Discovery of novel potential protein diagnostic biomarkers for Prostate Cancer in serum and tears

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A thesis submitted for the degree of
Doctor of Philosophy

St George Clinical School
Faculty of Medicine
The University of New South Wales
June 2011

PLEASE TYPE

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: You

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Abbreviation for degree as given in the University calendar: PhD

School: St George Clinical School Faculty: Medicine

Title: Discovery of novel protein diagnostic biomarkers for prostate cancer in serum and tears

Abstract 350 words maximum: (PLEASE TYPE)

Prostate cancer (CaP) is a heterogeneous multifocal cancer with high prevalence worldwide, particularly in developed countries. The introduction of the prostate specific antigen (PSA) blood test for CaP diagnosis saw a dramatic increase in the reported incidence rate of CaP, but only a slight decrease in mortality, highlighting the importance of developing a more accurate CaP diagnostic test. Discovery of novel biomarkers has been the focus of cancer diagnostic research, with protein biomarkers of particular relevance due to their direct reflection of phenotype changes, resulting from pathophysiological conditions and their presence in the easy accessible body fluids.

The aim of this PhD project was to detect, identify and verify potential novel CaP specific protein biomarkers that could distinguish CaP from benign prostatic hyperplasia (BPH) and healthy control groups; ideally identifying novel molecules that have the potential to improve the screening accuracy of the current CaP diagnostic test. Serum and tears were used as the sources of biomarkers in this study. Various proteomic approaches including gel based, mass spectrometry based and targeted antibody based techniques were used in this study to examine the serum and tear proteomes as well as for protein identification and quantitation.

The key finding of the present study was the identification of five serum proteins (albumin, fetuin A, IGHM, hemopexin and C4BPA) and one peptide (VPSHAVVAR) derived from the tear protein, lactoferrin, as potential biomarkers for CaP. From these proteins, albumin and fetuin A were evaluated in a separate small sample group of subjects, with these results further indicating their potential for differentiating between CaP and control groups. The findings from this study suggest that, using larger sample size to verify the results presented, the development of more accurate and non-invasive clinical tests for the diagnosis of CaP may be possible.

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Abstract

Prostate cancer (CaP) is a heterogeneous multifocal cancer with high prevalence worldwide, particularly in developed countries. The introduction of the prostate specific antigen (PSA) blood test for CaP diagnosis saw a dramatic increase in the reported incidence rate of CaP, but only a slight decrease in mortality, highlighting the importance of developing a more accurate CaP diagnostic test. Discovery of novel biomarkers has been the focus of cancer diagnostic research, with protein biomarkers of particular relevance due to their direct reflection of phenotype changes, resulting from pathophysiological conditions and their presence in the easy accessible body fluids.

The aim of this PhD project was to detect, identify and verify potential novel CaP specific protein biomarkers that could distinguish CaP from benign prostatic hyperplasia (BPH) and healthy control groups; ideally identifying novel molecules that have the potential to improve the screening accuracy of the current CaP diagnostic test. Serum and tears were used as the sources of biomarkers in this study. As a circulatory body fluid, serum can reflect the molecular changes due to the presence of CaP. The tear film is of particular interest in CaP research, as both the prostate gland and the major tear producing gland, the lacrimal gland, are androgen regulated. Various proteomic approaches including gel based, mass spectrometry based and targeted antibody based techniques were used in this study to examine the serum and tear proteomes as well as for protein identification and quantitation.

The key finding of the present study was the identification of five serum proteins (albumin, fetuin A, IGHM, hemopexin and C4BPA) and one peptide (VPSHAVVAR) derived from the tear protein, lactoferrin, as potential biomarkers for CaP. From these proteins, albumin and fetuin A were evaluated in a separate small sample group of subjects, with these results further indicating their potential for differentiating between CaP and control groups. Moreover, this was also the first study which has used MRM to validate presence of a novel tear protein dermcidin, to relatively quantify six tear proteins and to detect a potential CaP biomarker peptide in tears. The findings from this study suggest

that, using larger sample size to verify the results presented, the development of more accurate and non-invasive clinical tests for the diagnosis of CaP may be possible.

Acknowledgments

It is a great thing that I finally come to the stage to officially thank people who have helped me through the whole PhD course.

Firstly, I would like to thank all my supervisors: Dr. Yong Li, Prof Mark Willcox and Dr. Brad Walsh. Thank you for giving me this opportunity to take on this journey, supporting me all the way and spent lots of time reviewing my thesis. Thank you, Yong, for giving me all the support and resources that I need during the PhD. Thank you, Mark, for your interest, good advices and positive attitude towards my work. Brad, you have been helping me all the way, giving valuable advices on my experiment design. I have learnt many from all of you.

To the Minomic crew, I cannot express enough my appreciations. All of you have been helping and supporting me. Belinda, you have been the strongest supporter of my work. You have spent exceptional amount of your time helping and supporting me. You are knowledgeable on proteomic skills such as DIGE analysis, antibody assays including Western blot and ELISA, statistical analysis etc. You shared your tips on writing thesis to me, helped me modifying the thesis, and de-stressed me with your cheerful attitude. I have learnt so much from you, and I am truly grateful that you guided me and we become friends. Anna, you helped me get into the proteomic world. Your advices were always helpful and right on the spot. You are always optimistic about my work, encourage me trying new technologies, keeping me on track and helping me make the right choice. Agnes, Brigitte, Carlos, Shamila, Shona, Stefanie, Tammy, and Terry, chatting with you were delightful and it took away my stress. I can feel your care, love and friendship, and these were the source of strength for me to finish this journey.

To the kind and great scientists from Bioanalytical Mass Spectrometry Facility, Brien Holden Vision Institute, Royal North Shore Hospital, School of Optometry and Vision Science, South Eastern Area Laboratory Service and St George Clinical school, I thank you all for your kindness and full support. Dr. Valerie Wasinger, you have taught me how to use various mass spectrometry

instruments; gave me your suggestions on my experiments and were always there when I needed help. I thank you very much. To A/Prof Mark Raftery, Dr. Ling Zhong, Dr. Li Ping, Dr. Michele Madigan, Dr. Thomas John, Dr. Varghese Thomas, Keith Westbury, Helen Hao and Hongtu Chao, thank you very much for giving up your own time to help me. I really appreciate it.

Finally to my dear family and friends, thank you for being patient with me, caring me and understanding my situation. My dear mum and dad, without your support, I would not finish the study. Even you were far away, I can always feel your love from the telephone line and internet. You both were the role model for me as you both were hard working and strong willed. To my friends, Joanne, Karen, Kim, Ying and Rachael, thank you so much for accompanying me all the time, taking me out for dinners, helping me finalising my thesis and cheering me up.

To my dearest husband, Kelvin, thank you so much for your understanding, consistent support and encouragement. Your love and care have accompanied me all the way, and I can always feel the warmth from the delicious meals prepared by you. Because of your understandings and support, I was able to concentrate on my study for all these years. I thank you and love you very much.

This PhD was not just my sole work. This was a group work and it showed me how important and how much people can achieve with the support, love and care from all around. It has become one of my most valuable memories. Thank you all very much.

Abbreviations

%fPSA	Percent free prostate specific antigen
1DGE	One dimensional gel electrophoresis
2D-DIGE	Two dimensional differential in gel electrophoresis
2DGE	Two dimensional gel electrophoresis
A1AT	Alpha-1-antitrypsin
ACN	Acetonitrile
AMACR	Autoantibodies against α-Methyl-Acyl-Coenzym A-Racemase
AMBIC	Ammonium bicarbonate
APS	Ammonium persulfate
AUC	Area under the receiver operating characteristic curve
BCP	Bromocresol purple
BMSF	Bioanalytical Mass Spectrometry Facility
BPE	Benign prostate enlargement
BPH	Benign prostate hyperplasia
bPSA	Benign prostate specific antigen
C15ORF21	Chromosome 15 open reading frame 21
C4BP	Complement 4b-binding protein
C4BPA	Complement 4b-binding protein alpha chain
CaP	Prostate cancer
CE	Capillary electrophoresis
C3	Complement component 3
cPSA	Complexed prostate specific antigen
CRP	C-reactive protein
CV	Coefficient of variation
DCD	Dermcidin
DMBT1	Depleted in malignant brain tumour 1
DMF	N,N-Dimethylformamide
DRE	Digital rectal examination
EACA	ε-amino-n-caproic acid
EAM	Energy absorbing matrix
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EPCA	Early prostate cancer antigen
ERG	v-ETS erythroblastosis virus E26 oncogene like
ESI	Electrospray ionisation
ETS	E26 transforming sequence
ETV1	ETS variant gene 1
FA	Formic acid
FDA	U.S. Food and Drug Administration
fPSA	Free PSA
FT	Fourier Transform
HERV-K	Human endogenous retrovirus
HFBA	Heptafluorobutyric acid
HIP1	Huntington-interacting protein 1
hK2 or KLK2	Human kallikrein 2
HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
HPLC	High performance liquid chromatography
HPT	Haptoglobin
HUPO	Human Proteome Organisation
ICATs	Isotope-coded affinity tags
IEF	Isoelectric point
Ig	Immunoglobulin
IGHM	Ig mu chain C region
IL	Interleukin
IMAC	Immobilised metal affinity capture
IPG	Immobilised pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
LCN1	Lipocalin 1
Lf	Lactoferrin
LIT	Linear ion trap
LMW	Low molecular weight
LNM	Lymph node metastatic
LTQ	Linear ion trap

MALDI	Matrix assisted laser desorption ionisation
MF	MiPrep F
mGPS	modified Glasgow prognostic score
MIDAS	MRM-initiated detection and sequencing
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MW	Molecular weight
NSW	New South Wales
NUCB2	Nucleobindin 2
PCA3	Prostate cancer gene 3
pI	Isoelectric point
PIN	Prostatic intraepithelial neoplasia
PIP	Prolactin induced protein
PPP	Plasma Proteome Project
PSA	Prostate specific antigen
PSAD	PSA density
PSA-DT	PSA doubling time
PSA-V	PSA velocity
PSMA	Prostate-specific membrane antigen
PTM	Post-translational protein modification
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
RILT	Radiation-induced lung toxicity
ROC	Receiver operation characteristic
RP	Radical prostatectomy
RT	Room Temperature
SCGB2A1	Secretoglobin, family 2A, member 1
SEALS	South Eastern Area Laboratory Services
SELDI-TOF	Surface-enhanced laser desorption/ionisation time-of-flight
SEM	Standard error of mean
SILAC	Stable isotope labelling by amino acids in cell culture

SLC45A3	Solute carrier family 45, member 3
SPSS	Statistical Package for the Social Sciences
TBP	Tributylphosphine
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TF	Tear film
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TIC	Total ion current
TMPRSS2	Transmembrane protease, serine 2 gene
TNFR1	Type 1 tumour necrosis factor receptor
tPSA	Total PSA
TRUS	Transrectal ultrasonography
Vhrs	Volt Hours

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

1.1 The prostate

The prostate is a small gland found only in males and is composed of both glandular and muscular tissue. In humans, it is located below the bladder, above the base of the penis and in front of the rectum (**Figure 1.1**). Two types of ducts pass through the prostate; the prostatic urethra passes through the prostate from the bladder while the ejaculatory ducts run from the seminal vesicles through the posterior of the prostate to open onto the prostatic urethra.

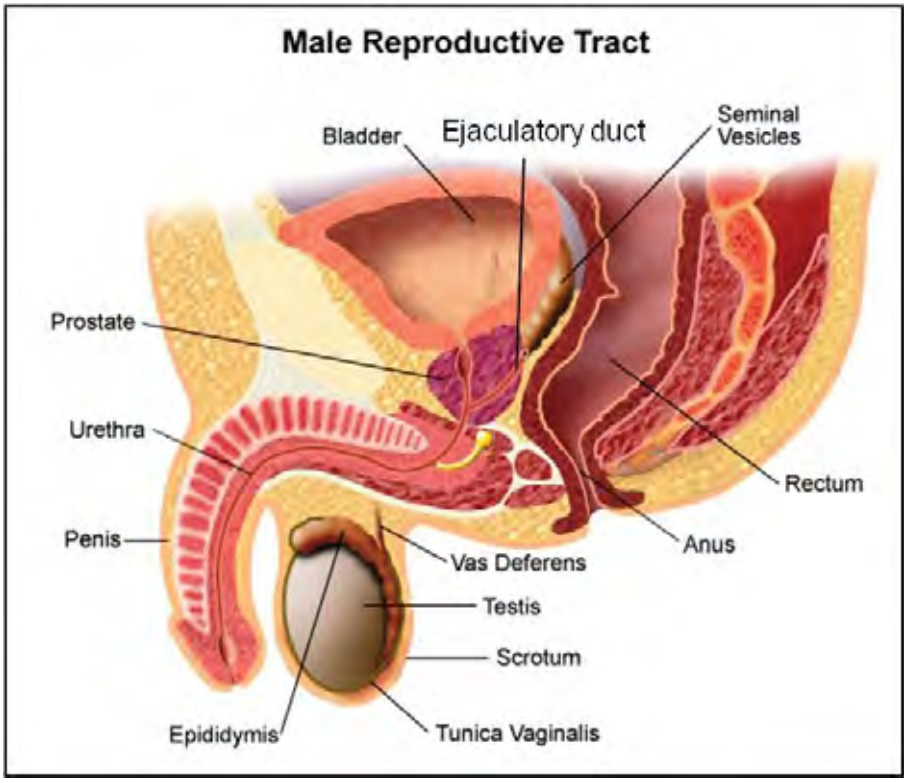


Figure 1.1: The anatomy of prostate gland. Modified from source: Overview of urinary/urogenital system disorders, Rush University Medical Center, http://www.rush.edu/rumc/page-1098987354057.html

The prostate is comprised of 30 – 50 small glandular units (acini) which empty into the prostatic urethra and the entire gland is encapsulated in a fibrous prostatic capsule. The primary function of the prostate is to add important nutrients to the seminal fluid that nourish, protect and transport sperm (Rashid, 2010). During ejaculation, the seminal vesicles and prostate gland contract to expel their contents into the prostatic urethra and towards the tip of the penis.

1.2 Prostate diseases

Diseases associated with the prostate gland can be divided into cancerous and non-cancerous conditions. Both prostate cancer (CaP) and non-cancerous prostate diseases are relatively common, however, while most non-cancerous conditions can occur in men of any age, the incidence of CaP is greatly increased in older men.

1.2.1 CaP

CaP is the fifth most commonly diagnosed cancer worldwide and approximately 70% of the disease occurred in males from developed regions in 2008 (Ferlay *et al.*, 2010). It is estimated that 217,730 new cases will be diagnosed in the USA in 2010 resulting in 32,050 deaths (Jemal *et al.*, 2010). In 2008, CaP was the most prevalent cancer in Australia/New Zealand when considering men alone or across both genders (Ferlay *et al.*, 2010). In New South Wales, Australia, the incidence rate of CaP most recently reported in 2007 was the highest among all cancers across both genders (Tracey *et al.*, 2009). Significantly, the 2007 figures regarding the prevalence of CaP and related mortality rates had increased from 2004 figures. Diagnosed cases of CaP increased from 5,477 in 2004 (Tracey *et al.*, 2006) to 6,665 in 2007 (Tracey *et al.*, 2009), while CaP related deaths increased from 905 in 2004 (Tracey *et al.*, 2006) to 977 in 2007 (Tracey *et al.*, 2009). Examining the overall CaP incidence and mortality trends in Australia, from 1982 to 2004, the incidence doubled with a slight decrease in mortality rate (**Figure 1.2**) (AIHW, 2007). These results suggested the importance of increased research associated with development of accurate CaP diagnostic, prognostic and treatment options.

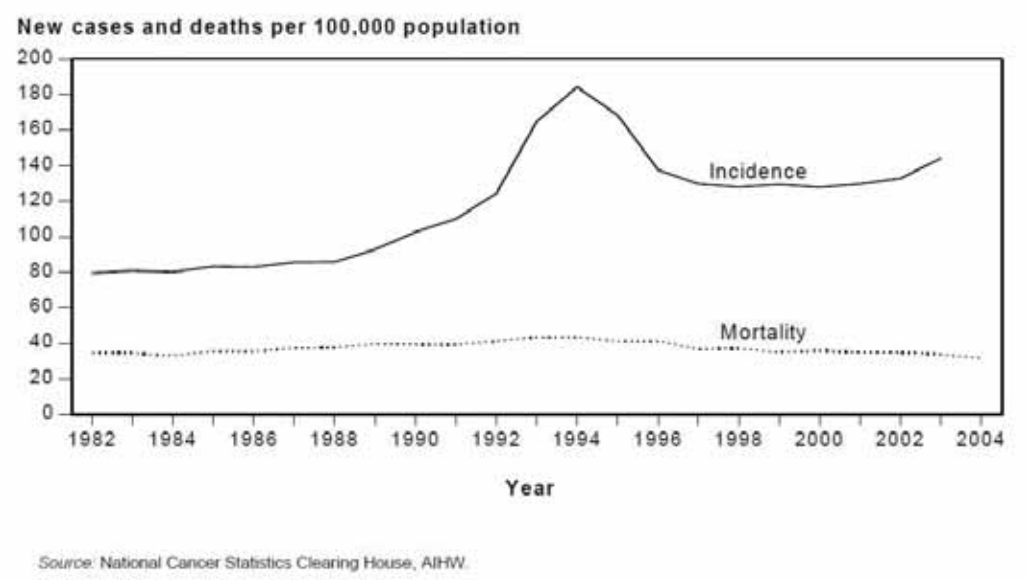


Figure 1.2: The trend of CaP incidence and mortality in Australia from 1982 to 2004. The figure is taken from the report, "Cancer In Australia: An Overview", 2006 published by Australian Institute of Health and Welfare (AIHW, 2007).

Whilst a single cause of CaP has not been elucidated, several risk factors have been identified and can be classified as either endogenous or exogenous factors (**Table 1.1**).

Table 1.1: Summary of the possible risks including the endogenous and exogenous risk factors for CaP.

Major endogenous risk factors	Heredity	Various population-based case-control studies have reported significant association between CaP and family history (Fincham *et al.*, 1990; Lesko *et al.*, 1996; Bratt *et al.*, 1999; Staples *et al.*, 2003). A study in Australia between 1994-1998 showed an increased risk of developing CaP, when a first degree family member had been affected (three times increase) or with an increased number of affected family members (Staples *et al.*, 2003).
	Hormones	Although androgen affects the prostate gland development and androgen deprivation is used as a treatment for CaP (Sharifi, 2009), studies have shown only a weak association between androgens and the risk of developing CaP (Vatten *et al.*, 1997; Travis *et al.*, 2007; Gill *et al.*, 2010).
	Age	Age is a significant risk factor for developing CaP. Around 85% of CaP is detected in men > 65 years old and less than 0.1% of CaP is detected in men < 50 years old (Grönberg, 2003). In NSW, the current figures suggest that by 75 years of age 1 in 7 men will suffer CaP and by 85 years of age this number will increase to 1 in 4 (Tracey *et al.*, 2009).
	Race	The mortality rate of CaP was reported to change considerably depending on geography, with high mortality rates in America and low rates in China and Japan (Ross, 1996). In the US, African Americans have the highest CaP incidence, follow by Caucasians, and Asian American and Pacific Islanders had the lowest incidence per 100,000 populations (Jemal *et al.*, 2010).
Major exogenous risk factors	Environmental agents	Cadmium contamination and pesticides have been studied in various epidemiological studies. Cadmium has shown a possible association with CaP risks (Elghany *et al.*, 1990;Nawrot *et al.*, 2010). Pesticides and CaP risk have also been studied, however the epidemiological studies on the linkage showed inconsistent results (Mink *et al.*, 2008).
	Dietary factors	Some epidemiologic studies suggested diet was associated with CaP (Whittemore *et al.*, 1995;Pienta *et al.*, 1996). The most recent study on dietary factors (lycopene, fat, vitamins, Calcium, Zinc, Selenium and fish oil) using 9,559 participants did not find an association between nutrition/supplements and overall CaP risks, but found that certain nutritional intakes (polyunsaturated fat, alcohol, calcium and zinc) were associated with the grade of CaP (Kristal *et al.*, 2010).

One of the major difficulties in the treatment and management of CaP is that diagnosis at an early stage is complicated due to very few, if any, early symptoms (Rashid, 2010). As CaP progresses some symptoms may emerge including common urinary problems, blood in the urine, pain in the pelvis or lower back, pain with ejaculation, urinary tract infections, incontinence and impotence. These symptoms, however, are not CaP specific and can be common to other non-cancerous prostate and urinary tract conditions. In particular, this includes benign prostate hyperplasia (BPH) which can coexist with CaP (Rashid, 2010).

1.2.2 Non cancerous prostate diseases

The three most commonly occurring non-cancerous prostate diseases are prostatitis, prostatodynia and benign prostate hyperplasia/enlargement (BPH/BPE) (Rashid, 2010). Prostatitis refers to prostatic inflammation and the subsequent symptoms, and can be acute or chronic. It is not in itself a life threatening condition, however it can lead to development of CaP (Rashid, 2010) and an associated increase in prostate specific antigen (PSA), which is a CaP biomarker.

Prostatodynia is a chronic prostate problem which causes discomfort of the entire pelvic area. Symptoms of this condition can be relieved by various treatments including antibiotics, non-steroidal anti-inflammatory agents and muscle relaxants. However, the condition itself is still poorly understood and recovery can occur if even untreated (Rashid, 2010).

BPH or BPE refers to the benign condition of prostate enlargement. It shares many similarities with CaP including an increase incidence rate with age, and common symptoms such as prostate enlargement and difficulty urinating. Presence of BPH is not itself a risk factor for development of CaP and in fact both disorders can be present in the one individual, further increasing the difficulty of accurate diagnosis of CaP (Rashid, 2010).

1.3 Challenges of detecting CaP and CaP biomarkers

At present, a major challenge in CaP diagnosis and treatment is the discovery of a diagnostic tool which is accurate at an early stage of the cancer. The Tumour-Node-Metastatsis (TNM) Staging system is a staging system used across many cancers and is used to record the development of CaP. The three scores T, N and M then make up an overall grading to reflect the stage of cancer related to the primary tumour (T), the spread to lymph nodes (N) and the presence or absence of metastases (M). The T staging can be determined by the digital rectal examination (DRE), and when the N and M scores are both 0, it reflects an early stage of CaP development, in which the cancer cells are still localised in the prostate gland (Heidenreich *et al.*, 2008). An N score which is other than 0 suggests lymph node involvement, while an M score of 1 is indicative of the most advanced stage of CaP with metastases present (Heidenrich *et al.*, 2008). It is important to identify CaP when it is classified on the TNM system with only a T score (N and M 0).

Non-invasive diagnostic tests are most suitable because they are usually easier to perform and have minimal side-effects for patients. These two advantages can encourage testing to be undertaken at a greater rate. Currently available diagnostic techniques for CaP include digital rectal examination (DRE), transrectal ultrasonography (TRUS), patho-histology of prostate biopsies and assaying serum PSA. DRE is widely employed but has a very limited ability to diagnose CaP. A recent European randomized study, which analysed 2218 men with a PSA $\geq$ 3.0 ng/mL and a negative biopsy result, showed 21% of them had a suspicious DRE (Gösselaar *et al.*, 2009). This study rescreened these patients within an eight year interval, and found that the initial suspicious DRE results did not have any significant prediction value in CaP detection (Gösselaar *et al.*, 2009). TRUS has similar accuracy to DRE in detecting CaP but at a higher cost than DRE (Palken *et al.*, 1991; Pepe *et al.*, 2003). Therefore TRUS is not widely used for CaP screening but is useful in guiding biopsy and evaluating certain clinical parameters (such as PSA density) required for diagnosing CaP (Pepe *et al.*, 2003).

Pathohistology of prostate tissue can definitively identify CaP in most cases and is also the most commonly used prognostic indicator for CaP. Diagnosis results in a grading called the Gleason score, which is based on the architecture of cancer tissue observed under a microscope (Epstein *et al.*, 2005). Five grade patterns (1 to 5) are assigned to the prostate cancer tissue structure as it progresses, and the Gleason score is the sum of the two most common observed patterns. The lower Gleason score (comprised of grade patterns lower than 4) indicates that the prostate cancer cells are well differentiated, *ie*. the CaP cells still have similar structures to normal prostate cells and thus the cancer itself is less advanced. Higher Gleason scores (comprised of grade patterns greater than 4) suggest poor differentiation of the prostate cancer cells, and therefore bear only limited resemblance to normal prostate cells (Epstein et al., 2005). Albertsen *et al.*, (1998) showed that Gleason scores from 7 to 10 have a higher risk of death from CaP than low Gleason score (< 4) when patients aged 74 were treated conservatively (Albertsen *et al.*, 1998). Between the ages of 55 and 74, when treated conservatively, patients with Gleason scores 5-6 are most likely to die from non-CaP conditions, however patients with scores greater than 6 are likely to die from CaP despite treatment (Albertsen *et al.*, 2005). However, there are limitations to this method of screening. A biopsy or similar invasive operation must be performed in order to obtain the cancer tissues for testing. Additionally, the Gleason grading scale is semi-quantitative as it is difficult to search every cell of every tissue slice. It is also subjective, resulting in a lack of concordance between the threshold of scoring by different pathologists (Bostwick *et al.*, 2000). For these reasons Gleason scores themselves have limited quantitative value.

The current biomarker-based diagnostic for CaP is measurement of PSA concentration in blood. However, the usefulness of PSA for diagnosis remains in question and its use for prognosis is not possible as it does not differentiate between the clinically significant stages of CaP (Draisma *et al.*, 2003). Prognostic biomarkers that can identify and predict clinically significant CaP in patients are also important in management of the disease. Ideally these prognostic/predictive

biomarkers would be non-invasive to obtain, and would guide clinicians for an appropriate treatment and provide maximum benefit whilst minimising the related risks.

In this chapter, biomarkers are grouped into three categories:

1) the PSA and PSA related biomarkers
2) the non-PSA based biomarkers found prior to the proteomic era
3) the biomarkers found using proteomic techniques.

Two important parameters to define the accuracy of the diagnostic tests are specificity and sensitivity, of which sensitivity is to measure the probability of the true positive of the diagnostic test and specificity is to measure the probability of the true negative (Weinstein *et al.*, 2005).

1.3.1 Current PSA test and related biomarkers

PSA is the only conventional biomarker accepted by the U.S. Food and Drug Administration (FDA) for CaP diagnosis. While it is not an ideal biomarker, its use as a screening biomarker for CaP has resulted in an increase in CaP detection and a decrease in mortality rates in many countries.

PSA is a 33 kDa glycoprotein that is a neutral serine protease from the human kallikerin family of proteins. It has several isoforms ranging in isoelectric point (pI) from 6.8 to 7.2 (Lukes *et al.*, 2001). Expression of PSA depends on the secretion of androgen and it is mostly found in prostatic tissues, although low concentrations of the protein can be found in other tissues (Papotti *et al.*, 1989; Clements, 1994).

In the prostate, PSA is secreted by the epithelium and the epithelial lining of the acini and prostatic ducts (Papsidero *et al.*, 1981; Armbruster, 1993) and it plays a role in liquefaction of the seminal fluids (Lee *et al.*, 1989). In order to enter the blood circulation the PSA molecules have to move through the prostatic basal

membrane, stroma, capillary basal membrane and capillary endothelial cells (Lukes *et al.*, 2001).

Once in the blood PSA can be measured as free PSA (fPSA) or bound to α1-antichymotrypsin (Wu *et al.*, 1995), α2-macroglobulin (Wu *et al.*, 1995) or α1-antitrypsin (Stenman *et al.*, 1991). The PSA test measures the total concentration of PSA in the blood. The generally accepted cut-off level of PSA for CaP detection is > 4.0 ng/mL (Antenor *et al.*, 2004), although age-specific levels have also been suggested as the concentration in blood naturally increases with age (Moul *et al.*, 2007). Levels above the cut-off do not provide a definitive diagnosis of CaP but provide a basis to suggest that a biopsy be carried out to determine presence/absence of CaP. The specificity of PSA is only 20%-30% between the range of 2.6 ng/mL and 10.0 ng/mL (Smith *et al.*, 1997); this means that 70-80% of men undergo unnecessary biopsies. Additionally, a USA based study has shown that 33.7% of men actually had CaP with PSA levels ≤ 2ng/mL, and 50.8% of men had CaP with levels ranging from 2.1- 4 ng/mL (Thompson *et al.*, 2004). Among those men who would remain undiagnosed using currently accepted PSA levels, 37.5% of them had high grade cancer. This finding is similar to that of a European based study conducted from 1997 to 2005, in which the clinical data of 855 men whose PSA was less than 4.0 ng/mL and who had normal DRE results were evaluated and analysed by log regression and found that 20.5% of them had high grade cancer (Ahyai *et al.*, 2008).

The evidence for a link between serum PSA levels and CaP has weakened over the past 20 years. A study by Stamey (2004) showed that serum PSA level was only well correlated with BPH but not CaP (Stamey, 2004). Multiple other factors have been shown to influence serum PSA concentration regardless of the presence of CaP, including age and body mass index as well as other diseases such as acute urinary retention and prostatitis (Tchetgen and Oesterling, 1997). The Mayo clinic has reported that the level of serum PSA is not proportional to positive biopsy rates but is proportional to the age of patients (Stewart *et al.*, 2001). Stamey (2004) reported

that in the USA, 8% of men in their twenties have CaP, and the incidence of the disease rises steadily and linearly within each decade of life until 80% of men in their seventies are afflicted (Stamey, 2004). Therefore it is probable that one universal cut off point to indicate normal levels may not be sufficient when considering factors such as age, which complicate the test. Moul *et al.* (2007) proposed that different PSA cut off points should be employed for different age groups (Moul *et al.*, 2007). An investigation of 11,861 men who all had had their PSA levels measured within two years reported that PSA levels varied with age and the recommendation was to use the threshold value of 2 ng/ml for men aged 50-59 (Moul *et al.*, 2007). PSA has also been found to be linked to body mass index in Korea. After an examination of 8640 Korean men without CaP aged between 40-79, PSA levels were found to be inversely correlated to body mass index among men aged 40 to 59 (Kim *et al.*, 2007). It was found that obesity can decrease the levels of PSA by approximately 0.13-0.18 ng/mL.

As a result of the specificity of the PSA test being challenged, addition of two PSA related measures (PSA isoforms and PSA paramaters) have been suggested (Ozen and Sozen, 2006). PSA isoforms consist of fPSA, proPSA, complexed PSA (cPSA) and benign PSA (bPSA) (Ozen and Sozen, 2006). PSA parameters involve analysis of percent free PSA (%fPSA) (Catalona *et al.*, 2000b), PSA density (PSAD) (Catalona *et al.*, 2000b), age-specific PSA ranges (Catalona *et al.*, 2000b), PSA velocity (PSA-V) (Sengupta *et al.*, 2005) and PSA doubling time (PSA-DT) (Sengupta *et al.*, 2005).

fPSA, which refers to the PSA not bound to plasma proteins, is the most studied PSA isoform. The percentage of fPSA over total PSA (tPSA; the sum of fPSA and complexed PSA) showed better accuracy than the PSA test alone in men with tPSA between 4 and 10 ng/mL, because the area under the receiver operating characteristic curve (AUC) of the percentage of fPSA test was 0.72, higher than the tPSA test which was 0.53 (Catalona *et al.*, 1998). This finding was supported by another study conducted in 2000 which focused on a comparison of the fPSA,

PSAD and age in a total of 773 samples (379 with CaP, 394 with BPH) from patients recruited from seven medical centers, aged between 50 and 75 with PSA from 4-10 ng/mL (Catalona *et al.*, 2000a). This study demonstrated that fPSA (sensitivity 95%, specificity 27%) has higher specificity compared to the normal PSA test (sensitivity 95%, specificity 15%) when the same sensitivity cut-off was chosen (Catalona *et al.*, 2000a).

Varied results have come from analysis of proPSA with studies showing that proPSA improved test specificity compared with tPSA and %fPSA in the PSA range of 4 to 10 ng/mL (Catalona *et al.*, 2003; Sokoll *et al.*, 2003); whereas a European multi-institutional trial including 2055 men showed no significant improvement comparing (-5) and (-7) proPSA (the number prefixes refer to the length of the pro-peptide sequence) over tPSA and %fPSA in the same range (Lein *et al.*, 2005). A study of men with PSA levels between 4-10 ng/mL has also shown that a combination of proPSA, fPSA and tPSA had the highest specificity compared to any of the individual tests (Khan *et al.*, 2003). However whether it is better than the %fPSA test has not yet been determined. More studies are needed to determine the specificity and sensitivity of proPSA for CaP detection.

Two multi-center studies have shown that cPSA has better specificity for detection of CaP than the current PSA test, but not of f/t PSA which is the ratio of fPSA to tPSA (Djavan *et al.*, 2002) and that the density of cPSA had the highest specificity when compared to tPSA and f/t PSA (Sozen *et al.*, 2005). Other PSA parameters such as PSA-V and PSA-DT have also been assessed in terms of sensitivity and specificity compared with the current PSA test. High PSA-V is associated with a higher risk of CaP recurrences (Patel *et al.*, 2005) and shorter recurrence intervals (D'Amico *et al.*, 2004), therefore, it could be used as a potential prognostic biomarker for CaP recurrence after radical prostatectomy (RP). D'Amico *et al.* (2004) investigated the association between preoperative PSA-V and the risk of death from CaP in 1054 patients. Those with an annual preoperative PSA-V > 2.0 ng/mL/yr seven years before RP were shown to have a higher risk of death from

CaP (D'Amico *et al.*, 2004). The linkage between PSA-V level before diagnosis and when a cure is still possible was examined by Cater *et al.* (2006) who proposed a cutoff level of PSA-V of 0.35ng/mL/yr to screen potentially lethal CaP in men with low PSA levels (Carter *et al.*, 2006). These findings suggested that the level of PSA-V could be used to guide the management of CaP. The combination of preoperative PSA-V and PSA-DT has also been shown to have significant predictive value in terms of the progression of CaP (Sengupta *et al.*, 2005), although this finding needs to be verified by others.

1.3.2 Non PSA-based biomarkers for CaP

At least 91 potential biomarkers have been suggested to be of use for diagnosis or prognosis of CaP (Tricoli *et al.*, 2004). Of these 91 potential biomarkers few have shown improved accuracy compared to the PSA test when the sample size and presence within human body fluids is taken into account. Thus none are currently used as clinical assays for CaP diagnostic/prognostic purposes. The following sections focus on the potential biomarkers that are well documented and have shown improved specificity and sensitivity compared to the PSA test within the sample size tested.

1.3.2.1 Human kallikrein 2 in CaP diagnosis

Human kallikrein 2 (hK2 or KLK2), like PSA, belongs to the serine protease family and has 80% amino acid sequence homology with PSA. hK2 was initially found to be over expressed in CaP epithelial cells by immunohistochemical staining, which also suggested that it could be a potential biomarker for CaP (Darson *et al.*, 1997). Several groups have now successfully detected hK2 in serum, where, like PSA, it is present in free and bound forms (Piironen *et al.*, 1996; Grauer *et al.*, 1998). However, Partin *et al,.* (1999) compared the sensitivity and specificity of %fPSA and the total hK2/fPSA ratio using 973 serum samples with PSA levels between 2-10 ng/mL (Partin *et al.*, 1999). The results showed that %fPSA alone had better diagnostic accuracy (AUC=0.67) than the total hK2/fPSA ratio (AUC= 0.61) (Partin

et al., 1999). A recent study which used 2,914 samples found that incorporating fPSA, intact PSA (an un-reactive single chain isoform of fPSA), and hK 2 to the standard PSA test can improve the detection accuracy (AUC was increased from 0.64 to 0.76), and resulted in a reduction of 513 unnecessary biopsies (Vickers *et al.*, 2010).

The potential prognostic value of hK2 has also been assessed. Both Haese *et al.* (2001) and Steuber *et al.*, (2006) found that the concentration of hK2 in blood was significantly associated with the risk of biochemical recurrence after RP when serum tPSA was ≤ 10 ng/mL (Haese *et al.*, 2001; Steuber *et al.*, 2006). When 294 men with tPSA ≤ 10 ng/mL were examined, hK2 showed higher predictive accuracy (0.815) for biochemical recurrence when compared to other individual tests, the base model (0.771) and fPSA (0.782) (Steuber *et al.*, 2007). The combination of hK2 and fPSA test had the highest predictive accuracy (0.835) among all (Steuber *et al.*, 2007). These findings suggest that hK2 might have value as an independent prognostic biomarker for biochemical recurrence with tPSA ≤ 10 ng/mL, however further validation is needed to confirm its prognostic ability.

1.3.2.2 Prostate cancer gene 3 in urine

Prostate cancer gene 3 (PCA3), also known as PCA3DD3 or DD3PCA3, is a new prostate-specific gene that is highly overexpressed in CaP tissue. It was first identified using differential display analysis comparing mRNA expression in normal and tumor-bearing prostate tissues (Bussemakers *et al.*, 1999). PCA3 is a prostate-specific noncoding mRNA that is highly overexpressed in more than 95% of primary CaP specimens and CaP metastases (Bussemakers *et al.*, 1999;de Kok *et al.*, 2002). PCA3 encodes a prostate-specific mRNA that has shown promise as a CaP diagnostic tool. Measurement of PCA3 mRNA normalised to PSA mRNA in urine has been proposed as a marker for CaP.

The possible usefulness of the urinary PCA3 assay as a CaP marker was suggested by de Kok *et al.* (2002). Hessels *et al.* (2003) found that the median up-regulation of

PCA3 in CaP tissue compared with normal prostate tissue was 66-fold and the sensitivity for the detection of CaP by the time-resolved fluorescence-based PCA3 test in urine was 67%, and the specificity was 83% (Hessels *et al.*, 2003). On the basis of these results, Groskopf *et al.*, (2006) developed a commercially available highly sensitive transcription-mediated amplification method (PCA3, Gen-Probe Incorporated) for urinary assay for general clinical use (Groskopf *et al.*, 2006). The method measures both PCA3 and PSA mRNA in first-pass urines collected following a DRE. The function of the DRE is to enhance the release of prostate cells through the prostate duct system into the urinary tract and thus into the urine. Recent studies have shown that the PCA3 urine assay has promise in improving the diagnostic accuracy of CaP detection, especially in the PSA gray zone (between 2 - 4 ng/mL) (Groskopf *et al.*, 2006; Marks *et al.*, 2007; van Gils *et al.*, 2007).

Van Gils *et al.*, (2007) showed that the time-resolved fluorescence-based PCA3 urine test in a total of 534 men (the largest numbers until now) from five different institutions, when used as a reflex test, can improve the specificity in CaP diagnosis and could prevent many unnecessary prostate biopsies (van Gils *et al.*, 2007). Clinical studies of urinary PCA3 levels have detected early CaP more accurately than serum PSA levels ($p < 0.01$) (Marks *et al.*, 2007). Nakanishi *et al.*, (2008) demonstrated that the PCA3 score is significantly associated with tumor volume and Gleason score in prostatectomy specimens, suggesting that the urinary PCA3 score may be a novel molecular marker not only for CaP detection, but also for the classification of men diagnosed with CaP (Nakanishi *et al.*, 2008). Deras *et al.*, (2008) also reported the quantitative urinary PCA3 score was directly related to the probability of positive biopsy and could improve diagnostic accuracy for CaP detection (Deras *et al.*, 2008). Although the results from some institutes are promising, the diagnostic value needs to be further validated in a multicenter setting and to be followed up to show if indeed the PCA3 urine test is able to predict the presence of CaP.

1.3.2.3 Early prostate cancer antigen (EPCA) and EPCA-2

Both EPCA and EPCA-2 are nuclear matrix proteins. The CaP diagnostic potential of EPCA was initially proposed after immunohistochemical staining of CaP tissue (Dhir *et al.*, 2004). This initial analysis indicated a sensitivity of 84% and specificity of 85% for CaP detection in prostate tissue (Dhir *et al.*, 2004). Another study found that EPCA expression was detected in 94% of localised CaP tissues (50 patient samples used) and 0% in bladder cancer patients (10 patient samples used) and suggested that EPCA could be used for improving the current diagnosis of early stage of CaP (Uetsuki *et al.*, 2005). However the specificity of the test was not evaluated. Paul *et al.*, (2005) later developed an enzyme-linked immunosorbent assay (ELISA) to detect the EPCA level in blood and showed 92% sensitivity and 94% specificity in CaP detection, however only 46 plasma samples were examined (Paul *et al.*, 2005). More samples are required to validate its diagnostic value for CaP. A five year follow up study of 112 men with isolated high-grade prostatic intraepithelial neoplasia (PIN, an intermediate stage from benign epithelium to carcinoma) found increased EPCA serum levels in patients who subsequently developed CaP, but not in the patients that did not develop CaP (Zhao and Zeng, 2010). This recent study suggested that EPCA could potentially predict CaP development in PIN patients.

EPCA-2 is also a nuclear matrix protein and its diagnostic value was reported from the same research group as EPCA but three years later (Leman *et al.*, 2007). Three epitopes, EPCA-2.19, 2.22 and 2.4 can be detected by specific antibodies (Leman *et al.*, 2007). Serum EPCA-2.22 levels were measured from 385 men using an indirect ELISA test which showed improved specificity (92%) compared to PSA (65%) (Leman *et al.*, 2007). An indirect ELISA test of serum EPCA-2.19 also showed high specificity (94%) in differentiating between BPH and CaP groups (Leman *et al.*, 2009). However Diamandis (2010) challenged the detection limit of the ELISA test, claiming it is unlikely that the indirect ELISA test can detect EPCA-2 at ng/mL levels (Diamandis, 2010). Independent research is needed to verify these tests.

1.3.2.4 Prostate cancer-specific autoantibodies

Cancer specific autoantibodies are generated when the immune system responds to over-expressed tumor associated antigens. The most prevalent circulating prostate cancer-specific antoantibodies that have been detected include autoantibodies against α-methyl-acyl-coenzymA-racemase (AMACR) (Sreekumar *et al.*, 2004) and Huntington-interacting protein 1 (HIP1) (Bradley *et al.*, 2005). Within the PSA range from 4-10 ng/ml, detection of immunoreactivity against AMACR in serum has higher sensitivity and specificity (77.8% and 80.6%, respectively) than those of PSA (45.6% and 50%, respectively) (Sreekumar *et al.*, 2004), while a test based on detection of both types of autoantibodies has been shown to increase specificity to 97% (Bradley *et al.*, 2005). AMACR autoantibodies have also been tested in urine collected from 276 patients before needle biopsy or RP (Laxman *et al.*, 2008). This resulted in no significant differences between BPH and CaP groups which suggested this biomarker might only have diagnostic value in serum (Laxman *et al.*, 2008). A serum anti-HIP1 test showed a specificity of 73% and a sensitivity of 46% using 81 CaP and 211 control samples (Bradley *et al.*, 2005). When combined with the AMACR test, the specificity increased to 97%, however the sensitivity was still low at 55% (Bradley *et al.*, 2005). The use of immunologically based molecules for diagnosis and prognosis of CaP has not been investigated to a large extent, but there is the potential for such molecules to be of use in detecting and monitoring cancer after the immune response has been induced.

1.3.2.5 Prostate-specific membrane antigen (PSMA)

PSMA was originally found to be located only on the membrane of prostate epithelium in 1987 (Horoszewicz *et al.*, 1987) and its expression was later seen to be increased in CaP tissue (Silver *et al.*, 1997). As a result, PSMA is proposed to be a potential biomarker for CaP and has been extensively studied. Its role as a serum prognostic CaP biomarker, however, is controversial. Studies using Western blots showed that PSMA could distinguish between late stage CaP and early stage disease (Rochon *et al.*, 1994; Murphy *et al.*, 1998), whereas other studies showed that

PSMA was not more effective than PSA (Douglas *et al.*, 1997; Beckett *et al.*, 1999). A study using a newly developed technology, the ProteinChip technique, demonstrated higher PSMA serum levels in CaP patients compared to BPH and normal groups (Xiao *et al.*, 2001). This pilot study suggested a new approach for examining serum PSMA.

However, a number of other factors such as secretion from normal tissues or other tumors and age are likely to contribute to the serum levels of PSMA and so might affect its relation to CaP (Kinoshita *et al.*, 2006). Overall, PSMA is regarded as a promising biomarker for CaP and further investigations and perhaps more sensitive detection methods are needed to fully evaluate its usefulness.

1.3.2.6 Gene fusions

Gene fusions resulting from chromosomal translocations and are commonly found in haematological malignancies but are rare in solid tumours. Recurrent gene fusions in CaP were first discovered by Tomlins *et al.*, (2005) using a self-developed algorithm named Cancer Outlier Profile Analysis to analyse DNA microarrays (Tomlins *et al.*, 2005). They discovered recurrent gene fusions involving the fusion of the 5' untranslated region of the prostate specific, androgen-regulated transmembrane protease, serine 2 gene (TMPRSS2) to two transcription factors of the erythroblastosis virus E26 transforming sequence (ETS) family, the ETS variant gene 1 (ETV1) and v-ETS erythroblostosis virus E26 oncogene homolog (ERG) (Tomlins *et al.*, 2005). After their discovery, the TMPRSS2:ETV1 and TMPRSS2:ERG gene fusions were validated by measuring the level of RNA expression in several prostate cancer cell lines and tissue specimen using PCR-based assays (Wang *et al.*, 2006; Lapointe *et al.*, 2007; Winnes *et al.*, 2007) and fluorescent in situ hybridization based assays (Lapointe *et al.*, 2007; Tomlins *et al.*, 2005; Perner *et al.*, 2007). Additional gene fusions have been reported involving another two ETS members, ETV4 and ETV5 and more 5' partners such as solute carrier family 45, member 3 (SLC45A3), human endogenous retrovirus (HERV-K) (22q11.3), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRPA2B1) and

chromosome 15 open reading frame 21 (C15ORF21) have also been identified in gene fusions with ETV1 with SLC45A3 also able to fuse to ETV5 (Tomlins *et al.*, 2007; Helgeson *et al.*, 2008). Another two 5' partners identified for ETV4 are hK2 and calcium-activated nucleotidase 1 (Hermans *et al.*, 2008). Among the gene fusions, TMRSS2:ERG is the most common one (Soller *et al.*, 2006).

The effect of gene fusions on CaP is still subject to investigation, however the fact that TMPSS2:ETS gene fusions can only be found in PIN, carcinoma and metastases but not in BPH or proliferative inflammatory atrophy suggested that gene fusions are likely to be genetic triggers of the development of invasion of CaP (Perner *et al.*, 2007).

The potential of gene fusions as CaP biomarkers has also been investigated, with TMRSS2:ERG being the main focus because it is the most common gene fusion in prostate cancer. The association between TMRSS2:ERG and Gleason score is controversial which is probably due to the limitation of technology sensitivity, relatively small sample sizes, varied populations and different experimental designs. Although Winnes *et al.*, (2007) found a significant association between positive TMRSS2:ERG fusion and lower Gleason score and better survival rates using 50 patient tissue samples (Winnes *et al.*, 2007), no association was found between TMRSS2:ERG fusion status and Gleason score on samples from 300 patients by Perner *et al.*, (2007). Several studies have suggested that TMRSS2:ERG gene fusion is associated with aggressiveness of prostate cancer and death from CaP. Mosquera *et al.*, (2007) found no association between the gene fusion and Gleason score but other morphological features linked to aggressive CaP were significantly related to the gene fusion status (Mosquera *et al.*, 2007). Perner *et al.*, (2006) has shown a significant association between positive TMRSS2:ERG and higher tumour stage using tissue samples from 211 patients (Perner *et al.*, 2006). Wang *et al.*, (2006) and Nam *et al.*, (2007) and both reported positive TMRSS2:ERG fusion was strongly correlated to the recurrence of CaP (Wang *et al.*, 2006; Nam *et al.*, 2007).

Demichelis *et al.,* (2007) reported TMRSS2:ERG is significantly associated with CaP specific death (Demichelis *et al.*, 2007).

In addition to the prognostic value of TMRSS2:ERG, it has helped to improve CaP detection when combined with other biomarkers, possibly due to its specificity for prostate cancer. One study used a multiplexed quantitative-PCR based test to analyse a panel of seven potential urinary CaP biomarkers, including TMRSS2:ERG. This showed improved specificity compared to the serum PSA test (Laxman *et al.,* 2008). Combining TMRSS2:ERG and PCA3 also improved the predictive value of CaP compared to PCA3 test alone (Hessels *et al.*, 2007).

Overall, despite a few investigations, most studies suggest that TMRSS2:ERG could be a prognostic biomarker for aggressive prostate cancer. It has also had great impact on improving CaP detection when coupled with other biomarkers. However, studies on the clinical implications of TMRSS2:ERG often involve relatively small sample sizes and different experimental designs. More studies are needed to validate its clinical significance on CaP.

1.3.3 CaP biomarkers found using proteomic techniques

With the development of highly accurate, high throughput proteomic techniques it is now possible to detect and identify novel protein biomarkers for the diagnosis and prognosis of disease states. Protein biomarkers can be identified from biofluids such as blood products, tears, urine and cerebrospinal fluid and can provide the basis of clinically relevant diagnostic/prognostic tests which are fast, non-invasive and highly accurate. Discovery of novel protein biomarkers for diseases such as CaP is a key step in improving disease detection and management and subsequently reducing related morbidity and mortality.

Much gel-based proteomic research has made use of either prostate tissue or CaP cell lines. This has resulted in several potential biomarkers being identified, but no clinically relevant diagnostic test has been produced based on these studies (**Table**

1.2). Even in general cancer biomarker research, only twelve protein biomarkers were approved by 2005, and most of them were generated from serum (Lufwig and Weinstein, 2005). Since 2005, FDA has approved a DNA microassay based test, MammaPrint, for breast cancer diagnosis (Lacal, 2007) and two protein tests for ovarian cancer, *i.e*. the human epididymis protein 4 for monitoring the progression of ovarian cancer (Andersen *et al.,* 2010), and the ovarian diagnostic blood test, OVA 1, using SELDI technique (Fung, 2010). Both of tests used proteins from blood.

Although proteomic techniques have been widely used to analyse human body fluids such as urine or serum, no clinical useful CaP biomarkers were found. Two factors affect the unsuccessful clinical applications of the discovered potential biomarkers. Firstly, there are not yet standardised procedures to evaluate the clinical usefulness of the potential biomarkers. For the biomarkers to be accepted and routinely used in clinical setting, their sensitivities and specificities needs to be objectively measured and validated, however these measurements are critically affected by the quality of the biospecimens and analytical assay performances through different laboratories (Rodriguez *et al*., 2010). In addition, various parties including the government, the commercial parties and the clinical research laboratories are all involved in the approval of a potential biomarker, and each represents different interests, as a result an effective communications between the groups are needed (Khleif *et al.,* 2010). Fortunately, these issues are being addressed and the American Association for Cancer Research (AACR), in partnership with FDA and the National Cancer Institute (NCI) (AACR-FDA-NCI) cancer biomarkers collaborative have been conducting work to build up a better pipeline to accelerate the translation of the potential biomarkers from bench to clinic (Khleif *et al.,* 2010). A new pipeline by incorporation of the assay verification step after the discovery of potential biomarkers and before the clinical qualification was proposed to screen for the high potential biomarkers, and MRM-MS, was proposed to be a reliable verification technique (Boja *et al.,* 2010; Rodriguez *et al.,* 2010).

Secondly, the difficulties of analyzing the proteome of human body fluids using proteomic technologies also hinder the discovery of large potential biomarkers. One issue with such research is that the concentration of potential biomarkers varies in human body fluids depending on their level of direct contact with the tumour tissue. Concentrations of tumour derived proteins are generally higher in tissue interstitial fluid as it directly contacts tumour cells, but these molecules are present at lower concentrations in circulating body fluids such as serum/plasma (Ahn and Simpson, 2007). Another major issue is that proteomic analysis of blood and blood products is challenging due to the large dynamic range of molecules present, both those of interest and those interfering with analysis (Rabilloud, 2002; Moritz *et al.*, 2004). Proteomic analysis of urine is complicated by the low concentration of proteins and high concentrations of salts (Norden *et al.*, 2004). Whilst these issues remain, with the enormous advances in proteomic technologies that continue to occur, the discovery of novel protein biomarkers from biofluid sources appears one of the most promising tools for development of clinically relevant, sensitive and specific diagnostic and prognostic tests.

Table 1.2: Potential CaP biomarkers found using proteomic techniques.

Samples studied	Techniques used	Potential biomarkers found	Followed up?	Study reference
Cell suspensions (35 prostatectomy specimens, 29 cancer samples and 10 benign samples)	Two dimensional gel electrophoresis (2DGE) coupled with mass spectrometry (MS)	Nucleoside diphosphate kinase 1, chromobox protein, 39S ribosomal protein L12, cytosol aminopeptidase, endopeptidase C1p, inorganic pyrophosphatase, metaxin 2, GST-pi, acyl-CpA dehydrogenase, lysophospholipase, NADH-ubiquinone oxidoreductase, 60 and 70 kDa GRP-78, β-actin, cytokeratins 7, 8, and 18 stomatin-like protein 2, a-actin and mutant desmin	No	Lexander *et al.*, 2006
Conditioned media (three human CaP cell lines, PC-3 , LNCaP, and 22Rv1)	Strong cation exchange high-performance liquid chromatography (HPLC)-tandem MS	PSA, hK2 ,PSMA, follistatin, chemokine (C-X-C motif) ligand 16, pentraxin 3 and spondin 2	ELISA confirmed with 42 serum samples from patients with or without CaP	Sardana *et al.*, 2008
Prostate tissue specimens (17 from CaP group and 10 from BPH group)	Two dimensional differential in gel electrophoresis (2D-DIGE) coupled with MS	Six biomarkers were identified for lymph node metastatic (LNM) CaP : epidermal fatty binding protein 5 (e-FABP5), methylcrotonoyl Coenzyme A carboxylase 2, inorganic pyrophosphatase 2, Ezrin, stomatinlikeprotein2, and smooth muscle protein	Western blot confirmed with 27 tissue samples. Serum e-FABP5 was confirmed by ELISA using 70 sera samples (20 patients with localized PCa, 20 patients with LNM CaP, and 30 patients with BPH)	Pang *et al.*, 2010
Urine (10 from CaP, 10 from BPH and 10 normal controls)	2DGE	Unidentified protein spots:A (36 kDa),B (23 kDa) and F (18-28 kDa)	No	Grover and Resnick, 1997
Urine (6 from CaP and 6 from BPH)	2DGE coupled with MS	Calgranulin B/MRP-14	No	Rehman *et al.*, 2004

Continued on next page

Samples studied	Techniques used	Potential biomarkers found	Followed up?	Study reference
Urine (51 from CaP, 35 from control)	Capillary electrophoresis (CE) coupled with MS	A panel of biomarkers consisting 12 polypeptides	Blind sample tested (total 264 samples). High sensitivity (89%), low specificity (51%)	Theodorescu *et al.*, 2008
Serum (16 CaP patients and 15 healthy individuals)	MS based mass profiling	Platelet factor 4	Western blot and ELISA confirmed with the same samples	Lam *et al.*, 2005
Serum (the work was based on Qu et al. (2002) study which used 197 with CaP, 92 with BPH, and 96 healthy individuals)	HPLC, reverse-phase chromatography, one dimensional gel electrophoresis (1DGE), Liquid chromatography (LC)-MS/MS and surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF)-MS	Apolipoprotein A-II (m/z at 8,946)	Only protein identity confirmed	Qu *et al.*, 2002; Malik *et al.*, 2005
Serum (19 patients with confirmed CaP bone matastasis and 19 patients without bone metastases)	SELDI-TOF-MS	Serum Amyloid A for bone metastasis	Only protein identity confirmed but no quantitation of results	Le *et al.*, 2005
Serum (11 localised CaP patients, 12 BPH patients and 12 controls)	SELDI-TOF-MS	Three unidentified putative protein markers with relative molecular weights of 15.2, 15.9 and 17.5 kDa	No	Lehrer *et al.*, 2003
Pooled serum samples (CaP serum pooled from 10 different CaP patients and a BPH serum sample pooled from 10 different BPH patients)	Anion displacement liquid chromatofocusing chromatography, 2D-DIGE coupled with MS	Squamous cell carcinoma antigen 1, calgranulin B and haptoglobin-related protein	No	Qin *et al.*, 2005
Serum samples (from 12 different grades of CaP patients)	2D-DIGE coupled with MS	Pigment epithelium derived factor and zinc-α-glycoprotein	Western blot and ELISA confirmation using 50 samples from different grades of CaP and BPH patients	Byrne *et al.*, 2009

1.4 Biofluid proteomics for biomarker discovery

One important criterion for the use of markers from biofluids is that they are present in samples which can be obtained in a non-invasive fashion. Urine, blood and tears can easily be sampled to provide material for initial research and for subsequent use for diagnosis/prognosis in the clinical setting. Both urine and serum have been used for CaP research (as indicated in **Table 1.2)**, however using tears to find CaP biomarkers is a novel approach.

1.4.1 Urine

1.4.1.1 The advantages of using urine for CaP biomarker research

Urine is the waste product generated by filtering blood that flows through kidneys. Analysis of pooled urine from healthy individuals (from both male and female) showed 70% of the urine proteins originated from the urinary system and the remaining 30% represented proteins filtered from the blood by the glomerulus (Thongboonkerd and Malasit, 2005). This high percentage of urinary system information present showed that urine could be a useful resource for searching biomarkers for urinary-related diseases including prostate cancer.

Urine has several distinct advantages as a reservoir for biomarkers. Among all the human body fluids, it is the most easily accessible and has the highest volume. Shortage of available human body fluid samples is a major problem and hinders the progress of searching for biomarkers. The large amount of urine available allows repetitive analysis from the same individual sample which in turn can improve the accuracy of the results. The large quantity also provides extra resources for validation purposes. The second advantage is that the urine proteome is relatively stable. One study using SELDI-TOF-MS to analyse the urine profile showed that the mid-stream urine proteome is stable up to four freeze/thaw cycles when samples were stored at -70 °C (Schaub *et al.*, 2004). Urine can be stored at -70 °C for >17 years and still allow for collection of high-quality data, using a gel-based proteomics approach (Thongboonkerd, 2007). As for searching for CaP biomarkers,

urine contains a high proportion of urinary tract information. Although urine does not pass through the prostate gland, it does contain secretions from the prostate gland and abnormalities in secretion products could shed light of the status of the prostate gland (Downes *et al.*, 2006).

1.4.1.2 The challenges of analysing urine proteome

The analysis of urine samples presents several challenges. First of all, the protein concentration in urine is very low. The normal protein secretion rate for adults is around 0.15-0.2 g per day, average 4.9 mg/dL (Ginsberg *et al.*, 1983). Using amino acid analysis, a later study found that the normal urine collected per 24 hours contained around 33 mg of protein (molecular mass greater than 10 kDa) excluding the Tamm-Horsfall protein, and 22 mg of peptides (between 750 Da and 10 kDa) (Norden *et al.*, 2004). In addition to this low protein amount, various interfering components are also present in urine including cells and debris, a high concentration of electrolytes, and highly abundant proteins such as albumin (Thongboonkerd, 2007). Removal of the interfering substances is important for urine proteome analysis, however this can result in a loss of proteins and possibly important disease-related information (Thongboonkerd, 2007).

Several studies have used proteomic techniques to analyse the urine proteome for CaP biomarkers. As listed in **table 1.2**, the most recent proteomic study of urine used capillary electrophoresis coupled with MS to investigate urine samples and found 12 polypeptides that could detect CaP using a 'blind' sample set with high sensitivity but low specifity (Theodorescu *et al.*, 2008). However, only several of the polypeptides were identified (Theodorescu *et al.*, 2008). Another two studies have identified several novel CaP biomarkers, but these have not yet been validated (Rehman *et al.*, 2004; M'Koma *et al.*, 2007).

Because of the low protein content and high interfering substances in urine, urine was not chosen for further analysis in this thesis. Serum and tears were chosen for this research and are discussed below.

1.4.2 Blood

1.4.2.1 The advantages of blood for biomarker identification

The advantages of blood-based biomarker discovery are:

1) the sample collection procedure is well established, rapid and economically viable
2) blood sampling is already widely used for other classical clinical chemistry assays
3) repeat, longitudinal sampling can be readily conducted from the same individual
4) samples can be easily aliquoted for long-term storage (McKay *et al.*, 2007)
5) it is relatively stable when compared to other biological fluids such as urine whose composition changes depending on fluid flow rates (Lundblad and White, 2005).

Most blood based biomarker research focuses on the blood products - plasma and serum. A plasma sample is prepared by withdrawing blood with the presence of an anticoagulant such as ethylenediaminetetraacetic acid (EDTA), sodium citrate or heparin, whereas a serum sample is collected after the blood clots and is centrifuged to remove cellular components such as erythrocytes, leukocytes and platelets (Hu *et al.*, 2006). During the clotting process, a number of proteins are removed into the clot, which contains the fibrin converted from fibrinogen, platelets, protease-serpin complexes, protein fragments such as D-dimer and prothrombin fragment 1. At the same time some components such as vascular endothelial growth factor are secreted into the serum by cellular elements (Lundblad and White, 2005).

1.4.2.2 Comparison of plasma and serum for biomarker searching

Both serum and plasma have been used extensively in biomarker research, and there are no clear terms determining a preference for one sample. The Human Proteome Organization (HUPO)/Plasma Proteome Project (PPP) Specimens Committee recommends using platelet-depleted or citrate plasma for peptidome analysis

(Tammen *et al.*, 2005). They elucidated that more than 40% of all peptides detected in serum samples are serum-specific and are due to the results of the coagulation cascades and other protease events, hence limiting the detection of real biomarkers (Tammen *et al.*, 2005). In a latter study, when comparing between the proteome displays of plasma and serum, only six serum specific proteins were found (Kim *et al.*, 2007). Additionally, no significant differences have been found between serum and plasma glycoproteomes, which is important for cancer-based biomarker discovery as many cancer specific proteins are glycosylated (Yang *et al.*, 2005). Removal of the abundant protein fibrinogen during the serum clotting process may aid proteomic studies as it simplifies the serum profile when compared to plasma (Lundblad and White, 2005). Whilst it is recognized that biomarkers can be identified from both plasma and serum samples, for the present CaP-based study serum was chosen as the sample type. Serum contains a higher amount of protein than urine, and the proteome of a serum sample can reflect the metabolic state, physiological and pathophysiological processes of an individual at the time of blood withdrawal (Tammen *et al.*, 2005), therefore it is likely that one may find reliable CaP biomarkers in serum.

At least ten proteins have been identified as potential CaP diagnostic markers in serum using proteomic techniques between 2002 and 2009 (**Table 1.2**). Among them, only two, pigment epithelium derived factor and zinc-α-glycoprotein, have been further verified by Western blot and ELISA, however no control group was included in this study (Byrne *et al.*, 2009). In all of the studies from which potential biomarkers were identified, the serum samples were pre-fractionated prior to biomarker discovery experiments. C18 tips have been utilised to extract low molecular weight serum proteins (MW < 20 kDa) for MS based mass profiling (Mobley *et al.*, 2004; Lam *et al.*, 2005). SELDI-TOF-MS was widely used for searching serum biomarkers (**Table 1.2**), as the various protein chips available can prefractionate serum proteins based on a range of physical or chemical properties. However, one drawback is that not all the proteins/peptides detected by this method can be identified (Lehrer *et al.*, 2003). In the present study, affinity removal of

highly abundance proteins and MF-10 prefractionation were used to simplify the serum profile. These techniques as well as the importance of serum prefractionation are discussed in the following section.

1.4.2.3 The challenges for analysing serum proteome

The major challenges of analysing the serum proteome are its complexity and the extreme dynamic range of proteins (Barnea *et al.,* 2005). A serum sample contains thousands of proteins and this can increase the difficulty of identifying each of them at the same time since some of them have similar molecular weight (MW) and charge (Chromy *et al.*, 2004), and can co-migrate with each other (Barnea *et al.*, 2005). The dynamic range of proteins in serum differs by at least ten orders of magnitude ranging from albumin and immunoglobulins (g/L range) to interleukins (pg/L range) (Anderson and Anderson, 2002). Twenty two proteins including albumin, immunoglobulin (Ig) G, IgA, transferrin, haptoglobin, and antitrypsin represent about 99% by weight of total serum proteins (Chromy *et al.*, 2004). These proteins may mask low abundance proteins and make them hard to detect using standard 2-D gel analysis (Zhang *et al.*, 2004).

Detection of low abundance serum proteins is important for biomarker discovery and sample complexity must therefore be reduced to facilitate their analysis using currently available proteomic techniques (Chromy *et al.,* 2004). Pre-purification steps such as enrichment of target analytes, depletion of high-abundance proteins, and pre-fractionation can reduce sample complexity and decrease the limits of detection (Makawita and Diamandis, 2010). Removal of highly abundant serum proteins is a common pre-fractionation technique and can be achieved using various antibody or ion-exchange based removal systems. The combination of 2DGE analysis and removal of six high-abundant proteins has shown an increase of 76% in serum protein spots detected in the depleted serum samples and a two fold increase in intensity of several protein spots when compared with the crude serum proteins (Chromy *et al.*, 2004). Antibody-based removal may be preferable to the ion-exchange method (Chromy *et al.*, 2004). However, removal of high abundance proteins such as albumin can cause co-depletion of biologically important proteins and peptides. Albumin is a major carrier protein for metabolites and fatty acids;

smaller proteins and peptides may also bind to albumin to avoid kidney clearance and have extended half-life (Dennis *et al.*, 2002). A study analysing both the depleted (removal of the six highly abundant serum proteins) and undepleted serum proteome found 142 proteins unique to depleted serum and 38 unique to the undepleted sample (Yocum *et al.*, 2005). It is therefore important that the depletion strategy chosen provides an adequate balance between depletion and associated loss.

An alternative pre-fractionation technique using an apparatus called MF-10 developed by NuSep, can separate proteins into specific pI and MW ranges under both native and denatured conditions (Wasinger *et al.*, 2008). The mechanism used by the MF 10 is illustrated in **Figure 1.3**. The major component of this machine involves a separation unit (an electrolytic cell) consisting of 2 to 6 chambers divided with separation membranes. Each separation membrane with a different MW cut-off can be inserted into the unit to produce a sequential gradient, for example 1000 kDa, 400 kDa, 150 kDa, 65 kDa, 30 kDa and 5 kDa. When a current is applied, the negatively charged proteins move through the chamber and become trapped between the membranes which define their MW, for example between 1000 kDa and 400 kDa chambers (Wasinger *et al.*, 2008). As a result, for size separation, a high pH buffer is usually used so that all proteins will be negatively charged and moved to the positive electrode and are thus separated only by MW (Wasinger *et al.*, 2008). For fractionation by pH, the pH buffer needs to be adjusted to between the pI of the target protein and any contaminating proteins so that the proteins migrate to different separation chambers (Wasinger *et al.*, 2008). The advantages of this technology include that:

1) the proteins from each fraction can be analysed directly by 2DGE
2) it reduces the sample complexity but allows analysis of the entire proteome (Fitzgerald and Walsh, 2010)

In the present study, size separation was used and proteins were fractionated in their native condition thus maintaining their biological structure and conformation.

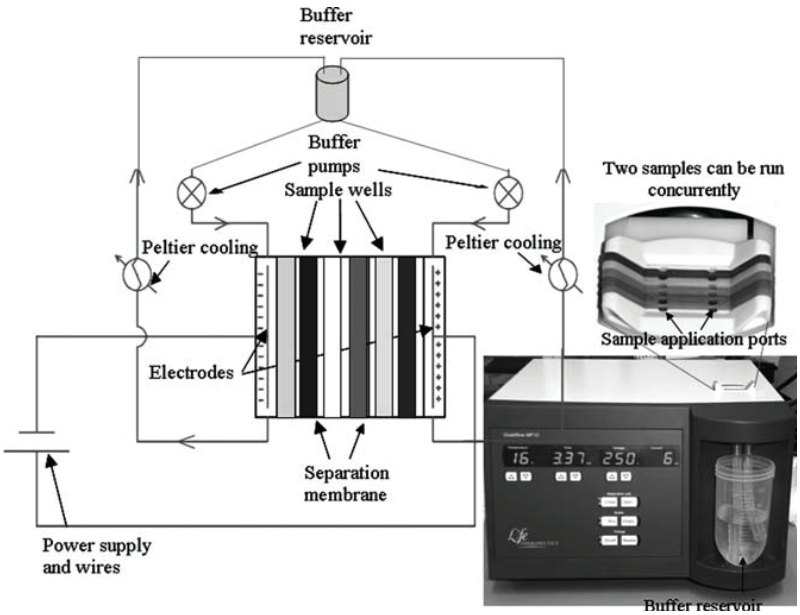


Figure 1.3: The schematic figure of MF 10. Samples can be loaded into the sample application ports in the separation unit containing six separation chambers that were divided by separation membranes that have various protein size restrictions. Figure is taken from Wasinger *et al.*, (2008).

1.4.3 Tears

1.4.3.1 Tears as a sample source for biomarker research

CaP is an androgen-dependent disease. Androgen levels can also change tear production (Sullivan *et al.*, 1999), implicating the possibility of finding CaP biomarkers in tears. Different tear protein contents have been observed for systemic diseases such as diabetes (Herber *et al.*, 2002) and a potential breast cancer biomarker has been found in tears (Evans *et al.*, 2001). In addition, tears also contain a higher amount of protein than urine. Nonstimulated tears contain around seven mg/mL protein (Fullard and Tucker, 1991).

The tear film (TF) is the outermost thin liquid layer covering the ocular surface. It is a unique body fluid that is predominantly composed of mucins, proteins, lipids and salts, and serves multiple functions in order to maintain the health of the eye. Presence of this fluid layer provides nutrition and oxygen to the cornea, lubricates

and moistens the eyes and eye lids, and allows smoothing of the corneal surface to increase refraction power (Craig, 2002). In addition, the TF protects the eye from debris and bacteria both by physically flushing unwanted material from the eyes and by the action of antibacterial constituents such as lysozyme (Craig, 2002). The constituents of tears are important for their essential functions and subsequent maintenance of ocular health. In comparison with the two most commonly studied body fluids, serum and urine, the tear composition is less complex. The tear proteome has been proposed to contain around 500 proteins (de Souza *et al.*, 2006), whereas the proteome of saliva contains around 1000 proteins (Guo *et al.*, 2006) and that of serum/plasma is estimated to contain up to 10,000 proteins (Wrotnowski, 1998). As Doughty (2002) summarised, the components of the TF can be grouped based on their origins into:

1) lacrimal proteins secreted from the main and accessory lacrimal glands
2) cellular debris and cell contents from degenerate cells, including from the meibomian gland
3) mucus from the bulbar conjunctiva goblet cells, the palpebral conjunctiva goblet cells and cell vesicles
4) proteins, inflammatory mediators (including various interleukins) from serum/plasma leakage from the conjunctival vasculature
5) inflammatory mediators from stressed cells and the mast cells under the conjunctiva (Doughty, 2002)

This diverse composition of the tear fluid can vary in response to pathophysiological conditions which affect other areas of the organism, making the tear fluid useful for biomarker research. Additionally, collection methods of this biofluid are non-invasive, and relatively easy, therefore subjects are more willing to provide tear samples.

1.4.3.2 The challenges of tear biomarker research

Variation in tear fluid sample compositions are observed between samples collected under different conditions such as stimulated/non-stimulated and eye open/closed. Tears collected without external stimulation are basal tears, while reflex tears are

produced with external irritation by odours such as onions or by emotion. Similar levels of major tear proteins including the lacrimal gland proteins, lactoferrin, tear specific prealbumin, and lysozyme have been observed in both types of tears collected using the capillary method, however secretory IgA, and serum proteins such as IgG, IgM, and transferrin were increased in non-stimulated tears compared with stimulated tears (Fullard and Snyder, 1990). Studies using the Schirmer strip collection method have also reported differences in protein composition between reflex, open and closed eye tears (Sack *et al.*, 1992; Sack *et al.*, 2000). Reflex tears were shown to contain mostly proteins from lacrimal or accessory gland secretion and a small amount of alpha and beta globulin. Open eye tears contained higher secretory IgA compared to reflex tears, while closed eye tears contain a high proportion of albumin and secretory IgA as well as inflammatory proteins such as cytokines (Sack *et al.*, 1992; Thakur *et al.*, 1998; Sack *et al.*, 2000). The use of absorbent material such as filter papers or cellulose sponges is invasive and the tear fluid sample collected contains a higher proportion of ocular surface cellular proteins (Craig, 2002; Green-Church *et al.*, 2008). As a result, analysis of tear fluid samples for protein biomarker discovery is complicated by the differences in composition associated with these various collection methods. These highlight the importance of using a standard operation protocol for sampling for biomarker research.

Another challenge associated with tear fluid biomarker research is the requirement of highly sensitive and reliable techniques for analysis of the small amounts of tears able to be collected to ensure reproducible and quantifiable results. With the development of very sensitive proteomic techniques, such as various MS techniques that require only 1-5 μL of tears, it is timely for tears to be considered as a useful source of biomarkers for various diseases. Studies of the tear proteome have found that it can reflect some local eye diseases (Grus *et al.*, 2001; Tiffany, 2003) and systemic diseases such as diabetes (Herber *et al.*, 2001) and cancers (Evans *et al.*, 2001).

1.4.3.3 2DGE analysis of tear samples

A common method used for TF analysis is 2DGE. While not the most efficient way to identify all proteins in tears, 2DGE is widely employed to examine changes in abundance and/or modifications to tear proteins between different sample groups. Molloy *et al.* (1997) sampled human reflex tears and detected all the major tear proteins using 2DGE, with subsequent identification of a new tear protein, lacryglobin (Molloy *et al.*, 1997). Lacryglobin is a protein displaying strong homology with mammaglobin, which has been found to be a potential marker for metastasis of breast cancer cells to lymph nodes (Ouellette *et al.*, 2004). A later study found that this protein could be a potential tear biomarker for prostate and breast cancer (Evans *et al.*, 2001). The tear proteome has also been analysed using 2DGE to detect differences between diabetic patients and healthy controls (Herber *et al.*, 2001) and between blepharitis patients and healthy controls (Koo *et al.*, 2005). Herber *et al.*, (2001) found the 2D protein profiles were significantly different between control and diabetic patients with significantly more protein spots in the diabetic group (Herber *et al.*, 2001). Koo *et al.*, (2005) successfully identified nine proteins (serum albumin, α1-antitrypsin, lacritin, lyzozyme, Ig-kappa Chain V III, prolactin inducible protein (PIP), cystatin-SA III, pyruvate kinase, unnamed protein) using 2DGE that were down-regulated in blepharitis (inflammation of the lids of the eye) (Koo *et al.*, 2005).

2DGE has also been used to study the post-translational protein modifications (PTM) of tear proteins. The two most common types of PTMs are phosphorylation and glycosylation (Mann and Jensen, 2003). Phosphorylation often results in changes in enzyme activity and protein signaling processes, while glycosylation can change cell-cell recognition/signaling and regulatory functions (Mann and Jensen, 2003). Changes of PTMs are closely associated with various diseases such as cancer (Krueger and Srivastava, 2006). Therefore, protein PTMs are relevant for biomarker detection and understanding the level of PTMs will allow better understanding of protein function and their role in diseases. The phospho- and glyco-protein profiles of basal normal tears have been studied using Pro-Q Diamond stain for

phosphoproteins and Emerald stain for glycoproteins (Zhao *et al.*, 2010; You *et al.*, 2010). These methods can be used to compare PTMs of proteins from control and disease patients' tears, such as the analysis of glycoproteins in diabetic and healthy control tear samples in which it was observed that there are more glycoproteins present in diabetic patients' tears than healthy controls (Koo *et al.*, 2005). Analysis of these modifications could therefore lead to possible identification of PTM biomarkers for cancers.

For 2DGE techniques to produce reliable results, one usually requires relatively large amounts of tear samples (greater than 5 μL) and proteins that are well separated and detected are generally those greater than 12 kDa. MS-based techniques now provide an alternative approach to tear protein analysis. Zhou *et al.* (2009a) used isobaric tags for relative and absolute quantitation (iTRAQ) to identify ten potential tear protein biomarkers for dry eye syndrome: α-enolase, α-1-acid glycoprotein1, S100A8 (calgranulin A), S100A9 (calgranulin B), S100 A4, S100 A11 (calgizzarin), PIP, lipocalin-1, lactoferrin and lysozyme (Zhou *et al.*, 2009a). In particular, α-enolase and S100 A4 were successfully verified by ELISA (Zhou *et al.*, 2009a). This technique is promising for future tear proteomic studies.

1.4.3.4 SELDI analysis of tear proteins

The SELDI-TOF-MS ProteinChip Array has also been introduced for biomarker research and has been used for tear studies. This technology utilizes affinity surfaces to retain adherent proteins based on their physical or chemical characteristics, which is then followed by direct analysis using TOF-MS (Hutchens and Yip, 1993). This simplified the prefractionation and enrichment steps required for analysing low abundance proteins from a mixture and has been used widely for identifying biomarkers in various cancers including breast cancer (Li *et al.*, 2005), renal cancer (Marshall, 2005), ovarian cancer (Moshkovskii *et al.*, 2005) and prostate cancer (Matharoo-Ball *et al.*, 2007) as well as other diseases such as infectious diseases (Hodgetts *et al.*, 2007), Parkinsonian disorders (Constantinescu *et al.*, 2010), and arthritis (de Seny *et al.*, 2008). Analysing tears from pterygium

patients pre- and post-ocular surface surgery, Zhou *et al.*, (2004) have successfully detected and verified three human α-defensins (HNP-1, HNP-2 and HNP-3) which were up-regulated after surgery suggesting these proteins could be involved in wound healing (Zhou *et al.*, 2004). Due to the small amount of tears required for SELDI- TOF-MS, other tear studies have applied this technology (Grus *et al.*, 2005; Hida *et al.*, 2005; Tomosugi *et al.*, 2005). The tear proteomic patterns from dry eye and healthy subjects were screened using SELDI-TOF-MS with three different chip surfaces, and seven peaks showed significant differences between the two groups (Grus *et al.*, 2005). Using these peak panels, the specificity and sensitivity of the detection test were 90%. Five biomarkers for dry eye syndrome were identified from the peaks: proline-rich protein 3, C-terminal fragment of proline-rich protein 4, C-terminal fragment of nasopharyngeal carcinoma-associated PRP4, C-terminal fragment of α-1-antitrypsin and calgranulin A (Grus *et al.*, 2005). Among them, calgranulin A has also been reported by Zhou *et al.* as a potential biomarker for dry eye syndrome (Zhou *et al.*, 2009a). Increased level of human α-defensin was detected by SELDI in tears from allergic patients with corneal lesions, a result then confirmed by ELISA (Hida *et al.*, 2005). In addition to ocular diseases, SELDI has also been used for finding biomarkers in TF for systemic diseases such as cancer. A biomarker panel in TF was successfully generated by SELDI to allow breast cancer patients to be discriminated from healthy women (Lebrecht *et al.*, 2009). However, whilst SELDI has been used in multiple studies, the major disadvantage is that significant further work is necessary to identify and verify biomarker proteins that are detected in the peptide fingerprint.

1.4.3.5 Tear protein identification by other forms of MS

A real advance in tear biomarker research has come with the use of MS technology to identify proteins and detect modifications. In a study examining the normal tear proteome, 54 proteins were identified using in gel digestion with matrix assisted laser desorption ionisation (MALDI) MS analysis. Fourty-four proteins were identified using only two microliters tears with liquid chromatography (LC)-MALDI-MS (Li *et al.*, 2005). In addition, an LC-MALDI-MS/MS approach was

able to detect variants and PTMs of tear proteins. Lipocalin 1 (von Eber's gland protein) and proline-rich protein 4 were found to have variants in their peptide sequences with one phosphorylation site of lipocalin1 specifically identified (Li *et al.*, 2005). Several O-linked glycosylated proteins such as lacritin, proline-rich protein 1 and proline-rich protein 4 were also detected using this method (Li *et al.*, 2005). Using more powerful MS instruments such as hybrid linear ion trap fourier transform (LTQ-FT) and a LTQ-Orbitrap, a total of 491 tear proteins were identified from four microliter of tears, of which less than 80 had been identified previously (de Souza *et al.*, 2006). As a result, this approach has greatly improved our understanding of the tear proteome and provides a promising method of analysis of low abundance tear proteins.

As previously mentioned, proteomic techniques have greatly enhanced analysis of biofluid proteome. Well developed proteomic techniques used in this study have allowed sensitive and accurate analysis of the whole serum and tear proteome as well as more specific analysis of PTMs and low molecular weight (LMW) proteins and are discussed in the following sections.

1.5 General introduction to major proteomic techniques

Common techniques used in proteomic research can be divided into gel-based and gel-free techniques. The most commonly used gel-based technique is 2DGE which can be used to separate and visualize thousands of proteins in a gel. In biomarker discovery this technique can be used to detect protein spots which are altered in abundance between disease and control samples. This is then followed by identification of spots of interest using MS.

MS is a gel-free technique which is widely used for both protein identification and detection/quantitation of proteins as well as peptides that are smaller than 20 kDa, which cannot be found on 2D gels. MS has three major components: ionisation, mass analysis and detection (Aebersold and Mann, 2003). The ionisation techniques include electrospray ionisation (ESI) (Fenn *et al.*, 1989), MALDI (Karas and

Hillenkamp, 1988) and SELDI (Hutchens and Yip, 1993), of which ESI and MALDI are the two most commonly used ones. The major application of SELDI, a modification of MALDI, is finding biomarkers (Issaq *et al.*, 2002). The four most common mass analysers are TOF, quadrupole mass analyser, quadrupole ion trap and fourier transform ion cyclotron resonance (Aebersold and Mann, 2003). In biomarker research, combinations of gel-based and gel-free techniques are commonly used.

1.5.1 2D-DIGE

2D-DIGE is based on classical 2DGE and was developed to overcome one of the major limitations, i.e. large experimental variation. Proteins are separated based on charge in the first dimension and then molecular mass in the second dimension. Ideally 2DGE is used to separate all proteins in a sample, however proteins at either end of the size and pI scales, as well as those that are difficult to solubilise such as membrane proteins, are under-represented and therefore not reproducibly detected. Additionally, experimental variation is large as each sample is processed, separated and detected separately. Consequently, triplicates are essential to generate reliable results from 2DGE, limiting the ability to analyse low volume samples and increasing the time and labour required (Choe and Lee, 2003; Wienkoop and Weckwerth, 2006).

2D-DIGE involves labelling samples with three different CyDyes (Cy2, Cy3 and Cy5) that excite at different wavelengths allowing three separate samples to be separated and detected together on one gel (**Figure 1.4**). Additionally, an internal control is included in all experiments allowing normalisation of all samples and therefore eliminating between-gel variations. The internal control is a combination of all samples that will be analysed in the one experiment and is labelled with Cy2. Samples for analysis are labelled with Cy3 or Cy5 and on each gel the same amount of protein from two different samples and the internal control are separated to allow reliable protein detection and analysis of changes in relative abundance. 2D-DIGE

is significantly more cost effective than 2DE particularly when analysing large sample size (Karp *et al.*, 2008).

This method has been used in both serum and tissue specimens for CaP research (as listed in **Table 1.2**). 2D-DIGE analysis of sera has detected two proteins, pigment epithelium derived factor and zinc-α2-glycoprotein, which have significantly different abundance in serum depending on the grade of CaP present (Byrne *et al.*, 2009). A recent study used 2D-DIGE of tissue specimens identified six potential biomarkers of LNM CaP group (Pang *et al.*, 2010). These findings were all confirmed by Western blot and ELISA in both studies for validation purposes suggesting the high reliability of DIGE results. However, none of the groups included control samples in their studies and further clinical assays are needed to assess the sensitivity and specificity of the potential biomarkers identified. 2D-DIGE was used in the current research project for serum analysis in an attempt to detect novel biomarkers for CaP.

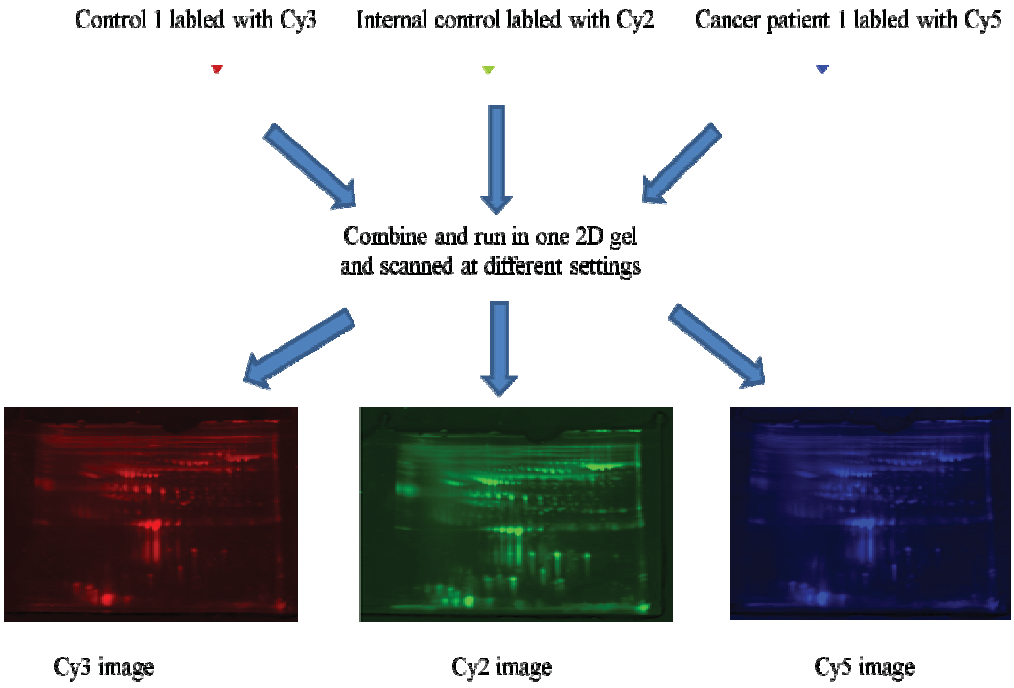


Figure 1.4: Working flow of DIGE technique. A single 2D gel can be used to separate two samples and an aliquot of the internal control sample. Reliable analysis

of protein abundance can occur within and between gels due to the presence of the same internal control on every gel to be analysed together.

1.5.2 ProteinChip/SELDI technology

SELDI-TOF-MS ProteinChip was first described by Hutchens and Yip in 1993 (Hutchens and Yip, 1993). This MS based technology utilises affinity surfaces on chips to retain certain adherent proteins based on their physical or chemical characteristics, and their direct analysis using TOF-MS. Such surfaces include normal phase for general protein binding, hydrophobic surfaces for reversed-phase capture, cation- and anion-exchange surfaces, and immobilized metal affinity capture (IMAC) for metal-binding proteins. Other surfaces can also be produced for specific targets such as antibody-antigen complexes, DNA-protein complexes, various receptors or drugs. An energy absorbing matrix (EAM) such as sinapinic acid is bound to the chip with the protein sample and absorbs energy when hit by the laser, allowing the proteins to desorb and ionise for MS analysis.

In comparison with 2DGE, SELDI-TOF-MS is more sensitive, requires only small amounts of sample (as little as two microliter) (Zhou *et al.*, 2004) and can detect small peptides (approximately 500 Da) (Grus *et al.*, 2005). The SELDI technique can serve many purposes such as development of protein profiles of different pathological states. However, the development of clinically viable diagnostic or prognostic tests usually requires further substantial research.

The main advantages of SELDI technology are:

1) that the development of protein peak profiles does not require protein identification which significantly reduces labour intensity
2) it has high throughput ability
3) it is able to detect LMW as well as truncated, modified or fragmented proteins and peptides (Petricoin and Liotta, 2004)

A crucial factor of using SELDI-TOF-MS in biomarker research is its reproducibility. Pattern based diagnosis has been described as a 'black box' approach with samples entering at one end and data coming out at the other end, with everything in between hidden in the 'box'. Therefore it is important to have a consistent response from the instrument for both peak mass and intensity from the instrument (Baggerly *et al.*, 2004). However, one main limitation of the instrument is its low resolution which can cause peak overlap causing problems in accurate detection of peak intensity and protein identification (De Bock *et al.*, 2010). Furthermore it has been recognised that different laboratories will have difficulty in achieving concordant data sets because of the evidence of experimental variation both from sample processing and different instruments used (Baggerly *et al.*, 2004).

Whilst the usefulness of SELDI in the clinical setting is at present limited, its ability to detect LMW protein/peptides using small sample amount and high sensitivity means that it can be used both as a starting point for peptide and LMW biomarker discovery and searching for biomarkers in human body fluids with limited volume such as tears.

1.5.3 Quantitative MS

MS-based technology remains one of the most powerful tools in proteomic research. MS has long been used for protein identification following protein separation and detection, however it is now developing as a valuable tool for detection and quantitation of proteins, and particularly of LMW proteins and peptides which can not otherwise be detected within a proteome. There are two types of MS based quantitation methods, labelling based and label free MS quantitation. Several commonly used reagents for labelling based quantitation via MS include isotope-coded affinity tags (ICATs) (Gygi *et al.*, 1999), stable isotope labelling by amino acids in cell culture (SILAC) (Ong *et al.*, 2002) and iTRAQ (Ross *et al.*, 2004). Multiple reaction monitoring (MRM) is now a promising label-free quantitation technique (Lange *et al.*, 2008).

1.5.3.1 ICATs

ICAT is a thiol specific reagent that labels only peptides with cysteine residues (Gygi *et al.*, 1999). There are two versions of the reagent: the light/ normal form and the heavy/ deuterated form. In this technique, the extracted proteins from treatment and control samples are labelled with either light or heavy ICAT reagents by reacting with cysteinyl thiols on the proteins. Peptides containing the labelled and unlabelled ICAT tags are recovered by avidin affinity chromatography and are then analysed by LC-MS/MS. Differential protein expression is determined by the isotope peak ratio of the peptide. Enrichment of low-abundance proteins can be performed through cell lysate fractionation (Graves and Haystead, 2002). In addition, the ICAT method can prefractionate protein mixtures by allowing only peptides containing cysteine to pass through the MS, which largely reduces the background noise. The ICAT technique has been widely used for protein identification and quantitation in breast cancer (Pawlik *et al.*, 2006; Kang *et al.*, 2010). However, it also has limits to its application as it cannot be used to analyse proteins that do not have a cysteine residue. Because this reagent only exists in two forms heavy (contains eight deuteriums) and light (no deuteriums attached), only two samples can be compared at a time (Gygi *et al.*, 1999).

Using ICAT with 2D LC-MS/MS, Meehan and Sadar (2004) found changes in a number of both known and unknown/uncharacterized proteins between androgen-stimulated and unstimulated LNCaP prostate cancer cells (Meehan and Sadar, 2004). These results provided an overview of the proteome of the LNCaP cells and the global changes that occurred in response to androgens in the CaP cell line *in vitro*. This technique is also used to analyse protein function. Using ICAT-LC-MS/MS to compare proteins in different organelle fractions of actinin-4 cDNA transfected and non-transfected 22RV1 CaP cell line, Hara *et al.*, (2007) found that actinin-4 was associated with change of protein expression and subcellular locations, and therefore plays an important role in intracellular molecular trafficking (Hara *et al.*, 2007). One recent study used ICAT as a non-invasive method to analyse the association of CaP and CD90 in patients' urine (True *et al.*, 2010). CD90 was found

overexpressed in the surface of CaP associated stromal fibroblastic cells. Urine from patients before operation (prostatectomy) and after operation were compared and CD 90 was absent in urine after operation (True *et al.*, 2010).

1.5.3.2 SILAC

SILAC can label all proteins and, as its name indicates, it incorporates isotopic labels into proteins via metabolic labelling (Ong *et al.*, 2002), In SILAC experiments cells representing two biological conditions can be cultured in amino acid-deficient growth media supplemented with ^{12}C- or ^{13}C-labelled amino acids. For example, one cell population is fed with regular amino acids, while the other is fed with ^{12}C- or ^{13}C-labelled amino acids. After several rounds of cell division, labelled amino acids will be incorporated into newly synthesized proteins. The proteins in these two cell populations effectively become isotopically labelled as "light" or "heavy." Upon isolation of proteins from these cells, samples can then be mixed in equal ratios and processed using conventional techniques for tandem MS. Given that corresponding light and heavy peptides from the same protein will co-elute during chromatographic separation into the mass spectrometer, relative quantitative information can be gathered for each protein by calculating the ratio of intensities of the two peaks produced in the peptide mass spectrum (MS scan). Furthermore, sequence data can be acquired for these peptides by fragment analysis in the product ion mass spectrum (MS/MS) scan and used for accurate protein identification. SILAC is widely used for biomarker discovery (Gronborg *et al.*, 2006), cell signaling dynamics (Guerrera *et al.*, 2007), identification of PTM sites (Soufi *et al.*, 2008), protein - protein interactions (Bose *et al.*, 2006; Foster *et al.*, 2006) and subcellular proteomics (Waanders *et al.*, 2007).

For prostate cancer research, Everley *et al.* (2004) have used SILAC to label two CaP cell lines (PC-3M: low metastatic potential; PC-3M-LN4: high metastatic potential) derived from the same cell line (PC-3) to analyse proteins involved in metastasis (Everley *et al.*, 2004). Membrane and membrane-associated proteins from the two cell lines were enriched and compared, and 440 proteins were

quantified, of which 60 were increased and 22 were decreased in PC-3M-LN4. However, because of its labelling nature, it can be used only for *in vitro* experiments and not human body fluids.

1.5.3.3 iTRAQ

This unique approach labels samples with eight independent reagents of the same mass that, upon fragmentation in MS/MS, give rise to eight unique reporter ions (m/z =113–121) (Phanstiel *et al.*, 2009). The reporter ions are subsequently used to quantify the eight different samples. In addition, the MS/MS fragmentation also produces strong signature y- and b- ions for each peptide detected, confirming the protein identification (Zieske, 2006).

Compared to ICAT and SILAC, iTRAQ has the ability to simultaneously label up to eight samples and analyse PTMs (Ross *et al.*, 2004). It has also showed higher quantitation sensitivity when compared to ICAT. Wu *et al.*, (2006) compared the quantitation sensitivity of DIGE, cleavable ICAT (one type of ICAT with labelling reagents containing ^{13}C isotopes and an acid-cleavable biotin group) and iTRAQ using both known protein mixtures and a cell lysate, and showed that iTRAQ was more sensitive than cICAT and similar to DIGE (Wu *et al.*, 2006).

iTRAQ has been applied to the analysis of a wide range of samples including human serum (Boylan *et al.*, 2010) or plasma (Song *et al.*, 2008), urine (Akkina *et al.*, 2009), saliva (Streckfus *et al.*, 2008), cerebrospinal fluid (Abdi *et al.*, 2006), disease tissues (DeSouza *et al.*, 2005), and cell lines (Keshamouni *et al.*, 2006). In terms of cancer biomarker studies, iTRAQ methodology has been employed to analyse breast cancer derived cells (Chen *et al.*, 2007), endometrial cancer tissues (DeSouza *et al.*, 2005) and saliva in breast cancer (Streckfus *et al.*, 2008) for cancer diagnosis and prognosis.

Glen *et al.*, (2008) used iTRAQ and 2D LC-MS/MS to profile potential prognostic biomarkers by comparing cell line LNCaP (poorly metastatic) and LNCaP-LN3

(highly metastatic) (Glen *et al.*, 2008). A total of 280 unique proteins (≥ 95% confidence) were identified, of which ten were significantly up-regulated and four were significantly down-regulated in LNCaP-LN3. Among the ten proteins, tumour rejection antigen (gp96) was subjected for immunohistochemistry staining using prostate tissues with a relatively large sample size (95 samples from BPH, 66 samples from malignant cases and 3 with metastasis CaP). A significant decrease of gp96 expression was observed in BPH samples when compared to CaP samples. Using the same technique, Garbis *et al.*, (2008) compared the proteomes of 10 BPH and 10 CaP tissue specimens and detected 65 proteins that changed between the two samples (Garbis *et al.*, 2008). These results demonstrated that iTRAQ can serve as a useful tool for global proteome comparison among different groups.

1.5.3.4 MRM

Alternatively, free labelling MS quantitation, particularly MRM, is an emerging and continually developing technology. MRM is a highly specific and sensitive technique for quantifying targeted protein/peptides. It refers to a tandem MS (MS/MS) scan mode that is coupled with triple quadrupole MS instrumentation and is able to select predefined ions for analysis (**Figure 1.5**). Before starting MRM, it is critical to design transitions for the target protein and this involves selecting peptides (also called precursor ions) and their corresponding product ions based on criteria as described in **Table 1.3.** Usually 2-4 transitions are selected for a target protein (Lange *et al.*, 2008). Based on the predesigned transition lists, the first quadrupole (Q1) of the MS will be able to select and transmit the precursor ions to be sent to the second quadrupole (Q2) for further fragmentation. The resultant product ions will be transmitted to the third quadrupole (Q3) which detects only product ions with selected pre-defined m/z (McKay *et al.*, 2007). Because of the two selection steps, the specificity of MRM is ensured. At least three y ions (produced by cleaving the CO-NH from the N-terminal side) are needed for MRM analysis to be successful and this can then specifically target a peptide/protein for both relative and absolute quantitation purposes (Kondrat *et al.*, 1978).

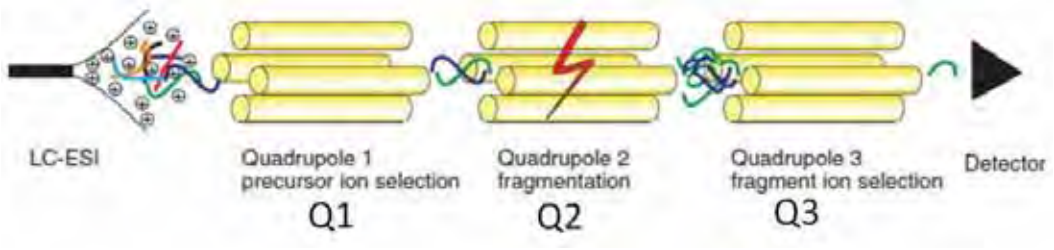


Figure 1.5: The filtering process of MRM. Predefined precursor ions are selected firstly in Q1, and then transferred to Q2 where they are fragmented to generate product ions. The predefined product ions are then selected by Q3 from all the product ions released from Q2. Finally, the selected product ions are measured by the detector. The picture is modified from Lange *et al.*, (2008).

Table 1.3: Summary of the general selection criteria for MRM transitions.

Transitions	Criteria	Detection Quadruple	Reference
Peptide/precursor ions	a) Unique for the target protein b) Should not have any PTMs either biologically or chemically induced c) Should not have missed cleavage sites d) Should be the predominant peptides detected by MS e) Peptide m/z within 600-2000	Q1	Lange *et al.*, 2008
Product ions	a) Singly charged y ions are preferable as they are the predominant ions generated from fragmentation b) Avoid product ions which have similar m/z to precursor ions; higher m/z than precursor ions are preferable c) Should be the predominant product ions	Q3	Lange *et al.*, 2008

A common application of MRM is to detect and quantify target molecules in a mixture which includes pesticides in crop extracts (Hiemstra and de Kok, 2007), and analysing drugs (Gergov *et al.*, 2003), hormones (Wu *et al.*, 2002) and proteins in blood (Anderson and Hunter, 2006). Addition of an internal control, such as a

stable isotope-labelled internal standard, allows MRM to be used for absolute quantitation of low molecular mass peptides (<1 kDa) (Gergov *et al.*, 2003). This method uses a series of isotopically labelled synthetic peptide standards to generate a standard curve. Based on the standard curve, MRM can be used to determine the concentration of target peptides in a wide dynamic range of $10^3 – 10^4$ (Anderson and Hunter, 2006; Ackermann and Berna, 2007). Unlike previously mentioned proteomic methods which are commonly used in the discovery phase of biomarkers, the ability to specifically quantify target peptides allows MRM to be a valuable tool for biomarker validation.

Compared to the widely used technology of immunoassay which is based on antigen-antibody interactions, the MRM technique has two advantages, it does not require the use of antibody thereby reducing the time required for antibody development, and it is capable of simultaneously quantifying up to 40 proteins. Kuzyk *et al.*, (2009) developed a protocol to successfully detect 45 plasma proteins using the MRM technique (Kuzyk *et al.*, 2009). It also showed high accuracy in quantifying the protein concentrations with 23 having the MRM determined concentration within previously reported ranges and 16 within double the range (Kuzyk *et al.*, 2009). Keshishian *et al.*, (2009) successfully quantified six cardiovascular injury protein biomarkers from six patients' sera (Keshishian *et al.*, 2009). In a prostate cancer study, PSA which is present in serum in the ng/mL range, was successfully quantified by MRM using nine depleted patients' sera (five with CaP and four with BPH) and showed high correlation with an ELISA test ($r^2 > 0.95$) (Fortin *et al.*, 2009). Reproducibility and accuracy of the MRM technique between laboratories has been studied. By assessing MRM assays across eight laboratories, Addona *et al.*, (2009) showed that the inter- and intra-laboratory reproducibility and accuracy, coefficient of variation (CV) < 25% for detecting proteins with concentrations above 2- 6 μg/mL in plasma and suggested MRM is a reliable tool for quantifying proteins in unfractionated plasma (Addona *et al.*, 2009), and is likely to be recongised by FDA as a reliable technique for biomarker verification (Boja *et al.*, 2010).

An additional application of MRM is to analyse the site of post-translational modification of proteins. Unwin *et al.,* (2005) has developed MRM-initiated detection and sequencing (MIDAS) and used it to successfully identify a novel serine phosphorylation site on both Cyclin-B and Hsp 60 isolated from *Schizosaccharomyces pombe* (Unwin *et al.,* 2005). This approach was also used to analyse the acetylation of proteins and successfully identified acetylated peptides from bovine serum albumin and a naturally modified protein cytokeratin 8 from a cell lysate (Griffiths *et al.,* 2007).

Moreover, Mayya *et al.,* (2006) has demonstrated that MRM was able to absolutely quantify four different forms (three phosphorylated and one unphosphorylated form) of cyclin-dependent kinases 1 and 2 which were highly consistent with their immunoblotting results (Mayya *et al.,* 2006). The ability to quantify glycosylated proteins was also shown by Hülsmeier *et al.,* (2007), where MRM was used to quantify the status of N-glycosylation sites in serum transferrin and α1-antitrypsins from healthy and congenital disorders of glycosylation related subjects (Hülsmeier *et al.,* 2007). The MRM results showed a reduction of N-glycosylation occupancy in diseased samples (Hülsmeier *et al.,* 2007).

Although MRM has many advantages, there are also several points of concern. First, different quantitation results from selected peptides from the same target proteins have been reported (Keshishian *et al.,* 2007; Fortin *et al.,* 2009; Kuzyk *et al.,* 2009). This inconsistent performance of peptides could be caused by many reasons including incomplete trypsin digestion of parental ions (Kuzyk *et al.,* 2009), different ionisation of individual peptides caused by instrument/interfering molecules (usually when the target peptides are in low abundance in a mixture with presence of other high abundance molecules) (Addona *et al.,* 2009), and modifications of the peptide either naturally or artifactually (Addona *et al.,* 2009). Previously mentioned selection criteria for MRM transitions could help avoid this problem. As mentioned, interfering substances in the mixture could affect the

accuracy of MRM quantitation, therefore appropriate fraction preparation is preferable for analysing mixtures containing high abundance proteins such as plasma or serum (Addona *et al.*, 2009). Compared to ELISA, which could detect proteins in plasma at ng/mL in a robust manner, the multi-center study of the reproducibility of MRM only confirmed its accuracy and reproducibility at μg/mL level in plasma. Although Fortin *et al.*, (2009) have successfully quantified PSA in serum (Fortin *et al.*, 2009), the samples used in this study were depleted sera and one PSA peptide transition selected was not detectable. As a result, MRM is a complementary tool to ELISA but not a replacement for the latter.

1.6 Current project

The aim of the current project was to use multiple proteomic techniques to detect, identify and verify potential diagnostic biomarkers for CaP in serum and tear film.

The two biofluid proteomes were analysed using different proteomic techniques as shown in **Figure 1.6**. Both analyses were involved in the key steps of biomarker discovery, identification and verification.

The long term outcome of this work would be development of a non-invasive diagnostic test for CaP based on these protein biomarker/s. Such a test would allow early, sensitive and specific detection of CaP which would ultimately improve treatment and management options, decreasing related morbidity and mortality.

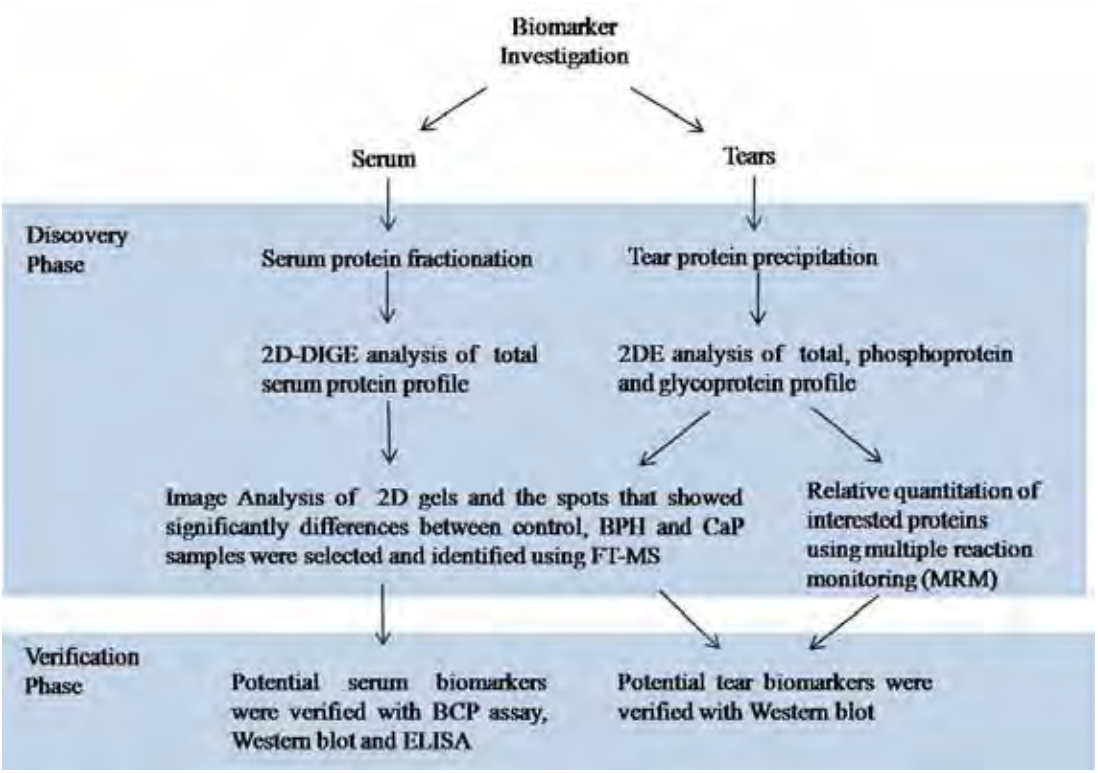


Figure 1.6: The techniques used to discover CaP biomarkers in serum and tears.

Chapter 2 Materials and methods

This chapter described the general materials and methods used in this study. Testing, optimisation and comparison between various sample preparation methods are also included and are described in section 2.3. All the samples used in each experiment were randomized but not assessed blinded, due to the fact that sample collection, preparation and analyses were all performed by the PhD candidate.

2.1 Sample collection

Ethics approval for sample collection was obtained from the South Eastern Sydney Area Health Service Ethics Committee (SEA HRCE #04/112) and informed consent was obtained from all participants prior to sample collection. The Declaration of Helsinki regarding the use of human subjects in research was adhered to. All BPH and CaP patient serum or tear samples were collected at St. George Private Hospital and Hurstville Community Hospital (NSW, Australia). Age and sex-matched control samples were collected from volunteers at three institutes: the St George Cancer Care Center (NSW, Australia), Brien Holden Vision Institute (NSW, Australia) and Minomic International Ltd. (NSW, Australia).

2.1.1 Serum sample collection

For each sample, 8 mL of blood was collected in a 9 mL Z serum clot activator Vacuette® tube (Greiner Bio-one, Austria) which was transported on ice to the laboratory of Minomic International Ltd (NSW, Australia). Tubes were left to stand at room temperature (RT) for half an hour to ensure maximum clotting and were then centrifuged at 2,500 rpm at 4 °C for 10 min to separate the serum. Serum was removed and 100 μL aliquots of each sample were frozen at -80 °C until used.

2.1.2 Tear sample collection

Basal tears were collected by placing a Blaubrand® intraMark micropipette (Brand, Germany) gently onto the corner of the interior lower lid margin of the tear meniscus for about 5 to 10 min. The collected tears were expelled into Eppendorf

tubes, transported on ice to the laboratory of Minomic International Ltd (NSW, Australia) and frozen at -80 °C until used.

2.2 Protein concentration determination methods and sample preparation

2.2.1 Bradford protein assay

Serum protein concentrations were determined by standard Bradford assay (Bio-Rad, USA) according to the manufacturer's instructions, using bovine serum albumin as a standard. Briefly, a serial dilution of standards (0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL and 0.5 mg/mL) was prepared with MilliQ water to construct a standard curve. Samples were diluted accordingly with MilliQ water (detailed in Chapter 3). Ten microliters of diluted standards, samples and blank (MilliQ water only) were pipetted into a 96 well flat bottom PS-microplate (Greiner Bio-one, Germany) in triplicate. Bradford reagent was diluted 1 in 5 with MilliQ water and 200 µL of the diluted dye was pipetted into each standard, blank and sample well. The microplate was read at 562 nm using a Kinetic Microplate Reader (Bioclone Australia, Australia) and protein concentrations of samples were determined based on the standard curve after subtraction of absorbance readings from the blank. This experiment was done in triplicate.

2.2.2 Fluoroprofile assay

Protein concentrations of tear samples were quantified using the FluoroProfile® Protein Quantitation Kit (Sigma-Aldrich, USA) following manufacturer's instructions. Briefly, a serial dilution of bovine serum albumin (0.048 µg/mL, 0.195 µg/mL, 0.781 µg/mL, 3.125 µg/mL, 12.5 µg/mL and 50 µg/mL) in MilliQ water was prepared to construct a standard curve. Tears were diluted 1 in 400 with MilliQ water, and 50 µL of each sample and standards were added into a 96 well flat bottom PS-microplate in duplicate. The Fluoroprofile™ Fluorescent reagent, quantitation buffer and ultrapure water were mixed in a 1:1:8 ratio, and 50 µL was added to the protein standards and tear protein samples. The plate was incubated at

4 °C in the dark for half an hour. The fluorescent intensities of the analytes were measured at excitation 510 nm and 620 nm band pass, using a Pharos FX™ Plus Molecular Imager (Bio-Rad, USA) and the Quantity One program (Bio-Rad, USA). Protein concentrations were calculated based on the standard curve. This experiment was done in triplicate.

2.2.3 Acetone precipitation of serum proteins

Two hundred microlitres of each serum sample were mixed with 100% ice-cold acetone in a ratio of 1:4 (sample : acetone). Samples were incubated at -20 °C for 1.5 hr to allow protein precipitation and then were centrifuged at 24,000 *x g*, 4 °C for 15 min. The supernatant was discarded and each serum sample pellet was air dried and resuspended in 200 µL of MiPrep F Isoelectric focusing (IEF) sample solution (MF solution, Proteome Pty Ltd, NSW, Australia). The MF solution contains the active ingredients, urea to help dissolving proteins and DTT to reduce the disulfide bonds of proteins, for analysis by gel electrophoresis. Precipitated serum samples were then stored at -80 °C until used.

2.3 Optimisation of sample preparation methods – preliminary experiments

2.3.1 Testing of reduction and alkylation methods for serum 2DGE analysis

Three 50 µL aliquots of one acetone precipitated serum sample were each mixed with 150 µL MF solution and labelled as sample A, B and C.

Sample A and C were reduced by mixing with 5 µL of tributylphsphate (TBP) and incubated for 30 min at RT. After reduction, the samples were alkylated by addition of 3.7 µL of 25% v/v acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution, Bio-Rad, USA) and incubated for 30 min at RT. Sample A was then frozen at -80 °C for 1 hr before use, sample C was incubated at RT for 1 hr before use. Sample B was neither reduced nor alkylated.

Each sample was analysed using 2DGE as in Sections 2.5.1.1 and 2.5.2. The gels were then stained with Sypro Ruby (Section 2.5.3.1) and scanned at an excitation wavelength of 532 nm and an emission wavelength of 605 nm (long pass filter) with the Pharos FX™ Plus Molecular Imager (**Figure 2.1**). Sample B showed the best separation with minimal vertical streaking and more distinct spots in the region pI 4.5 – 6 and MW 40 – 100 kDa. As a result, reduction and alkylation were not performed on acetone precipitated serum samples prior to 2DGE analysis.

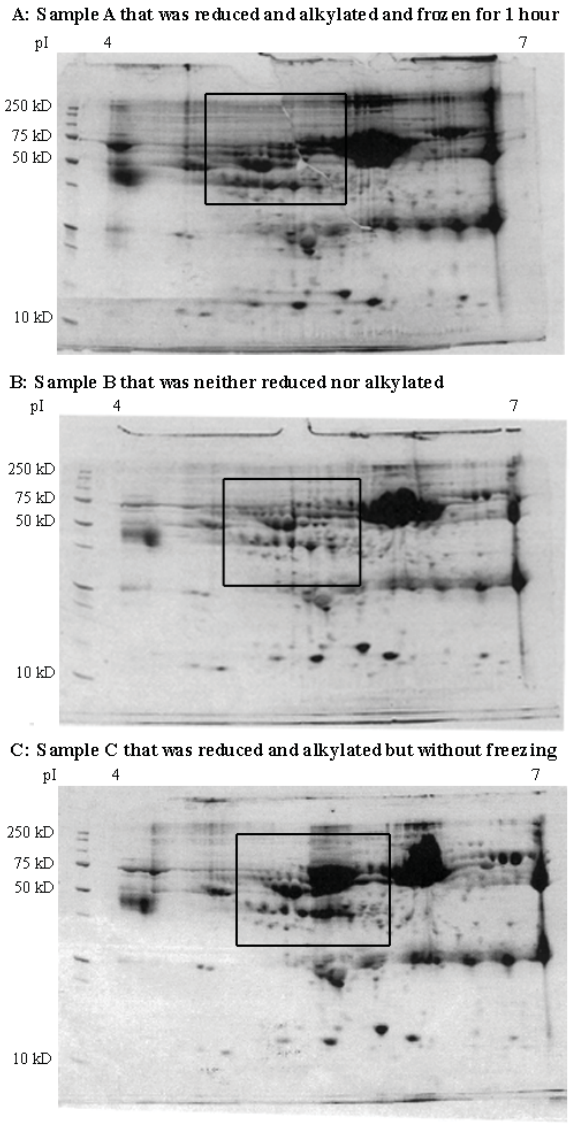


Figure 2.1: 2D gel images of three samples separated in the pI range 4 – 7 and MW range 10 – 250 kDa stained with Sypro Ruby. A : Sample A was reduced and alkylated and frozen for 1 hr. B: Sample B was neither reduced nor alkylated, and C: Sample C was reduced and alkylated and incubated at RT for 1 hr. Acetone precipitation without subsequent reduction and alkylation produced the most distinct spots and best separation across the pI and MW ranges assessed (B).

2.3.2 Testing of optimal pI range for serum proteome anaylsis

Separation of serum proteins in the ranges pI 3-10 and pI 4-7 were assessed to determine the most suitable range of study for this project. IEF was carried out on two acetone-precipitated serum samples using ReadyStrip 11 cm, pI 4-7 and pI 3-10 immobilized pH gradient pre-cast gel (IPG) strips (Bio-Rad, USA, Section 2.5.1.1) (A&C were from sample one and B&D were from sample two, **Figure 2.2**). Second dimension separation was carried out to separate proteins according to MW (Section 2.5.2) and the gels were stained with Sypro Ruby (Section 2.5.3.1) and imaged using the Pharos FX™ Plus Molecular Imager at an excitation wavelength of 532 nm and an emission wavelength of 605 nm (long pass filter).

Separation using the pI 4-7 IPG strip was deemed most suitable for the present study because there was little protein observed outside this range and there was better separation of the serum proteins within this range (**Figure 2.2**). As a result, pI 4-7 IPG strips were used in all 2DGE experiments in this study.

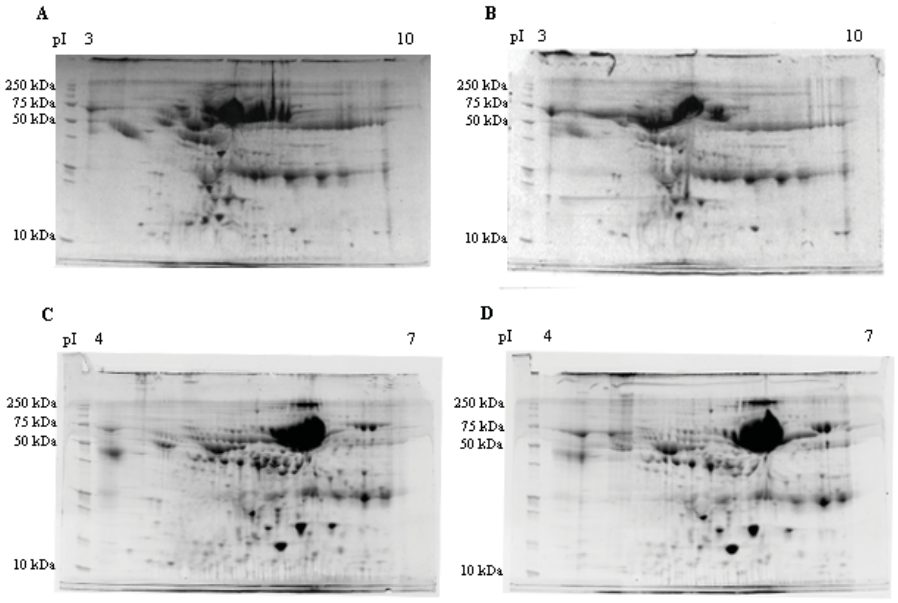


Figure 2.2: 2D gel images of the two acetone precipitated serum samples separated in the pI ranges of 3-10 and 4-7 and MW range 10 – 250 kDa and stained with Sypro Ruby. A & B: Few protein spots were observed at pI < 4 and pI > 7. C & D: Better separation of serum proteins was observed using pI 4 – 7 IPG strips.

2.3.3 Gradiflow MF10 electrophoresis of serum samples

Pooled normal human serum (Innovative Research, Michigan, USA) was used for optimisation of Gradiflow MF10 (Life Therapeutics, Sydney, Australia) electrophoresis for protein fractionation. The protein concentration of the pooled serum sample was 72 μg/μL, as determined by Bradford assay (Section 2.2.1).

Serum proteins were fractionated in the separation unit assembled with six separation chambers (**Figure 2.3**). Each chamber was formed by inserting a specific size restriction membrane: Chamber 1, 5 -1000 kDa; Chamber 2, 1000 – 500 kDa; Chamber 3, 500 -150 kDa; Chamber 4, 150 - 65 kDa; Chamber 5, 65 - 45 kDa; Chamber 6, 45 – 5 kDa (**Figure 2.3**).

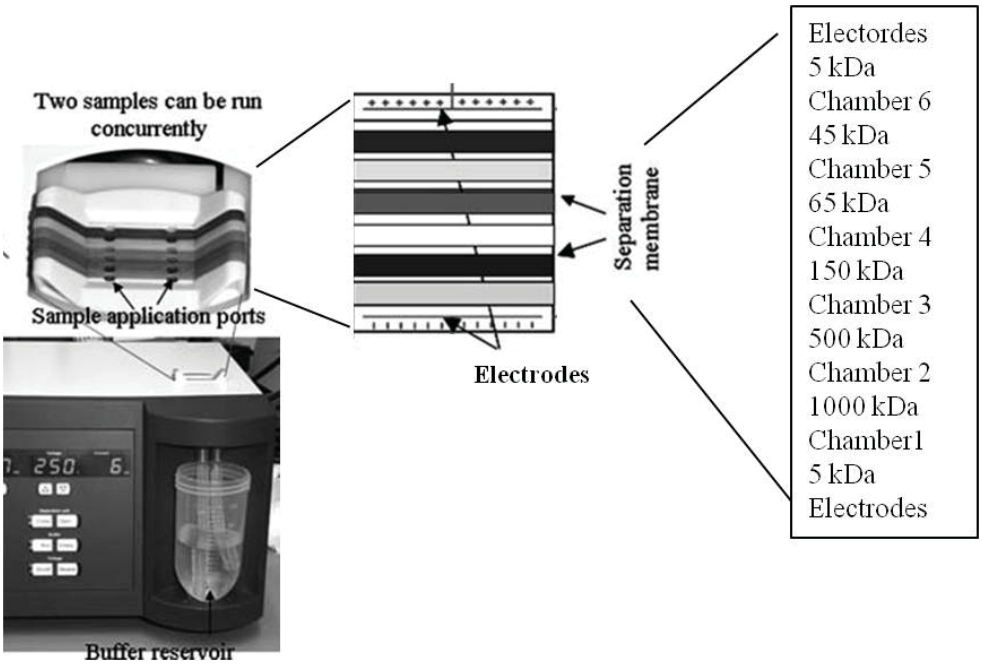


Figure 2.3: Assembly of the MF10 separation chambers using six different pore size membranes: 5 kDa, 1000 kDa, 500 kDa, 150 kDa, 65 kDa and 45 kDa. The figure is modified from Wasinger *et al.*, (2008).

The optimal quantity of serum proteins for fractionation were determined by mixing with 1x Tris/e-amino-n-caproic acid (EACA) separation buffer (0.02M Tris and 0.015M EACA, pH 10.2) to give a final volume of 60 µL. The amount of buffer loaded into each chamber was adjusted to minimise the electro-endoosmosis effect when MF10 was operated for 3 hr (data not shown). Two hundred and fifty milliliters of separation buffer were added into the buffer reservoir to allow its constant circulation throughout the MF 10. Fractionation run time varied according to the amount of protein present (**Table 2.1**). The performance of each run, as indicated by sample loss and separation quality, was assessed using 1DGE analysis (Section 2.4) of the different protein fractions collected from the chambers. All the tests were performed in duplicate.

Initial protein amount loaded to the MF10 was not lower than 390 µg to ensure sufficient protein was collected from each chamber for subsequent protein analysis

(such as 1D and 2DGE). Following this initial testing, however, when several patient serum samples were tested in duplicate, results of this method of fractionation were not at all reproducible (**Figure 2.4**). This method was not used for fractionating serum samples in this study due to the poor quality of results from this low throughput technique.

Table 2.1: Records of each MF10 test run with varying protein content, current and run time. The quality of fractionation that was achieved was dependent on these factors, as noted.

Total protein loaded	Current	Running Time (hr)	Separation	Note*
2400µg	8-10mA	1h30min-2h45min	Poor	Contaminating proteins were found in the 150 -500 kDa chamber as a thick band at a size of approximately 65 kDa in the 1D gel.
1800 µg	8-10mA	2h-3h47min	Poor	The 1DGE of solution collected from the 5 -1000 kDa chamber showed obvious bands less than 1000 kDa indicating poor fractionation quality due to possible aggregation of proteins in the chamber.
1200 µg	4-7mA	2h-3h30min	Poor	Poor reproducibility between runs.
1000 µg	5mA	NA	N/A	Run aborted for unkown reasons both times tested.
810 µg	6mA	1h40min-2h	Poor	The 1DGE analysis of solution collected from the 150 - 500 kDa chamber showed a weak band of approximately 65 kDa indicating insufficient separation.
600 µg	4-9mA	2h30min-3h30min	Good	Good separation only occurred when the running time was ≥ 3 hr. However, buffer in the last chamber overflowed after 3 hr resulting in sample loss.
557 µg	4-6mA	3h	Good	Fractionation was good. However buffer in the last chamber overflowed at the end of the 3 hr run resulting possible sample loss.
480 µg	4-7mA	2h30min	Poor	Contaminating proteins were found in the 150 -500 kDa chamber as a thick band of approximately 65 kDa in the 1D gel
390 µg	4-6mA	3h	Good	Good separation observed from the 1DGE image of each chamber, and no overflow of buffer from chambers. This setting was applied when using patient serum samples.

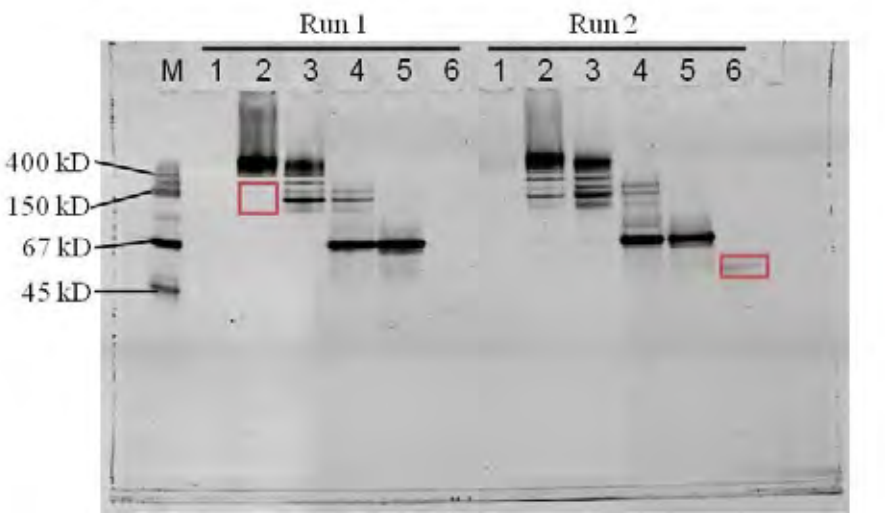


Figure 2.4: A representative 1DGE image of protein fractions collected from the duplicate MF10 electrophoresis of a single patient serum sample. M: Marker. 1: Chamber 1, 5 - 1000 kDa. 2: Chamber 2, 1000 – 500 kDa. 3: Chamber 3, 500 -150 kDa. 4: Chamber 4, 150 – 65 kDa. 5: Chamber 5, 65 – 45 kDa. 6: Chamber 6, 45 - 5 kDa. The band separation in each lane represents the protein separation collected from each chamber, and they should be identical. Obvious differences, particularly the absence of a band in lane 2 (MW ~150 kDa) and in lane 6 (MW ~ 45 kDa) from run 1 compared to run 2 as indicated in the two red rectangular box can be seen, therefore this technique was not used further for fractionation prior to 2DGE in the present study.

2.3.4 Albumin and IgG depletion of serum samples

Serum samples were depleted using a ProteoPrep® immunoaffinity albumin and IgG depletion kit (Sigma-Aldrich, USA) according to manufacturer's instructions. The reproducibility of the depletion was tested by using 50 µL of a pooled normal human serum sample. Briefly, the beads within the column were equilibrated three times by addition of 400 µL equilibration buffer and centrifugation at 5000 *x g* for 10 sec. Samples were mixed with a suitable amount of equilibration buffer to give a final volume of 100 µL and then were applied to the equilibrated column, incubated at RT for 10 min and then centrifuged at 8000 *x g* for 1 min. This step was repeated

once more with the eluant and the column was then washed to ensure complete recovery of unbound proteins. The combined eluant and wash sample was aliquoted into three Eppendorf tubes and stored at -80 °C until used.

The bound proteins were extracted from the column by addition of 150 µL extraction buffer (supplied) and centrifuged at 8000 *x g* for 1 min. This step was repeated and the eluants combined and diluted with MilliQ water in a 1:2 ratio. The diluted bound protein solution was stored in 300 µL aliquots at -80 °C for further analysis. The experiment was repeated twice.

The depleted and bound fractions were analysed using 2DGE (Section 2.5.1.1 and 2.5.2) to check the depletion quality (**Figure 2.5**) and 1DGE (Section 2.4) to check reproducibility (**Figure 2.6**). Significant reduction of proteins in the pI range 5 – 6 and the MW range 50 - 60 was observed (**Figure 2.5**). The reproducibility was reliable as identical band patterns were observed for each run on the 1D gel (**Figure 2.6**). As a result, all serum samples were fractionated using this albumin and IgG depletion kit.

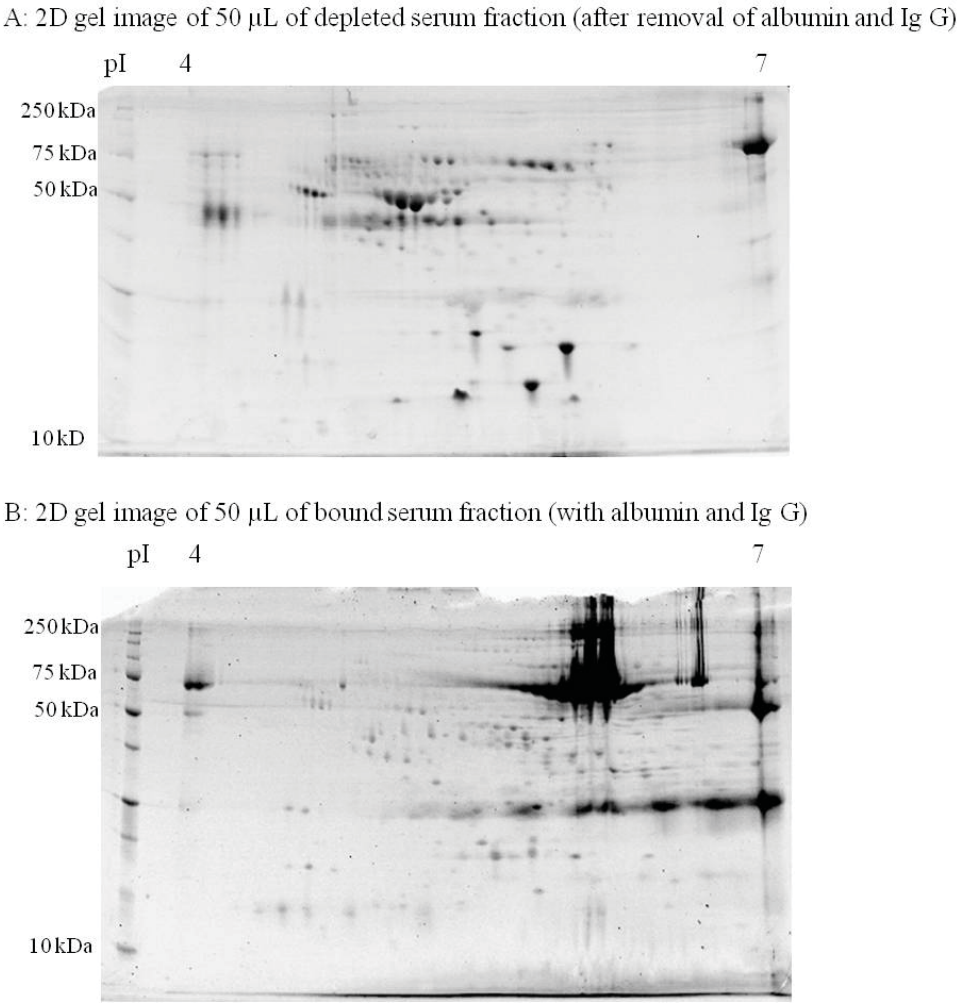


Figure 2.5: Representative 2D gel images of depleted and bound serum sample separated in pI range 4 – 7 and MW 10 – 250 kDa. Image A had significant less proteins in the 50 -75 kDa range compared to image B.

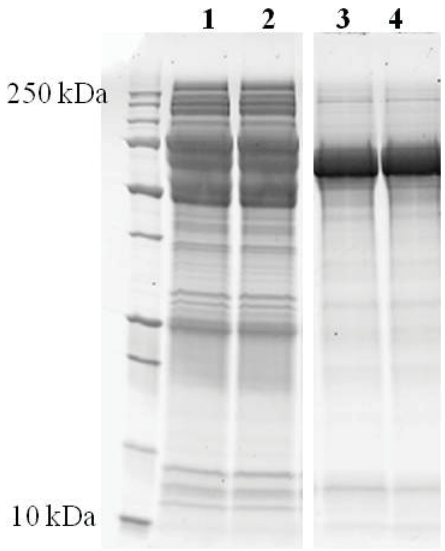


Figure 2.6: 1D separation of the serum sample fractions in the MW range 10 – 250 kDa. Lane 1 and 2 were the duplicate runs of the depleted proteins with albumin and IgG removed by the ProteoPrep® immunoaffinity albumin and IgG depletion kit. Lane 3 and 4 were the extracted serum proteins bound to the ProteoPrep® immunoaffinity albumin and IgG column from the same runs.

2.3.5 Optimisation of tear protein precipitation methods

Precipitation of tear proteins is required prior to 2DGE to ensure samples are free from salts and contaminants and separation is as efficient as possible. Three precipitation protocols were tested to determine the optimal precipitation method. A neat tear sample underwent reduction and alkylation for direct comparison.

2.3.5.1 Reduction and alkylation of neat tear samples

Thirty-five microliters of pooled tear sample was mixed in a ratio of 4:1 with 2.5% v/v TBP in MF solution and reduced for 30 min at RT. Each tear sample was then alkylated with 25% v/v acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution) at a ratio of 14:1 (tear sample : acrylamide) for 40 min at RT. After the reduction and alkylation, the tear sample was labelled as sample A and was analysed by 2DGE immediately.

2.3.5.2 Acetone precipitation

Thirty-five microliters of pooled tears were mixed with 100% ice-cold acetone in a ratio of 1:4 (sample : acetone). These samples were incubated at -20 °C for 1.5 hr to allow protein precipitation and then centrifuged at 24,000 *x g*, 4 °C for 15 min. The supernatant was discarded and the tear protein pellet was air dried and incubated in 140 µL of 2.5% v/v TBP in MF solution for 30 min at RT for reduction of disulfide bonds. The sample was then alkylated with 25% v/v acrylamide (30% v/v Acrylamide/Bis, 29:1 solution, Bio-Rad, USA) at a ratio of 14:1 (tear sample : acrylamide) for 40 min at RT. This was labelled sample B and analysed by 2DGE immediately.

2.3.5.3 TCA precipitation

Thirty-five microliters of pooled tears were incubated with 90% v/v trichloroacetic acid (TCA) in a ratio of 1:4 (sample: TCA) and precipitated at -20 ^{o}C for 2 hr. The precipitated samples were centrifuged at 24,000 *x g*, 4 ^{o}C for 15 min. The supernatant was discarded and the pellet was resuspended in 500 µL 100% ice-cold acetone. Samples were sonicated with a probe sonicator to ensure complete resuspension and then centrifuged again at 24,000 *x g*, 4 ^{o}C for 15 min. The protein pellet was air-dried and incubated in 140 µL of 2.5% v/v TBP in MF colution for 30 min at RT for reduction of disulfide bonds. The sample was then alkylated with 25% acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution) at a ratio of 14:1 (sample : 25% acrylamide/bis) for 40 min at RT. This was labelled sample C and analysed by 2DGE immediately.

2.3.5.4 Chloroform and methanol precipitation

This method is modified based on the protocol developed by (Wessel and Flügge, 1984) to effectively recover proteins from detergents and lipids without additional reduction and alkylation. Six hundred microlilters of methanol were added to 35 µL of pooled tear samples and the sample vortexed. One hundred and fifty microlitres of chloroform was then added and the mixture vortexed, followed by addition of

450 µL of MilliQ water with a final vortex to ensure complete resuspension. The solution was centrifuged at 14,000 *x g* for 5 min and the upper phase discarded. The white precipitate disc and lower phase were mixed with 450 µL of methanol and centrifuged at 14,000 *x g* for 5 min. The supernatant was again discarded and the pellet dried in a CentriVap Centrifugal Vacuum Concentrator (Labconco, Missouri, USA) for 10 min. The dried pellet was resuspended in 100 µL of MF solution and was labelled as sample D and analysed by 2DGE immediately.

2.3.5.5 2DGE analysis of precipitated tear samples

Tear samples A-D were analysed by 2DGE (Section 2.5.1.2 and 2.5.2) to determine which method of precipitation allowed for the most efficient total protein and phosphoprotein separation by pI and MW, detected with Sypro Ruby (Section 2.5.3.1) and Pro-Q diamond (Section 2.5.3.2) respectively.

Decreased protein concentration in the high molecular weight region occurred as a result of TCA precipitation (C, **Figure 2.7**). Both neat tear and TCA precipitated samples (A and C, **Figure 2.7**) had less spots present across the entire gel when compared with all other samples. Both acetone and chloroform methanol precipitated samples showed high protein intensity, and chloroform methanol precipitated samples showed the best combination of both protein spot intensity and separation (B and D, **Figure 2.7**). As a result, chloroform: methanol precipitation was used for all 2DGE analyses in the present study.

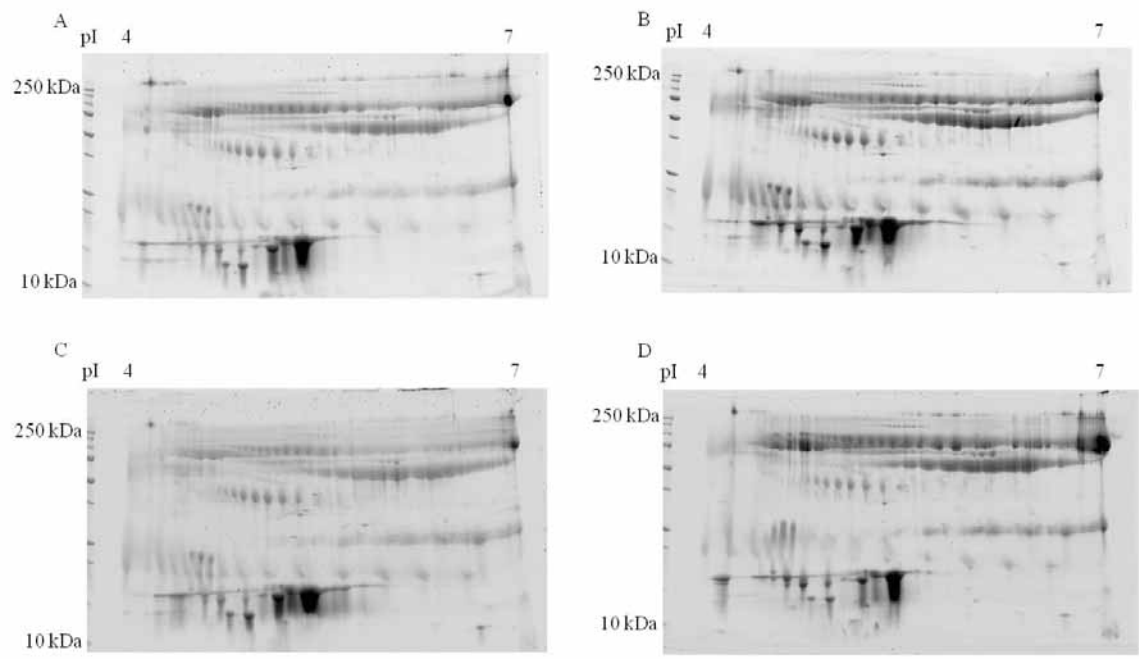


Figure 2.7: Sypro Ruby stained image of 2DGE analysis following precipitation by various methods. A: Pooled tear samples reduced and alkylated with no precipitation, B: pooled tear samples precipitated with acetone, C: pooled tear samples precipitated with TCA, and D: pooled tear samples precipitated with chloroform and methanol.

2.4 1DGE analysis

All 1DGE analyses were carried out using pre-cast 8-16 % Tris-HCl 1D gels (Bio-Rad, USA) in a Criterion Cell tank (Bio-Rad, USA). Sixty millilitres of Tris-Glycine non-reducing running buffer (192 mM glycine and 24.8 mM tris in MilliQ water, final pH~8.2) was poured into the upper buffer chamber of each 1D gel.

For 1DGE of native protein samples, 10 µL of sample was mixed with 10 µL of native loading buffer (62.5 mM Tris-HCl, 40% glycerol (v/v), 0.01% Bromophenol Blue (w/v), pH 6.8, Bio-Rad, USA) and pipetted into a single lane of the gel. Four microlitres of native molecular weight standards (Life Therapeutics, Australia) were pipetted into the marker lanes.

For 1DGE of reduced protein samples, samples were first reduced by mixing with 2X reducing loading buffer (0.3M Tris-HCl, 20% v/v glycerol, 5% w/v SDS, 100 mM DTT and few grains of Bromophenol blue dye to add colour) in a ratio of 1:1 (sample : reducing buffer) and incubated at 100 °C for 10 min. The reduced samples were then loaded into single lanes of a gel and 4 µL of Precision Plus Protein™ Standards (Bio-Rad, USA) were pipetted into the marker lanes.

Tris-Glycine running buffer (192 mM glycine, 24.8 mM tris and 0.1% w/v SDS in MilliQ water, final pH ~8.2) was poured to the maximum fill line marked on the side of the gel tank. Gels were run at 200 V for 1 hr or until the dye front reached the bottom of the gel. Each gel was fixed for 2 hr with 10% v/v ethanol and 7% v/v acetic acid, and then stained with Sypro Ruby (Invitrogen, USA) overnight. The gels were destained with the same fixing solution for 2 hr, and scanned with a Pharos Imager at an excitation wavelength of 532 and an emission wavelength of 605 nm (long pass filter) with 100 µm resolution.

2.5 2DGE analysis

2.5.1 IEF

2.5.1.1 In-gel rehydration of serum samples

Serum proteins were separated according to pI using 11 cm, pI 4-7 IPG strips. Each serum sample was mixed with MF solution to a final volume of 200 µL to rehydrate each IPG strip overnight (detailed in Chapter 3). IEF was conducted using an IPGPhor (GE Healthcare, UK) at 100 V for 2 hr, 300 V for 3 hr, 1000V for 2 hr, 2500 V for 1 hr and 7500 V for 7.5 hr to reach a final of 61,850 VHrs.

2.5.1.2 IEF using cup loading rehydration of tear samples

Precipitated tear proteins were separated according to pI using 11 cm, pI 4-7 IPG strips. The IPG strips were rehydrated with 200 µL of MF solution for a minimum of 6 hr. Two sample cups (GE Healthcare, UK) were then placed on each end of a

rehydrated IPG strip and 50 µL of precipitated tear sample was loaded into each cup for a single strip (100 µL total sample per strip). IEF was conducted using an IPGPhor at 100 V for 2 hr, 300 V for 3 hr, 600 V for 2 hr, 1000 V for 2 hr, 2500 V for 1 hr, 3500 V for 1 hr, 8000 V for 5 hr, to reach a final of 50, 000 VHrs.

2.5.2 Second dimension separation

After completion of IEF, IPG strips were equilibrated with reducing solution (urea 6 M, 2% w/v SDS, 20% v/v glycerol, 65 mM DL-Dithiothreitol) for 15 min, followed by alkylating solution (urea 6 M, 2% w/v SDS, 20% v/v glycerol, 2.5% w/v acrylamide, pH 8.8) for 15 min, each with shaking. The strips were then placed on top of 4-16% gradient Tris-HCl precast 2D gels (11 cm x 20 cm) (Bio-Rad, USA) with the strip in contact with the gel and covered with 350 µL of 0.5% w/v agarose in 0.37 M Tris-HCl (pH 8.8) to secure the strip in place. Sixty milliliters of Tris-Glycine-SDS running buffer (192 mM glycine, 24.8 mM tris and 0.1% w/v SDS in MilliQ water) was poured into the upper chamber of each 2D gel and the gels were run in a Criterion Dodeca Cell tank (Bio-Rad, USA) filled with Tris-Glycine-SDS buffer (approximately 5L) for 1 hr at 200 V or until the dye front reached the bottom of the gel.

2.5.3 Gel imaging

2.5.3.1 Sypro Ruby total protein stain

The 1D and 2D gels were taken out of their gel cassettes and incubated in fixing solution (10% v/v ethanol and 7% v/v acetic acid) for a minimum of 2 hr. Fixed gels were stained with Sypro Ruby overnight and then destained with fixing solution for 2 hr to reduce background staining. Gel images were obtained by scanning the gels using a Pharos Imager (Bio-Rad, USA) with UV-light to excite the fluorescence of Sypro Ruby at an excitation wavelength of 532 nm and an emission wavelength of 605 nm (long pass filter) with 100 µm resolution.

2.5.3.2 Gel staining for phosphoproteins and glycoproteins

Pro-Q Diamond (Invitrogen, USA) was used to stain 2DGE gels specifically for phosphoproteins. Gels were fixed with 250 mL fixing solution (50% v/v MeOH and 10% v/v acetic acid) overnight, and then were incubated with fresh fixation solution for 1 hr to ensure all of the SDS was washed out of the gels. The gels were washed four times for 15 min with 500 mL of MilliQ water, and then incubated in 350 mL of Pro-Q Diamond for 2.5 hr. After staining, the gels were destained with 350 mL of 20% v/v acetonitrile (ACN) and 50 mM sodium acetate (pH 4.0) for 1 hr. The destain step was repeated for 45 min another four times. The gels were then washed twice with 500 mL of Milli-Q water and scanned using a Pharos Imager at an excitation wavelength of 532 nm and an emission wavelength of 605 nm long pass filter with 100 μm resolution. After image acquisition, gels were washed in 3% v/v acetic acid overnight in preparation for Pro-Q Emerald staining of glycoproteins.

All gels to be stained with Pro-Q Emerald 488 (Invitrogen, USA) were oxidised in 160 mL of 0.5% w/v periodic acid/3% v/v acetic acid for 1 hr. The gels were washed three times for 20 min each with 250 mL of 3% v/v acetic acid and then incubated with 85 mL of Pro-Q Emerald 488 for 2 hr. The gels were destained with 250 mL 3% v/v acetic acid for 30 min once, for 45 min twice and then overnight. Spots were visualised by the Pharos Imager at an excitation wavelength of 488 nm and an emission wavelength of 530 nm with 100 μm resolution. After destaining, the gels were stained with Sypro Ruby (Section 2.5.3.1) for comparison of the three staining patterns.

2.5.3.3 Coomassie staining for MS analysis

Gels were stained with Coomassie blue G250 (Sigma-Aldrich, USA, 0.1% w/v in 17% ammonium sulfate, 34% v/v methanol and 3% v/v phosphoric acid) overnight, destained in 6% v/v acetic acid and washed with 1% v/v acetic acid followed by MilliQ water. Destained gels were then ready for mass spectrometry analysis of protein spots.

2.6 2D-DIGE analysis of depleted serum samples

2.6.1 Constitution of CyDye and sample labelling

Minimal labelling Amersham CyDye DIGE Fluors (5 nM Ettan DIGE kit, GE Healthcare, UK) were used to label serum samples according to the manufacturer's instructions. Briefly, CyDye stocks were reconstituted with 5 µL of anhydrous N,N-Dimethylformamide (DMF), 99.8% (Sigma-Aldrich, USA) to make the final dye concentration 1 mM. Stock solution was stored at -20 °C until just before use when a 200 pmol working solution was prepared by further diluting with DMF.

The entire labelling process was carried out on ice in the dark. The pH of samples was checked to ensure it was between 8.5 and 9. Fifty microgram of protein, as determined by Bradford assay (Section 2.2.1), were mixed with 200 pmol of either Cy3 or Cy5 dye for 30 min. Dye swapping was employed such that all samples from a single experimental group were not labelled with the same dye to further limit experimental variation based on differences in labelling efficiency. The labelling reaction was quenched by addition of 1 µL of 10mM lysine (Sigma-Aldrich, USA) for 10 min.

The internal standard was generated by combining equal amounts of every sample to be analysed together with a total volume which would allow it to be run on every gel. This standard was labelled with Cy2 as described above.

All labelled samples were stored in the dark at -80 °C until used and all the samples were used within one month of labelling.

2.6.2 2D-DIGE

Randomly paired Cy3 and Cy5 labelled samples were pooled with an aliquot of the internal control and resuspended in MF solution to a final volume of 200 µL. The pooled samples were separated by pI and MW (Section 2.5.1.1 and 2.5.2) with all

equipment used covered in aluminum foil to ensure the entire 2D separation was carried out in the dark.

2.6.3 DIGE gel imaging

Following 2D-DIGE, the gels were immediately scanned using a Pharos Imager. Multiplex DIGE-Cy2 (excitation wavelength: 488nm and emission wavelength: 530 nm), multiplex DIGE-Cy3 (excitation wavelength: 532 nm and emission wavelength: 605 nm) and multiplex DIGE-Cy5 (excitation wavelength 635 nm and emission wavelength: 695nm) with 100 μm resolution were selected in different channels for continuous scanning of one gel. The images generated were then used for image analysis (section 2.8).

2.7 Large format 2DGE analysis

2.7.1 IEF for large format 2DGE

Serum proteins were separated according to pI using 17 cm, pI 4-7 IPG strips. Each sample (detailed in Chapter 3) was mixed with MF solution to a final volume of 400 μL to rehydrate each IPG strip overnight. IEF was conducted using an IPGPhor to a final of 87,800 VHrs with the optimised program parameters (listed in **table 2.2**). After IEF, the strips were stored in -80°C until second dimension separation.

Table 2.2: IPGPhor IEF Program Settings for 17 cm IPG strips.

Steps	Voltage	Hours
1	100	1 hr at gradient
2	100	2 hr at step-n-hold
3	300	1 hr at gradient
4	300	2 hr at step-n-hold
5	1000	1 hr at gradient
6	1000	2 hr at step-n-hold
7	2500	1 hr at gradient
8	2500	1 hr at step-n-hold
9	7500	11 hr at step-n-hold
		Final 87,800 VHrs

2.7.2 Large format 2DGE

Large format 2D gels were cast manually using the PROTEAN® II Xi Multi-Gel Casting Chamber (Bio-Rad, USA) to produce gels 18.5 cm × 20 cm × 0.1 cm (width x height x thickness). The separation gel was prepared with a solution of 12% v/v acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution), 0.375 M Tris-HCl, 0.1 % w/v SDS, 0.05% w/v ammoniumpersulfate (APS) (Bio-Rad, USA) and 0.05% v/v PlusOne™ Tetramethylethylenediamine (TEMED) (GE Healthcare, UK). The separation gel solution was then poured into the chamber, overlaid with approximately 2 mL of water-saturated butanol (butanol : water, 4.5 : 1), and left for at least 2 hr to polymerize. After the gel had polymerized, the butanol was poured from the top of the gel and a stacking gel (4% v/v acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution), 0.125 M Tris–HCl, 0.1 % w/v SDS, 0.05 % w/v APS and 0.01% v/v TEMED) was carefully poured on top of the separation gel and left to polymerise as for the stacking gel.

IEF strips were thawed at RT and then equilibrated with reducing solution (urea 6 M, 2% w/v SDS, 20% v/v glycerol, 65 mM DL-Dithiothreitol) for 15 min, and then alkylating solution (urea 6 M, 2% w/v SDS, 20% v/v glycerol, 2.5% v/v acrylamide/bis (30% v/v acrylamide/bis, 29:1 solution), pH 8.8) for 15 min, with shaking. During equilibration, the second dimension gel sandwiches were removed from the chamber and rinsed with water to remove excess gel. Clamps were put on each side of the gel sandwich, the equilibrated strips were placed carefully onto the top of each gel and held in place with boiling 0.5% w/v agarose (Sigma-Aldrich, USA) in tris-glycine-SDS running buffer (192 mM glycine, 24.8 mM tris, 0.1% w/v SDS in MilliQ water and few grains Bromophenol blue). When the agarose buffer set, the gels were placed into the PROTEAN II Xi multi-cell containing 5 L of Tris-Glycine-SDS running buffer. The upper buffer chambers of the gels were filled with fresh Tris-Glycine-SDS running buffer. Precision Plus Protein™ Standards (10 μL for each gel) were run on the side of each gel and gels were run at 250 V and 10 mA/gel for 1 hr and then 40 mA/gel until the dye front reached the bottom of the gel.

2.7.3 Gel staining and analysis

Gels were stained with Sypro Ruby (Section 2.5.3.1) and then destained with 7% v/v ethanol and 10% v/v acetic acid overnight and stained with Coomassie blue (Section 2.5.3.3) for MS analysis (Section 2.9).

2.8 Analysis of differentially detected protein spots using 2DGE

Gel images were analysed using the Non-linear Progenesis SameSpots version 3 (Nonlinear Dynamics Ltd., UK). For 2DGE gel analysis, the gel with the highest number of detected spots was used as the reference for normalisation. For 2D-DIGE analysis, only Cy2 labelled gels were used as the reference for normalisation. All other gels in an analysis were aligned and spots were matched with the reference gel. After grouping gels into their experimental groups, the Progenesis software used an in-built algorithm to do a discrimination analysis to automatically calculate the differences in abundance of matched spots between different groups, and one way ANOVA was used to determine the p value of these differences in abundance between groups for each spot. Each spot profile was then checked manually and statistically significant spots were selected. A report containing details of the spots of interest and a “picking” gel were generated and these spots underwent mass spectrometry for protein identification.

2.9 Mass spectrometry for protein identification

Mass spectrometry was performed for protein identification of spots of interest from Coomassie stained 2D gels of depleted serum samples (following 2D-DIGE analysis) and precipitated tear samples (following analysis of PTMs).

2.9.1 Spot cutting and digestion

Coomassie stained gels were washed with MilliQ water to remove excess Coomassie stain. Protein spots of interest were excised using a sterile scalpel and were dehydrated in Eppendorf tubes with 100% ACN for 10 min and then dried

completely using a CentriVap Centrifugal Vacuum Concentrator (Labconco, USA). Gel pieces were rehydrated at 4 ^{o}C for 45 min with 50 ng trypsin (Promega, Madison, USA) in 50 mM ammonium bicarbonate (AMBIC, pH 9), and digested overnight at 37 ^{o}C. The following morning supernatant that remained was pipetted into clean Eppendorf tubes for full recovery of peptides. The remaining peptides in each gel piece were extracted with one 20 min wash of 20 mM AMBIC and three 20 min washes of 5% v/v formic acid (FA)/50% v/v ACN. Supernatant was collected after each wash and combined with the supernatant recovered after the overnight digestion. The pooled supernatant containing tryptic peptides from a single gel piece was dried completely using a CentriVap Centrifugal Vacuum Concentrator.

2.9.2 Stage tipping

Dried peptide samples were resuspended in 5% v/v FA and then purified using StageTips, C18, 200 µL tips (Proxeon, Odense, Denmark) as per manufacturers' instructions. Briefly, each Stage Tip was initialised with 20 µL of 50% v/v ACN and 5% v/v FA and then re-equilibrated with 20 µL of 5% FA. The sample was loaded into the Stage Tip and slowly passed through the tip to allow peptide binding to the C18 column. The tip was washed with 20 µL of 5% v/v FA twice and then the sample was eluted into a clean Eppendorf tube with 20 µL of 80% v/v ACN and 5% v/v FA. The eluate was then dried completely in the CentriVap Centrifugal Vacuum Concentrator and frozen at -80 °C for further use.

2.9.3 Protein identification

Protein digests were analysed at the Bioanalytical Mass Spectrometry Facility (BMSF), UNSW, using a LTQ-FT Ultra mass spectrometer (Thermo Electron, Germany).

Peptides were resuspended in 5 µL of buffer A (2% v/v acetic acid, 0.1% v/v FA) and separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Netherlands) and then concentrated and desalted onto a micro C18 pre-

column (500 μm × 2 mm, Michrom Bioresources, USA) with 0.05% v/v heptafluorobutyric acid (HFBA) at 20 μL/min. After a 4 min washing the pre-column was automatically switched (Valco 10 port valve, Houston, Texas, USA) into line with a fritless nano column manufactured according to Gatlin *et al.* (1998). Mobile phase buffers A and B were prepared (A: 2% v/v CH_3CN/ 0.1% v/v FA/98% deionised water and B: 80% v/v CH_3CN/0.1% v/v FA/20% v/v deionised water) and peptides were eluted using a linear gradient of A:B 98:2 to 50:50 over 30 min at a flow rate of ~300 nL/min and then washed with 100% B for 1 min at a flow rate of ~300 nL/min. The LTQ-FT was operated as described previously (Ly and Wasinger, 2008). Peak lists were generated using 'Mascot Daemon/extract_msn' (Matrix Science) using default parameters and submitted to the protein database search program Mascot (Matrix Science, Boston, USA).

All MS/MS spectra were searched against the human non-redundant NCBI database (2010) using Mascot (www.matrixscience.com). In-gel digests analysed on the LTQ-FT Ultra were searched with the following criteria: trypsin digestion, precursor and product ion tolerances ± 4 ppm and ±0.4 Da, respectively; variable modifications of methionine oxidation, carbamidomethyl and acrylamide; and one missed cleavage allowed. Identifications were accepted based on the MOWSE scores with a score > 44 indicating significant homology ($p < 0.05$). A search using a reverse database was also performed to determine the false positive rate of identifications according to (Peng *et al.*, 2003) and to increase confidence of protein identifications.

2.10 SELDI-TOF-MS Analysis

2.10.1 SELDI chips

The reverse phase surface (H50) SELDI chip (Bio-Rad, USA) was used for SELDI-TOF-MS experiments, and this was based on the quality of spectra produced in preliminary comparisons with weak cation exchange surface and strong anion exchange surface chips (data not shown).

2.10.2 Sample preparation

H50 SELDI chips were pretreated according to the manufacturer's instructions. Forty four tear samples were analysed in total and were applied to a chip in duplicate. One microlitre of tear sample was mixed with four microlitres of H50 binding buffer (10% v/v ACN and 0.1% v/v trifluoroacetic acid (TFA)). Each spot was washed twice for 2 min with 5 μL of binding buffer. After washing, 5 μL of prepared sample was pipetted carefully on to each spot and the chips were incubated in a humidity chamber for 30 min on an OM7 orbital mixer (Ratek Instruments Pty Ltd., Australia). Each spot was then washed three times for 2 min with 5 μL of binding buffer and air-dried for 5 – 10 min. One microlitre of the energy absorbing saturated sinapinic acid solution (Sigma-Aldrich, USA) was applied to each spot and the chip left to dry for 5 min. This step was repeated once more and the chip was then stored in a 15 mL Falcon tube wrapped covered in foil at RT until analysed. Analysis was performed within 24 hr of sample binding and was completed at Royal North Shore Hospital (NSW, Australia).

2.10.3 Data Acquisition and Preprocessing

Each SELDI chip was analysed on a PBS-IIc ProteinChip Reader using the ProteinChip Software version 3.2 (Ciphergen Biosystems Inc., USA). The samples were read with the optimised setting which had a laser intensity of 200, a deflector setting of 1,500 Da, a detector sensitivity of eight and a molecular mass detection range of 2,000 to 200,000 Da. The raw data were transferred to the CiphergenExpress Data Manager Software version 2.1 (Ciphergen Biosystems Inc, USA) for analysis. The baseline was subtracted using the default setting which was 12 times the expected peak width. The spectra were calibrated with external calibrants (bovine insulin, 5,733.6 Da; ubiquitin, 8,654.8 Da; cytochrome *c*, 12,360.2 Da), and spectral intensities were normalized by total ion current (TIC). In this study, the low mass cutoffs were 1500 m/z, and spectra with normalization factors that were 100% above the mean were deleted if their normalization factors

were more than double the value calculated for the replicate spectrum. In the case where both spectra were above the cutoff, both spectra were kept. At least one replicate was retained for every sample. Automatic peak detection was performed using the settings of five times the signal-to-noise ratio for the first pass of peak detection and two times the signal-to-noise ratio for the second pass. The Biomarker Wizard feature in the CiphergenExpress Data Manager Software was used to generate peak clusters and only the peak clusters that have a p value lower than 0.05 were selected for further evaluation.

2.11 MRM analysis

2.11.1 Sample preparation for MRM

Tear samples were digested with sequencing grade modified trypsin (Promega, USA) in a ratio of 50 : 1 (protein : trypsin, w/w) overnight at 37 °C (detailed in Chapter 4). Five microliter of 5% FA was added to stop the reaction (final pH ~ 3), and the digested samples were dried completely in a CentriVap Centrifugal Vacuum Concentrator (Labconco, USA) and purified using C18, 200 μL StageTips (Proxeon, Denmark) (Section 2.9.2). The purified samples were dried again using a CentriVap Centrifugal Vacuum Concentrator and stored at -80 °C until use.

2.11.2 Constructing MRM transition lists using Skyline

The FASTA formatted sequences of target proteins were directly pasted to the Skyline program Skyline v0.5 (MacCoss Lab, University of Washington, Seattle, USA) which generated a full list of peptides and corresponding product ions. Based on the MRM precursor and product ions selection criteria (**Table 1.3**, Chapter 1), and the previous MS/MS spectra of the target proteins, several peptides and product ions were selected for each target protein to form a transition list (detailed in Chapter 4). The transition list also included the appropriate collision energy and declustering potential which was determined by Skyline based on the equipment used for MRM analysis. The dwell time 50 ms for quantitation and 25 ms for verification, was also added to the transition list file. The list was then saved as a

*.csv format and exported into the 4000 QTRAP hybrid triple quadrupole linear ion trap (LIT) mass spectrometer (Applied Biosystems, USA).

2.11.3 Using MRM for peptide validation

This work was performed at BMSF by Dr. Ling Zhong, on a 4000 QTRAP hybrid triple quadrupole LIT mass spectrometer interfaced with a nanospray ion source and operating in positive ion mode. Prepared samples were concentrated and desalted onto a micro C18 precolumn (500 μm x 2 mm, Michrom Bioresources, USA) with H_2O:CH_3CN (98:2, 0.05 % v/v TFA) at 15 μl/minute. After 4 min washing, the pre-column was automatically switched (Valco 10 port valve, Houston, USA) into line with a fritless nano column manufactured according to Gatlin *et al.*, (1998). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1 % v/v FA) to H_2O:CH_3CN (36:64, 0.1 % v/v FA) at ~300 nl/min over 30 min. The pre-column was connected via a fused silica capillary (25 cm, 25 μm) to a low volume tee (Upchurch Scientific, USA) and introduced into the 4000 QTRAP mass spectrometer. Samples were analyzed with an ion spray voltage of 2.4 kV, curtain gas flow of 12 and nebulising gas flow of 5. For MRM analysis, quadrupoles were operated in low resolution, and the dwell time was 25 ms. For validation runs, the MRM experiment triggered subsequent MS/MS spectral acquisition. MS/MS spectra were acquired in trap mode (enhanced product ion) with dynamic fill time. Q1 was operated in low resolution.

2.11.4 Using MRM for peptide quantitation purpose

MRM for peptide quantitation was similar to that described for peptide validation, however the dwell time was set at 50 ms which was optimised for the 4000 QTRAP hybrid triple quadrupole LIT mass spectrometer. The files generated by MRM were imported to Skyline open source program for quantitation analysis. The Skyline v0.5 program automatically generated the chromatogram, retention time and peak area for each peptide and its corresponding product ions.

2.12 Targeted protein analysis

Changes in abundance of proteins observed using 2D-DIGE were investigated using targeted proteomic techniques.

2.12.1 Cobas® 6000 Bromcresol purple (BCP) albumin test

Unfractionated serum samples (detailed in Chapter 3), each with a required volume between 200 and 250 µL, were sent to Biochemistry Laboratory, South Eastern Area Laboratory Service (SEALS), St George Hospital for analysis with the help of clinical projects coordinator, Keith Westbury. The albumin concentrations were measured by laboratory staffs in SEALS using the automatic clinical chemistry analyser Cobas® 6000 (Roche diagnostics, USA) with BCP (Roche diagnostics, USA).

2.12.2 Human Fetuin A ELISA

The concentration of Fetuin A in unfractionated serum was determined using a Human Fetuin A ELISA kit (BioVendor, Czech Republic) according to the manufacturer's instructions. Reagents including dilution buffer, wash solution, quality controls and master standard were prepared as according to a supplied protocol. Serum samples were diluted (detailed in Chapter 3) and 100 µL of diluted standards, quality controls and samples were pipetted into the appropriate wells of the ELISA plate in duplicates. The plate was incubated for 1 hr on the OM7 orbital mixer at 40 rpm at RT, and then washed three times with wash solution. Excess wash solution was completely removed and 100 µL of conjugate solution was pipette carefully into each well and the plate incubated at RT for 1 hr on the orbital shaker (40 rpm). The plate was washed as before three times and excess wash solution removed completely. One hundred microlitres of substrate solution was carefully pipetted into each well and the plate incubated in the dark for 10 min or until a blue colour developed. The reaction was stopped by addition of 100 µL of stop solution into each well and the plate was read at 450 nm by Kinetic Microplate Reader within 5 min of addition of stop solution. Concentrations of protein samples

were determined based on the standard curve obtained and statistical analysis was carried out to assess differences between sample groups (detailed in Chapter 3). This experiment was done in duplicate.

Chapter 3 Finding diagnostic biomarkers for CaP in serum using proteomic techniques

3.1 Introduction

PSA is the only FDA approved biomarker for CaP, but it is widely recognised that this biomarker is limited in its diagnostic utility. As a result, a lot of men go through unnecessary biopsy tests, which are not only unpleasant but also place needless burden on limited medical resources. Whilst other molecules have been investigated in relation to CaP diagnosis (reviewed in Section 1.3), no effective diagnostic marker has yet been validated for clinical use. Discovery of biomarkers that can accurately and non-invasively detect CaP are urgently needed to aid early detection and allow appropriate treatment, thereby reducing the related rates of morbidity and mortality.

Blood has long been considered a valuable source of information regarding the physiological state of an organism due to its constant circulation to all organs and tissues in the body. The proteins that are contained within a blood sample, or its derivatives plasma and serum, can provide an insight into the metabolic, physiological and pathophysiological processes of an individual at the time of blood withdrawal (Tammen *et al.*, 2005). Although platelet-depleted or citrate plasma is preferred by the HUPO / PPP Specimens Committee (Tammen *et al.*, 2005), serum was used in this study because it is the sample type most often collected and stored for clinical analyses. Whilst the serum proteome can be a source of diagnostic, prognostic and therapeutic markers, the complexity of the serum protein profile in combination with the limitations of conventional proteomic techniques such as 2DGE have restricted the rate of biomarker discovery.

In this study, fractionation of serum samples was performed to simplify the complex proteome by depletion of the highly abundant proteins albumin and IgG. Each fraction was then separated by either large format 2DGE (albumin and IgG bound

fraction) or 2D-DIGE (albumin and IgG depleted fraction) to detect protein spots and potential CaP biomarkers (**Figure 3.1**). Mass spectrometry was used to identify potential CaP biomarkers, followed by targeted protein assays such as ELISA. This step allowed verification of gel based results as well as quantitation of protein concentration. Quantitation of the potential biomarkers allows evaluation of their diagnostic utility, indicated by sensitivity and specificity, within a small sample size. The work presented in this chapter moves from full proteomic screening of serum to targeted quantitation of potential and novel CaP biomarkers.

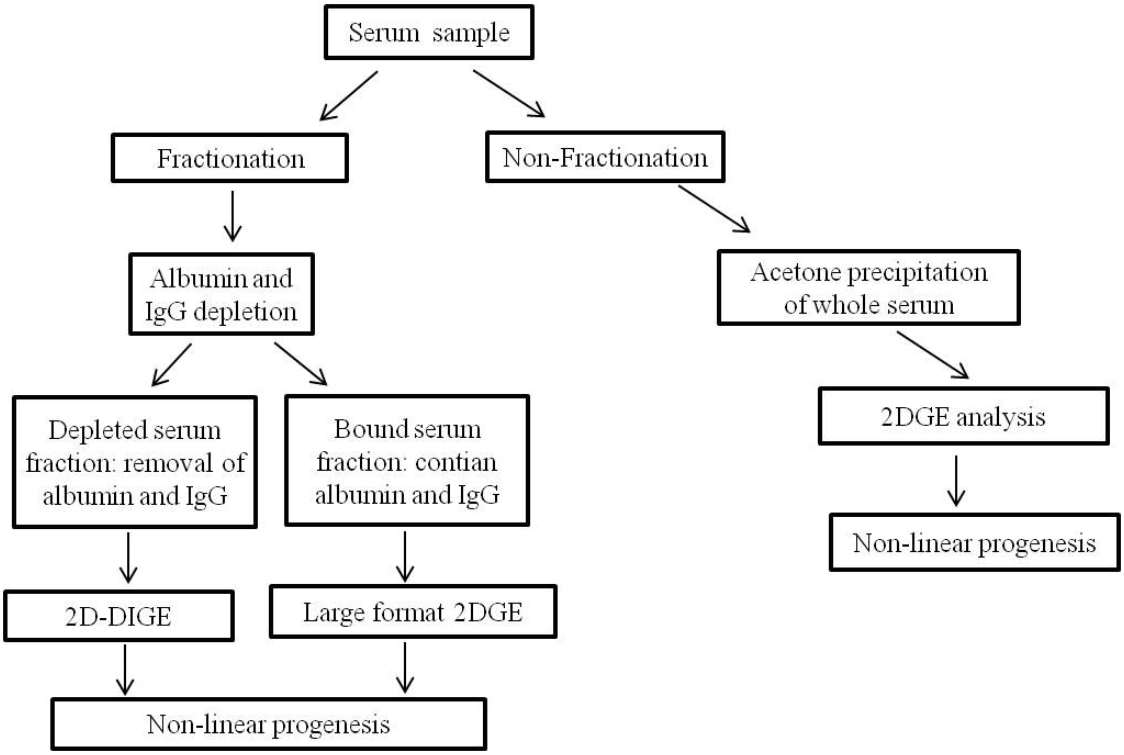


Figure 3.1: Schematic chart showing the gel based proteomics approach that was used to analyse serum for protein biomarkers in order to differentiate between CaP, BPH and control samples. Serum that was unfractionated was analysed using traditional 2DGE. Fractionated serum resulted in a depleted fraction (with albumin and IgG removed) and a bound fraction (with albumin and IgG) which were subject to 2D-DIGE and large format 2DGE, respectively, for increased coverage of the proteome.

3.2 Methods and Materials

The following methods have been described in Chapter 2:

- Serum sample collection (Section 2.1.1)

  Different sample numbers were required for the different methods of serum proteome analysis (**Table 3.1**). All the samples were collected from patients who have been fasting for 12 hours.

Table 3.1: The number of samples in each group (CaP, BPH and control) used for different analyses.

Method of analysis	CaP serum samples	BPH serum samples	Control serum samples
2DGE (Unfractionated serum)	6	6	n/a
2D-DIGE (Depleted serum)	15	15	15
Large format 2DGE (Bound serum)	5	5	n/a
Cobas 6000® BCP albumin test (Unfractionated serum)	15	13*	15
Fetuin A ELISA (Unfractionated serum)	8	8	7

*: Albumin concentration was measured in 15 BPH samples, however two of the patients were diagnosed with CaP at a date after sample collection, therefore their results were not included in this study. The samples used for albumin and fetuin A assays were not the same as used in 2D-DIGE experiment, and these two excluded samples were not used in 2D-DIGE experiment.

- Acetone precipitation (Section 2.2.3)

- 2DGE analysis of unfractionated acetone-precipitated serum samples (Section 2.5.1 and Section 2.5.2)

- Sypro Ruby Staining (Section 2.5.3.1)

- Image analysis (Section 2.8)

- Albumin and IgG depletion of serum samples (Section 2.3.4)

- Large format 2DGE analysis of albumin and IgG serum fractions (Section 2.7)
  Six hundred micrograms of each bound serum fraction was mixed with MF solution to a final volume of 400 μL. Protein concentration was determined using the standard Bradford assay (Section 2.2.1). Protein spots which separated at a pI and MW different to those of albumin and IgG were excised from the Coomassie stained gels and identified by MS. This experiment was repeated twice.

- 2D-DIGE analysis of depleted albumin and IgG depleted serum samples (Section 2.6)
  Samples were labelled and paired so as to maximise dye swapping within each group (**Table 3.2**).

Table 3.2: Samples were labelled with Cy3 and Cy5, the internal standard was labelled with Cy2. Each group contained samples labelled with each of Cy3 and Cy5 to minimise error due to differences in labelling efficiency or detection.

Gel#	Cy3	Age	Cy5	Age
Gel1	C1 (control)	51	85 (BPH)	62
Gel2	C2 (control)	51	89 (BPH)	74
Gel3	C3 (control)	64	84 (BPH)	68
Gel4	C6 (control)	49	75 (BPH)	65
Gel5	C7 (control)	54	537 (CaP)	57
Gel6	C10(control)	52	327 (CaP)	68
Gel7	C12(control)	56	121 (CaP)	60
Gel8	87 (BPH)	65	595 (CaP)	68
Gel9	456 (BPH)	75	C13 (control)	66
Gel10	94 (BPH)	66	C14 (control)	78
Gel11	471 (BPH)	70	C15 (control)	54
Gel12	74 (BPH)	64	C16 (control)	49
Gel13	134 (BPH)	66	146 (CaP)	73
Gel14	88 (BPH)	77	566 (CaP)	65
Gel15	552 (CaP)	72	151 (CaP)	61
Gel16	169 (CaP)	66	86 (BPH)	63
Gel17	425 (CaP)	62	95 (BPH)	77
Gel18	539 (CaP)	67	59 (BPH)	72
Gel19	157 (CaP)	62	424 (BPH)	63
Gel20	114 (CaP)	70	C20 (control)	59
Gel21	263 (CaP)	56	C18 (control)	60
Gel22	491 (CaP)	63	C19 (control)	68
Gel23	491 (CaP)	63	C17 (control)	50

- MS identification of protein spots of interest

  One depleted serum sample from each group (319.2 µg protein per sample) was separated by 2DGE (Section 2.5.1.1 and 2.5.2) and stained with Coomassie blue (Section 2.5.3.3). Protein spots of interest were excised and identified by MS (Section 2.9).

- Targeted protein assays

  A Cobas® 6000 BCP albumin test (Section 2.12.1) and fetuin A ELISA (Section 2.12.2) were performed using unfractionated serum samples. Cobas® 6000 BCP albumin test was performed by an accredited laboratory SEALS. Only samples for which the average fetuin A concentration across their duplicates had CV% less than 10% were selected for further statistical analysis.

- Frequency Histogram

  Distributions of albumin and fetuin A concentration in the three sample groups (CaP, BPH and control) were displayed using frequency histograms to allow visual detection of different trends in concentration.

- Statistical analysis of the diagnostic utility of serum albumin and fetuin A

  The one tailed Student's t-test adjusted with Dunn-Bonferroni correction for multiple comparison ($p < 0.017$ was considered to be significant) was used to determine whether patient's age was significantly different between the sample groups used for both albumin and fetuin A assays.

  The albumin concentrations of control, BPH and CaP groups with and without age adjustments were compared using post-hoc analysis with Dunn-Bonferroni correction for multiple comparisons and the difference was significant when $p < 0.05$. The analysis was performed using the software SPSS (Statistical Package for the Social Sciences) by Dr. Varghese Thomas, biostatistician in the Brien Holden Vision Institute.

Concentrations of fetuin A were compared between CaP, BPH and control groups using the two tailed Student's t-test adjusted with Dunn-Bonferroni correction for multiple comparisons. Statistically significant differences were those for which the test returned a result of $p < 0.017$.

Receiver operation characteristic (ROC) curves were used to determine the diagnostic accuracy of albumin and fetuin A based on their concentrations. The optimal cutoff threshold for each protein was that which produced the maximum combination of sensitivity and specificity (Weinstein *et al*., 2005).

- Evaluation of the association between Gleason score, administered therapeutics and serum albumin/fetuin A concentration

  Information regarding the Gleason score and administered therapeutics in control, BPH and CaP subjects were recorded for those whom this data was available (patient data obtained following the terms of the approved Ethics Protocol). These data were arranged in relation to the serum albumin/fetuin A concentration of each patient using a tabular display for comparison purpose.

- Mass spectrometric analysis of protein spot 11

  The *.raw files obtained from FT-MS/MS analysis of the protein spot 11, identified as fetuin A and cut from 2D gels from each control, BPH and CaP samples, were subjected to Mascot search with the variable modification option, phosphorylation of S, T, and Y amino acids selected. The MS data file was also analysed in Thermo Xcalibur, v.2.1.0.1139 (Thermo Fisher Scientific, USA) searching for the neutral fragment 98 Da (H3PO4) which results from the loss of the phosphate group during collision-induced dissociation (CID).

3.3 Results

3.3.1 2DGE analysis of unfractionated serum

2DGE analysis of unfractionated serum was carried out to detect differences in the abundance of individual protein spots between CaP and BPH samples. No protein spots were significantly altered in abundance between the CaP samples (A-F, **Figure 3.2a**) and the BPH samples (G-L, **Figure 3.2b**). However, a large area of the gel could not be analysed due to the high concentration of albumin which obscured other protein spots and distorted surrounding regions of the gel (**Figure 3.2**).

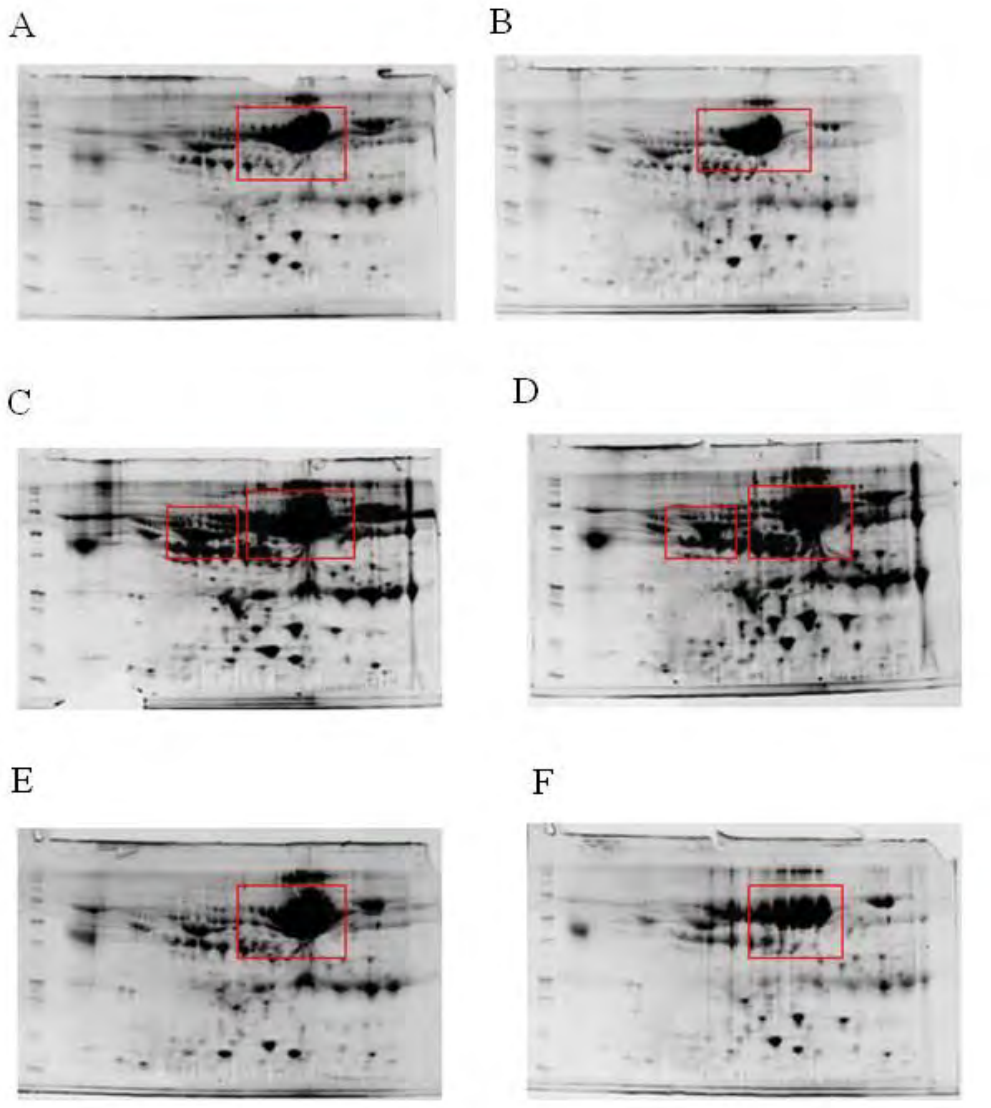


Figure 3.2a: The 2D gel images of unfractionated CaP serum samples (A-F). The red rectangle highlights the area of the gel to which albumin migrates causing distortion of surrounding areas; neither albumin nor the protein spots within this field could be accurately assessed for differences in abundance.

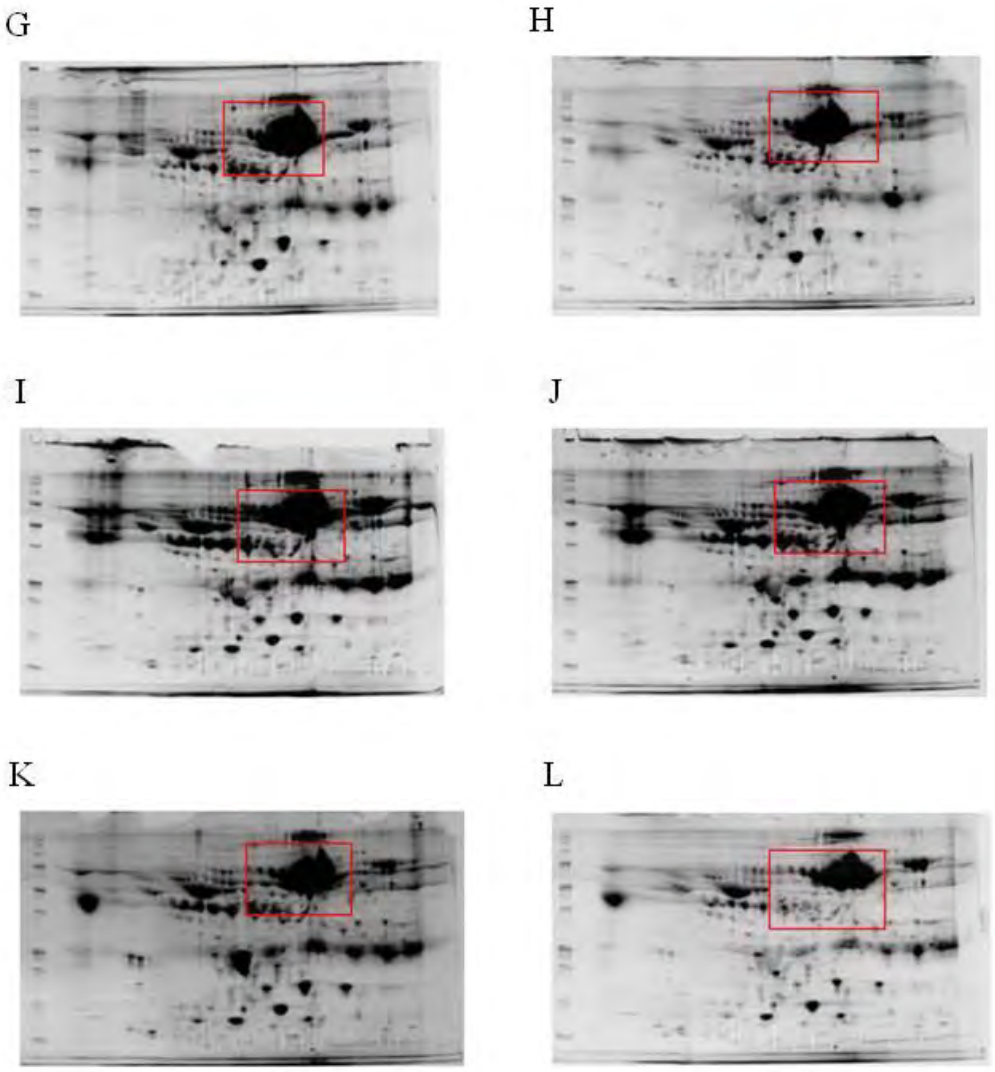


Figure 3.2b: The 2D gel images of unfractionated BPH serum samples (G-L). No protein spots were detected as significantly altered in abundance following comparison between CaP (A-F) and BPH (G-L) serum samples. The red rectangle highlights the area of the gel to which albumin migrates causing distortion of surrounding areas; neither albumin nor the protein spots within this field could be accurately assessed for differences in abundance.

3.3.2 Proteomic analysis of serum fractions

3.3.2.1 Large format 2DGE analysis of the albumin and IgG bound fraction

Analysis of the albumin and IgG bound fraction allowed detection and identification of proteins that co-depleted, as well as detection of any significant differences in abundance of these proteins between the BPH and CaP groups.

One protein spot from each of the other major trains, distinct from the albumin and IgG region, were identified to determine which other proteins were removed concurrently with albumin and IgG (**Figure 3.3**). Eight protein spots were identified as four individual proteins (albumin, alpha-1-antitrypsin (A1AT), complement component 3 (C3) and haptoglobin (HPT)) (**Table 3.3**). Two spots from the lower molecular weight region (spots 9 and 10, **Figure 3.3**) could not be identified, most likely due to their low concentation present.

Similar to whole serum analysis, the albumin area of each gel was unable to be aligned. Spots which were aligned successfully in all other regions of the gel showed no significant difference in abundance ($p < 0.05$, and fold change ≥ 2) between CaP and BPH groups.

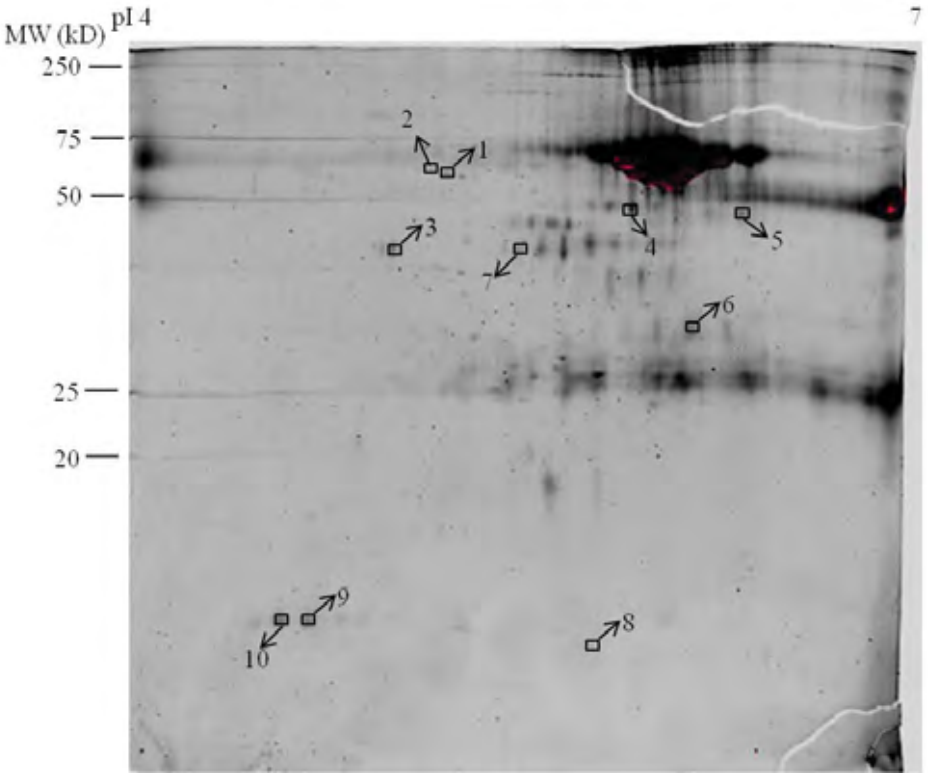


Figure 3.3: Albumin and IgG were depleted from serum samples, other proteins were also removed during this fractionation and ten protein spots were identified to confirm this.

Table 3.3: Eight protein spots from the bound fraction of serum samples were identified as four proteins, confirming concurrent removal of proteins other than IgG and albumin during the depletion process.

Spot ID	Identification	MOWSE score	Sequence coverage	Theoretical MW (kD)/pI	NCBI accession #
1	A1AT	94	18%	46.7/5.37	gi|1703025
2	A1AT	110	12%	46.7/5.37	gi|1703025
3*	C3	125	6%	187.0/6.02	gi|119370332
4#	Albumin-like	117	58%	53.4/5.69	gi|763431
5#	Albumin	120	14%	69.3/5.92	gi|113576
6#	Albumin	59	14%	69.3/5.92	gi|113576
7	HPT	70	42%	38.95/6.27	gi|1212947
8*	Albumin	147	5%	69.3/5.92	gi|113576
9^	Not identified				
10^	Not identified				

* Fragment of whole protein detected

Identification of other proteins/peptides present obscured by presence of albumin through gel

^ Only keratin and trypsin contaminants were detected. The amount of protein was most likely too low to detect.

3.3.2.2 2D-DIGE analysis of depleted serum samples

Spot discriminant analysis using the Progenesis software was performed to detect protein spots present at significantly different levels of abundance ($p < 0.05$, fold change ≥ 1.5) for the following comparisons: CaP versus BPH, CaP versus control and BPH versus control. Statistical analysis of differences between the three Gleason score groups (Gleason score < 7, Gleason score =7 and Gleason score >7)

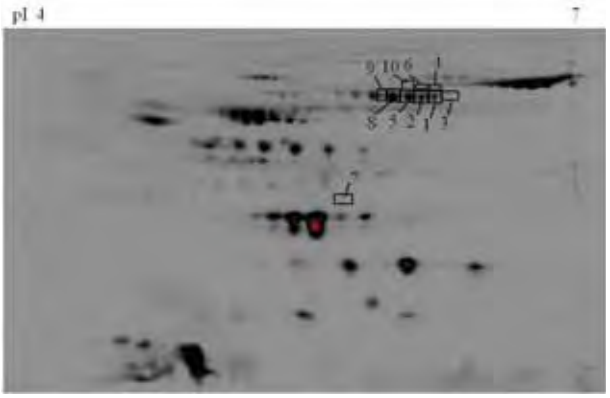
was not performed due to the small numbers within each group (n = 3, n = 9 and n = 3, respectively).

A total of 11 spots were detected at significantly different levels of abundance in at least one of the three comparisons (**Figure 3.4**). A significant decrease in abundance of spot 11 was observed in the BPH group compared with both CaP and control groups. Spots 1, 2, 3, 5, and 6 were significantly decreased in abundance in both CaP and BPH groups when compared to the control group. Spots 4, 7, 8, 9 and 10 were significantly decreased in abundance in the CaP group compared with the control group only; the BPH group was not significantly different from either control or CaP groups.

A

Spots	Anova (p)	Fold	Average Normalised Volumes	
			CaP (±SD)	BPH (±SD)
11	0.004	1.6	0.499 ±0.14	0.311 ±0.11

B



Spots	Anova (p)	Fold	Average Normalised Volumes	
			CaP (±SD)	Control (±SD)
1	6.3×10^{-7}	2.9	0.665 ±0.16	1.935 ±0.12
2	1.7×10^{-6}	2.6	0.688 ±0.15	1.795 ±0.13
3	5.8×10^{-6}	2.4	0.700 ±0.16	1.663 ±0.09
4	0.003	2.2	0.612 ±0.22	1.339 ±0.10
5	2.2×10^{-6}	2.1	0.609 ±0.12	1.273 ±0.11
6	3.8×10^{-5}	1.9	0.697 ±0.13	1.312 ±0.10
8	1.2×10^{-4}	1.6	0.596 ±0.11	0.973 ±0.09
9	0.004	1.6	0.657 ±0.10	1.025 ±0.11
10	3.1×10^{-4}	1.6	0.627 ±0.10	0.977 ±0.09
7	0.003	1.5	0.714 ±0.11	1.077 ±0.12

Continued on next page

C

Spots	Anova (p)	Fold	Average Normalised Volumes	
			Control (±SD)	BPH (±SD)
1	1.8×10^{-4}	2.0	1.935 ±0.16	0.945 ±0.12
2	5.6×10^{-4}	1.9	1.795 ±0.15	0.963 ±0.13
3	5.8×10^{-4}	1.9	1.663 ±0.16	0.896 ±0.09
5	8.8×10^{-4}	1.6	1.273 ±0.12	0.793 ±0.11
11	0.005	1.6	0.491 ±0.14	0.311 ±0.11
6	0.002	1.6	1.312 ±0.13	0.838 ±0.10

Figure 3.4: Eleven protein spots were significantly increased or decreased in abundance in at least one comparison of A: CaP and BPH samples, B: CaP and control samples, C: BPH and control samples.

Spots which were significantly increased or decreased in abundance across more than one comparison (Spots 1, 2, 3, 5, 6, and 11) were deemed to represent potential biomarkers and underwent MS analysis for identification (**Figure 3.5)**. Spots 1, 2, 3 and 5 were from the same train of spots and were all identified from control serum as albumin (**Table 3.4**). Only spot 1 from this train was excised and analysed from BPH and CaP serum and two proteins, albumin and hemopexin were identified from both sample types (**Table 3.4**). Spot 6 was identified from all three sample types as component 4 binding protein alpha chain (C4BPA) and Ig mu chain C region (IGHM) while spot 11 was identified from all three sample types as alpha-2-HS-glycoprotein (fetuin A) (**Table 3.4**). Albumin and fetuin A, the two proteins consistently identified across the three sample types, were quantified using targeted

proteomic approaches to verify the significant differences in abundance that were observed using 2D-DIGE analysis.

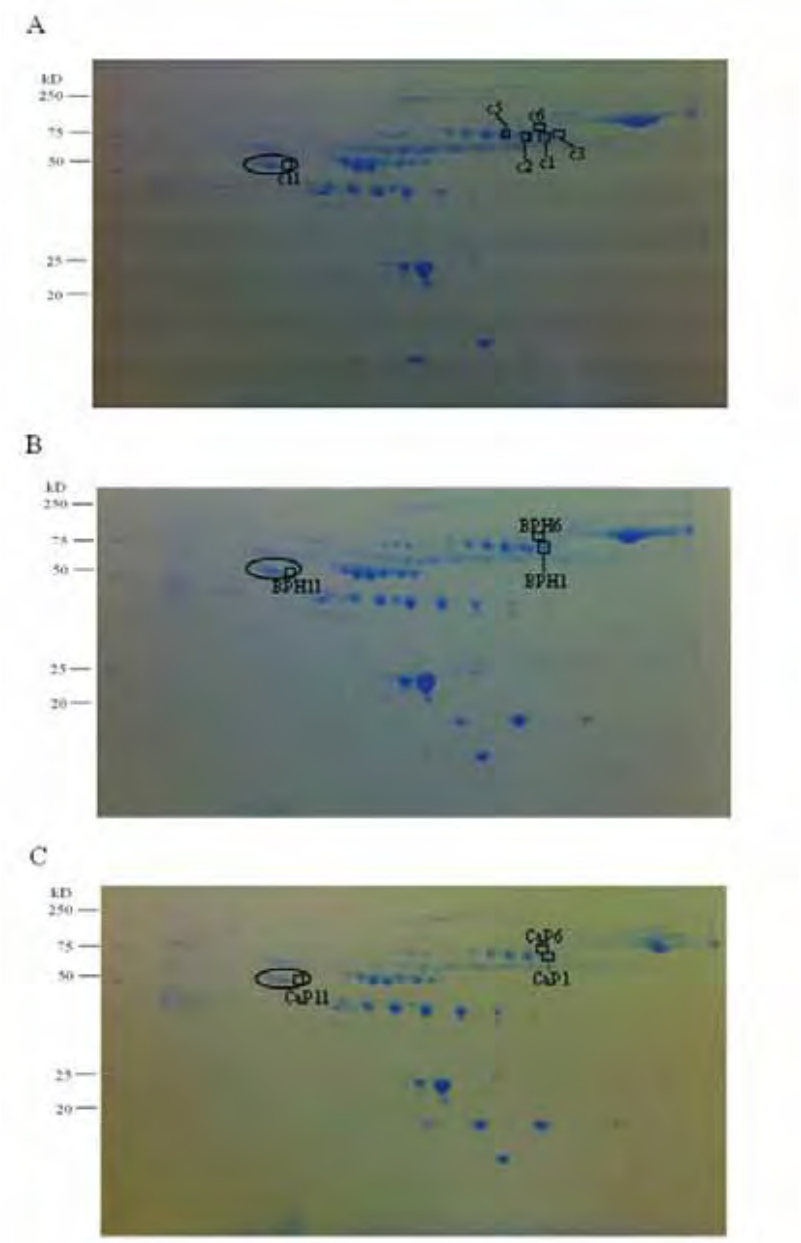


Figure 3.5: All protein spots of interest were excised from a preparative gel of control serum (A), while spots 1, 6 and 11 were also excised from preparative gels of BPH (B) and CaP (C) serum samples.

Table 3.4: Five proteins were identified from six spots that were excised from at least one preparative gel of control, BPH or CaP serum.

Spot ID	Identification	MOWSE score	Sequence coverage	Theoretical MW (kD)/pI	NCBI accession #
C1	Albumin	357	32%	69.3/5.92	gi|113576
C2	Albumin	332	34%	69.3/5.92	gi|113576
C3	Albumin	336	29%	69.3/5.92	gi|113576
C5	Albumin	311	32%	69.3/5.92	gi|113576
C6	C4BPA	457	37%	66.9/7.15	gi|416733
	IGHM	165	16%	49.3/6.35	gi|193806374
	Albumin	153	20%	69.3/5.92	gi|113576
C11	Fetuin A	210	33%	39.3/5.43	gi|112910
BPH1	Albumin	420	38%	69.3/5.92	gi|113576
	Hemopexin	219	36%	51.6/6.55	gi|1708182
BPH6	C4BPA	268	32%	66.9/7.15	gi|416733
	IGHM	192	20%	49.3/6.35	gi|193806374
BPH1	Fetuin A	168	35%	39.3/5.43	gi|112910
CaP1	Albumin	452	41%	69.3/5.92	gi|113576
	Hemopexin	134	26%	51.6/6.55	gi|1708182
CaP6	IGHM	155	19%	49.3/6.35	gi|193806374
	C4BPA	115	18%	66.9/7.15	gi|416733
CaP11	Fetuin A	133	27%	39.3/5.43	gi|112910

3.3.3 Targeted protein concentration

Concentrations of albumin and fetuin A were measured in undepleted serum samples from each of the three groups CaP, BPH and control. The average concentrations and their associated standard deviation (SD) of the serum albumin and fetuin A were calculated (**Table 3.5**). The average albumin concentrations was highest in the control group, followed by that of BPH group and then lowest among the CaP group (**Table 3.5**). Fetuin A concentration was highest in the control group, but lowest in BPH group (**Table 3.5**). The high SD of fetuin A concentration measured in CaP group, is because one of the samples had fetuin A three times higher than the rest of samples (**Figure 3.6, B).**

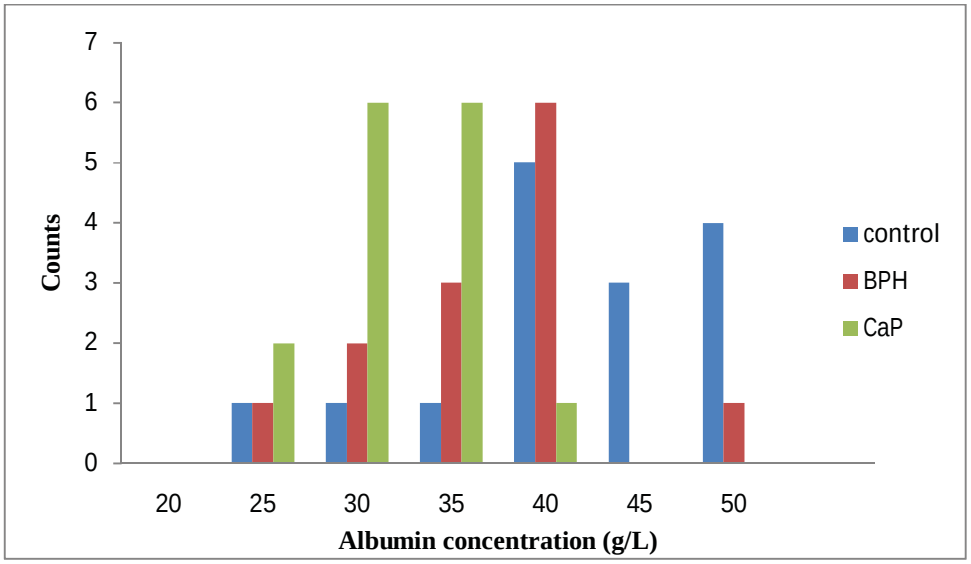
Table 3.5: Average concentration of the albumin and fetuin A in undepleted serum samples were quantified in control, BPH and CaP serum groups (average ± SD).

Sample group	Cobas® 6000 albumin concentration (g/L)	Fetuin A concentration (µg/µL)
Control	39.0 ± 7.20	0.387 ± 0.11
BPH	34.1 ± 5.23	0.303 ± 0.08
CaP	29.9 ± 4.30	0.365 ± 0.36

Frequency histograms were constructed to allow preliminary detection of any trends in albumin and fetuin A concentration which might differentiate between CaP, BPH and control serum samples when larger sample sizes are assessed. The frequency histogram indicates that the albumin concentrations of control samples were mostly skewed to the right of the graph (≥ 40 g/L), whereas the albumin concentrations of CaP samples were mostly below the control samples' concentration range (A, **Figure 3.6**). Similarly, the fetuin A concentrations of most control samples are observed > 0.3 µg/µL, whereas concentrations in most BPH and CaP samples fell below this concentration (B, **Figure 3.6**). It must be noted that the fetuin A concentration in one CaP serum sample was three fold higher than the maximum

fetuin A concentration from all other samples (B, **Figure 3.6**) and this was taken into account in further analyses.

A:



B:

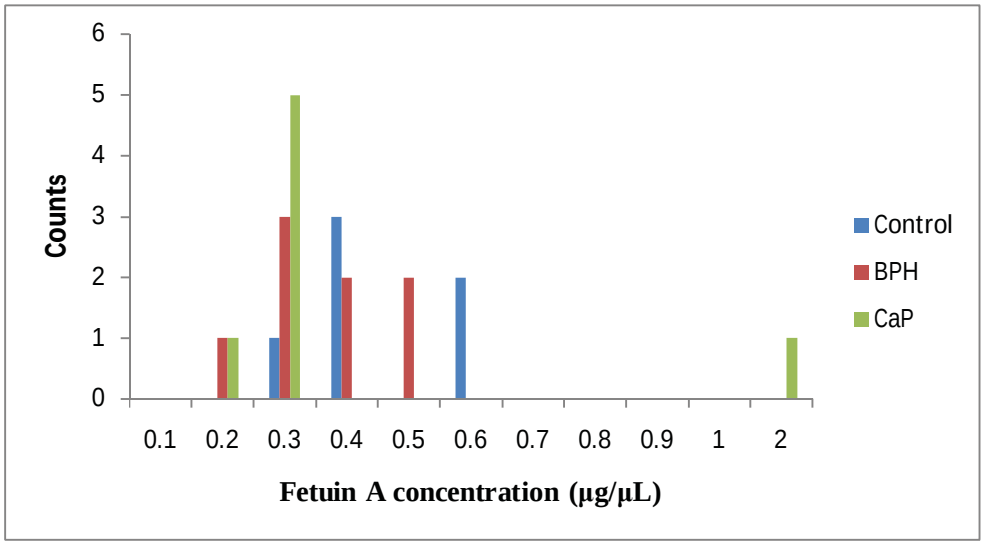


Figure 3.6: Frequency histograms of albumin and fetuin A concentration in CaP, BPH and control serum samples. A: The distribution of albumin concentrations in CaP, BPH and control serum samples shows a trend of lower concentration in CaP samples compared with control samples. B: The distribution of fetuin A concentrations in CaP, BPH and control serum samples indicates an overall trend towards lower concentration in CaP serum than control serum.

3.3.4 Assessment of diagnostic utility of serum albumin and fetuin A

3.3.4.1 Albumin

The ages of subjects from which samples were obtained were found to be significantly lower in the control group than in both the BPH and CaP groups (**Table 3.6**). However, post-hoc analysis showed that albumin concentration was significantly lower in the CaP group compared with the control group both with and without age adjustment, suggesting that this difference was not simply an effect of age (**Table 3.7**). No significant differences in albumin concentration were detected between the BPH group and either control or CaP groups, both with and without age adjustment (**Table 3.7**).

Table 3.6: The control group was comprised of subjects with significantly lower age than those from both the BPH and CaP groups. No significant age differences were detected between BPH and CaP groups.

Student's t-test for age difference	
Comparisons	**p ***
control vs BPH	0.0001
control vs CaP	0.0005
BPH vs CaP	0.1078

* Two tailed Student's t-test with Bonferroni correction, significance level $p < 0.017$.

Table 3.7: Post-hoc analysis of albumin concentration differences between control, BPH and CaP groups with and without age adjustment. A: samples analysed without age adjustment, B: samples analysed with age adjustment.

A:

Multiple Comparisons

Dependent Variable: Albumin Concentration

Group		Group	Mean Difference	Std. Error	p*	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	BPH	Control	-4.400	2.2480	.171	-10.012	1.211
		CaP	4.706	2.2480	.128	-.905	10.318
	Control	BPH	4.400	2.2480	.171	-1.211	10.012
		CaP	9.107*	2.2089	.001	3.593	14.620
	CaP	BPH	-4.706	2.2480	.128	-10.318	.905
		Control	-9.107*	2.2089	.001	-14.620	-3.593

*. The mean difference is significant at the .05 level.

B:

Pairwise Comparisons

Dependent Variable: Albumin Concentration

Group	Group	Mean Difference	Std. Error	p*	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
BPH	Control	-5.715	2.849	.155	-12.833	1.403
	CaP	4.435	2.288	.179	-1.282	10.152
Control	BPH	5.715	2.849	.155	-1.403	12.833
	CaP	10.150*	2.613	.001	3.622	16.679
CaP	BPH	-4.435	2.288	.179	-10.152	1.282
	Control	-10.150*	2.613	.001	-16.679	-3.622

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

3.3.4.2 Fetuin A

In the small sample size analysed it is unclear whether the extremely high concentration of fetuin A in one CaP sample (B, **Figure 3.6**) represents an outlier or is indicative of the maximum concentration that might be observed in a larger sample size. As a result, statistical analysis was completed both with and without this sample included (**Table 3.8)**. A significant difference in concentration of fetuin A was only detected between CaP and control serum samples excluding this sample ($p < 0.017$). There was no significant difference between age of subjects from whom samples were collected suggesting that the significant difference of fetuin A concentration detected was not affected by age.

Table 3.8: Fetuin A concentration was significantly increased in control serum samples when compared with CaP serum samples.

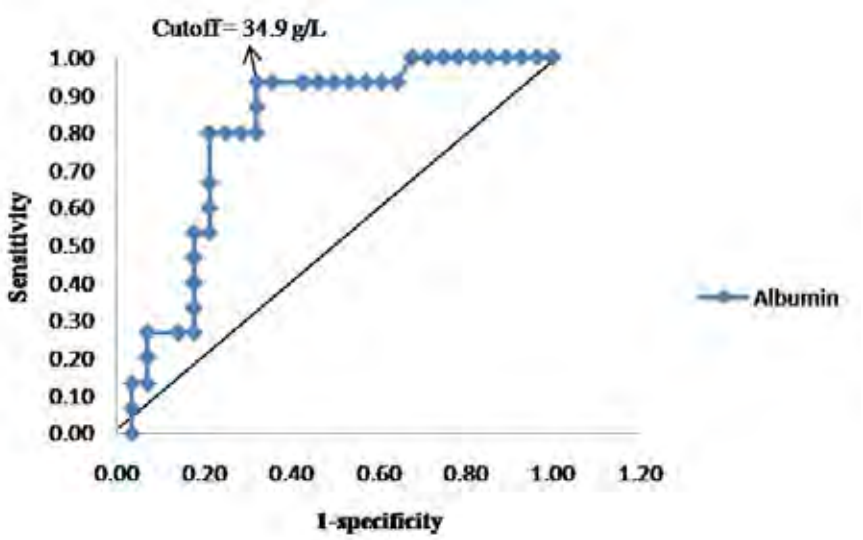
Student's t-test	
Fetuin A analysis with outlier	p*
control vs BPH	0.053619
BPH vs CaP	0.698936
control vs CaP	0.361974
Age analysis with outlier	
control vs BPH	0.034939
BPH vs CaP	0.063382
control vs CaP	0.193633
Fetuin A analysis without outlier	
control vs BPH	0.053619
BPH vs CaP	0.140770
control vs CaP	0.006278
Age analysis without outlier	
control vs BPH	0.034939
BPH vs CaP	0.107456
Control vs CaP	0.189171

* Significance level $p < 0.017$.

3.3.4.3 ROC curve analysis for albumin and fetuin A

ROC curves were constructed based on the concentrations of albumin and fetuin A that were measured, to analyse and compare the diagnostic accuracy of albumin and fetuin A for distinguishing CaP from a control group which comprised both control and BPH serum samples (**Figure 3.7**). Results from a total of 43 samples were included to construct the albumin ROC curve, and results from 23 samples were used for the fetuin A ROC curve. Albumin had an AUC of 0.79 (A, **Figure 3.7**), and the AUC for fetuin A was 0.75 (B, **Figure 3.7**). Based on the ROC curve, an optimal cutoff of 34.9 g/L for albumin allowed CaP samples (< 34.9 g/L) to be distinguished from controls (> 34.9 g/L) with 93% sensitivity and 67% specificity (A, **Figure 3.7)**. An optimal cutoff concentration of 0.257 μg/μL for fetuin A allowed CaP samples (< 0.257 μg/μL) to be distinguished from controls (> 0.257 μg/μL) with 86% sensitivity and 79% specificity (B, **Figure 3.7**).

A



B

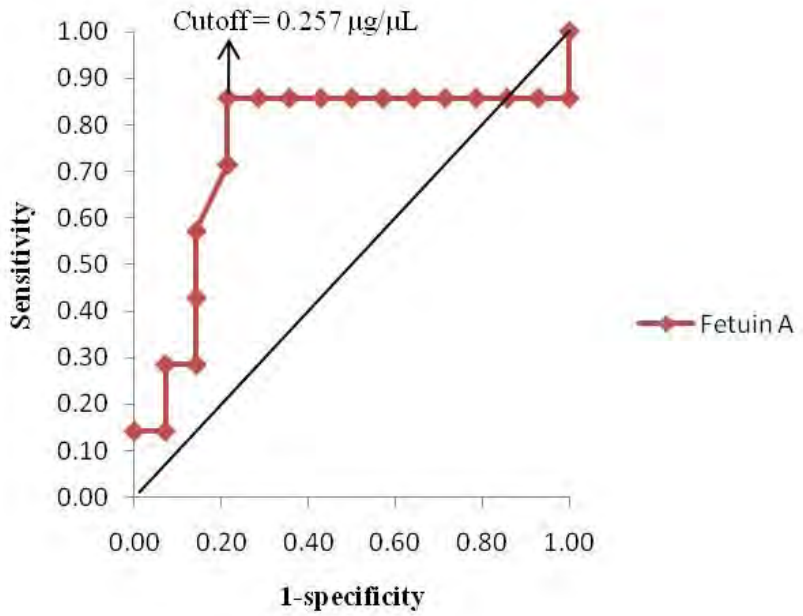


Figure 3.7: ROC evaluation of albumin and fetuin A as a diagnostic test for CaP. At the cutoff of 34.9 g/L, the sensitivity and specificity of albumin for CaP diagnosis were at 93% and 67% respectively. Fetuin A has a sensitivity and specificity for CaP diagnosis of 86% and 79% respectively, at the cutoff of 0.257 µg/µL.

3.3.5 Association between Gleason score, administered therapeutics and serum albumin/fetuin A concentration

Two variables relevant to prostatic disease samples, the Gleason score of CaP and administered therapeutics, were assessed to determine their potential effects on albumin and fetuin A concentrations that had been measured. Samples were divided into three Gleason score groups (Gleason score <7, Gleason score = 7 and Gleason score >7), however due to the small sample size particularly in the Gleason score < 7 and >7 groups, statistical analysis could not be performed to determine significant relationships between Gleason score and albumin and fetuin A concentrations (**Table 3.9**). Whilst statistical analysis was not possible, there is no trend present to indicate a relationship either between Gleason score and serum albumin concentration (A, **Table 3.9**) or between Gleason score and fetuin A concentration (B, **Table 3.9**).

Table 3.9: The Gleason score of each CaP patient in relation to the decreasing concentrations of serum albumin and fetuin A. A: CaP samples used for serum albumin test. B: CaP samples used for fetuin A test.

A:

ID	Gleason score	Albumin Concentration (g/L)
595	7	23.1
539	7	23.5
507	<7	26.1
566	<7	26.6
368	<7	28.1
558	7	28.2
401	7	28.5
327	7	28.6
169	>7	30.7
263	7	31.6
554	7	32.2
425	<7	32.2
552	>7	34.5
370	7	34.9
114	7	39.5

B:

ID	Gleason Score	Fetuin A Concentration (µg/µL)
157	7	1.248
146	>7	0.257
566	<7	0.247
327	7	0.234
151	7	0.232
121	7	0.216
263	7	0.118

In order to assess whether the significant albumin or fetuin A difference observed between control and CaP subjects were due to the effect of administered therapeutics, the information regarding the administered therapeutics for control, BPH and CaP subjects were tabulated in terms of condition targeted and the effect of the treatment on liver (as both fetuin A and albumin are synthesised in the liver, **Table 3.10 -3.11**). The order of the data was arranged based on the concentration of albumin and fetuin A from lowest to highest. Medical treatments were categorised based on the severity of potential impacts on the liver: hepatotoxic effect (red in **Table 3.10 - 3.11**), possible impairment of liver function (orange in **Table 3.10 - 3.11**), improvement of liver function (green in **Table 3.10 – 3.11**) and no reported effect on the liver (**Table 3.10 - 3.11**).

Only two control subjects were under medical treatments, and most of BPH and CaP patients had various administered therapeutics targeting other conditions rather than BPH or CaP (**Table 3.10 – 3.11)**. No obvious associations between administered therapeutics and albumin concentration were observed (**Table 3.10**), and the information available does not suggest that the significant decrease in albumin concentration observed in the CaP group when compared with the control group is a reflection of administered medical treatments.

Table 3.10: The effect of medical treatment on liver function and the association between the medical treatment and serum albumin. A: control samples, B: BPH samples and C: CaP samples.

A:

Control sample ID	Albumin (g/L)	Medical treatment	The effect of the medicine on liver
c7	21.2	None	None
c9	27.6	None	None
c12	32.6	None	None
c19	35.4	None	None
c6	37.0	None	None
c3	37.5	Calcium channel blockers and peripheral vasodilator: blood pressure tablets for controlling blood pressure.	Although the exact type of these tablets were unknown, no association between antihypertension drugs and hepatotoxicity has been established (Triantafyllou *et al.*, 2010)
c17	39.5	None	None
c1	39.8	None	None
c2	41.6	None	None
c20	43.0	Thyroxine for underactive thryoid (Malik and Hodgson, 2002)	Can reverse liver dysfunction caused by hypothyroidism (Malik and Hodgson, 2002)
c14	44.8	None	None
c13	45.2	None	None
c15	45.6	None	None
c16	46.5	None	None
c18	47.6	None	None

Continued on next page

B:

BPH sample ID	Albumin (g/L)	Medical treatment	The effect of the medicine on liver
87	25	Tamsulosin hydrochloride for BPH (Miyazawa *et al.*, 2001)	No significant effect to liver function (Miyazawa *et al.*, 2001)
88	27.6	N/A	N/A
84	27.8	Tamsulosin hydrochloride for BPH (Miyazawa *et al.*, 2001); Ciprofloxacin: an antibiotic drug (Zimpfer *et al.*, 2004)	Ciprofloxacin: cause severe liver injury (Zimpfer *et al.*, 2004)
75	30.5	Solifenacin, an antimuscarinic drug for overactive bladder (Kuipers *et al.*, 2006)	Could effect liver function (Kuipers *et al.*, 2006)
92	32.5	Norvasc®	Possible liver damage
86	33.6	N/A	N/A
424	35.2	N/A	N/A
85	35.4	ZOCOR® (simvastatin) for lower cholesterol and triglycerides in blood; ZOLOFT® (sertraline HCl) for depression and anxiety treatment.	ZOCOR®: Increased level of serum transaminases produced by liver; ZOLOFT®: Rare effect to liver function
73	36.1	N/A	N/A
94	36.4	COVERSYI® for lowering blood pressure	Possible liver damage
456	36.8	N/A	N/A
95	39	Tamsulosin hydrochloride for BPH (Miyazawa *et al.*, 2001); Solifenacin, an antimuscarinic drug for overactive bladder (Kuipers *et al.*, 2006)	Solifenacin: could effect liver function (Kuipers *et al.*, 2006)
74	47.7	Tamsulosin hydrochloide for BPH (Miyazawa *et al.*, 2001); NEXIUM® for high blood pressure	NEXIUM®: Possible liver inflammation

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C:

CaP sample ID	Albumin (g/L)	Medical treatment	The effect of the medicine on liver
595	23.1	N/A	N/A
539	23.5	Lipitor® (atorvastatin calcium) for reduction of MI, stroke; Naprosyn®: an anti-inflammatory drug	Lipitor®: rare effect on liver; Naprosyn®: Severe hepatic reaction
507	26.1	Solifenacin: an antimuscarinic drug for overactive bladder (Kuipers *et al.*, 2006)	Could effect liver function (Kuipers *et al.*, 2006)
566	26.6	N/A	N/A
368	28.1	Bricanyl Turbuhaler for breath difficulties; COVERSYL® PLUS for lowering blood pressure; LOSEC® for reflux oesophagitis; VIAGRA® for erectile dysfunction; ZANIDIP® for hypertension	LOSEC®: possible effect to liver inflammation; ZANIDIP®: rare effect on liver
558	28.2	Tamsulosin hydrochloide for BPH (Miyazawa *et al.*, 2001)	No significant effect to liver function (Miyazawa *et al.*, 2001)
401	28.5	N/A	N/A
327	28.6	Stilnox (zolpidem) for insomnia; Micardis® (telmisartan) for hypertension (Stangier *et al.*, 2000)	No significant effect on liver function
169	30.7	N/A	N/A
263	31.6	N/A	N/A
554	32.2	N/A	N/A
425	32.2	ACTONEL COMBI (risedronate sodium, colecalciferol, calcium carbonate): for prevention of bone breakdone	No significant effect on liver function
552	34.5	Zoladex® to suppress androgen, used in men for CaP treatment	No significant effect on liver (Miller and Frank, 1992)
370	34.9	N/A	N/A
114	39.5	N/A	N/A

Note: Where literature is unavailable for the administered therapeutics, information was taken from the consumer medicine information sheets published by their manufacturers.

An association between medical treatment, liver effect and measured serum fetuin A concentration was also assessed (**Table 3.11**). A possible association between the medication administered and the fetuin A concentrations measured in the present study was observed in the one patient, for whom fetuin A concentration was outside the range of all other samples measured. This patient (157) was treated with medication known to be hepatotoxic and this may have influenced the extremely high fetuin A concentration measured.

Table 3.11: The effect of medical treatment on liver function and the association between the medical treatment and serum fetuin A. A: control samples, B: BPH samples and C: CaP samples

A:

Control Sample ID	Fetuin A (µg/µL)	Medical treatment	The effect of the medicine on liver
c16	0.24	N/A	N/A
c20	0.327	Thyroxine for underactive thyroid (Malik and Hodgson, 2002)	Can reverse liver dysfunction caused by hypothyroidism (Malik and Hodgson, 2002)
c14	0.351	N/A	N/A
c1	0.368	N/A	N/A
c2	0.501	N/A	N/A
c17	0.508	N/A	N/A

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B:

BPH Sample ID	Fetuin A (µg/µL)	Medical treatment	The effect of the medicine on liver
75	0.426	Solifenacin antimuscarinic drug for overactive bladder (Kuipers *et al.*, 2006)	Moderate effect liver function (Kuipers *et al.*, 2006)
88	0.406	N/A	N/A
456	0.365	N/A	N/A
85	0.331	ZOCOR® (simvastatin) for lower cholesterol and triglycerides in blood (Vormfelde *et al.*, 1999); ZOLOFT® (sertraline HCl) for depression and anxiety treatment.	ZOCOR®: Increased level of serum transaminases produced by liver (Vormfelde *et al.*, 1999); ZOLOFT: Rare effect to liver function
471	0.275	Tamsulosin hydrochloride for BPH (Miyazawa *et al.*, 2001)	No significant effect to liver function (Miyazawa *et al.*, 2001)
89	0.247	N/A	N/A
134	0.229	N/A	N/A
87	0.146	Tamsulosin hydrochloride for BPH (Miyazawa *et al.*, 2001)	No significant effect to liver function (Miyazawa *et al.*, 2001)

Continued on next page

C:

CaP Sample ID	Fetuin A (µg/µL)	Medical treatment	The effect of the medicine on liver
157	1.248	Aspirin: prevention of cardiovascular disease and analgesic purpose; Betaloc® (metoprolol tartrate) for hypertension, angina pectoris, heart failure and maintenance treatment after myocardial infarction; Lipitor® (atorvastatin calcium) for reduction of myocardial infarction, stroke	Aspirin: Hepatotoxic effect (Seaman and Plotz, 1976); Lipitor®: increase of serum transaminases secreted by liver in clinical trials (Black *et al.*, 1998)
146	0.257	N/A	N/A
566	0.247	N/A	N/A
327	0.234	Stilnox (zolpidem) for insomnia; Micardis ® (telmisartan) for hypertension (Stangier *et al.*, 2000)	Stilnox: elevated liver enzymes but no significant effect on liver function reported. Micardis: no significant effect on liver function (Stangier *et al.*, 2000)
151	0.232	Centrum	Certain vitamins could improve the liver function (Tsiaousi *et al.*, 2008)
121	0.216	Amiloride hydrochloride is an antikaliuretic-diuretic agent in congestive heart failure or hypertension; Slow-K® (potassium chloride) for low potassium deficiency	Amiloride hydrochloride: no metabolised by liver. Slow-K®: no effect to liver function.
263	0.118	N/A	N/A

Note: Where literature is unavailable for the administered therapeutics information was taken from the consumer medicine information sheets published by their manufacturers.

3.3.6 Mass spectrometry analysis of fetuin A identified from spot 11

The Mascot search of the typtic digested spots from control, BPH and CaP gels showed that one peptide CDSSPDpSAEDVR (position 132 – 143) was phosphorylated at serine-138 (**Figure 3.8**). The observed $[M+2H]^{2+}$ of the phosphorylated peptide was 716.26 which differed from the unphosphorylated form by 80 Da (Fiogure 3.8 $[M+2H]^{2+}$ 676.27) indicating the addition of an HPO_3. This phosphorylation was supported by the findings of the Xcalibur analysis in which the MS file was searched for the 98 Da neutral fragment resulting from loss of a H_3PO_4 during CID. The only fragment detected in this analysis showed the unphosphorylated peak 676.27 was eluted at 20.03 min, and the phosphorylated peak $[M+2H]^{2+}$ 716.26 at retention time of 19.82 min (**Figure 3.9**). The FT-MS/MS spectrum of these two doubly charged ions had more than half of the b and y ions detected matching the Mascot data, confirming the identity of the phosphorylated peptide.

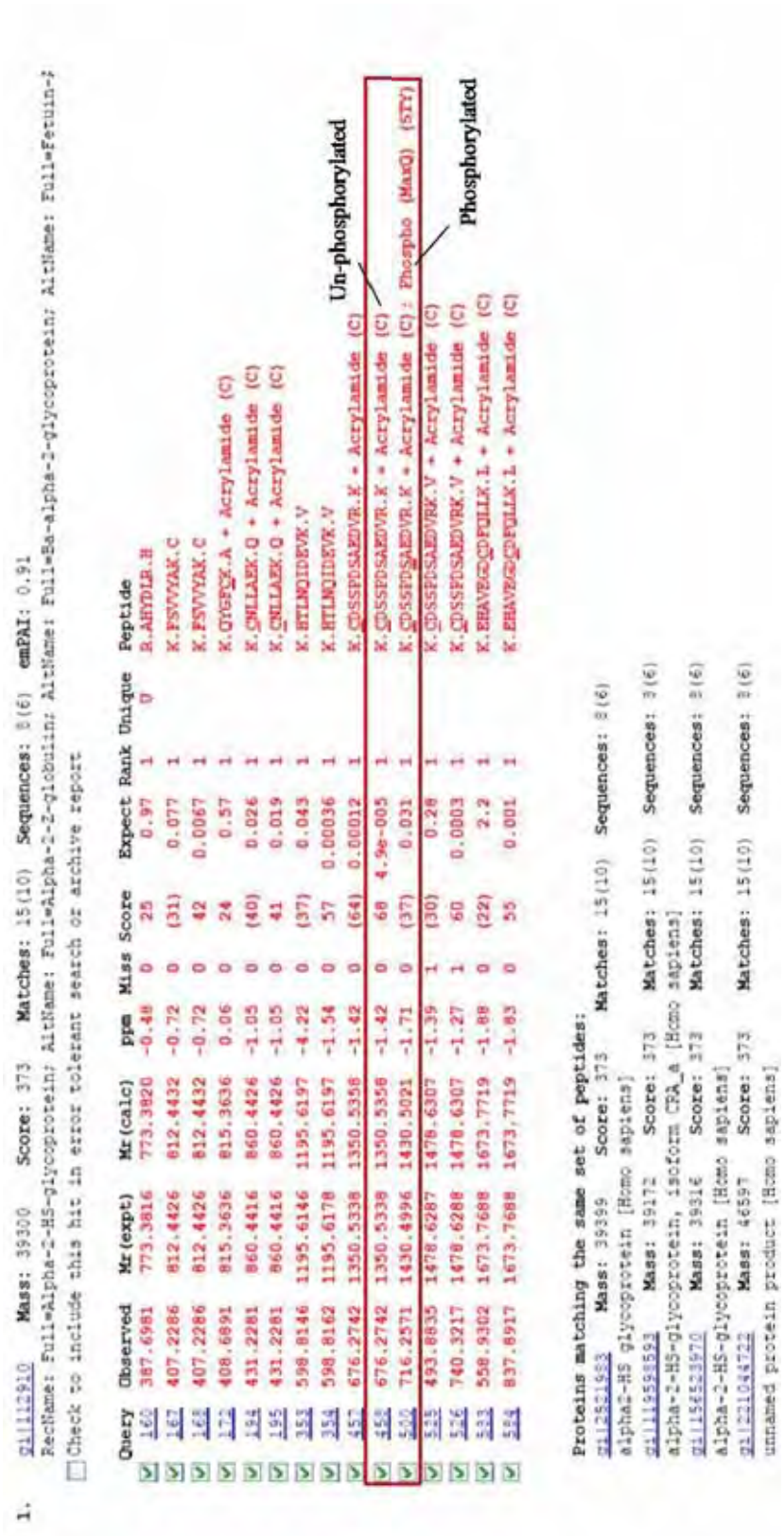


Figure 3.8: Mascot search has detected one phosphorylated peptide CDSSPDpSAEDVR with observed m/z of 716.2571 and its unphosphorylated m/z of 676.2742.

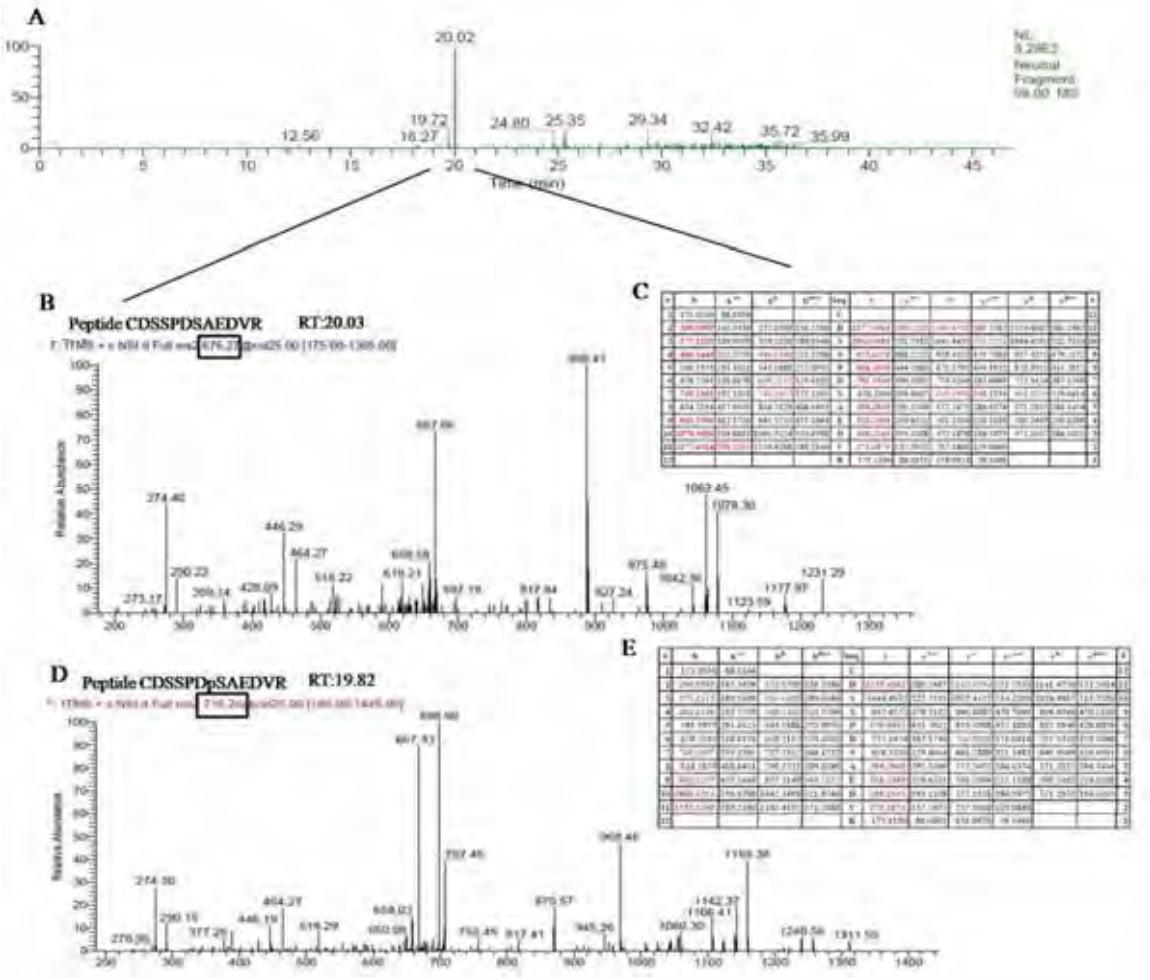


Figure 3.9: Xcalibur analysis of the FT-MS/MS spectrum of M+2H$^+$ 716.26 and 676.27. A: The peak detected when searching for neutral fragment of 98 Da. B: FT-MS/MS of the detected peak, M+2H$^+$ 676.27 at 20.03 min. C: The Mascot list of ions generated from fragmentation of peak 676.27, which showed more than half of y and b ions were matching the actual MS data. The matching ions were shown in red. D: FT-MS/MS of M+2H$^+$ 716.26 eluted at 19.82 min. E: The Mascot list of ions generated from fragmentation of peak 716.26, which showed more than half of y and b ions were matching the actual MS data. The matching ions were shown in red.

3.4 Discussion

The results presented in relation to fractionation of serum, using an albumin/IgG depletion kit in this study, support the widely accepted idea that pre-fractionation of the complex serum proteome improves the ability to detect novel biomarkers using gel based proteomic techniques. Using this technique combined with 2D-DIGE and MS analysis have allowed the identification of five proteins (albumin, fetuin A, IGHM, hemopexin and C4BPA) as potential diagnostic biomarkers for CaP. Albumin, hemopexin, C4BPA and IGHM were identified from protein spots which were significantly increased in control samples compared with both CaP and BPH samples, whereas fetuin A was identified from a protein spot which was significantly decreased in BPH samples compared with both control and CaP samples and was phosphorylated at serine 138. Therefore, a combination of these proteins could be useful in the diagnosis of CaP as well as differentiation from BPH, which shares similar symptoms to CaP. Notably, the protein spots showing significant differences in abundance between the three groups were all detected within trains of spots, suggesting only specific isoforms are associated with prostatic diseases.

The concentration of albumin and fetuin A were measured in CaP, BPH and control serum samples using targeted proteomics to provide preliminary verification of their usefulness as diagnostic biomarkers. At the cut-off levels determined most appropriate by construction of ROC curves, detection of prostate cancer using albumin concentration (cut off of 34.9 g/L) had a sensitivity of 93% and specificity of 68% and fetuin A concentration (cut off 0.257 µg/µL) had a sensitivity and specificity of 86% and 79%, respectively. These values, particularly the specificity, are higher than those of the current gold standard PSA test which has a sensitivity of 79% and a specificity of 59% (Catalona *et al.*, 1991). Analysis using larger group of subjects is required to determine whether these values are maintained, but the data presented in this chapter has provided evidence that novel biomarkers for CaP can be measured within serum samples.

3.4.1 Potential biomarkers for CaP

None of the serum proteins that have previously been suggested as potential CaP biomarkers in the literature (as reviewed in Chapter 1) were identified in the present study. It is not possible to detect the entire serum proteome using 2D-DIGE or any currently available single proteomic technique, since each technique has its limitations (as reviewed in Chapter 1). In addition, proteins detected and identified will differ between studies depending on many factors including characteristics of sample groups compared and methods of sample collection, storage, fractionation, separation, detection, analysis and identification. Finally, as the current study aimed to identify novel protein biomarkers for the diagnosis of CaP, the previously reported potential biomarkers were not targeted in conjunction with those identified here.

Of the five proteins identified, albumin and fetuin A were identified as the only protein in at least one spot across the three sample types, while hemopexin, C4BPA and IGHM were identified with other proteins. For this reason, albumin and fetuin A were verified using targeted proteomic analysis.

3.4.1.1 Albumin

Albumin, a 66 kDa protein synthesised by liver cells, is the most abundance protein found in blood with 90% of albumin synthesised secreted directly into the blood (Rothschild *et al.*, 1988). It can also be found in the extravascular space due to leakage from the vascular vessels (Rothschild *et al.*, 1988). The major functions of human serum albumin are as a carrier of a variety of essential molecules and to maintain osmotic pressure (Rothschild *et al.*, 1988). The average albumin concentration measured in 1000 healthy male was between 40 to 44 g/L, slightly decreasing with age (Campion *et al.*, 1988).

In the present study, when albumin and IgG were depleted from serum samples, the remaining albumin generated a train of distinct spots in all of the 2D gels. This

indicated that different isoforms/modifications of albumin were present in all control, BPH and CaP sera. Different isoforms of albumin are known to occur in serum (Rothschild *et al.*, 1988), and the 2D-DIGE results presented here suggest that some of these are present at significantly lower abundances in both BPH and CaP sera than in control. Due to multiple isoforms being altered in abundance, the concentration of total human serum albumin was quantified using BCP assay to provide preliminary data relating to the usefulness of the protein as a diagnostic biomarker.

Human serum albumin concentration is a well established clinical test for various conditions, with a cutoff concentration of 35 g/L widely used. A serum albumin concentration less than 35 g/L is referred as hypoalbuminemia (Salive *et al.*, 1992) and is used to indicate malnutrition of dialysis patients (used in > 90% dialysis centers in US) (Thomson, 2004) and the elderly (Omran and Morley, 2000). In Australia, a serum albumin test is also regularly performed as part of liver function tests, with low serum albumin suggestive of severe liver dysfunction as well as other conditions including inflammation, malnutrition and protein deficiency states (Coates, 2011), but there is no reported association between cancer diagnosis and the serum albumin test.

This is the first study that has demonstrated a significant association between serum albumin concentration and CaP. The total serum albumin concentration was significantly reduced in CaP patients compared to healthy controls, independent of age. The administered therapeutics of all participants in this study were also examined in terms of their effect on the liver, however no clear association of albumin and aministered therapeutics were detected. This suggested that in this sample set, the significantly reduced albumin concentration observed in the serum of CaP patients was not simply related to medical treatments administered. However, a larger sample size is necessary to assess potential significant associations between the therapeutics and albumin concentration. No information regarding liver dysfunction of the participants was available for the present research and thus the

direct effect of liver damage on the observed albumin concentrations could not be examined. However, patients with liver disease would be a useful control group to include as a separate group for validating the albumin CaP diagnostic test when a larger study is conducted.

The advantages of using albumin for potential CaP diagnosis include the measurement of serum albumin concentration is a well-established and widely used clinical test. However, the fact that the cutoff established in the present work, 34.9 g/L is very close to the threshold (35 g/L) used for clinical detection of other medical conditions suggests that the significant differences observed between the control and CaP groups could be a result of the overall systemic progression of the condition, including malnutrition, protein loss and inflammation. Moreover, Proctor *et al.*,(2010) examined the albumin concentration in 267 CaP patients and found more than half had a serum albumin concentration > 35 g/L; higher than the cut off for CaP diagnosis determined in the present study (Proctor *et al.*, 2010). This suggests that in a larger sample size, the total albumin concentration could actually have low CaP specificity. Considering the difference in the results between the total albumin measurement and the initial 2D-DIGE results, as well as multiple reports that various albumin isoforms are associated with disease states (discussed below), it is likely that the specific albumin isoforms detected as significantly altered on 2D-DIGE gels could have better specificity to prostatic diseases than total albumin. Further analysis of the specific isoforms which do alter in CaP, BPH and control serum is an important step for development of a sensitive and specific diagnostic test and may also lead to increased knowledge of processes involved in prostatic conditions.

Modified forms of albumin have been identified and investigated in relation to a variety of pathologies. Glycation, oxidation and nitrosylation in particular have been shown to be associated with different pathologies, indicating functional differences resulting from these modifications. Under normal conditions, around 10% of serum albumin is glycosylated (Shaklai *et al.*, 1984). Shaklai *et al.*,(1984) studied the

affect of non-enzymatic glycosylation (glycation) of albumin on its ligand binding properties and found 50% reduction in binding affinity to bilirubin and 20 fold reduction in binding affinity to the long chain fatty acid *cis*-parinaric acid compared with the non-glycosylated form (Shaklai *et al.*, 1984). In addition, glycation has been shown to effect the oxidation activity of bovine serum albumin with decreased antioxidant capability compared to non-glycated bovine serum albumin (Bourdon *et al.*, 1999). Increased levels of glycated albumin has been detected in hyperglyceamia caused by diabetes (Koga and Kasayama, 2010) and in the retinal capillaries of diabetic patients (Schalkwijk *et al.*, 1999) suggesting decreased ligand binding and antioxidant effects may follow these developments in these conditions. Glycated albumin has been shown to induce apoptosis in a retinal pericyte cell line while apoptotic cell death was decreased by addition of antioxidants, consistent with the idea that oxidative stress could be part of the cytotoxicity caused by glycated albumin (Kim *et al.*, 2002).

The oxidation state of human serum albumin has also been studied in relation to pathophysiological conditions, with an isoform of increased mass (by ~48 Da) and decreased pH shown to occur in the serum of nephrotic syndrome patients but not healthy controls (Musante *et al.*, 2006). This is due to the oxidation of the free sulphydryl group of Cys 34 to cysteic acid (Musante *et al.*, 2006), and the residue has also been found to bind to nitric oxide to form S-nitrosolylated albumin *in vivo* (Stamler *et al.*, 1992), a modification which has been suggested to induce tumour cell apoptosis and thus inhibit tumour growth (Katayama *et al.*, 2010). Another form of the protein arising from oxidative stress, ischaemia-modified albumin, also decreases the metal binding ability of albumin and this form has been found significantly elevated in the serum of BPH patients when compared to control serum, as opposed to total albumin which decreases in association with inflammation (Mastella *et al.*, 2009).

These previous studies all suggest that albumin is a complex molecule with differences in function and concentration of multiple isoforms under various

physiological and pathophysiological conditions. Therefore, results of the present study are most likely indicative of variations to specific modifications due to the presence of CaP, modifications which were not be reflected by the measurement of the total albumin concentration. Further work following from this study must provide characterisation of the albumin protein spots detected from the 2D-DIGE experiments. This will allow determination of the specific isoforms of albumin that are altered in concentration in CaP and also allow a more sensitive and specific diagnostic/prognostic test to be developed.

Finally, although the value of the total serum albumin for diagnosis of any cancer has not been examined previously, it has been reported by various research groups as an independent prognostic indicator of survival in various cancers such as ovarian cancer (Gupta *et al.*, 2009), breast cancer (Lis *et al.*, 2003), lung cancer (Lam *et al.*, 2007), pancreatic cancer (Siddiqui *et al.*, 2007), colorectal cancer (Heys *et al.*, 1998) and gastric cancer (Onate-Ocana *et al.*, 2007). Due to the small sample size used in CaP patients, the prognostic value of albumin could not be analysed, however in a larger sample size its value as a prognostic as well as diagnostic indicator of CaP could be assessed.

3.4.1.2 Fetuin A

This study is also the first study to report a significantly lower fetuin A concentration in the serum of CaP patients compared with that from healthy controls. Notably, it is also the first preliminary analysis that has verified the use of fetuin A to differentiate CaP serum from healthy serum with better performance (sensitivity 86% and specificity 79%) than the current PSA test. This result shows potential for the use of fetuin A in a CaP diagnostic, however a larger study is required to confirm the results and to determine other variables which might influence a fetuin A based test.

Like albumin, fetuin A is synthesised in the liver and secreted directly into the blood (Triffitt *et al.*, 1976). Other than blood, calcified tissues (such as bone and dentine) also contain high concentrations of fetuin A, probably due to its high affinity for calcium (Triffitt *et al.*, 1976). It is also a multifunction protein involved in bone formation (Szweras *et al.*, 2002), prevention of systemic calcification (Schafer *et al.*, 2003; Hendig *et al.*, 2006), regulation of serum phosphates (Osawa *et al.*, 2005), enhancement of macrophage deactivation (Wang *et al.*, 1998), inhibition of insulin induced insulin receptor autophosphorylation (Mathews *et al.*, 2000) and prevention of overproduction of pro-inflammatory cytokines (Gangneux *et al.*, 2003). Significant inverse relationships between serum fetuin A with the inflammatory markers C-reactive protein (CRP) and interleukin (IL) -6 in end stage renal disease patients have been reported resulting in the unconfirmed hypothesis that it is a negative acute phase protein (Ketteler *et al.*, 2003; Stenvinkel *et al.*, 2005; Hussein *et al.*, 2010).

Although serum fetuin A concentration has not previously been specifically investigated in relation to CaP, it has been shown to be altered in concentration in liver, lung and breast cancers, but in different ways. Kalabay *et al.*,(2002) reported decreased serum fetuin A levels in liver cirrhosis and liver cancer, with these levels also associated with high mortality (Kalabay *et al.*, 2002). In contrast, increased concentrations of serum fetuin A have been shown to promote tumour cell growth in mouse models of Lewis lung carcinoma (Kundranda *et al.*, 2005) and breast cancer (Guillory *et al.*, 2010). In this present study, it was also shown to be in significantly higher abundance in CaP than control sera.

Due to the association between fetuin A and liver function, a preliminary assessment of fetuin A concentration and medical treatments that impact on liver function was conducted. The results showed that patients undergoing treatments which have known affects on liver function tended to have higher fetuin A serum concentration than subjects from the same group who were not under such treatments. This suggested a possible association between fetuin A and liver

function, however in the small sample size investigated the significant difference does not appear to be simply due to this effect, as the significant difference is between CaP and control samples but not control and BPH. Additionally, the idea that fetuin A is a negative acute phase protein also raises the question of whether it is associated with presence or degree of any inflammation unrelated to CaP. As suggested for albumin, patient groups to control for liver function and inflammation should be included when validating the diagnostic usefulness of serum fetuin A in a larger sample size study.

Although no significant differences in the total fetuin A concentration were detected between the BPH group and either CaP or control groups, the fetuin A protein spot detected by 2D-DIGE showed significantly lower abundances in BPH when compared to CaP and control and a phosphorylation at serum 138. These different findings in the different experiments indicate that fetuin A isoforms could provide more specific diagnosis for CaP than its total serum level. Moreover, studies have reported specific fetuin A modifications are associated with different diseases (discussed below) and thus further characterisation of the fetuin A isoform detected by 2D-DIGE may be required for development of a CaP specific test.

Two forms of fetuin A are secreted from its site of synthesis in the liver, single-chain and double-chain polypeptide forms, however only the double-chain form was detectable in plasma by previous studies (Kellermann *et al.*, 1989; Jahnen-Dechent *et al.*, 1994). The double chain forms consists of a heavy chain (A chain and connecting peptide, 321 amino acids) and a light chain (B chain, 27 amino acids) linked by a disulfide bond whereas in the single-chain form, chain A and B form a single chain through a connecting peptide (Jahnen-Dechent *et al.*, 1994). Both the A and B chains are cystatin like making fetuin A a member of the cystatin superfamily (Kellermann *et al.*, 1989). In addition, the mature protein also has a calcium phosphate binding site (Schinke *et al.*, 1996) and a transforming growth factor (TGF) - β cytokine-binding motif (Demetriou *et al.*, 1996) which are responsible for calcium regulation.

Fetuin A has also been reported to have the potential to undergo multiple phosphorylation and glycosylation events. A phosphorylated peptide CDSSPDpSAEDVR (position 132-143) of which the serine 138 is likely to be the site of phosphorylation, was detected in the present study. This is consistent with a previous finding in which Haglund *et al.*, (2001) detected serine 138 and an additional serine 328 as being phosphorylated. In Haglund's study, purified human plasma fetuin A was analysed and around 20% of circulating plasma fetuin A was established as phosphorylated. Recently, additional phosphorylation sites have been identified on at serine residues at position 330 and 334 (Zhou *et al.*, 2009b). Whilst the significance of each specific phosphorylation sites remains unclear, it is known that presence of the modification does effect the biological function of the protein with rat fetuin A shown to only inhibit the mitogenic effect of insulin in its phosphorylated form (Akhoundi *et al.*, 1994).

Glycosylation of fetuin A included both N-linked (Hayase *et al.*, 1992) and O-linked glycosylations (Gejyo *et al.*, 1983). The glycosylation status of fetuin A has been studied in relation to multiple pathologies with indications that the specific structure of the protein plays an important functional role. Structural analysis of serum fetuin A from chronic pancreatitis patients detected increased levels of sialyl Lewis X (an O-glycan tetrasaccharide carbohydrate) and a higher degree of N-glycan branching compared with healthy control serum, but these differences were not observed in the serum of pancreatic cancer patients (Sarrats *et al.*, 2010). The changes in glycosylation pattern of the protein can also be detected at the tissue level, with high mannose structures present on fetuin A from lung cancer tissue but not control lung tissue, even though overall fetuin A protein and mRNA is decreased in the cancerous tissue (Rho *et al.*, 2009). Additionally, overall glycosylation of the B chain of fetuin A has been shown to be related to diet, with a MALDI-TOF MS based study detecting a 25% lower abundance of the glycosylated form resulting from basal diet than basal with cruciferous diet.

Both of the modifications that have been reported to be associated with fetuin A appear to be functionally relevant and related to pathological conditions, suggesting further work is required at the peptide and PTM level to identify its potential as a biomarker which differentiates CaP patients from BPH patients and/or controls. The identified phosphorylated peptide CDSSPDpSAEDVR will be the priority for further peptide analysis.

3.4.1.3 C4BPA, IGHM and hemopexin

Another two proteins identified in the 2D-DIGE experiments of this study, C4BPA and IGHM were identified from the same spot which formed part of a train of spots. C4BPA is the alpha chain of the C4BP (Scharfstein *et al.*, 1978; Sanchez-corral *et al.*, 1995), and IGHM is the heavy chain (mu chain) of IgM (Arnold *et al.*, 2005). C4BPA and IGHM were identified in the same spot from all three sample types, however at a position of the gel which does not precisely match either theoretical pI or MW. This would suggest that they have migrated together through the gel and are both present in this single spot, perhaps due to natural association in the serum or due to association as a result of sample processing. The presence of both proteins in the single spot across the three different sample types makes it unclear as to whether one or both proteins are responsible for the significant differences in abundance observed, and thus due to time restrictions and lack of targeted assays available, these proteins were not further investigated in the present study. However, targeted proteomic analysis of these two proteins in the future will indicate whether one or both are potential biomarkers for prostatic conditions as indicated by the 2D-DIGE study (significantly increased abundance of this spot in control serum compared with both BPH and CaP serum). Additionally, as both identifications were specific chains distinct from the entire proteins, peptide analysis using MRM will also be a relevant method for their further assessment and validation.

C4BP is a serum glycoprotein which modulates the complement pathway by binding to C4b (Barnum and Dahlback, 1990). Three isoforms have been detected

in serum including the major isoform α7β1, and two minor isoforms: α7β0 and α6β1 (Sanchez-corral *et al.*, 1995). C4BPA is present in the α7β1 isoform, which consists of seven copies of an α-chain (C4BPA, encoded by C4BPA gene) and one β-chain (C4BPB, encoded by C4BPB gene) (Scharfstein *et al.*, 1978; Hillarp and Dahlbäck, 1988; Sanchez-corral *et al.*, 1995). C4BPA plays an important role in the human immune system particularly clear through the presence of the C4b and heparin binding sites for the entire protein (Ogata *et al.*, 1993; Blom *et al.*, 2001). Although only one study has investigated C4BPA in relation to cancer, it showed significantly higher expression in non-small-cell lung carcinoma patients with radiation-induced lung toxicity (RILT) than without (Cai *et al.*, 2010). This suggests there maybe further scope for investigation of C4BPA as a novel biomarker for prostatic conditions.

While the structure and function of IgM have been well studied, the specific functions of IGHM, particularly in disease, have not been elucidated. IgM is present in serum either in the pentameric form consisting five subunits and a single J chain (Askonas and Parkhouse, 1971), or the hexameric form consisting of six subunits without J chain (Arnold *et al.*, 2005). Each subunit is made up of two heavy (IGHM) and two light chains, and five N-linked glycosylation sites can be found on each IGHM (Arnold *et al.*, 2005). Brändlein and his colleagues found that only IgM isotypes as opposed to IgG and IgA were isolated from the lymph nodes or spleen of different cancer patients including stomach, colon, esophagus, pancreas, lung, prostate and breast cancer suggesting close associations between IgM and epithelial cancers (Brändlein *et al.*, 2002; Brändlein *et al.*, 2003). However, no studies have focused on the roles of the specific chains of the protein under physiological nor pathophysiological conditions and thus the relationship of IGHM with CaP is unclear at present.

One additional protein, hemopexin, was also identified from the albumin protein spot from BPH and CaP samples only. This protein was not further analysed because the 2D-DIGE results showed decreased abundances of this protein spot in

BPH and CaP sera compared with that from controls, however only albumin was identified in this spot from control serum. This protein remains one of interest, the constraints on the present study meant its relevance to BPH and CaP could not be further investigated. Further work to quantify hemopexin and specific isoforms should be undertaken to assess whether it may provide additional diagnostic information.

3.4.2 Overall experimental discussion and recommendations

In the present study, the 2D-DIGE proteomic technique was used to discover potential novel CaP biomarkers in serum and five proteins were identified showing potential for CaP diagnosis. Among the five proteins, the serum concentration of total albumin and fetuin A were quantified. The diagnostic usefulness of albumin and fetuin A were verified in a small sample size, which showed higher sensitivity and specificity than the current PSA test. However, a larger study will be required if these proteins are to be verified as diagnostic markers. This larger study should also include assessment of CaP and control subjects known to have coincident liver dysfunction and inflammatory disorders to investigate specificity of these markers. Both proteins appear to be of most use when detected as specific isoforms, and together with the detection of the specific peptides C4BPA and IGHM from entire proteins, it would also be desirable to conduct further work assessing peptides from these proteins using techniques such as MRM and isoform specific experiments such as glycosylation mapping.

No potential biomarker detected was CaP specific; both albumin and fetuin A are also associated with overall systemic pathophysiological conditions such as inflammation. Lack of detection of CaP specific proteins could be due to the heterogeneity of CaP and/or the limited protein detection range of 2D-DIGE. CaP is a multifocal cancer and both inter-tumour and intra-tumour heterogeneity are recognised to complicate molecular profiling (Sboner *et al.*, 2010). Whilst the present study focused on analysis of individual proteins and protein isoforms using

screening and targeted proteomic techniques, due to the heterogeneous nature of CaP it is not clear that an individual protein is sufficient to reflect the abnormalities of CaP, and perhaps only a collective group of proteins can reflect CaP specific changes. This implies that a biomarker panel instead of a single biomarker would have greater use for CaP diagnosis. Various combinations of potential CaP biomarkers identified in this study, using their specific protein isoforms as well as inclusion of other biomarkers such as the PSA, should be examined to find out the best panel of markers which could improve the current CaP diagnosis.

In addition to difficulties surrounding biomarker research of CaP itself, 2DGE proteomic techniques, including 2D-DIGE, only allow analysis of a fraction of all proteins in any sample. In particular, proteins which are extremely under-represented are those outside the specific MW and pI range, as well as those that are hydrophobic, highly acidic, highly basic, and do not denature and solubilise easily. Potential biomarkers of CaP would most likely be altered in abundance in serum as a result of protein presence reflecting the systemic influence of CaP, and protein secretion from the CaP tumour microenvironment. The latter ones are likely to be small and present at low abundance, for example, PSA is only 36 kDa and has a concentration at ng/mL in serum (serum protein concentration currently measurable range from pg/mL to mg/mL (Anderson and Anderson, 2002)). Therefore analysis of low molecular weight serum proteins as well as serum peptidomes could complement the current research. One approach could be using a 20 kD cut-off centrifuged concentrator to fractionate the serum proteins and analyse the low molecular weight peptides/proteins through in solution digestion followed by MS based proteomics. This work would allow detection, identification and quantitation of low molecular weight proteins, degradation products and peptides, which might prove highly specific markers for CaP.

The work of this chapter also highlights the importance of analysing specific protein isoforms, and individual peptides from these forms, which could have closer association to disease states than total protein measurements. The 2D-DIGE

approach allowed detection of individual isoforms and detected a significant change in abundance of multiple, but not all, isoforms of albumin and fetuin A. On the other hand, the targeted proteomic approach allowed quantitation of total albumin and fetuin A concentrations and detected significant changes in concentration again, but only between control and CaP groups, the differences observed related to BPH groups from the 2D-DIGE experiments were not detected. Further characterisations of the modified albumin and fetuin A isoforms are required to determine their role in the progression of CaP and to allow development of the most specific test for CaP diagnosis. Mass spectrometry based proteomics will be of most use for such characterisation, with analysis of mass shift and characterisation of specific modifications which are present/absent. Further analysis for quantitation of specific isoforms and peptides can then occur using techniques such as MRM and development of specific ELISAs using antibodies raised to the relevant isoforms/peptides. MRM would be advantageous over ELISA in this regard, because it targets the specific peptide of interest, once known, and does not require production of highly specific antibodies which is both costly in terms of labour and time. Additionally, further work to assess the usefulness of hemopexin, C4BPA and IGHM as diagnostic biomarkers may allow production of a serum protein biomarker based diagnostic test using a panel of markers, thus increasing sensitivity and specificity further.

In conclusion, the work presented here suggests novel protein and peptide biomarkers for the diagnosis of CaP can be identified from serum. With further work in a larger but similar study, the proteins identified have potential for the development of a non-invasive and highly sensitive and specific diagnostic test for CaP.

Chapter 4 Finding diagnostic biomarkers for CaP in tears using proteomic techniques

4.1 Introduction

With the use of proteomic techniques, up to 500 proteins have been detected in tears (de Souza *et al.*, 2006). This high protein number suggests an excellent possibility of finding biomarkers for systemic diseases such as diabetes and cancer in tears. Herber *et al.* (2001) found that 2D protein profiles were significantly different between control and diabetic patients showing significantly more protein spots in the diabetic group (Herber *et al.*, 2001). A biomarker panel consisting of 20 proteins in tears was successfully generated by SELDI to allow breast cancer patients to be discriminated from healthy women, and the successful detection rate was 71% (Lebrecht *et al.*, 2009). Investigations of tear proteins have shown that the tear protein lacryglobin (also named secretoglobin family 2A member 1) is homologous to lipophilin C which is involved in androgen regulation and could therefore reflect changes to the prostate gland (Molloy *et al.*, 1997; Evans *et al.*, 2001).

PTMs of tear proteins are also a focus in this study. PTMs are critical in various biological process, and changes in PTMs are closely associated with various diseases such as cancer (Krueger and Srivastava, 2006). The two most common types of PTMs are phosphorylation and glycosylation. Phosphorylation often results in changes in enzyme activity and protein signaling processes and glycosylation can change cell-cell recognition/signaling and regulatory functions (Mann and Jensen, 2003). Analysis of protein PTMs is relevant for biomarker detection, and understanding the level of PTMs will allow better understanding of protein functions and their roles in disease. Various studies have investigated the tear protein PTMs (Kuizenga *et al.*, 1991; Zhao *et al.*, 2010), however no systemic detection of both phosphorylated and glycosylated tear proteins have been performed.

Complementing 2DGE analysis of proteins, SELDI technology has the advantage of detecting low molecular weight proteins and peptides (around 500 Da) and was also applied to examine the tear proteome. However, one major disadvantage of SELDI is the poor reproducibility (Baggerly *et al.*, 2004). In this study, the reproducibility of SELDI was tested before it was used for comparative analyses. MRM was used to verify the presence of novel tear proteins detected in the 2DGE experiments, as well as relatively quantifying peptides of interest from control, BPH and CaP tear samples.

The aim of this section was to systematically examine and map the tear proteins that have undergone two of the major post-translational modifications and to detect novel tear proteins, whose specific peptides were then quantified and compared between the control, BPH and CaP groups to identify novel CaP biomarkers.

4.2 Materials and Methods

The following methods have been described in Chapter 2.

- Tear sample collection (Section 2.1.2)

  Different sample sizes were used for different experimental techniques (**Table 4.1**).

  Table 4.1: The number of tear samples (CaP, BPH and control) used for each experimental analysis.

Method of analysis	CaP samples	BPH samples	Control samples
2DGE	3	3	3
SELDI	2	N/A	4
MRM	13	12	11

- Chloroform and methanol precipitation (Section 2.3.5.4)

  Three sets of pooled tear samples were prepared from 15 randomly selected individual samples from each group (control, BPH and CaP). Each pooled sample comprised 2 µL of tears from five samples of a single group.

- 2DGE analysis of the pooled tear samples (Section 2.5.1.2 and 2.5.2)

- Gel staining for phosphoproteins and glycoproteins (Section 2.5.3.2)

- Image matching

  Non-linear Progenesis SameSpots version 3 (Nonlinear Dynamics Ltd, UK) was used to align the total, phospho- and glyco-protein images of the same gel to ensure unambiguous spot matching, and to examine whether one protein spot was both phosphorylated and glycosylated, modified only by one of the PTMs, or was not modified in either way. The total protein gel with the highest number of spots was used as the reference gel to which all other gels were aligned and matched.

- Spot cutting and protein identification (Section 2.9)

  Tear protein spots that had not been identified in previous 2D gel publications, as well as those were glycosylated and/or phosphorylated were all selected for MS analysis for protein identification.

- SELDI analysis (Section 2.10)

  Six samples were randomly selected for preliminary SELDI analyses using H50, reverse phase protein chip. Two samples (P1 and P2) were tear samples from CaP patients, and the remaining four samples (C3-1, C4, C5, C6, and C3-2) were tear samples from control subjects. C3-1 and C3-2 were collected from the same subject but C3-1 was collected at the beginning of the study, whereas C3-2 was collected a week before the experiment was conducted.

  The SELDI experiments comprised two parts: optimisation (five tear samples) and reproducibility testing (four samples) (**Figure 4.1**). Inter-chip variations were assessed by comparing the intensity of major peaks derived from a single sample between two different chips. Inter-run variations were assessed by comparing duplicate spectra generated at different times from a single chip. Spectra generated from each run were labelled a or b (**Figure 4.1**). In addition,

the comparison of the spectra generated from C3-1 and C3-2 indicated whether storage time affected the SELDI result.

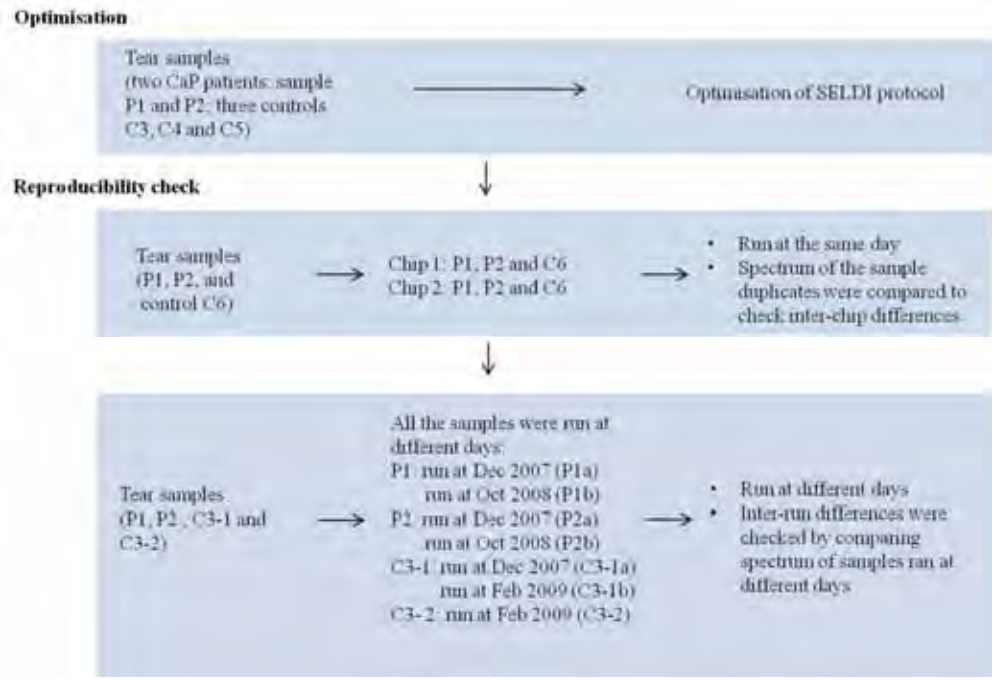


Figure 4.1: SELDI experimental workflow to optimise and test reproducibility.

- MRM validation of dermcidin (DCD) (Section 2.11.3)

  Six MRM transitions (**Table 4.2**) were selected to detect two DCD peptides. The doubly charged ions selected were based on the spectral data obtained from the initial MS protein identification and its product ions were selected by Skyline (section 2.11.2).

Table 4.2: A set of MRMs designed and tested for the detection of dermcidin in basal tear fluid.

Peptide sequence	RT	Q1[a]	Q3[b]
ENAGEDEPGLAR	19.7	564.8	885.4/ 814.4/757.3/628.3/513.3/246.1
DAVEDLESVGK	29.5	581.3	975.5/876.4/747.4/519.3/390.2/204.1

a. Peptide precursor ion *m/z*.
b. Product ion *m/z*.

- MRM quantitation of targeted proteins (Section 2.11.4)

  Thirty-six tear samples were used for MRM quantitation experiments; 11 samples from the control group, 12 samples from the BPH group and 13 samples from the CaP group (**Table 4.1**).

  Fifty micrograms of protein were measured (Section 2.2.2) from individual tear samples where possible and from pooled tear samples where required, and used for duplicate MRM experiments (**Table 4.3).**

  Six target proteins were selected including two highly abundant proteins lactoferrin (Lf) and lipocalin 1 (LCN1), and four relatively low abundance proteins depleted in malignant brain tumour 1 (DMBT1), nucleobindin 2 (NUCB2), DCD and secretoglobin, family 2A, member 1 (SCGB2A1). These proteins were all identified from 2DGE gels followed by FT-MS/MS in the present study, therefore the mass spectral information for all peptides was available. A total of 31 MRM transitions were constructed, of which the parental ions were selected based on their mass spectral information and the product ions were selected as previously described in section 2.11.2 (**Table 4.4**).

  When performing a quantitative MRM, peptide concentration can be normalised against a known concentration of internally spiked peptide (synthetic) to prepare standard curve for each peptide tested. This allows for absolute quantitation. In the present study, quantitation of each peptide in a sample group was carried out relative to the same peptide in a different sample group. Following consultation with Dr. Thomas John (Biostatistician in Brien-Holden Vision Institute), the normalisation step was achieved by dividing the average peak area of target peptide by a known peptide whose concentration remained consistent between control, Cap, and BPH. The control peptides were selected by two criteria, firstly no significant differences were detected between (or within) groups based on analysis by MRM, and secondly concentration in

tears has not been reported in the literature to be associated with cancer. As a result, the quantitation that was achieved was a relative quantitation rather than absolute quantitation.

Table 4.3: Samples used for MRM quantitation were either made up of 25 µg of protein from a single tear sample or from multiple tear samples combined.

Sample Type	Sample used (ID number)
Control	164
	219
	251
	571,190,181 pooled
	444
	449
	541
	231, 294 pooled
BPH	420
	85
	134,456
	234,86,424,92,96 pooled
	99,94,85 pooled
CaP	141
	380
	586
	182,401
	333,366,362 pooled
	179,440,225 pooled
	156,39 pooled

Table 4.4: MRM transition lists were designed for two peptides from DCD and SCGB2A1, one peptide from DMBT1, NUCB2 and LCN1, and three peptides from Lf.

Target proteins	Peptide Sequence	Precursor ions (Q1) $[M+2H]^{2+}$	Product ions (Q3) $[M+1H]^{1+}$
DCD	DAVEDLESVGK	581.3	876.4 747.4 632.4
DCD	ENAGEDPGLAR	564.8	757.4 628.3 513.3
DMBT1	FGQGSGPIVLDDVR	730.4	926.5 829.5 938.6
NUCB2	EYENIIALQENELK	853.4	1057.6 944.5 873.5
SCGB2A1	ELLQEFIDSDAAAEAMGK	969.5	1325.6 1178.5 1065.5
SCGB2A1	TINSDISIPEYK	690.4	849.5 736.4 536.3
Lf	DGAGDVAFIR	510.8	777.4 720.4 605.4
Lf	LRPVAAEVYGTER	730.9	995.5 924.4 853.4
Lf	VPSHAVVAR	468.3	836.5 739.4 652.4 515.3
LCN1	NNLEALEDFEK	661.3	851.4 667.3 780.4

4.3 Results

4.3.1 Mass spectrometry identification of protein spots

Proteins that had not previously been identified from 2DGE separation of tear film were detected first using Sypro Ruby stain and were then excised from Coomassie stained gels (**Figure 4.2**) and identified by mass spectrometry (**Table 4.5**). Peptides from four of the protein spots matched multiple proteins. The most interesting identification was that of DCD (spot 8, **Figure 4.2**) which has not previously been identified in 2DGE analysis of tears. All other proteins have been previously identified (Molloy *et al.*, 1997; Green-Church *et al.*, 2008).

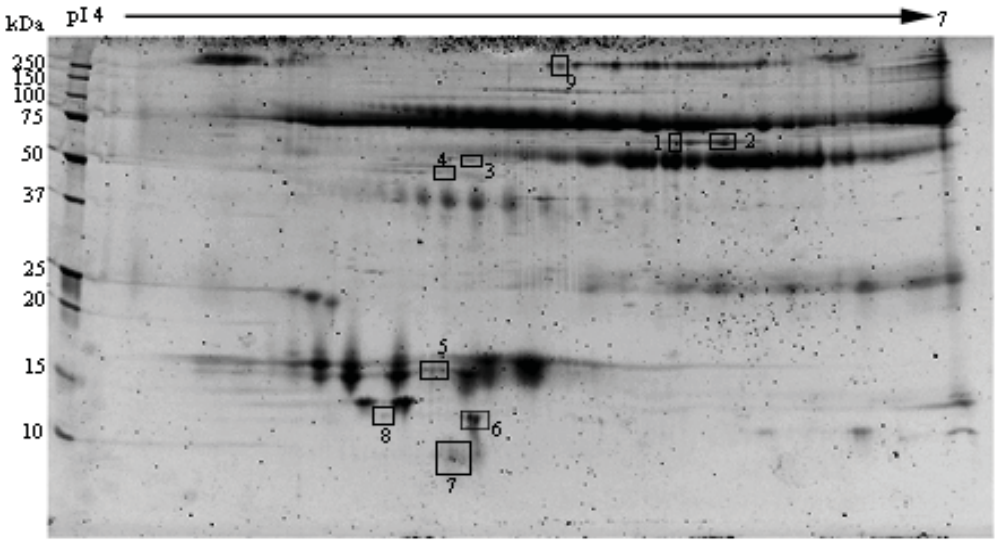


Figure 4.2: A representative Sypro Ruby stained 2D gel of tear samples showing the spots identified by MS analysis. Proteins identified in this figure are summarised in table 4.5.

Table 4.5: Summary of proteins identified using mass spectrometry.

Spot ID	Protein identification	Sequence coverage%	MOWSE Score	Accession no	Theoretical MW (kDa)/pI	Phospho(P)/Glyco(G) observed in this study
1	Serum albumin	23	867	gi|28592	69.3/6.05	P and G
2	Serum albumin	26	1129	gi|28592	69.3/6.05	P and G
3	α-1 Antitrypsin	8	170	gi|177927	46.6/5.42	P
4	Nucleobindin 2	8	271	gi|4826870	50.2/5.03	P
5	Lipocalin 1 precursor	31	303	gi|4505821	19.2/5.39	P and G
5	Prolactin-induced protein	19	174	gi|4505821	16.6/8.26	P and G
6	Lipocalin 1 precursor	28	211	gi|4505821	19.2/5.39	P and G
6	Prolactin-induced protein	26	209	gi|4505821	16.6/8.26	P and G
6	Secretoglobin, family 2A, member 1	21	81	gi|4505171	10.9/5.48	P and G
6	Dermcidin	10	57	gi|16751921	11.277/6.08	P and G
7	Secretoglobin, family 2A, member 1	40	214	gi|4505171	10.9/5.48	P and G
8	Cystatin S precursor	23	182	gi|4503109	16.2/4.95	**Not detected**
8	Cystatin SA-III	27	174	gi|235948	14.1/4.74	**Not detected**
8	Secretoglobin, family 2A, member 1	21	107	gi|4505171	10.9/5.48	**Not detected**
9	Deleted in malignant brain tumors 1	3	315	gi|157423470	180.9/5.22	G

4.3.2 Comparison of the total, Phospho- and Glycoprotein profiles of tears

Total protein spots were detected using Sypro Ruby and an average of 333 ± 27 (mean ± SD) protein spots were detected using Progenesis SameSpot (A, **Figure 4.3**). An average of 157 ± 10 spots (mean ± SD) were detected on gels stained specifically for glycoproteins (B, **Figure 4.3**), and 215 ± 20 spots (mean ± SD) detected on gels stained specifically for phosphoproteins (C, **Figure 4.3**).

Protein spots 4, 7 and 9, (**Figure 4.3**) were detected as phosphorylated (4 and 7) and/or glycosylated (7 and 9), and were identified using FT-MS/MS (**Table 4.5**). Protein spots 10, 11 and 12 (**Figure 4.3**) were detected with glycostaining, and these protein spots have previously been identified in our lab (unpublished work) as lactoferrin, IgA and zinc-α-2-glycoprotein respectively (**Table 4.6**). Protein spots 13, 14 and 15 (**Figure 4.3**) were detected with both glyco- and phospho- stains; previous results from our lab have identified proteins within these regions as PIP, LCN1 and Cystatin S (**Table 4.6**). Cystatin S was also identified in spot 8 (**Figure 4.3**), and thus it is possible that spot 14 (**Figure 4.3**) is post-translationally modified form of this protein. FT-MS/MS identification did not detect presence of phosphorylated peptides.

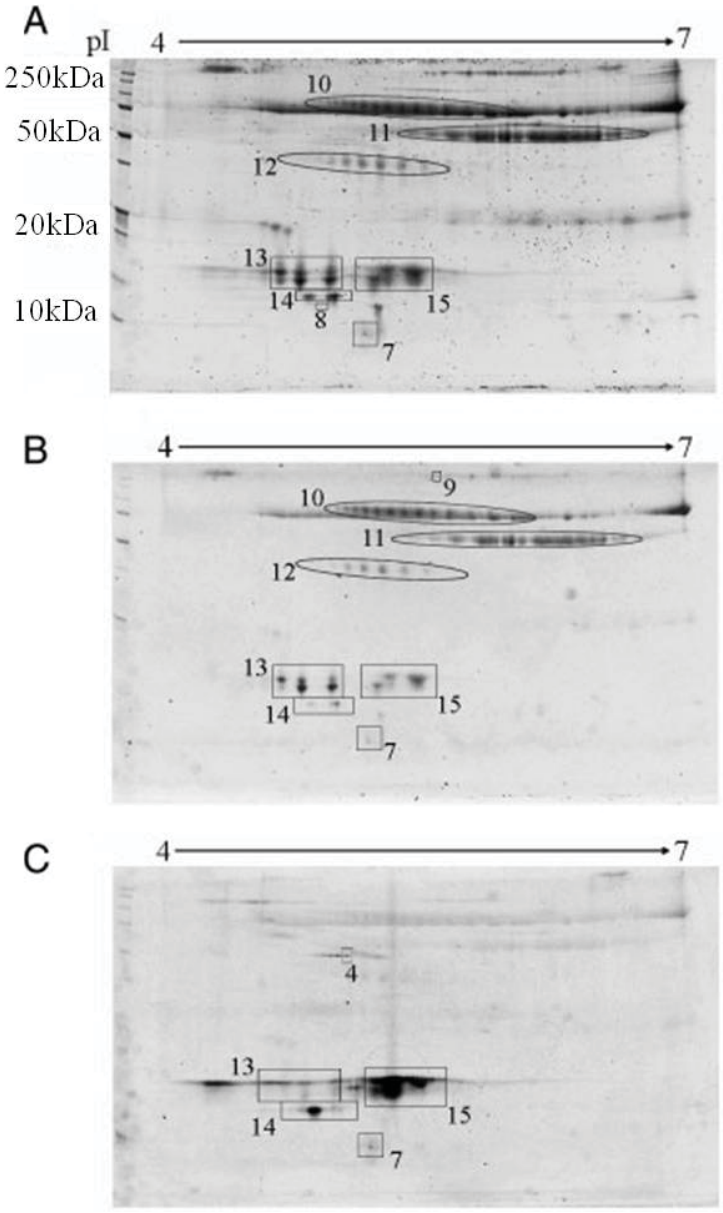


Figure 4.3: Representative gel images used to detect the total, phospho- and glyco-protein profiles of human tears. A: total protein profile stained by Sypro Ruby; B: glycoprotein profile stained by Pro-Q Emerald; C: Phosphoprotein profile stained by Pro-Q Diamond. Protein spots marked on these gels are those that were identified in the present study (**Tables 4.5** and **4.6**) or have previously been identified from 2DE gels in the literature (**Table 4.6**).

Table 4.6: Proteins identified from tear samples separated using 2DGE in the present study (spots 4-9, Figure 4.3) or in previous work (spots 10-15, Figure 4.3).

ID	Proteins	Sources of Identification
4	NUCB2	Present study
7	SCGB2A1	Present study
8	Cystatin S precursor, cystatin SA-III	Present study
9	DMBT1	Present study
10	Lf	Previous work
11	IgA	Previous work
12	Zn-α-2-glycoprotein	Previous work
13	Prolactin-induced protein	Previous work
14	Two isoforms of Cystatin S	Previous work
15	LCN1	Previous work

4.3.3 SELDI-MS analysis of tear samples

The SELDI method (Section 2.10) was tested initially using tear samples from two CaP patients (P1 and P2) and three controls (C3, C4 and C5), to ensure peptide detection. The spectra from the tear samples applied to a reverse phase, H50 Protein Chip showed distinct peaks across the m/z detection range from 2,000 to 50,000 Da, with the majority of peaks detected at m/z < 20,000 (**Figure 4.4**).

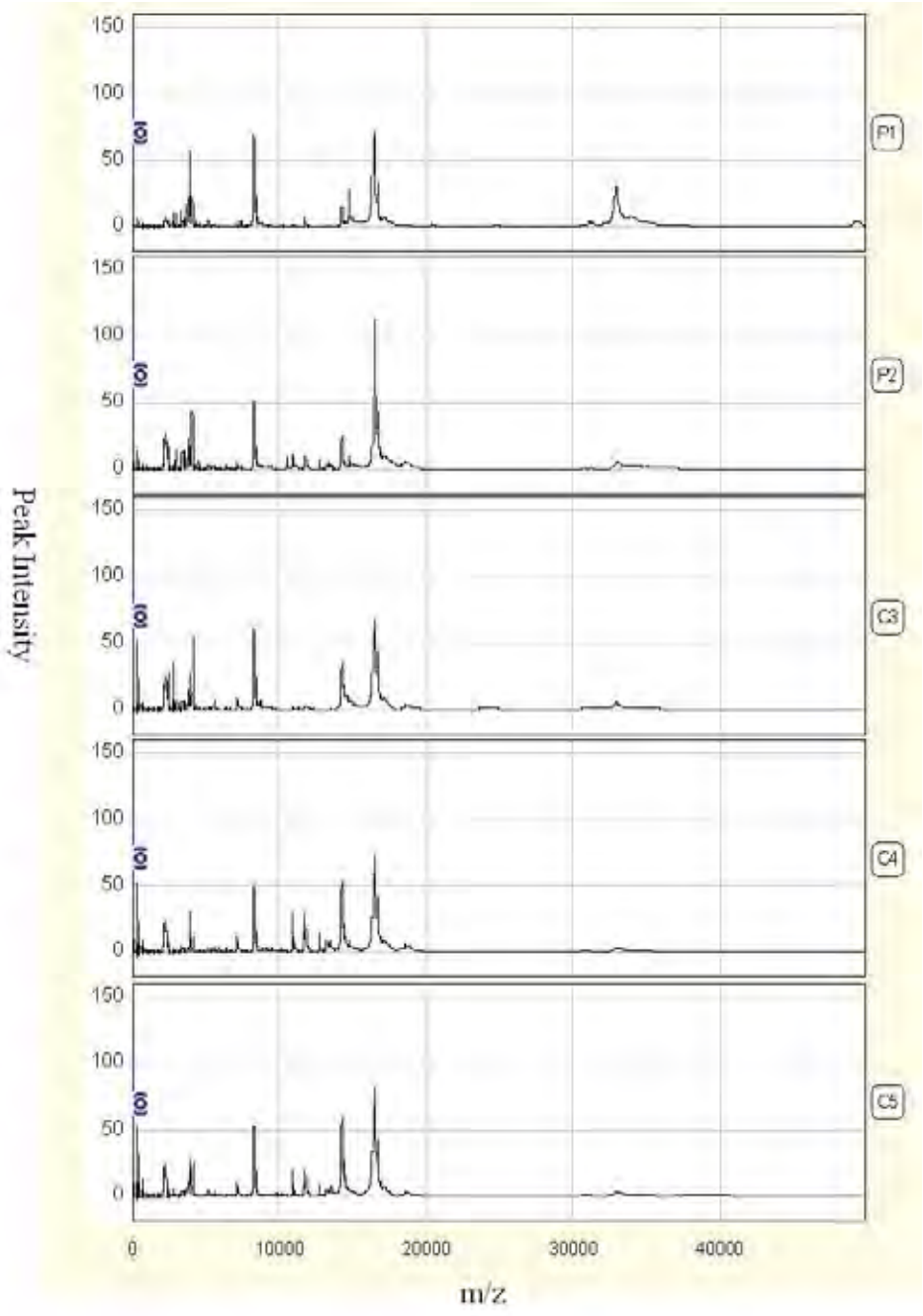


Figure 4.4: Spectra obtained from five tear samples applied to an H50 ProteinChip using the optimised SELDI protocol described in Section 2.10 showed distinct peaks which had m/z < 20,000, but few peaks with m/z > 20,000.

4.3.3.1 Inter-chip variation

Variation due to the use of multiple chips was assessed following protocol optimisation, as comparison of multiple samples would require the use of numerous chips. The results of Biomarker Wizard analysis (ProteinChip Software version 3.2; Ciphergen Biosystems, Inc.) for duplicate spectra of three samples were presented as two lists of peak intensities for each sample. Each list consisted of the log normalised intensity of peaks detected from a single sample on Chip 1 (red dot) or Chip 2 (blue square) (**Figure 4.5**). Most peaks showed log normalised intensities between ± 2 (**Figure 4.5**), and only the clusters which had m/z > 2000 and peak intensities > 10 were considered for analysis. Peaks from sample P1 had higher intensities when detected from Chip 1 than from Chip 2 (**Figure 4.5 A**). However, both P2 and C6 did not show this trend (**Figure 4.5 B and C**). CV% of each peak that had an intensity greater than 10 (which had better chances to be purified at a later stage if needed) was examined to more accurately assess the inter-chip reproducibility.

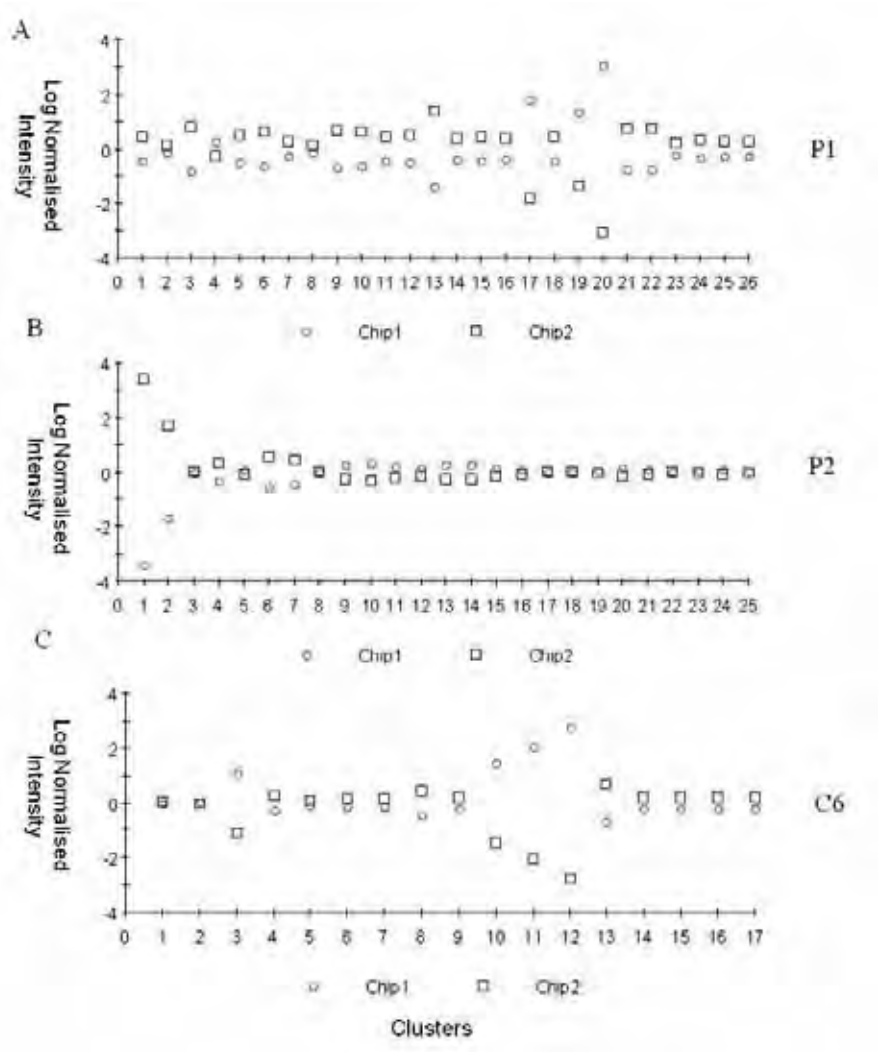


Figure 4.5: Cluster results of each sample generated by Biomarker Wizard.

The CV% of all peaks detected from sample P1 were greater than 10%, with the highest CV% close to 50% (**Table 4.7**). Five clusters that had peak intensities over 10 were detected from sample P2, and most of them had a CV% of less than 5%. Two of them had a CV% of greater than 10% and the highest CV% was 21% (**Table 4.8**). Sample C6 also had five clusters with peak intensities greater than 10, and only one had a CV% less than 10% with the highest CV% at 22% (**Table 4.9**). None of the samples showed a CV% less than 10% of all the peaks detected. Therefore the inter-chip variations were high.

Table 4.7: CV% of clusters which were detected from P1 and had peak intensities greater than ten.

Cluster #	m/z	Peak intensity - Chip1 (P1)	Peak intensity - Chip2 (P1)	CV%
2	3874.69	36.99	46.01	15%
5	4018.02	11.17	22.10	46%
14	8217.92	36.97	63.66	38%
15	8323.15	12.00	22.52	43%
23	16437.5	56.21	76.94	22%
24	16644.3	21.87	35.57	34%

Table 4.8: CV% of clusters which were detected from P2 and had peak intensities greater than ten.

Cluster #	m/z	Peak Intensity - Chip 1 (P2)	Peak Intensity - Chip 2 (P2)	CV%
3	3701.89	12.10	12.68	3%
11	8222.84	48.77	36.38	21%
12	8328.69	15.53	12.50	15%
22	16449.97	72.07	74.79	3%
23	16654.54	30.08	28.11	5%

Table 4.9: CV% of clusters which were detected from C6 and had peak intensities greater than ten.

Cluster #	m/z	Peak Intensity - Chip 1 (C6)	Peak Intensity - Chip 2 (C6)	CV%
2	3874.09	10.75	10.57	1%
5	8219.77	39.07	45.27	10%
6	8324.59	12.82	16.06	16%
14	16440.88	63.64	85.09	20%
15	16645.42	25.52	34.91	22%

4.3.3.2 Inter-run variation

Between run variations were also assessed using three samples on three different dates. Qualitative comparison found that three regions of the spectra at m/z < 5,000

had major pattern differences between runs (**Figure 4.6**). When compared with the analyses conducted on Oct-2008 and Feb-2009, the samples analysed on Dec-2007 had many more peaks detected and higher peak intensities observed (**Figure 4.6**). To test whether the differences in spectra were due to sample degradation overtime, C3-1 and 2 (C3-1 was collected from the same patient C3 two years prior to analysis and C3-2 collected one week prior to analysis) were analysed together (Feb-2009) and no differences in spectra were observed (**Figure 4.6**).

Notably, this was only preliminary work to assess whether SELDI-MS would provide results with the accuracy and reproducibility required for the present tear biomarker research. The variations observed both between Protein Chips and between experimental runs were too large to verify the use of this technology in the present study. A different MS based technique, MRM analysis, was thus used to relatively quantify proteins and peptides of interest in tear samples.

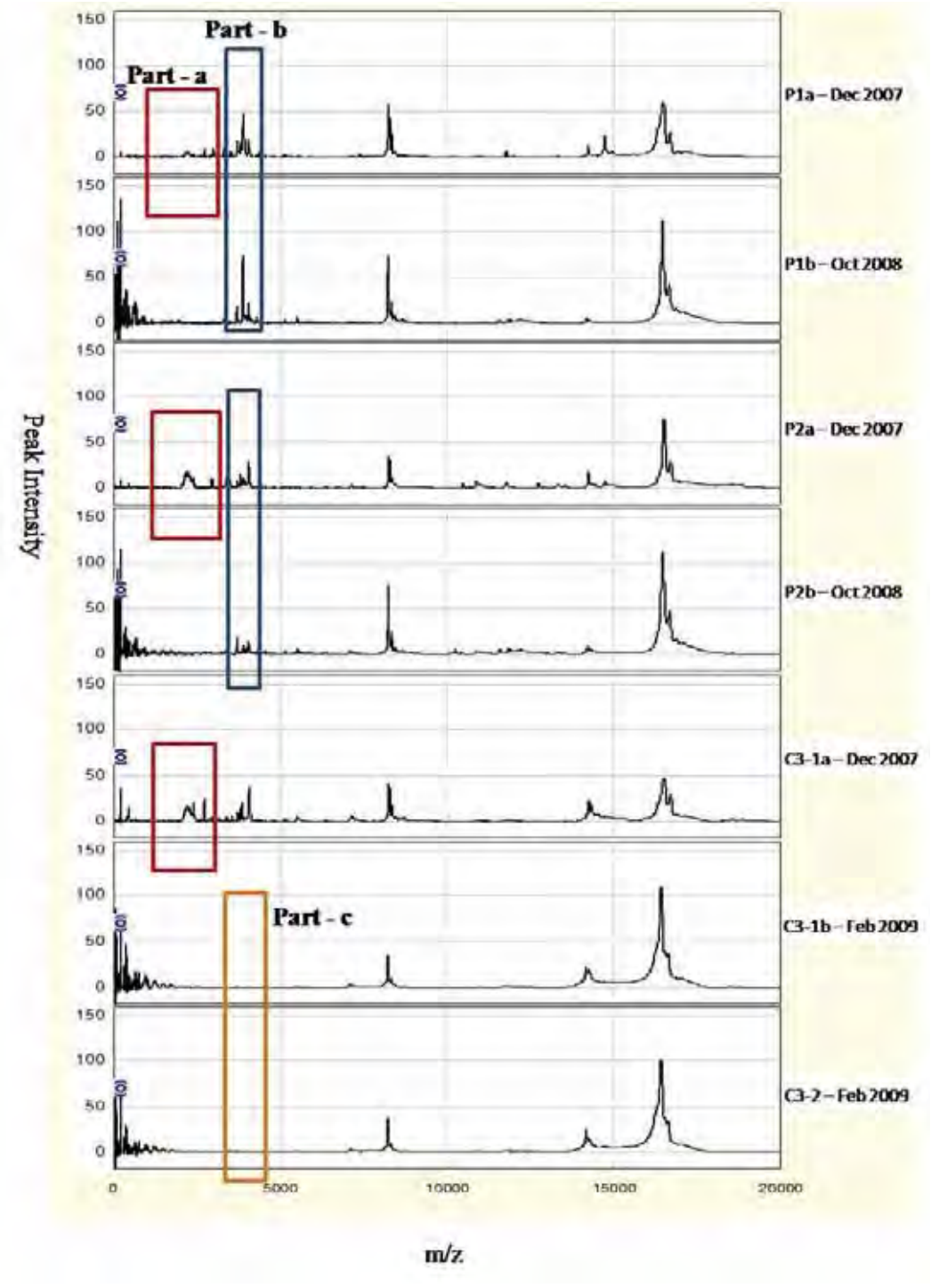
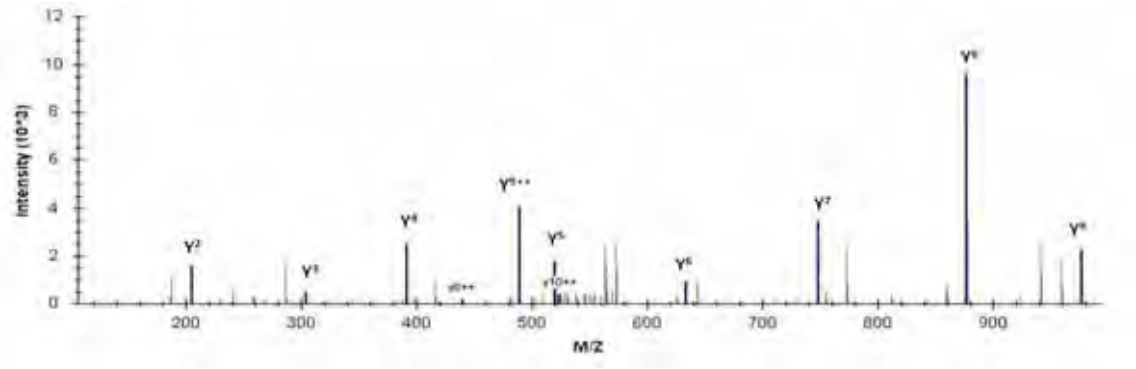


Figure 4.6: Spectra of tear samples (P1, P2 and C3) generated on different dates (Dec- 2007, Oct-2008 and Feb-2009) to assess inter-run variation. Three major areas of difference are highlighted as part-a (red rectangle), part-b (blue rectangle) and part-c (yellow rectangle).

4.3.4 Validation of DCD in Tear samples using MRM

DCD was the only protein identified in the present study whose presence was not confirmed previously. Analysis of a tryptic digest of basal tear fluid verified the presence of the two peptides targeted, with all 12 transitions detected by MRM (**Figure 4.7**). After the MRM method was constructed, each MRM transition and respective retention time were validated again to ensure they were indicative of the specific target peptides. Full scan MS/MS was acquired upon the detection of the MRM signal with manual inspection of each resultant spectrum to substantiate a match to the target peptide (**Figure 4.7**). A search of the international protein index human protein database using the MS/MS spectra confirmed that dermcidin (with a score of 53, individual ions scores > 35 indicate identity or extensive homology ($p<0.05$)) was the only protein candidate for the two peptides.

A



B

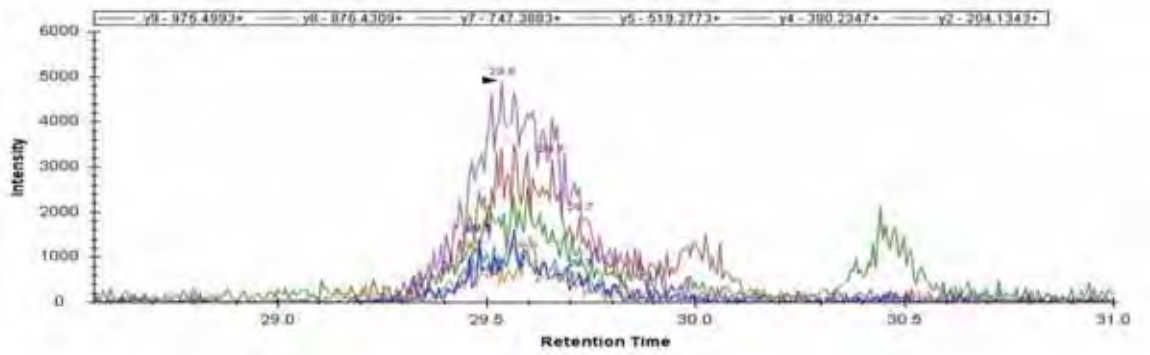


Figure 4.7a: Transition development and MRM-triggered MS/MS validation of the identity of the peptide representing DCD in a digest of basal tear fluid (A-B). A: tandem mass spectra of peptide DAVEDLESVGK for DCD. The six MRM transitions subsequently chosen for this peptide were 975.5 (y9), 876.4 (y8), 747.4 (y7), 519.3 (y5), 390.2 (y4), 204.1(y2), B: extracted ion chromatogram traces of six MRM transitions 581.3 and 975.5/876.4/747.4/519.3/390.2/204.1 (Q1 and Q3) developed for peptide DAVEDLESVGK.

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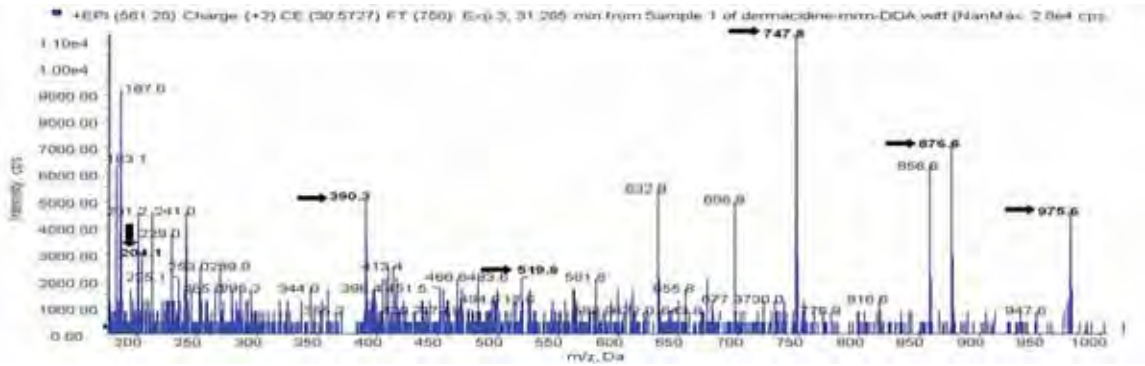
C



D

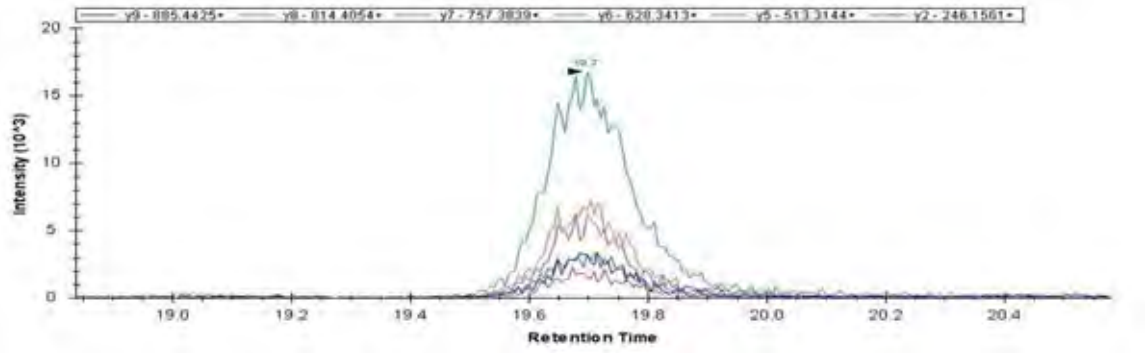
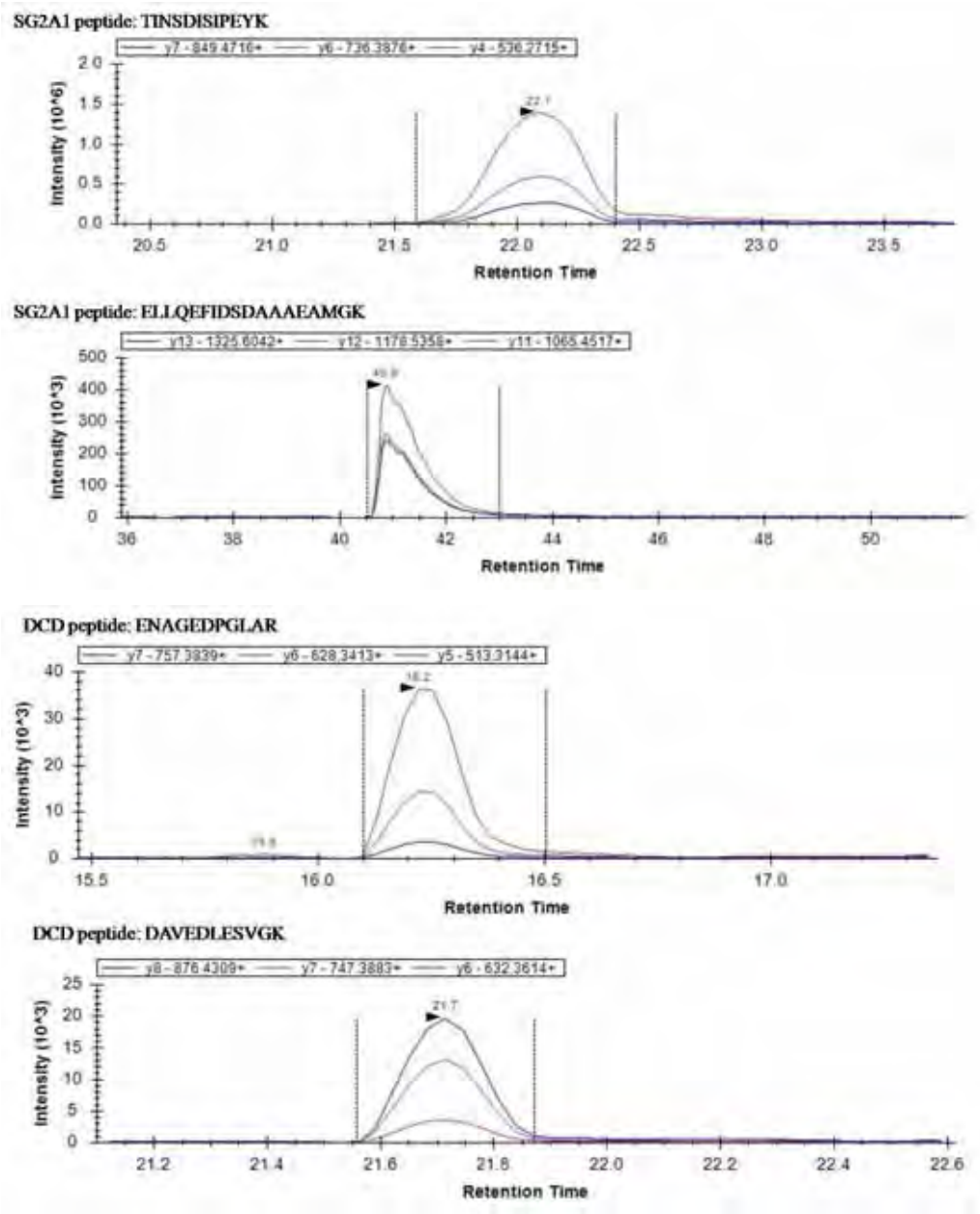


Figure 4.7b: Transition development and MRM-triggered MS/MS validation of the identity of the peptide representing DCD in a digest of basal tear fluid (C-D). C: MS/MS spectrum of peptide fragment taken at the time of the peak shown in panel B. Arrows indicate fragments monitored in this MRM. cps, counts per second. D: extracted ion chromatogram of six transitions 564.7 and 885.4/ 814.4/757.3/628.3/513.3/246.1 developed for peptide ENAGEDEPGLAR.

4.3.5 MRM quantitation of peptides of interest

All transitions designed for target peptides successfully detected the peptides (**Figure 4.8**) and no significant differences in retention times, relating to a single peptide, between samples were detected, suggesting the peptides detected in each sample were indeed the target peptides. Peptide quantitation was based on the log of total peak area (sum of all transitions) for each peptide. The average of the log form of total peak area for each peptide from each group, *ie*. control, BPH and CaP was calculated with only a small variation observed (**Figure 4.9**).



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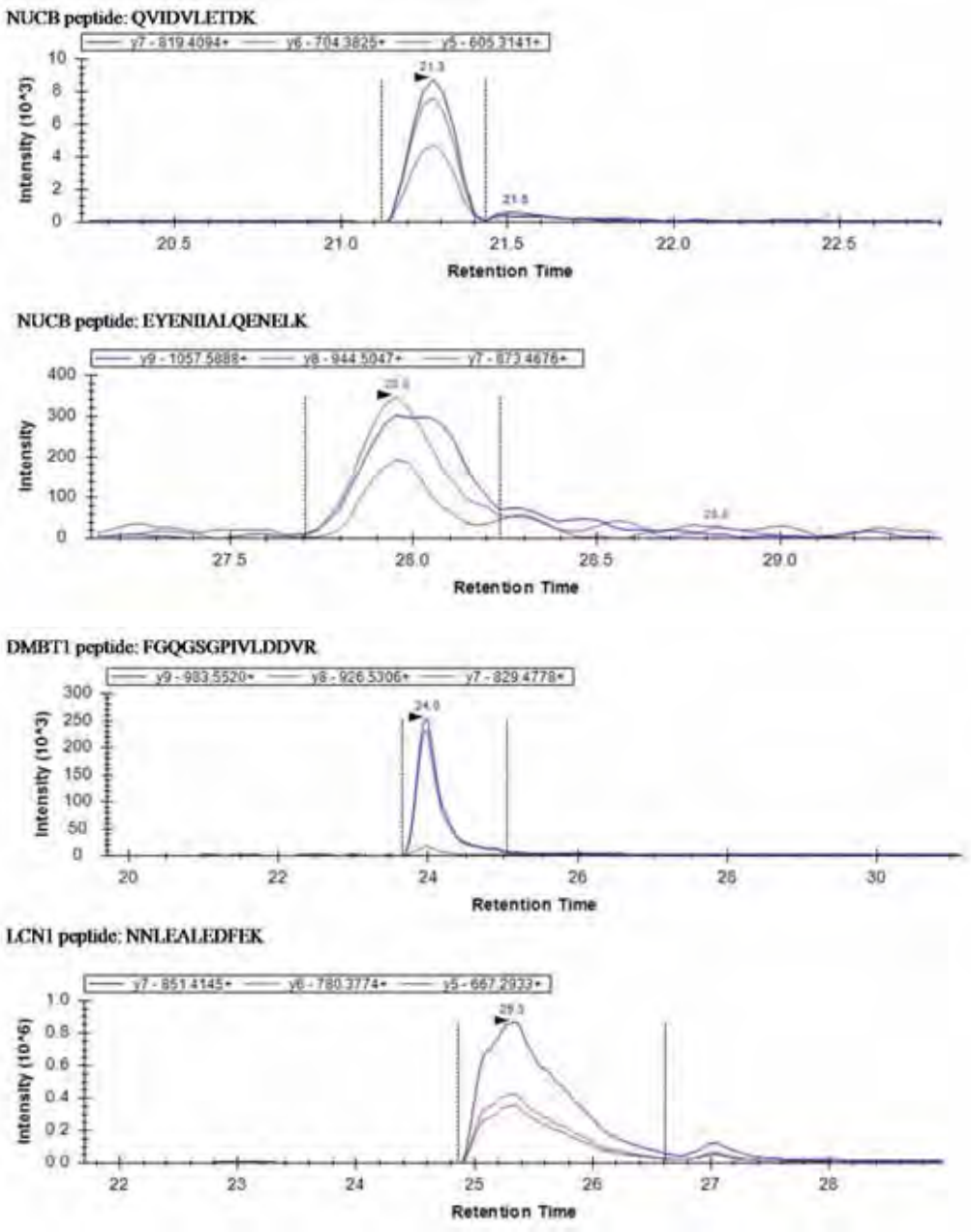

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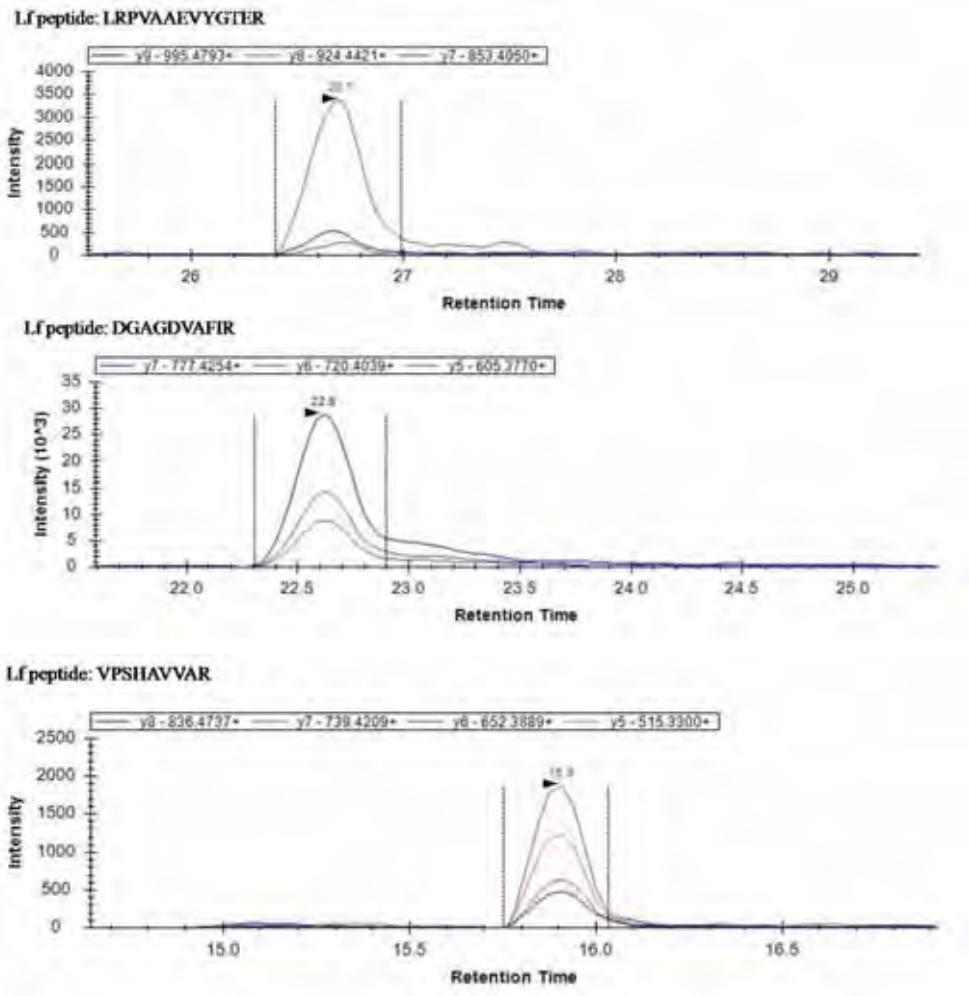

Figure 4.8: The extracted ion chromatogram of 11 transitions detected by MRM.

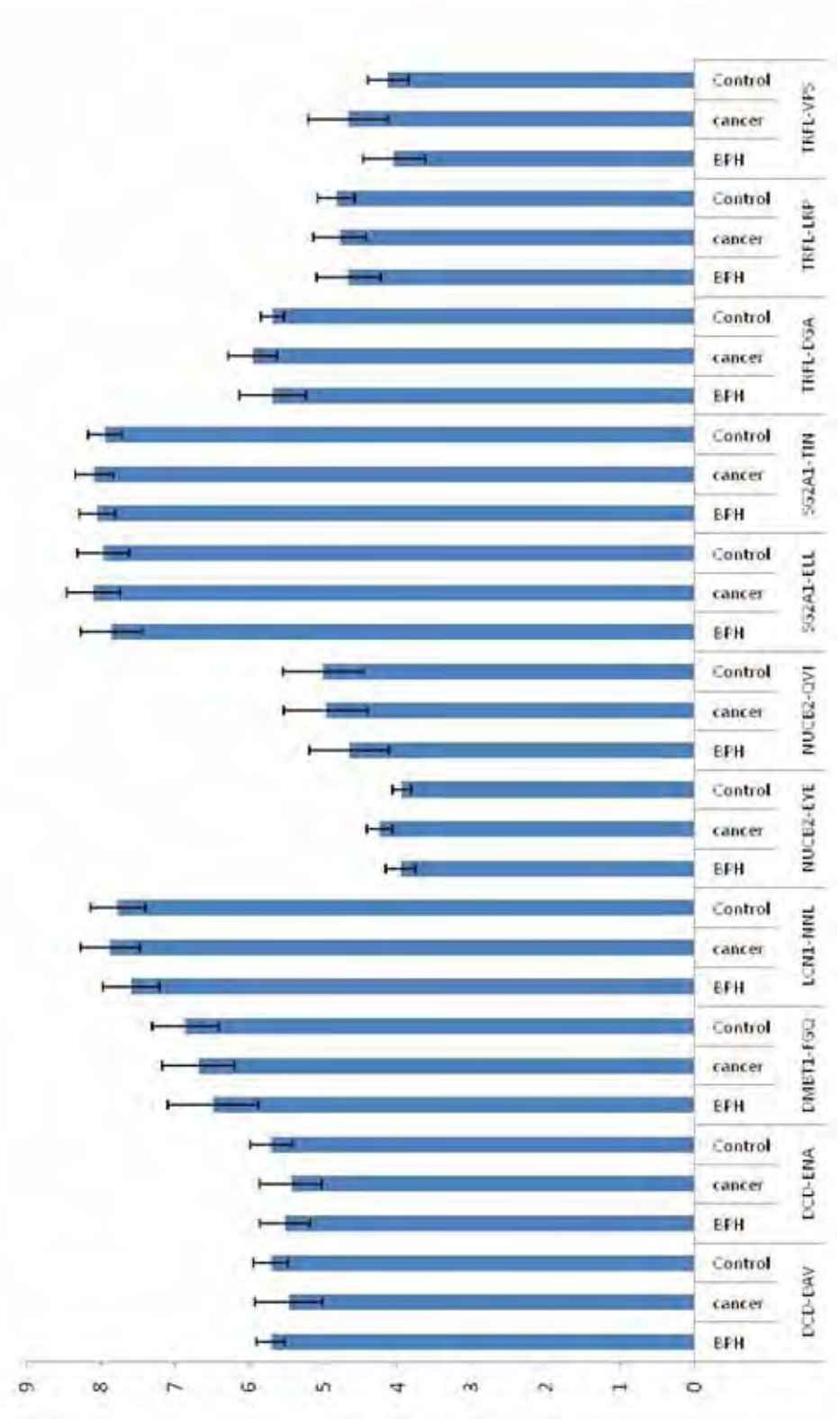

Figure 4.9: The average total peak area of each peptide for each group (the error bars were the SD). Each peptide was named by the first three amino acids.

Peptides from SCGB2A1 were deemed unsuitable as control peptides because the SCGB2A1 protein has previously been suggested to be associated with CaP (Evans *et al.*, 2001) and thus peptide levels might reflect this association and would not be suitable for use as controls. Peptide EYENIALQENELK from NUCB2 was also unsuitable for use as a control because, without normalisation, MRM results showed significant differences between CaP samples and both BPH and control samples when analysed using Tukey's test (**Table 4.10)**. The peptide which was selected as the control peptide was that from LCN1 because it satisfies the two crucial criteria that no significant differences were detected between (or within) groups in the present study and its concentration in tears has not previously been reported in the literature to be associated with cancer, and this peptide also had the lowest and most consistent CV% (**Table 4.11**).

Table 4.10: Statistical analysis to detect differences in peptide abundances between control, BPH and CaP groups suggests that only peptide EYENIALQENELK from NUCB2 varied significantly.

Peptide Sequence	Dependent Variable	Groups	p*		
			Groups		
			BPH	CaP	Control
DAVEDLESVGK (from DCD)	RetentionTime_mean	BPH		.940	.984
		CaP	.940		.980
		control	.984	.980	
	TotalArea_Log_mean	BPH		.405	1.000
		CaP	.405		.317
		control	1.000	.317	
DGAGDVAFIR (from Lf)	RetentionTime_mean	BPH		.929	.965
		CaP	.929		.990
		control	.965	.990	
	TotalArea_Log_mean	BPH		.328	.999
		CaP	.328		.268
		control	.999	.268	
ELLQEFIDSDAAAEAMGK (from SCGB2A1)	RetentionTime_mean	BPH		.886	.646
		CaP	.886		.900
		control	.646	.900	
	TotalArea_Log_mean	BPH		.573	.888
		CaP	.573		.808
		control	.888	.808	
ENAGEDPGLAR (from DCD)	RetentionTime_mean	BPH		.921	.935
		CaP	.921		.998
		control	.935	.998	
	TotalArea_Log_mean	BPH		.913	.636
		CaP	.913		.328
		control	.636	.328	
EYENIIALQENELK (from NUCB2)	RetentionTime_mean	BPH		.989	.502
		CaP	.989		.558
		control	.502	.558	
	TotalArea_Log_mean	BPH		.035	.986
		CaP	.035		.015
		control	.986	.015	
FGQGSGPIVLDDVR (from DMBT1)	RetentionTime_mean	BPH		.680	.370
		CaP	.680		.853
		control	.370	.853	
	TotalArea_Log_mean	BPH		.792	.432
		CaP	.792		.809
		control	.432	.809	

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PeptideSequence	Dependent Variable	Groups	p*		
			Groups		
			BPH	CaP	Control
LRPVAAEVYGTER (from Lf)	RetentionTime_mean	BPH		.949	1.000
		CaP	.949		.942
		control	1.000	.942	
	TotalArea_Log_mean	BPH		.815	.672
		CaP	.815		.965
		control	.672	.965	
NNLEALEDFEK (from LCN1)	RetentionTime_mean	BPH		.977	1.000
		CaP	.977		.964
		control	1.000	.964	
	TotalArea_Log_mean	BPH		.446	.683
		CaP	.446		.883
		control	.683	.883	
QVIDVLETDK (from NUCB2)	RetentionTime_mean	BPH		.938	.756
		CaP	.938		.921
		control	.756	.921	
	TotalArea_Log_mean	BPH		.618	.527
		CaP	.618		.991
		control	.527	.991	
TINSDISIPEYK (from SCGB2A1)	RetentionTime_mean	BPH		.691	.340
		CaP	.691		.808
		control	.340	.808	
	TotalArea_Log_mean	BPH		.957	.787
		CaP	.957		.583
		control	.787	.583	
VPSHAVVAR (from Lf)	RetentionTime_mean	BPH		.893	.900
		CaP	.893		.999
		control	.900	.999	
	TotalArea_Log_mean	BPH		.054	.948
		CaP	.054		.055
		control	.948	.055	

* Significance level $p < 0.05$.

The relative quantitation ratio of all other peptides was then generated by dividing peak area of target peptide by the peak area of the LCN1 peptide. The two tailed Student's t-test adjusted with Dunn-Bonferroni correction was used to analyse significant differences in quantitation ratio between CaP, BPH and control groups. Only peptide VPSHAVVAR from Lf showed a significant difference between control and CaP groups. The peptide was of highest relative concentration in CaP samples compared to control and BPH samples with a significant increase observed between CaP and control ($p = 0.012$) (**Figure 4.10**).

Table 4.11: Within sample CV% of peptides analysed using MRM for determination of a normalisation control.

Protein	Peptide	Groups	CV%
DCD	DAVEDLESVGK	BPH	3%
	DAVEDLESVGK	CaP	8%
	DAVEDLESVGK	Control	4%
	ENAGEDPGLAR	BPH	6%
	ENAGEDPGLAR	CaP	8%
	ENAGEDPGLAR	Control	5%
DMBT1	FGQGSGPIVLDDVR	BPH	9%
	FGQGSGPIVLDDVR	CaP	7%
	FGQGSGPIVLDDVR	Control	6%
LCN1	NNLEALEDFEK	BPH	5%
	NNLEALEDFEK	CaP	5%
	NNLEALEDFEK	Control	5%
NUCB2	QVIDVLETDK	BPH	12%
	QVIDVLETDK	CaP	11%
	QVIDVLETDK	Control	11%
Lf	DGAGDVAFIR	BPH	8%
	DGAGDVAFIR	CaP	6%
	DGAGDVAFIR	Control	3%
	LRPVAAEVYGTER	BPH	9%
	LRPVAAEVYGTER	CaP	7%
	LRPVAAEVYGTER	Control	5%
	VPSHAVVAR	BPH	10%
	VPSHAVVAR	CaP	12%
	VPSHAVVAR	Control	7%

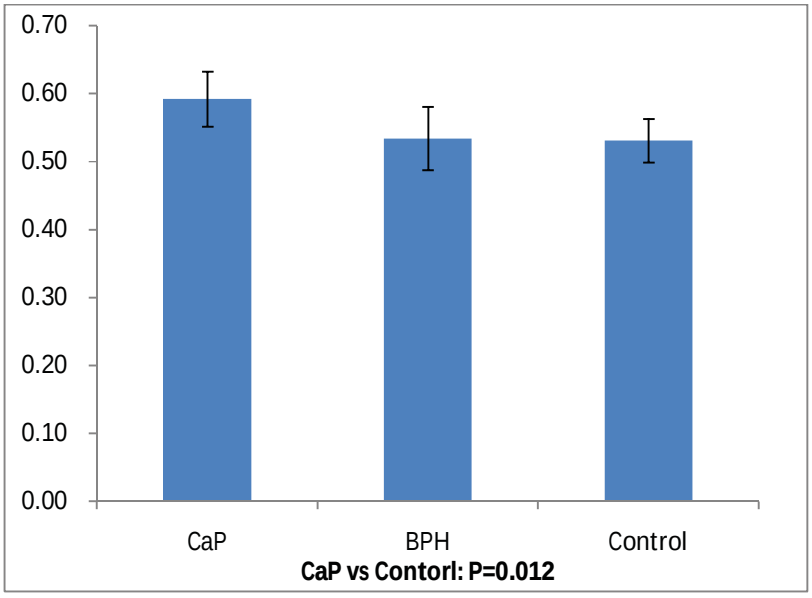

Figure 4.10: A significant increase in the target peptide : control peptide ratio of VPSHAVVAR from Lf was observed in CaP tear samples compared with control tear samples (the error bars were the SD).

4.4 Discussion

This chapter presents the first study that has mapped the phosphorylation, glycosylation and total 2D tear protein profiles, as well as applied a relative quantitative MRM technique, for tear biomarker research. Among the PTMs detected, previously reported phosphorylation (Zhao *et al.*, 2010) and glycosylation (Molloy *et al.*, 1997) of SCGB2A1, and glycosylation of Lf, IgA and Zn-α-2-glycoprotein (Kuizenga *et al.*, 1991) have been confirmed in this study. Phosphorylation of NUCB2 has also previously been identified in tear samples (Zhao *et al.*, 2010); however, no glycosylation of NUCB2 was detected in this study. In addition to the findings of PTMs of tear proteins, an antimicrobial protein, DCD, was detected and verified in tears using MRM for the first time, suggesting a possible new mechanism in tear defence. Most importantly, a potential CaP biomarker, a particular Lf peptide (VPSHAVVAR) was detected by MRM using the relative quantitation approach, which involved measurement of a control peptide to

allow a ratio of all target peptides to be calculated, these ratios were then compared between groups. This novel finding emphasised the potential of detecting cancer biomarkers in tears. Further investigations on this particular peptide as well as tear proteome may lead to the development of a better CaP diagnostic test.

4.4.1 Total, phospho- and glycoprotein profile of Tears

A significantly higher number of total protein spots (333 ± 27, mean ± SD) were detected using 2DGE separation of tear fluid in the present study when compared with a similar tear study previously reported. Herber *et al*. (2001) compared the protein profiles of tears from healthy controls (166 ± 11, mean ± SEM) and those from diabetic patients (243 ± 31, mean ± SEM). However in the present study a larger sample volume was used for IEF (10 μL versus 5 μL), and additional methanol/chloroform precipitation was used to remove lipids and salts.

Of the proteins identified in the present study, DCD was the only one that has not previously been identified in tear 2DGE analysis. Rieg *et al.* (2006) was unsuccessful in attempts to identify DCD from saliva and tears using SELDI-TOF-MS (Rieg *et al.*, 2006), although de Souza *et al.*, (2006) detected DCD in tears by analysing digested 1D gel fragments using LTQ-FT and LTQ-Orbitrap, the result was not verified (de Souza *et al.*, 2006). Compared to the work of Rieg *et al*. (2006), it is likely that the identification of DCD in the present study was made possible by the use of basal rather than the relatively protein poor stimulated reflex tears, and the use of the more sensitive FT-MS/MS approach compared with SELDI-TOF-MS. Notably, mRNA of DCD has been detected on corneal and/or conjunctival epithelial cells (McIntosh *et al*., 2005).

DCD is known to be constitutively produced by sweat glands and secreted into sweat where it plays an important role in the innate defence system (Schittek *et al.*, 2001). Following secretion, the DCD precursor becomes functionally active via proteolytic cleavage into a survival promoting peptide (position 20 – 49,

YDPEAASAPGSGNPCHEASAAQKENAGEDP) and at least one other peptide (Schittek *et al.*, 2001). The number of DCD peptides present in sweat varies between individuals and can be as high as 14, due to continual processing of the C and N terminal regions after secretion (Rieg *et al.*, 2006). DCD has been shown to act against both Gram - positive and Gram - negative bacteria (Schittek *et al.*, 2001). DCD-1L (consisting of 48 amino acids) and DCD-1 (47 amino acids lacking the terminal leucine) were the first two peptides identified and are active against pathogens such as *Staphylococcus aureus, E. coli, Entercoccus faecalis,* and *Candida albicans* over a broad pH range (5.5-7.4) and in high salt concentrations (25-150mM NaCl) (Schittek *et al.*, 2001). In addition to its antimicrobial action, tissue and cell studies of the survival peptide of DCD demonstrated phosphatase activity in the presence of manganese ions. It also exhibited a protective effect on neuronal (Cunningham *et al.*, 1998) and hepatic cells (Lowrie *et al.*, 2006) by suppressing oxidative stress, which could involve its phosphatase ability (Cunningham *et al.*, 1998; Lowrie *et al.*, 2006). DCD protein expression was also found increased in advanced breast cancer (Porter *et al.*, 2003) and hepatocellular carcinoma (Shen *et al.*, 2011).

In the present study, the two DCD peptides semi-quantified by MRM showed no significant differences in abundance between control, CaP and BPH groups. It is worth noting that one of the peptides analysed, ENAGEDPGLAR, linked the survival promoting peptide and DCD-1, as its first seven amino acids (ENAGEDP) was the last seven amino acids of the survival peptide, and the rest of sequence was the beginning of DCD-1. The successful detection of this peptide in both initial MS protein identification using FT-MS and MRM indicates the presence of the un-proteolysed DCD protein in tears. Further studies may focus on detection and quantitation of the survival peptide of DCD in tears whose phosphatase ability was found beneficial to normal cells; however conditions in which the response to chemical stress and microbial invasion should also be considered as they could also cause alterations in the concentration of DCD present.

The phosphorylation of LCN1 detected in the present study has confirmed the results of a recent study which used the same phospho-staining (Pro-Q diamond) and titanium dioxide column for phosphorprotein enrichment (Zhao *et al.*, 2010). The glycoprotein stain Pro-Q emerald also detected LCN1, whereas Kuizengo *et al.* (1991) did not detect any interaction between LCN1 and various lectins (ConA, PNA, SBA, PHA-E, WGA, Jacalin and PSA). It should be noted that this difference is likely due to Pro-Q Emerald glycostaining being less specific than lectin-glycoprotein interactions and thus the possible glycosylation state of LCN1 detected in the present study would require confirmation by a more specific technique, preferably mass spectrometric analysis which can provide evidence of glycan structures present. Although LCN1 is an abundant tear protein, its precise function in tears remains unclear. It is known to bind to small lipophilic molecules, acting as a lipid transporter in tears (Yamada *et al.*, 2005) and it is also suggested to be a cysteine protease inhibitor (van`t Hof *et al.*, 1997). Further characterisation of its PTM sites may provide information regarding the state of the molecule under various conditions and during different functions.

Cystatin S is the predominant cysteine protease inhibitor in tears and its activity is tightly regulated by a group of small proteins of the cystatin superfamily (Gachon and Lacazette, 1998). Two cystatin S isoforms were observed in the present study and although cystatin S is a known phosphoprotein (Isemura *et al.*, 1991), it was also detected here in a glycosylated form. The glycosyaltion should be further confirmed by mass spectrometry analysis which can give information of potential glycan structures. The fact that its phosphorylation was not detected by the phosphostaining but from titanium dioxide enrichment (Zhao *et al.*, 2010) suggesting that titanium dioxide enrichment is more sensitive than staining. Furthermore, Peptides of the cystatin S precursor and one of its variants, cystatin SA-III, were detected within one spot together with SCGB2A1 (spot 8, Figure 4.2 and Table 4.5), suggesting that both native and post translationally modified forms are present together in tears. These various forms suggest the actions of cystatin S

may be determined by the presence of multiple PTMs, and that further functions may exist for this protein in tears that have not yet been elucidated.

A phosphorylated form of NUCB2 has previously been identified from tear fluid samples (Zhao *et al.*, 2010) and the current work also showed that NUCB2 is phosphorylated but not glycosylated in tears. NUCB2 was originally isolated from plasma membrane, cytosol and culture medium of the human acute lymphoblastic leukemia cell line KM3 (Barnikol-watanabe *et al.*, 1994) and is a Ca^{2+}/DNA binding EF-hand protein involved in multiple signaling pathways (Valencia *et al.*, 2008). It consists of a signal peptide, a leucine/isoleucine rich region, a DNA binding domain and two EF-hand motifs which enable NUCB2 to perform multiple biological functions (Valencia *et al.*, 2008). Studies have suggested NUCB2 can act to suppress appetite in rats (Oh-I *et al.*, 2006), regulate the Ca^{2+} concentration or Ca^{2+} regulated physiological process in intracellular organelles in mice (Taniguchi *et al.*, 2000), affect the secretion of extracellular type 1 tumor necrosis factor receptor (TNFR1) in human vascular endothelial cells (Islam *et al.*, 2006). It is also proposed to be important for anti-DNA autoimmunisation in lupus erythematosus mice as the DNA-NUC complex could induce anti-DNA antibodies (Miura *et al.*, 1992; Barnikol-watanabe *et al.*, 1994). The functions and origin of NUCB2 in tears remain unclear, however it is possible that secreted NUCB2 is involved in regulating the Ca^{2+} concentration in tears. The cleavage of NUCB2 during apoptosis by caspases (Valencia *et al.*, 2008) and its association with TNFR1 secretion (Islam *et al.*, 2006) suggest that NUCB2 in tears may be associated with cell stress and apoptotic cell death.

Among the proteins identified in this study, DMBT1 was detected as glycosylated, consistent with the work of Schulz *et al.*, 2002. Although no phosphorylation was detected for this protein using the phosphostain method, Zhao *et al.*, (2010) was able to detect a phosphopeptide of DMBT1 using titanium dioxide column suggesting it is also phosphorylated in tears. The fact that this phosphorylated peptide was not detected by the phosphostaining again suggests that titanium

dioxide enrichment is a more specific method for this detection. DMBT1 is also known as salivary agglutinin or lung glycoprotein-340 and has been shown to interact with a broad range of pathogens, including streptococci, *Helicobacter pylori*, influenza viruses and HIV suggesting it plays a role in innate defense. It can also interact with IgA, surfactant proteins and mucin-5B and plays a role in epithelial and stem cell differentiation (Ligtenberg *et al.*, 2007). Inactivation of the gene encoding this protein may lead to the formation of brain tumors (Mollenhauer *et al.*, 1997), digestive tract cancer (Mollenhauer *et al.*, 2001), lung cancer (Mollenhauer *et al.*, 2002), intrahepatic cholangiocarcinoma (Sasaki *et al.*, 2003), breast cancer (Mollenhauer *et al.*, 2004), salivary gland tumors (Bikker *et al.*, 2004) and pancreatic cancer (Cheung *et al.*, 2008).

Finally, SCGB2A1 is a known glycoprotein in tears (Molloy *et al.*, 1997) and it is also known as mammaglobin-B, lipophilin-C, mammaglobin-2 and lacryglobin. In addition to its glycosylation, it was also detected in phosphorylated form in this study confirming the results from (Zhao *et al.*, 2010). Although the function of this protein in tears is unknown, it has been measured at increased levels in patients with breast, lung and colon cancer (Evans *et al.*, 2001).

4.4.2 SELDI analysis of tears

To complement the 2DGE technique, which is unable to detect proteins of MW less than 10kDa, SELDI-MS was originally chosen to generate spectra from CaP, BPH and Control groups and assess low molecular weight species and differences between the three groups for potential biomarkers. Given the high variations in the preliminary inter-chip and inter-run tests with SELDI-MS, the use of SELDI was decided against in favour of the emerging MS based technology, MRM.

Several other studies have assessed SELDI reproducibility in a large sample pool. Baggerly *et al.*, (2004) analysed three serum SELDI datasets from Petricoin's group who reported a distinct diagnostic spectral pattern for ovarian cancer and found that their results were not reproducible (Baggerly *et al.*, 2004). The possible reasons put

forward were that baseline correction was not completed and results could not withstand the differences of baseline noise, the protocol used in the study was inconsistent, and external calibration was possible but was not performed (Baggerly *et al.*, 2004). Inter-laboratory reproducibility of SELDI has also been evaluated in one study examining data from six laboratory sites and concluded that automatic sample preparation and instrument calibration were critical for ensuring reproducibility (Semmes *et al.*, 2005). Although automatic sample preparation could not be used in the present study, technical error was minimised by strictly following the standard tear sample collection and storage protocol, and using only optimised instrument settings. The calibration of the SELDI machine was performed on a weekly basis by Royal North Shore Hospital (Sydney, Australia), where the instrument was owned and kept.

The present work is consistent with that of Zhong *et al.*, (2010), who reported large peak variations when analyzing the same samples on single and multiple SELDI chips. Large within- and between-chip variation is an inherent problem when using SELDI for discovery phase research, as this requires output of accurate and repeatable data. In the current study, half of the peaks detected from all three samples analysed using SELDI had CV% of peak intensities > 20. Biological assays, such as protein assays, and clinical assays such as ELISA, are expected to have replicates with CV% < 20 and 10 respectively (Addona *et al.*, 2009; Zhong *et al.*, 2010). The problem of reproducibility means that results obtained from a single sample cannot be accurately compared.

The present work also examined the inter-run differences, comparing the spectra of stored samples and fresh samples to determine the impact of sample storage and instrument stability. Inter-run differences were not simply a result of sample storage type or time (**Figure 4.6**), but most likely caused by instrument shift. It must be noted that in the present study the inter-run experiments were performed more than half a year apart because the laser of the PBS-IIc ProteinChip Reader required changing twice during this time. The laser of the SELDI machine used was changed

once between Dec-2007 and Oct-2008, and again between Oct-2008 and Feb-2009. The change of laser may explain the pattern differences observed over time, further reflecting the inherent lack of reproducibility of this particular instrument. Nevertheless, a recently approved FDA ovarian diagnostic test, OVA1, was derived from SELDI research (Fung, 2010), suggesting that reliable instrumentation, adequate time for developing a highly accurate protocol and equipment optimisation with this method is possible in biomarker research.

4.4.3 Relative quantitation of tear proteins using MRM

This is the first study to report relative quantitation of multiple human tear peptides using MRM and as such has demonstrated the potential of the MRM technique in tear film analysis. The only other published tear study that used MRM was to absolutely quantify natamycin (an antibiotic used for the treatment for fungal keratitis) in rabbit tears (Bhatta *et al.*, 2011). Instead of using one product ion for the parental ion in Bhatta's study, at least three product ions for each parental ion were used in this study (**Table 4.4)** which increased the specificity to detect the target peptide (Keshishian *et al.*, 2007).

The six tear proteins that were assessed using MRM in the present study (DCD, DMBT1, NUCB2, SCGB2A1, Lf and LCN1) were chosen for the following reasons:

1) The mass spectral information for all peptides was available as they were identified by MS in the present study. This allowed accurate construction of MRM transition lists, as peptide ionisation does not always occur as *in silico* methods would predict (Anderson and Hunter, 2006).
2) DCD, DMBT1, NUCB2 and SCGB2A1 have been previously reported to be associated with various types of cancer (Section 4.4.1) with tear SCGB2A1 reported to be associated with CaP (Evans *et al.*, 2001).
3) More than one peptide was selected for DCD and SCGB2A1 because their verification and quantitation were of highest interest; DCD was identified

for the first time in tear fluid and SCGB2A1 has been reported to associate with CaP (Evans *et al.*, 2001).

4) Lf and LCN1 are two of the most abundant proteins in tears and thus their detection was essential to confirm the success of the MRM assays. Multiple peptides were analysed from the Lf protein to ensure good sequence coverage of the high MW protein (Lf MW 82 kDa; LCN1 MW 19.2 kDa).

MRM can be used for either absolute or relative quantitation. Performance of absolute quantitation was outside the scope of the present study. This approach requires the presence of either a well established control protein/peptide within the samples, which adjusts for relative differences in overall concentration between samples *ie*. error of protein assay calculated for determining equal amounts of starting material; or a labelled synthetic control peptide introduced into the samples. As this is the first MRM based study to examine peptide abundance in tear samples in relation to CaP the focus was on development of optimal instrument settings, construction of relevant MRM transition lists and relative quantitation for preliminary work towards biomarker discovery. Whilst various methods could be used for relative quantitation, the chosen analyses were completed under advice from the Dr. Thomas John (Biostatistician at the Brien Holden Vision Institute) as it was deemed most relevant to the experimental assay and samples under investigation.

This study successfully established a reproducible MRM method for the simultaneous detection and relative quantitation of peptides from two highly abundant proteins and four low abundance proteins. With the capability of absolute quantitation of 47 high to medium abundance serum proteins (more than 100 transitions) in a single MRM run (Anderson and Hunter, 2006), this emerging technology has great potential as a rapid method for screening proteins and peptides, and the work presented in this study provides a starting point for absolute quantitation of tear proteins using MRM.

4.4.4 Potential peptide biomarkers for CaP detected using MRM assay

Relative quantitation of abundance of Lf peptide VPSHAVVAR (position 250- 258, (Anderson *et al.*, 1989)) showed an increase in CaP samples when compared with Control and BPH samples, with a significant difference between CaP and Control. This was the only peptide from those analysed for which a significant difference in abundance was observed. It should be noted that a larger sample size and larger range of peptides tested will be required to confirm these results.

Although no previous studies have reported any PTMs associated with this peptide, VPSHAVVAR, it contains serine (S) residue for potential phosphorylation (Mann and Jensen, 2003), proline (P) for potential hydroxylation (Mann and Jensen, 2003) and arginine (R) for potential methylation (Mann and Jensen, 2003). This Lf protein could therefore be present in modified and unmodified forms with this peptide containing modification/s, and thus this peptide in unmodified form being detected atdifferent abundances between CaP and control groups. Notably, the only other difference between the peptide for which a difference in abundance was observed and the two peptides which did not change in abundance is that VPSHAVVAR contains an iron binding ligand at histidine (H, position 253) (Anderson et al., 1989). This suggests that peptides containing such functional sites could be expressed in a different manner. The iron content and iron binding proteins in tears between control, BPH and CaP groups could be further examined to determine the relevance of this to prostatic disorders and potential biomarkers.

The peptides of the only tear protein SCGB2A1 that has been previously suggested to associate with CaP did not show any significant difference in abundance between the three groups. The proposed potential association of SCGB2A1 with CaP was initially due to its homology with lipophilin C which is involved in androgen regulation (Molloy *et al.*, 1997). Further study by Evans *et al.*, (2001) did not detect this protein in all samples investigated, however the protein was detected in a higher percentage of cancer patients and controls having family history of cancer than in control samples, suggesting this protein maybe cancer associated. This conclusion

was based on absence or presence of the protein rather than quantitation and analysis between groups. The major difference between the present and previous study is the use of the peptide centric technique MRM in the current study than the protein orientated technique 2DGE. Other studies using healthy tear samples have also detected SCGB2A1 (de Souza *et al.*, 2006; Green-Church *et al.*, 2008). However, the sample sizes used in all studies were small; as a result, population based study and absolute quantitation of this protein needs to be performed to confirm the result.

4.4.4.1 Structure and function of Lf

Lf is an 80 kDa glycoprotein which can be found in various secreted body fluids such as human milk, tears, saliva and nasal-gland secretions (Kanyshkova *et al.*, 2001). Low levels of Lf have also been detected in blood, likely orginiating from the Lf producting cells; neutrophils (Birgens, 1985). Tear Lf is mainly produced by the lacrimal gland and has a constant concentration (at around 1.6 μg/μL) in both non-stimulated and stimulated conditions (Fullard and Tucker, 1991). The protein contains two identical iron binding sites consisting of four amino acids (two Tyr, one Asp and one His) and one $CO3^{2-}$ (Anderson *et al.*, 1989). It has much higher iron binding capacity than serum transferrin with three states of purified milk Lf reported based on the number of iron molecules bound. Ninety five percent of the protein was found to be iron free (apolactoferrin) or with one molecule bound (monoferric lactoferrin), while five percent was iron saturated (diferric lactoferrin) (Makino and Nishimura, 1992).

The major functions of Lf in tears are the multiple antimicrobial effects it exerts including inhibition of bacterial growth by creating an iron deprived environment (Arnold *et al.*, 1977), production of antimicrobial peptides through cleavage of the N-terminal region (Bellamy *et al.*, 1992; Haney *et al.*, 2009), and increased permeability of bacterial cell membrane to aid entry of lysozyme (Leitch and Willcox, 1999; reviewed in Jenssen and Hancock, 2009). In addition to antimicrobial activity, the iron binding ability of Lf allows it to decrease the iron-

catalysed production of hydroxyl radicals and thus help protect the ocular surface from oxidative stress (Shimmura *et al.*, 1998). Lf has also been shown to decrease the inflammatory reaction by suppression of the classical complement pathway (Veerhuis and Kijlstra, 1982) possibly through interactions of components of the classical pathway with the Lf N- terminal sequence (Samuelsen *et al.*, 2004; reviewed in Flanagan and Willcox, 2009).

Although, a train of glycosylated Lf spots were observed on 2DGE tear protein maps (spot 10, **Figure 4.3b**), knowledge of Lf isoforms is very limited. Analysis of Lf in milk has shown three isoforms that differ in MW and function, with two isoforms displaying RNAse activity but an inability to bind iron (Furmanski *et al.*, 1989). Lf produced by neutrophils has also been shown to occur has three disctint isoforms (MW 85 kDa, 80 kDa and 75 kDa) (Deriy *et al.*, 2000; Avram *et al.*, 2004). Unfortunately, due to the lack of structural information (such as MW), it is hard to know if the isoforms identified from neutrophil derived Lf is the same as milk derived Lf. Investigations on the PTMs of Lf could help better understand the functions of Lf.

4.4.4.2 Lf and diseases in particular CaP

Numerous studies have also investigated the association between Lf and cancer in both biofluid and tissue samples. In a mouse model of lung cancer, Lf was found to stimulate the activity of natural killer cells and significantly inhibit the growth and metastasis of lung tumour, independent of the iron binding ability (Bezault *et al.*, 1994). A decrease in Lf protein expression has been observed in CaP in both tissue specimens and serum when compared with control samples (Shaheduzzaman *et al.*, 2007). This finding was supported by evidence that Lf inhibited the growth of two CaP cell lines, LNCaP and LAPC4 (Shaheduzzaman *et al.*, 2007). Conversely, the present study showed significant higher abundance of one Lf peptide in tears from CaP patients when compared to control samples. With an increase in abundance in one peptide out of three from a single protein, this result suggested that distinct forms of a single protein could be expressed differently and the detection and

quantitation of various protein isoforms could provide insight that would not be achieved were the protein analysed as a single entity. The results of the present study also highlight the distinct nature of the proteomes of the various biofluids. In tears, Lf is mainly produced by the lacrimal gland, whereas neutrophils are the source of Lf in serum. Similar increases in the abundance of Lf have been detected in faeces samples from patients with inflammatory gastrointestinal disorders and colon cancer, likely resulting from a large discharge of neutrophils during the inflammatory response to these conditions (Uchida *et al.*, 1994). One of the functions of tear Lf is to control ocular inflammation. Although analysis of serum showed the presence of systemic inflammation in some of CaP patients (46%) (Proctor *et al.*, 2010), it will be also helpful to assess the ocular inflammation level of the subjects from whom tear samples are collected using, for example the membrane-bound antibody array which has successfully detected 80 cytokines in tears (Sack *et al.*, 2005), to further understand the association between tear proteins/peptides and prostatic diseases.

Whilst it is possible that altered levels of Lf peptides in tears is a response to systemic inflammation, association with Lf levels in multiple biofluids and sites of cancer suggest there may be a more specific relationship with various types of cancer. Shaheduzzaman *et al.* (2007) has suggested CaP cells might induce silencing of the Lf mRNA expression through DNA methylation and thus decrease the local expression of the Lf protein (Shaheduzzaman *et al.*, 2007). The increased levels of the particular Lf peptide detected in CaP patients' tears suggests altered regulation of specific isoforms of the protein and/or pathways related to the particular CaP pathophysiology and their effects on the lacrimal gland.

There is also evidence of hormonal regulation of Lf, with estrogen shown to interact with the promoter region of the gene and thereby induce protein expression in the endometrium (Teng, 2002). Both lacrimal and prostate glands have been reported to be regulated by the hormone testosterone (Sullivan and Edwards, 1997; Heemers *et al.*, 2003). Although lacrimal gland inflammation or aqueous tear deficiency have

not been detected in androgen deficient mice (Sullivan *et al.*, 1999), significant increases in IgA have been detected in the presence of testosterone which suggests an increase in lacrimal gland activity (Sullivan and Edwards, 1997). Significantly higher levels of testosterone were detected in serum from CaP patients compared to non-CaP patients in a pilot study of 408 subjects that had either urinary voiding difficulties, erectile dysfunction or increased PSA levels (Mydlo *et al.*, 2001), however decreased testosterone levels have been reported in CaP patients' sera compared with sera from BPH patients (Mearini *et al.*, 2008). The complex and multiple roles of testosterone and other molecules of the endocrine system in CaP and benign prostatic conditions remain unknown, however results of the present study suggest that the altered abundance of Lf peptides in the tear film could differentiate CaP from non-CaP subjects. Whilst the exact roles of testosterone and other molecules of the endocrine system in CaP and benign prostatic conditions remain unknown, it is possible that the differences in Lf peptide abundance observed are due to the pathophysiological changes derived from CaP, and thus further work to elucidate the related hormonal regulation might provide insights into potential diagnostic biomarkers and therapeutic targets.

4.4.4.3 Conclusions and next steps

Analysis of phosphorylation and glycosylation states of the detectable tear proteins on 2D gels produced an overall tear protein profile, the first evidence of the presence of DCD in tears using the MRM technique and detection of PTMs that have not previously reported. The detection and validation of DCD assists in understanding the pathways that might be involved in the antimicrobial mechanisms of the tear film. In addition, the novel work to relatively quantify tear proteins/peptides using MRM technology now provides a platform for further work in this area, with potential for detection of biomarkers which might not be identified using other proteomic techniques.

One Lf peptide has been highlighted to be of particular further interest, with a significantly higher level of abundance detected in CaP tear film samples compared

with control tear film samples. This altered abundance of a single and specific peptide could be due to the altered function of the lacrimal gland affected by the possible abnormal androgen levels and the inflammation in CaP patients. The fact that only one Lf peptide showed a significant increase in abundance also highlights the importance of analysing the PTMs of Lf as it is likely that a single form of the protein, potentially a post-translationally modified form, rather than total protein production that is affected by the disease. This also highlights the necessity to recognise that, with emerging technologies such as MRM that allows detection and quantitation of both protein and peptide species, biomarker research can extend from the protein level to the peptide level.

As a result, further work on this particular peptide such as raising specific antibodies to help detection, quantitation and characterisation of its associated isoform(s) is of high interest. Additionally, measurement of the testosterone and inflammation levels in the tear film samples from CaP, BPH and control groups will be of value to elucidate the relationship between this protein and prostatic conditions. Finally, absolute MRM quantitation using the corresponding synthetic peptide as an internal control in a larger sample size to validate its diagnostic accuracy is a key step to confirming the value as a diagnostic marker for CaP.

Chapter 5 General Discussion

The aim of the current project was to use multiple proteomic techniques to detect, identify and verify potential diagnostic biomarkers for CaP in serum and the tear film. The major part of the study was focused on the discovery stage, in which different approaches were taken to investigate the two proteomes due to their different composition and functions. Potential biomarker proteins and peptides for CaP have been identified from both serum and tears in the present work and this chapter will summarise and discuss the major findings, the relevance of the current study both clinically and more generally to the field of cancer biomarker research, and future work required to progress this study.

5.1 Novel findings of the present study

The key finding of the present study is the identification of multiple proteins, from both serum and the tear film, which show potential as diagnostic biomarkers for CaP. Additionally, the use of various traditional and emerging proteomic techniques to analyse the two biofluid proteomes produced multiple novel findings. All significant findings are summarised and discussed below:

1) Discovery of novel potential diagnostic CaP biomarkers. Five serum proteins (albumin, fetuin A, IGHM, hemopexin and C4BPA) and one tear peptide (VPSHAVVAR derived from lactoferrin) were identified as potential diagnostic biomarkers due to detection of their altered abundance in CaP and/or BPH patients when compared with healthy controls. Within the sample size tested, serum concentrations of total albumin and fetuin A can differentiate between CaP and control samples with higher sensitivity and specificity than the currently accepted PSA test. The relative abundance of the specific VPSHAVVAR peptide of Lf in tears can differentiate between CaP and control samples; further study is required to determine sensitivity and specificity of this peptide.

2) One specific fetuin A isoform detected by DIGE analysis showed significant differences in abundance between BPH and CaP groups, with decreasing abundance in the BPH group. However this difference was no longer detectable when total fetuin A concentration was measured using antibody based proteomics. This emphasises the importance of characterising this particular isoform as it may help overcome one of the major limitations of CaP diagnosis at present, the differentiation of CaP from other prostatic conditions. The other potential biomarkers all showed significant differences between control and CaP groups, with both increases and decreases in concentration observed. Thus, with differences in the nature of change observed, the groups differentiated and the biofluids they are present in, a combination of these biomarkers has the potential of generating a more accurate CaP diagnostic test than measurement of a single molecule alone.

3) Analysis of tear and serum samples in terms of total protein, protein isoforms and peptides derived from proteins produced distinct results. In the present study, differences in the serum proteome were observed between DIGE experiments which allow assessment of individual protein isoforms as protein spots and antibody based protein assays which detect all forms of the protein with a specific epitope present. Differences in the tear film proteome and peptidome were clear from gel based experiments targeting proteins and their isoforms and MS based experiments targeting peptides comprising the proteins. This study highlights the requirement for specific design of protein and peptide based research to ensure results are truly indicative of the hypothesis being tested. This also emphasises the importance of future investigation of specific protein isoforms and single peptides to improve the accuracy of a diagnostic test for CaP.

5.2 Relevance of the present work to the field of biomarker research

Biomarker research continues to be an expanding field, particularly in terms of quantitation of molecules from biofluids for non-invasive diagnostics. Whilst multiple techniques used in the present study are well established, the current work also included many novel features in terms of design and emerging proteomic and peptidomic techniques:

1) Currently, the majority of CaP biomarker studies include only samples from healthy control and CaP groups, adequate for discovery of biomarkers for prostatic pathologies but not allowing differentiation between prostatic conditions which are often confused clinically. The present study included serum and tear samples from BPH patients to allow detection of biomarkers which adequately differentiate between malignant and benign prostatic conditions. Results from this study have also highlighted the importance for including relevant control groups such as patients with coincident conditions of inflammation and liver dysfunction.

2) This is the pioneer study which specifically examined the tear proteome for CaP biomarkers with the subsequent detection of one potential CaP peptide biomarker. This work shows the potential of developing a CaP diagnostic test based on measurement of molecules from tear film. This study also analysed both phosphorylated and glycosylated tear proteins using 2DGE and provided the most complete information on the PTM analysis currently available in the literature. The work of the present study suggests that tear film may be a source of diagnostic and prognostic biomarkers for a variety of pathologies, which has been largely overlooked in favour of the commonly analysed biofluids such as serum and urine.

3) A semi-quantitative MRM approach has been successfully applied to biomarker discovery/verification in this study. With the capability of analysing more than 100 transitions in a single MRM run (Anderson and Hunter, 2006) and without the requirement of an isotopic labelled peptide, as demonstrated in the present

work, this approach can be used as a rapid screening tool for proteins and peptides before further analysis such as absolute quantitation.

4) Experimental design of antibody based proteomics has been shown to be extremely important, with variations in abundance of specific protein isoforms not necessarily reflective of total protein levels. More specific immunoassays such as using antibodies raised to certain isoforms or certain specific peptides of interest should be used for quantitation and verification based work.

5.3 Future experiments and conclusions

The present project has shown that the concentration of certain isoform(s) of a protein might allow development of a more sensitive and specific CaP diagnostic than that based on measurement of the concentration of the total protein. Therefore, characterisation of the PTMs of protein spots selected by DIGE will provide more information of the functions of these proteins as well as their relationship to CaP. MS/MS based techniques provide sensitive and efficient methods for determination of PTMs (Jensen, 2004). The general principle is to define the mass differences between the calculated mass of the modified protein and its corresponding native form and then to determine the amino acids involved (Jensen, 2004). The most common and simpler PTMs will produce mass increases that are known, such as phosphorylation which shows an increase of 80 Da (Steen *et al.,* 2001). More complex modifications can produce various mass differences, such as that from glycosylation which depends on the glycan that is attached to the peptide (Huddleston *et al.,* 1993). Once the PTMs are determined, quantitation of these protein isoforms can be achieved by MRM techniques using the determined signature peptide/s. It would be the best to give priority to characterisation of the fetuin A isoform which was the only one that could differentiate CaP from BPH serum samples. Interestingly, fetuin A, the potential marker identified from serum, has been reported in tears previously (de Souza *et al.,* 2006), although no other studies have validated this finding. Its potential presence in tears suggests that,

should further work detect a significant difference in concentration of fetuin A from total protein or a specific isoform in CaP serum compared with BPH and/or control serum, it should also be investigated in tears which can be collected non-invasively compared to serum.

The present study focused on analysing the depleted serum proteome using 2DGE, which only assessed proteins within the range of 20 to 200 kDa and discovered several potential CaP serum biomarkers that were all synthesied from liver. It is likely that prostate gland associated proteins/peptides are at the low molecular weight range (less than 20 kDa), therefore further studies could focus on the investigation of the serum peptidome fraction, where proteins/peptides that are directly secreted by the prostate gland are more likely to be found. Microcon Y-20 (Millipore, USA) with a 20 kDa cut-off membrane can be used to extract and enrich serum proteins that are lower than 20 kDa for MS analysis.

Due to the high potential of using the Lf peptide in CaP diagnosis, further evaluation of this particular peptide should be performed to validate its diagnostic result. The amino acid sequence of this particular peptide is known and thus an accurate quantitation using MRM and a corresponding synthetic peptide can be performed to determine the absolute concentration of this peptide between the three groups to validate the semi-quantitative results reported. Moreover, specific antibodies can be raised against this peptide to purify the associated Lf protein isoform(s), which can be characterised by MS/MS and subsequently provide some insight into the function of Lf and its CaP associated functions and pathways.

Gel based techniques, as demonstrated in this study, have both advantages and disadvantages for detection of novel protein biomarkers. On one hand, it provides information relating to proteins detected such as pI, MW and presence of PTMs. On the other hand, it has a higher limit of detection compared to MS based techniques and is therefore not able to detect proteins in the pg to ng range. Based on the successful use of MRM presented here, a technique which has the potential to

analyse up to 40 proteins at once, it is suggested that this technique could be further used as a screening tool for biomarker identification by firstly detecting and constructing MRM transitions for all previously reported serum and tear proteins, and then relatively quantifying each of them between the three groups to select the proteins that show significant discriminative power for further validation.

Finally, based on the potential protein/peptide biomarkers identified, it is essential that further studies include analysis of the subjects characteristics such as liver function, presence of inflammation, and the testosterone levels (particularly in terms of tear film as the lacrimal gland is androgen regulated) in all CaP, BPH and control groups. Changes to the serum proteome as a result of systemic inflammation has been examined in more than 200 CaP patients based on the modified Glasgow scoring system (mGPS), and while no significant associations were observed, no direct assessment within the three groups (CaP, BPH and control) has been made (Proctor *et al.*, 2010). Unlike the mGPS scoring system used to measure the inflammation level and related changes in serum, there is not yet a standard method to evaluate this in terms of tear film. Numerous inflammation related molecules such as various interleukins are present and measureable within the tear film, however these may be measured in a preliminary work. Sack *et al.*, (2007) has developed a tear specific antibody membrane array that successfully detected 16 inflammatory mediators using 7.5 μL of tears and has been used to compare the inflammation level between open and closed eye tear fluids collected from normal control subjects and patients with various ocular allergies (Sack *et al.*, 2007). In contrast to the traditional ELISA test in which only one protein is tested at one time, this array based method allows simultaneous measurement of a variety of inflammatory mediators.

In conclusion, the current study suggests that a novel diagnostic test for CaP can be developed based on proteins and their components detectable from serum and tear samples. Validation studies are required to further investigate albumin, fetuin A and the specific Lf peptide detected here in a large size samples. However, our

preliminary work in the current studies shows the potential of these molecules to be used as a diagnostic test which has improved sensitivity and specificity when compared with the current PSA test. Development of a highly sensitive and specific, non-invasive diagnostic test, particularly one which can differentiate CaP from other prostatic pathologies as well as healthy samples, can allow for largely increased levels of worldwide CaP screening, earlier detection and, most importantly, reduced morbidity and mortality associated with CaP disease.

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Appendix

1. YOU, J., FITZGERALD, A., COZZI, P. J.*, et al.* 2010. Post-translation modification of proteins in tears. *Electrophoresis,* 31**,** 1853-1861.