velocity desktop centrifuge. They were then incubated in the dark for thirty minutes. After incubation, plasma samples were diluted with sterile phosphate-buffered saline to a volume of 300 µl.

Samples were then analysed using an Apogee A50 nanoscale flow cytometer. Laser thresholds were set above the level of approximately 100 nm size, based on silicon bead calibration, to reduce noise levels. Events in the two different spectra (orange for PSMA-PE and green for Polysialic acid-FITC) were enumerated using wide gating to capture all potential multiple positives. Single and dual positive MP concentrations were enumerated (as events/µl). Total event events and total events per µl were also recorded. Using the total MP counts per specimen as a denominator, proportional single and dual positive events were calculated and recorded.

For each sample run, there were 7 data points generated that were considered in the statistical analysis. These were:

- Total MP count (events/µl) – all events captured by the MP ROI, which were MPs within the threshold of ±100-1000 nm in size.

- Absolute PSMA count (events/µl) – all MPs with orange fluorescence due to PE above the threshold.
- Absolute polysialic acid count (events/µl) – all MPs with green fluorescence due to FITC above the threshold.
- Absolute dual positive count (events/µl) – all MPs with orange and green fluorescence above the threshold.
- Proportional PSMA count (%) – PSMA count as a proportion of total MPs.
- Proportional polysialic acid count (%) – Polysialic acid count as a proportion of total MPs.
- Proportional dual positive count (%) – Dual positive count as a proportion of total MPs.

CD31 and CD41a: To address questions about the origin of some of the MP populations that arose during the study, selective samples within the cohort were tested for CD31 and CD41a antigen reactivity. These surface antigens are present on endothelial cell and platelet MPs, respectively.

Optimisation of these markers for analysing plasma samples with the flow cytometer was previously done by Janice Gomes, and that protocol was used for this experiment.

Platelet-derived MPs were labelled with anti-CD41a-FITC (clone HIP8, BD Biosciences Cat 555466) at 25 µg/ml. The isotype control was IgG1-kappa-FITC. 1 µl of antibody solution was added to 10 µl plasma, mixed, incubated for 30 minutes in the dark, and then diluted 30:1 with sterile PBS. The samples were analysed with the Apogee A50 flow cytometer using the same laser thresholds and gates as for the PCMP experiments.

The same protocol was used to investigate for endothelium-derived EVs using anti-CD31-FITC (BD Pharminogen Cat # 555445) at 50 µg/ml. The isotype control was IgG1-kappa. 1 µl of antibody was also used in this experiment.

Patient Characteristics: 22 men were enrolled in the study between October 2016 and April 2017 (Table 2). One participant (DRE005) did not have a Plasma Specimen 1 drawn prior to DRE. One patient (DRE022) had no follow-up bloods (Specimen 4) taken. Both participants' data were still included in the analysis where possible.

Mean age was 59.5 years. Preoperative data showed median PSA value and median prostate volume on TRUS were 6.15 ng/ml and 35 ml, respectively. 6 men had ISUP grade group 1 PCa on biopsy, 10 had grade group 2, while 8 had grade group ≥3. 19 of the men had normal prostates on DRE, while 3 had clinical T2 disease.

Characteristics of Study Participants

Participants Recruited		n = 22
Age (mean)		59.5 years
Pre-op Data	PSA (median)	6.15 ng/ml
	Prostate volume on TRUS (median)	35 ml
	Number of cores taken at Bx (median)	12
	Number of cores positive at Bx (median)	6
	ISUP Grade Group on Bx (1; 2; 3; 4; 5)	6; 10; 3; 2; 1
	Clinical T stage (T1c; T2a; T2b; T2c)	19; 1; 1; 1
	D'Amico Risk Group (Low; Intermediate; High)	6; 13; 3
Operative Pathology	Prostate mass (median)	42.5 g
	Path T stage (pT2c; pT3a)	13; 9
	ISUP Grade Group (1; 2; 3; 4; 5)	1; 16; 4; 0; 1
	ISUP Grade concordance (Down; Same; Up)	4; 12 ;6
	% Tumour volume (median)	15%
	Calculated tumour mass (median)	5.575 g
	Lymph nodes examined (median)	12
	Pathological N stage	All pN0
	Prostatitis	9/22
F/U	Time to follow-up bloods (3 weeks; 3 mo)	
	3 month PSA (<0.1; >0.1)	21; 1

Bx = Biopsy; F/U = Follow-up; ISUP = International Society of Uro-Pathologists; TRUS = Transrectal ultrasound

Table 2: Characteristics of Study Participants

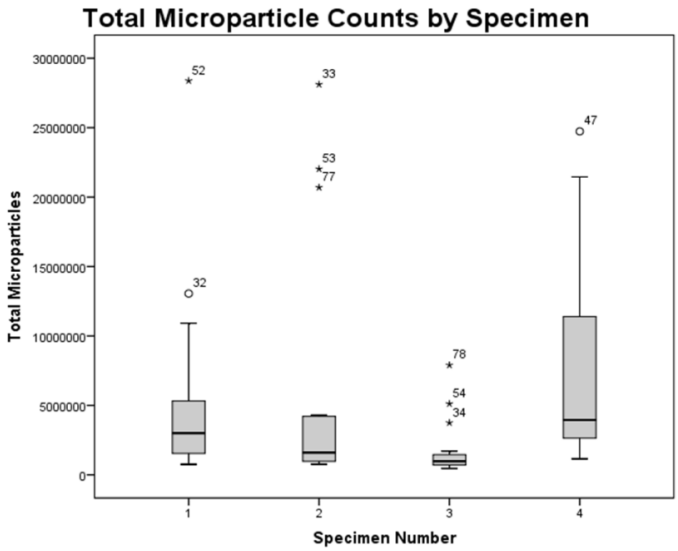
Statistical Analysis: Data was entered onto a spreadsheet using Microsoft Excel 2010. Statistical analysis was performed using IBM SPSS version 23.

Variables were tested for normality using the Shapiro-Wilk test. Since almost all of the variables were not from normal distributions, non-parametric statistical tests

were used for the analysis. For testing the significance of differences in a variable across all four samples, Friedman's two-way ANOVA test was used. The Mann-Whitney U test was used to test for significant difference across all four samples related to dichotomous categorical differences, namely histological grade, prostate volume, tumour volume, and presence of prostatitis. The Wilcoxon signed rank test was used individually to test the significance of changes from Plasma Specimen 1 (the baseline) to each of the other three specimens. Each of these individual comparisons was also tested based on groups using the Kruskall-Wallis test.

Results

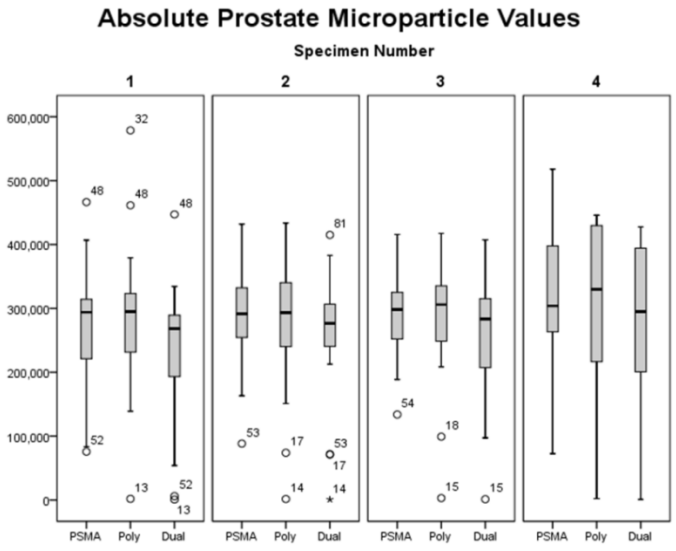
All events in the 100-1000 nm size range, fluorescent or non-fluorescent, were considered as the "total microparticle count". This total MP count showed a characteristic pattern that was consistent between participants (Figure 6). Firstly, the total MPs from the intraoperative bloods (specimens 1-3) were all significantly lower than the MPs in the follow-up sample (specimen 4) ($p<0.001$). Median MPs were 2 610 600 for all intraoperative samples versus 3 951 400 for post-operative samples. Venous samples from healthy volunteers showed numbers of MPs per µl similar to that of Specimen 4. Secondly, among the intraoperative specimens, there was a consistent drop in total MP counts from Specimen 1 to 2, and again from 2 to 3 (Figure 6). Median total MP counts for specimens 1, 2, and 3 were 3 005 500, 1 600 600, and 976 353, respectively. This represented drops from baseline of 46.7% and 67.5%, respectively. The difference between the MP counts from specimen 1 (median 3 005 500), which were the highest of the intraoperative samples, and MP counts from specimen 4 (median 3 951 400) approached statistical significance ($p=0.079$).



1 = Pre-DRE; 2 = Post-DRE; 3 = Post-prostatectomy; 4 = Follow-up

Figure 6: Total MP counts by specimen.

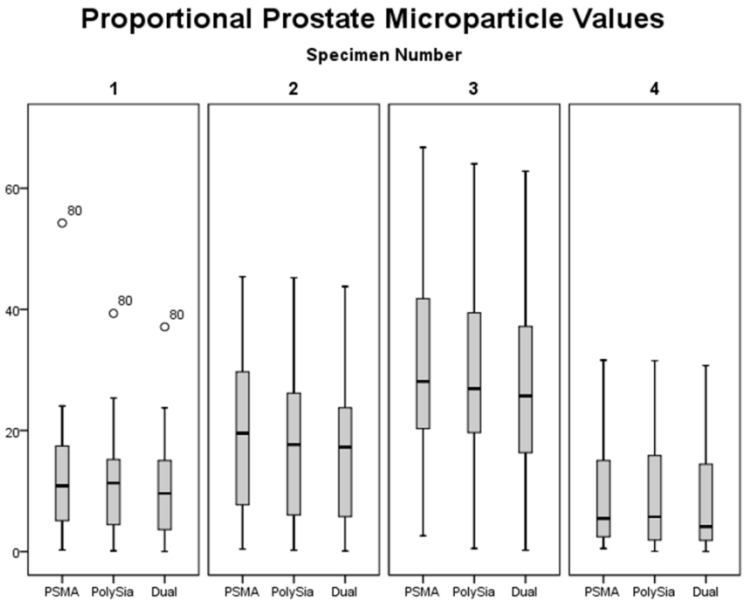
When considering only the fluorescent events, i.e. the PSMA, polysialic acid, and dual positive MPs, there was an increase in median number of events progressively from specimen 1 through to specimen 4 (Figure 7). This increase in absolute numbers occurred in the intraoperative specimens despite the reduction in overall MP levels. The increase was more pronounced for the dual positives (p=0.054 for Sp1 vs Sp2 and p=0.016 for Sp1 vs Sp3). The positive MPs in specimen 4 were higher than in specimen 1, significantly so with PSMA and dual positives (p=0.006 and p=0.013), and approaching significance for polysialic acid (p=0.058).



(1 = Pre-DRE; 2 = Post-DRE; 3 = Post-prostatectomy; 4 = Follow-up)

Figure 7: Absolute PCMP counts by specimen number and MP population.

The most dramatic differences were seen when comparing the positive MPs as percentages of the total MP counts in each specimen (Figure 8). For example, proportional dual positive values increased from a median of 9.6% in specimen 1 to 17.3% in specimen 2 ($p<0.001$), and then increased again to 25.7% in specimen 3 ($p<0.001$). The relative number of dual positive MPs dropped back down to 4.1% post-operatively ($p=0.170$). The differences between the intraoperative specimens were all highly statistically significant. The median postoperative proportional positive values were all lower than at baseline, but those values were not statistically significant.



(1 = Pre-DRE; 2 = Post-DRE; 3 = Post-prostatectomy; 4 = Follow-up)

Figure 8: Proportional PCMP counts by specimen number and MP population.

The data were interrogated to determine whether various clinical factors had any impact on either baseline PCMP levels or change from baseline after DRE, immediately after prostatectomy, or at follow-up after surgery. Participants were categorised dichotomously on histological grade (ISUP grade group 1-2 vs 3-5), total prostate volume (<40 g vs >40 g), tumour volume (<6 g vs >6 g), and presence or absence of prostatitis on postoperative pathology. There were no significant differences in any absolute MP counts or proportional values based on these

categories. There was also no difference in levels of PCMPs or change from baseline between men who had follow-up bloods taken at 3 weeks versus 3 months.

Within individual specimens, the values of PSMA, polysialic acid, and dual positives were closely correlated, and one marker did not obviously perform particularly better than the other. The combination of the two also did not give appreciably different readings compared to the baseline.

	Specimen 1	**Specimen 2**	**Specimen 3**	**Specimen 4**
Microparticle count (events/µl)	**3 005 500** (1,534 – 5,564 x10^6)	**1 600 600** (0.964 – 4.218 x10^6) p=0.016	**976 353** (0.708 – 1.476 x10^6) p<0.001	**3 951 400** (2.532 – 12.28 x10^6) p=0.079
PSMA count (events/µl)	**293 881** (0.217 – 0.324 x10^6)	**291 400** (0.254 – 0.334 x10^6) p=0.170	**298 244** (0.248 – 0.328 x10^6) p=0.058	**303 807** (0.248 – 0.397 x10^6) p=0.013)
Polysialic acid count (events/µl)	**295 002** (0.214 – 0.332 x10^6)	**302 854** (0.243 – 0.356 x10^6) p=0.092	**305 930** (0.244 – 0.339 x10^6) p=0.211	**330 030** (0.199 – 0.430 x10^6) p=0.058
Dual positive count (events/µl)	**268 314** (0.181 – 0.293 x10^6)	**276 664** (0.234 – 0.311 x10^6) p=0.054	**283 443** (0.206 – 0.316 x10^6) p=0.016	**295 013** (0.178 – 0.394 x10^6) p=0.006
%PSMA	**10.9%** (4.6% – 18.2%)	**19.6%** (7.5 – 29.8%) p=0.001	**28.1%** (19.8 – 42.2%) p<0.001	**5.5%** (2.4 – 15.7%) p=0.099
%Polysialic acid	**11.3%** (4.1 – 15.5%)	**17.7%** (5.9 – 26.2%) p<0.001	**26.9%** (16.8 – 40.2%) p<0.001	**5.7%** (1.7 – 16.0%) p=0.181
%Dual positive	**9.6%** (3.3 – 15.2%)	**17.3%** (5.4 – 24.3%) p<0.001)	**25.7%** (14.0 – 38.3%) p<0.001	**4.1%** (1.7 – 14.7%) p=0.170

Table 3: Median values of total, # positive (events/µl), and % positive MPs (p-values denoting significance of changes relative to specimen 1). Inter-quartile ranges in parentheses.

The discrepancy of reduced numbers of total MPs from the intraoperative arterial blood samples compared to the postoperative venous blood samples was not expected. In order to explore whether these differences were due to a global drop of MPs or the reduction of a specific population of MPs, the plasma samples from seven patients were analysed for CD41a and CD31 positivity. These markers are specific for platelet and endothelial EVs, respectively.

While it appeared that there might be an increase in platelet MPs during surgery, these results were not statistically significant (p=0.323).

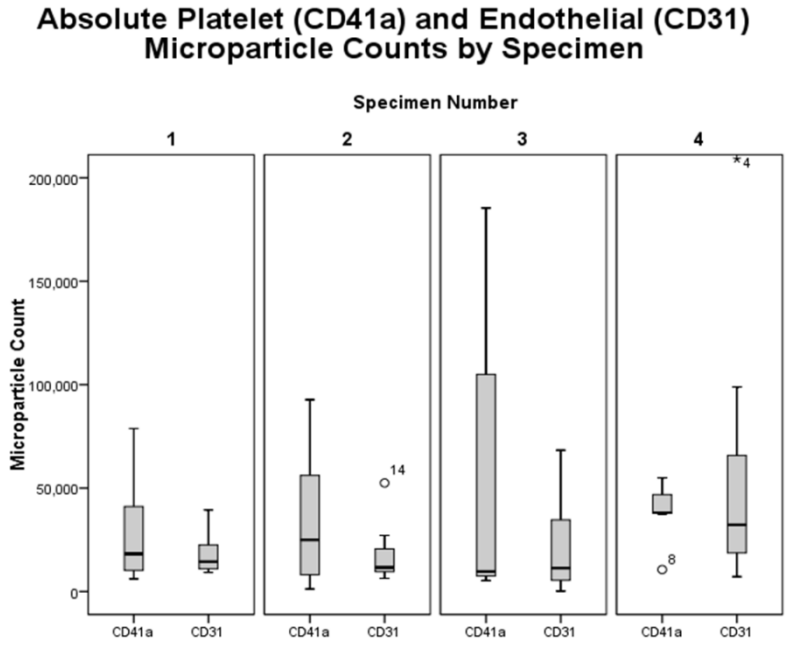


Figure 9: Absolute CD41a and CD31 positive microparticles.

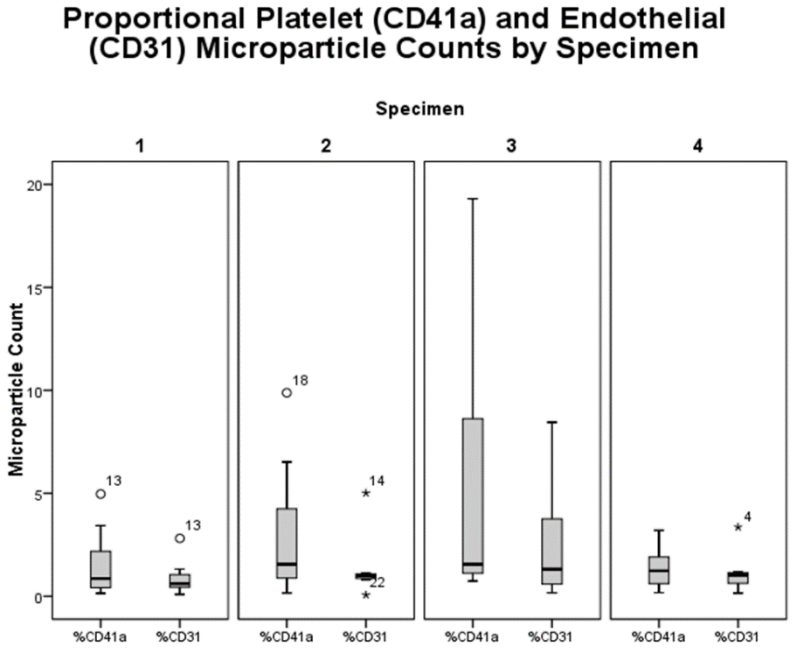


Figure 10: Proportional CD41a and CD31 positive microparticles

See chapter 5 for discussion of these results.

Chapter 4: Urinary Prostate Cancer Microparticles in Men with Prostate Cancer

Background and Rationale

The prostate gland is an exocrine gland with a number of secretory products that contribute to the ejaculate. Specialised extracellular vesicles, sometimes called prostasomes, are secreted by the normal prostate. These subcellular structures have several physiological functions in the semen, including support of the spermatozoa and modulation of the female immune response to the sperm. They have been detected in the plasma, and plasma levels have been found to be increased in prostate cancer (PCa). Studies analysing urinary prostate EVs have shown potential for diagnosis and risk stratification of PCa. Most of these studies have characterised the contents of urinary EVs, such as miRNA or proteins. We are unaware of any biomarker studies that have used flow cytometry to enumerate urinary prostate EVs.

Prostasomes are secreted directly into the urinary tract, and collection of urine is less invasive than collecting blood, which together make the urine an appealing potential substrate for the detection of PCa-specific MPs. Detection, enumeration, and characterisation of PMP populations using flow cytometry in conjunction with markers specific for prostate cancer has potential to be a totally non-invasive test for prostate cancer.

A DRE is required prior to PCA3 testing because it increases the urinary concentrations of the PCA3 mRNA. If MPs are detectible in the urine by flow cytometry, then it is also plausible that a DRE would similarly improve urinary concentrations of these MPs and increase the accuracy of a test based on MP quantification.

Aims and Objectives

The primary objective of this component of the study was to determine whether urinary prostate cancer microparticles (PCMPs) could be detected in the urine of men known to have prostate cancer (PCa).

Secondary objectives were to determine whether PCMP counts in the urine are affected by DRE, or whether pre- or post-DRE urinary PCMP counts correlated with clinical factors such as prostate volume, tumour grade, or tumour volume. The working hypothesis was that digital rectal examination (DRE) would increase the levels of urinary PCMPs in men with PCa.

A highly accurate urine test for PCa would be of great benefit in the field of urologic oncology.

Materials and Methods

Study Design: The study was prospectively planned and the protocol was approved by the institutional research ethics board of Western University (REB #107043). The study was designed and carried out in accordance with local ethics regulations, as well as with the Declaration of Helsinki.

Participants: All patients who were booked for robotic prostatectomy at an academic referral centre (London, Ontario) were approached for participation in the study. There were no further inclusion or exclusion criteria. Twenty-two (22) potential participants were eligible for the study and were approached. All men agreed to participate in the study. The analysis of urine for prostate MPs was an exploratory component of the DRE study and not the primary objective. Therefore, sample size determination did not take anticipated urinary MP levels into account.

Sample Collection Protocol: Study ID numbers were assigned sequentially as DRE001 to DRE022, in order to immediately de-identify specimens and case report forms. An encrypted master list was kept behind the hospital firewall. Each participant had two urine sample taken as part of the study. Each participant was asked to give a voided sample during the pre-operative preparation. The participant was requested to give at least 40-50 ml, and to include the initial voided urine component (rather than midstream urine). Those who were unable to pass a voided sample immediately pre-operatively had an in-out catheterisation performed immediately after induction of anaesthesia. The first urine specimen was labelled “Urine Specimen A”.

A vigorous digital rectal examination (DRE) was then performed for 2 minutes, with firm pressure anteriorly, and sequential strokes from lateral to medial on alternating sides. This DRE was followed by routine surgical preparation and sterile draping. The second urine specimen was collected on insertion of the transurethral catheter prior to starting the surgical procedure.

The samples were then centrifuged at 3000 g for 15 minutes. Supernatant was aliquoted into ±10 labelled cryo tubes, each containing 1.5 ml urine. Samples were then stored at -80°C.

Satisfactory urine samples were obtained for all 22 participants.

Specimen Testing Protocol: See Chapter 1 for more complete details on sample handling, antibody preparation, flow cytometer settings, and antibody optimisation.

Since the analysis of urine in the DRE study was the exploratory component of the study, the testing protocol was changed with serial experiments. Running of patient and healthy volunteer urine supernatant without antibody showed that there were 10-20 fold fewer events in the 100-1000 nm size range than in human plasma.

Initial experiments used an exact duplication of the protocol used for plasma, with 10 µl of urine supernatant with 2 µl of the anti-PSMA and anti-polysialic acid antibodies. However, because of the very low density of MPs, the presumed positive signals in the 10 µl sample were lost when diluting with 290 µl of PBS. An alternative strategy attempted was to use a greater volume of urine, up to 300 µl, to stain with antibody and run undiluted. The large volume of urine with such a small quantity of antibody also did not seem optimal for antibody binding. In these experiments, the active antibody produced similar populations to the isotype control, presumably from non-specific binding.

Much higher concentrations of urinary MPs were achieved using ultracentrifugation of urine. Comparative analysis of the pellets and supernatant from ultracentrifuged samples confirmed significant concentration of MPs. The protocol that was developed and used to test the entire cohort was as follows:

Separate samples of 1,000 µl of urine were centrifuged at 14,000 G for 45 minutes. 950 µl of supernatant was carefully removed from the surface of the specimen and the pellet resuspended in the remaining 50 µl of supernatant. 10 µl of resuspended centrifuged urine was pipetted into a new centrifuge tube. Antibody was carefully

pipetted into the urine and mixed thoroughly by gentle backwards and forwards pipetting. 0.75 µg of anti-PSMA-3E7-PE (2 µl @ 0.375 mg/ml) and 0.8 µg of anti-polysialic-acid-FITC (2 µl @ 0.4 mg/ml) were used.

Specimens were mixed with a vortex mixer and then spun down in a low velocity benchtop centrifuge. They were then incubated in the dark for thirty minutes. After incubation, urine samples were diluted with sterile phosphate-buffered saline (PBS) to a volume of 300 µl. Isotype controls were performed on several of the urine samples to exclude non-specific staining. Since non-specific binding did not seem to be a problem at any point, with ROI gates being predominantly clear , we did not perform isotype controls on all samples.

Samples were then analysed using an Apogee A50 nanoscale flow cytometer. Laser thresholds were set above the level of approximately 100 nm size, based on silica bead calibration, to reduce noise levels. Events in the two different spectra (orange for PSMA-PE and green for Polysialic acid-FITC) were enumerated using wide gating logic to capture all potential multiple positives. Single and dual positive MP concentrations were enumerated (as events/µl). Total event events and total events per µl were also recorded. Using the total MP counts per specimen as a denominator, proportional single and dual positive events were calculated and recorded.

Similar to the plasma samples run, each sample generated 7 data points for statistical purposes. Those were: Total MPs, absolute PSMA, absolute polysialic acid, absolute dual positives, proportional PSMA, proportional polysialic acid, and proportional dual positives.

Statistical Analysis: Data was entered onto a spreadsheet using Microsoft Excel 2010. Statistical analysis was performed using IBM SPSS version 23.

Variables were tested for normality using the Shapiro-Wilk test. Since almost all of the variables were not from normal distributions, non-parametric statistical tests were used for the analysis. For testing the significance of differences between the pre-DRE and post-DRE urine samples, the Wilcoxon signed rank was used. The Mann-Whitney U test was used to test for significant differences between pre- and post-DRE samples in relation to dichotomous categorical differences, namely as histological grade, prostate volume, and tumour volume.

Patient Characteristics: 22 men were enrolled in the study between October 2016 and April 2017. See the "materials and methods" section in chapter 3 and the accompanying Table 2 for a complete description of the cohort.

Results

Comparison of ultracentrifuged urine pellets from specimens A and B showed that the total MP counts differed significantly (Table 4). Median events per µl were 667 453 before DRE and 2 793 500 after DRE. This difference was highly significant (p=0.001).

	Total MPs	PSMA	Polysia	Dual	% PSMA	% Polysia	% Dual
Specimen A – Pre-DRE	667 453 (416 323 – 1 357 350)	28 165 (14 472 – 45 226)	12 837 (4 220 – 31 421)	11 876 (3 875 – 24 271)	3.946% (1.629 – 8.391%)	1.574% (0.667 – 4.060%)	1.321% (0.485 – 3.686%)
Specimen B – Post-DRE	2 793 500 (1 231 125 – 7 107 925)	28984 (14 228 – 45 602)	16141 (5 591 – 36 852)	12780 (4 436 – 23 834)	0.864% (0.347 – 2.007%)	0.884% (0.238- 1.470%)	0.321% (0.196- 0.944%)
p-value	0.001	0.907	0.489	0.907	0.002	0.037	0.011

Table 4: Median values of total, # positive (events/µl), and % positive urinary MPs (p-values for difference from Spec A to Spec C). Inter-quartile ranges in parentheses.

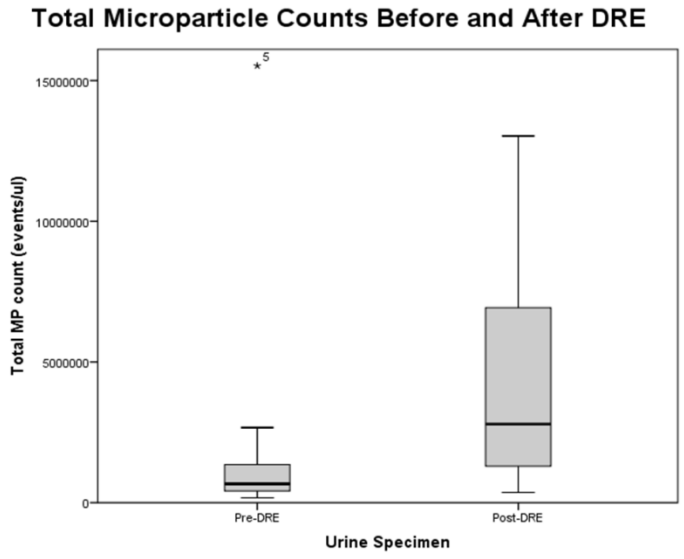


Figure 11: Total urinary microparticle counts before and after DRE.

The numbers of MPs positive for PSMA and polysialic acid, as well as the dual positives, were very similar between specimen A and B. None of the differences were statistically significant (Figure 12).

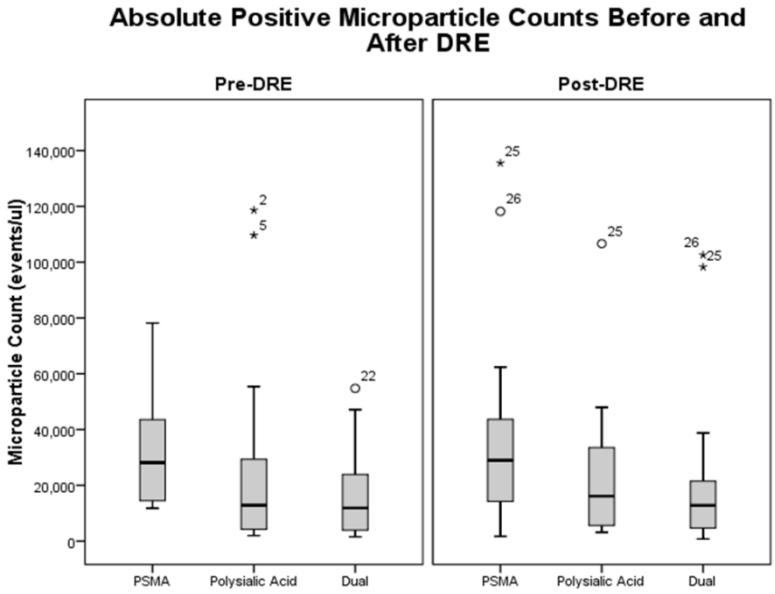


Figure 12: Absolute positive urinary microparticles before and after DRE.

As a result of the large discrepancy in the numbers of absolute MPs with the static numbers of positives, the proportional numbers of positive MPs were all significantly lower post-DRE (Figure 13).

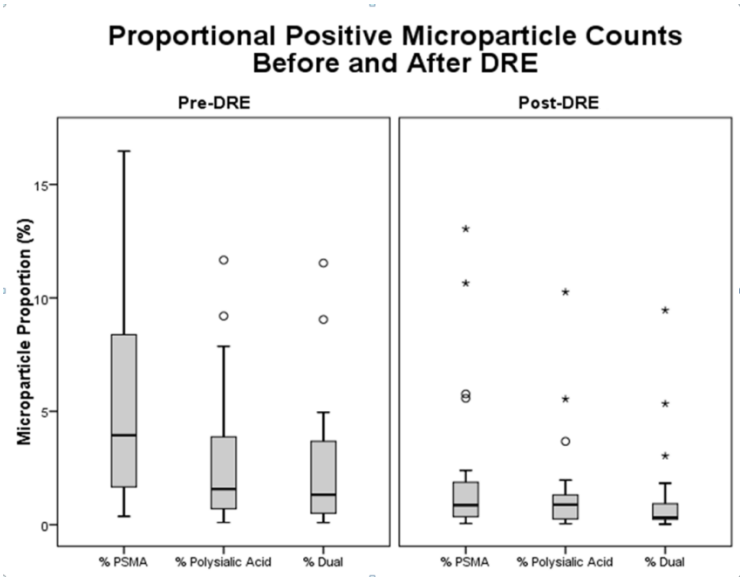


Figure 13: Proportional positive urinary microparticles before and after DRE.

Chapter 5: Discussion

Plasma Microparticles

All of intraoperative total MP counts were significantly lower than the post-operative venous blood draw. This phenomenon was first observed when analysing plasma samples from the first two patients after their three week follow-up. The immediate concern was that there had been a drop in the stored plasma samples, since they were three weeks older and had up to that stage been stored at -20°C. Subsequent experimentation in the laboratory (not discussed here) as well as the subsequent experience with the remainder of the cohort reassured us that this was not the case. One potential explanation is that these samples were arterial and venous blood, respectively, and that the difference we saw is truly representative of true *in vivo* physiology. There were no studies identified in the literature review that compare EV concentrations in arterial versus venous blood. Some studies examining EV clearance *in vivo* have shown that they have half very short half-lives in the circulation, as little as 2 minutes, and are found up to several hours later sequestered in the spleen, liver, and lymph nodes (185–187). These findings, combined with the findings that EVs are stable in non-circulating blood, suggest that they are cleared by the reticuloendothelial system (RES). Other studies have shown longer half-lives, up to 5.8 hours, and that EVs expressing different surface markers might be preferentially captured within the RES. Also, none have mentioned whether lung tissue was examined, which would most easily account for the very short half-life and the discrepancy between arterial and venous blood.

In addition to the differences in total MP counts between the intra- and post-operative specimens, this study found that total MP counts dropped from the first time-point a few minutes after induction of anaesthesia, to the second time-point, usually about 10 minutes later. The levels were consistently further decreased in the third specimen, taken two to three hours after the first two samples. This phenomenon is also not explained. It is quite possible that the underlying cause of this progressive decline is the same as for the low levels among the intra-operative specimens as a whole. The participants were all under anaesthesia, and there was therefore an inverse correlation between total MP counts and duration under

anaesthesia. MP levels may have been influenced by intravenous fluid administration, the subcutaneous heparin given at induction, or other drugs such as the volatile agents, propofol, opioids, or antibiotics. Explanations related to procedural error in sample processing, such as time to processing, also do not account for the difference. Sample processing took place either twice during a case (specimen 1 and 2 together, then specimen 3 later), or once all together at the end of the case. The pattern of MP decrease persisted despite the procedure followed. When processing was done at two time points, the third specimen's MPs were still appreciably lower. When processed all together (4-5 hours after the beginning of the case), there was still a large discrepancy between specimens 1 and 2, taken only 10 minutes apart.

Another uncertainty was whether the changes in overall MP concentrations were the result of all MPs in the specimen being reduced from baseline, or if there was a selective reduction of one or more specific populations within the blood. It is possible that the heparin given at induction of anaesthesia might cause a progressive reduction in platelet-derived MPs. Another possibility is that a venous blood draw, where slow-moving blood is sampled that has already moved past the needle's point of entry into the vein, could contain a significant number of MPs derived from endothelial trauma and platelet activation. Conversely, arterial blood is taken from a cannula that points *towards* a much more rapid flow of blood, which would make these samples much less likely to be influenced by the local vascular trauma. To test these hypotheses, a previously optimised pair of antibodies against CD41a and CD31 was used to examine seven sets of patient plasmas. These markers are specific for platelet and endothelial cell MPs, respectively (157). It was found that neither MP population mirrored the total MP population, indicating that isolated differences in either of these MP populations were not the cause of the overall differences in MP counts that were seen. Unfortunately, limitations to time and antibody precluded examination of the whole cohort. Concerns about the validity of this protocol were that the antibody concentrations used were extremely low, and positive populations for platelets and endothelial MPs were both less than 1% of total MP counts, when they would be expected to account for >70% and >10% respectively.

The effects of fasting might also have influenced total MP levels. All men were fasted for at least 6 hours prior to surgery, but many would not have had food since the evening before. Additionally, it might be assumed that most men were not similarly starved at their follow-up appointments. If these assumptions were true then there would be a negative correlation between total MPs and duration of fasting. The effect of fasting on EV concentrations is not known (188), but it is possible that a reduction in small chylomicrons in the 100-1000 nm size range might account for the observed discrepancy. This explanation, however does not account for the significant difference in MP levels between Specimens 1 and 2, taken only 10 minutes apart.

Looking at the MP populations positive for PSMA, polysialic acid, and both, the concentrations of all remained fairly stable, despite the differences in overall MP concentration. The only consistent significant differences were between baseline (specimen 1) and follow-up (specimen 4), where the absolute positive MPs actually rose. The higher PMP levels in these men is difficult to explain, considering their lack of prostates, and presumably in most cases, prostate cancer. If the assumption is made that whatever accounted for the large differences in total MP counts between specimens also affected all MP populations equally, then it would be rational to consider the concentration of positive MPs as a proportion of the concentration of total MPs. If one does this, then the changes in the relative PMP confirm the hypothesis of the study very well. Relative PMP levels increase significantly after a DRE, then increase significantly further over the duration of surgery, and then drop back down to a level below baseline at follow-up (although the latter change not being statistically significant). There was no difference between PMP levels at 3 weeks and at 3 months.

None of the secondary objectives of the study, related to plasma analysis, achieved statistical significance – most likely because of low power from the small numbers of participants. Gleason score, prostate volume, or tumour volume did not correlate with PSMA, polysialic acid, or dual positive MP levels.

Urine Microparticles

Analysis of patient urine samples generated large numbers of positive events, which likely represent MPs derived from the prostate gland.

Wide variations in event counts were noted between different patients, which was most likely due to differing urinary concentrations. Also, some of the participants had significant amounts of debris in their urine samples, which may have contributed "nano-debris" to samples, and accounting for the increases in non-specific MPs. There were significantly more total microparticles in post-DRE urine, which was either the result of the DRE itself, or the catheterisations used to retrieve the second (and sometimes the first) urine samples. A set volume of first-void urine should ideally have been used for both pre- and post-DRE urine samples to optimally catch the prostatic secretions. Unfortunately, the design of this study precluded such a collection method.

In the context of a urine biomarker, normal variation in urine concentration would make absolute concentrations an unreliable continuous variable for diagnosis or risk stratification. Having to ultracentrifuge small quantities of urine would also introduce room for error. Urine tests for PCA3 or TMPRSS:ERG RNA also measure the concentration of PSA mRNA. This serves several important purposes: It tests whether the urine specimen is adequate for analysis and it serves as a denominator to allow the PCA3 or T2 to be reported as a ratio. This latter function is most important, since a baseline PSA mRNA concentration is relatively reliable and predictable, so it compensates for differing concentrations of urine and prostate secretions in the sample (37). The MP analysis in the DRE study lacked such a "control", which was a shortcoming in the study design. Duijvesz et al. have reported using urinary PSA and protein concentrations as a baseline for urinary MP quantification (154) . Many blood and tissue RNA tests use a similar baseline by testing for "housekeeper" genes that are known to be expressed in all cell types at a predictable level.

The small number of participants in our study made it impossible to correlate findings of MP concentrations with clinical factors, such as PSA, histological grade, tumour volume, or prostate volume.

Challenges and Shortcomings

The original study design specified a triple test, that included STEAP1 along with the PSMA and polysialic acid. Unfortunately, the STEAP1 antibody that was available from two different suppliers did not reliably produce MP populations the study samples. This problem could not be resolved during the study period, and the final analysis of the samples therefore needed to be performed using only two antibodies. Work done by other investigators in the Leong laboratory suggests that elimination of the anti-STEAP1 from the protocol would reduce the specificity of the test by a small amount. Although this was less than desirable, the study compared changes in MP counts within the same participants, so the omission might not have impacted on the primary outcome of the study, which was determining the change in concentration with DRE. However, performing a dual instead of a triple antibody test could have reduced the ability to detect differences between subgroups of participants, such as histological grade or tumour volume. It is more likely that the study would still have been underpowered for these secondary outcomes.

The many processes in the protocol of the DRE study were made up of many individual steps that all had to be tightly controlled to reduce error as much as possible. Preparation of samples for analysis, for example, involved pipetting multiple small volumes, each prone to error. Much of the protocol had to be developed during the study. Decisions on standardising certain aspects were based as far as possible on experimentation (such as optimisation of antibody concentration and volume used), but several decisions were sometimes fairly arbitrary, with little data in the literature to guide decisions. It is not known whether these impacted negatively on the study. Examples of these include the freezing and thawing of specimens, whether or not to use cryo-protectants, the volume of plasma to use for each sample tested, and many of the calibration settings on the flow cytometer.

For all of the antibodies used during the study, there was the potential for batch to batch variation in antibody characteristics. This proved to be a critical problem for STEAP 1, but there was likely some variation with the other antibodies. There could also have been variation in the conjugation reactions, as well as degeneration of the

antibodies and fluorophores during storage. To circumvent this, the whole cohort of samples was re-run close to the end of the study period using the same batches of PSMA and polysialic acid antibodies. While this strategy would have produced more reliable results, it might also limit the reproducibility of the study.

Including a cohort of men without prostate cancer, such as men undergoing other urological surgery, might have allowed determination of the degree to which MPs were increased due to prostate cancer versus benign prostate tissue. Such a follow-up study would be a logical next step.

Conclusions

This study demonstrated that total plasma microparticle concentrations were significantly lower in plasma samples taken from arterial lines of men under anaesthesia than in venous blood taken from the same men at follow-up. Whether this difference was due to the arterial nature of the intraoperative specimens, or some factor related to the anaesthesia itself, is uncertain. It is also not certain whether the reduced microparticle concentrations represent a global reduction in plasma MPs or if it represents a reduction of a particular MP subpopulation.

Microparticles that express PSMA and polysialic acid can be successfully labeled using ligand specific antibodies conjugated to fluorophores, and these MPs can then be detected using nano-scale flow cytometry. Expression of both of these cell surface markers is known to be increased in prostate cancer, and work done in our laboratory has shown that the presence of these MPs is sensitive and specific for prostate cancer. The proportion of MPs per specimen that are positive for these markers increases significantly following a two minute vigourous digital rectal examination, and further increases three to four hours after the DRE, and once the prostate has been removed. Proportional levels then decrease to a level at or below the pre-operative level after a period of at least three weeks.

Prostate microparticles can also be detected in the urine after ultracentrifugation and a similar process of antibody labeling and flow cytometry. The concentration of these microparticles does not seem to be significantly increased following DRE, although a shortcoming of the study might be that catheter specimens were taken instead of first voided specimens. Catheterisation did seem to increase the total numbers of MPs, presumably due to disruption of the mucosa.

Further studies are necessary to properly understand the differences between MP concentrations in arterial versus venous blood, the effects of anaesthesia on MP concentrations, as well as the lifespan and metabolism of MPs. When viable MP-based tests for prostate cancer become available, it will be important to determine whether a DRE prior to blood sampling could increase the accuracy of those tests, since there is now some evidence to suggest that plasma prostate MP numbers are proportionally increased after DRE. Enumeration of prostate MPs in the urine is also

a promising potential test for PCa. With a formal protocol involving ultracentrifugation of specimens prior to analysis, these can be reliably detected with fluorescent antibodies and flow cytometry. There is sometimes wide variation in total microparticle concentration in both plasma and urine. Quantification of organ-specific MPs would therefore be more useful if there was a reliable background MP population against which to compare. Further work in this direction is also needed.

Prostate-specific MP quantification is a promising biomarker for the presence of prostate cancer, as well as a potential indicator of disease severity. Future studies should be performed toward developing and validating quantitative PMP measurement as a biomarker for prostate cancer. In that setting, pre-test DRE should be considered to increase circulating PMP concentrations, and to determine whether concentrations are similarly increased in men without prostate cancer.

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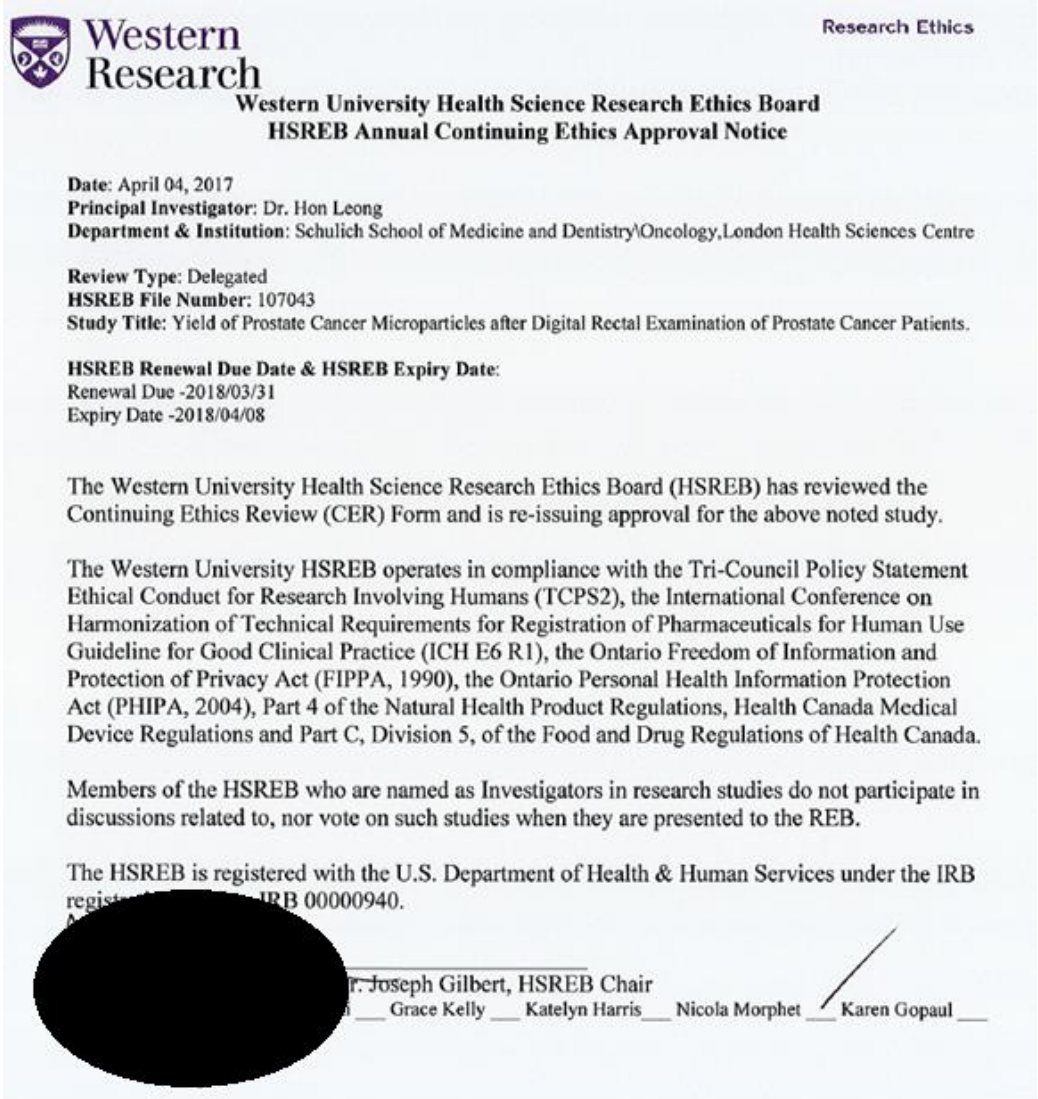
Appendices

Appendix 1: Glossary of Terms

- Extracellular vesicle (EV) – Subcellular phospholipid-bound particle, originating from a cell, between 30-1000 nm in diameter.
- Microparticle (MP) – Interchangeable term with “Ectosome”. EV originating from the outer plasma membrane of a cell by a process of membrane budding. Generally 100-1000 nm in diameter.
- Exosome – EV arising from a process of exocytosis from MVB. Generally 30-100 nm in diameter.
- Endosome – Not an EV. Membrane-bound structure within the cytoplasm resulting from endocytosis.
- Multivesicular body (MVB) – Membrane-bound structure within cytoplasm, containing multiple intraluminal vesicles (ILVs). Fuses with the outer cell membrane to release the ILVs as exosomes.
- Apoptotic body – EV produced as a result of apoptosis, which are fragments of the apoptotic cell. These are easily phagocytosed by phagocytes, and thereby prevent an immune response.
- Flow cytometry – a process to analyse the particulate components of a fluid by passes a stream through a narrow channel and measuring either electrical impedance or scattered laser light.
- Prostate-specific membrane antigen (PSMA) – transmembrane protein found mostly in glandular cells of the prostate that is involved in folate metabolism.
- Six-transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) – transmembrane protein located primarily in prostatic glandular cells, responsible for reduction of metals such as iron, copper, and zinc.
- Polysialic acid – a complex carbohydrate post-translational modification of the neural cell adhesion molecule (NCAM), which is important for growth and development, cell-cell adhesion, and neural regulation. Overexpressed in many malignant cell lines.

- Radical prostatectomy – a treatment for prostate cancer with curative intent, that is surgical removal of the entire prostate, its fascial coverings, and usually the seminal vesicles.
- Phycoerythrin – a fluorophore derived from red algae, with peak absorption at 565 nm, peak emission at 575 nm, and a high extinction coefficient of 1.96×10^6 $M^{-1}cm^{-1}$.
- Fluorescein isothiocyanate – commonly used fluorophore with peak absorption at 494 nm, peak emission at 521 nm, and extinction coefficient of 9.24×10^4 $M^{-1}cm^{-1}$ at a pH of 7.

Appendix 2: Ethics Approval Notifications



Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: April 04, 2017
Principal Investigator: Dr. Hon Leong
Department & Institution: Schulich School of Medicine and Dentistry\Oncology,London Health Sciences Centre

Review Type: Delegated
HSREB File Number: 107043
Study Title: Yield of Prostate Cancer Microparticles after Digital Rectal Examination of Prostate Cancer Patients.

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2018/03/31
Expiry Date -2018/04/08

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB regist[redacted]IRB 00000940.

[redacted]

r. Joseph Gilbert, HSREB Chair

[redacted] Grace Kelly Katelyn Harris Nicola Morphet Karen Gopaul

Appendix 3: Case Report Form for DRE Study

Investigator: Malcolm Dewar (+1-519-XXX-XXXX) **Subject ID:** DRE___
REB# : 107043
Study: Yield of PCMP's after DRE of Prostate Cancer Patients **Initials:** ___

Case Report Form

Clinical Information

Date:		**Age (yrs):**	
PSA (ng/ml):		**Clinical stage:**	T1c T2a T2b T2c T3
Biopsy date:		**Prostate volume (TRUS):**	
Cores taken:		**Gleason Score:**	3+3 3+4 4+3 4+4 ≥9
Cores positive:		**Perineural invasion:**	No Yes
Max core positive:		**Variant histology (eg. neuroendocrine):**	No Yes _____

Sample tracking

Blood Specimens:	**Date and Time**		**Date and Time**	**Spec Storage**
Specimen 1: Pre-DRE		**Specimen 3:** 15-180 min post-DRE	T=_____min	Plasma 1,2,3
Specimen 2: 5 min post-DRE	T=_____min	**Specimen 4:** 3 weeks to 3 months post-RP	T=_____days	Plasma 4

Urine Specimens:	**Date and Time**		**Date and Time**	**Spec storage**
Specimen A: Pre-DRE		**Specimen B:** 5-30 min post-DRE	T=_____min	Urine A + B

Post-operative histology

pT stage and margins:	pT2a pT2b pT2c pT3a pT3b R0 R1	**pN stage:** **(# pos / # examined)**	X 0 1 (_____/_____)
Gleason score	3+3 3+4 4+3 4+4 ≥9	**Perineural invasion**	No Yes
Prostate mass (g):		**Prostatitis:**	No Yes
Tumour volume (%):		**Comments:**	

Investigator: Malcolm Dewar (+1-519-XXX-XXXX) **Subject ID:** D R E _ _ _
REB# : 107043
Study: Yield of PCMP's after DRE of Prostate Cancer Patients **Initials:** _ _

Lab results

All results to be dated. Control values in brackets:

	PSMA	STEAP1	PolySi	PSMA + STEAP1	PSMA + PolySi	STEAP1 + PolySi	Triple pos	Other
Specimen 1								
Specimen 2								
Specimen 3								
Specimen 4								
Specimen A								
Specimen B								
Post-op PSA								

Appendix 4: Letter of Information for DRE Study

London Health Sciences Centre

Letter of Information

Yield of Prostate Cancer Microparticles After Digital Rectal Examination of Prostate Cancer Patients.

Principal Investigator: Hon Leong

You have been scheduled for radical prostatectomy of the prostate gland. All patients who are scheduled for this procedure are eligible to participate in this research study. We invite you to participate in this study. Our ultimate goal is to clinically validate a blood test (fluid biopsy) to improve diagnosis and prognostication of prostate cancer.

Please read the following information carefully and discuss it with your family and/or family doctor, if you wish. If you wish to participate, you will be asked to sign the consent form.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy in males in Canada. We have developed a blood test that enumerates prostate cancer cell fragments released by the tumor into the bloodstream. We have previously shown that this blood test can distinguish low risk prostate cancer patients from high risk prostate cancer patients. To improve the efficacy of this blood test, digital rectal examination (DRE) may cause increased blood levels of prostate cancer cell fragments (also known as prostate cancer microparticles) or a raised blood test called PSA (Prostate Specific Antigen) and to confirm the diagnosis, a biopsy is mandatory. However, DRE and PSA test are not specific for the prostate cancer. Currently, 75% of men with high PSA levels do not have PCa according to their biopsy. If we are able to refine our criteria for biopsy we will be able to avoid unnecessary biopsies of the prostate.

Prostate cancer fragments, also known as microparticles are present in the blood of prostate cancer patients in a very small quantity. This is an emerging topic in oncological research. Our laboratory has developed the capacity to measures prostate cancer fragments in the blood. This test may be better than the PSA test which may be raised in both benign prostatic hyperplasia (BPH) and PCa patients. The blood test for prostate cancer microparticles may prevent a large majority of men from being submitted to unnecessary prostate biopsy.

The focus of this work is to validate the clinical utility of our prostate cancer microparticle test as a screening tool and to compare its accuracy with PSA test.

Study Procedures

If you agree to participate we may collect demographic data including date of birth and the pre-biopsy PSA results. We will ask you to provide us with a urine sample before going into the OR. While you are under anesthesia you will have three 8 ml tubes of blood drawn by the surgeon as well as 10 ml of urine collected by the surgeon. At three weeks to three months after the surgery, we will request one last tube of 8 ml blood drawn by a qualified health care professional when you visit your urologist for your first follow-up visit. All blood and urine samples will be analysed for the presence of prostate cancer microparticles and correlated with the biopsy report and your follow-up PSA result.

Your ongoing care will be unchanged and will continue as usual in consultation with your urologist. We will be enrolling 74 patients over the next year; all study participants will be patients of Dr. Stephen Pautler or Dr. Nicholas Power. Please note that you will not receive the results of any of the tests that will be performed for research purposes.

Initial: _____

Potential Risks
There is no known major risk in participation except the minor discomfort of giving the blood during your first followup visit. As veins may vary in size from one patient to another obtaining a blood sample from some people may be more difficult than from others. Some patients may feel light-headed but fainting is extremely rare. There is remote chance of forming a hematoma or infection.

Potential Benefits
The main benefit from this study is to patients in the future. We might be able to better select patients for prostate biopsy.

Treatment Alternative(s)
This study will not alter your treatment other than the potential benefit of mentioned in the preceding section.

Funding:
None.

Confidentiality:
The information collected will be used for research purposes only, and neither your name nor information which could identify you will be used in any publication or presentation of the study results. All information collected for the study will be kept confidential; however, representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research. While we will do our best to protect your information there is no guarantee that we will be able to do so. The inclusion of your initials and date of birth may allow someone to link this data to you.

Reimbursement:
There is no reimbursement for participation in this study.

Participation:
Participation in research is voluntary. If you choose to participate in this study you may withdraw at any time. If you do not wish to participate, you do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled to or are presently receiving. You do not waive any of your legal rights by signing the consent form.

Questions about Database or Treatment
If you have any concerns in regards to the recording of your details or wish to withdraw from this study please either mention this to your Urologist or alternatively contact:
Dr. Hon Leong, Division of Urology, Department of Surgery, 268 Grosvenor St., London, Ontario

Questions about Your Rights or This Studies Conduct
If you have any questions about your rights as a research participant or the conduct of the study you may contact: Dr. David Hill, Scientific Director, Lawson Health Research Institute at (519) xxx-xxxx.
If you have any concerns regarding this study you can contact the Principal Investigator, Dr. Hon Leong at 519-xxx-xxxx or the Urology Research Office at 519-xxx-xxxx.

Initial: _____

Consent for Participation

Study Title**: Yield of Prostate Cancer Microparticles After Digital Rectal Examination of Prostate Cancer Patients.**

Principal Investigator **Hon Leong**

I have read the letter of information, have had the nature if the study explained to me and all questions have been answered to my satisfaction and ***I agree to participate***

Name (please print)_____

Signature_____

Date_____

Person Obtaining Consent_____

Signature_____

Date_____

Appendix 5: Curriculum Vitae

Name: Malcolm James Dewar

Undergraduate Medical Training

- Bachelor of Medicine and Bachelor of Surgery (MBChB) 1998-2003, University of Cape Town, Cape Town, South Africa

Postgraduate Education and Training

- Registrar in General Surgery at the University of Cape Town – Nov 2008 to Jan 2012 (39 months)
- Registrar in Urology at the University of Cape Town – Feb 2012 to May 2016 (52 months)
- **FCUrol(SA)** – South African College of Urologists, Awarded May 2015.
- **MMed(Urol)** – University of Cape Town, Awarded December 2016
- **SUO Fellow in Urologic Oncology** – London Health Sciences Centre and Western University, July 2016 to June 2018
- Enrolled in MSc (Surgery) programme – Western University, Sept 2016 to Aug 2017.

Related Work Experience

- Registrar in Urology - University of Cape Town, Cape Town, South Africa, Feb 2012 to May 2016
- SUO Fellow in Urologic Oncology – London Health Sciences Centre and Western University, July 2016 to June 2018

Honours and Awards

- **Phillip Smith Prize** for the Best Post-Graduate Student in Urology, University of Cape Town, 2015.
- **Discovery Health Prize** for the best paper/presentation by a South African Urologist no longer in training at the SAUA biennial meeting, for the presentation "Investigating racial differences in clinical and pathological characteristics of prostate cancer in South African men" (Nov 2016).

Registrations and Affiliations

- Registered as a Urologist with the Health Professions Council of South Africa
- Registered as a Clinical Fellow with the College of Physicians and Surgeons of Ontario
- Fellow of the South African College of Urologists
- Member of the South African Urological Association
- Member of the Society of Uro-Oncology

Publications

- Bao Y, Al KF, Chanyi RM, Whiteside S, Dewar M, Razvi H, Reid G, Burton JP. *Questions and challenges associated with studying the microbiome of the urinary tract.* Ann Transl Med. 2017 Jan; 5(2):33. PMID: 28217698 PMCID: PMC5300849 DOI: 10.21037/atm.2016.12.14

- Chin JL, Dewar M, Siddiqui K. *High Intensity Focused Utrasound in the Management of Prostate Diseases.* AUA Update Series: American Urological Association, 2017.
- Dewar M, Izawa J, Li F, Chanyi R, Reid G, Burton JP. "Microbiome" Future Perspective in Bladder Cancer. Chapter 32. Elsevier publishing, Amsterdam, Netherlands, 2017.