

**THE IDENTIFICATION OF PROSTATE
CANCER ASSOCIATED TUMOUR
ANTIGENS AND BIOMARKERS**

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Dedicated to Uncle Brian. Thank you for believing in me.

*Brian (Coco) Heath.
23rd October 1943 - 25th August 2014.*

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ABBREVIATIONS

1-DE	1 Dimensional gel electrophoresis
2-DE	2 Dimensional gel electrophoresis
Ab	Antibody
ACN	Acetonitrile
AMACR	α -Methylacyl-CoA racemase
AMIDA	Autoantibody-Mediated IDentification of Antigens
APS	Ammonium persulphate
AR	Androgen receptor
ATP	Adenosine triphosphate
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CAV-1	Caveolin-1
CCD	Charged coupled device
CD	Cluster of differentiation molecules
cDNA	Complementary deoxyribonucleic acid
CHCA	α -cyano-4-hydroxycinnamic acid
Da	Dalton
DCIS	Ductal carcinoma <i>in-situ</i>
ddH ₂ O	Double distilled water
DIGE	Difference gel electrophoresis (Differential in-gel electrophoresis)
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide Hybri-Max
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffer saline
DRE	Digital rectal examination
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's Minimal Essential Media
EN-2	Engrailed-2
ENOA	Alpha enolase
ESI	Electrospray Ionisation

FA	Formic acid
FC	Flow cytometry
FCS	Foetal calf serum
FDA	Food and drug administration
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FGFs	Fibroblast growth factors
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate-dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GSTP1	Glutathione S-transferase P1
h	hours
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HTLV-1	Human T cell leukaemia virus type 1
hv	High voltage
IEF	Isoelectric focussing
IF	Immunofluorescence
Ig	Immunoglobulin
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IL-2	Interleukin 2
IPG	Immobilised pH gradient
kDa	Kilodalton(s)
KLK2	Human kallikrein 2
LC MS	Liquid chromatography coupled to mass spectrometry
LC	Liquid chromatography
LCM	Laser capture microdissection
<i>m/z</i>	Mass to charge ratio
mAb	Monoclonal antibody
MALDI-TOF	Matrix assisted laser desorption ionisation time of flight mass spectrometry

min	minutes
MOWSE	MOlecular Weight Search
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
NCBI	National centre for Biotechnology information
NCBI	National centre of biotechnology information
NEAA	Non-essential amino acids
NK	Natural killer
NRES	National research ethics service
NSCLC	Non-small cell lung cancer
NTU	Nottingham Trent University
°C	degrees celcius
OD	Optical density
OGP	Octyl β -D-glucopyranoside (OGP)
PCa	Prostate cancer
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGDF	Platelet derived growth factor
pI	Isoelectric point
PIN	Prostatic intraepithelial neoplasia
PIP	Prostatic inhibin-like peptide
PMF	Peptide mass fingerprint
PPAP	Prostatic acid phosphatase
pRB	Retinoblastoma protein
PROTEOMEX	Proteomics and SEREX
PSA	Prostate-specific antigen
PSMA	Prostate membrane-specific antigen
QTOF	Quadrupole time of flight
rcf	Relative centrifugal force
RNA	Ribonucleic acid
RT	Room temperature

s	seconds
SC	Sequence coverage
SD	Standard deviation
SDS PAGE	Sodium Dodecyl Sulphate PolyacrylAmide Gel Electrophoresis
SDS	Sodium dodecyl sulphate
SERPA	Serological proteome analysis
SLE	Systemic lupus erythematosus
SPON2	Spondin-2
T&V	Trypsin/Versene
TAA	Tumour associated antigens
TBST	Tris-buffered saline and Tween-20
T _C	Cytotoxic T cells
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tf	Transferrin
TFA	Trifluoroacetic acid
TGF α	Tumour growth factor α
T _h	T Helper cells
TMA	Tissue microarray
TMPRSS2	Transmembrane protease serine 2
TNF	Tumour necrosis factor
TNM	Tumour node metastasis
TPTB	Transperineal template prostate biopsy
TRUS	Transrectal ultrasonography of the prostate
TSA	Tumour specific antigens
UHL	University Hospitals Leicester
V	Voltage
VEGF	Vascular endothelial growth factor
WB	Western blotting
WHO	World Health Organisation
ZA2G	Zinc alpha-2-glycoprotein

ABSTRACT

The widespread use of Prostate Specific Antigen (PSA) testing has resulted in the over detection and over treatment of potentially indolent disease due to the lack of specificity of PSA for prostate cancer (PCa). PROTEOMEX, a method of tumour associated antigen (TAA) identification, combines the separation of tumour proteins by conventional proteomic methods (2-DE and mass spectrometry) with serological screening using serum antibodies, to identify immunogenic proteins in cancer. This project aims to identify TAAs and/or biomarkers for PCa which on subsequent validation, can be utilised as an improved diagnostic screening test.

In pilot studies, SDS PAGE, 2-DE and OFFGEL electrophoresis were performed to identify immunogenic urine TAAs using PCa and healthy control sera. Proteins within the serum-reactive spots were either identified by Liquid Chromatography coupled to Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight mass spectrometry (MALDI-TOF MS) or Electrospray Ionisation mass spectrometry (ESI MS). Among the urinary proteins separated by SDS PAGE, 2-DE and OFFGEL respectively, exclusive autoreactivity was identified in PCa sera to serum albumin and existing PCa biomarkers - human prostatic acid phosphatase & zinc-alpha-2 glycoprotein. Differential autoantibody responses were also identified to various TAAs in the PC-3 and DU-145 PCa cell lines using PCa and healthy sera. The presence of a differential TAA and autoantibody PCa serum response to one of the proteins identified by MALDI-TOF, alpha enolase, was further verified in a subset of PCa samples using immunohistochemistry, Western blotting and ELISA.

In a larger sample cohort, the cytoplasmic and nuclear alpha enolase expression in a PCa TMA was assessed, where statistical significance was observed between benign controls and PCa ($p=0.000003$ and $p=0.003$ respectively), although protein expression did not correlate with any important clinico-pathological variables. Alpha enolase autoantibody expression was statistically significant between PCa and healthy controls ($p=0.0038$), where its expression correlated with D'Amico risk classification, indicating that alpha enolase may serve as a potential indicator of biochemical recurrence in PCa.

PROTEOMEX represents a valuable approach for the identification of tumour biomarkers which may have diagnostic and/or prognostic value in PCa. Further work should identify more TAAs and autoantibodies associated with PCa, alongside a validation of the diagnostic utility of the identified biomarkers from this study.

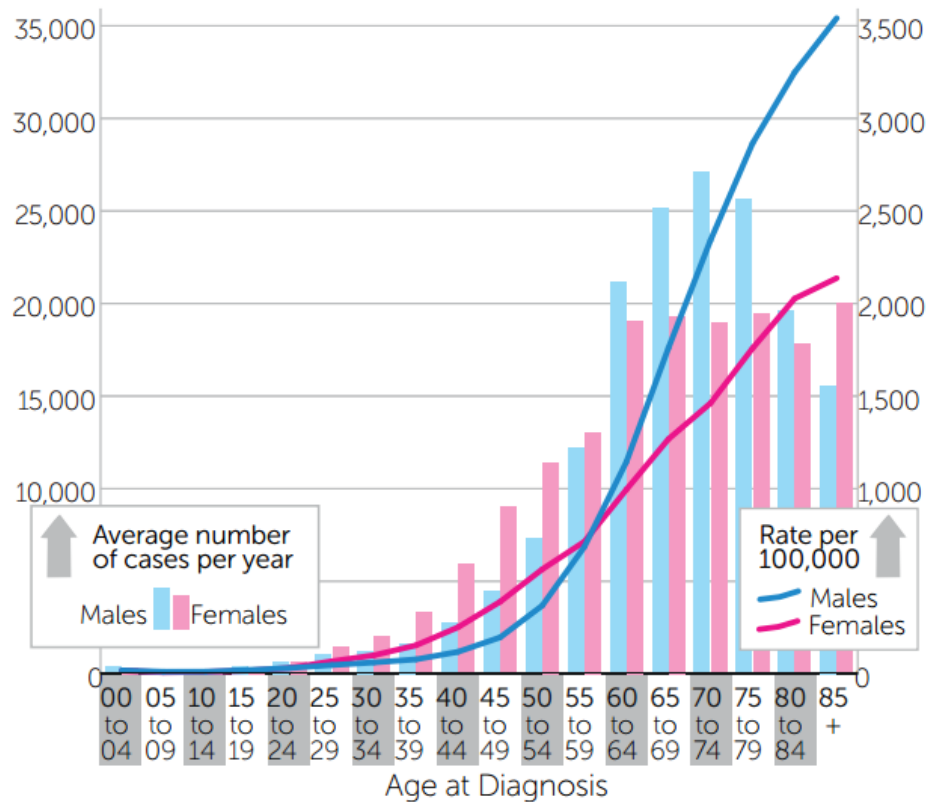
CHAPTER 1: INTRODUCTION

1.1 Cancer

1.1.1 Epidemiology

Despite advances in medical research, cancer remains a significant cause of morbidity and mortality worldwide. According to the World Health Organisation (WHO), cancer is one of the global leading causes of death, accounting for over 8.2 million cancer-related deaths and 14 million cases in 2012 alone (WHO, 2015). The WHO expects that annual cancer incidences will rise from 14 million in 2012 to 22 million in the next few years. In addition, WHO statistics from 2012 show that the most causes of cancer related deaths are from lung (1.59 million deaths), liver (745,000 deaths), stomach (723,000 deaths), colorectal (694,000 deaths), breast (521,000 deaths), oesophageal (400,000 deaths) and prostate cancers (307,000 deaths) (WHO, 2015).

In the UK, cancer is diagnosed every two minutes, where more than 331,000 new cancer cases were diagnosed in 2011 alone (Cancer Research UK, 2015a). In 2011, research gathered by Cancer Research UK suggests that more than half (54%) of all new cancer cases were diagnosed in breast, lung, prostate or bowel cancer; where more than a third (36%) of cancers are diagnosed in people aged 75 or over (Cancer Research UK, 2015a) (figure 1.1). Cancer Research UK also proposes that 42% of all cancers are preventable, while 50% of patients survive cancer for more than 10 years (Cancer Research UK, 2015a).



All cancers excluding non-melanoma skin cancer (C00-C97 excluding C44), annual average number of new cases and age-specific incidence rates, UK, 2009-2011.

Figure 1.1: UK cancer incidences by age and gender.

The incidence rates for most cancers increases with age, where 36% of all cancers in the UK are diagnosed in patients aged 75 and above. More cancer cases are diagnosed in men (approximately 60,828) than women (57,221). Incidence rates shown occurred between 2009 and 2011. Source: Cancer Research UK (Cancer Research UK, 2015a).

1.1.2 The nature of cancer

‘Cancer’ is a broad term given to a group of diseases that involves dynamic changes in the genome, resulting in conferring a survival advantage to cells. This eventually leads to uncontrolled cell replication and eventually to invasion and tissue metastasis in somatic or germinal cells (Pelengaris and Khan, 2009). In a normal multicellular organism, tissue mass is tightly regulated by a network of overlapping molecular mechanisms that govern the rate of cell proliferation and cell death (by apoptosis) (Bertram, 2000) (figure 1.2). Thus, any factor that can alter the balance between cell birth and death, if not controlled has the ability to cause disease in a particular organism.

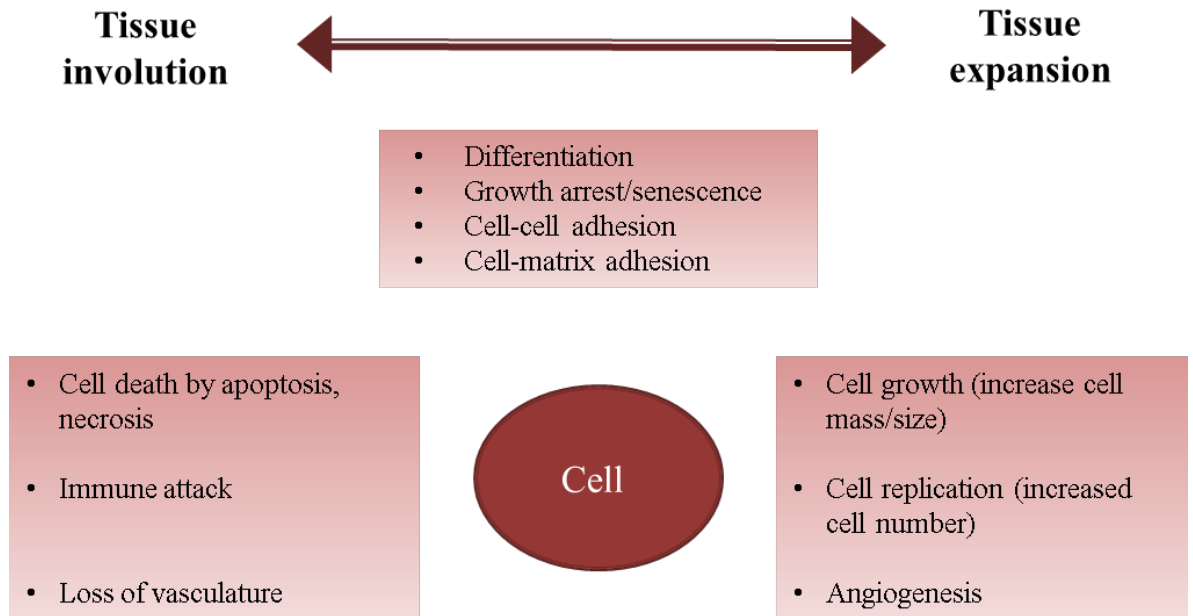


Figure 1.2: Regulation of tissue mass in multicellular organisms.

Tissue mass is tightly regulated by the balance of cellular processes which cause cell replication and cell death. Any disturbances to these processes results in diseases. When cell loss exceeds cell renewal, the result is involution or degeneration, while the opposite effect results in hyperplasia, tissue expansion or neoplasia. Adapted from Pelengaris and Khan (2009).

Cancer cells must evolve to prevent any limitation on their proliferation potential, in order to multiply and allow tumour development (Weinberg, 2014) (figure 1.3). Most cancers evolve from a single somatic cell where the initiation and progression of tumourigenesis is dependent on the genetic changes that occur to the cell (Pelengaris and Khan (2009) and Weinberg (2014). Advanced tumours are monoclonal growths, descending from a single normal progenitor that can migrate from their anatomical site of origin to distant sites (metastasis), establishing new colonies, where it becomes life threatening (Bertram, 2000).

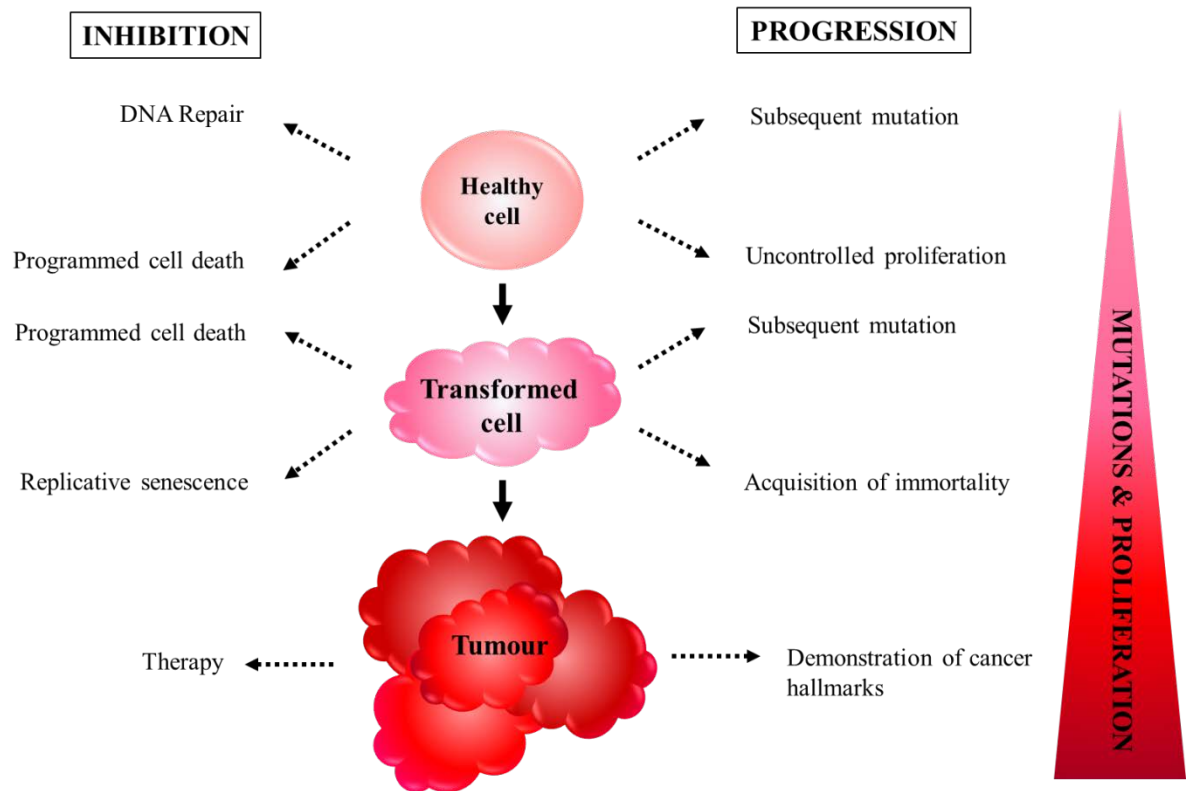


Figure 1.3: Causes of cancer.

Cells accumulate mutations which cause the progression from a pre-neoplastic stage, where it acquires features required for survival (listed in the ‘progression’ heading). At each stage, cancer cells must overcome the features that govern the rate of cell proliferation which aim to eliminate mutated cells from the host (listed in the ‘inhibition’ heading). Adapted from Bertram (2000).

1.1.3 Carcinogenesis and carcinogens

Carcinogenesis is a multistep process that arises when cells become irresponsive to stringent growth control signals; thereby proliferating excessively due to alterations in the genes controlling cell proliferation, survival and other traits consistent with the malignant phenotype (Bertram, 2000). Most of the mutations (substitutions, frame-shift mutations or mutations to stop codons etc.) that give rise to cancer occur spontaneously due to chemical damage to DNA, altering the function of crucial genes. Repeated exposure to either chemical or physical exogenous agents (such as infection by viruses, radiation and tobacco) are generally carcinogenic as they can act as mutagens, responsible for some human cancers (Hesketh, 2013).

For example, a prolonged infection of the retrovirus, human T cell leukaemia virus type 1 (HTLV-1), gives a 3-4% risk of developing adult T-cell leukaemia (Uchiyama *et al.*, 1977 and Poiesz *et al.*, 1980). Studies have shown that HTLV-1 encodes a viral *tax* gene whose products activate the transcription of the growth factors interleukin 2 (IL-2) and granulocyte macrophage colony stimulating factor (GM-CSF), inducing proliferation of haematopoietic cells, increasing the frequency of neoplastic cell variants (Weinberg, 2014). More recently, studies have found that an increased mRNA expression of endogenous viruses especially the provirus HERV-K (HML-2) is associated with prostate cancer (Wallace *et al.*, 2014).

In addition, UV light is a well-known cause for skin cancers where UVB (with a wavelength of 280-320 nm), is well known to form pyrimidine dimers in DNA. Epigenetic aberrations (e.g. histone modification, nucleosome remodelling, DNA methylation, chromatin remodelling and RNA interference) may also occur, induced by hormones such as oestradiol (which is important in the initiation and progression of breast cancer); leading to the alteration in chromatin condensation, regulating gene activation and promoting malignant behaviour (Pelengaris and Khan, 2009 and Weinberg, 2014). The initiated cell is now able to form a tumour although initiation alone is not sufficient enough for tumour formation (Hesketh, 2013).

1.1.4 Oncogenes and tumour suppressor genes

It is well known that mutations occurring in genes responsible for cell proliferation or apoptosis - oncogenes, tumour-suppressor genes and DNA repair enzymes are implicated in the tumourigenic process. Such mutations provide cancerous cells the ability to evade normal homeostatic mechanisms, further driving carcinogenesis (Bertram, 2000).

Mutations in oncogenes contribute to the malignant process because they acquire a *gain of function* mutation which provides a dominant effect on cell growth due to an enhanced cell replication and avoidance of cell death (Hesketh, 2013 and Weinberg, 2014). Genes which promote autonomous growth in cancer cells are termed *oncogenes* while their non-mutated counterparts are called *proto-oncogenes*. Proto-oncogenes function as growth factors or receptors, cell cycle components, signal transducers or transcription factors while their mutated equivalents also carry out the same function with an additional trait allowing them self-sufficiency in growth (Hesketh, 2013). For example, the normal form of the epidermal growth factor gene (ERBB1) is overexpressed in 50% of glioblastomas, 80% of squamous cell carcinomas and 80-100% of head and neck tumours, contributing to the malignant phenotype in the proliferating cell population (Kumar *et al.*, 2007).

While oncogenes drive cell proliferation, tumour suppressor genes control proliferation, differentiation or cell death by acting as cell cycle inhibitors, transcription factors, cell surface receptors, signal transduction molecules, transcription factors and regulators of cell response to DNA damage. One main tumour suppressor gene, p53, the 'molecular policeman' of the cell, prevents the transformation to neoplasm by triggering apoptosis or inducing a permanent or temporary cell cycle arrest (Bertram, 2000 and Hesketh, 2013). Homozygous loss to p53 is evident in nearly every cancer – lung, colon and breast, the three leading causes of cancer mortality worldwide. Homozygous loss of p53 has been identified in prostate cancer (PCa) and has been demonstrated to facilitate the invasion and metastasis of PCa cells (Wang *et al.*, 2013). In addition, Ecke *et al* (2010) has shown that p53 mutations in exon 7 and exon 8 are factors of tumour progression, where p53 mutations were detected in 35.6% of patients with PCa. Tumour suppressor genes have also been shown to be hypermethylated in various tumours (e.g. MGMT in colorectal cancer and DAPK in bladder cancer), allowing uncontrolled cell division of the malignant population (Kumar *et al.*, 2007).

1.1.5 The hallmarks of cancer

In 2000, Hanahan and Weinberg outlined six main essential alterations present in cell physiology which combine to drive malignant transformation (figure 1.4). These 'hallmarks' are features that enable tumours grow and metastasise and are shared in common by most tumours (Hanahan and Weinberg, 2000). Each of these changes are acquired during tumour development and signify successful breaching of an anti-cancer defence mechanism by the tumour. Hanahan and Weinberg (2000) suggest that most cancers have acquired the same set of features during their development, even if via various strategies. The 6 hallmarks of cancer (figure 1.4) are described below.

1. Self-sufficiency in growth signals

Normal cells are dependent on mitogenic growth signals before they can move from a quiescent state to an active proliferative state. Diffusible growth factors, extracellular matrix components and cell-to-cell adhesion/interaction molecules bind to transmembrane receptors and transmit growth signals to healthy cells. Normal cells cannot proliferate in absence of these growth signals (Hanahan and Weinberg, 2000). However, oncogenes in cancer produce and respond to their own growth, reducing their dependence on growth factor stimulation from their normal tissue microenvironment. For example glioblastomas and sarcomas produce PDGF (platelet derived growth factor) and TGF α (tumour growth factor α) respectively to which they become responsive, creating autocrine stimulation (Fedi *et al.*, 1997).

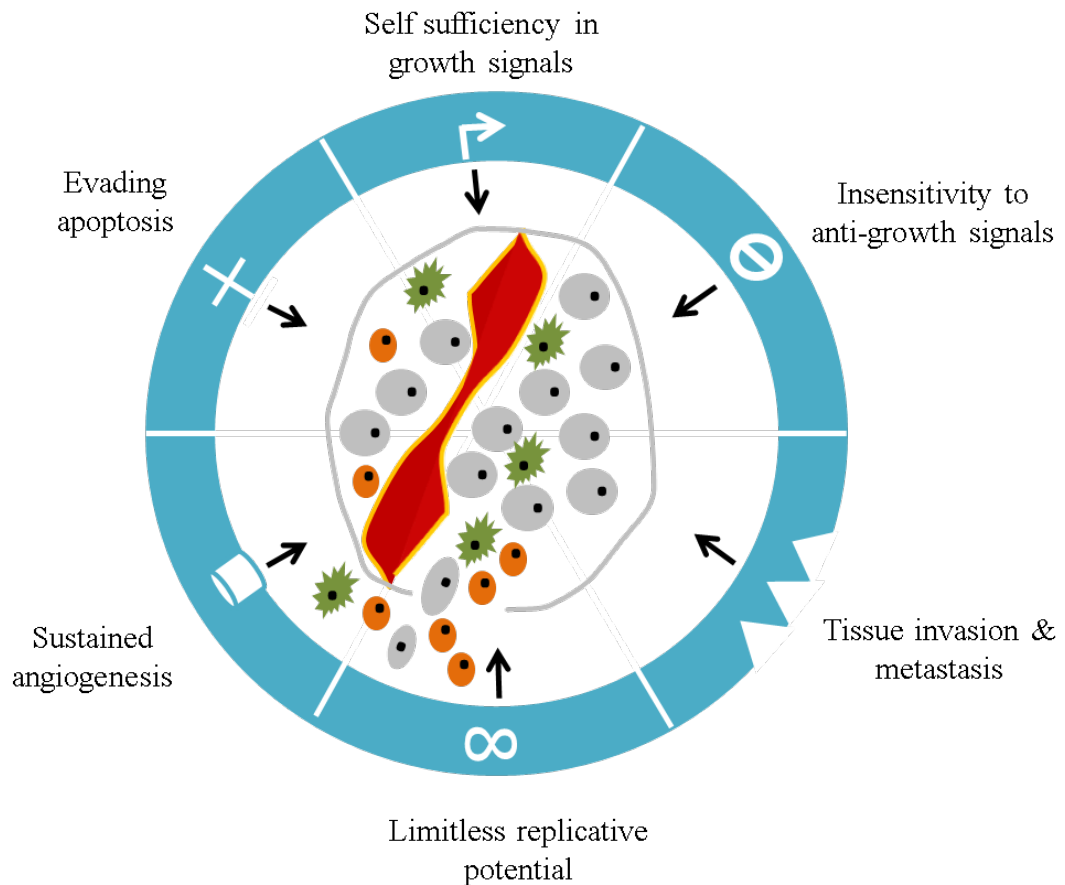


Figure 1.4: The original hallmarks of cancer.

Hanahan and Weinberg (2000) outline 6 main hallmarks that are acquired by cancer cells. They propose that most cancers acquire these functional characteristics during neoplastic development, although via various mechanisms. Adapted from Hanahan and Weinberg (2000).

2. Insensitivity to growth inhibitory (antigrowth) signals

To maintain tissue homeostasis, two distinct mechanisms occur to prevent proliferation in normal cells. Antigrowth signals may force healthy cells into the quiescent (G_0) state from an active state or force cells to permanently abandon their proliferative state, entering into a postmitotic state (Weinberg, 2014). Hanahan and Weinberg (2000) outline that emerging tumour cells must evade anti-proliferative signals in order to drive malignant growth. Inactivation of tumour suppressor genes that limit cell growth and proliferation have been reported in many human and animal cancers. The retinoblastoma protein (pRb) governed by $TGF\beta$ and central to the decision-making of anti-proliferative signals is disrupted in

various cancers (Fyran and Reiss, 1993) liberating E2F transcription factors, driving cell proliferation, thereby rendering cells insensitive to antigrowth signals.

3. Evasion of programmed cell death (apoptosis)

Tissue growth in normal tissues is maintained by balancing the rate of cell proliferation and cell death. Programmed cell death (apoptosis) is triggered in response to a variety of physiological signals which trigger regulatory proteins to disrupt the cell membrane, break down the cytoplasmic and nuclear skeletons, extrude the cytosol, degrade the chromosomes and fragment the nucleus. Mounting evidence indicates that most, if not all tumour cells develop an ability to evade apoptosis by down regulating proapoptotic factors, (Bax, Bim, Puma), increasing the expression of anti-apoptotic regulators (Bcl-2, Bcl-x_L) or that of survival signals (Igf1/2) (Hanahan and Weinberg, 2000).

4. Limitless replicative potential

Normal cells have a finite replicative potential and once they have reached a certain number of doublings, they lose their ability to divide and stop growing – senescence (Hanahan and Weinberg, 2000). An increasing body of evidence has shown that human tumours can prevent senescence by inactivating their tumour suppressor proteins (p53 and pRb) enabling their uncontrolled cell division and infinite expansion which results in tumour formation (Weinberg, 2014). In addition, most tumour cell types propagated in culture, are immortalised, demonstrating that a limitless, replicating potential is essential for their malignant growth. Furthermore, a progressive shortening of telomeres by a 50-100 bp loss of telomeric DNA after each cell cycle is characteristic of normal cells (Counter *et al.*, 1992 and Shay and Bacchetti, 1997). Tumour cells preserve their immortality by maintaining their telomeres at a length above a certain threshold, permitting unlimited cell multiplication.

5. Sustained angiogenesis

The formation of new blood vessels from pre-existing vessels, (angiogenesis) is an essential process in normal cells, as oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. After tissue formation, the process of angiogenesis is

tightly regulated (Hanahan and Weinberg, 2000). However, tumours activate the angiogenic switch by increasing the gene transcription of angiogenic inducers such as vascular endothelial growth factors (VEGF) and/or fibroblast growth factors (FGFs) (Fedi *et al.*, 1997; Veikkola and Alitalo 1999) or downregulating the expression of endogenous angiogenic inhibitors such as thrombospondin-1 or β -interferon (Singh *et al.*, 1995; Volpert *et al.*, 1997).

6. Tissue invasion and metastasis

The capability for tissue invasion and metastasis occurs providing that transformed cells possess all the previously aforementioned characteristics. Tissue invasion and metastasis after tumour development occurs where cancer cells escape the primary tumour and colonise a new area where nutrients and space are abundant in supply, enabling them to form new colonies (Hanahan and Weinberg, 2000). This distant settlement is responsible for mortality in over 90% of cancer cases (Sporn, 1996). Downregulation and occasional mutation of E-cadherin, an important cell-to-cell adhesion molecule, which helps to assemble adjacent epithelial cells is another feature of human carcinomas (Christofori and Semb, 1999). Furthermore, in some invasive tumours, an upregulation of N-cadherin, an adhesion molecule associated with cell migration is observed while genes encoding cell-to-extracellular matrix adhesion molecules are demonstrably downregulated or mutated in highly aggressive carcinomas.

In 2011, Hanahan and Weinberg revisited the original hallmarks and added four new concepts which emerged as important for tumour development (Hanahan and Weinberg, 2011). The new hallmarks are described below:

7. Genomic instability and mutation

Certain mutant genotypes confer a selective advantage on subclones of cells allowing their outgrowth and dominance in a tissue microenvironment. Multistep tumour progression is believed to be a succession of clonal expansions, each triggered by the acquisition of a mutant genotype (Hanahan and Weinberg, 2011). Genome maintenance systems detect and resolve any defects in the DNA to ensure that the rate of spontaneous mutations are kept low throughout each cell generation. In order to form tumours, cancer cells increase the

rates of mutation by increasing the sensitivity to mutagenic agents and/or the breaking down one or more of the components of the genomic maintenance machinery (Negrini *et al.* 2010, and Salk *et al.* 2010). Furthermore, Jackson and Bartek (2009), Kastan (2008) and Sigal and Rotter (2000) have also reported that surveillance systems that normally monitor genomic integrity and force cells into apoptosis or senescence (e.g. p53) have been compromised in various cancers.

8. Tumour promoting inflammation

Most tumours contain cells of the immune system that are present in various amounts from subtle infiltrations to gross inflammations (Pages *et al.*, 2010). Inflammation may contribute to many of the hallmarks of cancer by supplying growth factors that sustain proliferative signals, proangiogenic factors, extracellular matrix-modifying enzymes to promote angiogenesis, invasion and metastasis, and survival factors that limit cell death (DeNardo *et al.*, 2010, Karnoub and Weinberg 2006, Grivennikov *et al.*, 2010 and Qian and Pollard 2010). In many patients, inflammation is evident at early stages in the neoplastic transformation and capable of driving the progression of incipient neoplasms into full blown cancers. Inflammation may also release reactive oxygen species that induce mutations for nearby cancer cells, thus promoting their genetic evolution into malignancy (Grivennikov *et al.*, 2010).

9. Deregulating cellular energetics

Under aerobic conditions, respiring cells convert glucose to pyruvate in the cytosol and eventually to carbon dioxide in the mitochondria. While during anaerobic conditions, glycolysis is favoured such that little pyruvate is taken up into the mitochondria. Otto Warburg (1930) observed that tumour cells reprogram their glucose metabolism (and thus energy production) even in the presence of oxygen by limiting their energy metabolism mainly to glycolysis, a term described as ‘aerobic glycolysis’. Reprogramming energy metabolism is unproductive, as tumour cells must compensate for the ~18-fold lower ATP produced by glycolysis compared to mitochondrial oxidative phosphorylation. This is achieved by upregulating glucose transporters (i.e. GLUT-1), increasing the glucose uptake into the cell (Hsu and Sabatini, 2008 and DeBerardinis *et al.*, 2008). Marked increases in the uptake and utilisation of glucose is documented in many tumour types and visualised

using positron emission tomography (PET) using a radiolabelled glucose analogue (^{18}F -fluorodeoxyglucose, FDG) as a reporter. Increased glycolysis also allows the diversion of glycolytic intermediates into various biosynthetic pathways including generating nucleosides and amino acids, facilitating the synthesis of macromolecules and organelles for assembling new cells (Hanahan and Weinberg, 2011).

10. Avoiding immune destruction

It is widely accepted that the immune system plays a role in preventing the formation of tumours (Dunn *et al.*, 2002). The theory of immune surveillance postulates that the immune system constantly monitors cells and tissues in order to eliminate nascent tumours (Dunn, Old and Schreiber, 2004). Solid tumours are thereby believed to be formed as a result of evading immune detection (Hanahan and Weinberg, 2011). Teng *et al.*, (2008) demonstrated that mice genetically engineered to be deficient for various components of the immune system, developed more frequent tumours compared to immunocompetent controls. In addition, they also showed that mice deficient in the development or function of CD8^+ cytotoxic T lymphocytes, CD4^+ $\text{T}_\text{h}1$ helper T lymphocytes or natural killer (NK) cells resulted in increased rates of tumour incidence, while mice with combined immunodeficiencies in both T cells and NK cells were more susceptible to the development of tumours, demonstrating the contribution of the immune system to tumour eradication.

1.1.6 The immune system and cancer

It is well-known that the immune system is a barrier to the growth of tumours (Dunn *et al.*, 2002), where it is well-accepted that the interaction between tumours and immunity is in three forms, termed the three 'E's of cancer immunity – elimination, equilibrium and escape (Dunn, Old and Schreiber, 2004). Cancer arises from an accumulation of genetic alterations in cells (Hanahan and Weinberg, 2011). Elimination of cancer consists of three stages – elimination, equilibrium and escape (figure 1.5).

During elimination, the adaptive and innate immune systems work together to destroy tumours in order to keep the host free of cancer. If this process fails, and a rare variant is not eliminated, it may lead to an equilibrium phase where tumour growth is kept in a state of dormancy by T cells, IL-12 and IFN- γ . Due to the constant immune selection pressures placed on genetically unstable tumours, tumour cell variants may arise that are insensitive to adaptive immunity, effector mechanisms or induce an immunosuppressive state within the tumour. This may then lead to a final, escape phase where growth of tumour cells is no longer inhibited by immunity. These cells are then able to cause clinically apparent disease (figure 1.5) (Schreiber, Old and Smyth, 2011).

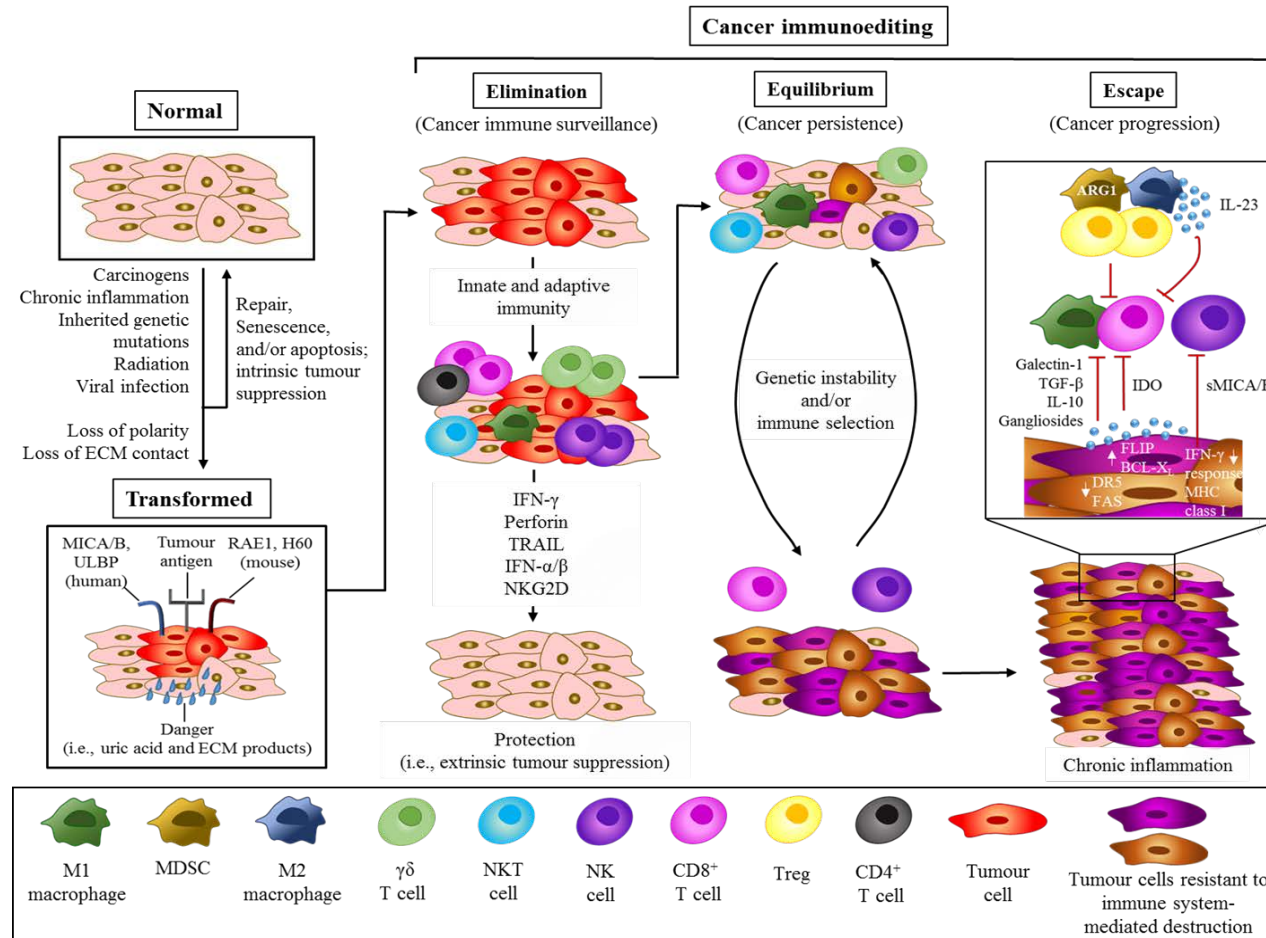


Figure 1.5: Cancer immunoediting: from immunosurveillance to immune escape

After cells become transformed from normal cells to cancerous cells, cells of the immune system may eliminate tumour cells (elimination/immunosurveillance). When elimination is unsuccessful, the immune system and cancer reach equilibrium where it can keep the cancer in check, but cannot eliminate it completely. The immune system also selects/promotes the generation of tumour variants with ability to survive immune attack. This may lead to escape where tumour cells expand in an uncontrolled manner in an immunocompetent host. Adapted from Strausberg (2005).

1.2 Prostate cancer (PCa)

1.2.1 Prostate anatomy

The prostate gland is a small, walnut shaped gland lying underneath the bladder (figure 1.6) and directly below the internal urethral orifice, around the beginning of the urethra. Adult prostatic parenchyma is divided into four anatomically and biologically distinct zones: the peripheral, central, transitional zones and the region of the anterior fibromuscular stroma (Kumar *et al.*, 2007). Histologically, the prostate is made up of glands lined by two layers of cells: a basal layer of low cuboidal epithelium covered by a layer of columnar secretory cells. Testicular androgens control the growth and survival of prostatic cells. The prostate gland is surrounded by the prostatic capsule and secretes an alkaline fluid containing prostate-specific antigen (PSA) which forms part of the ejaculate, aiding the nourishment and motility of sperm (Spickett and Robertson, 2010).

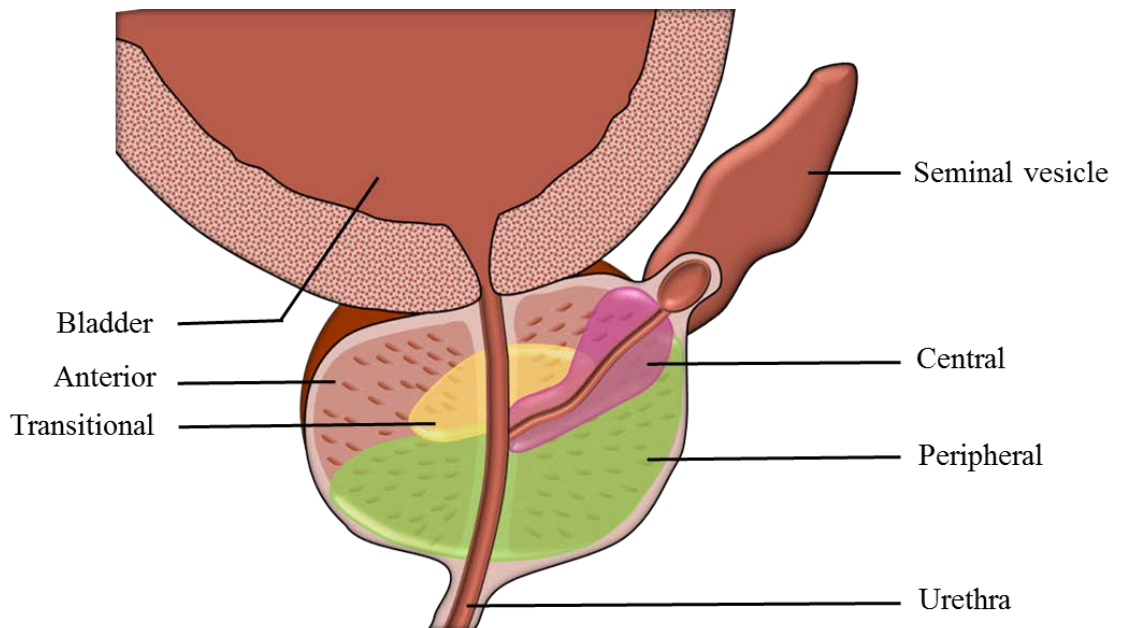


Figure 1.6: Prostate anatomy.

The prostate gland lies under the bladder and is made up of two tissue types – the glandular or epithelial cells which produce seminal fluid (including prostate-specific antigen; PSA) and stromal tissue. The stromal tissue is comprised of smooth muscle and connective tissue that contract to release prostatic secretions during sexual activity. The prostate gland is divided into 4 anatomical zones – the transitional, central, peripheral and anterior zones. Adapted from WholeLifeProstate (2015).

1.2.2 Incidence, risk factors, clinical presentation and treatment of PCa

Prostate cancer (PCa) is the most common cancer in men above 65 years (Drewa and Styczynski, 2008), where three quarters of PCa deaths occur in men above 75 years (Cancer Research UK, 2015b) (figure 1.7). In 2011, approximately 41,700 new cases of PCa were diagnosed in the UK causing approximately 11,000 deaths in 2012 (Cancer Research UK, 2015b). According to the American Cancer Society, approximately 1 in 7 men will be diagnosed with PCa in their lifetime, where 1 in 38 men will die from PCa in their lifetime (American Cancer Society, 2015). PCa incidences at autopsy are high, increasing from 20% in 50 year old men to 70% in men between 70 and 80 years.

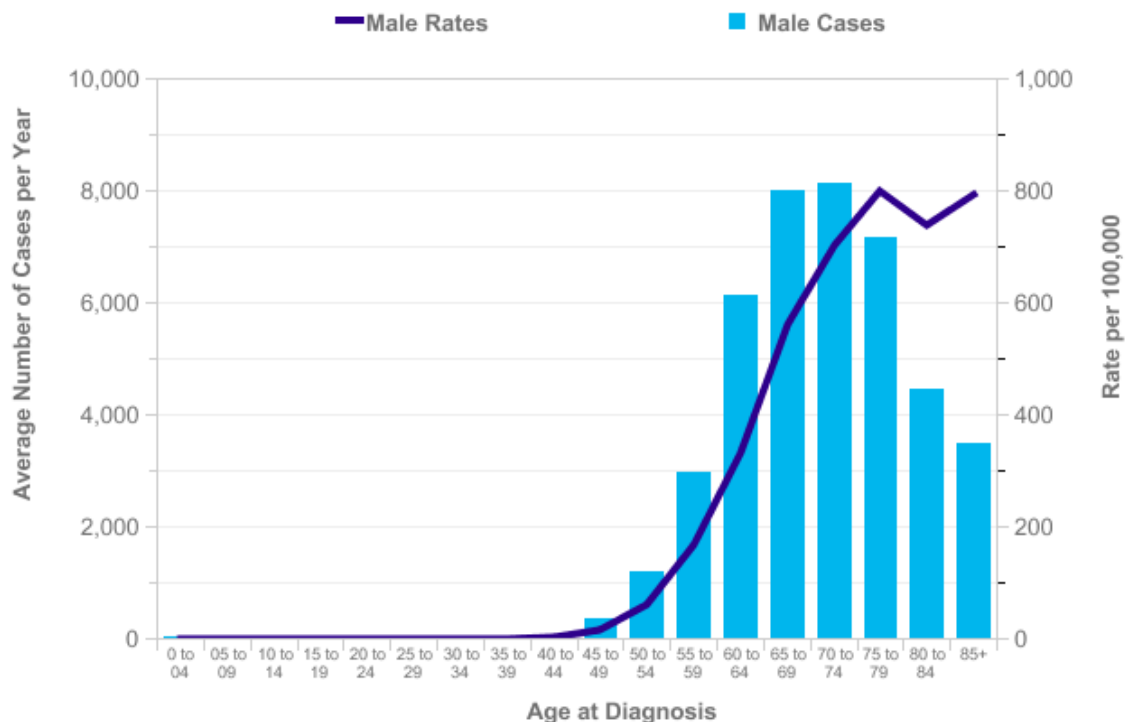


Figure 1.7: UK PCa incidences by age at diagnosis.

The highest incidence rate of PCa occurs in older men between the ages of 65 and 74 where it occurs in approximately 8,000 per 100,000 men. Source: Cancer Research UK (2015b).

Risk factors for PCa include advanced age, family history (in the father or brother) and race where African American men have the highest risk of developing PCa (Kohli and Tindall, 2010 and Hunchareck *et al.*, 2010). According to the American Cancer Society, 27,130 cases were diagnosed in African American populations, which accounted for 34% of all the cancers diagnosed in this population (American Cancer Society, 2015). In addition, men from African American descent are generally diagnosed with more advanced disease at an earlier age compared to men from Caucasian or Asian populations (Odedina *et al.*, 2009). PCa can be eradicated or treated effectively if detected at early stages. As a result, methods of detecting PCa at earlier stages of the disease are of prime importance for cancer patient management (Madu & Lu, 2010 and Kumar *et al.*, 2007).

PCa is generally asymptomatic in early stages of disease, however it may exhibit a wide range of clinical behaviours, ranging from incidentally discovered, clinically insignificant cancers to more aggressive lethal tumours (Roth-Kauffman, 2011). Lower urinary tract symptoms may be present such as weak stream, frequency, urgency, hesitancy, straining, incomplete emptying, nocturia, intermittency and various degrees of incontinence. Men with advanced PCa may present with pain in the hips, back and pelvis as a result of metastatic disease (Litman *et al.*, 2007).

Tumours originating in glandular, prostatic epithelia are referred to as ‘adenocarcinomas’ which account for more than 95% of PCa cases. Of these, 70% occur in the peripheral zone, 15-20% in the central zone while 10-15% occur in the transitional zone (Bostwick *et al.*, 2004). PCa tumours metastasise through the bloodstream and lymphatic system, forming secondary tumours mainly in the bones and lymph nodes. As a result, a dramatic decline in the cure rates occurs in metastatic tumours (Thalman *et al.*, 1994).

When PCa is confined to the prostate gland, curative treatment options include various forms of radiation therapy (external beam and/or brachytherapy), radical prostatectomy, and active surveillance (Roth-Kauffman, 2011). In advanced stages, where the tumour has metastasised beyond the prostatic capsule, the chances of cure are decreased. Treatment options for metastatic PCa include local therapy combined with adjuvant radiation therapy or androgen deprivation therapy (Widmark *et al.*, 2009). Survival rates for men with early stage PCa is prolonged. Studies have reported up to 60-90% occurrence of 10 year survival

rates after radical prostatectomy or radiation therapy (Inmann *et al.*, 2008 and Sanda and Kaplan, 2009).

1.2.3 Diagnosing PCa

PCa is asymptomatic until it becomes advanced or metastatic disease, thus, patient screening to aid early PCa diagnosis is mandatory (Smith *et al.*, 2003). PCa screening is carried out in order to detect tumours before symptoms have developed. In early stages of PCa, screening is achieved through PSA blood test, digital rectal examination (DRE) and transrectal ultrasound of the prostate (TRUS) (Mettlin *et al.*, 1991 and Catalona *et al.*, 1991) while diagnosis is achieved by a histological examination of prostate tissue obtained from a prostate needle biopsy, carried out using a transrectal ultrasound of the prostate (TRUS).

1.2.3.1 Prostate specific antigen (PSA)

PSA is the most important biomarker for PCa detection, staging and monitoring (Catalona *et al.*, 1991). PSA, a serine protease of approximately 30 kDa, is produced in normal prostatic epithelium and secreted into the semen to cleave and liquefy seminal coagulum after ejaculation (Spickett and Robertson, 2010). Normal serum levels of PSA range from 0 – 4.0 ng/mL, where elevated levels are frequently observed in localised and advanced PCa (Roth-Kauffman, 2011). A PSA value of >4.0 ng/mL may require follow up depending on age, ethnicity and family history of PCa.

Although PSA can increase the detection rate in men with no symptoms (Catalona *et al.*, 1993 and Mettlin *et al.*, 1991), and men at an early age and stage (Balducci *et al.*, 1997), PSA has resulted PCa overdiagnosis. PSA lacks specificity and sensitivity for PCa as it is raised in other non-malignant conditions such as Benign Prostatic Hyperplasia (BPH), prostatitis, or regular processes such as ejaculation, while reduced PSA levels can be observed in castration, surgery to the prostate or radiation therapy (Dimakakos *et al.*, 2014). In addition, only 25% of men who display elevated PSA are associated with PCa (Andriole, 2012, Barry, 2001 and Schroder, 2009). These false positive rates, resulted in the use of invasive procedures leading to over diagnosis and overtreatment of potentially indolent

disease, or delayed treatments for more advanced, aggressive and life threatening tumours (Oh *et al.*, 2003).

Recently, variations of PSA have been introduced to increase the sensitivity and specificity of PSA for PCa, such as free vs. total PSA (Stenman, 1995), age of patient (Carter *et al.*, 1992), PSA velocity and doubling time, pro-PSA (Mikolajczyk *et al.*, 2003), and PSA density of the transition zone. In 1991, a study by Stenman *et al* demonstrated that free PSA may be better at detecting BPH while bound PSA could more accurately detect PCa.

Although each of these variations have shown some added diagnostic value to PCa, serum PSA alone cannot accurately predict pathological stage as it varies with tumour volume, grade and tumour site of origin (Partin *et al.*, 1990 and Partin *et al.*, 1993). In addition, studies have shown that for each patient benefitting from PSA diagnosis-initiated treatment, unnecessary biopsy and treatment is carried out on 47 patients due to false positive PSA results (Andriole *et al.*, 2009). Furthermore, over diagnosis rates of up to 29% for Whites and 44% for Blacks have been reported (Oh *et al.*, 2003), suggesting the need for more accurate biomarkers for PCa.

1.2.3.2 Digital rectal examination (DRE)

A DRE involves feeling the prostate through the rectum for irregularities in prostate size, shape and texture. DRE is a routine screening method for PCa, which can detect cancer in men with small, well differentiated tumours, and cancers missed by other tests (Basler and Thompson, 1998). It has 59% accuracy and 94% specificity in detecting PCa (Hoogendam *et al.*, 1999) and is relatively well tolerated by patients and inexpensive to perform. DRE is useful for the detection of other abnormal prostate conditions such as BPH (Yedema *et al.*, 1993).

On the other hand, a DRE is invasive, subjective and cannot detect tumours that are non-palpable (usually early stage tumours) or tumours located in regions of the gland that are distant and obscure from digital palpitation (Mahon, 2005). Furthermore, studies have showed that DRE is more accurate in combination with other diagnostic indicators such as PSA (Harris and Lohr, 2002). Despite its limited sensitivity, a DRE along with elevated

PSA levels are the primary indicators for a prostate biopsy, although both markers still result in overdiagnosis (Gomella *et al.*, 2011). This highlights the need for novel biomarkers which can detect PCa particularly aggressive from indolent diseases.

1.2.3.3 Transrectal ultrasonography (TRUS) of the prostate

PSA use in clinics has resulted in an increase in the number of patients undergoing TRUS (Punnen *et al.*, 2009). A transrectal ultrasound (TRUS) involves the use of an ultrasonograph which sends out high energy sound waves to the rectum of a patient to allow the imaging of the entire prostate gland (Keyhole Urology, 2015). Using ultrasonography, areas of the prostate with varying morphology produce different images, which results in a high sensitivity, paramount for early PCa diagnosis. In addition, TRUS can be used to guide a needle biopsy in order to confirm PCa diagnosis after PSA and/or DRE (Oh *et al.*, 2003). This involves isolating 10-12 cores of the prostate, which is histologically analysed for the morphological assessment of tumour cells and the extent of spread.

It is well documented that TRUS should not be utilised alone as a primary screening tool due to its low specificity. One study by Catalona *et al.*, (1994) demonstrated that nearly 40% of tumours would have been missed if biopsies were performed only in men with abnormalities in TRUS. Furthermore, it is not feasible as a screening test in primary care clinics, but rather to guide biopsy for diagnostic purposes. In addition, studies have shown that TRUS guided biopsy have sensitivities ranging from 39-52% (Norberg *et al.*, 1997), PCa detection rate of 25% on initial biopsy (Naughton *et al.*, 2000) and 18-32% detection rate on repeated biopsies (Yuasa *et al.*, 2008 and Aganovic *et al.*, 2011).

1.2.3.4 Transperineal template prostate biopsy (TTPPB)

Unlike TRUS, transperineal template prostate biopsy (TTPPB) is associated with a high detection rate for PCa in men who have rising PSA and negative TRUS biopsy. A study by Pal *et al.*, (2012) showed a higher PCa detection rate in 40 men who underwent a standardised 36-core TTPPB despite two sets of negative TRUS biopsies. It involves using a brachytherapy template grid to allow the sampling (every 5 mm of prostate) and isolation of 30-50 tissue cores from various parts of the prostate, through the perineum. This allows

the detection of small cancers compared to other methods of prostate biopsy (Nafie *et al.*, 2013). The absence of a sensitive and specific marker for the diagnosis and prognosis of PCa creates a demand for new, reliable markers which can be used in a clinical setting.

1.2.4 Staging & grading PCa

1.2.4.1 Grading system (Gleason grade)

PCa biopsies are carried out to histologically determine the stage and grade of the prostate tumour, as these are the best prognostic indicators for PCa (Fielding *et al.*, 1992). The grading of PCa refers to the microscopic description of the glandular patterns of differentiation (aggressiveness of PCa) and is achieved using the Gleason scoring system, outlined by Donald Gleason *et al.*, 1966 and refined in 1974 and 1977 (Gleason 1966 and Gleason and Mellinger, 1974). The Gleason scoring system classifies PCa into one of five grades - grade one represents well differentiated tumours, where the neoplastic glands are round in appearance, uniform, and packed into well-defined nodules (figure 1.8). Grade 5 tumours have no glandular differentiation and tumour cells infiltrate the stroma in forms of cords sheets and nests (Gleason *et al.*, 1974).






1		Small, uniform glands (nearly normal cells)	Well differentiated
2		More stroma between glands (some abnormal cells loosely packed)	
3		Distinctly infiltration of cells from glands at margins (many abnormal cells)	Moderately differentiated
4		Irregular masses of neoplastic cells with few glands (very few normal cells left)	
5		Lack of occasional glands, sheets of cells (completely abnormal cells)	Poorly differentiated

Figure 1.8: Gleason pattern scale.

Gleason scoring system grades tissues collected at biopsy, where pathologists determine the two most common tissue patterns giving them a score of 1 (well differentiated) to 5 poorly differentiated) tumours. The Gleason sum is a sum of both most common patterns observed under the microscope. Adapted from Prostate Health Organisation (Prostate Health Organisation, 2015).

Tumours may contain more than one pattern where a primary grade is assigned to the most dominant pattern, and a secondary grade to the second most dominant pattern (Kumar *et al.*, 2007). The numeric values of both patterns are added up to make a Gleason score or sum. Gleason scores with a similar biological behaviour are classified into groups. Grades 2 – 4 are classified as well differentiated tumours, while 5 – 6 are regarded as intermediate tumours. Gleason grades 7 tumours are termed moderate to poorly differentiated cancers while 8 -10 are high grade tumours (Dunn and Kazar, 2011) and figure 1.7.

1.2.4.2 Staging system (TNM staging)

The stage and grade classification of PCa indicates the tumour size, extent of spread, depth of penetration, organ of metastasis and effect on organ in relation to stage (Yano *et al.*, 2007). The pathologic staging system used for PCa is the TNM system based on the grade and extent of the tumour size (T), lymph node involvement (N) and possible metastasis (M) (American Joint Committee on Cancer, 1997). Staging is established on a scale of 0 to IV (cancer is staged as progressing from Stage 0 to Stage IV), where each stage is subdivided into more specific alphanumeric categories based on the primary tumour, regional nodes and distant metastasis (Bostwick *et al.*, 1994). The extent of the tumour involvement is divided into T1-T4, where higher T values indicate more prostate involvement (figure 1.9). Although PCa staging is essential, it cannot be carried out independently. Staging of PCa is carried out alongside knowledge of the PSA level, DNA ploidy, nuclear morphometry as well as cellular, molecular and genetic factors (Montie *et al.*, 1995).

Incorrect staging of PCa may result in overdiagnosis, overtreatment and a reduction in a patient's survival chances (Yano *et al.*, 2007). As staging is based on results from a DRE, PSA and TRUS, this highlights the need to utilise accurate markers for PCa screening and diagnosis. Nevertheless, other clinical tests such as CT scans, X-rays, bone scans and MRIs can be used to further determine the stage of PCa and/or detect localised tumours beyond the prostate.

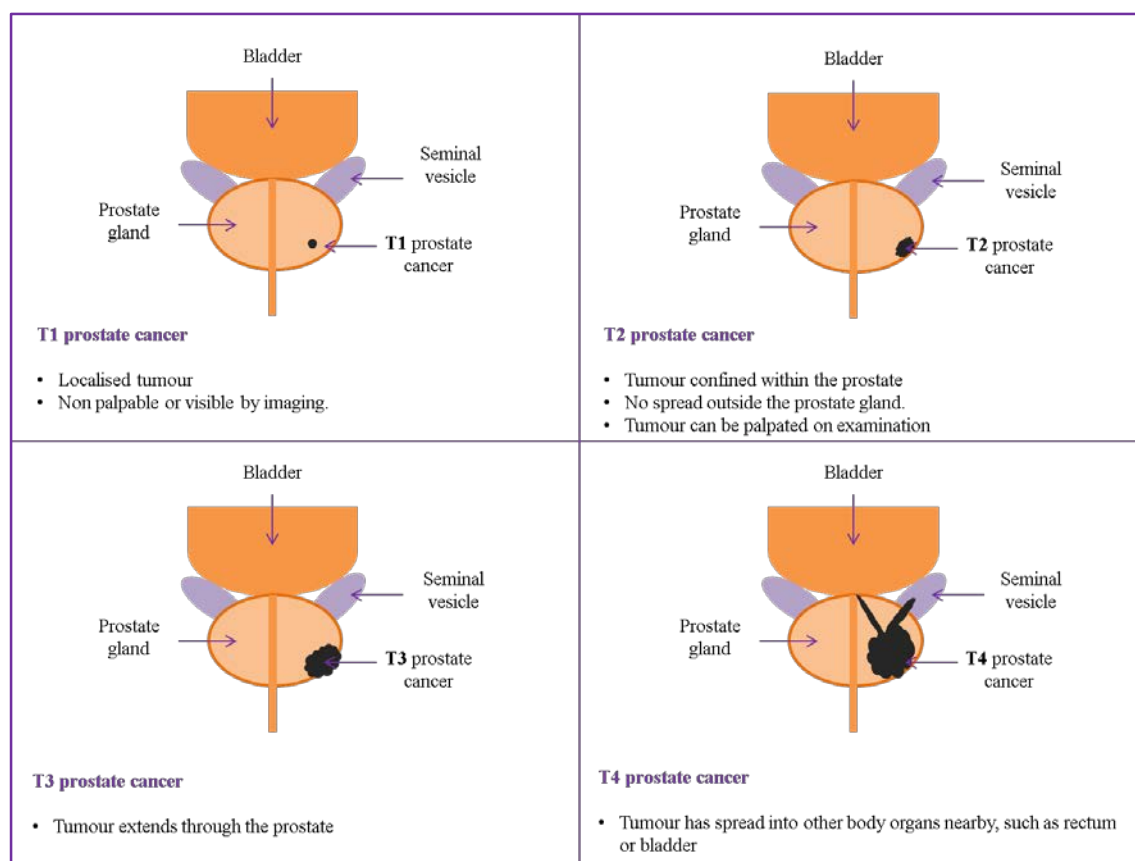


Figure 1.9: Staging prostate cancer – the ‘T’ system.

The figure above shows the pathological staging of PCa depicting the extent of the tumour size at various stages.

1.2.4.3 D’Amico risk classification system

In 1998, D’Amico *et al.* stratified patients into groups with low, intermediate or high risk of biochemical recurrence after radical prostatectomy, based on their stage, Gleason grade and PSA level (table 1.1) (Hernandez *et al.*, 2007).

Table 1.1: D’Amico risk classification system

	Gleason score	Clinical stage	Serum PSA (ng/mL)
Low risk	≤6	T1 or T2a	<10
Intermediate risk	≤ 6 - 7	T1 or T2a or b	10 – 20
High risk	≤ 7 8 - 10	T1 or T2a, b or c T1 or T2a, b or c	>20 Any PSA

D’Amico risk classification system to determine patients with a low, intermediate or high risk of biochemical recurrence of PCa after radical prostatectomy. Risk system is based on clinical stage, Gleason score and serum PSA. Adapted from: D’Amico *et al.*, 1998.

1.3 Cancer biomarkers and biomarker discovery

Epidemiologic and intervention studies have shown that early detection decreases the incidence of cancer morbidity and mortality (Brooks, 2012). Thus, tremendous effort is currently placed on cancer detection in early, curable stages in order to decrease the rates of cancer mortality, worldwide. Consequently, identifying serum biomarkers which manifest prior to the onset of cancer is the Holy Grail for biomarker discovery (Tan *et al.*, 2009).

1.3.1 Biomarkers

According to the National Cancer Institute, a biomarker is:

*‘Any biological molecule found in blood, or other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition of disease.’
- (National Cancer Institute, 2015).*

Cancer biomarkers are biochemical indicators of the presence of a tumour in a patient (Goedegebuure *et al.*, 2004). They are produced by or present in tumours or produced by a host in response to a tumour, and used to differentiate the presence of a tumour via measurements of the analytes in serum, tissue or urine (Diamandis 2002 and Sokoll and Chan, 2004). Biomarkers may be altered qualitatively or quantitatively in precancerous or cancerous conditions, making them detectable by an assay (Schrohl *et al.*, 2003). According to the National Cancer Institute, the ideal cancer screening and detection biomarker should be present in an easily available sample source (e.g. serum or urine) (Madu and Lu, 2010).

In symptomatic patients, cancer biomarkers (tumour markers) may be useful in determining tumour stage, grade and response to therapy (Ludwig and John, 2005) as well as for screening for early signs of malignancy in asymptomatic patients (table 1.2). After diagnosis, and treatment, cancer biomarkers may be used in assessing patient prognosis, therapy prediction, postoperative surveillance and response to systemic therapy (Duffy, 2001 and Duffy, 2007).

Table 1.2: Biomarker types based on their clinical application

Type of biomarker	Biomarker application
<i>Screening/detection</i>	Predict the potential occurrence of disease in a asymptomatic patients or those with non-disease specific symptoms
<i>Diagnostic</i>	Make predictions for patients suspected of having a disease. Required to have high sensitivity and specificity.
<i>Prognostic</i>	Predict the overall outcome of a patient, including recurrence and aggressiveness, regardless of therapy.
<i>Predictive</i>	Used to identify patient subpopulations that are most likely to respond to therapy. May also be used as a target for therapy.

Common uses of some tumour markers. Adapted from (Rigau *et al.*, 2013).

1.3.2 The ideal PCa biomarker

Pin *et al.* (2013) suggest that PCa biomarkers should ideally originate from the stromal or epithelial cells in the tumour and correlate to the tumour microenvironment (Pin *et al.*, 2013). Furthermore, they outline that levels of the tumour marker should be present at detectable levels in healthy controls, but significantly increased in cancer where its concentration reflects tumour burden. The ideal PCa biomarker should also be specific to PCa, yielding a high diagnostic sensitivity and specificity (Kulasingam and Diamandis, 2008). It should also have a short half-life which enables frequent serial measurement and correlate with tumour burden, indicating tumour progression. The clinical value of such a biomarker must be validated in a large patient cohort, where assays of tumour markers should be standardised, reproducible, cheap and simple to perform. They must also have clearly defined reference ranges to allow screening in a large population cohort (Duffy, 2013).

Despite advances in medical research, the ideal biomarker does not exist and only a few FDA approved serum biomarkers are currently available in clinical settings for example CA125 (for ovarian cancer), PSA (for prostate cancer), carcinoembryonic antigen (for colon cancer) and HER2/neu (for breast cancer). However, of these markers, PSA is the only serological marker utilised in cancer screening; having a sensitivity of 90% and specificity of 21% for prostate cancer making its use as a screening tool controversial (Pedersen and Wandall, 2011). Although these screening methods are available, they all

pose issues with sensitivity and specificity, indicating the need for more sensitive, accurate biomarkers for PCa detection.

1.3.3 Sample choices for PCa biomarker discovery (sources of PCa biomarkers)

Biomarker discovery is challenged by the necessity to use large sample numbers in order to obtain statistically significant data; which is essential for the robustness of the identified biomarkers (Rabilloud and Triboulet, 2013). The other main difficulty lies in electing the most appropriate sample source for biomarker discovery studies (Alvarez-Chaver *et al.*, 2014). Samples which can be utilised for biomarker identification include clinical samples such as patient serum, tissue and urine or other biological samples such as PCa cell lines (Fliser *et al.*, 2007). This section discusses the types of biological sample sources for biomarker discovery. A summary of diagnostic PCa biomarkers is outlined in table 1.3.

1.3.3.1 Serum and plasma

The most commonly used biological fluid for biomarker research is human blood as it is obtained via a minimally invasive procedure, is abundantly available, and changes in the blood, serum and plasma reflect diverse pathological states (Even-Desrumeaux *et al.*, 2011). However, serum and plasma have a heterogeneous mixture of proteins which are derived from various tissues, making it difficult to attribute differentially expressed proteins in cancer biomarker discovery studies to tissue specific tumours (Alvarez-Chaver *et al.*, 2014).

One of the PCa biomarkers identified in serum is urokinase plasminogen activator (uPA). The inactive precursor of the serine protease urokinase plasminogen activator (uPA), binds to a cell surface receptor (uPA receptor [uPAR]) and promotes the conversion of plasminogen to plasmin, which degrades the extracellular matrix proteins by activating proteases (Otero *et al.*, 2014). Various forms of uPA have been measured in patients to improve PCa detection, including serum cleaved uPAR domain I (uPAR [I]) and uPAR [II-III]) which are increased in PCa patients compared to benign controls. Furthermore, Piironen *et al.* (2006) showed that in patients with a total PSA of 2-10 ng/mL, combining the percentage of free PSA with uPAR (I)/uPAR (I-III) ratio increased specificity (0.73 ROC score) for PCa than using a free PSA alone (0.68). In addition, immunohistochemical

studies by Gupta *et al.* (2009) showed that overexpression of uPA, its inhibitor (PAI-1) and uPAR were associated with aggressive PCa recurrence in patients who had a radical prostatectomy, while Kumano *et al.* (2008) also found the same correlation with poor pathological features, including stage, Gleason grade, lymph node metastasis, surgical margin status and lymphatic invasion.

1.3.3.2 Urine

The formation of human urine occurs in the kidney, via processes resulting in ultrafiltration of plasma proteins and proteins of the urinary tract, selective reabsorption of water, glucose, sodium, and nitrogenous wastes, tubular secretion of urea, uric acid, drugs and creatinine, and the concentration of water (Kumar *et al.*, 2009). The normal urinary proteome contains a significant amount of proteins and peptides consisting of the soluble proteins and solid components of urine (figure 1.9) (Pisitkun *et al.*, 2006). As urine eliminates bodily waste products and maintains homeostasis, it is widely accepted that urine may contain information on urogenital and systemic diseases (Gonzalez-Buitrago and Ferreira, 2007). Thus, medical evaluation of renal function and the pathophysiology of the kidney and other distant organs rely on urinary analysis. Urinary biomarkers identified for PCa include DNA based markers e.g. Glutathione-S-Transferase P1 (GSTP-1), RNA based α -methylacyl coenzyme-A (AMACR) or protein based such as telomerase, calgranulin, and AMACR (Ludwig and Weinstein, 2005).

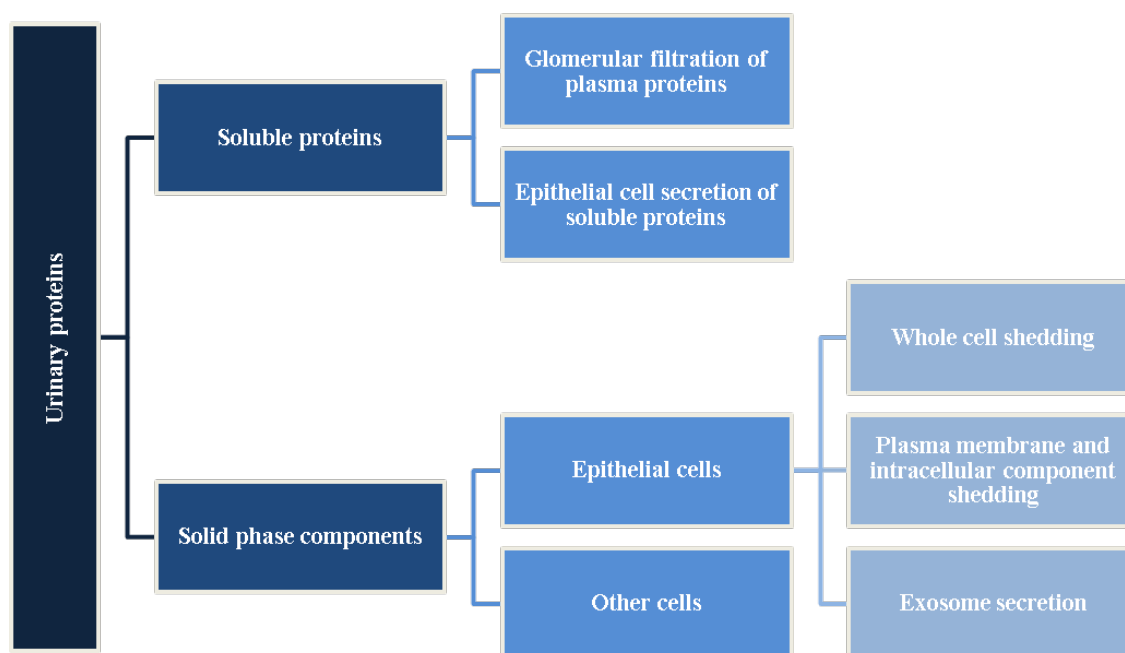


Figure 1.10: Sources of urinary proteins.

Urinary proteins include soluble proteins from glomerular filtration and membrane bound proteins, solid phase components such as epithelial cells and casts. Adapted from Pisitkun *et al.* (2006).

Exosomes may also serve as biomarkers from urine. The formation of exosomes occurs in multivesicular bodies by almost all tissues and utilises an inward budding mechanism that encapsulates cytoplasmic components to create small (50-150 nm), secreted membranous vesicles (Rigau *et al.*, 2013). Exosomes represent their tissue of origin and are present in most body fluids – urine, serum and ascites and in some cell lines, where they have a role in inter-cellular communication (Duijvesz *et al.*, 2013). Tumour derived exosomes are essential in growth and tumour progression and an increase in exosomal release has been shown to facilitate the communication between the tumour microenvironment and the tumour cell (Roberson *et al.*, 2013).

Urinary exosomes have been described as a treasure chest of information and potential source of new biomarkers for PCa, because purification of prostate derived exosomes will allow the identification of new biomarkers for the early diagnosis of PCa (Duijvesz *et al.*, 2011). Duijvesz *et al.* (2013) identified PDCD6IP, fatty acid synthase, exportin and alpha enolase as new candidate biomarkers for cancer where a higher abundance of fatty acid synthase, exportin and PDCD6IP was observed in PCa exosomes.

1.3.3.3 Tumour tissues

The analysis of biopsy-obtained tissue samples from cancer patients allows the comparison of protein profiles between adjacent healthy tissues and tumour tissue for the identification of cancer biomarkers (Alvarez-Chaver *et al.*, 2007). Tumour tissues are ideal for biomarker discovery studies because not all proteins altered in tumours, and therefore tumour marker candidates are secreted from the tumour cells into the blood or urine (Alvarez-Chaver *et al.*, 2014). Furthermore, because altered proteins originate in the tumours themselves, in theory tumour tissue samples have the richest concentration of tumour proteins, providing a more accurate representation of the tumour microenvironment, making them the ideal source for cancer biomarkers (Chen and Yates, 2007 and Even-Desrumeaux *et al.*, 2011).

Using tissue samples for cancer biomarker discovery has many limitations. Firstly, tumour tissue samples are obtained via an invasive biopsy procedure. Furthermore, it is difficult to obtain sufficient sample quantities, especially adjacent normal counterparts for comparison with tumour tissue for biomarker discovery (Even-Desrumeaux *et al.*, 2011). Tumours are also complex by nature and heterogeneously composed of stromal cells and surrounding neoplastic cells, therefore using them for cancer biomarker identification studies involves overcoming the challenges with tissue heterogeneity and cellular diversity (Pin *et al.*, 2013). One of the methods to overcome this has been the use of Laser Capture Microdissection (LCM) which isolates histologically pure cancer cells from surrounding a complex heterogeneous tissue microenvironment using laser-assisted micro-dissection (Hussain *et al.*, 2013). However, protein yield from LCM are low, limiting its use prior to biomarker discovery. A summary of tissue biomarkers can be found in table 1.3.

1.3.3.4 Immortalised PCa cell lines

Cell lines used in cancer biomarker discovery are isolated from primary tumours or tumour metastasis (e.g. pleural effusion or aspirates), while those derived from metastatic cancers may not always represent tumour diversity, thus using cell lines in translational studies may introduce bias (Hussain *et al.*, 2013). However, cell lines obtained from tumours are useful in biomarker research because they provide information on tumour biology and molecular characteristics such as cell-matrix and cell-cell interactions and gene expression (Lacroix and Leclercq, 2004). They offer an advantage over clinical samples due to their

ease of availability and homogeneous populations and the fact that they can be rapidly propagated *in vitro* and independently validated (Johnson *et al.*, 2014). Furthermore, it is also easy to obtain subcellular fractions such as plasma membrane, nucleus, secretome and exosomal fractions from cell cultures, reducing the sample complexity which aids the identification of cancer biomarkers (Alvarez-Chaver *et al.*, 2014).

Using cell lines for PCa biomarker research has some drawbacks. Most of the PCa cell lines were derived from prostate tumours, whereas cell lines from normal epithelia do not exist, limiting the study of the malignant transformation in PCa. In addition, each cell line represents only one tumour, and their cells may have been subjected to clonal drift and *in vitro* selection (Even-Desrumeaux *et al.*, 2011). Furthermore, it is difficult to mimic the tumour microenvironment in cell lines as cell cultures lack features of an *in situ* tumour such as the interaction with other tumour cells, stromal cells and the cells of the immune system, thus making them a poor representation of the tumour *in vivo*. For this reason, it has been argued that it may be difficult to extrapolate candidate biomarkers found in cell lines to clinical samples. As a result, it is of prime importance that candidate biomarkers identified in cell lines are validated in clinical samples from cancer patients (Alvarez-Chaver *et al.*, 2014).

Most studies investigating the identification of cancer biomarkers using PCa cell lines have been limited to the use a single cell line in isolation, rather than a panel of cell lines, which makes the validation of identified biomarkers difficult because these candidate markers may not be expressed by other PCa cells, in order to establish biomarker reproducibility or have the ability to distinguish between PCa and non-malignant cells (Johnson *et al.*, 2014). Johnson *et al.* (2014) compared multiple PCa cell lines (22RVI and LNCaP) with benign PNT1a and PNT2) and found a high expression of the lysosomal protein LIMP-2 protein and gene expression in PCa cell lines in comparison to benign controls, highlighting the significance of analysing more than one PCa cell line for biomarker discovery.

Conditioned media (secretome) also serves as a source of PCa biomarkers. Secreted proteins from cultured tumour cells into the cell supernatant media are thought to mimic the proteins released by tumours into the microenvironment where they can reach proximal fluids and circulation (Pin *et al.*, 2013). Conditioned media from tumour cells are an attractive source for biomarkers because proteins which are specific for and directly linked

to tumour cells are easily obtained from this simple matrix which is easier to analyse compared to serum.

Chen *et al.*, (2011) analysed the conditioned media of the androgen sensitive PCa cell line (LNCaP) compared to BPH cells (BPH-1) by two-dimensional electrophoresis and MALDI-TOF MS. They identified differential expression of eleven proteins (6 up regulated including: creatine kinase brain (CKB), triosephosphate isomerase 1 (TPI1), isocitrate dehydrogenase 2 (IDH2) and 5 down regulated proteins including: glutathione S-transferase pi (GST-pi) some of which were verified using real time polymerase chain reaction. They also found a similar expression pattern between mRNA levels of the conditioned media levels of CKB, TPI1, IDH2 and GST-pi in BPH and LNCaP.

Table 1.3: Diagnostic PCa biomarkers

S/N	Biomarker	Source	Biological Function	Reference
1	Prostate specific antigen (PSA)	Serum	Secreted protein that hydrolyses semenogelin-1 thus leading to the liquefaction of the seminal coagulum.	Ferro <i>et al.</i> , (1987)
2	Prostate membrane-specific antigen (PMSA)	Serum	Possesses folate hydrolase function. Cell stress reaction, signal transduction, cell migration, nutrient uptake. May possess questionable receptor function.	Rigau <i>et al.</i> , (2011)
3	Zinc alpha-2-glycoprotein (ZA2G)	Serum	Induces lipid decline in adipocytes and therefore implicated as possibly acting in cachexia	Katafigiotis <i>et al.</i> , (2012)
4	Human kallikrein 2 (KLK2)	Serum	Secreted serine protease. Splits pro-PSA to create PSA	Recker <i>et al.</i> , (2000)
5	Early prostate cell antigen-1, -2	Serum & tissue	May be involved in early prostate carcinogenesis; however, has uncertain contribution to nuclear morphology	Gu <i>et al.</i> , (2000)
6	α -Methylacyl-CoA racemase (AMACR)	Serum, urine & tissue	Engaged in bile acid synthesis, stereoisomerization, and β -oxidation of branched-chain fatty acids	Jiang <i>et al.</i> , (2001)
7	Thymosine β -15	Urine	Regulation of actin polymerization and immune response, key role in facilitating tumour metastasis and angiogenesis.	Huchinson <i>et al.</i> , (2005).
8	Telomerase	Urine	Protects the linear ends of eukaryotic chromosomes against degradation and fusion, thus maintaining genome stability	Botchkina <i>et al.</i> , (2005)
9	Prostatic inhibin-like peptide (PIP)	Urine	Suppresses the synthesis and release of follitropin (follicle-stimulating hormone)	Tremblay <i>et al.</i> , (1987)
10	Transferrin (Tf)	Urine	Iron metabolism. Key role in erythropoiesis/active cell division occur.	van Dieijen-Visser <i>et al.</i> , (1988)
11	Sarcosine	Urine	Catalyses the oxidative demethylation of sarcosine to glycine.	Sreekumar <i>et al.</i> , (2009)
12	PCA3 (DD-3)	Tissue & Urine	non-coding RNA	De Kok <i>et al.</i> , (2002)
13	Engrailed-2 (EN-2)	Urine	Developmental protein; transcriptional repressor	Morgan <i>et al.</i> , (2011)
14	Glutathione S-transferase P1 (GSTP1)	Tissue & urine	Conjugate reactive substrates with reduced glutathione (GSH) and are involved in detoxification	Köllermann <i>et al.</i> (2003) & Goessl <i>et al.</i> , (2001)
15	Caveolin-1 (Cav-1)	PCa tissue & cells	May act as a scaffolding protein within caveolar membranes. Interacts directly with G-protein alpha subunits and can functionally regulate their activity	Yang <i>et al.</i> , (1999)
16	Transmembrane protease serine 2 (TMPRSS2):ERG	Tissue, urine	Androgen-regulated, type II transmembrane-bound serine protease	Leyten <i>et al.</i> , (2012)
17	Transforming growth factor β 1	Tissue	Growth factor involved in cellular differentiation, immune response, angiogenesis and proliferation.	Shariat <i>et al.</i> , (2004)
18	Spondin-2 (SPON2)	Tissue, serum	Cell adhesion protein, promotes adhesion & outgrowth of hippocampal embryonic neurons. Essential in initiating innate immune response	Qianet <i>et al.</i> , (2012)

The table outlines a list of diagnostic PCa biomarkers identified from various biological sample sources. Although these biomarkers have been identified, none of them have been shown to accurately detect PCa. In addition, PCa is a heterogeneous malignancy such that a panel of markers would be more realistic than a single marker to indicate the presence of PCa (Downes *et al.*, 2006). Adapted from Madu and Lu (2010).

1.3.4 Proteomics & mass spectrometry for biomarker discovery

Mass spectrometry (MS) is an analytical technique that can provide qualitative (structural) and quantitative (concentration or molecular mass) information on analyte molecules after their conversion into ions (Ho *et al.*, 2003). Samples for mass spectrometry are ionised in the ion source to acquire positive or negative charges. The ions can then travel through the mass analyser, arriving at various parts of the detector depending on their mass-to-charge (m/z) ratio. Ions that make contact with the detector generate signals recorded by a computer system (figure 1.11), which displays these signals as a mass spectrum indicating the relative abundance of the detected signals according to their m/z ratio. The various components of mass spectrometers will be discussed below.

1.3.4.1 Ion source

Samples are analysed prior to analysis in the source using various ionisation techniques, depending on the instrument used. Some ionisation techniques are energetic, causing excessive fragmentation while others are softer, only producing ions of the molecular species (Hoffman and Stroobant, 2007). Ion sources produce ions by ionising a neutral molecule in the gas phase by electron ejection, protonation, deprotonation, adduct formation, electron capture or transferring a charged species from a condensed phase onto a gas phase. Examples of ion sources used in biomarker discovery include Electrospray Ionisation (ESI) or Matrix Assisted Laser Desorption Ionisation (MALDI).

1.3.4.1.1 Electrospray Ionisation (ESI)

Electrospray ionisation (ESI) was first reported in 1984 by Yamashita and Fenn. It is achieved when a strong electric field is applied to a liquid passing through a capillary tube with weak flux under atmospheric pressure (Ho *et al.*, 2003). At the liquid surface located at the end of the capillary, the electric field induces a charge accumulation which breaks to form highly charged droplets. At this point, a gas is injected at a low flow rate which allows the spray dispersion to be limited in space. The droplets then pass through a curtain of heated nitrogen, or via a heated capillary to remove the last solvent molecules (Hoffman

and Stroobant, 2007). The solvent contained in the droplets evaporates, causing them to shrink, increasing their charge per unit volume. These small, highly charged droplets continue to lose solvent such that when the electric field on their surface is large enough, desorption of ions from the surface occurs (Kearle and Tang, 1993).

ESI is different to MALDI as it may produce multiply charged ions, which effectively extends the mass range of the analyser (most commonly ion trap and quadrupole instruments) in order to accommodate kDa – Mda orders of magnitude commonly observed with proteins and polypeptide fragments (Ho *et al.*, 2003). ESI carries out ‘soft ionisation’ as little fragmentation occurs. The detection limit of ESI depends on the sample analysed (its preparation and purity), the type of instrument used and the skill of the operator. ESI is the ion source of choice coupled to liquid chromatography with mass spectrometry.

1.3.4.1.2 Matrix Assisted Laser Desorption Ionisation (MALDI)

In MALDI, compounds to be analysed are dissolved in a solvent containing small organic molecules called a matrix, which has a strong absorption at the laser wavelength. The mixture is dried before analysis, creating a ‘solid solution’ deposit of analyte-doped matrix crystals. In the source, rapid heating of the matrix molecules by the laser causes sublimation of the matrix into the gas phase (Hoffman and Stroobant, 2007). Although the origins of ions produced by MALDI isn’t fully understood, the most widely accepted pathway suggested for ionisation reactions include proton transfer in the solid phase before desorption or gas phase proton transfer from photoionised matrix molecules.

MALDI is a very sensitive laser ionisation technique. The matrix acts to minimise sample damage from the laser pulse by absorbing most of its energy. It also allows the desorption and ionisation of analytes with high molecular masses >100,000 Da. MALDI has also been applied in imaging biological materials to map the distribution of targeted biomolecules, allowing the direct study of proteins and peptides on tissue samples. MALDI is also characterised by an easy sample preparation method which has a high tolerance to contaminants such as salt, buffers and detergents, making it suitable for biomarker discovery studies (Chen *et al.*, 1998).

1.3.4.2 Mass analysers

After ions have been produced in the gas phase, they are separated according to their mass-to-charge ratio (m/z). The differences between various types of mass analysers are based upon the way magnetic/electric fields are applied to achieve mass separation. The most important characteristics for measuring the performance of a mass analyser is its analysis speed, mass accuracy, resolution and mass range limit (Hoffman and Stroobant, 2007).

1.3.4.2.1 Time of Flight (TOF)

The Time of Flight (TOF) mass analyser is well suited to the pulsed nature of laser desorption ionisation, separating ions according to their velocities as they drift in a free field region, known as a flight tube. After expulsion from the source, ions are accelerated towards the flight tube by a potential difference applied between electrode and an extraction grid (Hoffman and Stroobant, 2007). Mass to charge ratios are then determined by measuring how long ions take to move through a region between the source and detector, where the lower the mass of the ion, the faster it reaches the detector. The speed of a TOF analyser is very fast, generating a spectrum over a broad mass range in microseconds. However, TOF instruments are limited by their mass resolution, which is affected by time, space and kinetic energy distribution. The development of TOF analysers has paved the way for new applications for biomolecules and synthetic polymers and polymer/biomolecule conjugates.

1.3.4.1 Liquid Chromatography (LC)

In order to analyse a complex sample mixture, chromatography separation techniques are coupled to a mass spectrometer, such that separated products are introduced one after the other into the mass spectrometer either in gaseous phase (in gas chromatography, GC) or in solution (liquid chromatography, LC). High Performance Liquid Chromatography (HPLC) is an analytical technique used to separate, quantify and identify each component in a mixture (Hoffman and Stroobant, 2007). Using HPLC, a liquid solvent containing the

sample to be separated is passed through a column filled with solid adsorbent material (usually silica or polymers about 2-50 μm in size). Due to the different degrees of interaction of each component in the sample with the adsorbent material, different flow rates are generated for each component resulting in the separation of components as they flow through the column. A typical HPLC instrument includes a sampler to carry the sample into the mobile phase stream, bringing it into the column, a pump to deliver the desired composition and flow of the mobile phase into the column and a detector to generate a signal proportional to the amount of sample component. This creates a quantitative analysis of each sample component. Some HPLC models are able to mix multiple solvents in ratios that change with time in order to generate a composition gradient in the mobile phase (Hoffman and Stroobant, 2007).

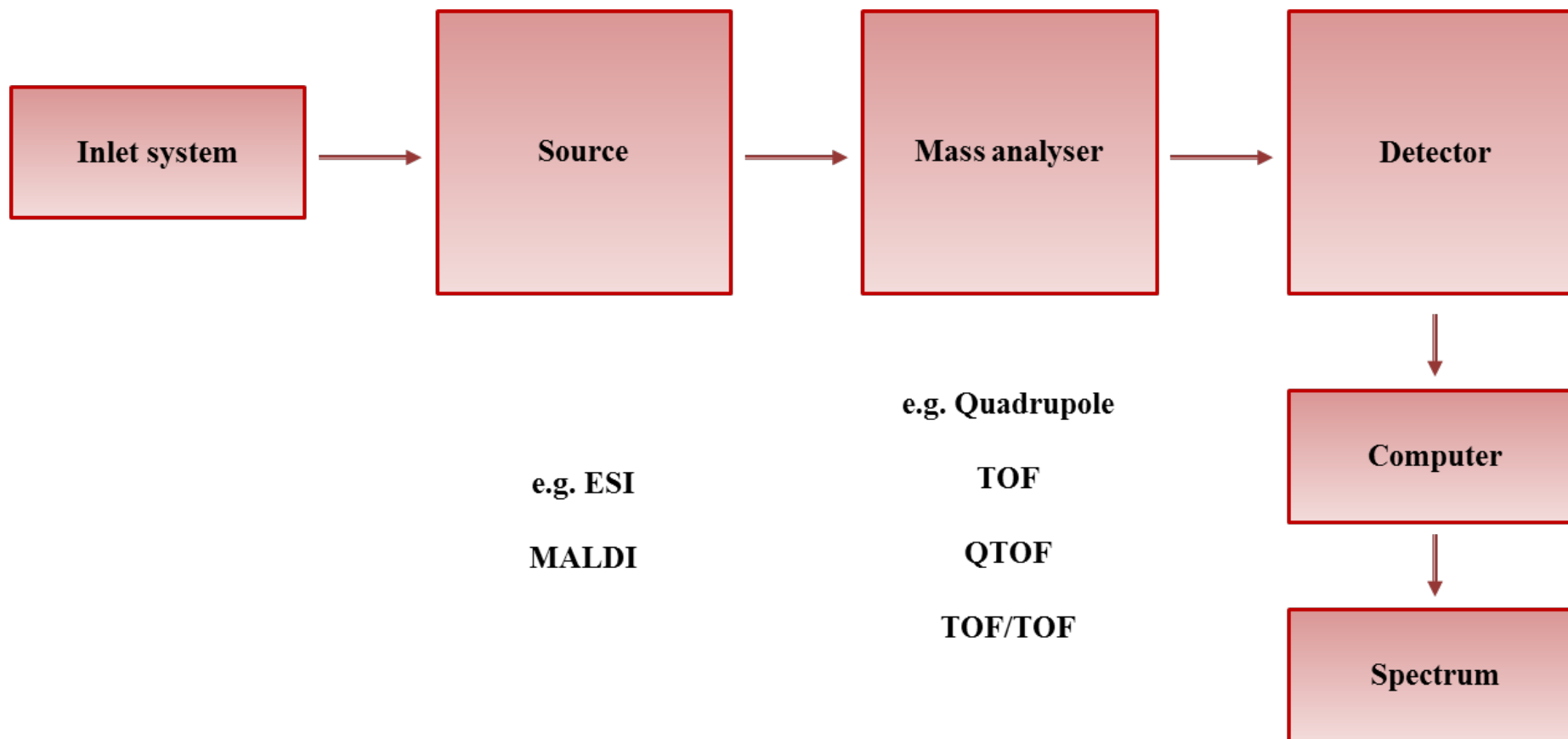


Figure 1.11: The basic features of a mass spectrometer. Samples are ionised in the ion source under vacuum or atmospheric pressure. Examples include Electrospray Ionisation (ESI) or Matrix Assisted Laser Desorption Ionisation (MALDI). Ions generated are then separated based on their mass-to-charge (m/z) ratio by the mass analyser, examples of which include quadrupole, ion trap and time of flight mass analysers. Detected ions are then used to produce a mass spectrum.

1.3.5 Tumour antigens and autoantibodies as biomarkers for cancer

It is well documented that immune responses against antigens produced by the tumour (tumour associated antigens; TAAs) occur in patients in cancer (Rauch and Gires, 2008). Autologous proteins expressed by tumour cells may be mutated, misfolded, over-expressed, aberrantly degraded or post-translationally modified in a way that renders them immunogenic (Forgber, *et al.*, 2009). TAAs that undergo post-translational modifications (e.g. oxidation, glycosylation) may generate neo-epitopes or enhance self-epitope presentation to the major histocompatibility complex of T-cell receptors, thus making them perceived as foreign by the immune system, consequently activating CD4⁺ T helper cells and plasma B cells (Spiotto *et al.*, 2003 and Spiotto *et al.*, 2002). Furthermore, proteins that are aberrantly localised during tumourigenesis may evoke a humoral immune response in the host. Suzuki *et al* (2005) showed that T-cell responses to cyclin B1 occurred when cyclin B1 was overexpressed and localised in the cytoplasm rather than the nucleus in breast, colon, pancreatic and lung cancer cells.

Although the mechanism by which TAAs induce a humoral immune response is not yet fully understood (as most of the TAAs identified so far have been intracellular proteins), one of the hypothesised mechanisms by which an immune response is generated to self-antigens postulates that immune responses are triggered to overexpressed TAAs (nearly 100 fold overexpression). Zinkernagel *et al.* (2001) suggests that the overexpression of self-antigens and/or the existence of neoantigenic peptides within autoantigens would overcome the antigen threshold required to mount an immune response. Consequently, the increased presentation of these neoantigenic epitopes and their continuous presentation to antigen presentation cells (e.g. dendritic cells, macrophages) leads to the activation of CD4⁺ T lymphocytes and the production of antibodies by plasma (B-cells) (Spiotto *et al.*, 2003 and Spiotto *et al.*, 2002). Furthermore, Winter *et al.* (1992) showed that tumours contained many different p53 mutations including frameshift mutations, stop codon and missense mutations, but it was the missense mutations with p53 overexpression and increased protein stability that was positively correlated with autoantibody production in lung cancer patients.

Another mechanism proposes that repeated exposure to modified intracellular proteins released in response to aberrant tumour cell death from defective apoptosis, ineffective clearance or necrosis leads to the production of a humoral immune response. Proteases released during cell death could also generate cryptic self-epitopes which could generate an autoimmune response (Tan *et al.*, 2009). For example, Le Naour *et al.* (2002) showed that Crt32, a truncated form of calreticulin elicits a humoral immune response in patients with hepatocellular carcinoma (HCC) compared to healthy controls and was found overexpressed in HCC tissues compared to healthy liver tissue, showing that TAAs can serve as potential biomarkers. It was also demonstrated that, although calreticulin induces a humoral response in autoimmune diseases such as systemic lupus erythematosus (SLE), the epitope recognised in HCC (C-terminal) is different to the one recognised in SLE patients (N-terminal), indicating that various mechanisms exist for autoantibody responses in autoimmunity and cancer (Le Naour *et al.*, 2002).

Furthermore, some TAAs have been shown to induce leukocyte migration, especially immature dendritic cells to damaged tissues by interacting with chemoattractant G-protein receptors in order to alert the immune system of danger signals from invaded and damaged tissues to facilitate tissue repair. The interaction with immature dendritic cells makes them immunogenic because they are liable to be sequestered and aberrantly presented to the cellular immune system (Oppenheim *et al.*, 2005).

In addition to TAAs, cancer patient sera contain autoantibodies to various aberrantly expressed cancer-related antigens involved with tumourigenesis (e.g. signal transduction, cell cycle progression, cell proliferation and apoptosis), making the serum antibody profiles for cancer patients and healthy controls vastly different (Mojtahedi *et al.*, 2011 and Zhang *et al.*, 2009). Thus, identifying TAAs and their corresponding autoantibodies may provide new insights into the interrelationship between the immune system and cancer and into the molecular pathology of tumour cells (Forgber *et al.*, 2009). As a result, identification of TAAs and autoantibodies, which serve as ‘reporters’ of tumourigenic processes will lead to earlier diagnosis, better prognosis and more efficient therapy for cancer (Mojtahedi *et al.*, 2011).

The ultimate utility of autoantibodies lies in the early detection of cancer. High titres of autoantibodies are identified in patients with early stage cancer and correlate with the progression of a malignant transformation, suggesting that the immune response to cancer occurs at an early stage during tumourigenesis and as such may serve as an indispensable biomarker for cancer. Furthermore, research has shown that autoantibodies can be detected in asymptomatic stages of cancer and in some cases, 5 years before the onset of disease (Fernandez 2005).

One of the advantages of the humoral response to cancer is that it is persistent. Although tumour antigens may only be transient in duration (due to short lived changes in tumours, transient shedding, rapid degradation or clearance) the corresponding antibody response is persistent and present in high concentrations (Imafuku *et al.*, 2004). In addition, even if the concentration of the TAAs is low (i.e. below the detection limit of the diagnostic assays or technology used to identify them), the nature of an autoantibody response to a single autoantigen is such that the autoantibody concentrations can be ‘amplified’ by the immune system, making it the autoantibody an easier biomarker target. Compared to TAAs which are subject to rapid degradation and clearance and are also rapidly secreted, this amplified response persists for months and years after the onset of disease, facilitating the detection of TAAs over a long period (Tan *et al.*, 2009).

Furthermore, obtaining serum samples from patients to determine the levels of an autoantibody response to a TAA present in the tumour tissue is less invasive than obtaining a biopsy. Antibodies are appealing as biomarkers because they have a stable structure, which do not commonly undergo proteolytic cleavage, simplifying sample handling. Finally, the biochemical properties of antibodies are well understood, making assay development easier. Since the primary antibody is available in the serum, only labelled species-specific secondary antibodies are required during the detection procedure with diagnostic assays (e.g. anti-human IgG), simplifying their commercial assay development (Tan *et al.*, 2009).

However, due to the complex nature of cancer, a single autoantibody assay lacks the sensitivity and specificity required for cancer screening such that a multi-marker panels of autoantibodies might be a more promising approach for early cancer detection (Werner *et*

al., 2014). Casiano *et al* (2006) suggests that autoantibodies to TAAs occur in between 10-30% of the patient population due to the heterogenic nature of cancer. Brichory *et al* (2001) has also shown that nearly 60% of lung adenocarcinoma patient sera have autoantibodies to annexin I or II (40% and 37% to respectively) with no positive autoantibody response in healthy controls, smokers or patients with non-malignant lung disease.

In addition, some TAAs occur in more than one tumour type making it difficult to discriminate between cancer subtypes using autoantibodies and TAAs. Autoantibody response is also notoriously induced in other autoimmune and non-malignant conditions, such as asthma, rheumatoid arthritis, autoimmune thyroid disease and type 1 diabetes, and sometimes in healthy patients, making it difficult to distinguish cancer from non-cancer using an autoantibody profile (Tan *et al.*, 2009).

Apart from its diagnostic utility, autoantibodies may also be used to selectively isolate and identify their cognate antigen protein. Target identification technologies which employ autoantibodies to identify potentially novel tumour antigens are of prime importance in immunoproteomics. One of the widely used approaches of TAA identification is the use of serum autoantibodies from cancer patients to immunoscreen for tumour antigens in patient tissue.

1.3.6 Serological screening methods for TAA identification

Robert W. Baldwin was the first to demonstrate the presence of an immune response to solid tumours. In the 1970's, Lloyd J. Old was the first to demonstrate the use of autoantibodies for the identification of cell surface antigens using autologous typing (Shiku *et al.*, 1977). Their method bound autologous serum antibodies to autologous tumour cell lines *in vitro* and the specificity of cancer reactivity was determined by antibody absorption analysis, to see whether pre-incubation of sera with other autologous or allogeneic cell lines would absorb out serum reactivity (Chen, 2012). After several rounds of autologous typing experiments, a panel of tumour antigens were identified including p53, the cell surface antigen on B-cell lymphomas, CD20 (Seliger and Gires, 2009).

In 1988, Boon *et al.* developed a method to identify TAA and TSA. They isolated cDNA libraries from tumours and recombinantly expressed them in bacteria before screening with tumour-specific CD8⁺ T cells. Their technique was modified by Falk *et al.* (1991) and Maldelboim *et al.* (1994). Over two decades later, due to the advances in molecular biology and genetic engineering, the value of using autoantibodies to identify and isolate TAAs is still an attractive one. The aim of this field of cancer biomarker discovery is to identify tumour relevant autoantibodies and their cognate TAAs and has been termed ‘cancer immunomics’ (Seliger and Gires, 2009 and Rauch and Gires, 2008). Currently, two proteome based methods can be used to serologically screen TAAs: PROTEOMEX and AMIDA.

1.3.6.1 PROTEOMEX

PROTEOMEX (an abbreviation of proteomics and SEREX; another serological screening method used to screen cDNA expression libraries in bacteria to identify and isolate TAAs) is also known as SERPA (Serological Proteomics Analysis) or SPEAR (Serological and Proteomic Evaluation of Antibody Response). For the sake of simplicity, the method will be referred to as PROTEOMEX throughout this thesis. It is the first TAA identification method that combines proteomics technology in order to separate proteins of interest and serological screening using serum antibodies (Rauch and Gires, 2008).

PROTEOMEX uses tumour cell lysates to screen for TAAs. Unlike SEREX, TAAs are screened in their ‘natural context’ using non-transformed tissues in their screening procedure, rather than after their recombinant expression in prokaryotes, which makes it possible for the post translational modification (PTM) of TAAs to be ‘considered’, which in some cases qualifies them as TAAs (Gunawardana and Diamandis 2007). These PTMs include sumoylation, acetylation, phosphorylation, glycosylation (Seliger and Gires, 2009).

Using PROTEOMEX, diseased and (adjacent) normal tissues are lysed and separated by 2-DE. IEF is commonly conducted in precast, immobilised IPG strips (18-24 cm) in pH ranging from 3-10 or 4-7, while SDS PAGE is run using large 24 x 24 cm polyacrylamide gels (figure 1.12). This separation of proteins over a large area allows a better resolution of TAAs compared to SDS PAGE alone (Gunawardana and Diamandis 2007). After

separation, proteins are transferred onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes before probing with cancer patient sera. TAA candidates are selected by comparing immunoblots of tumour and healthy tissue screened with cancer sera. Protein spots exclusively identified by cancer serum antibodies in tumour lysates are potential TAA candidates to be identified (Mojtahedi *et al.*, 2011). A replica gel which contains tumour samples is also run in parallel and stained with a MS compatible stain (usually Coomassie blue or silver) in order for proteins of interest to be isolated for MS/MS identification (Rauch and Gires, 2008). After their identification, the expression pattern of TAAs must be validated in a cohort of normal, pre-malignant and diseased tissues alongside the investigation of serum auto-reactivity in multiple patient sera.

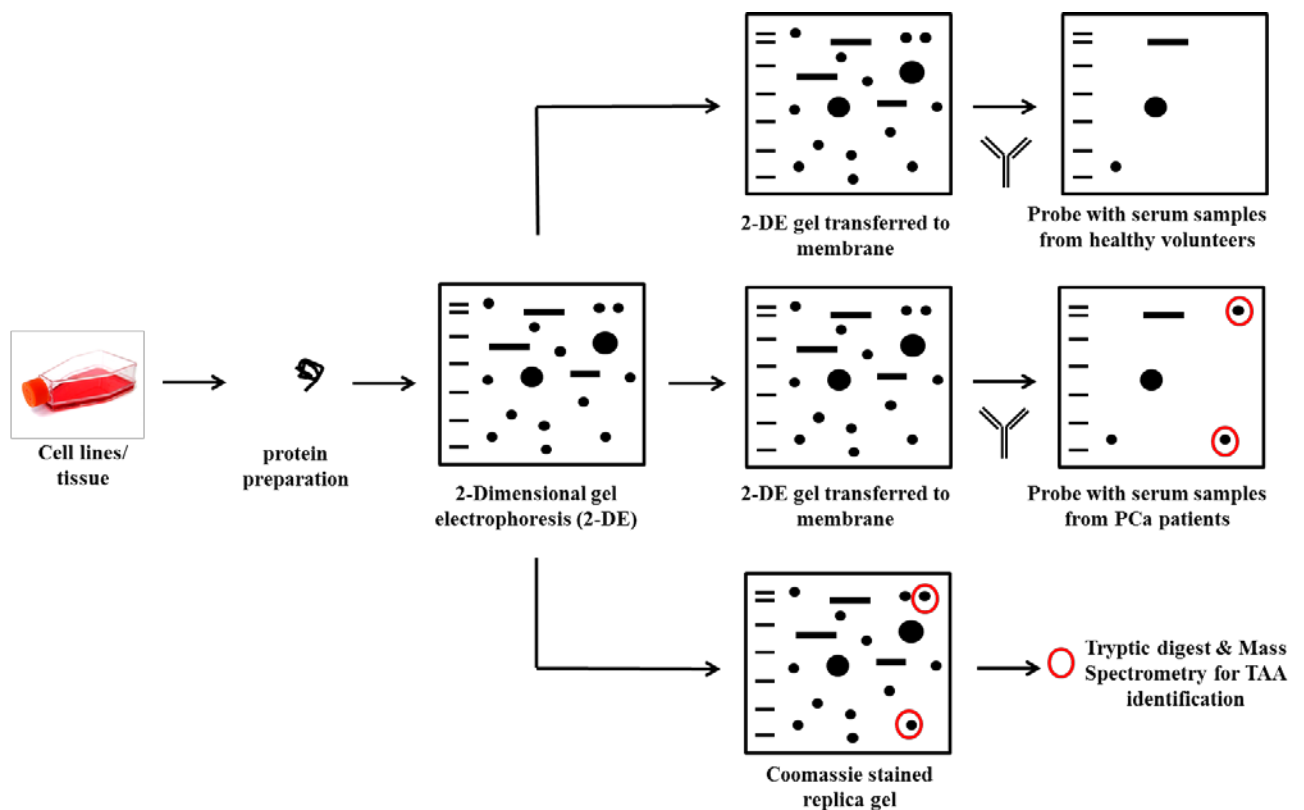


Figure 1.12: The PROTEOMEX methodology.

Protein lysates prepared from tumour cell lines or tissues (used as a source of TAA) are separated by 2-DE, transferred onto a membrane and screened either with patient or control sera. Proteins exclusively reacting with sera from PCa patients are identified from stained replica gels by mass spectrometry as potential TAA candidates.

PROTEOMEX is however a robust technique for TAA identification which has been successfully implemented in tumour antigen discovery for many cancers including the identification of UBL1 in renal cell carcinoma (Lichtenfels *et al.*, 2003) and RS/JD-1 in breast cancer (Le Naour *et al.*, 2011). PROTEOMEX has also identified TAAs in other cancers such as the metabolic enzyme superoxide dismutase in melanoma (Lichtenfels *et al.*, 2003) and the glycolytic enzyme glucose -6-phosphate-dehydrogenase (G6PD) in pancreatic adenocarcinoma (Tomaino *et al.*, 2007).

PROTEOMEX is not without its limitations. One of the main drawbacks with PROTEOMEX is the use of 2-DE for separation of tumour lysates. Without any pre-fractionation, 2-DE is limited to identifying abundant proteins due to its limitation in sensitivity and sample capacity (Gunawardana and Diamandis 2007). Furthermore, it is unable to separate proteins which co-migrate on polyacrylamide gels due to their post-translational modification, making the identification of spots difficult (Rauch and Gires, 2008). This problem is further compounded by the use of wide pH ranges for biomarker discovery studies. It is also notoriously challenging to separate cell membrane or very acidic or basic proteins using 2-DE due to their insoluble nature in aqueous buffers (Martin *et al.*, 2011). Finally, PROTEOMEX is a labour-intensive method due to the inherent lack of reproducibility of 2-DE gels and the accurate excision of immunoreactive spots from replica gels and identification by MS.

1.3.6.2 AMIDA

AMIDA stands for Autoantibody-Mediated IDentification of Antigens and was first described in 2004. AMIDA immunoprecipitates TAAs from tumour lysates using serum antibodies and these antibody-antigen complexes are further separated by 2-DE (Rauch and Gires, 2008). TAAs immunoprecipitated using patient sera are compared to those precipitated using healthy sera and selected protein spots are in-gel digested before analysis by MS. Like PROTEOMEX, gels are stained with a MS compatible stain, usually Coomassie blue or silver nitrate and protein spots exclusively immunoprecipitated out of tumour sera are identified as potential TAA candidates by MS (table 1.4). Additional controls can be introduced into AMIDA by omitting immunoglobulins during immunoprecipitation so that non-specific binding can be excluded from samples to be identified by MS (Seliger and Gires, 2009).

One of the advantages of using AMIDA is that immunoprecipitation using serum antibodies acts as an ‘immunological filter’ which isolates proteins that are assumed to be directly associated with cancer. In contrast, other proteomic and genomic methods have the added challenge of determining which of the recovered proteins are of importance in cancer. The original technique was performed in an autologous fashion where sera and cell lysates were obtained from the same donor. Problems associated with the heterogeneity of tumour tissue alongside the amount of protein required for the sufficient representation of low-abundant proteins on 2-DE limit the use of autologous tumour lysates for TAA identification. Thus, an allogeneic AMIDA method was developed by Rauch *et al.* in 2004 using tumour cell lines as a source of TAA in order to overcome the limitation of low number of proteins and allow for experiment replication. However, one main drawback of this method is that there is a biased protein expression in cultured cell lines compared to primary tumour cells, stating the need for a thorough validation of all TAAs identified using AMIDA.

Table 1.4: A comparison between PROTEOMEX and AMIDA.

Feature	PROTEOMEX	AMIDA
Protein source	Protein lysates from primary tumours or cell lines	Protein lysates from primary tumours or cell lines
Screened proteins	Proteins resolved by 2-DE (<3,000)	Whole lysate
Properties of TAA	Natural, PTM proteins, immobilised on membrane, urea/SDS denatured	Natural, PTM proteins, native conformation in solution
Detection	2-DE immunoblot using diluted sera	Immunoprecipitation (IP) with immobilised serum autoantibodies
Specificity control	2-DE immunoblot with healthy tissue, 2-DE immunoblot screened with healthy sera	IP using healthy tissue, IP using healthy donor immunoglobulins
Isolation and identification of TAAs	Comparison of immunoblot with 2-DE replica gel, tryptic digestion and MS	Tryptic digestion & MS
Sensitivity	Dependent on the number of copies per protein	Limited by stain sensitivity

Similarities and differences between PROTEOMEX and AMIDA which use proteome based approaches to identify TAAs using protein lysates. Adapted from Rauch And Gires (2008).

1.4 Study rationale and aims

Previous studies have demonstrated that TAAs and autoantibodies are present in the sera of cancer patients. PROTEOMEX, a method of TAA identification, has successfully been utilised in the discovery of TAAs for various cancers such as HSP-60 and RS/DJ-1 in breast cancer (Hamrita et al., 2008, Desmetz et al., 2008 and Le Naour et al., 2011), cytokeratin 8 and thioredoxin in renal cell carcinoma (Kellner et al., 2002 and Lichtenfels et al., 2003), eukaryotic elongation factor, alpha enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase and heterogeneous nuclear ribonucleoproteins A2B1 and galectin-3 in melanoma (Suzuki et al., 2010 and Forgber et al., 2009) and alpha enolase in pancreatic adenocarcinoma (Tomaino et al., 2007). Till date, no study has identified PCa TAAs using the PROTEOMEX methodology.

It is well known that urine obtained after a DRE may contain prostatic secretions and cells originating from the prostate tumour, which may serve as early PCa biomarkers (Downes *et al.*, 2006). Thus, an analysis of the urinary proteome of patients with PCa may yield potential diagnostic or prognostic biomarker targets.

Thus, the aim of this project was to utilise the PROTEOMEX technique to identify TAAs from urine and PCa cell lines that elicit autoantibody responses, which following additional validation may serve as potential PCa biomarkers. The objectives of this study are as follows:

1. Optimise a reproducible protein preparation method for urine and PCa cell lines analysis prior 2-DE.
2. Optimise conditions for PROTEOMEX and antigen discovery (e.g. sera dilution, serum titre, protein concentration).
3. Screen and identify TAAs associated with PCa using an optimised PROTEOMEX method.
4. Verify and validate all biomarkers identified in a separate cohort of samples from PCa, benign and normal patient samples, using Western blotting, ELISA and immunohistochemistry.

CHAPTER 2: IDENTIFICATION OF TUMOUR ANTIGENS IN URINE AS BIOMARKERS FOR PROSTATE CANCER

2.1 INTRODUCTION

Despite advances in medical research, PCa is still the most common form of cancer in males above 60 years (Johnson *et al.*, 2014). Current methods of PCa cancer diagnosis are limited in specificity and sensitivity - unable to accurately discriminate patients with a more favourable from a less favourable prognosis. In addition, PSA cannot distinguish aggressive from non-aggressive PCa, resulting in over diagnosis, unnecessary treatments and increased clinical costs (Dijkstra *et al.*, 2014). Consequently, there is a pressing need to identify more accurate biomarkers which can be used in addition to, or in place of the current PCa biomarkers.

Urine is an excellent source for biomarker discovery for PCa due to the proximity of the bladder to the prostate gland (Hessels and Schalken, 2013). During ejaculation, prostatic fluid combines with seminal vesicle derived fluid to promote the activation and function of the male sperm (Drake *et al.*, 2009). In patients who have PCa, the gentle massage of both sides of the prostate gland during DRE, stimulates the release of prostatic fluids and detached epithelial cells (expressed prostate secretions) into the urethra. As a result, urine obtained after DRE in patients with PCa may contain prostatic secretions and cells originating from the prostate tumour, which could serve as early biomarkers for PCa (Downes *et al.*, 2006) and figure 2.1. A summary of urine biomarkers for PCa can be found in table 1.3.

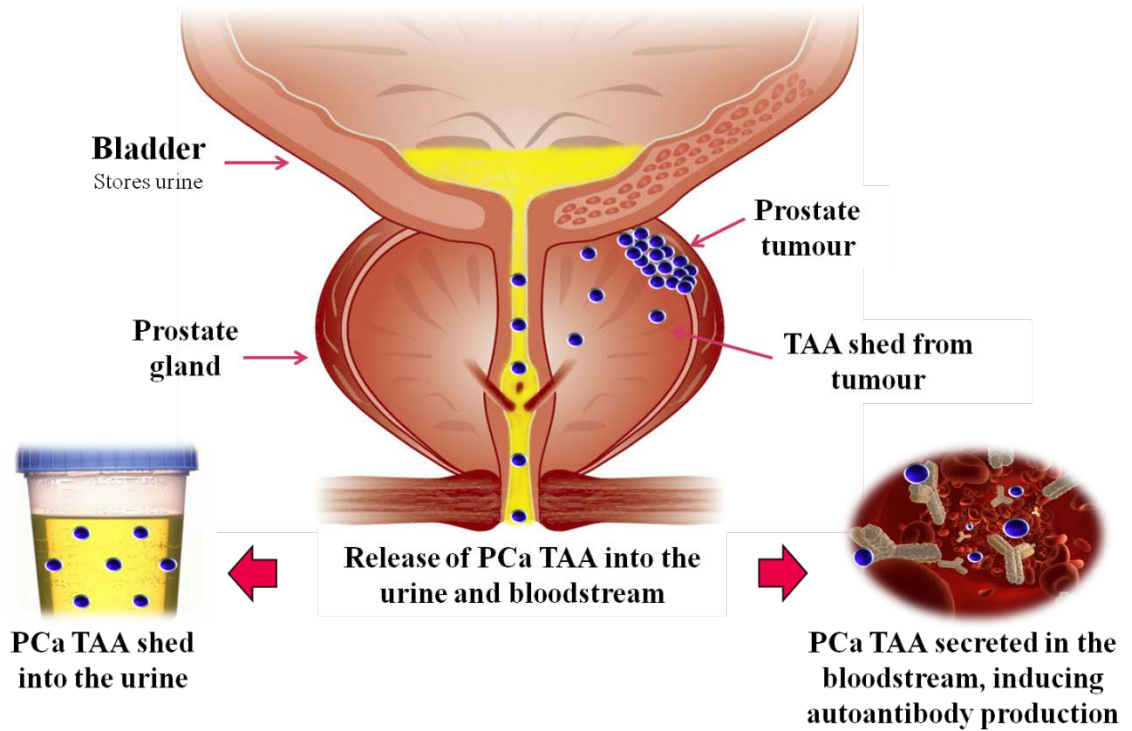


Figure 2.1: PCa TAA production and secretion into the urine and serum inducing autoantibody responses.

After a prostatic massage, expressed prostate secretions are released into the urine which is believed to contain prostate fluids and detached epithelial cells from the prostate tumour. Simultaneously, these TAAs are also released from the prostate tumour into the bloodstream where they induce an immune response, which results in the production of serum autoantibodies against the TAA. Image adapted from Hospital Vita (2015).

The analysis of urine for PCa biomarkers is not without its challenges – urine has a low overall protein content compared to serum (1000 fold lower than in plasma, typically <0.1 mg/mL), high intra- and inter-individual variability and contains high salt and cell debris which interfere with proteomic research (Kiprijanovska *et al.*, 2014). In spite of this, the use of proteomic technologies to identify potential urinary biomarkers for cancer in recent years has been on the rise. As a result, many attempts have been made to identify cancer biomarkers in urine for diagnostic or prognostic use due to the non-invasive nature and ease of collection of urine (from most patients) (Hessels and Schalken, 2013).

In 1979, the first proteomic profiling of urine was carried out on healthy controls by Anderson *et al.* (1979), in order to determine differences and similarities in 2-DE spot patterns of urine. Since then, many studies have been attempted to characterise the urinary

proteome, employing an array of techniques such as 1-DE SDS PAGE and 2-DE or LC coupled to high resolution mass spectrometry, facilitating the identification of between 1300 (Adachi *et al.*, 2006) to 1800 proteins in normal human urine (Marimuthu *et al.*, 2011). From these studies, it is evident that we do not yet have a comprehensive view of the urinary proteome. In addition, the identification of all proteins present in the urinary proteome will greatly aid the discovery of biomarkers for various cancers. A number of studies have been carried out in urine to identify biomarkers for many urological related disorders and malignancies such as diabetic nephropathy (Rossing *et al.*, 2008 and Sharma *et al.*, 2005), prostate (Okamoto *et al.*, 2009), colon (Ward *et al.*, 2008) and bladder cancers (Gkialas *et al.*, 2008).

To date, candidate PCa urine biomarkers identified include Engrailed-2 (Morgan *et al.*, 2011), semenogelin and uromodulin (M'Koma *et al.*, 2007), calgranulin/MRP-14 (Rehman *et al.*, 2004), annexin A3 (Schostak *et al.*, 2009), CD90 (True *et al.*, 2010) and inter-alpha-trypsin inhibitor heavy chain 4 (Jayapalan *et al.*, 2013). These proteins have been identified using various proteomic techniques in independent studies, where the utility of these markers are yet to be validated in a larger, independent sample cohort. In addition, PCa is a heterogeneous malignancy in that, from a diagnostic perspective, it is unlikely that a single marker will have enough specificity and sensitivity for accurate cancer detection (Davalieva *et al.*, 2015). Thus, it is well accepted that a panel of markers may be more likely to diagnose cancers (Coronell *et al.*, 2012). For example, O'Rourke *et al.* (2012) identified that a panel of autoantibodies consisting of TLN1, TARDBP, LEDGF, CALD1 and PARK7 were better able to distinguish PCa from BPH, increasing the sensitivity from 12.2% using PSA alone to 95% and 80% specificity for the panel. The aforementioned study thus highlights the necessity of carrying out more extensive analysis into well-defined samples to identify a reliable diagnostic PCa biomarker or biomarker panel.

In addition to urine proteins serving as biomarkers, the existence of neoantigenic peptides within a prostate tumour and the overexpression of TAAs, induces immune responses in patients, resulting in the production of specific autoantibodies (figure 2.1) and a selection of cytotoxic and T_h (T helper) cells (Rauch and Gires 2008). These autoantibodies have a high affinity and specificity for their cognate antigenic protein and are present even when the TAA concentration is low, making them more suitable for use as early cancer biomarkers (Massoner *et al.*, 2012). More recently, autoantibodies against alpha 2-HS

glycoprotein (Yi *et al.*, 2009), NY-ESO-1 (Fujiwara *et al.*, 2013), HSP60 (He *et al.*, 2007) and AMACR (Sreekumar *et al.*, 2004), have been identified in breast, gastric, colorectal and prostate cancer. Although many candidate markers have been identified, efforts are still made to discover new biomarkers for PCa. The identification of specific autoantibodies to tumour markers shed in the urine may aid in sensitive, early disease detection and therefore improved prognosis.

PROTEOMEX, a method of tumour antigen identification, which combines serological screening using serum autoantibodies with proteomic identification by mass spectrometry, has been successfully utilised in the discovery of many TAAs (Lichtenfels *et al.*, 2003; Le Naour *et al.*, 201 and Tomaino *et al.*, 2007 (section 1.3.7). In most of these studies however, the source of tumour antigens has been tumour tissue lysates and tumour cell line extracts (Rauch and Gires, 2008). To date, only one study by Yi *et al.* (2009) has screened sera from breast cancer patients for autoantibodies that may serve as biomarkers to urinary proteins. Due to the increased likelihood that urinary TAAs may be present in PCa, this chapter aimed to screen urinary proteins with autologous or allogeneic sera containing autoantibodies, for the identification of novel PCa associated TAAs. Autoantibodies reacting exclusively to proteins in urine are potential candidates which require further validation. To our knowledge, this is the first attempt to identify urinary TAAs in PCa using the PROTEOMEX approach.

2.1.1 Aims & objectives

The aim of this chapter is to identify urinary TAAs which can serve as potential PCa biomarkers following subsequent validation. This will be carried out by:

1. Confirming that urine, a sample readily obtained from most PCa patients, contains PCa associated TAAs that react with PCa associated serum autoantibodies.
2. Confirming that these PCa associated autoantibodies can be used to isolate their cognate antigenic protein using the PROTEOMEX method.
3. Developing and optimising reproducible protein preparation, separation and immunoblotting conditions for PROTEOMEX.
4. Identifying TAAs and autoantibodies associated with PCa using an optimised PROTEOMEX method.

2.2 METHODS

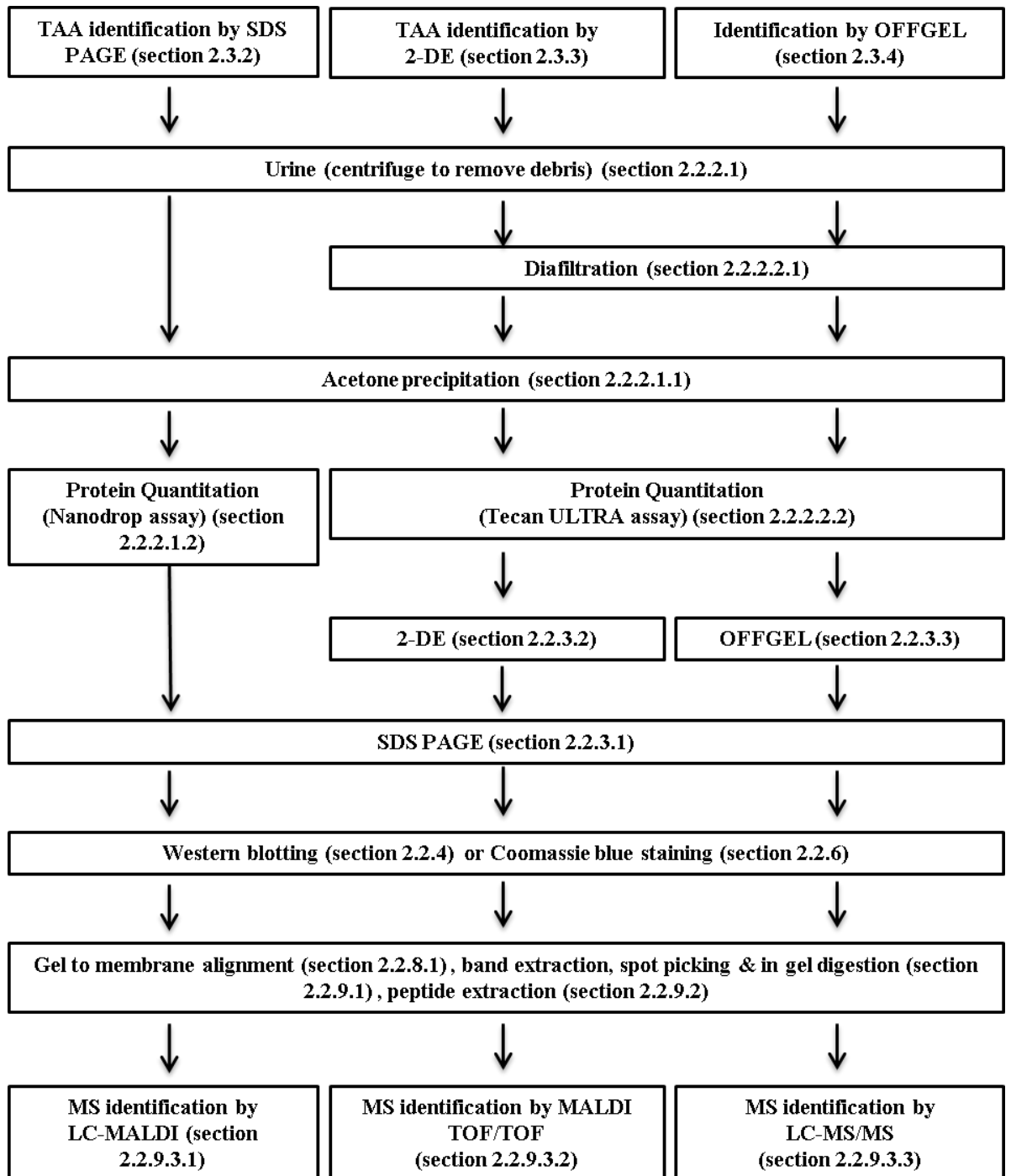


Figure 2.2: Method outline for urinary TAA identification.

A flowchart summary of the methods used for the identification of urinary TAAs in this chapter. All equipments used throughout this study are listed in appendix I.

2.2.1 Prostate cancer serum and urine

Matched serum and urine samples from PCa patients were collected and banked at the Urology Centre, Nottingham City Hospital, Nottingham, UK after obtaining ethical approval from the National Research Ethics Service Committee (NREC #09/H0S04/6). Males (n=26) aged 46-83 years, referred by their GP for high PSA levels (>4 ng/mL) and attending the urology clinic, were recruited after informed consent forms had been signed. Control serum and urine from matched patients were also provided by healthy volunteers (above 35 years) with no known history of PCa after ethical approval from Nottingham Trent University (Ethical number 165) and informed consent forms had been signed.

In each case, 4 yellow top Vacutainer (Becton Dickinson, Oxford, UK) tubes containing a silicone clotting activator were filled with whole blood from PCa patients or healthy volunteers. Whole blood was thoroughly mixed in the Vacutainer tubes by inverting the tube 5 times and serum was obtained by allowing whole blood to coagulate at room temperature for 30 min. Clotted blood was removed by centrifugation at 300 g for 15 min at room temperature and 1 mL of each serum sample was aliquoted into sterile 1.5 mL Eppendorf tubes using a Pasteur pipette. Each sample was assigned a unique identification number, sent to the laboratory on dry ice, stored at -80°C and thawed only prior to processing.

First flow urine from PCa and healthy donors was collected into sterile 25 mL tubes and aliquoted into 1 mL aliquots. Each sample was assigned a unique identification number, stored at -80°C and thawed only prior to processing.

2.2.2 Urinary protein isolation and quantitation

2.2.2.1 Urinary TAA identification in SDS PAGE

Prior to protein harvesting from urine, urine samples were centrifuged at 500 g for 3 min at 4°C, to remove debris.

2.2.2.1.1 Protein harvesting from urine: Acetone precipitation

Acetone precipitation of urinary proteins was performed in order to further concentrate and purify low volumes of urine samples which may still contain some contaminants such as salts, into a protein pellet which could be resuspended in a small volume of a solution compatible for downstream analysis.

Prior to acetone precipitation, 200 mL of HPLC grade acetone was left at -20°C overnight to ensure that the buffer was cold. 4 volumes of cold acetone were then mixed with 1 volume of ultrafiltrated urine sample, and vortexed for 20 s. The mixture was chilled at -80°C for 10 min and then at -20°C for 1 h. Precipitated proteins were isolated by centrifugation at 3000 g for 10 minutes at 4°C. The supernatants were carefully aspirated and the pellet was allowed to air dry for 20 min at room temperature. Protein pellets were resuspended in 40 µL of PBS before protein concentration determination using the mini-Bradford Assay.

2.2.2.1.2 Urinary protein quantitation: Nanodrop mini Bradford protein assay (TECAN Ultra)

Proteins from urine samples were quantified using a mini Bradford protein assay using the Nanodrop 8000 (Nanodrop 8000, Thermo Scientific). The mini Bradford assay has a linear range of 15-100 µg/mL making it ideal to quantify proteins in urine, which have a low protein concentration.

To generate a linear standard curve, a 1:1 protein standard to dye ratio was prepared by diluting the BSA standard of 2 mg/mL in ddH₂O to generate a serial dilution of protein standards of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.125 µg/mL and 0 µg/mL (blank). 10µL of dye reagent and 10 µL of sample or standard was added into PCR 96 well plates and vortexed before incubating for 5 min at room temperature for colour development. The Nanodrop 8000 was calibrated by pipetting a 2.5 µL of ddH₂O to initialise the Nanodrop machine. Standard curves and samples were then measured by pipetting 2.5 µL of the appropriate samples onto the sample pedestal before measuring absorbance at 595 nm. A blank sample was also assayed containing ddH₂O and dye reagent, which was subtracted from each sample to generate a corrected absorbance at 595

nm. Each sample and standard was assayed in triplicate to obtain an average absorbance reading. Only R^2 values greater than 0.98 were considered acceptable for protein estimation.

2.2.2.2 Urinary TAA identification in 2-DE & OFFGEL/SDS PAGE

2.2.2.2.1 Protein harvesting from urine: Diafiltrate columns

In order to isolate and concentrate proteins from urine, urine samples were processed using a combination of Vivaspin 20 protein concentrators and acetone precipitation. First, proteins from urine were isolated using Vivaspin 20 columns (2,000 MWCO, Sartorius Stedim, UK). Vivaspin concentrators use membrane ultrafiltration techniques and centrifugal force to concentrate and purify biological samples through a semi-permeable membrane. Proteins are concentrated using membrane pores which retain proteins greater than the molecular weight cut off (MWCO) of the concentrator, while water and salts pass through the membrane. As urine has a high salt (chloride 1.87 g/L; sodium 1.17 g/L), low protein (<0.1 mg/mL) and high water content (95% urine composition), ultrafiltration by membrane centrifugation was chosen to concentrate urinary proteins.

Typically, 10 mL of PBS was pipetted onto a Vivaspin 20 columns and centrifuged at 3000 g at 4°C until all the PBS had passed through the membrane in the permeate. Urine samples were diluted 1:4 in PBS and 20 mL of this solution was loaded onto the Vivaspin column and centrifuged as before until 200 μ L of urine was left in the column. This procedure was repeated until all the diluted urine samples had been filtered through the columns and the retentate was approximately 200 μ L. The retentate was then pipetted out of the membrane concentrator into 1.5 mL Eppendorf tubes for further concentration by acetone precipitation.

2.2.2.2.2 Urinary protein quantitation: Bradford protein assay

Protein concentration of cell lysates was performed using the Bio-Rad Bradford protein assay according to the manufacturer's instructions. This Coomassie based method is based on the colour change that occurs to proteins in the presence of the Coomassie dye. A 2

mg/mL BSA protein standard solution (Pierce, UK) was diluted to a 0.5 mg/mL working concentration and used to plot a standard curve with concentrations ranging from 0.05 - 0.5mg/mL (diluted according to table 2.1).

10 μ L of BSA standard solution or protein sample was added into a flat bottom 96 well plate and mixed with 200 μ L of Bio-Rad dye reagent (diluted 1 in 4 with ddH₂O). After a 5 min incubation at room temperature, the absorbance of the plates were measured at 570 nm using a Tecan Ultra spectrophotometer (Tecan Group Ltd, UK). The calibration curve constructed with the BSA standard curve was used to determine the concentration of the unknown protein samples. Each sample and standard was assayed in triplicate.

Table 2.1: BSA protein standards for Bio-Rad protein assay

BSA concentration (mg/mL)	ddH₂O (μL)	0.5 mg/mL BSA solution (μL)
0.5	0	40
0.4	8	32
0.3	16	24
0.2	24	16
0.1	32	8
0.5	38	2
0	40	0

A standard curve was plotted using the above dilutions of BSA and was used to determine the protein concentration of the unknown samples. R² values greater than 0.98 were considered acceptable for protein estimation.

2.2.3 Gel based protein separation

2.2.3.1 SDS PAGE

In all cases, 10% SDS PAGE gels were prepared as outlined in table 2.2 and cast using the mini gel system (Bio-Rad, UK). The solution was mixed in the order shown. First, 5 mL of the gel solution was poured into the casting frame and covered with a layer of 2-propanol to prevent dehydration and provide the gel with a flat surface. Gels were allowed to polymerise for 30 min at room temperature before the upper surface was rinsed with distilled water. 1 mL of the stacking gel solution was poured above the resolving gel and added with the 10 well comb (for 1-D SDS PAGE) or without combs (for 2-DE). Gels were left to polymerise for 30 min at room temperature. For 2-DE, a layer of isopropanol

was added to the top of the stacking gel to provide a flat surface for the IPG strips, instead of the addition of a 10 well comb.

Table 2.2: The composition of 10% SDS PAGE resolving and stacking gels

Solution	Resolving gel Volume (mL)	Stacking gel Volume (mL)
Distilled H₂O	6.0	2.44
Protogel 30% Acrylamide solution (cat: EC-890)	4.94	0.52
1.5 mM Tris-HCl 0.4% SDS pH 8.8 (cat: EC-892)	3.74	-
0.5 mM Tris-HCl 0.4% SDS pH 6.8 (cat: EC-893)	-	1.0
10% (w/v) ammonium persulphate (cat: EC-504)	0.150	0.02
TEMED (cat: EC-503)	0.015	0.004

All solutions are electrophoresis grade and purchased from Geneflow National Diagnostics, UK. This solution can be used to prepare 2 resolving gels (approximately 15 mL) and 2 stacking gels (approximately 3 mL) for the Bio-Rad mini gel system.

Glass plates were placed into the running tank containing 1x running buffer and the combs were removed. Protein lysates were prepared by mixing 20 µg of urinary proteins (or 50 µg of PCa cell lysate) with approximately 10-20 µL of sample reducing buffer, in a 3:1 volume ratio, making a final volume of 25 µL to load onto each well of the polyacrylamide gel. The solution was left to denature at 95°C for approximately 5 min, before loading into the wells of the polymerised SDS PAGE gels. 5 µL Precision Plus Protein molecular weight standards were also loaded into one well of each gel. To run the gels, gel tanks were filled with 1 L of 1 x running buffer (Geneflow National Diagnostics, UK) (diluted 1 in 10 as per manufacturer's instructions) and the sample was run for approximately 50 min at 200 V until the sample dye front reached the anodic side of the gel.

2.2.3.2 2-DE

To separate proteins by 2-DE, 20 µg of protein (urinary proteins or cell lysate) was mixed with sample rehydration buffer (Bio-Rad, UK) by aspirating and dispensing the solution 6 times using a pipette. The lysates were vortexed for 5 min and subsequently pipetted along the length of the mini IPG chamber (Bio-Rad, UK). One 7 cm 3-10L IPG strip (Bio-Rad, UK) per experiment was placed gel face down over the sample and allowed to passively

rehydrate for 1 h at room temperature. After the incubation, each strip was overlaid with 1.5 mL of mineral oil to prevent the strip from dehydration and urea crystallisation before placing it on the PROTEAN IEF cell chamber (Bio-Rad, UK).

Strips were actively hydrated for 13 h 40 min at 20°C at 50 μ A/strip using a PROTEAN IEF cell followed by focussing at 250 V for 20 min (linear), 4,000 V for 2 h (linear), 4,000 V for 10,000 V/h (rapid) and 500 V for 25 h (rapid). IPG strips were then transferred onto an equilibration tray where they were incubated for 15 min in equilibration buffer I (6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 50 mM Tris pH 8.8 in nanopure water and 2% DTT) (Bio-Rad, UK), and a further 15 min in the same buffer replacing DTT for iodoacetamide (2.5% w/v) (Bio-Rad, UK).

Next, the surface of the gels and IPG strips were rinsed in distilled water and 1 x running buffer respectively. 5 μ L of precision plus protein molecular weight standards (Bio-Rad, UK) was pipetted onto a 50 mm x 50 mm Whatmann filter paper (SLS, Nottingham UK) and left to dry for 5 min before inserting in between the glass plates (above the polymerised gels). 100 μ L of melted agarose (Bio-Rad, UK) was overlaid on the gels and the strips were inserted in between the glass plates. 300 μ L of the melted agarose solution was then added above the IPG strip and left to set for 10 min before being mounted onto the Bio-Rad mini gel running tank and filled with 1 x running buffer and run as outlined in section 2.2.3.1.

2.2.3.3 OFFGEL electrophoresis

Sample fractionation prior to protein identification by mass spectrometry is essential for biomarker discovery studies. OFFGEL electrophoresis utilises immobilised pH gradient based isoelectric focussing (IPG IEF) and recovers separated proteins in liquid phase. Fractionated proteins can be digested and analysed by LC/MS or separated by SDS PAGE for protein identification.

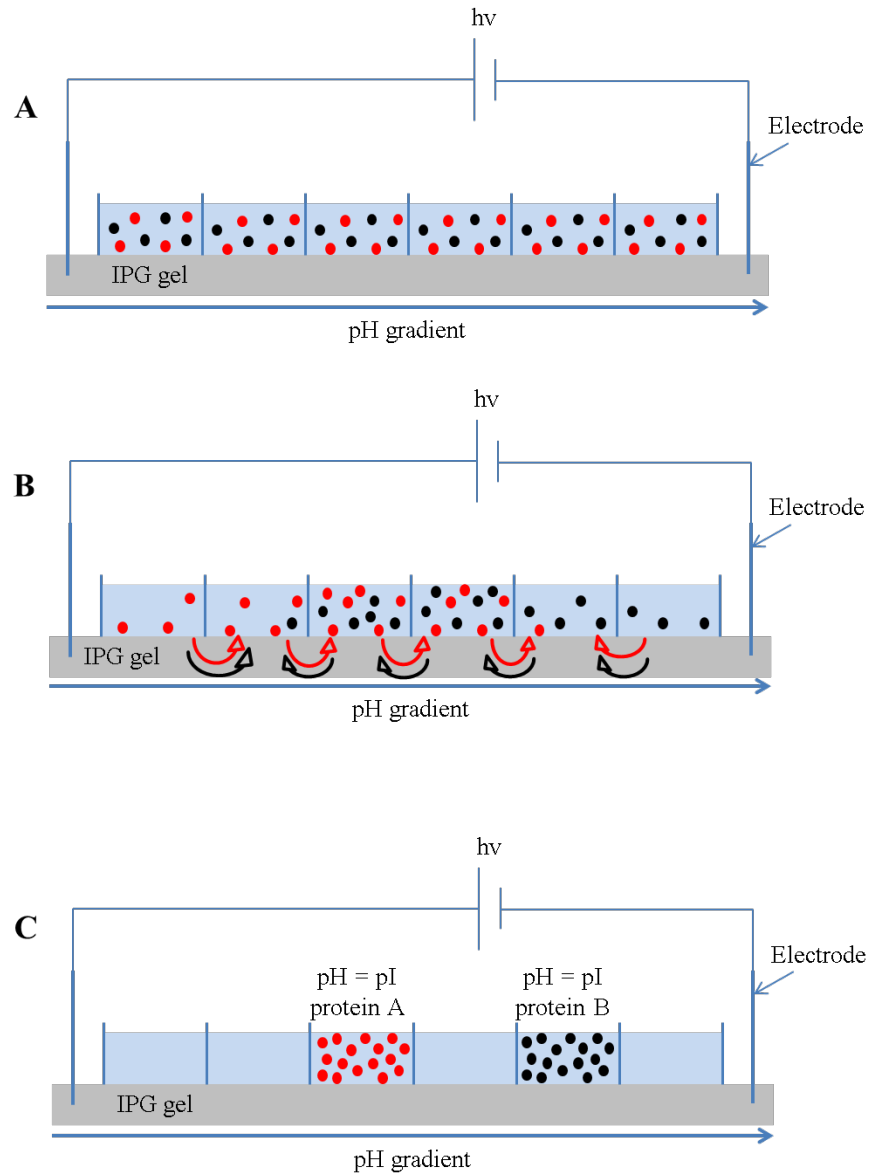


Figure 2.3: The principles of OFFGEL fractionation.

Samples are separated and retrieved in liquid phase, where high voltage is applied to proteins allowing them to migrate until they reach a point where the pH equals the pI of the molecule. hv; high voltage. A: At the start, micro-wells are filled with sample and diluted in buffer. B: during the run, peptides/proteins migrate until they reach their isoelectric point. C: after the run, separated proteins remain in solution and can be removed using a pipette.

OFFGEL fractionation was performed using an OFFGEL fractionator kit (Agilent Technologies cat: 5188-6425) according to the manufacturer's instructions with minor modifications (Agilent 3100 OFFGEL Fractionator, Agilent Technologies, Cheshire, UK). Typically, OFFGEL stock solution was prepared by mixing the urea, thiourea, DTT and OFFGEL buffers provided with 50 mL ddH₂O. This stock solution was used to prepare a

3-10 L IPG strip (Agilent Technologies cat: 5188-6431) rehydration and sample solution depending on the strip length as indicated below:

Table 2.3: Preparing an OFFGEL protein IPG strip rehydration solution

	24 cm strip	12 cm strip
Protein OFFGEL stock solution	0.96 mL	0.56 mL
ddH₂O	0.24 mL	0.14 mL
Total solution	1.2 mL	0.7 mL

The OFFGEL stock solution was mixed in ddH₂O to make a rehydration buffer for the IPG strips.

Table 2.4: Preparing an OFFGEL sample solution

	24 cm strip	12 cm strip
Protein OFFGEL stock solution	2.88 mL	1.44 mL
Sample	0.72 mL	0.36 mL

The OFFGEL stock solution was mixed in sample an OFFGEL sample solution to aid separation by IEF focussing.

For urine, 7 mg of protein (typically from 100 mL urine; or 5 mg from cell lysates) was prepared into pellets. Depending on the strip length, each pellet was resuspended in the appropriate volume of OFFGEL sample solution and left to vortex for 5 min at room temperature. Each 12 or 24 cm 3-10 (low resolution) IPG strip (Agilent Technologies, Cheshire, UK) was placed gel side up on an OFFGEL tray underneath a 12 or 24 well sample frame. 2 electrode pads were wet with rehydration solution and placed on each protruding end of the IPG strip. 40 µL of the rehydration solution was added into each well and the IPG strips were allowed to passively rehydrate for 15 min at room temperature. After the incubation, cover seals were applied onto the 12 or 24 well frames and 200 µL of mineral oil (Agilent Technologies, Cheshire, UK) was applied onto the anode ends of the gel and 400 µL to the cathode end (24 cm strip) or 200 µL and 200 µL (12 cm strip) to prevent the samples from drying up.

After a min incubation, 200 µL of mineral oil was applied to both anodic and cathodic ends (12 and 24 cm strip) and after 3 min, 200 µL of mineral oil was pipetted onto the anodic

end (24 cm strip only) before allowing the samples to run using a manufacturer's predefined method (table 2.5 below). The upper electrode pads were replaced with fresh pads wetted with deionised water every 24 h, and protein fractionation was performed until the samples had reached their isoelectric point; indicated by a flashing light on the OFFGEL fractionator.

Table 2.5: Method for performing a 24 fraction OFFGEL strip

	Volt Hours	Volts	Current	Power	Time
Focussing	64.0 kVh	4500 V	50 μ A	200 mW	100 h
Hold	-	500 V	20 μ A	50 mW	-

OFFGEL fractionation using high resolution protein separation (pH 3-10, 24 cm strip) was run using the manufacturer's default methods on the Agilent 3100 OFFGEL fractionator as described above.

Table 2.6: Method for performing a 12 fraction OFFGEL strip

	Volt Hours	Volts	Current	Power	Time
Focussing	20.0 kVh	4500 V	50 μ A	200 mW	100 h
Hold	-	500 V	20 μ A	50 mW	-

OFFGEL fractionation using low resolution protein separation (pH 3-10, 12 cm strip) was run using the manufacturer's default methods on the Agilent 3100 OFFGEL fractionator as described above.

After OFFGEL fractionation, the cover seals were removed and each liquid fraction was carefully removed from the chamber using a pipette and transferred into a 1.5 mL Eppendorf tube. Samples were stored at -20°C until their analysis by SDS PAGE (described in section 2.2.3.1).

2.2.4 Western blotting

Separated proteins were transferred electrophoretically onto a nitrocellulose membrane (Amersham, UK) using a wet transfer method as follows. For each gel, a 9 x 6 cm square of nitrocellulose membrane and four pieces of 9 x 6 cm filter cards (SLS, Nottingham UK) were pre-soaked in transfer buffer (Geneflow National Diagnostics, UK) before

assembling the transfer stack in the following order: transfer sponge pad, 2 x filter paper, polyacrylamide gel, nitrocellulose membrane, 2 x filter paper and transfer sponge pad (as shown in figure 2.3).

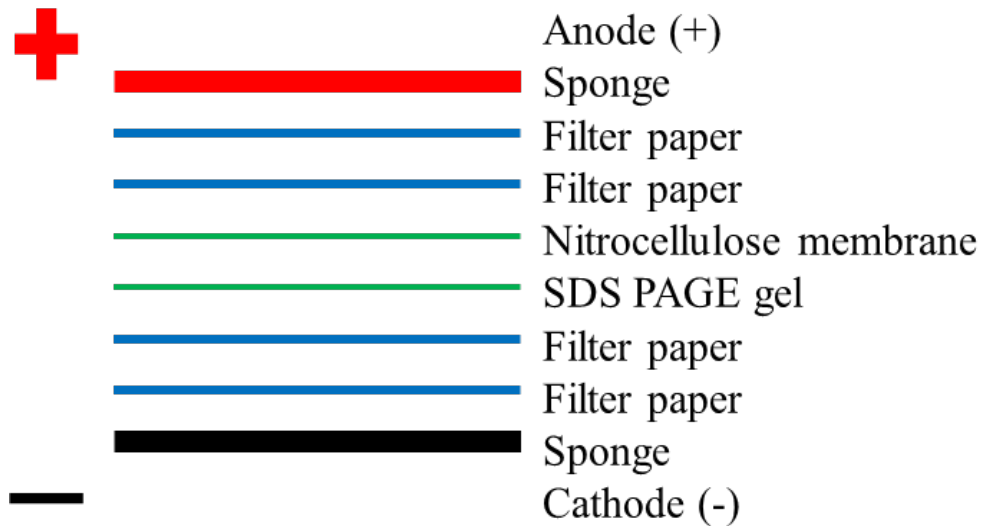


Figure 2.4: Assembling the transfer stack.

Western blots were arranged in the order shown with the nitrocellulose and SDS PAGE gel sandwiched in between 2 filter paper cards.

Before the transfer procedure, the transfer sponge was submerged in transfer buffer in an appropriately sized glass container and gels were removed from the running tank before rinsing once in distilled water to remove excess running buffer and then once in transfer buffer. The ‘*transfer sandwich*’ was assembled as described above and air bubbles were eliminated from the ‘*transfer sandwich*’ by rolling an empty glass pipette over the nitrocellulose membrane. The transfer sandwich was placed into the transfer tank with cold transfer buffer solution and transferred electrophoretically at a constant voltage of 100 V for 60 min. Following transfer, the blotting efficiency was assessed by staining the nitrocellulose membrane with a ponceau S solution prior to immunoprobng.

2.2.5 Ponceau S membrane staining

After proteins had been transferred onto nitrocellulose membranes, they were visualised using a Ponceau S solution (Sigma, UK). To do this, nitrocellulose membranes were rinsed once in distilled water to remove excess transfer buffer before placing them into a plastic

tray containing 10 mL of Ponceau S solution. Membranes were allowed to incubate with the stain at room temperature for 5 min on a rocker before rinsing 4 times with 10 mL of distilled water until excess background stain was removed.

To visualise the proteins, membranes were placed on a white laminated paper and visualised using a CCD camera (Fujifilm intelligent dark box, UK). After visualising stained bands or spots (1-DE or 2-DE respectively), each membrane was further destained by rinsing the membranes using distilled water until the bands were no longer visible.

2.2.6 Coomassie blue staining

In order to visualise stained proteins on polyacrylamide gels, gels were stained with a Coomassie brilliant blue solution (R-250, Phi-Bio, UK). First, polyacrylamide gels were removed from the Bio-Rad running tanks and rinsed once with distilled water to remove excess running buffer. Each gel was placed in an appropriately sized plastic tray and incubated with 10 mL Coomassie blue solution and left rocking on an orbital plate shaker (VWR, Leicestershire, UK) overnight at room temperature.

The following day, the stain was removed by pouring off excess Coomassie stain and the gel was rinsed 3 times in distilled water. Stained polyacrylamide gels were placed in a Coomassie destain solution and incubated again at room temperature for 1 h on a rocker. After this, gels were rinsed in distilled water and further incubated in fresh destaining solution. This procedure was repeated 3 times until the excess Coomassie blue had been eliminated and gel spots/bands were distinguishable from the background.

2.2.7 Immunoprobng

After proteins had been electrophoretically transferred onto nitrocellulose membranes, each membrane was incubated in a 3% blocking solution (3% Marvel milk powder in TBS 0.1% Tween-20, Premier Brands, UK) to minimise non-specific binding of antibodies. Membranes were blocked for 1 h at room temperature on an orbital plate shaker and the blocking solution was subsequently discarded. To probe membranes with antibodies or sera for biomarker discovery, each blocked membrane was incubated in a pre-optimised antibody concentration (as stated in table 2.4), prepared in a fresh blocking solution containing 3% milk in TBS-0.1% Tween solution and left shaking at 4°C overnight.

Table 2.7: Optimised dilutions of primary antibody solutions for biomarker discovery

Primary antibody	Antibody dilution	
	SDS PAGE	2-DE & OFFGEL
Patient sera	1:8	1:80
Monoclonal anti-alpha enolase antibody (L-27)	1:1000	1:1000

Antibody dilutions used in Western blotting for biomarker discovery using SDS PAGE, 2-DE & OFFGEL experiments. These dilutions were used for urinary proteins and PCa cell lines.

Following immunoprobings, nitrocellulose membranes were rinsed twice in a TBST wash solution (TBS-0.1% Tween-20) to remove excess antibody solution. After this, membranes were washed 5 times for 10 min each by incubating them in the wash solution whilst being agitated on a rocker at room temperature, to remove unbound primary antibodies. To detect primary antibodies bound to proteins of interest, each membrane was incubated for 1 h in a HRP conjugated secondary antibody solution (1:1000 Dako polyclonal goat anti-mouse IgG for alpha enolase, or Dako polyclonal 1:1000 rabbit anti-human IgG for human sera). A 1:5000 dilution of Precision Plus molecular weight reference standard (Bio-Rad, UK) was also added to detect binding of the molecular weight ladder. Immunoprobed membranes were rinsed and washed as before to remove unbound secondary antibodies.

After immunoprobings, nitrocellulose membranes were sprayed with a chemiluminescent substrate reagent spray (Calbiochem, UK) to detect the presence of horseradish peroxidase on probed immunoblots. Detection of chemiluminescent images was performed using a CCD camera (Fujifilm intelligent dark box, UK).

2.2.8 Gel to membrane alignment: initial attempts

Gel to membrane alignments were initially attempted using the Progenesis SameSpot software (Nonlinear Dynamics, Newcastle, UK), which automates the image alignment of 2D gels with any type of staining including DIGE and secondary staining. However, after numerous attempts, this software was unable to align an immunoprobed membrane to a stained gel. This meant that manual alignments of gel to membranes had to be performed.

2.2.8.1 Gel to membrane alignment: 2-DE

In order to match potential tumour antigen spots on nitrocellulose membranes to their corresponding spots on the Coomassie stained gels, probed membranes were stained with Ponceau S solution and images were taken using a CCD camera. Before spot comparison, it was ensured that the images of the probed membrane and the stained gels membrane were identical in size and that the molecular weight ladders on both the polyacrylamide gel and nitrocellulose membranes were aligned to each other. Following ladder alignment, the most intense 2-DE spots (marker spots) on the membrane were matched to their corresponding spots on the stained polyacrylamide gel by comparing the spot to the nearest molecular weight standards. To do this, the vertical distance (distance a, figure 2.4) from the spot of interest to the nearest molecular weight standard was calculated and recorded along with the horizontal distance (distance b, figure 2.4) from the spot of interest to the nearest molecular weight standard.

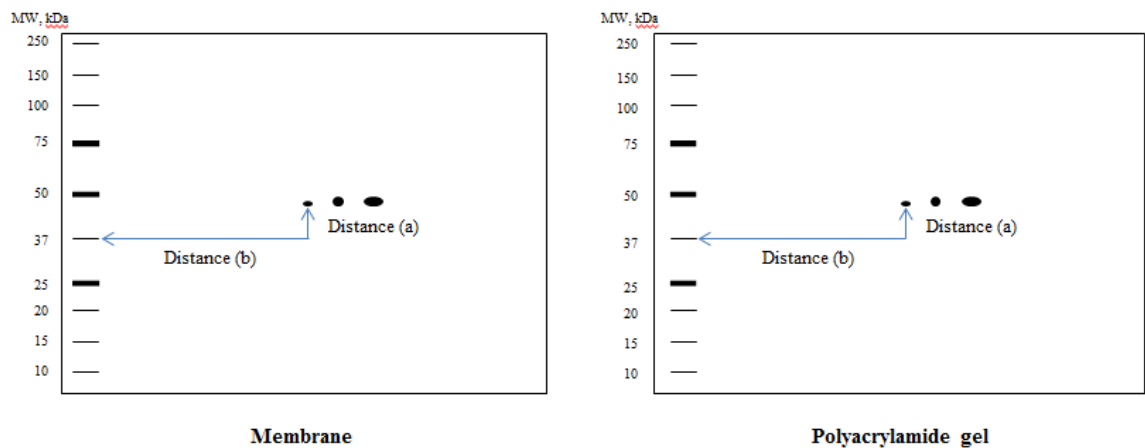


Figure 2.5: Gel to membrane alignment.

Alignment of 2-DE spots of interest on nitrocellulose membranes with polyacrylamide gels to facilitate TAA identification.

Following alignment, the spot pattern of the membrane and the Coomassie stained gel were matched. This was achieved by comparing the size and shape of the protein spots on the stained gel image to the corresponding spot on the membrane. The spots were only considered the same if:

- a.) The spot size and shape were similar in both the polyacrylamide gel and the stained membrane.
- b.) The antigen spot pattern surrounding the antigen spot of interest on the gel was matched to the spots on the blots.
- c.) The spot pattern of neighbouring spots resembled those on the immunoblots.

After gel staining, immunoprobng and gel to membrane alignment, spots of interest were assigned a unique identification number to aid identification by MS.

2.2.9 Identification of urinary TAAs

2.2.9.1 Band extraction, spot picking and in-gel digestion

After labelling spots of interest, polyacrylamide gels were rinsed twice in mass spectrometry grade water to remove excess Coomassie stain and a pipette tip was used to excise relevant spots for mass spectrometry. Each gel spot was cut into small 20 mm pieces using a sterile scalpel in order to increase the surface area for enzymatic digestion. Gel spots were placed in 1.5 mL Eppendorf tubes and stored at -20°C until analysis.

Upon analysis, gels were rinsed twice in mass spectrometry grade water and left agitating for 1 h at room temperature to eliminate excess protein stain. To further destain spots, 200 μL of 200 mM of NH_4HCO_3 was added to each gel spot and left to shake with the spots for a further 15 min at room temperature, and subsequently discarded. In a fume hood, a 50:50 ratio of 200 mM NH_4HCO_3 :100% ACN was also added to each Eppendorf to aid the destain procedure. The solution was left shaking on a rocker for 15 min and then heated for 2 min in a 37°C incubator, to enhance the destain process. Heated gel spots were left to shake for a further 15 min at room temperature and the NH_4HCO_3 :100% ACN mixture was then added to the previous sample and left shaking for a further 15 min.

To allow access of the digestive enzyme (trypsin) to the gel matrix, gel spots were subject to a series of hydration and dehydration steps. First, 100 μL 100% ACN was added to the Eppendorf tube which was left to shake for 2 min before a further 100 μL NH_4HCO_3 was added and left to shake for a further 5 min. The solution was discarded and a further 100 μL 100% ACN was added, to completely dehydrate the spots. As before, this was left to shake for 2 min, and then all the ACN was removed. To rehydrate the spots, 200 μL of

NH_4HCO_3 was added into Eppendorf tubes and left to shake for 5 min. The same volume of 100% ACN was added to the previous mixture and left shaking for 15 min. The liquid was discarded and 100% ACN was added to shrink the gel pieces, which was finally removed and gel spots were allowed to air dry in a fume hood.

To allow the identification of potential tumour antigens, enzymatic digestion of proteins into peptides was employed using the enzyme trypsin (MS grade trypsin, Promega, UK). Trypsin digests proteins by cleaving polypeptide chains at the C terminal end of Lysine (K) or Arginine (R) residues except when followed by proline (P). To digest the gel spots, 100 ng of trypsin diluted in 200 mM NH_4HCO_3 was added to the gel spots and left at 4°C for 2 h to allow the diffusion of trypsin into the gel matrix. Then, 10 μL of NH_4HCO_3 was added with an additional 6.25 ng of trypsin and then the solution was incubated at 37°C overnight (for a minimum of 15 h).

2.2.9.2 Peptide extraction, Zip-Tip C_{18} sample clean-up and sample spotting

After sample digestion, peptides were extracted from the gel matrix using several steps. First, 7 μL of 1% TFA was added to the digestion mix. The supernatant, containing most of the peptides was collected and left on ice and a further 3.5 μL of 0.1% TFA was added to the gel spots and vortexed. The supernatant was collected from the gel spots and mixed with the previous supernatant to increase the yield of peptides by approximately 5-10%.

Zip-Tip C_{18} pipette tips (Millipore, UK) contain a small bed of C_{18} chromatography media at their ends to purify and concentrate proteins and peptide samples before analysis by MALDI-TOF MS. Sample clean-up was performed using Zip-Tip C_{18} reverse phase chromatography columns according to the manufacturer's instructions. First, pipettes were set to aspirate and dispense 10 μL solutions and ZipTip columns were wet for 4 cycles in 80% ACN in 0.1% TFA to hydrate the columns. The columns were then equilibrated for 4 cycles in 0.1% TFA. For maximum binding of peptides, peptides from the gel spots were aspirated and the liquid was expelled back into the sample for 20 cycles before 4 wash cycles in 0.1% TFA. Peptides bound to the ZipTip columns were eluted by aspirating and dispensing the tips for 20 cycles into a solution of 80% ACN in 0.1% TFA. During the binding cycle, air bubbles were avoided by pipetting the solution slowly to maximise

binding. The pipette plunger was prevented from fully being released during the elution steps.

Eluted peptides were spotted onto a 384 MALDI ground steel target plate for analysis by mixing 1 μL of eluted peptides with the same volume of CHCA matrix (10 mg/mL). 1 μL of this mixture was spotted manually onto the MALDI target plate and allowed to air dry at room temperature until sample spots had crystallised on the target plate. For calibration spots, 1 μL of an external calibrant standard solution (Bruker peptide calibration mix II; Bruker Daltonics, Coventry, UK) was mixed with 9 μL of CHCA matrix and 1 μL of this mixture was spotted onto one spot of a ground steel target plate (Bruker Daltonics, Coventry, UK).

2.2.9.3 Mass spectrometry

2.2.9.3.1 Identification of 2-DE gel spots: MALDI TOF/TOF

Extracted and digested gel spots were analysed on an ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Coventry, UK) in positive reflectron mode. Matrix suppression was carried out in deflection mode, suppressing up to 650 Da at a sample rate of 4 GS/s. Data was collected over an m/z range of 800-4000 and calibration was performed using external standards of Bruker peptide calibration mix II (Bruker Daltonics, Coventry, UK). Monoisotopic peptide masses were recorded.

Using a flexAnalysis software (Bruker Daltonics, Coventry, UK), MS spectra were passed to the BioTools software (Bruker Daltonics, Coventry, UK) where a Mascot PMF search (local Mascot Server version 2.2.) was initialised. The BioTools search programme was used to search the NCBI database. Typical search parameters were as follows; species: *Homo sapiens*, database: SwissProt, Oct 2012, enzyme: trypsin, variable modifications of carbamidomethylation of cysteine residues and oxidation of methionine residues. MS mass tolerance of 100 ppm and partials (missed cleavages): 2.

Monoisotopic peptide peaks were selected in flexAnalysis and passed to flexControl software as precursor ions for MSMS. The MALDI-TOF instrument was set to positive LIFT mode and fragmentation data was collected for each selected peak. Data was then processed in flexAnalysis software to create an m/z and intensity list which was passed to

BioTools where a Mascot MSMS ion search was carried out with the following parameters: MS mass tolerance of 100 ppm and MSMS tolerance of 0.8 Da. All other parameters were the same as previously described.

2.3 RESULTS

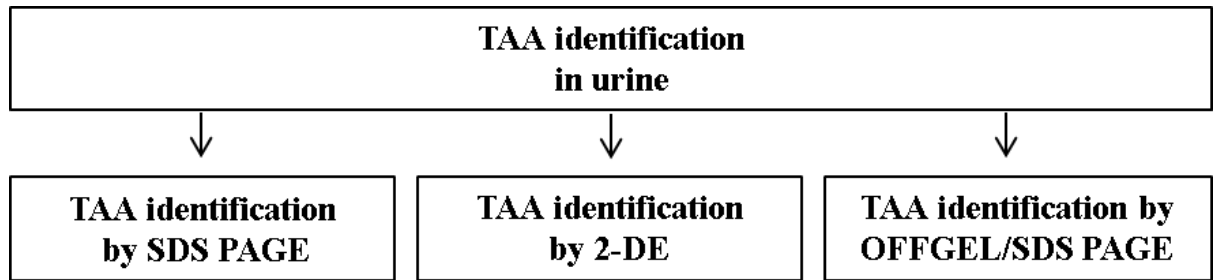


Figure 2.6: Overview of the identification of urinary TAAs.

The identification of urinary TAAs in this chapter is divided into 3 sections - identification using SDS PAGE, 2-DE and OFFGEL/SDS PAGE.

2.3.1 Selection of the appropriate urine samples

To date, no studies have demonstrated the presence of urinary TAAs in PCa using the PROTEOMEX method. However, this study was carried out with a hypothesis that TAAs are present in urine which can be isolated and identified using the PROTEOMEX technique. The hypothesis also proposed that urinary TAAs originating from the prostate tumour can be investigated as potential PCa biomarkers. To support these hypotheses, urine and matched serum from males attending the urology clinic at Nottingham City Hospital, were obtained prior to diagnosis. Although urine collected after a gentle prostatic massage is known to contain detached epithelial cells and prostatic secretions, making it an excellent biomarker source for PROTEOMEX, massaged patient urine was unavailable from this patient cohort. Urine samples from healthy volunteers at NTU were also collected, which served as a control.

As this was a pilot experiment to establish the presence of PCa specific autoreactivity to urinary TAAs, it was important to demonstrate that the PROTEOMEX technology could isolate and identify differential urinary TAAs in a small sample cohort. Immunoscreening would be carried out using a larger sample cohort if pilot studies were successful. As a result, a small number of patients were randomly selected from the samples available. Table 2.8 outlines the samples utilised for this study, consisting of PCa (n=3), benign (n=3) and healthy control cases (n=3).

Table 2.8: Urine samples used for TAA identification by SDS PAGE

Patient category	Sample no.	Age	PSA (ng/mL)	Gleason 1	Gleason 2	Gleason grade	D'Amico Risk	TNM staging
PCa	PC01	71	5.4	3	4	7	Intermediate	T2N0M0
PCa	PC02	69	7.9	3	4	7	Intermediate	T2cN0M0
PCa	PC03	65	178	4	5	9	High	T4N1M1b
Benign	BE01 ¹	56	4.9	3	4	7	Intermediate	-
Benign	BE02	62	8	-	-	Benign	-	-
Benign	BE03	67	23.2	-	-	Benign	-	-
Healthy	HE01	33	-	-	-	-	-	-
Healthy	HE02	36	-	-	-	-	-	-
Healthy	HE03	48	-	-	-	-	-	-

All urine samples were collected prior to any diagnosis. ¹ BE01 was initially diagnosed with BPH when samples were collected for this study. Subsequent examination of patient notes after study completion showed that BE01 was eventually diagnosed with Gleason 7 PCa.

Before studies were conducted, a series of optimisation experiments were performed to determine the optimum conditions for desalting and concentrating urine prior to PROTEOMEX, the optimum protein concentration to load on SDS PAGE gels, and the serum dilution that showed the lowest background after spot detection. Optimisation experiments were also performed to determine stains which could detect proteins on replica gels and facilitate protein identification by mass spectrometry. All subsequent experiments were carried out using these pre-optimised conditions.

2.3.2 TAA identification in urine by SDS PAGE

In pilot experiments, urine was separated using 1-DE SDS PAGE and screened with allogeneic sera to determine the presence of PCa autoantibody specific reactivity to urinary proteins. In brief, 200 μ L of urine was collected from each patient: PCa (n=3), benign (n=3) and healthy control subject (n=3) and pooled into 3 separate pools, to create a PCa, benign and healthy control pool. Each pool was acetone precipitated (section 2.2.2.1.1), and quantified using a BCA protein assay using a Nanodrop mini assay method (section 2.2.2.1.2). 20 μ g of total urinary protein from each pool was loaded in duplicate on 10% SDS PAGE gels, and run under denaturing conditions before transferring onto a nitrocellulose membrane. The transferred membrane was incubated with pooled allogeneic PCa sera (n=3) at a 1:8 dilution to detect the presence of PCa serum IgG based immunoreactivity to urinary proteins.

Figure 2.7A shows the immunoreactivities of PCa sera to allogeneic PCa, benign and healthy control urine, where most of the immunoreactivity to urinary proteins occurred in the 37-75 kDa molecular weight regions (highlighted in the red rectangle and enlarged in figure 2.7B). Figure 2.7A & 2.7B shows that pooled PCa sera detected multiple bands in allogeneic urine compared to reactivity in benign and healthy controls. An intense band was observed at 50 kDa, and this band was common to PCa, benign and healthy subjects. The 50 kDa band was thought to represent normal autoreactivity to PCa serum IgG, although this band was more intense in benign urine (figure 2.7A & 2.7B). Two fainter bands and a slightly more intense band were detected exclusively to allogeneic PCa urine at approximately 40, 44 and 48 kDa. At the same molecular weight (40–50 kDa) these bands were absent from benign and healthy urine (figure 2.7A and 2.7B).

This study demonstrated differential reactivity patterns of pooled PCa sera to proteins in urine, which may be potentially of interest in PCa. As a result, all immunoreactive bands were excised from Coomassie stained replica gels and stored for subsequent identification by mass spectrometry.

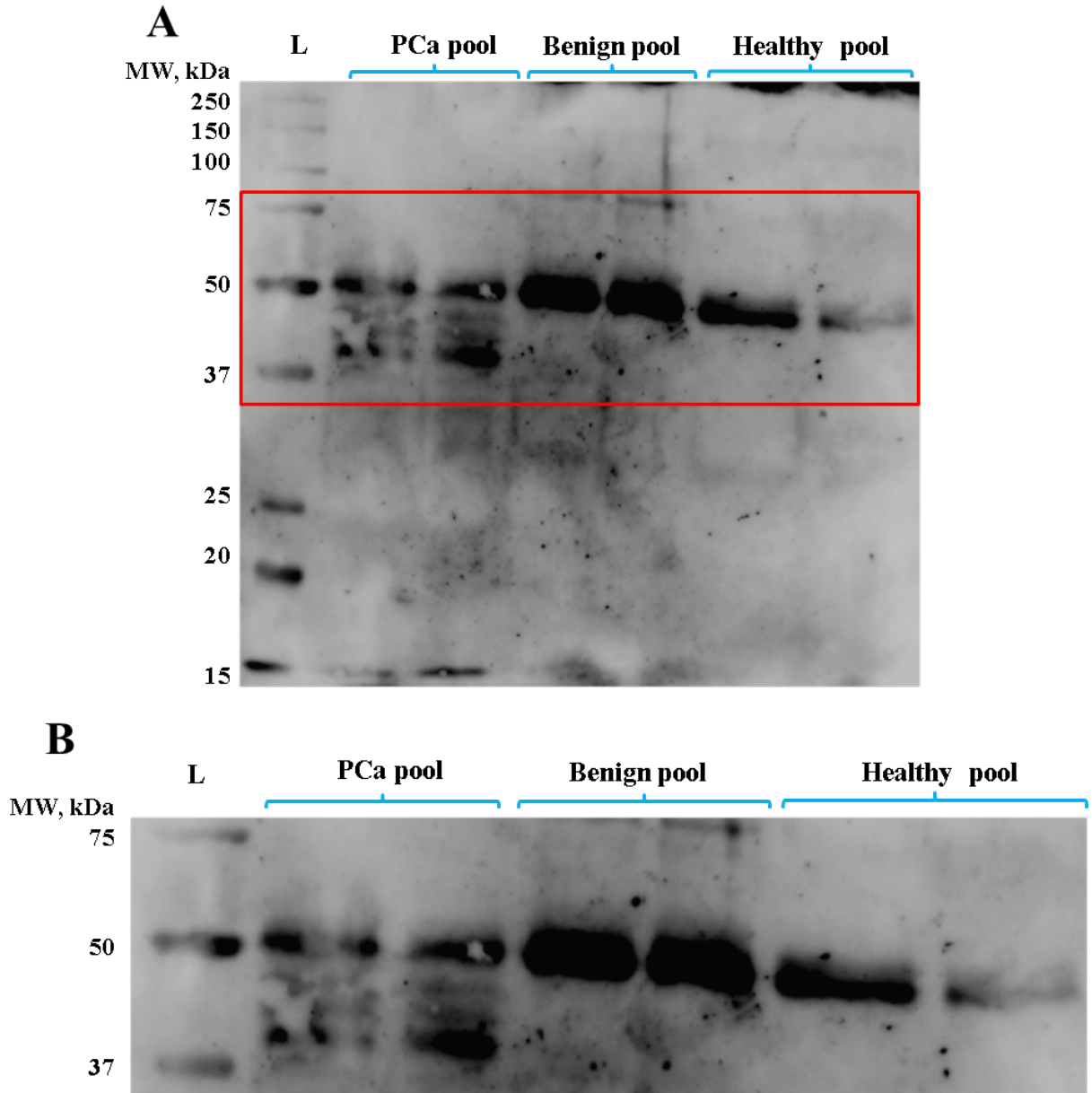


Figure 2.7: Western blotting showing the screening of PCa serum autoantibodies to urinary proteins by SDS PAGE.

Urine samples from 3 patients each with PCa, benign disease or healthy controls were pooled into 3 distinct pools – a PCa, benign or healthy pool and acetone precipitated. 20 μ g of total protein from each pool was loaded in duplicate on a 10% polyacrylamide gel and immunoscreened for PCa autoantibody specific reactivity to urinary proteins. A representative immunoblot is shown in (A), while a cropped blot showing the most immunoreactive region 37-75 kDa is depicted in (B). L; molecular weight ladder.

It became apparent after inspection of the patient database that the benign samples utilised for this investigation and some of the benign patients in our sample database were diagnosed '*benign with a possibility of cancer*'. This caused some confusion as to whether these samples were truly benign or at a pre-malignant phase. Consequently, these samples were omitted from subsequent studies, in order to make the patient categories more stringent.

2.3.2.1 Identification of frequency and specificity patterns in autoreactivity

PCa specific reactivity to urine proteins was observed in section 2.3.2, indicating that TAAs may be present in urine which can be utilised as potential biomarkers for PCa. Before immunoreactive bands were analysed by MALDI-TOF MS, it was important to determine the frequency and specificity patterns of serum autoantibody responses to urinary TAAs, as pilot experiments utilised pooled patient samples. To this end, two separate experiments were performed simultaneously.

First, urine from each PCa patient and healthy control was separated by 10% SDS PAGE and probed with pooled allogeneic PCa sera (n=3) (same samples selected in section 2.3.1) (figure 2.8A). Figure 2.8A, shows a band at 50 kDa, present in all PCa and control urine samples. As before, this band was thought to represent normal autoreactivity to PCa serum IgG, the 50 kDa band was more intense in PC03 urine (Gleason 9). Most of the bands identified in figure 2.8A occurred in PC02 urine (Gleason 7; 3+4), where 4 moderately intense bands were observed at 50, 47, 43 and 40 kDa, and a much fainter band at approximately 39 kDa. A moderately intense band showing immunoreactivity of pooled PCa sera occurred at 40 kDa in PC03 urine (Gleason 9), while a less intense band was also observed at 39 kDa in urine from the same patient. Similarly, a faint band at 40 kDa was identified in PC01 (band observed in the 1st lane).

Secondly, urine samples were separated as before, and probed with allogeneic PCa sera (figure 2.8B). Figure 2.8B shows that immunoblot 1 (PC01 urine screened with allogeneic PC01 sera) only detected bands at 50 kDa in both PCa and healthy urine, while immunoblot 2 (PC02 urine screened with PC02 sera) detected bands at 66 kDa and 50 kDa in both cancer and healthy urine. More intense bands were observed exclusively in PCa sera at approximately 46, 43 and 40 kDa, while a strong band was observed at 49 kDa

exclusively in healthy urine (HE02). Immunoblot 3 (PC03 urine screened with screened with PC03 sera) detected intense bands at 50 kDa in both cancer and control urine, moderately intense bands at 75 kDa and fainter bands at 42 and 40 kDa in PCa urine only. PC02 and PC03 in figure 2.7B display prominent bands in PCa urine that are not found in the healthy control urine or are much stronger than those of the control group. In figure 2.8, red arrows to the right of the immunoblots indicate bands that are detected in both PCa and healthy urine. Blue arrows indicate proteins in PCa urine reacting exclusively to allogeneic sera while green arrows indicate healthy urine proteins which immunoreact exclusively with PCa sera.

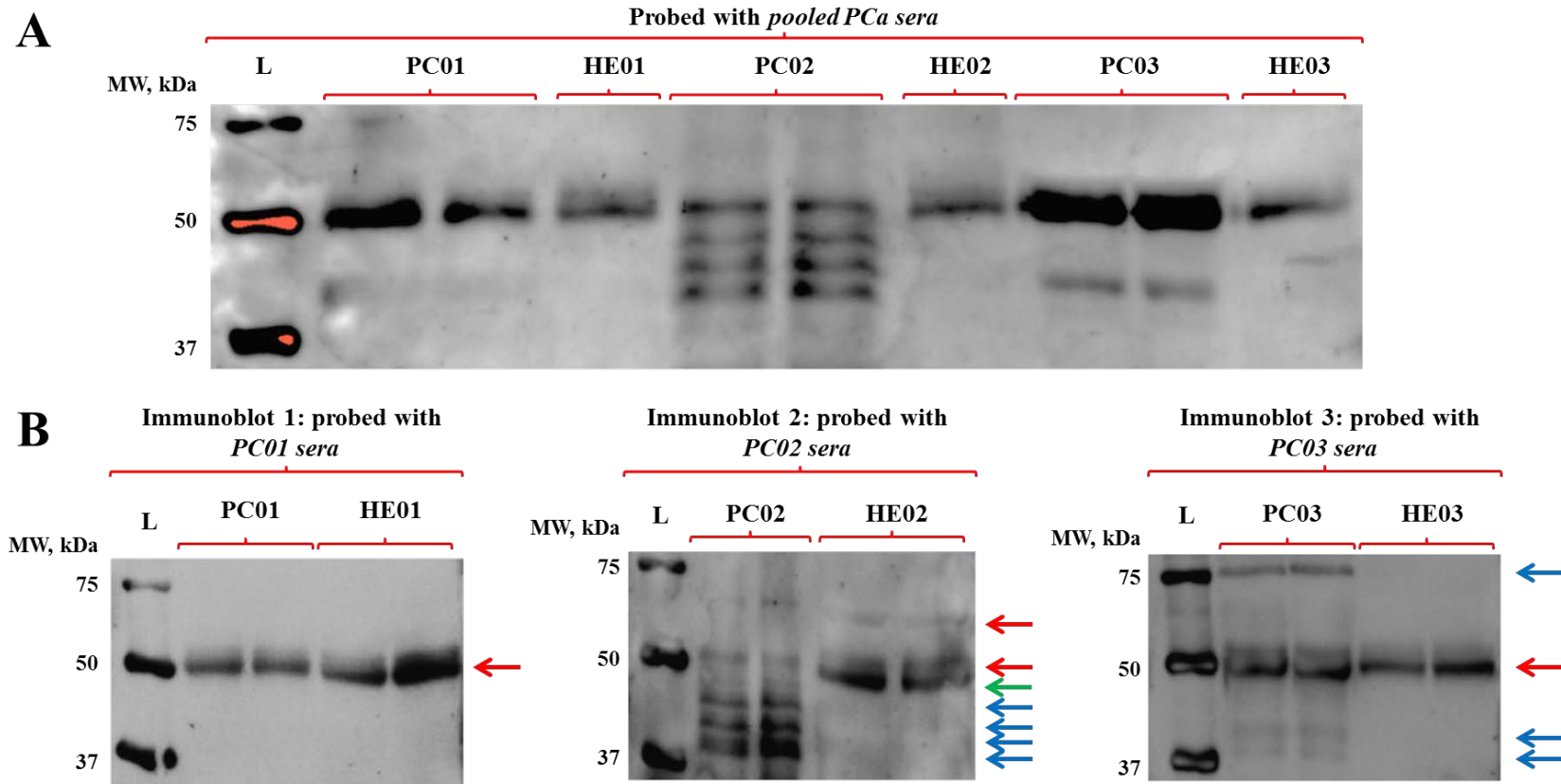


Figure 2.8: Western blotting showing the allogeneic PCa sera screening of urine proteins to identify patterns and frequencies in immunoreactivity.

Urinary proteins from each PCa and healthy subject was separated on 10% SDS PAGE gels and transferred onto nitrocellulose membranes, before probing with pooled PCa sera (1:8 dilution) (figure 2.8A). In figure 2.8B, urinary proteins were separated as before, and probed with allogeneic PCa sera (1:8 dilution). Red arrows on the right of the immunoblot indicate shared TAAs identified in PCa and healthy urine, green arrows indicate immunoreactivity exclusive to healthy urine, while blue arrows depict TAAs identified in PCa urine alone. A cropped blot at the most immunoreactive region (37 – 75 kDa) is shown for each Western blot.

2.3.2.2 Identification of TAAs that raise PCa autoantibody responses in urine by SDS PAGE

Section 2.3.2.1 documents the reactivity patterns obtained from the allogeneic combinations of PCa sera and urine for PROTEOMEX studies, highlighting the importance of screening patients individually after a pooled PROTEOMEX study. Figure 2.8B showed bands at 43 kDa and 40 kDa which were immunoreactive in PC02 and PC03 urine (both bands were more intense in PC02 than PC03). As these were the most frequently observed bands showing exclusive reactivity to PCa sera, they were selected first for identification by LC-MS/MS. The PC03 43 kDa band was excised from a Coomassie stained replica gel and subject to an overnight in-gel digest with Trypsin. Peptides were cleaned up before spotting on an anchorchip target plate for fractionation by LC MS/MS. The acquired PMF was searched against the SwissProt database using the Mascot search engine to identify potential TAA candidates. Table 2.9 shows a list of significant protein hits identified from this band.

Table 2.9: Urinary proteins identified from PC03 urine at 43 kDa

S/N	Protein	UniProt Accession	Score	MW [kDa]	pI	SC [%]	# Peptides
1	Prostatic acid phosphatase	PPAP_HUMAN	626.94	44.54	5.81	25.39	14
2	Ig gamma-1 chain C region *	IGHG1_HUMAN	577.32	36.08	9.36	21.82	5
3	Alpha-1-antitrypsin *	A1AT_HUMAN	515.04	46.71	5.27	23.92	5
4	Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	483.27	34.24	5.66	28.19	7
5	Ig gamma-4 chain C region *	IGHG4_HUMAN	460.01	35.92	7.8	21.71	5
6	Serum albumin *	ALBU_HUMAN	385.78	69.32	5.88	14.61	10
7	Ig gamma-2 chain C region *	IGHG2_HUMAN	373.95	35.88	8.8	11.04	5
8	Leucine-rich alpha-2-glycoprotein	A2GL_HUMAN	308.18	38.15	6.51	23.92	4
9	Vitamin D-binding protein	VTDB_HUMAN	278.07	52.93	5.28	12.03	4
10	Alpha-1-acid glycoprotein 1 *	A1AG1_HUMAN	221.3	23.5	4.79	25.37	4
11	Fibrinogen beta chain *	FIBB_HUMAN	217.52	55.89	9.34	11	3
12	Alpha-1-acid glycoprotein 2 *	A1AG2_HUMAN	199.99	23.59	4.89	11.94	3
13	Fibrinogen gamma chain *	FIBG_HUMAN	187.53	51.48	5.27	7.73	2
14	Monocyte differentiation antigen CD14	CD14_HUMAN	164.35	40.05	5.82	15.73	4
15	Haptoglobin *	HPT_HUMAN	128.61	45.18	6.13	12.07	3
16	Gelsolin	GELS_HUMAN	124.28	85.64	5.86	3.84	2
17	Complement C4-A *	CO4A_HUMAN	106.35	192.65	6.69	5.79	4
18	Protein AMBP	AMBP_HUMAN	95.21	38.97	5.91	3.41	1
19	EGF-containing fibulin-like extracellular matrix protein 1	FBLN3_HUMAN	78.65	54.60	4.81	4.67	1
20	Complement C3 *	CO3_HUMAN	78.05	187.03	5.98	4.57	4
21	Angiotensinogen	ANGT_HUMAN	66.53	53.12	5.86	7.01	2
22	Clusterin	CLUS_HUMAN	46.51	52.46	5.86	3.79	1
23	Ig heavy chain V-III region BRO *	HV305_HUMAN	39.01	13.22	7	15.83	1
24	Ankyrin repeat and SOCS box protein 18	ASB18_HUMAN	32.49	50.77	6.53	4.94	1
25	Keratin, type I cytoskeletal 9	K1C9_HUMAN	32.11	62.03	5	5.14	1

25 urinary proteins were identified from the immunoreactive 43 kDa band in PC03 urine. Protein hits are arranged in descending order of their Mascot ion score, where a Mascot ion score >30 = significant. Score; Mascot ion score. SC; sequence coverage, #peptides; number of peptides identified. Proteins in asterisks indicate the top 20 abundant serum proteins.

The aim of this chapter was to determine whether urine, a sample easily obtained from most PCa patients contained TAAs, which could be utilised as a source of biomarkers for PCa following additional investigation. Using PROTEOMEX by SDS PAGE, this study demonstrated that differential bands were exclusively present in PCa urine compared to healthy (and benign) controls, which warranted their identification by mass spectrometry. One of the differential bands subject to MALDI TOF MS yielded 25 significant protein identities, making it difficult to determine which of the proteins in the list was responsible for the immune response observed. Table 2.9 shows that 52% of the identified proteins are high abundant proteins in serum (Millioni *et al.*, 2011); and are likely to be present in urine due to the ultrafiltration of plasma proteins which occurs normally in urine, rather than due to a neoplastic process.

From the 43 kDa gel band, prostatic acid phosphatase was identified as the protein with the most identified peptides (14 peptides) and the protein with the highest Mascot ion score (score 626.24 where a score >30 is significant), indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$) and the protein with the 2nd highest peptide sequence coverage. In addition, the theoretical molecular weight of prostatic acid phosphatase is 44.5 kDa, close to the observed molecular weight of the immunoreactive band at 43 kDa in PC03. As a result, a conclusion was made that prostatic acid phosphatase was likely to be present in the 43 kDa band in PC03 urine. However, due to the limitation of the technology utilised in this study, it was not possible to determine whether or not prostatic acid phosphatase was responsible for eliciting the immune response in the patients studied. Zinc alpha-2-glycoprotein, a well-studied PCa biomarker was also identified as the protein with the third highest number of peptides and 4th most significant protein identified by MOWSE score (table 2.9).

2.3.2.3 Identification of the most significant protein hit (by Mascot score) as prostatic acid phosphatase

Prostatic acid phosphatase was identified from 14 peptides (10 of which were considered significant) with a MOWSE score of 626.94 (where a score of >30 is significant), indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$) (table 2.10). Figure 2.9A shows the complete protein sequence of human prostatic acid phosphatase where bold letters indicate all matched peptides by mass spectrometry. Tandem mass spectrometry of one of fourteen sequenced peptides of prostatic acid phosphatase, **LSGLHGQDLFGIWSK** is also shown in figure 2.9B, where the sequenced peptide has a Mascot ion score of 105 where a score of >30 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$) (figure 2.9C).

Table 2.10: Prostatic acid phosphatase peptides identified from PC03 urine by LC MS/MS

Protein	Mascot ion score	Sequence	Variable Modifications
Prostatic acid phosphatase	30.24	FVTLVFR	
	76.23	LHPYKDFIATLGK	
	105.08	LSGLHGQDLFGIWSK	
	62.51	RLHPYKDFIATLGK	
	90.93	LQGGVLVNEILNHMK	
	35.36	LQGGVLVNEILNHMK	Oxidation (M)
	83.08	LQGGVLVNEILNHMK	Oxidation (M)
	101.06	ELSELSLLSLYGIHK	
	36.96	LQGGVLVNEILNHMKR	
	27.85	LQGGVLVNEILNHMKR	Oxidation (M)
	108.73	LRELSELSLLSLYGIHK	
	1.69	SRLQGGVLVNEILNHMKR	Oxidation (M)
	13.51	ESSWPQGFGQLTQLGMEQHYELGEYIR	
	10.25	ESSWPQGFGQLTQLGMEQHYELGEYIR	Oxidation (M)

Using LC MS/MS, fourteen peptides were identified as prostatic acid phosphatase (where 10 were considered significant). The Mascot ion score for each sequenced prostatic acid phosphatase peptide is shown in table 2.10, alongside any variable modifications. Mascot ion scores >30 indicate identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

Prostatic acid phosphatase protein sequence: Matched peptides shown in **bold red**:

A

```

1   MRAAPLLLAR AASLSLGFLF LFFWLDRSV LAKELKFVTL VFRHGDRSPI
51  DTFPTDPIKE SSWPQGFQQL TQLGMEQHYE LGEYIRKRYR KFLNESYKHE
101 QVYQRSTDVD RTLMSAMTNL AALFPPEGVS IWNPILLWQP IPVHTVPLSE
151 DQLLYLPFRN CPRFQELESE TLKSEEFQKR LHPYKDFIAT LGKLSGLHGQ
201 DLEGIWSKVY DPLYCESVHN FTLPSWATED TMTKLRELSE LSLLSLYGIH
251 KQKEKSRLQG GVLVNEILNH MKRATQIPSY KKLIMYSAHD TTVSGLQMAL
301 DVYNGLLPPY ASCHLTELYF EKGEYFVEMY YRNETQHEPY PLMLPGCSPS
351 CPLERFAELV GPVIPQDWST ECMTTNSHQG TEDSTD
  
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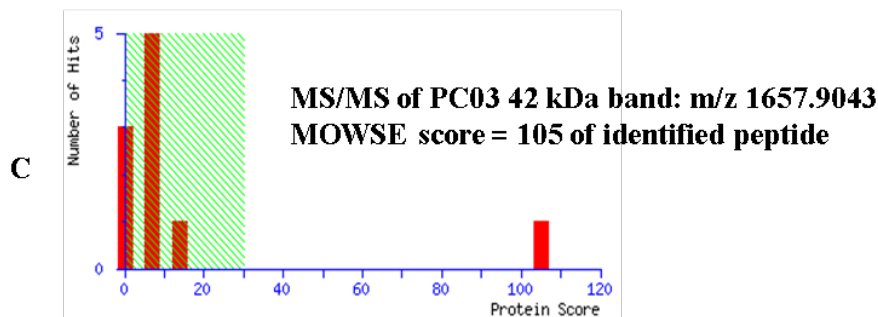
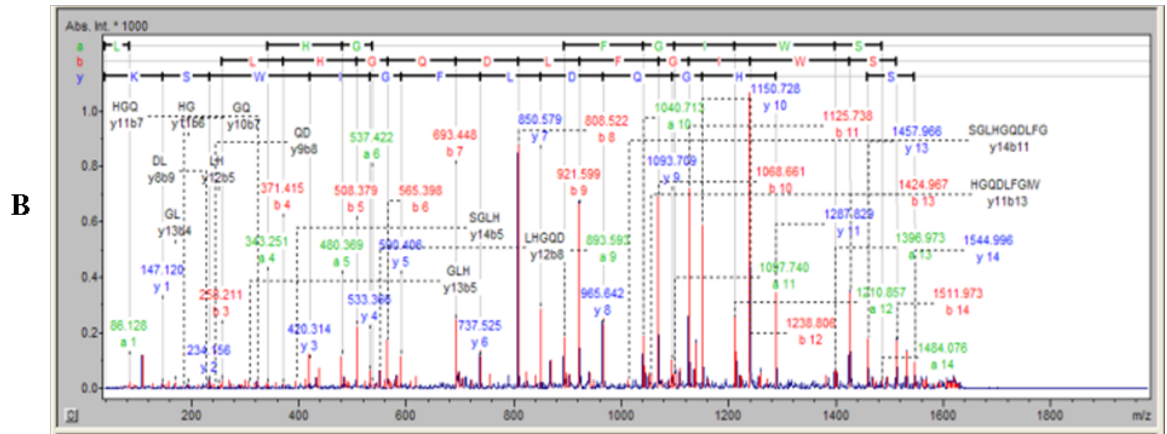


Figure 2.9: Identification of the immunoreactive 43 kDa band as prostatic acid phosphatase by LC MS/MS.

A band at 43 kDa showing exclusive immunoreactivity to PCa sera (patient 03) was excised from a Coomassie stained replica gel and subject to identification by LC MS/MS. 25 significant proteins were identified from this band where the most number of peptides and the highest MOWSE score was identified from prostatic acid phosphatase. Panel (A) shows the full length protein sequence of prostatic acid phosphatase where bold letters indicate all matched peptides by mass spectrometry, while (B) shows the identification for one of 14 sequenced peptides for prostatic acid phosphatase, **LSGLHGQDLFGIWSK** with an m/z 1657.9043. Panel (C) shows the Mascot ion score of **LSGLHGQDLFGIWSK** after matching the SwissProt database. The sequenced peptide has a MOWSE score of 105 where a score >30 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

This pilot study demonstrated that differential TAA candidates are present in urine, which immunoreact with autoantibodies in PCa sera and can be isolated and identified using the PROTEOMEX technique in combination with mass spectrometry. However, the study highlighted the difficulty in identifying TAAs responsible for immune responses using this crude separation technique (SDS PAGE combined with identification by LC MS/MS). As a result, no further samples were analysed or identified by mass spectrometry until more fractionation was carried out prior to TAA identification.

2.3.2.4 Verification of prostatic acid phosphatase expression in urine

As this was the first attempt at PROTEOMEX in our laboratory, it was important to verify the presence of prostatic acid phosphatase in the samples studied. To this end, 20 µg of urinary proteins were isolated from samples that made up the discovery pool (n=3 PCa; n=3 healthy controls) and separated using 10% SDS PAGE gels. Separated proteins were then immunoblotted with a mouse-anti-prostatic acid phosphatase antibody (PASE/4LJ: SC-52354, Santa Cruz, UK) by Western blotting.

Figure 2.10 demonstrates an intense band in just one sample (PC03) at 66 kDa while a moderately intense band was detected at 50 kDa. A similar expression pattern was observed in HE02 where low prostatic acid phosphatase expression was identified at 70 kDa and a similar expression 50 kDa. No prostatic acid phosphatase expression was found in other PCa urine samples (PC01 & PC02) or healthy controls (HE01 & HE03).

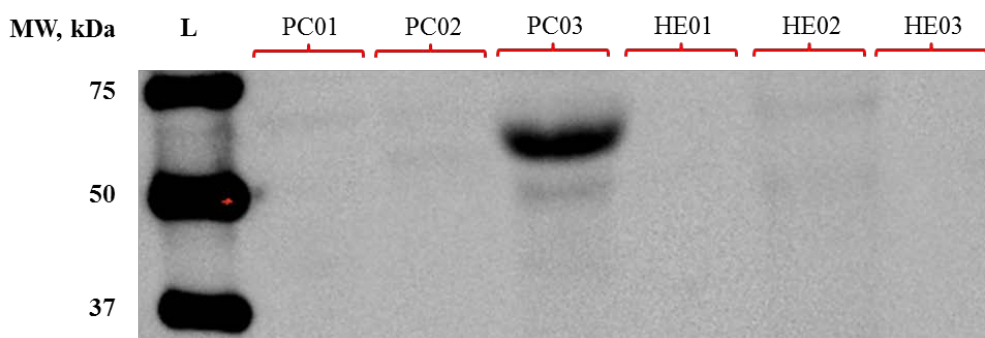


Figure 2.10: Verification of prostatic acid phosphatase expression in urine.

Urinary proteins from PCa patients and healthy controls were separated on 10% SDS PAGE gels and immunoblotted with a monoclonal mouse anti-prostatic acid phosphatase antibody (PASE/4LJ:SC-52354, Santa Cruz). An intense band at 66 kDa and a moderately intense band at 50 kDa was observed in one PCa patient (PC03). A similar expression

pattern was observed in HE02, where faint bands were detected at 70 kDa and 50 kDa respectively.

2.3.3 TAA antigen identification in urine by 2-DE

Section 2.3.2 demonstrates that direct identification of TAAs responsible for eliciting immune responses is not possible due to the complexity of protein mixtures which are present at every position in the lanes. This is further compounded by the difficulty in aligning Western blots to Coomassie stained SDS PAGE gels. As a result of the limitations of SDS PAGE, and because pilot studies showed promising results, the next phase of the study aimed to determine whether a fractionation step prior to SDS PAGE would offer the identification of more TAAs. The study therefore aimed to separate proteins from urine of patients with PCa and healthy controls by 2-dimensional electrophoresis (2-DE) using mini-gels with a pH gradient of 3 – 10. In theory, 2-DE would offer better separation of proteins, allowing a more accurate identification of TAAs that induce an immune response in PCa using PROTEOMEX and mass spectrometry.

2.3.3.1 Selection of clinical samples for TAA identification in urine by 2-DE

PCa urine and matched patient sera collected from Nottingham University Hospital and Nottingham Trent University were utilised for this investigation. At the time of the study, only 3 volunteers had been recruited for our investigation and thus were available for biomarker discovery. These control samples could not be age matched to cancer patients due to the limited number of volunteers available. In addition, due to sample volume restraints, the same patient samples used to screen urinary proteins by SDS PAGE (section 2.3.1) could not be used for 2-DE screening. Thus, urine from another set of patients recruited at the time of diagnosis, were chosen from the available samples collected. Patient samples utilised are outlined in table 2.11 - PCa n=26: Gleason 7 (3+4) (n=11), Gleason 7 (4+3) (n=10), Gleason 8 (n=2), Gleason 9 (n=3) and healthy controls (n=3).

Table 2.11: Patient samples used for TAA identification in urine by 2-DE

Patient category	Sample no.	Age	PSA (ng/mL)	Gleason 1	Gleason 2	Gleason grade	D'Amico Risk	TNM stage
PCa	PC04	56	4.9	3	4	7	Intermediate	-
PCa	PC05	65	8.9	3	4	7	Intermediate	-
PCa	PC06	71	5.4	3	4	7	Intermediate	T2N0M0
PCa	PC07	69	7.9	3	4	7	Intermediate	T2cN0M0
PCa	PC08	58	5.3	3	4	7	High	T2cN0M0
PCa	PC09	57	3.6	3	4	7	Intermediate	T2aN0M0
PCa	PC10	58	4.3	3	4	7	Intermediate	-
PCa	PC11	63	10.5	3	4	7	Intermediate	-
PCa	PC12	57	4.6	3	4	7	Intermediate	-
PCa	PC13	68	6.8	3	4	7	Intermediate	T2cN0M0
PCa	PC14	63	7.0	3	4	7	Intermediate	-
PCa	PC15	62	11.3	4	3	7	Intermediate	T2cN0M0
PCa	PC16	78	42.1	4	3	7	Mets	-
PCa	PC17	68	5.3	4	3	7	High	-
PCa	PC18	68	16.2	4	3	7	Intermediate	T3bN0M0
PCa	PC19	72	9.3	4	3	7	Intermediate	T2cN0M0
PCa	PC20 ¹	72	7.3	4	3	7	Intermediate	T1/2N0M0
PCa	PC21	64	4.4	4	3	7	Intermediate	T2N0M0
PCa	PC22	68	10.5	4	3	7	Intermediate	T2cN0M0
PCa	PC23	63	13	4	3	7	Intermediate	T1N0M0
PCa	PC24 ²	65	0.05	-	-	-	-	-
PCa	PC25	67	7	4	4	8	High	T2aN0M0
PCa	PC26	65	178	4	5	9	Mets	T4N1M1b
PCa	PC27	73	8.9	4	5	9	Mets	T3aN1M0
PCa	PC28	69	82.5	4	5	9	High	T3N0M1
PCa	PC29	68	10.4	4	4	8	High	T3a/bN0M0
Healthy	HE01	33	-	-	-	-	-	-
Healthy	HE02	36	-	-	-	-	-	-
Healthy	HE03	48	-	-	-	-	-	-

Urine samples were collected prior to any diagnosis. To identify autoantibodies to TAAs present in urine of PCa patients using 2-DE, patients from the following categories were selected at random and chosen for this study. A total of 26 PCa patients were chosen: Gleason 7 (3+4) (n=11), Gleason 7 (4+3) (n=10), Gleason 8 (n=2), Gleason 9 (n=3) and healthy controls (n=3). ¹; post biopsy haemorrhage, ²; prostatectomy sample.

2.3.3.2 PCa and healthy sera immunoreactivity to urinary proteins

To screen for TAAs, urinary proteins from a pool of PCa (n=26) or healthy (n=3) controls were prepared and separated by 2-DE as described in section 2.2.3.2. In brief, proteins were concentrated using molecular weight concentrators and acetone precipitated before protein quantification using a Bradford protein assay. 20 µg of pooled urinary proteins from PCa or healthy controls were separated by isoelectric focussing in the first dimension and SDS PAGE in the second dimension. Resolved proteins were visualised by Coomassie brilliant blue for spot picking or transferred onto nitrocellulose membranes for

immunoblotting. Membranes were incubated with a 1:80 dilution of pooled allogeneic PCa (n=26 patients) or healthy (n=3) sera as a source of primary antibody.

Sera from PCa patients reacted with numerous spots, some of which were observed in the control group and thus considered representative of non-specific reactivity (figure 2.11). Although a lot of spots were observed from immunoblots, this study aimed to isolate the most obvious spots for TAA identification. From both immunoblots, differences in 8 spots were observed and circled in red, where 3 out of 8 spots (37%) reacted exclusively with PCa sera (spots 6, 7 and 8). One out of 8 spots (circled in red) showed exclusive reactivity with healthy sera (spot 1), while 50% of the spots reacted with both PCa and healthy sera (spots 2, 3, 4 and 5; figure 2.11). Of the immunoreactive spots occurring in PCa and healthy controls, 3 out of 4 spots (75% of the spots) were more intense in PCa (spots 2, 3, 4), while spot 5 appeared more intense in healthy controls only (figure 2.11). Spots identified by both patient groups mainly occurred in the 40-100 kDa molecular weight range.

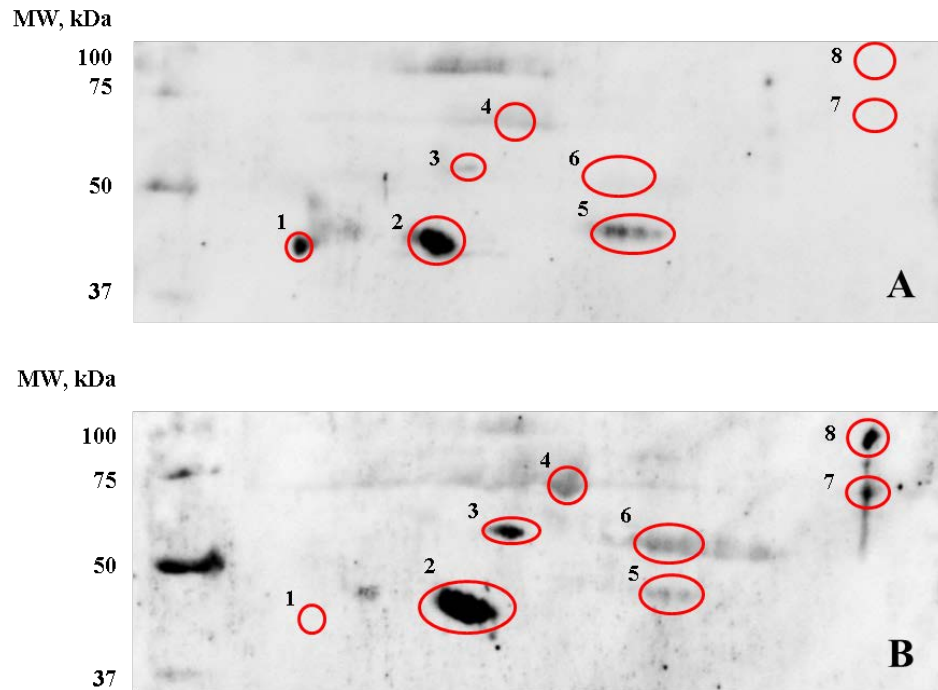


Figure 2.11: Screening PCa serum autoantibodies against 2-DE separated urinary proteins.

Urinary proteins from PCa (n=26) and healthy controls (n=3) were separated by 2-DE, transferred to nitrocellulose membranes and immunoblotted with sera from a pool of allogeneic PCa patients (Gleason 7, 8 and 9; n=26) or healthy controls (n=3). Immunoblots from healthy urine are shown in (A) and from PCa urine in (B) at the most immunoreactive region of the probed membrane (40-100 kDa). Circled spots indicate those chosen for mass spectrometry.

2.3.3.3 Identification of urinary TAAs that raise autoantibody responses in PCa

In order to identify immunoreactive TAAs, all circled spots were aligned to a Coomassie stained replica gel (figure 2.12) and corresponding spots were excised from the gel to allow identification by MALDI-TOF MS. Proteins were subject to an overnight in-gel tryptic digest (methods described in section 2.2.9.1) and peptides were cleaned up and spotted on a ground steel target plate. The acquired PMF was searched against the SwissProt database using the Mascot search engine to identify potential TAA candidates (search criteria described in section 2.2.9.3.1).

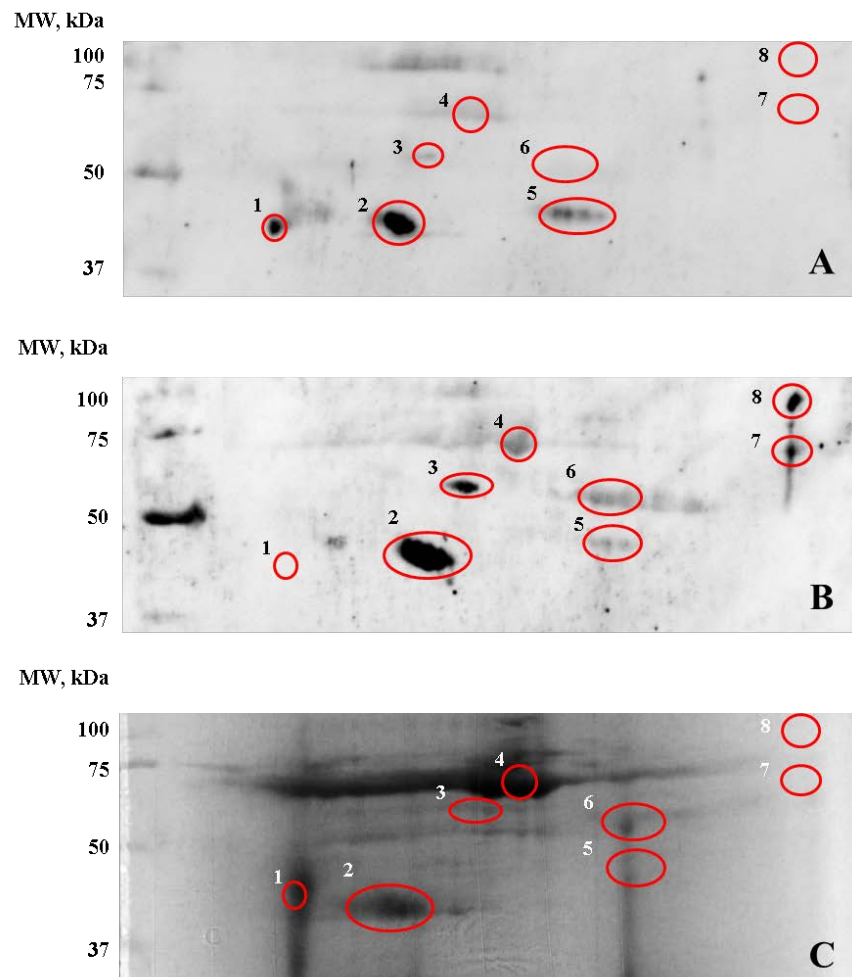


Figure 2.12: Gel to membrane alignment for TAA identification by 2-DE.

Eight protein spots from healthy (A) or PCa urine (B) immunoreactive with pooled allogeneic PCa or healthy sera were labelled as shown above and aligned to a Coomassie stained replica gel (C). Immunoblots and replica gel shown are cropped around the molecular weight where immunoreactivity occurred (40–100 kDa). Each spot was excised from the Coomassie stained gel to facilitate protein identification by MALDI TOF MS/MS.

Although 8 spots were isolated from the replica gel, only 6 of them were matched to proteins on the SwissProt database (table 2.12). Among these, spots 3, 4, 6 and 7 were identified as the same protein, serum albumin with high significance (MOWSE score 65, 119, 82 and 196 respectively; where a MOWSE score >56 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$) (table 2.12).

Table 2.12: Identified urinary TAAs from 2-DE immunoreactive to PCa or healthy sera

Spot no.	Protein	UniProt Accession	e. pI	t. pI	e. MW (kDa)	t. MW (kDa)	MOWSE Score	Immunoreactivity with sera
1	Alpha-1-acid glycoprotein 1	A1AG1_HUMAN	3.0	4.97	44	23.50	63/56	Healthy only
2	Zinc-alpha-2 glycoprotein	ZA2G_HUMAN	5.0	5.66	42	34.24	82/56	Healthy & PCa
3	Serum albumin*	ALBU_HUMAN	5.7	5.88	66	69.32	65/56	Healthy & PCa
4	Serum albumin*	ALBU_HUMAN	6.0	5.88	70	69.32	119/56	Healthy & PCa
5	No protein identified	-	-	-	-	-	-	Healthy & PCa
6	Serum albumin*	ALBU_HUMAN	7.0	5.88	55	69.32	82/56	PCa only
7	Serum albumin*	ALBU_HUMAN	9.0	5.88	73	69.32	196/56	PCa only
8	No protein identified	-	-	-	-	-	-	PCa only

Identified urinary proteins from 2-DE immunoblots immunoreactive with allogeneic PCa or healthy sera. Only 6 spots were identified out of 8 analysed by PMF using MALDI-TOF MS/MS. 63/56 = Mascot ion score of 63 where a score of >56 is considered significant. t; theoretical, e; experimental, pI; isoelectric point, MW; molecular weight. Proteins in asterisks indicate the most abundant protein in human serum.

2.3.3.4 Identification of the 42 kDa tumour antigen as zinc alpha-2-glycoprotein by mass spectrometry

The most obvious difference between PCa and control immunoblots occurred in spot 2, which separated at 42 kDa and an approximate isoelectric point of 5 from its migration distance by 2-DE. Using PMF, spot 2 was identified as zinc alpha 2 glycoprotein with a MOWSE score of 82 (where a MOWSE score of >56 is considered significant) (table 2.12). Although 6 peaks were identified as zinc-alpha-2 glycoprotein by PMF and chosen for sequence analysis, only 4 of them were matched to zinc-alpha-2 glycoprotein by MS/MS (table 2.13). Figure 2.13A shows the whole sequence of zinc-alpha-2 glycoprotein, where bold letters indicate all four matched peptides by mass spectrometry. B shows the tandem mass spectra of one out of 4 peaks sequenced (m/z 2304.2483). The peak at m/z 2304.2483 was identified as zinc-alpha-2 glycoprotein by the peptide sequence **HVEDVPAFQALGSLNDLQFFR**. Figure 2.13C shows that the fragmented peak yielded a MOWSE score of 208 where a score of >29 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

Zinc alpha-2 glycoprotein protein sequence: Matched peptides shown in **bold red**:

1 MVRMVPVLLS LLLLLGPAVP QENQDGRYSL TYIYTGLSKH **VEDVPAFQAL**
 51 **GSLNDLQFFR** YNSKDRKSQP MGLWRQVEGM EDWKQDSQLQ KAREDIEMET
 A 101 LKDIVEYYND SNGSHVLQGR FGCEIENRS SGAFWKYYYD GKDYIEFNKE
 151 IPAWVPFDPA AQITK**QKWEA** **EPVYVQRAKA** **YLEEECPATL** **RKYLKYSKNI**
 201 LDRQDPPSVV VTSHQAPGEK KKLKCLAYDF YPGKIDVHWT RAGEVQEPPEL
 251 RGDVLHNGNG TYQSWVVAV PPQDTAPYSC HVQHSSLAQP LVVPWEAS

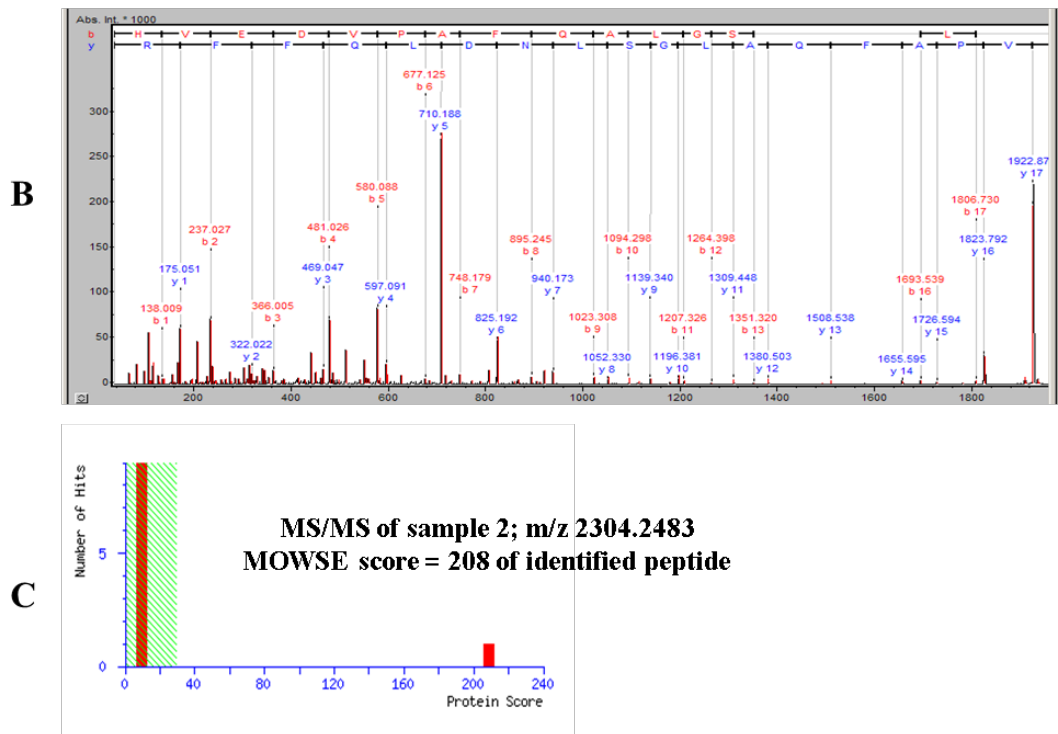


Figure 2.13: Identification of immunoreactive spot 2 as zinc-alpha-2 glycoprotein.

A single spot (spot 2) showing increased immunoreactivity in PCa sera was excised from a Coomassie stained replica gel and subject to identification by PMF using MALDI TOF MS/MS. Panel A shows the full length protein sequence of the only identified protein in this spot, zinc-alpha-2 glycoprotein, where bold letters indicate all 4 matched peptides by mass spectrometry. One of 4 peptides were chosen from PMF and subject to sequence analysis by MS/MS. Panel B shows fragmentation spectra of m/z 2304.2483 identified as zinc- α -2 glycoprotein by the peptide **HVEDVPAFQALGSLNDLQFFR**. Panel C shows the Mascot ion score of the peptide **HVEDVPAFQALGSLNDLQFFR** after matching the SwissProt database. The sequenced peptide has a MOWSE score of 208 where a score >29 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

Table 2.13: Zinc- α -2 glycoprotein peptides identified from PCa urine by MALDI-TOF MS/MS

Sample & protein	<i>m/z</i> peak	Mascot ion score	SC (%)	Peptide
Spot 2: Zinc-α-2 glycoprotein	2403.2483	208/29	7%	K.HVEDVPAFQALGSLNDLQFFR.Y
	1451.6976	53/30	4%	K.AYLEEECPATLR.K
	1532.8020	71/30	4%	K.QKWEAEPVYVQR.A
	1276.6438	59/29	3%	K.WEAEPVYVQR.A
	2779.8086	-	-	No significant protein hit
	804.2823	-	-	No significant protein hit

Six peaks identified as zinc- α -2 glycoprotein by PMF were subjected to MALDI-TOF MS/MS. Four out of six peaks were matched to the zinc- α -2 glycoprotein protein on the SwissProt database. The Mascot ion score for each *m/z* peak is shown as well as its corresponding sequenced peptide. SC; sequence coverage. 208/29; indicates a peptide with a MOWSE score of 208, where a score >29 is considered significant.

2.3.4 Tumour antigen identification in urine using a combined OFFGEL & SDS PAGE approach

In an attempt to identify additional TAAs in urine and combat the issues of low reproducibility of 2-DE, another pI based protein separation method was employed using OFFGEL fractionation. This method was employed to determine if another method of separating proteins prior to PROTOMEX would yield more TAA candidates.

2.3.4.1 ‘Proof of principle’ of the OFFGEL SDS PAGE approach in urine

As this was a ‘proof of principle’ experiment to determine if OFFGEL electrophoresis could be utilised for PROTEOMEX, it was necessary to carry out pilot experiments to determine if urinary proteins could be fractionated by OFFGEL electrophoresis. In brief, 100 mL of urine from a healthy male (HE03; table 2.8) was centrifuged to remove debris and concentrated using molecular weight cut-off columns (section 2.2.2.2.1). Urine was precipitated with acetone and quantified before mixing with an OFFGEL sample buffer and run as suggested by the manufacturer (pH 3-10, low resolution OFFGEL strips). Each fraction was separated using 10% SDS PAGE gels and transferred onto nitrocellulose membranes before immunoprobng with a 1:1000 dilution of mouse monoclonal anti-human alpha enolase antibody (L-27, Santa Cruz, UK).

Figure 2.14 shows intense bands at 47 kDa in fraction 8 while weaker expression was identified in fraction 6, 7, 9, 10 and 11. Strong expression of alpha enolase was also detected at approximately 66 kDa. Efficient OFFGEL fractionation had also been demonstrated using the metastatic PCa cell line, DU-145 (characteristics of this cell line is outlined in table 3.1), which was utilised as a control against OFFGEL fractionation in urine. The same pattern of expression for alpha enolase was observed in DU-145 using a monoclonal mouse anti alpha enolase antibody, where alpha enolase was only detected in fractions 7, 8, 9, 10 & 11 and 12 (appendix II).

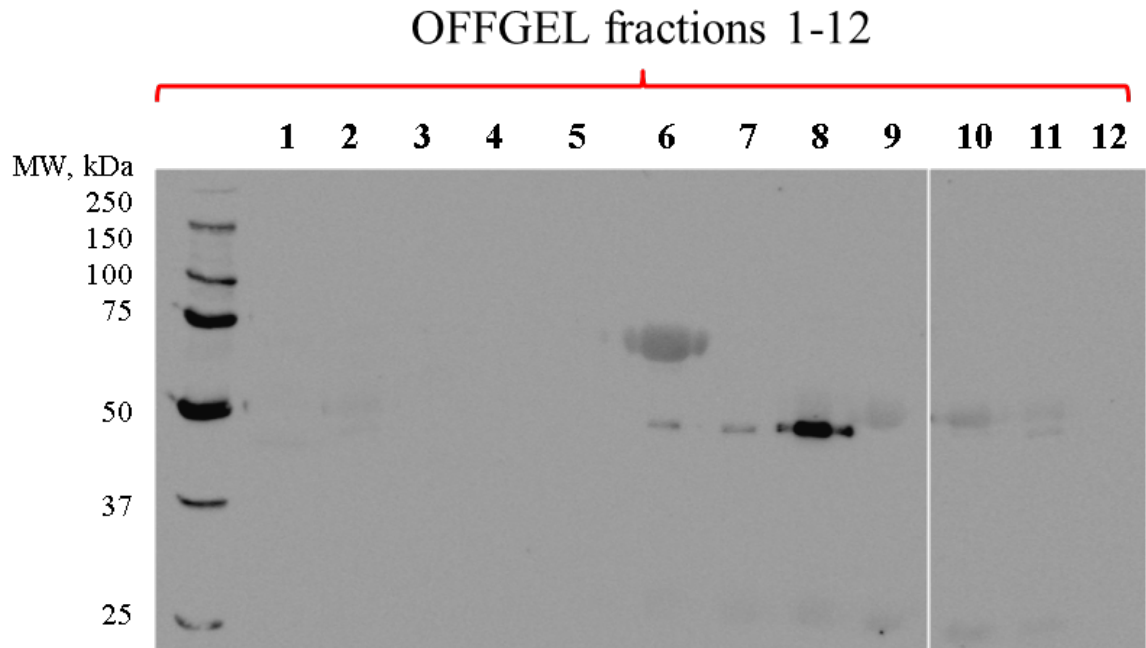


Figure 2.14: Proof of principle of OFFGEL SDS PAGE methodology in urine.

100 mL of healthy male urine was concentrated and acetone precipitated before fractionation by OFFGEL electrophoresis (pH 3-10, low resolution IPG strips). OFFGEL fractions were separated using a 10% SDS PAGE gel and probed with a monoclonal mouse anti-alpha enolase antibody. Strong expression of alpha enolase at 47 kDa was observed in fraction 8, while weaker expression was identified in fraction 6, 7, 9, 10 and 11 using this antibody. Alpha enolase expression was also identified in fraction 6 at 66 kDa.

2.3.4.2 Identification of urinary TAAs and serum autoantibodies using OFFGEL and SDS PAGE

'Proof of principle' studies using alpha enolase (section 2.3.4.1) demonstrated that OFFGEL fractionation can successfully be applied to urine samples, showing the separation of urinary proteins into discrete fractions based on their pI. By Western blotting, correct expression of alpha enolase at specific fractions (pI 6-9) corresponding to its theoretical pI (pI 7.01) and molecular weight (47 kDa) was observed. However, successful OFFGEL electrophoresis requires a minimum of 0.5-5 mg total protein (manufacturer's recommendations), typically requiring 5-50 mL of patient urine for a single OFFGEL strip. Due to the limited volumes (and as a result, protein concentration) of urine collected for this study, it was not feasible to carry out OFFGEL electrophoresis for PROTEOMEX.

However, an attempt was made to screen sera from PCa and healthy controls for autoantibodies, which may serve as potential biomarkers that are immunoreactive towards

proteins in urine. To screen for PCa associated TAAs and autoantibodies, a combined OFFGEL and SDS PAGE approach was applied. Samples utilised for this study are outlined in table 2.14.

Table 2.14: Urine samples used for TAA/autoantibody identification by OFFGEL & SDS PAGE

Patient category	Sample no.	Age	PSA (ng/mL)	Gleason 1	Gleason 2	Gleason grade	D'Amico Risk	TNM
PCa	PC30	76	20.5	4	5	9	High	-
PCa	PC31	71	39.6	4	5	9	High	-
PCa	PC32	51	10.9	4	5	9	High	T3aNOM0
PCa	PC33	74	8.7	4	5	9	High	-
PCa	PC34	59	86.8	4	5	9	High	-
Healthy	HE04	38	-	-	-	Healthy	-	-
Healthy	HE05	35	-	-	-	Healthy	-	-
Healthy	HE06	51	-	-	-	Healthy	-	-
Healthy	HE07	38	-	-	-	Healthy	-	-
Healthy	HE08	60	-	-	-	Healthy	-	-

Urine samples were obtained prior to any diagnosis. To identify TAAs and autoantibodies for PCa, patients from the following categories were selected at random and chosen for this study. A total of 5 PCa patients were chosen: Gleason 9 (n=5) and healthy controls (n=5).

As in the previous section, urinary proteins from healthy controls (n=5) were prepared (as described in section 2.2.2) and fractionated using a low resolution IPG strip (pH 3-10). Acidic fractions (fractions 1-9) were then separated using 10% SDS PAGE gels and immunoprobed with pooled PCa (n=5) or allogeneic healthy sera (n=5). Figure 2.15 shows that a similar pattern of reactivity was observed in immunoblots probed with PCa sera (figure 2.15A) and healthy sera (figure 2.15B), where bands were detected at 50 kDa in PCa and healthy urine (fraction 1 and 2). From figure 2.15A and 2.15B, bands at 45 kDa were observed exclusively to PCa sera in both fraction 3 and 4, where no autoreactivity was observed towards healthy urine at the same molecular weight and pI. In the 6th, 7th, 8th and 9th fraction, the same immunoreactivity pattern was observed towards PCa and healthy sera with proteins at 50 kDa, 49 kDa, 48 kDa and 48 kDa respectively, which was thought to represent normal autoreactivity to serum IgG. No bands were detected in fraction 5 in either immunoblots.

From figure 2.15, the most obvious difference between PCa and healthy immunoblots occurred in fractions 3 and 4, where a moderately intense band was detected exclusively at 45 kDa in PCa urine (fraction 3, figure 2.15). Similarly, a less intense band was observed

exclusively in fraction 4 from pooled PCa urine. Each immunoreactive band was subdivided into two (figure 2.15C) in order to reduce the protein complexity during identification. All 4 bands were excised from Coomassie stained replica gels and subject to a tryptic overnight in-gel digest.

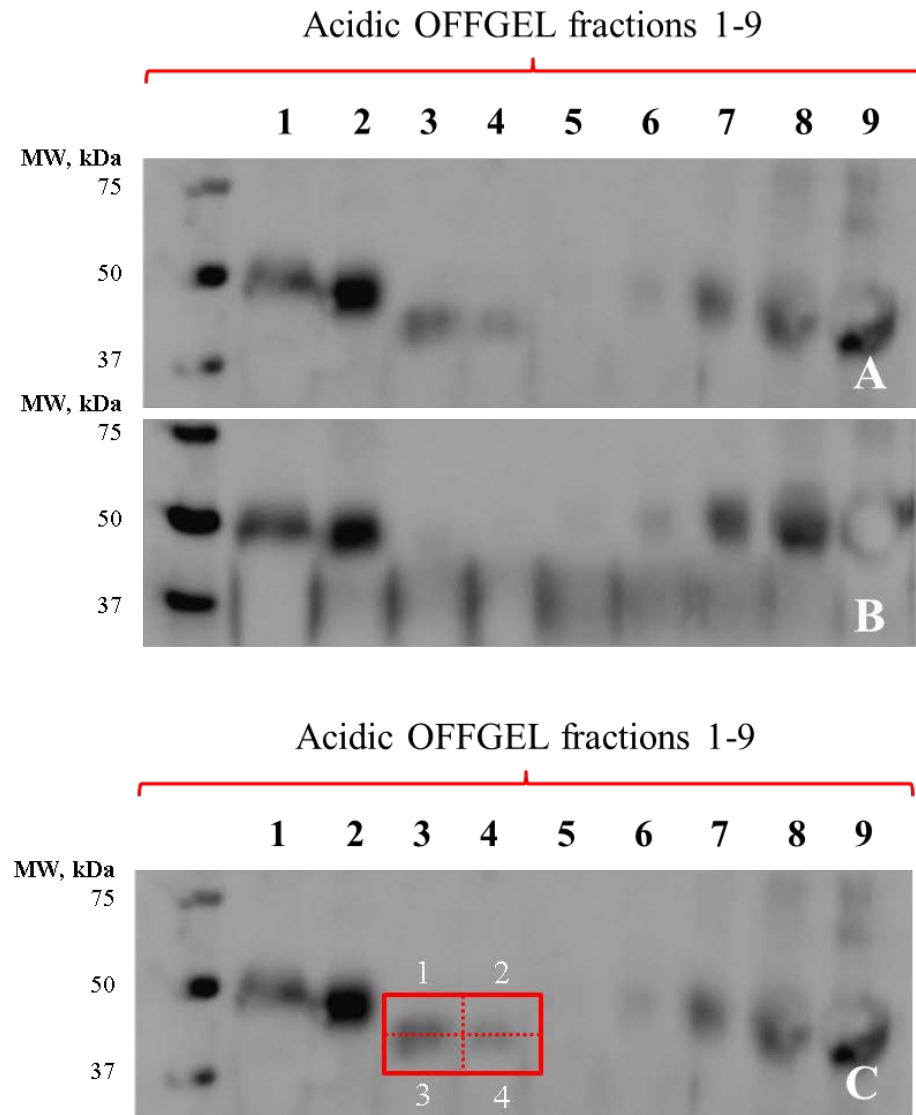


Figure 2.15: PROTEOMEX screening of urinary TAAs separated by a combined OFFGEL and SDS PAGE approach, using allogeneic PCa and healthy sera.

Urinary proteins from PCa (n=5) and healthy controls (n=5) were separated by low resolution OFFGEL (3-10) and then by 1-D SDS PAGE (10% gels). Gels were transferred to nitrocellulose membranes and immunoblotted with sera from a pool of PCa sera (A) or allogeneic healthy sera (B). C shows highlighted bands chosen for mass spectrometry.

2.3.4.3 Identification of urinary TAAs by electrospray LC-MS/MS

ESI MS/MS was the mass spectrometry method chosen to identify potential TAA candidates due to the previous issues determining which protein was responsible for the immune response. In addition, this was a new instrument acquired by our laboratory and as such, this was the first time it was used to demonstrate its compatibility with PROTEOMEX.

After an in-gel digest, samples were cleaned up using a C₁₈ ZipTip column and subjected to identification by electrospray coupled to a LC system. As shown in figure 2.14, 2 immunogenic bands, which separated at approximately 45 kDa in fractions 3 and 4, were isolated for TAA identification. Tables 2.15-2.17 show the proteins identified from each gel band at the appropriate FDR cut off level, corresponding to bands 1, 2 and 4. No proteins were identified from immunogenic band 3.

Table 2.15: Urinary proteins present in the immunogenic 48 kDa band; fraction 3 (band 1)

S/N	Protein name	UniProt Accession	MW (kDa)	SC (95%)	# Peptides
1	Actin, cytoplasmic 2	ACTG_HUMAN	41.79	21.87	8
1	Actin, cytoplasmic 1	ACTB_HUMAN	41.74	21.87	8
2	Uromodulin **	UROM_HUMAN	69.76	9.06	6
3	Osteopontin	OSTP_HUMAN	35.42	14.97	5
4	Trypsin-1	TRY1_HUMAN	26.56	17.41	7
4	Putative trypsin-6	TRY6_HUMAN	26.54	13.36	6
4	Trypsin-2	TRY2_HUMAN	26.49	9.31	5
5	Protein AMBP	AMBP_HUMAN	39.00	14.49	4
6	Protein YIPF3	YIPF3_HUMAN	38.25	5.43	3

Urinary proteins identified from the immunoreactive band at 45 kDa (1). Fifteen proteins and 43 peptides were identified by LC ESI-MS at the appropriate 1% FDR cut-off. SC; sequence coverage at a 95% confidence interval. #peptides; number of peptides identified. Proteins in double asterisks indicate the most abundant protein in human urine.

Table 2.16: Urinary proteins present in the immunogenic 48 kDa band; fraction 4 (band 2)

S/N	Protein name	UniProt Accession	MW (kDa)	SC (95%)	# Peptides
1	Actin, cytoplasmic 2	ACTG_HUMAN	41.793	19.73	6
1	Actin, cytoplasmic 1	ACTB_HUMAN	41.737	19.73	6
2	Trypsin-1	TRY1_HUMAN	26.558	22.67	5

Urinary proteins identified from the immunoreactive band at 45 kDa (2). 0 proteins and 8 peptides were identified by LC ESI-MS at the appropriate 1% FDR cut-off. SC; sequence coverage at a 95% confidence interval. #peptides; number of peptides identified.

Table 2.17: Urinary proteins present in the immunogenic 43 kDa band; fraction 4 (band 4)

S/N	Protein name	UniProt Accession	MW (kDa)	SC (95%)	# Peptides
1	Serum albumin*	ALBU_HUMAN	69.37	22.50	14
2	Trypsin-1	TRY1_HUMAN	26.56	17.81	13
3	Prostaglandin-H2 D-isomerase	PTGDS_HUMAN	21.03	31.58	7
4	Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	34.26	28.86	6
5	Prostatic acid phosphatase	PPAP_HUMAN	44.57	20.73	6
6	Gelsolin	GELS_HUMAN	85.70	13.17	6
7	Actin, cytoplasmic 2	ACTG_HUMAN	41.79	18.13	5
7	Actin, cytoplasmic 1	ACTB_HUMAN	41.74	18.13	5
8	Vesicular integral-membrane protein VIP36	LMAN2_HUMAN	40.23	14.89	3
9	Kininogen-1	KNG1_HUMAN	71.96	5.28	3
10	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_HUMAN	103.36	6.67	3
11	Clusterin	CLUS_HUMAN	52.50	11.58	3
12	Beta-glucuronidase	BGLR_HUMAN	74.73	4.76	3
13	Ig alpha-1 chain C region*	IGHA1_HUMAN	37.66	5.38	2
14	Apolipoprotein D	APOD_HUMAN	21.28	10.58	2
15	Protein AMBP	AMBP_HUMAN	39.00	7.67	4
16	Endothelial protein C receptor	EPCR_HUMAN	26.67	10.92	2
17	Cathepsin B	CATB_HUMAN	37.82	7.67	2
18	Ig kappa chain C region*	IGKC_HUMAN	11.61	49.06	2
19	Polymeric immunoglobulin receptor	PIGR_HUMAN	83.28	3.53	2
20	D-3-phosphoglycerate dehydrogenase	SERA_HUMAN	56.65	3.94	2
24	Putative trypsin-6	TRY6_HUMAN	26.54	17.81	12

Urinary proteins identified from the immunoreactive band at 45 kDa (4). 34 proteins and 105 peptides were identified by LC ESI-MS at the appropriate 1% FDR cut-off. SC; sequence coverage at a 95% confidence interval. #peptides; number of peptides identified. Proteins in asterisks indicate the top 20 most abundant proteins in human serum.

In this study, ESI MS/MS identified previously discovered proteins of interest - zinc-alpha 2 glycoprotein, prostatic acid phosphatase and serum albumin (table 2.17).

2.4 DISCUSSION

A PROTEOMEX based approach was implemented to identify proteins that elicit a humoral immune response in PCa. Using SDS PAGE, 2-DE, OFFGEL and Western blotting, urine from PCa, benign and healthy controls were screened with pooled allogeneic PCa sera to identify TAAs that react to autoantibodies in PCa. Unlike other studies that utilise cell lines and tumour tissues as an antigenic source (Rauch and Gires, 2008), this study employed urinary proteins to study PCa induced immune responses. Although urine collected after a DRE contains prostatic secretions and detached epithelial cells, making massaged urine excellent for TAA identification & biomarker discovery (Downes *et al.*, 2006), this study was carried out to identify if PCa specific immune responses could be observed, allowing the identification of PCa specific TAAs in non-massaged urine.

2.4.1 TAA identification by SDS PAGE

Urinary proteins from PCa, benign and healthy controls were screened for seropositivity to pooled allogeneic PCa sera. Figure 2.6 demonstrates the presence of differential bands exclusive to allogeneic PCa urine at 40, 44 and 48 kDa, which was absent from the control groups (figure 2.6A and 2.6B), suggesting the specificity of this immune response to PCa. In order to determine the frequency and specificity patterns of the immune responses observed, urine from all patients were separated by SDS PAGE (section 2.3.2.1) and screened with pooled allogeneic PCa sera (figure 2.7A) or autologous sera (figure 2.7B). Figures 2.7A & 2.7B illustrates that most immunoreactivity was detected in PC02 (Gleason 7 PCa), with a minimum of 2 bands observed per seropositive patient screened (figure 2.7A). Figure 2.7B shows that apart from the bands that occurred in all patients at 50 kDa, no other bands were detected in PC01, while two additional bands were observed in PC03 and 3 bands in PC02, demonstrating that no two patients displayed the same pattern of antigenicity.

Although it would have been of interest to screen urinary proteins with healthy sera to determine autoreactivity patterns of urinary proteins to healthy serum IgG, healthy serum was not available at the time of the study. Nevertheless, carrying out various combinations

of allogeneic screening (figure 2.7A and 2.7B) demonstrated the varying reactivity patterns in the allogeneic combination of urine and serum.

A 42 kDa band in PCa urine was exclusively recognised by sera from PC03 (Gleason 9, figure 2.7B), and identified by LC MALDI TOF/TOF. Among the 25 significant proteins identified by this band using LC-MALDI TOF/TOF, a well-studied PCa biomarker, prostatic acid phosphatase was identified (and verified in figure 2.9) with the most number of peptides and the highest MOWSE score (table 2.9). Human prostatic acid phosphatase is a prostate epithelium-specific differentiation antigen with significantly higher activity in PCa patients, especially those with bone metastasis compared to its expression in healthy controls (Gutman *et al.*, 1936). This is consistent with findings from this study where prostatic acid phosphatase was identified and verified in the only patient with metastasis (figure 2.9). In PCa, prostatic acid phosphatase activity correlates with tumour progression and may also serve as an indicator of treatment response (Huggins & Hodges, 1941). Prostatic acid phosphatase expression has also been shown to serve as an independent predictor of biochemical failure and tumour recurrence following radical prostatectomy (Veeramani *et al.*, 2005). As a result, prostatic acid phosphatase was the preceding PCa biomarker prior to PSA. Prostatic acid phosphatase is secreted glycoprotein enzyme (100 kDa) synthesised by prostate columnar epithelium secretory cells (Vihko *et al.*, 1978) where it may be involved in fertility and the liquefaction of semen (Afzal *et al.*, 2003).

It is tempting to hypothesise that prostatic acid phosphatase could be responsible for the immune response observed in this study - it is well documented that prostate cancer is an immunogenic tumour and humoral immune responses to prostatic acid phosphatase have previously been identified by many researchers, similar to this study (McNeel *et al.*, 2000 and GuhaThakurta *et al.*, 2015). However, due to the limitation of the technology utilised in this study, such conclusions cannot be drawn. What can be concluded is that prostatic acid phosphatase is present in the urine of PC03 as identified and verified by mass spectrometry and Western blotting (figures 2.9 & 2.10). In order to demonstrate the presence of autoantibodies to prostatic acid phosphatase and whether prostatic acid phosphatase was responsible for the immune response observed in PC03, it would have been beneficial to carry out an ELISA assay using a recombinant prostatic acid phosphatase protein screened with PC03 sera. This, in combination with results by mass spectrometry would determine whether prostatic acid phosphatase was responsible for the

immune response observed at 42 kDa in PC03; as implied by the findings from this study. Only then can conclusions be made to whether prostatic acid phosphatase is responsible for the immune response observed. Furthermore, in order to determine if prostatic acid phosphatase could serve as a potential PCa biomarker, an ELISA assay could be carried out to screen a range of PCa patient and healthy urine for differences in the concentration of prostatic acid phosphatase protein.

Other proteins identified from this gel band represent the top 20 high abundant serum proteins (52% of proteins identified) (high abundant proteins indicated by asterisks in table 2.9), likely present in urine due to the ultrafiltration of plasma proteins which occurs normally in urine, rather than due to a neoplastic process. The identification of diagnostic biomarkers in human urine or serum using mass spectrometry based proteomics, is hindered by the large dynamic range of serum based proteins and because 22 of the most abundant proteins in serum make up approximately 99% of the total serum proteome (Prieto *et al.*, 2014). Thus, although immunodepletion of these high abundant proteins (e.g. albumin) facilitates the detection of lower abundant biomarkers, high abundant proteins serve as a potential source of biomarkers due to the protein fragments or peptides that interact with this high abundance group (Dowling *et al.*, 2014). As a result, it is likely that these bound, low abundant peptides/fragments may be responsible for eliciting the immune response in PC03. However, without an added immunodepletion step to eliminate these high abundant proteins, it is not possible to identify the TAA of interest using this technology.

Amongst the 25 significant proteins identified, zinc-alpha-2 glycoprotein (identified with the 4th highest MOWSE score, 4th highest sequence coverage and 3rd highest number of peptides); and this has been established to have diagnostic significance in PCa (Hale *et al.*, 2001). Hale *et al.* (2001) identified that high grade tumours expressed significantly less tissue zinc-alpha-2 glycoprotein than moderate grade tumours. In addition, they found that in men with zinc-alpha-2 glycoprotein positive tumours had higher serum zinc-alpha-2 glycoprotein levels compared to their race and age matched controls. They also demonstrated that tumour produced zinc-alpha-2 glycoprotein contributes to an elevated concentration of serum zinc-alpha-2 glycoprotein, by detecting human zinc-alpha-2 glycoprotein in the serum of mice bearing an hZAG-B16 transfected tumour. Their study demonstrates that zinc-alpha-2 glycoprotein production can be useful for diagnostic

purposes (Hale *et al.*, 2001). As zinc-alpha-2 glycoprotein was identified by 3 separate MS identification methods, it would have been beneficial to determine if zinc-alpha-2 glycoprotein could serve as a potential PCa biomarker. This could be achieved by screening urine samples from a range of PCa and healthy controls for differences in zinc-alpha-2 glycoprotein protein expression using a quantitative proteomic method such as ELISA assay. However, due to time and feasibility constraints, this was not carried out in this study.

Overall, TAA identification by SDS PAGE demonstrated that direct identification of antigens that elicit an immune response is not possible due to the complexity of protein mixtures which are present within every lane on SDS PAGE gels (Forgber *et al.*, 2009). In addition, one of the main drawbacks to using pooled samples is that we cannot be sure how many patients in the pool have the specific reactivity patterns observed. Due to time, feasibility and the limitation in technology utilised, it was not possible to screen each patient individually. However, the pilot study has identified PCa relevant biomarkers, establishing that these TAAs are present in urine and can be isolated and identified using PROTEOMEX in combination with more protein fractionation prior to mass spectrometry.

2.4.2 Urine TAA identification by 2-DE

The combination of low resolution 2-DE (pH gradient of 3-10) for isoelectric focussing, with serum immunoblotting proved to be a suitable technique to reduce the complexity of proteins identified after the PROTEOMEX screen. Although 2-DE showed a separation of protein bands into spots, which potentially contain a smaller number of proteins compared to SDS PAGE, only 8 spots were immunoreactive to PCa or healthy sera from this study and thus were shortlisted for identification by mass spectrometry (figure 2.10). Comparing both studies; TAA identification by SDS PAGE (section 2.3.2) and using 2-DE (section 2.3.3), it is evident that more immunoreactivity was observed in the 2-DE than with SDS PAGE alone (at molecular weights 37-75 kDa, 4 unique bands were observed in SDS PAGE (figure 2.6) and 8 unique spots (figure 2.10) by 2-DE). All 8 spots were aligned to protein spots on Coomassie stained gels, where only 6 of them were identified as 3 proteins after matching them to the SwissProt database – alpha-1-acid glycoprotein 1 (spot 1), zinc-alpha-2 glycoprotein (spot 2) and serum albumin (spot 3, 4, 6 & 7) (table 2.12).

Interestingly, these same proteins were identified previously in the 1-D SDS PAGE screen (proteins #4, #6 and #10, table 2.9) as potential PCa TAAs; though a different set of patient samples had been used. Spots 3, 4 (appeared in healthy & PCa urine), 6 and 7 (PCa urine only; figure 2.10) were identified using PMF as albumin, the most abundant protein in serum, which binds many hormones, proteins, chemicals and drugs and also accounts for 60% of total serum proteome (Dowling *et al.*, 2014). Many of the albumin bound proteins are likely to be released directly from diseased tissues and as such may be responsible for eliciting the immune responses observed in this study (Grundy *et al.*, 2007 and Grundy *et al.*, 2009 and Camaggi *et al.*, 2010).

Zinc-alpha-2 glycoprotein demonstrated a more intense differential expression in PCa urine, compared to healthy controls (figure 2.10). Zinc-alpha-2 glycoprotein, synthesised in the prostate and secreted in various body fluids, is known to stimulate lipolysis by the depletion of fatty acids from adipose tissues. Frenette *et al.* (1987) first identified zinc-alpha-2 glycoprotein in prostatic tissues. Since then, many studies have suggested that it may serve as a potential serum marker for PCa due to its elevation in prostatic adenocarcinoma (Gagnon, *et al.*, 1990). Hale *et al.* (2001) also found that the majority of tested PCa cells react with anti-zinc-alpha-2 glycoprotein antibodies, while also demonstrating that high grade tumours express minimal zinc-alpha-2 glycoprotein compared to moderate grade tumours. These studies alongside Hassan *et al.* (2007), which showed an increased expression of zinc-alpha-2 glycoprotein in the seminal fluid of prostate cancer patients, indicate that zinc-alpha-2 glycoprotein may serve as a potential biomarker for PCa.

One of the limitations of this study is that mini gels were used for screening, which gives a poor resolution and increases the likelihood that excised spots may contain overlapping spots. PROTOMEX studies are typically carried out in large-scale gels for IEF (18-24 cm length strips) and SDS PAGE (24 x 24 cm gels) to allow better separation of the spots and thus a better resolution of proteins (Rauch and Gires, 2007). Also, the global proteome was studied using a pH 3-10L IEF strip. It may have been advantageous to use a narrower pH range (e.g. pH 4-7 or pH 5-8 IEF strips) as a means of reducing the complexity of the samples loaded on the 2-DE gel, in order to increase the visibility of lower abundant proteins and increase the loading capacity of the IPG strips (Catsimpoolas, 1976).

2.4.3 Urine TAA identification by OFFGEL and SDS PAGE

The next study aimed to determine whether more TAAs could be identified from urine using a modified OFFGEL and SDS PAGE approach. To our knowledge, this is the first study to utilise OFFGEL and SDS PAGE for the identification of urinary TAAs using PROTEOMEX. As a ‘proof of principle’ of this technology, a monoclonal antibody to alpha enolase, a well-studied protein utilised for proteomics research, demonstrated the presence of alpha enolase in certain fractions corresponding to its theoretical molecular weight (47 kDa) and pI (figure 2.13).

From the PROTEOMEX screen, an obvious differential expression of two bands (fractions 3 and 4 at 45 kDa) was observed in PCa immunoblots compared to healthy controls. In a bid to reduce the complexity of the sample, each immunogenic band was spilt into two. Similar to the SDS PAGE study, a list of proteins were identified from each gel band where it was not possible to determine which protein(s) were responsible for the immune response observed. From this study, the following proteins were present in all 3 bands – actin, cytoplasmic 2, actin, cytoplasmic 1, trypsin-1, vimentin, eukaryotic initiation factor 4A-I, all representing structural proteins involved with cell adhesion, except trypsin, which may be present from the digestion mixture. Putative trypsin, protein AMBP and serum albumin were present in 2 out of 3 bands identified, while zinc-alpha-2 glycoprotein and prostatic acid phosphatase represent proteins which were identified in band 4 (table 2.14), but were also identified in previous studies using SDS PAGE or 2-DE.

From this chapter, the best technology for urinary TAA identification using PROTEOMEX appears to be 2-DE in combination with MALDI MS/MS, where single or a smaller number of proteins are identified from each gel spot. This does not negate the need however, to validate these identified TAAs in a larger sample cohort or repeat the study using more fractionation and immunodepletion to determine if these same proteins are identified, albeit from the same sample set. Further studies need to carry out more fractionation and immunodepletion to the urine before 2-DE to identify more TAAs associated with PCa. This study however aimed to determine whether TAAs were present in urine and if they could be isolated and identified using PROTEOMEX. The chapter has identified prostatic acid phosphatase and zinc-alpha-2-glycoprotein as potential urine TAA candidates that require further investigation. Interestingly, both proteins identified are well-

known PCa markers, which validates the utility of PROTEOMEX as a robust method, useful for narrowing down and identifying potentially relevant biomarkers requiring further validation.

CHAPTER 3: IDENTIFICATION OF TUMOUR ANTIGENS IN CELL LINES AS BIOMARKERS FOR PROSTATE CANCER

3.1 INTRODUCTION

Early detection and diagnosis are essential for the management of PCa. Current diagnosis of PCa is based on the identification of raised serum PSA (>4 ng/mL) levels alongside an invasive and subjective DRE; where more invasive procedures such as a transrectal ultrasonography of the prostate and biopsy are carried out if cancer is suspected (Madu & Lu, 2010). However, the lack of specificity of PSA combined with the subjectivity of DRE leads to over diagnosis and overtreatment of patients, resulting in increased clinical costs and unnecessary patient distress. As a result, more stringent biomarkers are required for accurate PCa diagnosis (Larkin *et al.*, 2010). It is therefore necessary to identify biomarkers which are more accurate, precise and clinically relevant, which can serve as early indicators for PCa.

As outlined in section 1.2.4.1, the grading of PCa uses the Gleason grade system which indicates the aggressiveness of tumours. It is well known that within Gleason grade 7, Gleason 3+4 tumours (i.e. tumours where pattern 3 is the most prevalent pattern, and pattern 4 is the second most prevalent observed under a microscope) have a better prognosis than Gleason 4+3 tumours (i.e. tumours where pattern 4 is the most prevalent pattern, and pattern 3 is the second most prevalent observed under a microscope) (Stephen and Kattan, 2006). In order to identify differences between both Gleason 7 phenotypes, clinicians associate Gleason grade with other prognostic factors such as biochemical progression (Chan *et al.*, 2000, Rasiah *et al.*, 2003 and Sengupta *et al.*, 2006) or the development of tumour metastasis (Lau *et al.*, 2001). Although biochemical recurrence is most popularly used in order to determine PCa risk prediction and the efficacy of treatment, studies have found that its definition varies (Amling *et al.*, 2001 and Freedland *et al.*, 2003), making it an imperfect model to discriminate between the two phenotypes. This

warrants the identification of more PCa markers that can distinguish between the two forms of Gleason 7 PCa.

The previous chapter utilised a PROTEOMEX approach to identify TAAs for PCa using urine as a biomarker source. Amongst all the separation methods used in combination with mass spectrometry, chapter 2 demonstrated that the best method for TAA identification was 2-DE in combination with MALDI-TOF mass spectrometry. The chapter also demonstrated that while PCa relevant TAAs could be identified from urine, more fractionation is required in order to detect low abundant proteins, which may contain potential PCa biomarkers. As a result, the current chapter aims to identify TAAs from another biomarker source – immortalised PCa cell lines.

It is not entirely clear how intracellular proteins induce autoimmune responses (Suzuki *et al.*, 2010). However, it is well documented that post transcriptional modifications enhance protein immunogenicity (Forgber *et al.*, 2009), and that certain foetal antigens are highly expressed in tumour cells (Ting *et al.*, 1972). As a result, cancer sera are a valuable source of TAAs (Rauch and Gires, 2008). Although there have been a lot of reported TAAs linked to the immune response in cancers, the challenge with TAA identification is that it is difficult to find TAAs which can be utilised for clinical diagnostic applications (Desmetz *et al.*, 2008). As a result, the aim of this study is to identify PCa relevant TAAs that can serve as cancer biomarkers using the same PROTEOMEX technology (detailed in section 1.3.7.1 and successfully utilised in the previous chapter).

Autoantibodies produced in response to TAAs can provide an alternative source of biomarkers (Desmetz *et al.*, 2008). During early tumour development, the levels of TAAs produced are usually too low to detect using conventional methods. However, the autoantibodies produced in response to TAAs are amplified, allowing cancer detection long before any clinical diagnosis (Fernandez 2005). Furthermore, high titres of autoantibodies are detectable in patients with early stages of cancer (Disis *et al.*, 1997) and have been shown to correlate with malignant transformation (Tan *et al.*, 2009). As a result, several techniques have been successfully employed to detect TAAs and their corresponding autoantibodies for biomarker use. One such method is PROTEOMEX which allows the identification of autoantibodies to TAAs as they occur in their natural

state, as opposed to SEREX which screens for autoantibodies to proteins expressed in bacteria (Rauch & Gires, 2008).

Using proteomic techniques in combination with serological screening, TAAs can be identified by matching differential antigen spots on membranes immunoprobed with cancer or patient sera against protein spots in stained replica gels. TAAs of interest are then excised from these replica gels and identified by PMF or *denovo* peptide sequencing using mass spectrometry (Rauch and Gires, 2008). This approach was employed in this chapter to determine the immunogenicity of PCa cell lines and the specificity of the corresponding patient autoantibody responses.

While cancer cell lines lack features of an *in situ* tumour, making them a poor representation of the tumour *in vivo* (Alvarez-Chaver *et al.*, 2014), immortalised cell lines can still provide a suitable source of TAAs for identifying autoantibodies associated with cancer. Using PROTEOMEX, many studies have identified relevant TAAs and autoantibodies for various cancers in tumour cell lines; such as HSP-60 and RS/DJ-1 in breast cancer (Hamrita *et al.*, 2008, Desmetz *et al.*, 2008 and Le Naour *et al.*, 2011), cytokeratin 8 and thioredoxin in renal cell carcinoma (Kellner *et al.*, 2002 and Lichtenfels *et al.*, 2003), eukaryotic elongation factor, alpha enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase and heterogeneous nuclear ribonucleoproteins A2B1 and galectin-3 in melanoma (Suzuki *et al.*, 2010 and Forgber *et al.*, 2009) and alpha enolase in pancreatic adenocarcinoma (Tomaino *et al.*, 2007). These studies have demonstrated the utility of cancer cell lines as a model to study the interrelationship between cancer and the immune system, also showing the value of PROTEOMEX as a means of narrowing down and identifying potentially relevant biomarkers requiring further validation.

Despite the utility of this approach, to date, only one study has utilised the PROTEOMEX technique to identify TAAs and autoantibodies for PCa using patient tissue lysates as a biomarker source (Ummanni *et al.*, 2015). Autoantibody responses to antigens PRDX2, PRDX6 and ANXA11 were identified, where a combination of PRDX6 and ANXA11 autoantibodies yielded a biomarker sensitivity of 90% for PCa and 100% for healthy controls. To our knowledge, this will be the first study utilising PCa cell lines as a biomarker source to identify TAAs for PCa using PROTEOMEX. Thus, it was important to demonstrate the utility of the PROTEOMEX technique in identifying relevant TAAs in

PCa using readily available cancer cell lines, before carrying out further validation experiments on tumour tissue samples. Table 3.1 outlines the PCa cell lines utilised for this investigation alongside their attributes.

Table 3.1: Attributes of PCa cell lines utilised for biomarker discovery

Cell Line	Attributes
PC-3	Derived from bone metastasis, grade IV prostatic adenocarcinoma 62 year (male), Caucasian, high tumourigenicity <i>Low prostatic acid phosphatase and testosterone-5-alpha reductase activities.</i>
OPCT-1	Androgen sensitive Prostate tumour epithelium (T1cN0M0, Gleason 3+3) Patient received no chemotherapy, no radiotherapy and no hormone treatment
DU145	Derived from brain metastasis, androgen resistant 69 year (male), Caucasian, moderate tumourigenicity <i>Weakly positive for prostatic acid phosphatase</i> <i>Does not express prostate specific antigen</i>
LNCAP.fcg	Derived from the left supraclavicular lymph node of a 50 year old patient with a confirmed diagnosis of metastatic prostate carcinoma Androgen/oestrogen receptors <i>Expresses prostatic acid phosphatase, prostate specific antigen positive</i>

General information and characteristics of all PCa cell lines. *Words in italics represent markers expressed by each cell line.*

3.1.1 Aims & objectives

The aim of this chapter is to identify TAAs in PCa cell lines which may serve as potential PCa biomarkers, following subsequent validation. This will be carried out by:

1. Identifying PCa TAAs and biomarkers in two well-characterised PCa cell lines using PROTEOMEX in combination with MALDI TOF MS.
2. Identifying biomarkers that can differentiate between the aggressive (4+3) vs. the non-aggressive (3+4) phenotype of Gleason grade 7, using PROTEOMEX in combination with MALDI TOF MS.
3. Confirming and verifying the MS identified PCa TAA(s) using immunofluorescence, flow cytometry and Western blotting.
4. Verifying the existence of a differential serum autoantibody response towards the identified TAA using ELISA.

3.2 METHODS

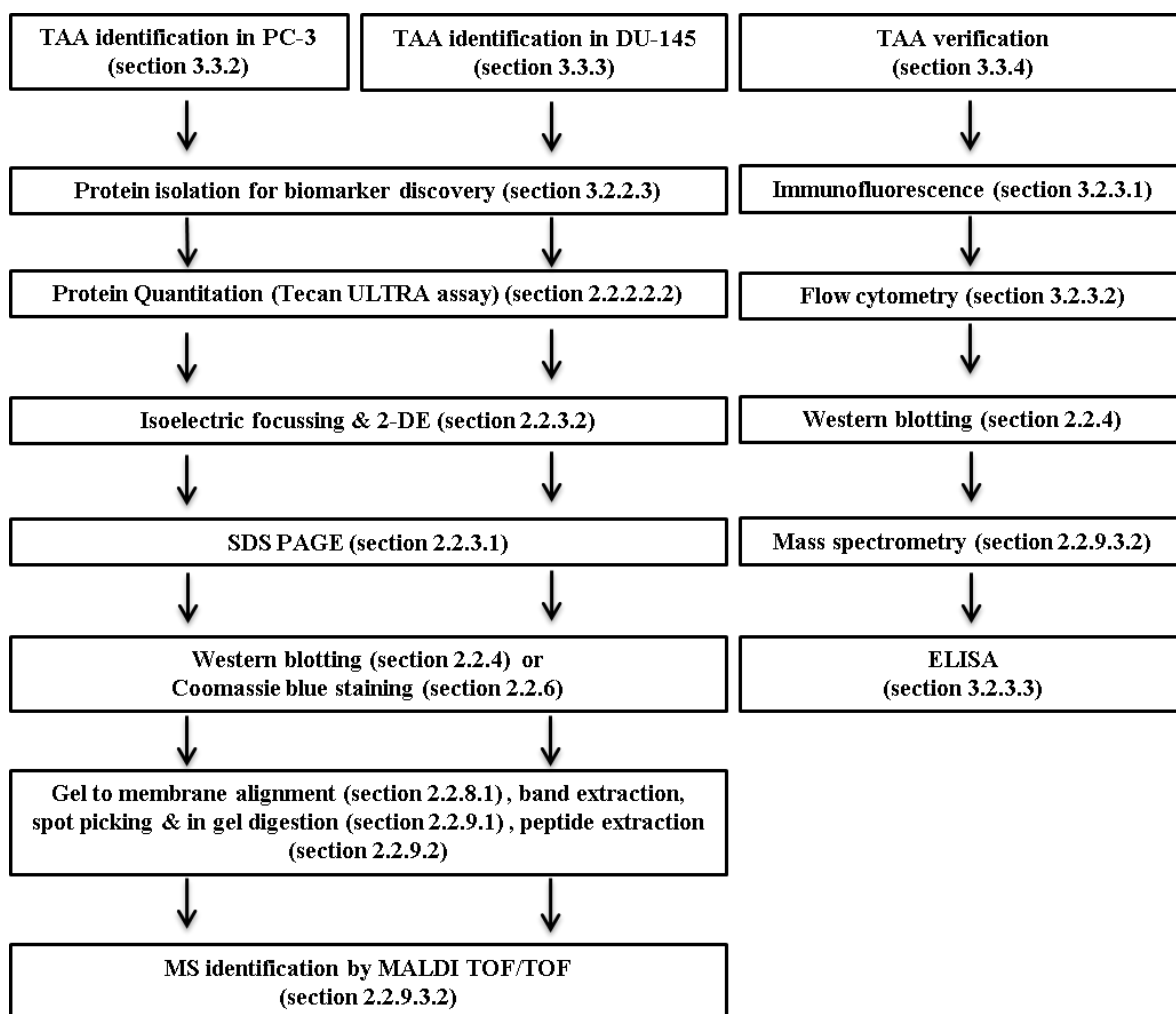


Figure 3.1: Method outline for TAA identification in PCa cell lines.

This chapter is divided into 3 sections – identification in PC-3 and DU-145 PCa cell lines and verification of the identified TAA. The flowchart shows the techniques used for TAA identification and verification in this chapter.

3.2.1 Selection of the appropriate PCa TAA tissue

3.2.1.1 Prostate cancer sera and urine

Sera and urine samples from PCa patients were collected and banked at the Urology Centre, Nottingham City Hospital, Nottingham, UK. Samples were prepared and stored as outlined in section 2.2.

3.2.2 Reactivity of patient and healthy sera with protein extracts from PCa cell lines

3.2.2.1 Cell lines and routine cell culture

Two commercially available PCa cell lines were purchased from ATCC and utilised in this study as a source of potential TAAs (PC-3 and DU-145). PC-3 were cultivated in Ham's F12K media supplemented with 1% sodium pyruvate, 1% non-essential amino acids and 10% foetal calf serum (FCS) while DU-145 were grown in DMEM media supplemented with 10% FCS and 1% L-glutamine. The attributes of all PCa cell lines utilised in this chapter are outlined in table 3.1.

All cell lines were maintained by serial *in vitro* passage and cultured at 37°C in an incubator containing 5% CO₂ and 95% humidified air. When the cultured monolayers had reached 80-90% confluence, they were passaged using a combination of trypsin and versene (T and V) as described below.

Spent culture media was discarded and adhered cells were rinsed twice with PBS to remove traces of culture media during routine cell culture. A 5 mL T and V solution was added to the T175 flask and left to incubate at 37°C for approximately 5 min. After the incubation, adherent PCa cells were detached from tissue culture flasks by gently tapping the flask 4 times. The dispersion of the adherent cell layer was observed under an inverted microscope, after which 10 mL of fresh culture media was added to the flask. The cell solution was aspirated by gentle pipetting into a universal tube and centrifuged at 400 g for 3 min at 20°C. After centrifugation, 20 mL of fresh culture media was added to new T175 flasks. The supernatant from the centrifuged cells were discarded and the cell pellet was

resuspended in 1 mL routine culture medium. Cells were re-seeded at a 1:20 ratio into fresh tissue culture flasks and left to incubate at 37 °C or left in suspension for protein isolation and cell counting.

3.2.2.2 Cell counting

In order to count live (viable) cells for downstream analysis, 10 µL of cell suspension was mixed with 90 µL of 0.4% (v/v) trypan blue solution and pipetted onto both chambers of a haemocytometer. Cell counting via the trypan blue dye exclusion method is based on the principle that non-viable cells take up certain dyes as opposed to viable cells, aiding the distinction of cells under a light microscope. Starting with the first chamber of the haemocytometer, all the cells in the 1 mm square are counted and an average is taken of all four 1 mm squares. The number of cells was calculated using the formula:

$$\text{Average number of cells/1 mm square} \times \text{dilution factor} \times 10^4 = \text{cell count per mL}$$

The number of cells required for each assay was then calculated from the total number of cells and the appropriate volume of cell suspension was collected.

3.2.2.3 Protein isolation from PCa cell lines for biomarker discovery

For proteomic studies, cell lines were harvested at 80-90% confluence and adherent cells were rinsed twice with cold PBS to remove spent culture media (as detailed in section 3.2.2.1). 7 mL of cold PBS was added to the flask and adhered cells were scraped using cell a scraper before centrifuging at 400 g for 3 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 1 mL of cold PBS before a further centrifugation at 400 g for 3 min at 4°C to obtain a dry pellet. The supernatant was discarded and a dry pellet was stored at -80°C until use or resuspended in 50 µL of cell lysis buffer (9.5 M Urea, 2% DTT, 1% OGP) for proteomic analysis. The solution was vortexed for 20 sec, sonicated at 37°C for 5 min and left on ice for a further 5 min. The sonication-ice step was repeated 3 times before centrifugation at 400 g for 3 min to obtain a supernatant, which was subsequently collected and stored at -80°C until the protein concentration was determined.

3.2.2.4 Protein concentration determination (protein assay)

Protein estimations were performed using a Bradford protein assay as described in section 2.2.2.2. In brief, serial dilutions of BSA protein standards were prepared and added in duplicate into designated wells of a flat bottom 96 well plate. Lysed PCa cells were diluted at 1:1000 in lysis buffer before pipetting into the remaining wells of a 96 well plate. 200 μ L of Bio-Rad dye reagent (diluted 1 in 4 with ddH₂O) was added to the lysate or BSA standard and incubated for 5 min at room temperature. The absorbance was measured at 570 nm using a Tecan Ultra spectrophotometer (Tecan Group Ltd, UK) and a BSA standard curve was constructed using the concentration and absorbance values of the standards.

3.2.2.5 2-DE, SDS PAGE and Western blotting

2-DE electrophoresis and SDS PAGE was performed according to the manufacturer's instructions as described in section 2.2.3.1 and 2.2.3.2. In brief, 20 μ g of cell lysate was mixed with sample dehydration buffer and pipetted along the length of a 7 cm 3-10L IEF strip (Bio-Rad, UK), before allowing passive rehydration of the strips for 1 h at room temperature. Each strip was run using the manufacturer's predefined programme for approximately 18 h, before loading onto 10% SDS PAGE gels. Each gel was run at 200 V until the sample dye front reached the anodic side of the gel. Separated proteins were then transferred electrophoretically onto a nitrocellulose membrane using a wet transfer method as described in section 2.2.4, or stained with a Coomassie brilliant blue solution. Membranes were then immunoprobed with human sera or a monoclonal antibody at a pre-optimised dilution (stated in table 2.7).

3.2.2.6 Coomassie, Ponceau S stain, gel to membrane alignment

SDS PAGE gels were stained overnight in a Coomassie brilliant blue solution and de-stained as outlined in section 2.2.6. Electrophoretically transferred membranes were also stained using a Ponceau S solution (section 2.2.5) and rinsed in dH₂O until stained spots were visible on the membrane. In order to carry out TAA identification, gels and membranes were manually aligned as described in section 2.2.81 and spots of interest were chosen for MS identification.

3.3.2.7 Band extraction, In-gel digestion, MS identification

Spots of interest were excised from the Coomassie stained replica gel as described in section 2.2.9.1 and digested overnight using trypsin (described in detail in section 2.2.9.1). Digested peptides were extracted from the gel matrix and cleaned up using C₁₈ pipette tips (Millipore, UK) (section 2.2.9.2). Eluted peptides were spotted onto a 384 MALDI ground steel target plate for analysis (section 2.2.9.2) and analysed using an ultraflextreme MALDI-TOF/TOF mass spectrometer (search parameters detailed in section 2.2.9.3.1).

3.2.3 Verification of identified TAAs

3.2.3.1 Immunofluorescence

For examination of protein expression in PCa cell lines, round glass coverslips were dipped into methanol, placed into individual wells of a flat-bottomed 24 well plate and allowed to air dry in the hood for approximately 15 min. Cells were counted as described in section 3.2.2.1, diluted accordingly at 5×10^4 cells/well and left to culture at 37°C in 5% CO₂ for 48 h until the cells had adhered to the coverslips.

To stain the cells, each well was washed twice for 10 min each with PBS on a rocking platform to remove the excess culture media. Following washes, 200 µL of cold 4% paraformaldehyde was added to each well and left on ice for 15 min to fix the cells. Each well was washed twice with PBS for 10 min each and non-specific binding sites were blocked using 200 µL of 10% BSA in PBS 0.1% Tween for 1 h at room temperature before a wash step for a further 10 min.

To detect protein expression in PCa cell lines, an optimal (1:50) dilution of mouse-anti-alpha enolase antibody (clone L-27, Santa Cruz, UK), or mouse isotype control (1:50 dilution (IgG mouse isotype control, Invitrogen, Paisley, UK) diluted in 2% BSA-PBS was added to relevant wells and incubated at room temperature for 1 h. Following incubation, unbound primary antibodies were removed by performing three 10 minute washes with PBS. An optimised dilution (1:1500 dilution) of a fluorescent Alexa-Fluor antibody (goat anti-mouse IgG AlexaFluor 488, Invitrogen, Paisley, UK) was diluted in blocking buffer

and allowed to bind to primary antibodies by shaking at room temperature for 1 h and the plates were wrapped in foil.

Following incubation, unbound secondary antibodies were removed by washing three times in a PBS wash buffer. To visualise the expression of alpha enolase by immunofluorescence, a drop of Vectafield containing DAPI mounting media (VectorLabs, Peterborough, UK) was placed on a microscope slide and the coverslips were gently removed from the wells and inverted onto the DAPI to aid visualisation of the cell nucleus during microscopy. The edges of the coverslips were sealed with a clear nail varnish (Rimmel 60 seconds nail varnish, UK) to maintain fluorescence. Slides were then wrapped in foil until visualisation by fluorescence microscopy using an Olympus BX51 fluorescence microscope (KeyMed medical & industrial equipment Ltd, Essex, UK).

3.2.3.2 Flow cytometry

To determine whether alpha enolase is expressed in PCa cell lines, cells were trypsinised and counted as detailed in section 3.2.2.1. In brief, and 5×10^5 cells were placed into FACS tubes for analysis by flow cytometry, to determine cell surface or intracellular staining of alpha enolase.

3.2.3.2.1 Cell surface staining

To demonstrate the presence of alpha enolase on the cell surface, 2 mL of FACS buffer (PBS 0.05% BSA 0.02% sodium azide) was added to each tube and centrifuged at 400 g for 5 min at room temperature to wash off excess culture media. After centrifugation, the supernatant was discarded and the pellet was resuspended in an optimised dilution (1:10) of a mouse anti-human alpha enolase antibody (clone L-27, Santa Cruz, UK) diluted in 100 μ L FACS buffer. This solution was vortexed for 20 sec and left to incubate in the dark for 30 min at room temperature to enable antibody binding.

After incubation, cells were washed with 2 mL FACS buffer, centrifuged and incubated with an optimised dilution of a secondary antibody (goat-anti-mouse IgG AlexaFluor 488). Any unbound secondary antibody was removed by washing twice with FACS buffer,

centrifuged and the pellet was resuspended in 500 μ L of isoton to aid analysis by flow cytometry.

3.2.3.2.2 Intracellular staining

Intracellular alpha enolase was demonstrated by washing cultured cells in 2 mL of cold PBS before centrifuging at 400 g for 5 min at 4°C. Cell pellets were resuspended in 500 μ L of cold PBS and an equal volume of cold 4% paraformaldehyde was added to each tube to fix the cells for 30 min at room temperature. 2 mL of ice-cold PBS was added to wash the cells and centrifuged at 400 g at 4°C for 3 min. 600 μ L of permeabilisation buffer was added to the cells and mixed before a further incubation at room temperature for 10 min. Cells were centrifuged as before and resuspended in permeabilisation buffer containing a pre-optimised dilution (1:10 dilution) of mouse anti-human alpha enolase antibody (clone L-27, Santa Cruz, UK) or isotype control. Each sample was mixed gently and incubated in the dark for 30 minutes at room temperature, after which another wash was performed before the addition of secondary antibodies as described in section 3.2.3.2.1). After this, cells were resuspended in a BSA/Azide buffer and analysis of cell surface and intracellular staining was performed using a Gallios flow cytometer (Beckman Coulter, UK).

3.2.3.3 ELISA

In order to detect autoantibody responses to alpha enolase in PCa and healthy sera, recombinant alpha enolase (Fitzgerald, USA) was coated overnight on NUNC 96 well plates. Recombinant alpha enolase was diluted to 100 ng/mL in coating buffer and left to adsorb overnight at 4°C onto the bottom of the wells. The next day, any unbound protein was washed away three times in a wash solution containing TBS containing 0.05% Tween-20. Each wash was carried out for 15 min at room temperature and plates were left shaking. Using a multichannel pipette, each well was blocked with 4% BSA for 90 min to prevent non-specific binding and washed again as described previously.

To determine alpha enolase autoantibody responses in sera, each serum sample was diluted 1:25 and plated in quadruplicate in 2% BSA in 0.05% Tween or 1:10,000 for mouse anti-human alpha enolase antibody, which served as a positive control. The solution was left to incubate in the wells for 90 min at room temperature to allow maximum binding of

antibodies to alpha enolase. Each well was then washed again as described previously to remove unbound primary antibodies.

Finally, a 1:3000 dilution of HRP labelled rabbit anti-human (for human sera) or goat anti-mouse antibody (for alpha enolase) secondary antibody was added to the wells. Secondary antibodies were allowed to bind to their respective primary antibodies for 90 min at room temperature. A final wash step was performed as described previously and to enable visualisation each well was incubated with 50 μ L TMB solution for 15 min. The reaction was stopped using the same volume of 1 N sulphuric acid before an absorbance reading at 450nm was measured.

3.3 RESULTS

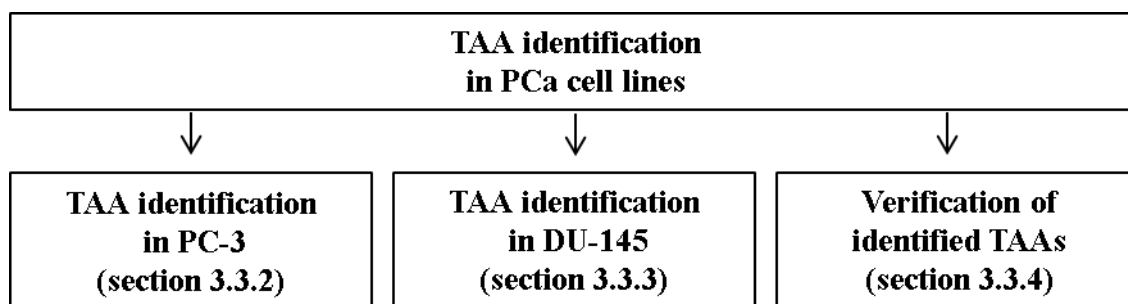


Figure 3.2: Overview of the identification of TAAs in PCa cell lines.

The identification of TAAs in this chapter is divided into 3 sections - identification using PC-3, DU-145 and the verification of identified TAAs.

3.3.1 Selection of appropriate PCa samples for biomarker discovery

Sera from healthy control male volunteers (above 35 years) at Nottingham Trent University (NTU) were collected and stored after ethical approval and informed consent forms had been signed (University ethical number 165). Only 3 volunteers had been recruited for our investigation at the time of the study and thus were available for comparison to patient sera (n=22). In addition, these healthy control samples could not be age matched to cancer and benign patients due to the limited number of volunteer sera available.

Sera from males aged 46-83 years who had been referred by their GP for high PSA levels and attending the urology clinic at the Urology Centre, Nottingham City Hospital, Nottingham, UK, were also obtained prior to diagnosis after ethical approval by the National Research Ethics Service Committee (NREC #09/H0S04/6). Although matched tumour tissue in the form of needle core biopsies were collected from these patients for pathological diagnosis and as part of this study, the amount of tissue (and protein) available was insufficient for 2-DE (20 μ L/IEF strip) and therefore not used as part of this experiment. As a result, PCa cell lines were explored as an alternative source of TAAs.

One of the sub-aims of this study was to identify serum derived biomarkers that could discriminate the so-called aggressive from the non-aggressive (tiger vs. pussycat) phenotype of Gleason 7 PCa. As a result, initial experiments aimed to identify biomarkers that could differentiate between both phenotypes. Consequently, a small subset of cancer and benign patients were chosen randomly from the samples available in the Nottingham City Hospital cohort to represent these patient categories (patients summarised in table 3.2).

Table 3.2: Patient serum samples used for TAA identification in PC-3

Patient category	Sample no.	Age	PSA (ng/mL)	Gleason 1	Gleason 2	Gleason grade	D'Amico Risk	TNM stage
PCa	PC04	56	4.9	3	4	7	Intermediate	-
PCa	PC05	65	8.9	3	4	7	Intermediate	-
PCa	PC06	71	5.4	3	4	7	Intermediate	T2N0M0
PCa	PC07	69	7.9	3	4	7	Intermediate	T2cN0M0
PCa	PC08	58	5.3	3	4	7	High	T2cN0M0
PCa	PC09	57	3.6	3	4	7	Intermediate	T2aN0M0
PCa	PC10	58	4.3	3	4	7	Intermediate	-
PCa	PC11	63	10.5	3	4	7	Intermediate	-
PCa	PC12	57	4.6	3	4	7	Intermediate	-
PCa	PC13	68	6.8	3	4	7	Intermediate	T2cN0M0
PCa	PC14	63	7.0	3	4	7	Intermediate	-
PCa	PC15	62	11.3	4	3	7	Intermediate	T2cN0M0
PCa	PC16	78	42.1	4	3	7	Mets	-
PCa	PC17	68	5.3	4	3	7	High	-
PCa	PC18	68	16.2	4	3	7	Intermediate	T3bN0M0
PCa	PC19	72	9.3	4	3	7	Intermediate	T2cN0M0
PCa	PC20 ¹	72	7.3	4	3	7	Intermediate	T1/2N0M0
PCa	PC21	64	4.4	4	3	7	Intermediate	T2N0M0
PCa	PC22	68	10.5	4	3	7	Intermediate	T2cN0M0
PCa	PC23	63	13	4	3	7	Intermediate	T1N0M0
PCa	PC24 ²	65	0.05	-	-	-	-	-
PCa	PC27	73	8.9	4	5	9	Mets	T3aN1M0
Benign	BE01 ³	56	4.9	3	4	7	Intermediate	-
Benign	BE02	62	8	-	-	Benign	-	-
Benign	BE03	67	23.2	-	-	Benign	-	-
Healthy	HE01	33	-	-	-	-	-	-
Healthy	HE02	36	-	-	-	-	-	-
Healthy	HE03	48	-	-	-	-	-	-

Sera was collected prior to diagnosis. To identify TAAs in PC-3, patients from the following categories were selected at random and chosen for this study. All patient samples were collected prior to diagnosis. A total of 21 PCa patients were chosen: Gleason 7 (3+4) (n=11), Gleason 7 (4+3) (n=10), benign (n=3) and healthy controls (n=3). ¹; post biopsy haemorrhage, ² prostatectomy sample, ³ BE01 was eventually diagnosed with Gleason 7 PCa, after the study was completed.

Two 'classic' PCa cell lines (DU-145 and PC-3) available in our laboratory were chosen for technique optimisation, initial TAA screening and biomarker identification. These were

chosen because they represent well characterised PCa cell lines which have been used for other biomarker identification studies in the literature (Saraon *et al.*, 2012). Thus, initial experiments aimed to identify TAAs in these well-characterised PCa cell lines, that elicit antibody responses to PCa patient or healthy sera. Although antibodies produced by an organism in response to antigens produced by their own tissues are referred to as 'autoantibodies', they will be referred to as 'autoantibodies' throughout this chapter regardless of the fact that the patient sera used in this study have not been produced in response to TAAs in PCa cell lines.

Before studies were conducted to investigate the presence of sera autoantibodies to PCa protein lysates, a series of optimisation experiments were performed to determine the best lysis buffer, optimum protein concentration to load on 2-DE, and the ideal serum dilution that showed low background after spot detection. Experiments were also performed to identify the best stain for detecting proteins on the replica gel which would facilitate spot identification by mass spectrometry, alongside the optimum incubation times for each experimental procedure. Subsequent experiments were carried out using these pre-optimised conditions (data not shown).

3.3.2 TAA identification in the PC-3 cell line

3.3.2.1 Reactivity of patient and healthy sera with protein extracts from the PC-3 cell line

To investigate the presence of antibodies against PC-3 proteins, the first experiment utilised a serological proteome analysis that combines 2-DE and Western blotting. In brief, 20 µg of total PC-3 protein lysate was separated by IEF in the first dimension and then by SDS PAGE in the second dimension. 2-DE separated proteins were transferred onto nitrocellulose membranes and probed with diluted sera (1:80 dilution) from either pooled healthy (n=3), benign (n=3) or PCa patients: Gleason 7 (3+4; n=10) or Gleason 7 (4+3; n=10) sera (figure 3.3). A replica gel was run simultaneously and visualised by Coomassie stain (150 µg total proteins) to facilitate spot identification by MALDI-TOF mass spectrometry.

As shown in figure 3.3, immunoblots probed with pooled PCa sera reacted with multiple spots (two red spots circled in figure 3.3) compared to pooled benign or healthy control sera. Both spots migrated at the same molecular weight (approximately 45 kDa) and an estimated pI of 6.0 and 6.5, showing strong reactivity to PCa patient sera [Gleason 7 (3+4) and Gleason 7 (4+3)] but not with sera from the control groups (benign and healthy controls). These spots were excised from the Coomassie stained replica gel, trypsin digested for analysis by MALDI-TOF MS. The other spots on the immunoblotted membrane in our experience are not spots of interest due to their fine, diffuse spot pattern which in our experience is consistent with milk deposits or incomplete washing of the nitrocellulose membrane. In addition, as this was the first study identifying TAAs in PCa cell lines, the most obvious spots were excised for MALDI-TOF MS analysis.

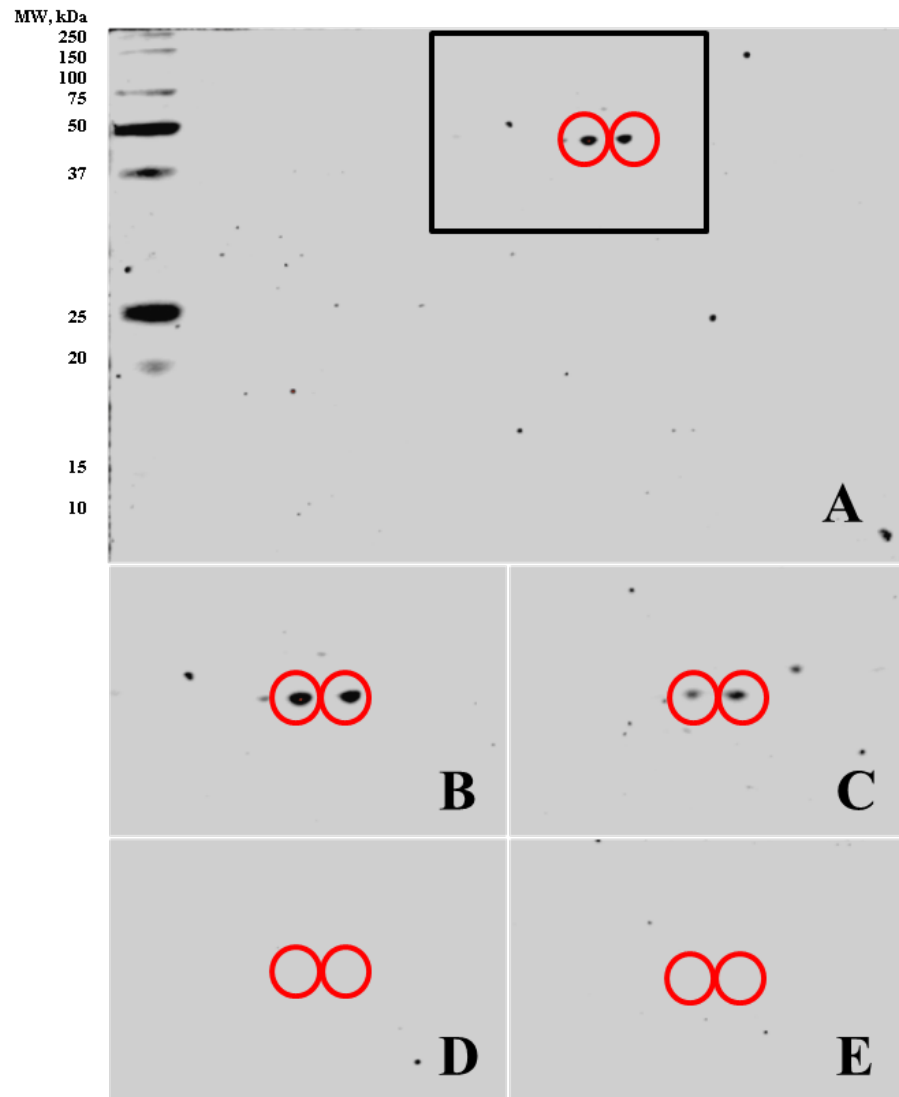


Figure 3.3: Immunoblot showing the reactivity of PCa, benign and healthy sera with protein extracts from the PC-3 cell line.

Protein lysates from PC-3 were separated by 2-DE, transferred to nitrocellulose membranes and immunoblotted with sera from a pool of Gleason 7 PCa patients, benign or healthy controls. An immunoblot probed with pooled Gleason 7 (4+3) sera (n=10) is shown in (A), where circled spots represent positive immunoreactivity. The most immunoreactive region of (A) is highlighted in the black square and enlarged in B, while immunoreactivity at the same regions of membranes probed with Gleason 7 (3+4), benign and healthy sera are depicted in (C), (D) and (E) respectively.

3.3.2.2 Identification of PC-3 TAAs that elicit antibody responses in PCa

To facilitate TAA identification in PC-3, Coomassie stained replica gels were manually aligned to the immunoprobed membrane (figure 3.4A & B). Spots corresponding to positive immunoreactivity from the immunoblots were excised from the replica gel and subjected to an overnight in-gel digest with trypsin. Tryptic peptides were cleaned up using C₁₈ ZipTip columns and spotted on a ground steel target plate for identification using MALDI-TOF MS. The acquired PMF was searched against the SwissProt human database (search criteria outlined in section 2.2.9.3.1) using the Mascot search engine to identify potential TAA candidates.

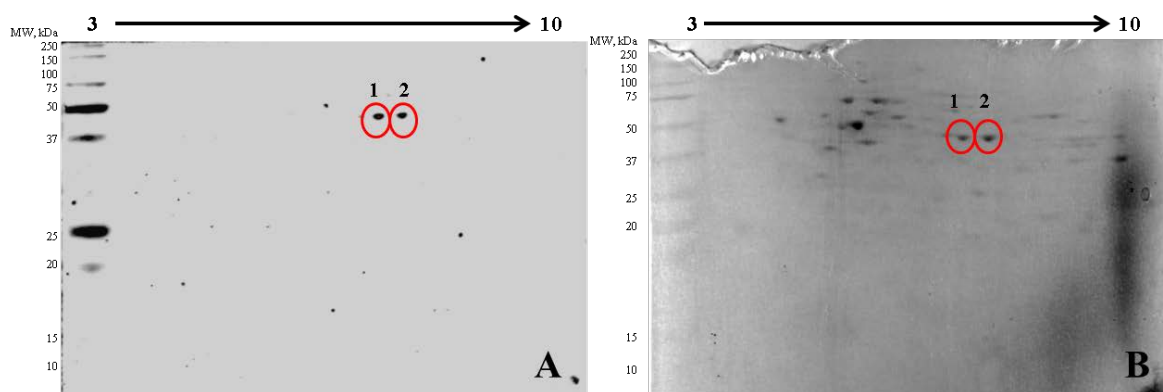


Figure 3.4: Image showing immunoblot to membrane alignment for immunoreactive TAAs identified in PC-3 PCa cell line.

Two spots showing positive immunoreactivity by Western blotting to PC-3 proteins (A) were manually aligned and to Coomassie stained replica gels (B). A is the same immunoblot from figure 3.3A. Spots were isolated from the corresponding region on replica gels and excised for identification by MALDI-TOF MS. Immunoreactivity to PCa Gleason 7 (4+3) sera is depicted in A.

After the SwissProt database search, both spots were identified with borderline significance as the same protein, alpha enolase (ENOA) by PMF. MOWSE scores of 41 and 61 were obtained for spot (1) and (2) respectively, where a score of >56 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$). In order to increase the confidence in the protein identified, peptides from each sample identified during PMF were fragmented using MALDI-TOF MS. As sample 1 yielded the lowest score by PMF, 6 peaks identified with low significance from sample 1

were subject to sequence analysis using MALDI-TOF/TOF (sequence analysis outlined in section 2.2.9.3.1) and are summarised in table 3.3.

Table 3.3: Identification of alpha enolase peptides from PC-3 by MALDI-TOF MS/MS

Protein	Observed <i>m/z</i> peak	Mascot score	SC (%)	Peptide
Alpha enolase	1406.7182	73/31	2%	R.GNPTVEVDLFTSK.G
	1804.9615	123/31	4%	R.AAVPSGASTGIYEALRLR.D
	1143.6204	42/31	2%	R.IGAEVYHNLK.N
	1556.7831	60/31	3%	K.VVIGMDVAASEFFR.S
	1425.7372	101/31	2%	R.YISPDQLADLYK.S
	1633.8877	127/31	3%	K.VNQIGSVTESLQACK.L

Six peaks identified with a low significance as alpha enolase by PMF were subject to sequence analysis by MALDI-TOF MS/MS. The Mascot ion score for each observed peak is shown in table 3.3 as well as the peptides they correspond to. SC; protein sequence coverage. 127/31; MOWSE score of peptide is 127, where a score >31 is considered significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$). R. or K. at the start or end of each peptide sequence represents tryptic cleavage sites for each peptide.

Figure 3.5 shows the full protein sequence of human alpha enolase, where bold peptides indicate all 6 matched peptides by MALDI TOF MS/MS. Tandem mass spectrometry of one of 6 matched peptides of alpha enolase **YISPDQLADLYK**, is also depicted in figure 3.5B, where the sequenced peptides was identified with a Mascot ion score (MOWSE score) of 101 (figure 3.5C) where a score of >31 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

Alpha enolase protein sequence: Matched peptides shown in **Bold Red**:

A

```

1      MSILKIHARE  IFDSRGNPTV EVDLETSKGL  FRAAVPSGAS TGIYEALELR
51     DNDKTRYMGK  GVSKAVEHIN  KTIAPALVSK  KLNVTEQEKI  DKLMIEMDGT
101    ENKSKFGANA  ILGVSLAVCK  AGAVEKGVPL  YRHIADLAGN  SEVILPVPFAF
151    NNINGGSHAG  NKLAMQEFMI  LPVGAANFRE  AMRIGAEVYH  NLKNVIKEKY
201    GKDATNVGDE  GGFAPNILEN  KEGLELLKTA  IGKAGYTDKV  VIGMDVAASE
251    FERSSGKYDLD  FKSPDDPSRY  ISPDQLADLY  KSFIKDYPVV  SIEDPFDQDD
301    WGAWQKFTAS  AGIQVVGDDL  TVTNPKRIAK  AVNEKSCNCL  LLKVNQIGSV
351    TESLQACKLA  QANGWGMVS  HRSGETEDTF  IADLVVGLCT  GQIKTGAPCR
401    SERLAKYNQL  LRIEELGSK  AKFAGRNFN  PLAK
    
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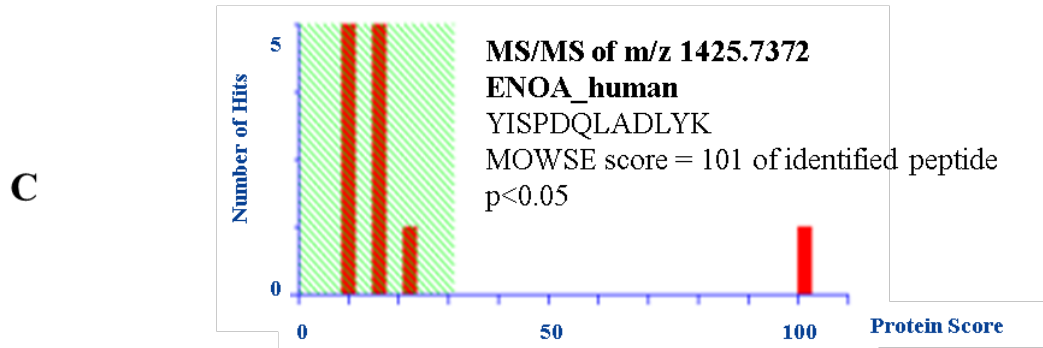
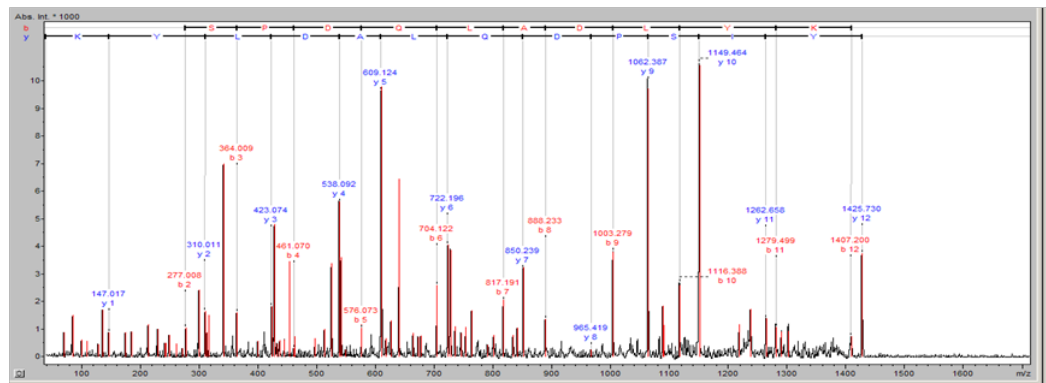


Figure 3.5: Identification of alpha enolase as a potential TAA from the PC-3 PCa cell line using MALDI-TOF MS/MS.

The figure shows the full length sequence of human alpha enolase where peptides in bold indicate all six matched peptides by MALDI-TOF MS (A). Panel (B) shows one of six sequenced peptides of alpha enolase **YISPDQLADLYK** with an m/z 1425.7372 by MALDI-TOF MS/MS. Panel (C) shows the Mascot ion score of the peptide **YISPDQLADLYK** after matching the SwissProt database. The sequenced peptide has a MOWSE score of 101 where a score >31 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

3.3.3 TAA Identification in DU-145

3.3.3.1 Selection of appropriate samples for PROTEOMEX screening in DU-145 PCa cell lines

Section 3.3.2 demonstrated that autoreactivity towards alpha enolase may occur in some PCa patients. The next study aimed to identify TAAs that may serve as biomarkers for PCa in DU-145 using the same method. Due to limited sample volumes, the same patient samples used to screen the PC-3 cell line could not be used to screen DU-145 lysates. Thus, another set of patients recruited at the time of diagnosis were chosen from the PCa sample cohort collected at Nottingham University Hospitals. Patients utilised for this study are listed in table 2.11 (section 2.3.3.1). This study was carried out at the same time as the 2-DE urine study (section 2.3.3), where it became apparent that the benign samples collected was classed as '*benign with a possibility of cancer*'. Thus, all benign samples were omitted from further analysis, in order to make the patient categories more stringent.

3.3.3.2 Reactivity of PCa and healthy sera with protein extracts from DU-145 PCa cell lines

To investigate the presence and patterns of serum IgG-based immunoreactivity against DU-145 cytosolic proteins, the same approach was used as in the PC-3 study. In brief, 20 µg of total DU-145 lysate was separated by 2-DE as described in section 3.3.2 and transferred onto nitrocellulose membranes to facilitate TAA screening. Serum (1:80 dilution) from a pool of PCa patients (n=26) or healthy control volunteers (n=3) were used for immunoscreening making up the discovery sample set. Figure 3.6 shows the immunoblots screened with PCa and healthy control sera, which yielded a larger number of spots (16 spots) compared to the PC-3 study.

Figure 3.6A-C demonstrates the immunoreactivity in a heterogeneous combination of sera, where six reactive protein spots were observed in control sera (circled in blue) and thus were considered to represent proteins expressed in normal patients and therefore not of interest. However, pooled PCa sera reacted exclusively and more intensely with twice the number of spots detected by healthy controls (circled in red). In addition, in the spots identified by both sample groups (circled in blue), spots from PCa sera were more prominent in 2 out of 6 spots, equally as reactive in both sample groups in 2 out of 6 spots,

and more reactive in healthy sera in 2 out of 6 spots. PCa and healthy sera immunoreactivity to DU-145 proteins mainly occurred in the 40 – 90 kDa molecular weight range with the strongest PCa detected spots around pI 6 – 7. As this was still a preliminary study to identify TAAs in PCa cell lines, only the most obvious spots which were clearly distinguishable from the background (circled spots) were chosen for MALDI-TOF analysis.

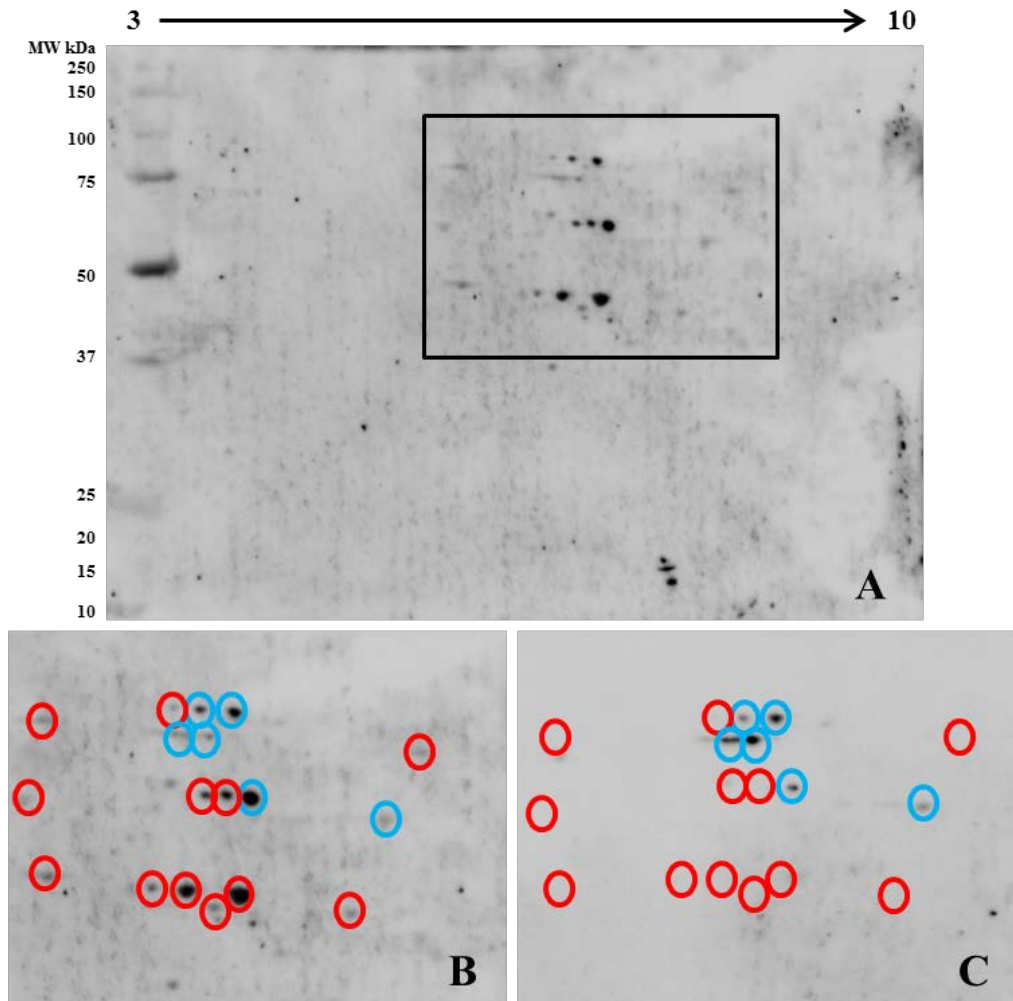


Figure 3.6: Immunoblots showing the screening of PCa and healthy sera against DU-145 PCa cell line proteins.

Protein lysates from DU-145 were separated by 2-DE, transferred onto a nitrocellulose membrane and immunoblotted with sera from a pool of PCa patients (Gleason 7, 8 and 9) or healthy controls. An immunoblot probed with pooled PCa sera is shown in (A), where the most immunoreactive region of the probed membrane (40 – 90 kDa) is highlighted in the black square and enlarged in (B). Immunoreactivity within the same region in healthy sera is depicted in (C). Spots identified by both healthy and PCa sera are circled in blue, whereas those identified exclusively by PCa patient sera are circled in red. All circled spots indicate those chosen for mass spectrometry.

3.3.3.3 Identification of TAAs from the DU-145 PCa cell line that elicit antibody responses in PCa sera

To facilitate TAA identification by MALDI-TOF MS, DU-145 lysates were separated by 2-DE and stained using Coomassie brilliant blue as described previously. 18 intense spots representing immunoreactivity with PCa or healthy sera were observed from immunoblots and circled in red or blue (figure 3.6); although only 16 of them could be assigned to protein spots on the Coomassie stained gels (figure 3.7). To identify these potential TAAs, the Coomassie stained replica gel and immunoprobed nitrocellulose membranes were manually aligned (figure 3.7) and corresponding spots were excised from the replica gel before being subjected to an overnight tryptic digest. Tryptic peptides were cleaned up and spotted on a ground steel target plate for identification using MALDI-TOF MS and the acquired PMF was searched against the SwissProt database using the Mascot search engine to identify potential TAA candidates. Identified proteins are summarised in table 3.4.

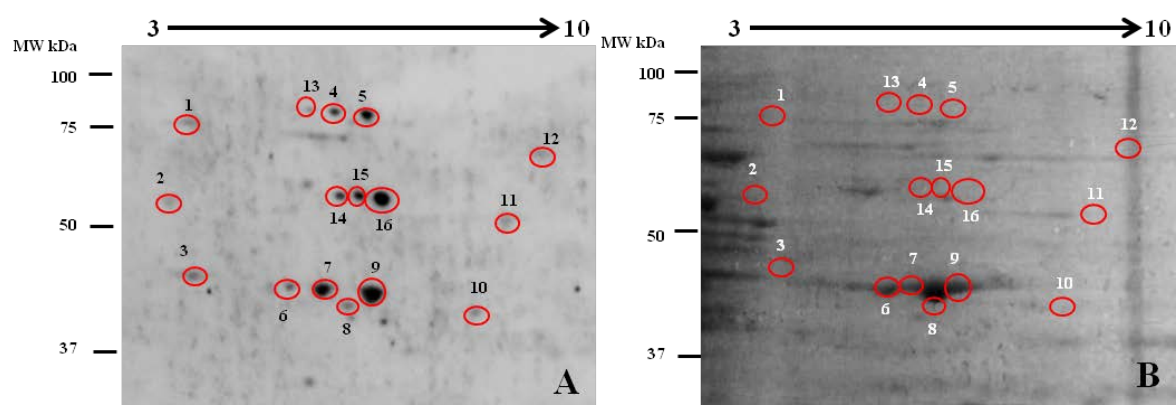


Figure 3.7: Image showing immunoblot to membrane alignment to identify immunoreactive TAAs in the DU-145 PCa cell line.

16 immunoreactive spots identified with pooled PCa (Gleason 7, 8 and 9) sera were labelled as shown in (A), and aligned to Coomassie stained replica gels (B) for spot picking and TAA identification by MALDI-TOF MS. Immunoblot and membrane shown are cropped around the molecular weight and pI where most of the immunoreactivity occurred, as shown by the black square in figure 3.6.

Table 3.4: DU-145 identified proteins immunoreactive to PCa or healthy sera

Spot no.	Protein	UniProt Accession	e.pI	t.pI	e. MW (kDa)	t. MW (kDa)	MOWSE score	Immunoreactivity with sera
1	No protein identified	-	-	-	-	-	-	PCa sera
2	Heat Shock Protein 60	CH60_HUMAN	5.0	5.7	57	61	68/29	PCa sera
3	Heterogeneous nuclear riboprotein	HNRFF_HUMAN	5.1	9.4	46	53	132/30	PCa sera
4	No protein identified	-	-	-	-	-	-	PCa & healthy sera
5	No protein identified	-	-	-	-	-	-	PCa & healthy sera
6	Alpha enolase	ENOA_HUMAN	6.5	7.01	45	48	65/56	PCa sera
7	Alpha enolase	ENOA_HUMAN	6.7	7.01	45	48	63/56	PCa sera
8	No protein identified	-	-	-	-	-	-	PCa sera
9	Alpha enolase	ENOA_HUMAN	7.0	7.01	45	48	88/56	PCa sera
10	WNT7A	WNT7A_HUMAN	8.0	9.05	43	39	37/30	PCa sera
11	ATP synthase subunit	ATPK_HUMAN	8.5	-	52	11	60/56	PCa & healthy sera
12	No protein identified	-	-	-	-	-	-	PCa sera
13	No protein identified	-	-	-	-	-	-	PCa sera
14	No protein identified	-	-	-	-	-	-	PCa sera
15	No protein identified	-	-	-	-	-	-	PCa sera
16	No protein identified	-	-	-	-	-	-	PCa & healthy sera

Identified TAAs from 2-DE immunoblots immunoreactive with PCa or healthy sera. Only 7 spots were identified out of 16 analysed by PMF using MALDI-TOF MS/MS. 65/56 = Mascot ion score of 65 where a score of >56 is considered significant. t; theoretical, e; experimental, pI; isoelectric point, MW; molecular weight.

3.3.3.4 Identification of the 47 kDa protein as alpha enolase by MALDI-TOF MS

16 proteins were shortlisted for MS identification from the PROTEOMEX screen in DU-145, however, only 7 spots yielded any protein identification by MALDI-TOF MS (table 3.4). Of these 7 proteins, three of them (spots 6, 7 and 9) separated at an estimated isoelectric point of 6.5, 6.7 and 7.0 and approximate molecular weight 47 kDa. All three proteins also showed strong reactivity with sera from PCa patients in comparison to controls (Figure 3.6 and Table 3.4) and were identified as the same protein, alpha enolase by PMF with significance (MOWSE score of 65, 63 and 88 for spots 6, 7 and 9 respectively, where a MOWSE score of 56 is considered significant).

In order to confirm the identification of alpha enolase by PMF, four peaks from sample 9 were chosen from the peptides identified by PMF and subjected to sequence analysis by MALDI-TOF/TOF (table 3.5). Figure 3.8A shows the whole sequence of alpha enolase where bold letters indicate all four matched peptides by MALDI TOF MS/MS. B shows the sequence analysis for one of four sequenced peptides of alpha enolase, **AAVPSGASTGIYEAL****ELR**, with a Mascot ion score of 114/30 (fig. 3.8C) where a score of >30 is significant, indicating identity or extensive homology to a known protein on the SwissProt database (p<0.05).

Table 3.5: Identification of alpha enolase peptides from DU-145 by MALDI-TOF MS/MS

Protein	Observed <i>m/z</i> peak	Mascot score	SC (%)	Peptide
Alpha Enolase	1804.9656	144/30	4%	R.AAVPSGASTGIYEAL ELR .D
	1924.0011	72/30	3%	K.LAMQEF <u>M</u> ILPVGAANFR.E
	1540.8016	102/31	3%	K.VVIGMDVAASEFFR.S
	1908.0095	108/30	3%	K.LAMQEF <u>M</u> ILPVGAANFR.E

Four peaks identified as alpha enolase in DU-145 by PMF were subjected to MALDI-TOF MS/MS. The Mascot ion score for each peak is shown as well as the peptide it corresponds to. SC; protein sequence coverage. 114/30; MOWSE score of peptide is 114, where a score >30 is considered significant; underlined M indicates an oxidation of methionine. R or K. at the start of the peptide sequence represents tryptic cleavage sites for each peptide.

Alpha enolase protein sequence: Matched peptides shown in **Bold Red**:

A

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1      MSILKIHARE  IFDSRGNPTV  EVDLFTSKGL  FRAAAVPSGAS TGIYEALELR
51     DNDKTRYMGK  GVSKAVEHIN  KTIAPALVSK  KLVNTEQEKI  DKLMIEMDGT
101    ENKSKFGANA  ILGVSLAVCK  AGAVEKGVPL  YRHIADLAGN  SEVILPVPFAF
151    NVINGGSHAG  NKLAMQEFMI LPVGAANFRE  AMRIGAEVYH  NLKNVIKEKY
201    GKDATNVGDE  GGFAPNILEN  KEGLELLKTA  IGKAGYTDKV  VIGMDVAASE
251    FFRSGKYDLL  FKSPDDPSRY  ISPDQLADLY  KSFIKDYPVV  SIEDPFDQDD
301    WGAWQKFTAS  AGIQVVGDDL  TVTNPKRIAK  AVNEKSCNCL  LLKVNQIGSV
351    TESLQACKLA  QANGWGMVVS  HRSGETEDTF  IADLVVGLCT  GQIKTGAPCR
401    SERLAKYNQL  LRIEELGSK  AKFAGRNFRN  PLAK
    
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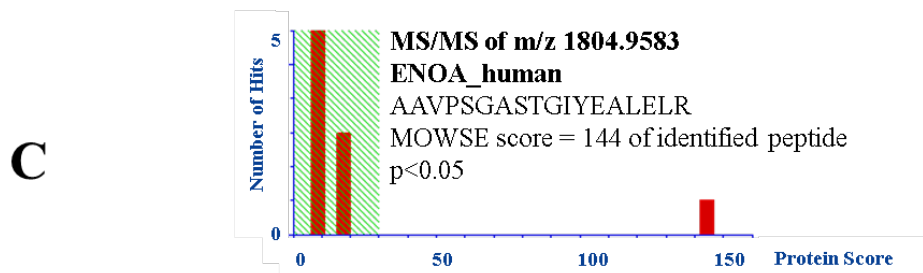
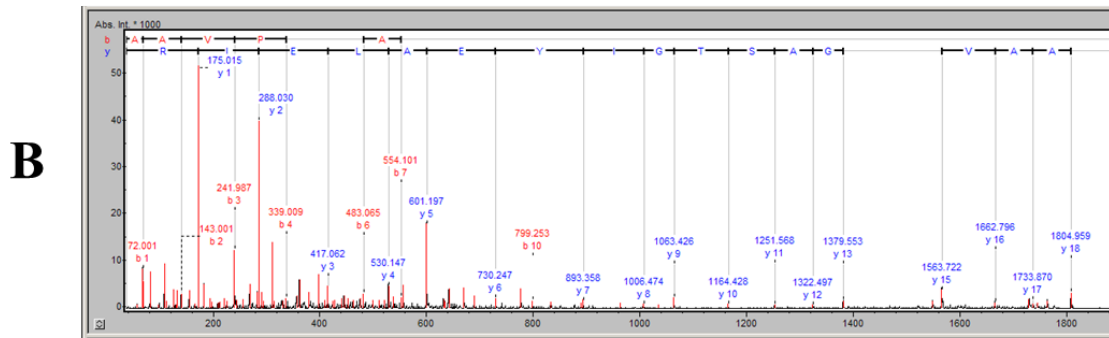


Figure 3.8: Identification of alpha enolase a as potential TAA from the DU-145 cell line using MALDI-TOF MS/MS.

The figure shows the full length sequence of human alpha enolase where peptides in bold indicate all four matched peptides by MALDI-TOF MS (A). Panel (B) shows one of four sequenced peptides of alpha enolase **AAVPSGASTGIYEALELR** with an m/z 1804.9583 by MALDI-TOF MS/MS. Panel (C) shows the Mascot ion score of the peptide **AAVPSGASTGIYEALELR** after matching the SwissProt database. The sequenced peptide has a MOWSE score of 144 where a score >30 is significant, indicating identity or extensive homology to a known protein on the SwissProt database ($p < 0.05$).

3.3.4 Confirmation of MALDI-TOF identification of alpha enolase in the DU-145 PCa cell line

Among the protein spots identified with high confidence by MALDI-TOF MS, this study focussed on alpha enolase as it was identified in more than one patient group and both PCa cell lines. However, before alpha enolase could be validated as a potential biomarker for PCa, it was important to confirm the MS identification of alpha enolase in PCa cell lines using other complementary techniques, and confirm the presence of a differential autoantibody response in the PCa and healthy sera samples analysed. Both studies were carried out using immunofluorescence, flow cytometry, western blotting, mass spectrometry and ELISA techniques.

3.3.4.1 Confirmation of alpha enolase expression in DU-145 using immunofluorescence, flow cytometry and Western blotting

To further demonstrate that alpha enolase is present in DU-145, the first experiments were carried out using immunofluorescence (method as described in section 3.2.3.1). In brief, DU-145 PCa cells were seeded and grown as a monolayer on round fluorescence compatible coverslips in 24 well plates. After a period of growth (about 48 hours), cells were examined to confirm the presence of alpha enolase by immunofluorescence using a commercially available specific monoclonal antibody raised against alpha enolase (clone L-27, Santa Cruz, UK) (figure 3.9A) or using a mouse IgG isotype control. Figure 3.9A shows intense cytoplasmic and nuclear staining for alpha enolase in DU-145 with some suggested staining observed around the outside of the cell (indicated by the red arrows on figure 3.9A). No staining was observed using the mouse IgG isotype control.

The presence of alpha enolase in DU-145 was also verified using flow cytometry. In brief, DU-145 cells were counted, washed and permeabilised before incubation with the same anti-alpha enolase monoclonal antibody (clone L-27, Santa Cruz, UK). Expression of alpha enolase in DU-145 was demonstrated by a Beckman Coulter Gallios flow cytometer which showed a positive intracellular stain, indicated by a large number of cells shifted to the right of the overlay plot (figure 3.9B). In addition, no staining was observed in any of the controls - cells alone, secondary alone or mouse IgG isotype (figure 3.9B). In order to confirm that alpha enolase potentially exists around the outside of the cell as implied by

immunofluorescence (red arrows in figure 3.9A), DU-145 cells were stained using flow cytometry to determine cell surface alpha enolase expression. Multiple attempts demonstrated no alpha enolase cell surface expression in DU-145 (appendix VI).

Finally, alpha enolase in DU-145 was verified using Western blotting. 20 μ g of DU-145 protein lysates were separated by 2-DE gels (3-10L IPG strips and 10% SDS PAGE) and transferred onto nitrocellulose membranes or stained with Coomassie blue as described in the discovery experiments (section 3.2.2.5). Membranes were probed with a monoclonal mouse-anti alpha enolase antibody at a 1:1000 dilution (clone L-27, Santa Cruz, UK) (figure 3.9C & D). Specific binding of the antibody was only observed at 49 kDa, and at a pH between 5 and 8. To further confirm the presence of alpha enolase in DU-145, a Coomassie stained replica gel was run alongside the analytical gel for spot picking. As with the discovery experiments, the membrane probed with the monoclonal mouse anti-alpha enolase antibody (figure 3.9C & D) was manually aligned to the stained gel (figure 3.9E). Two spots representing positive alpha enolase expression by Western blotting were chosen from the immunoblots (circled in red in figure 3.9C&D) and excised from the stained replica gel before analysis by MALDI-TOF MS.

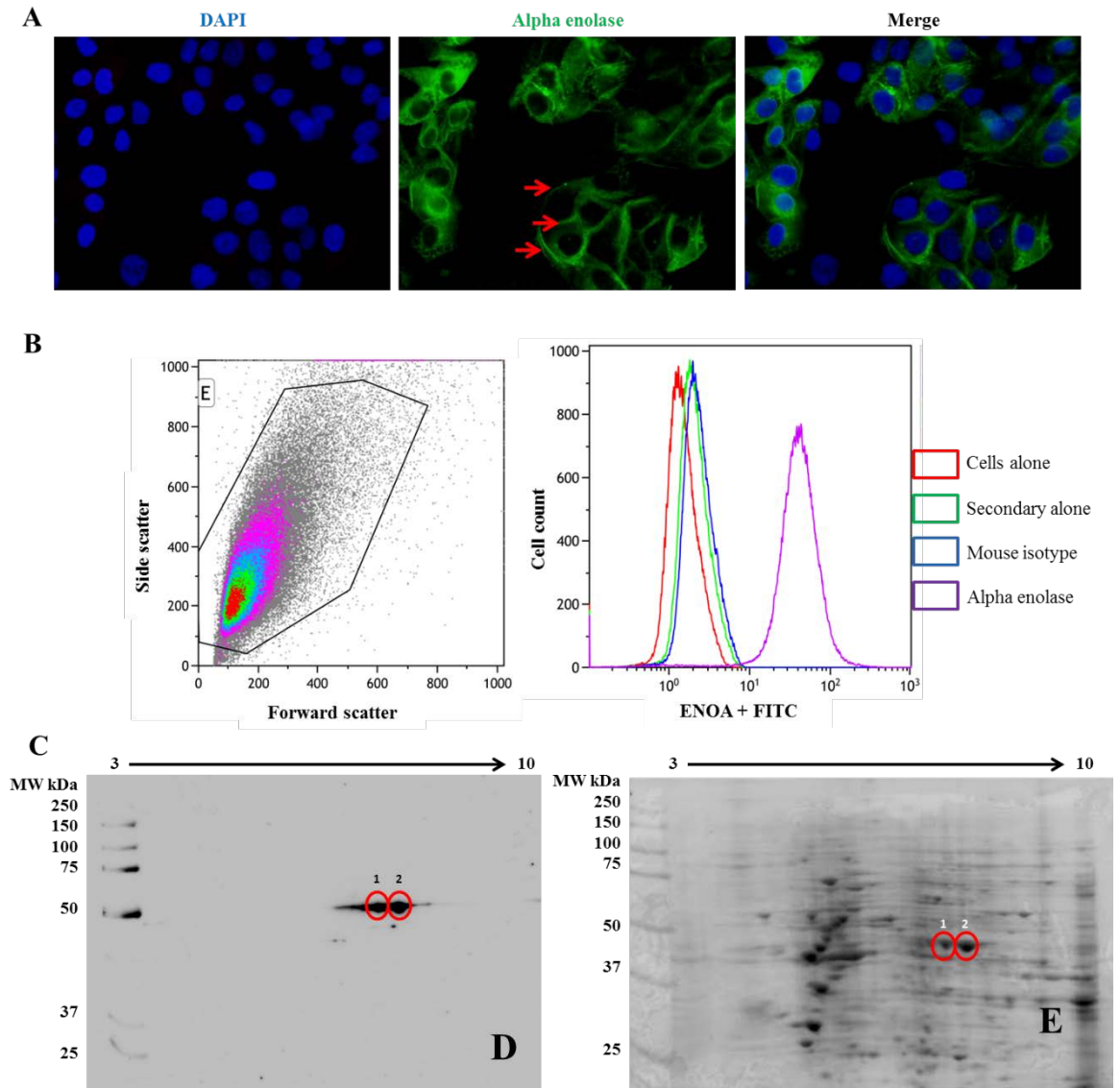


Figure 3.9: Verification of alpha enolase expression in DU-145 PCa cell line by immunofluorescence, flow cytometry and Western blotting.

The presence of alpha enolase in DU-145 was determined by immunofluorescence staining using a specific monoclonal mouse anti-alpha enolase antibody, with membrane staining indicated by red arrows (A) and using flow cytometry (B). Alpha enolase expression was also determined by Western Blotting where DU-145 lysates were separated by 2-DE and immunoblotted with a monoclonal alpha enolase antibody (clone L-27, Santa Cruz, UK). Positive reactivity enclosed by the red circle on the blotted membrane (D) indicates the presence of alpha enolase protein in DU-145. Spots corresponding to alpha enolase were excised from the Coomassie stained replica gel (E) for the confirmation of alpha enolase in DU-145 by mass spectrometry. *Objective magnification x 40.*

3.3.4.2 Confirmation of alpha enolase expression in DU-145 using mass spectrometry

Two spots at an estimated molecular weight of 49 kDa and a pI of 6.5 and 7.0 respectively showing positive expression of alpha enolase by Western blotting (figure 3.9C, D & E), were excised from a stained Coomassie gel and subjected to an overnight in-gel trypsin digest for TAA identification by MALDI-TOF MS (as described in section 2.2.9). Tryptic peptides were identified by PMF and used to search the SwissProt databases using the Mascot search engine (search criteria outlined in section 2.2.9.3.1). Identified proteins are summarised in table 3.6). Both spots were identified as the same protein, alpha enolase by PMF with a MOWSE score of 82 and 100 for spots 1 and 2 respectively (where a score >56 is considered significant).

Table 3.6: Verification of alpha enolase from DU-145 2-DE spots by MALDI-TOF MS/MS

Spot no.	Protein name	Accession	t. Isoelectric point	t. MW (Da)	Mascot score
1	Alpha enolase	ENOA1_HUMAN	7.01	47,841	82/56
2	Alpha enolase	ENOA1_HUMAN	7.01	47,841	100/56

Corresponding spots recognised by the monoclonal mouse anti-alpha enolase antibody were identified by MALDI-TOF by PMF or MS/MS of one or more of the peaks identified by PMF. 82/56 = Mascot score of 82 where a score >56 is considered significant. t; theoretical.

Nine peaks from one of the spots, (spot 2) were chosen from the PMF and subject to sequence analysis by MALDI-TOF/TOF. Figure 3.10A shows all nine peptides identified as alpha enolase using MALDI-TOF MS/MS, while figure 3.10B shows the whole sequence of alpha enolase where bold letters indicate all nine matched peptides by MALDI-TOF mass spectrometry.

Sample & protein	Observed peak	Mascot score	SC (%)	Peptide
A Spot 2: Alpha Enolase	1425.7435	90/30	2%	R.YISPDQLADLYK.S
	1540.7921	99/31	3%	K.VVIGMDVAASEFFR.S
	1556.7959	75/31	3%	K.VVIGMDVAASEFFR.S + Oxidation (M)
	1804.9647	178/31	4%	R.AAVPSGASTGIYEALRL.D
	1924.0048	88/31	3%	K.LAMQEFMILPVGAANFR.E + Oxidation (M)
	1908.0024	124/31	3%	K.LAMQEFMILPVGAANFR.E
	2033.0824	148/31	4%	K.FTASAGIQVVGDDLTVTNPK.R
	2353.1863	176/31	5%	R.SGETEDTFIADLVVGLCTGQIK.T + Carbamidomethyl (C)
	2510.1475	159/31	4%	K.DYPVVSIEDPFDQDDWGAWQK.F

Alpha enolase protein sequence: matched peptides shown in **bold red**.

1	MSILKIHARE	IFDSRGNPTV	EVDLFTSKGL	FR AAVPSGAS	TGIYEALRL
51	DNDKTRYMGK	GVS KAVEHIN	KTIAPALVSK	KLVNTEQEKI	DKLMIEMDGT
101	ENKSKFGANA	ILGVSLAVCK	AGAVEKGVPL	YRHIADLAGN	SEVILPVPF
151	NVINGGSHAG	NK LAMQEFMI	LPVGAANFR	AMRIGAEVYH	NLKNVIKEY
B 201	GKDATNVGDE	GGFAPNILEN	KEGLELLKTA	IGKAGYTDK V	VIGMDVAASE
251	FFR SGKYDLLD	FKSPDDPSRY	ISPDQLADLY	KSFIKDYPVV	SIEDPFDQDD
301	WGAWQKFTAS	AGIQVVGDDL	TVTNP KRIAK	AVNEKSCNCL	LLKVNQIGSV
351	TESLQACKLA	QANGWGMVS	HR SGETEDTF	IADLVVGLCT	GQIKT GAPCR
401	SERLAKYNQL	LRIEELGSK	AKFAGRNFNRN	PLAK	

Figure 3.10: Verification of alpha enolase by MALDI-TOF mass spectrometry.

Panel (A) shows the nine peaks identified as alpha enolase by PMF, which were then subjected to MALDI-TOF MS/MS. The Mascot ion score for each peak is shown as well as the peptides they correspond to. Panel (B) shows the full length protein sequence of alpha enolase where bold letters indicate all nine matched peptides by mass spectrometry. SC; protein sequence coverage. R or K. at the start of the peptide sequence represents tryptic cleavage sites for each peptide.

3.3.4.3 Confirmation of the presence of alpha enolase autoantibodies in pooled discovery sera

To confirm that pooled PCa and healthy discovery sera contained autoantibodies to alpha enolase, a full-length commercially available recombinant alpha enolase protein was purchased from Fitzgerald (USA) to serve as a positive control. Recombinant alpha enolase was separated by 10% SDS PAGE and transferred onto nitrocellulose membranes before probing with pooled (discovery) sera from PCa and healthy subjects (i.e. same patient samples used in the DU-145 discovery/identification experiments).

Various concentrations of recombinant alpha enolase (1000 ng, 100 ng, 50 ng, 25 ng & 10 ng) were separated by SDS PAGE and immunoprobed with PCa or control sera (1:80 dilution; same as the discovery experiments). Expression of alpha enolase was demonstrated at the expected 47 kDa molecular weight using a mouse anti-alpha enolase antibody probed against the recombinant protein, which served as a positive control (figure 3.11). Figure 3.11A showed that pooled PCa sera was able to detect as little as 25 ng of recombinant alpha enolase, whereas pooled healthy sera could not detect beyond 1000 ng by Western blot analysis (figure 3.11A). No bands were observed when PCa or healthy sera were screened with various concentrations of BSA, which served as a negative control.

A similar study was carried out using an ELISA assay to demonstrate that PCa and healthy sera contained differential titres of alpha enolase autoantibodies. A pre-optimised concentration of alpha enolase (100 ng/mL) was coated on each well of a 96 well plate overnight and incubated with various dilutions of pooled PCa or healthy (discovery) sera. Using a two way ANOVA, statistical significance ($p=0.001$) was observed at each dilution between PCa and control sera at $p<0.05$ (figure 3.11B). This study was also carried out in order to determine the optimum sera dilution required for subsequent verification and validation experiments by ELISA (chapter 5). Similar to figure 3.11A, figure 3.11B shows that PCa sera contain more autoantibodies to alpha enolase than healthy sera even at the same antigen concentration. Results from both studies indicate that pooled PCa (discovery) sera contain more autoantibodies against recombinant alpha enolase compared to healthy sera.

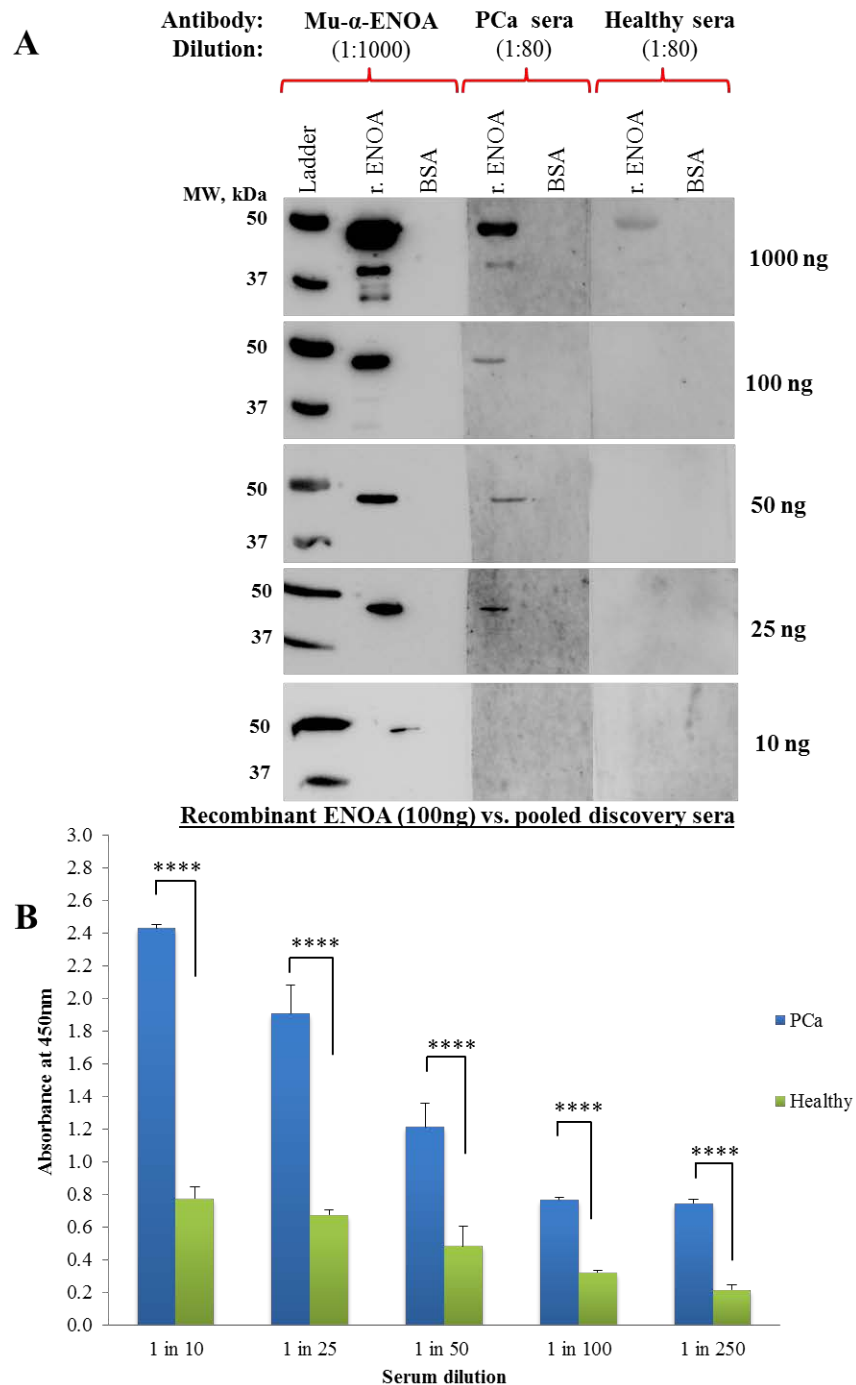


Figure 3.11: Detection of autoantibodies to various concentrations of recombinant alpha enolase and various dilutions of pooled PCa and healthy sera.

Panel A shows recombinant alpha enolase protein titrated at various concentrations (10 – 1000 ng) and loaded onto SDS PAGE gels to verify the presence of autoantibodies to recombinant alpha enolase in pooled patient or healthy (discovery) sera. Sera were used at the same dilution as those used in the discovery experiments. r; recombinant alpha enolase. Panel B shows the mean optical density of pooled PCa and healthy sera from the discovery sample set to 100 ng of recombinant alpha enolase. Statistical significance ($p=0.001$) was observed at each dilution between PCa and control sera at $p<0.05$.

3.3.4.4 Confirmation of the prevalence of alpha enolase sera autoreactivity in patients with PCa

Section 3.3.4.3 demonstrated alpha enolase specific reactivity patterns to pooled PCa sera, indicating that autoantibodies to alpha enolase may serve as a potential PCa biomarker following additional validation. It was important to then determine the frequency of alpha enolase autoantibody occurrence in the patients studied. To do this, all patients that made up the discovery set were analysed using an ELISA assay. Serum from each patient – PCa (n=26) or healthy controls (n=3) (1:25 dilution) was added to 96 well plates coated with recombinant alpha enolase. Sera from individual PCa patients were compared against pooled healthy sera (n=3), due to insufficient volumes of healthy sera. Wells containing recombinant alpha enolase protein and incubated with a monoclonal anti-alpha enolase antibody served as a positive control, while wells containing secondary alone or recombinant protein alone served as a negative control. No increase in absorbance was observed in wells containing only recombinant alpha enolase or secondary antibody (data not shown).

Figure 3.12 shows an increase in the average absorbance values of various PCa samples to recombinant alpha enolase, compared to pooled healthy controls, indicating a differential serum autoantibody titre to alpha enolase in the samples studied. Due to the limited volume of sample available, alpha enolase expression could only be studied in a pool of healthy control sera compared to individual PCa sera (n=26 patients). As a result, the conventional statistical test could not be performed between the two patients groups to determine whether there was a significant difference between both groups. Nevertheless, the average optical density of the pooled healthy control samples was 0.34 and 21 out of the 26 PCa patients studied (81%) had an average optical density >0.34 (mean optical density of healthy sera is indicated by a red dashed line in figure 3.12). Figure 3.12 also shows the average absorbance of all patients studied when arranged according to their Gleason grade, where there seemed to be no association between Gleason grade and alpha enolase autoantibody quantity.

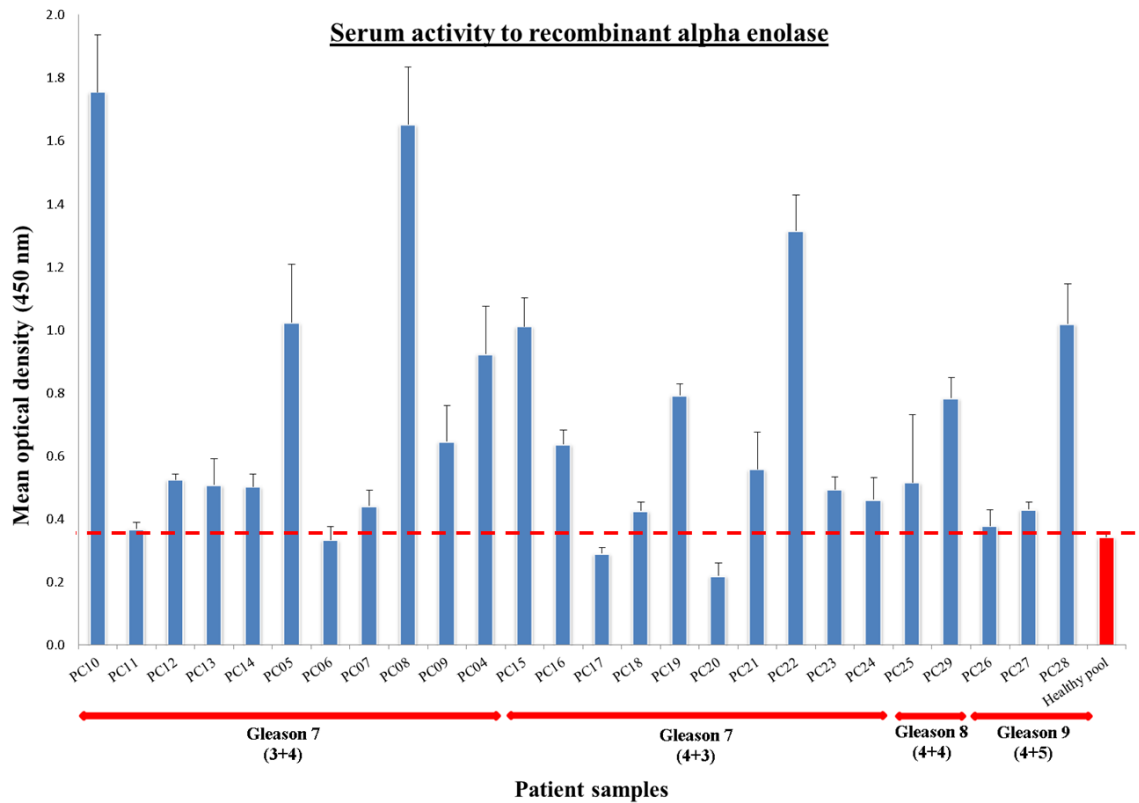


Figure 3.12: ELISA assay showing the mean optical density for PCa or pooled healthy sera towards recombinant alpha enolase.

The graph shows the mean optical density (at 450 nm) of 26 PCa or pooled healthy sera towards 100 ng recombinant alpha enolase. Samples which made up the discovery set were utilised in this study - Gleason 7 (3+4) (n=11), Gleason 7 (4+3) (n=10), Gleason 8 (n=2) and Gleason 9 (n=3). Vertical error bars indicate standard deviation of the replicates (n=3) from each sample. Dashed red line indicates the average absorbance at 450 nm of healthy control sera. As healthy sera was only available in a small pool to compare with individual PCa samples, it was not possible to perform the conventional statistical test to determine the significance of autoantibody titres between PCa and healthy control groups.

3.4 DISCUSSION

The current study aimed to identify TAAs and autoantibodies which can serve as biomarkers for PCa. To this end, a serological analysis of the proteome (PROTEOMEX), which combines 2-DE and Western blotting to detect autoantibodies in the sera of patients to TAAs in two classic PCa cell lines was utilised. Although PROTEOMEX is more usually carried out using tumour tissues, the lack of sufficient tissue material allowed PCa cell lines to be investigated as a source of biomarkers.

3.4.1 TAA identification in the PC-3 cell line

Initial screening in the PC-3 PCa cell line used a small subset of Gleason 7 (3+4), (4+3), benign and healthy controls samples to identify TAAs in PC-3. A sub-aim of the study was to identify biomarkers which could distinguish the aggressive from non-aggressive form of Gleason 7 PCa. Using PROTEOMEX, two intense spots reacted with sera from newly diagnosed PCa patients and not from healthy controls, suggesting that these proteins evoke a humoral immune response in PCa (figure 3.3). Using PMF and MALDI-TOF MS/MS (figure 3.5 and table 3.3), both spots were identified as the same protein, alpha enolase. Figure 3.3 also shows that both spots were more intense in Gleason 7 (4+3) compared to Gleason 7 (3+4) although the same amount of protein was loaded on both gels. This suggests that there may be a higher titre of autoantibodies to alpha enolase in the aggressive form of Gleason 7 PCa (Gleason 7 4+3) compared to the less aggressive form (Gleason 7 3+4). However, to further validate this finding, additional studies utilising an ELISA assay need to be carried out on a large cohort of patients with both Gleason 7 (4+3) and Gleason 7 (3+4) PCa to determine if there is a relationship between alpha enolase expression and PCa aggressiveness.

Alpha enolase is one of the most abundantly expressed cytosolic proteins in many organisms (Diaz-Ramos *et al.*, 2012). It is expressed by most cells, where its level of expression is dependent on the pathophysiological, developmental and metabolic cell state (McAlister and Holland, 1982). Enolase catalyses the dehydration of 2-phospho-D-glycerate (2-PG) to phosphoenolpyruvate (PEP) in the 9th step of glycolysis (Tomaino *et al.*, 2011). Many studies have reported the use of a similar immunoproteomic approach to

identify autoantibodies to alpha enolase. He *et al* (2007) identified autoantibodies to alpha enolase in 27.7% of non-small cell lung cancer (NSCLC) patients compared to 1.7% of healthy controls. Immunohistochemical tissue staining also found an increased alpha enolase expression in NSCLC tumour tissues, which was confirmed by flow cytometry showing alpha enolase expression on cancer cell surfaces. Using a proteomics approach, Tomaino *et al* (2011) also identified circulating autoantibodies to two acidic forms of phosphorylated alpha enolase in 62% of patients with pancreatic cancer compared to 4% of healthy controls.

3.4.2 TAA identification in the DU-145 cell line

The DU-145 study (section 3.3.3) utilised another classic PCa cell line to screen for PCa sera immunoreactivity. Due to sample volume limitations, the same set of samples used for the PC-3 screen was not used for the DU-145 screen. Furthermore, in comparison to other studies in the literature who have carried out TAA identification using PROTEOMEX, the number of immunoreactive spots yielded from the PC-3 study was low (2 spots in our study, compared to 25 spots on average in the literature). Thus, the number of patients utilised for screening was increased. The new discovery sample set consisted of PCa patients with a medium to high Gleason grade (Gleason 7, 8, 9); because it was hypothesised that sera from these patients would contain more TAAs and autoantibodies than those with lower grades, because they had more aggressive tumours. At this point in the study, no more healthy control male volunteers had been recruited for this investigation leaving the number of control sera from the PC-3 experiment the same as the one used to screen the DU-145 lysates (n=3).

DU-145 immunoscreening revealed more immunoreactive spots than PC-3 (figure 3.6; 18 immunoreactive spots in DU-145 compared to 2 spots in PC-3). In addition, PCa sera reacted exclusively and more intensely with twice the number of spots compared to healthy sera, suggesting increased autoantibody responses in PCa compared to healthy controls (figure 3.6). The visualisation of spots in DU-145 occurred within a similar molecular weight region as PC-3 (40 – 90 kDa) range. Other studies using PROTEOMEX have also found this molecular weight region the most immunoreactive (Desmetz *et al.*, 2008 and Forgber *et al.*, 2009).

Although 18 spots were differentially expressed on immunoblots, only 16 could be matched to proteins on the replica gel due to difficulties in aligning immunoblots to mini-gels. Out of the 16 spots analysed by MALDI-TOF MS, only 7 spots could be identified as 5 different proteins (summarised in table 3.4). This could be due to insufficient protein in the sample, insufficient tryptic cleavage sites on the protein, sample contamination which masks peptide signals in mass spectrometry or protein sequences not being present in the MASCOT database searched. In terms of biological processes however, the TAAs identified belong to several groups; immune response, B-cell activation and cell surface binding (CH60 or HSP-60), cell-cell signalling (WNT7A), metabolic enzymes (alpha enolase) and energy metabolism (ATP synthase subunit). HSP-60 belongs to a family of chaperone proteins and has previously been shown to induce an immune response in hepatocellular carcinoma (Hong *et al.*, 2015), colorectal cancer and ductal carcinoma in situ (Desmetz *et al.*, 2008). Desmetz *et al.* (2008) also demonstrated that autoantibodies to HSP-60 could serve as a potential breast cancer biomarker, where 32% of patients with ductal carcinoma in situ (DCIS) had autoantibodies to HSP-60 compared to 4.3% of healthy controls. Furthermore, a significantly higher frequency of autoantibodies to HSP-60 was found in high grade DCIS patients compared to patients with lower grade cancers. WNT7A part of the WNT signalling pathway, has been found to be over-expressed in the epithelium of serous ovarian carcinomas, but undetected in normal ovary, benign and endometrial carcinomas, suggesting its role as a TAA (Yoshioka *et al.*, 2012).

Out of several candidate TAAs identified, this study observed for the first time that alpha enolase was capable of inducing a specific sera reactivity in a subset of PCa patients, compared to healthy controls. Interestingly, although two sets of patient samples (and two separate cell lines) were utilised for TAA identification, both studies identified reactivity to alpha enolase in PCa, warranting further investigation into the potential utility of alpha enolase as a biomarker for PCa. It is also well documented that the overexpression of alpha enolase in various tumours and its ability to induce a humoral and/or cell mediated immune response in various cancers, allows it to be classified as a true TAA (Capello *et al.*, 2011).

3.4.3 Tumour antigen confirmation and verification

Although alpha enolase was identified with a high level of significance in PC-3 and DU-145, it was important to demonstrate that its identification was not due to false discovery. Thus, verification experiments aimed to confirm results obtained by immunoblotting and MALDI-TOF mass spectrometry (sections 3.3.1 and 3.3.2), to demonstrate the presence of alpha enolase in DU-145 and a differential expression of serum autoantibodies to alpha enolase in the discovery cohort using immunofluorescence, flow cytometry, Western blotting and mass spectrometry.

DU-145 cells were immunofluorescently screened with a monoclonal anti-alpha enolase antibody which demonstrated correct alpha enolase expression in the cytoplasm and some potential membrane expression (figure 3.9). Alpha enolase expression was also verified by flow cytometry, Western blotting and MALDI-TOF MS (figure 3.9 and 3.10); where extracellular staining flow cytometry staining failed to detect membranous alpha enolase (appendix VI). It is well documented that alpha enolase is expressed on the membrane of cells, where it acts as a plasminogen receptor in cancer cells (Lopez-Aemany *et al.*, 1994), leukocytic cell lines (Lopez-Aemany *et al.*, 2003) and monocytoid cells (Miles *et al.*, 1991). The expression of membranous alpha enolase is likely to be dependent on the activation status or pathophysiological conditions of the cells (Fontan *et al.*, 2000 and Arza *et al.*, 1997). However, this study failed to confirm membranous alpha enolase expression in DU-145, possibly due to cell surface proteins cleaved off during cell harvesting by Trypsin & Versene treatment.

The presence of autoantibodies to alpha enolase in sera was also verified (section 3.3.4.3). Using the same pooled sera from the DU-145 discovery study, Western blotting and ELISA showed a differential expression of sera autoantibodies in PCa sera compared to controls (figure 3.11). When patients in the PCa pool were screened individually using an ELISA assay, a higher level of autoantibody response was detected in PCa patients compared to a pool of healthy controls (figure 3.12). Due to sample volume limitations, it was not possible to carry out a statistical test to determine if the observed differences in autoantibody responses were statistically significant. Studies to demonstrate significance were carried out in subsequent chapters (chapter 5).

Previous research has identified alpha enolase autoantibodies in PCa. Autoantibodies to alpha enolase were first demonstrated in one PCa patient by Adamus *et al.* (1996) and subsequently in another PCa patient by Weleber *et al.* (2005). Alpha enolase has also been identified as a TAA in chronic myeloid leukaemia (Zou *et al.*, 2005), HER2+ breast cancer (Mojtahedi *et al.*, 2011) non-malignant diseases such as rheumatoid arthritis (Saulot *et al.*, 2002) and 41% of asthma patients (Nahm *et al.*, 2006).

To our knowledge, this is the first report outlining the presence of alpha enolase autoantibodies in a subset of (Gleason 7, 8 and 9) PCa patients. However, the occurrence of alpha enolase autoantibodies in a large sample cohort needs to be experimentally determined in order to validate our findings, which constitutes part of the penultimate chapter (chapter 5). The identification of alpha enolase as a TAA and potential PCa biomarker in this study demonstrates the robustness of the methodology developed in identifying relevant TAAs for PCa.

CHAPTER 4: EVALUATION OF ALPHA ENOLASE PROTEIN EXPRESSION IN OTHER BIOLOGICAL SAMPLES

4.1 INTRODUCTION

Early diagnosis and treatment of PCa increases the possibility of curing the disease by avoiding the progression and development of micro metastasis in localised tumours (Smith *et al.*, 2003). PSA, the first line test for PCa detection currently lacks the sensitivity and specificity required for accurate cancer diagnosis, resulting in an increase in PCa incidence due to the widespread use of PSA screening (Stanford *et al.*, 1999). This emphasises the need for the identification of more accurate markers which can detect the presence of prostate tumours but more importantly, distinguish indolent from aggressive diseases.

Using MALDI-TOF mass spectrometry, the previous chapter identified antibody responses to alpha enolase in two metastatic PCa cell lines (PC-3 and DU-145; section 3.3.2 & 3.3.3), suggesting that alpha enolase may serve as a potential PCa biomarker following additional investigation. However, before studies could be carried out to validate its utility as a potential PCa biomarker, it was important to evaluate the expression of alpha enolase in more than one prostate relevant biological sample. This would further confirm studies reported in the literature regarding its expression pattern and justify its validation in other biological samples in the subsequent chapter.

Discovered in 1934 by Lohmann and Meyerhoff, enolase (phosphopyruvate hydratase) is a homodimeric metalloenzyme which catalyses the dehydration of 2-phospho-D-glycerate (2-PG) to phosphoenolpyruvate (PEP) in the 9th step of the glycolytic pathway (Pancholi, 2001). During gluconeogenesis, enolase also catalyses the reverse reaction in the anabolic pathway which hydrates phosphoenolpyruvate to 2-phospho-D-glycerate (World, 1971). Enolase is a metalloenzyme, requiring Mg^{2+} ions for its catalytic activity (Brewer, 1981 and Brewer and Ellis, 1983). Three isoforms of the enzymes exist in mammals: α -enolase (ENO1), γ -enolase (ENO2) and β -enolase (ENO3), all encoded by three separate genes

ENO1, ENO2, ENO3, with high sequence identity. The isoform expression of enolases is tissue specific: ENO1 is found in most adult tissues while ENO2 is present in neurons and neuroendocrine tissues and ENO3 in muscle tissues (Craig *et al.*, 1990, Feo *et al.*, 1990 and Rider and Taylor, 1975).

Depending on its cellular location, alpha enolase plays a functional role in several physiological processes. Using an alternative start codon, the ENO1 gene can be translated into a 37 kDa protein, c-myc promoter binding protein (MBP-1). In the nucleus, MBP-1 binds to the c-myc P2 promoter acting as a transcription repressor which leads to tumour suppression by its interaction with histone deacetylase (Feo *et al.*, 2000). Furthermore, studies have shown the association of ENO1 with MBP-1 in the transcriptional regulation of the c-myc oncogene (Subramanian and Miller, 2000). In addition to its role in transcriptional regulation, alpha enolase acts as a stress protein, upregulated by the activation of hypoxia-inducible factor (HIF-1) and increasing the protection of cells by increasing anaerobic metabolism (Semenza *et al.*, 1996 and Aaronson *et al.*, 1995).

In pancreatic, breast and lung cancers, alpha enolase has been identified on the surface of tumour cells (Capello *et al.*, 2009, Seweryn *et al.*, 2009 and He *et al.*, 2007), while it is secreted by exosomes in melanoma and non-small cell lung cancer (Mears *et al.*, 2004 and Yu *et al.*, 2006). Alpha enolase lacks a transmembrane domain; as a result, it is not clear how it translocates to the cell surface. However, it is well documented that the binding of alpha enolase to its plasminogen receptor leads to the activation of plasminogen to plasmin by tissue-type (tPA) or urokinase type (uPA) plasminogen activators, further contributing to physiological and pathophysiological processes such as embryogenesis and tissue remodelling, inflammatory responses, cell invasion and tissue metastasis (Felez 1998, Moscato *et al.*, 2000; Lopez-Aleman *et al.*, 2003 and Plow *et al.*, 1995).

Alpha enolase is overexpressed in many tumours (table 4.1), where it induces a humoral and/or cell mediated immune response, classifying it as a true TAA (Capello *et al.*, 2011). Otto Warburg observed that cancer cells consume more glucose than their normal counterparts and thereby generate ATP by converting pyruvate to lactic acid, even in the presence of a normal supply of oxygen (Warburg, 1930 and Vander Heiden *et al.*, 2009). This mechanism, termed the 'Warburg effect' is observed in many tumours where glycolytic enzymes are upregulated by hypoxia-inducible factor. As alpha enolase contains

a hypoxia responsive element, an upregulation of alpha enolase has been identified at both the mRNA and protein level in many cancers, as summarised in table 4.1.

Table 4.1: Increased expression of alpha enolase in cancer

Cancer	Alpha enolase mRNA or protein expression
Brain	mRNA (Altenberg and Greulich, 2004)
Breast	mRNA (Tu <i>et al.</i> , 2010), protein (Somari <i>et al.</i> , 2003, Malorni <i>et al.</i> , 2006 and Kabbage <i>et al.</i> , 2008)
Cervix	mRNA (Altenberg and Greulich, 2004), protein (Bae <i>et al.</i> , 2006 and Bae <i>et al.</i> , 2005)
Colon	mRNA (Altenberg and Greulich, 2004), protein (Katayama <i>et al.</i> , 2006 and Wong <i>et al.</i> , 2008)
Eye	mRNA (Altenberg and Greulich, 2004)
Gastric	mRNA (Altenberg and Greulich, 2004), protein (Qi <i>et al.</i> , 2005 and Zhao <i>et al.</i> , 2007)
Head & neck	mRNA (Tsai <i>et al.</i> , 2010), protein (Govekar <i>et al.</i> , 2009)
Kidney	mRNA (Altenberg and Greulich, 2004)
Leukaemia	protein (Lopez-Pedraza <i>et al.</i> , 2006)
Liver	mRNA (Altenberg and Greulich, 2004, Hamaguchi <i>et al.</i> , 2008), protein (Takashima <i>et al.</i> , 2005)
Lung	mRNA (Altenberg and Greulich, 2004), protein (Li <i>et al.</i> , 2004, Li <i>et al.</i> , 2006, Huang <i>et al.</i> , 2006, Rubporn <i>et al.</i> , 2009, Chang <i>et al.</i> , 2006)
Muscle	mRNA (Altenberg and Greulich, 2004)
Ovary	mRNA (Altenberg and Greulich, 2004), protein (Cao <i>et al.</i> , 2010)
Pancreas	mRNA (Altenberg and Greulich, 2004), protein (Shen <i>et al.</i> , 2004, Capello <i>et al.</i> , 2009, Mikuriya <i>et al.</i> , 2007)
Prostate	mRNA (Altenberg and Greulich, 2004), protein (Rehman <i>et al.</i> , 2004)
Skin	mRNA (Suzuki <i>et al.</i> , 2010)
Testis	mRNA (Altenberg and Greulich, 2004)

The expression of alpha enolase at the mRNA or protein level reported in various cancers. Adapted from Capello *et al.* (2011).

4.1.1 Aims and objectives

The aim of this chapter is to evaluate the expression of alpha enolase in various biological samples, in order to validate its potential utility as a PCa biomarker. This will be carried out by:

1. Demonstrating and confirming the expression of alpha enolase in four available PCa cell lines – PC-3, LNCaP, OPCT-1 and DU-145 using immunofluorescence, flow cytometry and Western blotting.
2. Demonstrating and confirming the expression of alpha enolase in the urine of PCa and healthy controls by Western blotting.
3. Demonstrating and confirming the expression of alpha enolase in normal organ tissues, multiple tumour tissues and cancer adjacent normal tissues and a small cohort of PCa patient tissues using immunohistochemistry.

4.2 METHODS

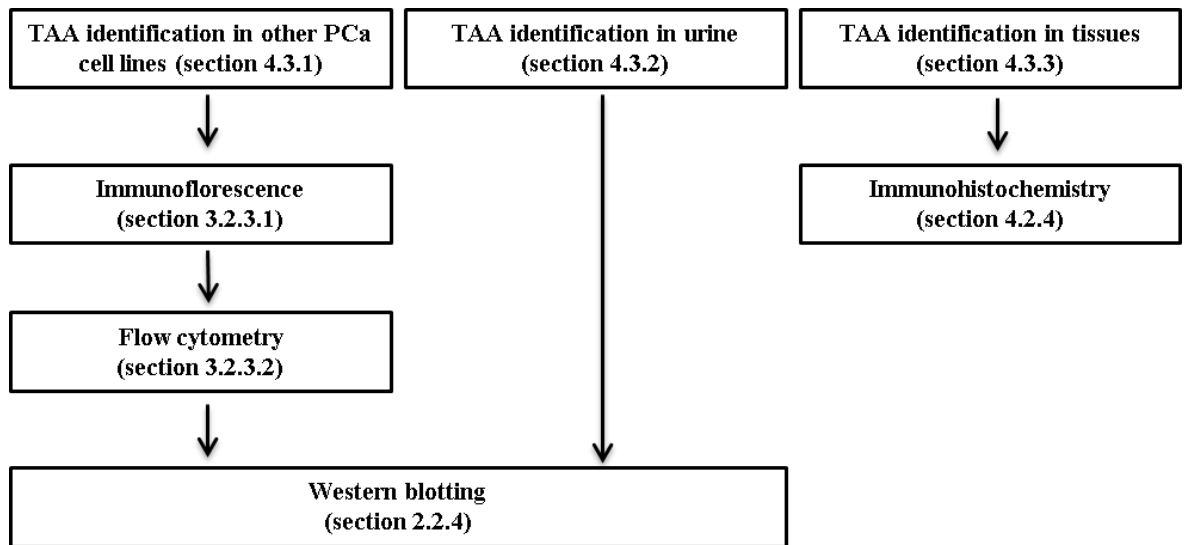


Figure 4.1: Method outline for the identification/verification of alpha enolase expression in other biological samples.

A flowchart summary of the methods used for the identification of alpha enolase expression in this chapter.

4.2.1 Prostate cancer sera and urine

Sera and urine samples from PCa patients were collected and banked at the Urology Centre, Nottingham City Hospital, Nottingham, UK. Samples were prepared and stored as outlined in section 2.2.

4.2.2 PCa cell lines

Four available PCa cell lines were purchased from ATCC and utilised in this study as a source of TAAs (PC-3, DU-145, LNCaP and OPCT-1). PC-3, DU-145 and LNCaP were purchased from ATCC while OPCT-1 was gifted from ONYVAX. PC-3 was grown in Ham's F12 media supplemented with 1% sodium pyruvate, 1% non-essential amino acids and 10% foetal calf serum (FCS). DMEM was supplemented with 10% FCS and 1% L-Glutamine for DU-145 cells, while RPMI-1640 media was supplemented with 5 ng/mL hydrocortisone, 5 ng/mL testosterone and 10% FCS for LNCaP cells. OPCT-1 was cultured in keratinocyte media supplemented in 2% FCS. The attributes of all PCa cell

lines utilised in this chapter are outlined in table 3.1. Cell lines were maintained by serial *in vitro* passage as described in section 3.2.1.1.

4.2.3 Immunofluorescence, flow cytometry, Western blotting

The verification of TAAs by immunofluorescence, flow cytometry and Western blotting was carried out as described in 3.2.3. TAAs were verified in all cell lines using the monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK).

4.2.4 Immunohistochemistry staining of alpha enolase TMAs

Tissue pre-staining: Tissue microarray slides (TMA) were purchased from US Biomax – slides BN243a, MC246 and T195B representative of normal organs, multiple cancer and prostate cancer tissues respectively (appendix III-V). Prior to immunohistochemical staining, paraffin wax on the TMA slides was melted at 60°C for 10 min to remove the wax and unmask the antigen epitopes, in order to facilitate the staining procedure. Each slide was then allowed to cool on the bench at room temperature for 5 – 10 min before being placed on an Autostainer ST5010 XL (Leica, Milton Keynes, UK), which carried out automated deparaffinisation/hydration process as follows:

- Slide immersed in a xylene bath (1) for 5 min
- Slide immersed in a xylene bath (2) for 5 min
- Slide immersed in a graded alcohol bath (1): (100%) ethanol for 2 min
- Slide immersed in a graded alcohol bath (1): (100%) ethanol for 2 min
- Slide immersed in a graded alcohol bath: (70%) ethanol for 2 min
- Slide immersed in dH₂O for 5 min

Slides were then placed into a bath containing citrate buffer for antigen retrieval (0.01 M citrate solution pH 6) and microwaved for 20 min at full power before carefully allowing to cool by dipping the slides into a bath of running tap water for 5 min. While slides were underwater, they were loaded onto sequenza immunostaining coverslips (Fisher Scientific, Loughbrough, UK) and inserted into sequenza immunostaining slide racks (Fisher Scientific, Loughbrough, UK). Each slide was then rinsed with a 1 x TBS solution which

was applied between the sequenza slide and its coverslip. This created a reservoir which allowed liquid to flow away from the bottom of the slide by capillary action.

Tissue staining: Tissue staining was carried out using a Novolink Polymer Detection System kit (Leica, Milton Keynes, UK) which allows the co-visualisation of mouse and rabbit antibodies on a single slide. The staining protocol was carried out as per the manufacturer's instructions. In brief, all staining solutions were applied in 100 μ L volumes each time and then followed up with after two 5 min washes in TBS. A peroxidase block step was carried out using the peroxidase block solution for 5 min to reduce non-specific tissue staining due to endogenous peroxidase activity. The blocking buffer was washed as described before and a protein block was added for 5 min at room temperature and washed, to reduce non-specific background staining. After this, an optimised dilution (1:3000) of mouse monoclonal alpha enolase antibody (clone L-27, Santa Cruz, UK) was added to the sequenza reservoir. Antibody was left to bind for 60 min at room temperature followed by washing, and the addition of a post-primary block for 30 minutes which was washed as before.

100 μ L of a Novolink polymer was added and left to incubate for 5 min followed by a wash. DAB working solution was added for another 5 min, washed and counterstained with haematoxylin in a 6 min incubation step. Excess haematoxylin was rinsed off the slide using dH₂O. Each slide was then removed from the sequenza reservoir and placed into the Autostainer as before, for a dehydration process which was carried out as follows:

- Slide immersed in dH₂O for 5 min
- Slide immersed in a graded alcohol bath: (70%) ethanol for 2 min
- Slide immersed in a graded alcohol bath (1): (100%) ethanol for 2 min
- Slide immersed in a graded alcohol bath (2): (100%) ethanol for 2 min
- Slide immersed in a xylene bath (1) for 5 min
- Slide immersed in a xylene bath (2) for 5 min

Coverslips were mounted onto each slide using DPX mounting medium.

4.3 RESULTS

As depicted in figure 4.1, the identification of alpha enolase in other biological samples is subdivided into 3 sections:

- i. Alpha enolase expression in other PCa cell lines (section 4.3.1)
- ii. Alpha enolase expression in urine (section 4.3.2)
- iii. Alpha enolase expression in tissues (section 4.3.3).

4.3.1 Alpha enolase expression in other PCa cell lines

Although alpha enolase was identified in PC-3 and DU-145 PCa cell lines in the previous chapter (sections 3.3.1 and 3.3.2), its expression was only verified in DU-145 due to feasibility restraints (section 3.3.4). Thus, this chapter aimed to determine its expression in other available PCa cell lines.

4.3.1.1 Alpha enolase protein expression in other PCa cell lines demonstrated by immunofluorescence

Three available PCa cell lines (PC-3, LNCaP and OPCT-1) were examined for the expression of alpha enolase in PCa. As detailed in section 3.2.3.1, cells were grown on a monolayer and alpha enolase expression was determined using a mouse monoclonal anti-alpha enolase antibody (clone L-27, Santa Cruz, UK). Figure 4.2 demonstrates a distinctive cytoplasmic expression in all 3 cell lines tested (DU-145 cells added for reference). Although these cell lines ranged from having moderate to high metastatic potential (table 3.1), there was no obvious relationship in the level of alpha enolase expression and metastasis as demonstrated by immunofluorescence. It is worth noting, that although membranous expression was identified in DU-145 by immunofluorescence (indicated by red arrows in figure 4.2) no membranous expression was identified in any of the other PCa cell lines tested. These 4 cell lines have however provided a model system which alpha enolase expression could be investigated, for example to study its interacting partners. No expression was identified in isotype controls.

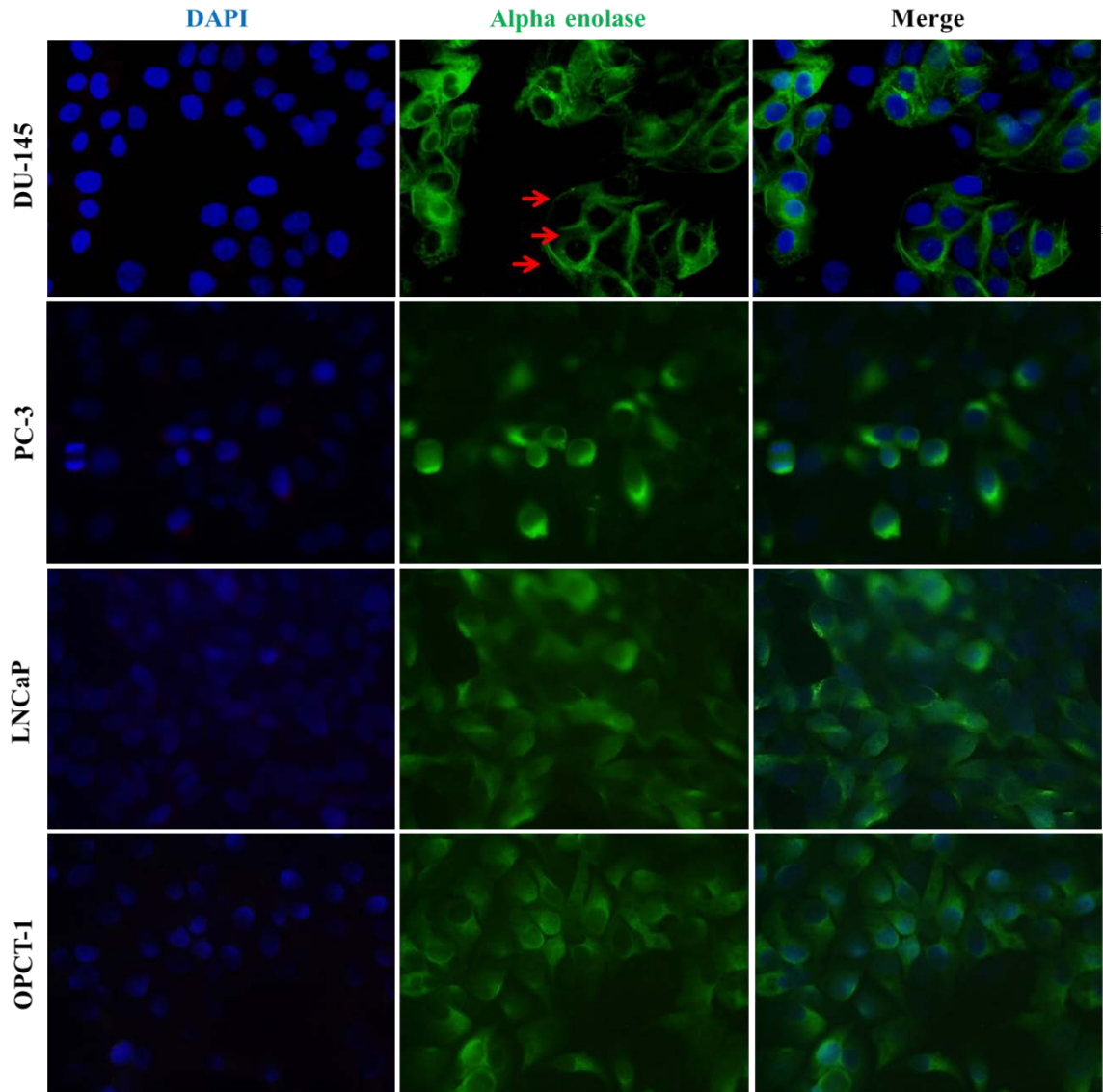


Figure 4.2: Alpha enolase expression in PCa cell lines by immunofluorescence.

Immunofluorescence staining of all available PCa cell lines (DU-145, PC-3, LNCaP and OPCT-1) to assess alpha enolase protein expression. All cells were stained using a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK). Cytoplasmic alpha enolase expression was identified in all PCa cell lines as shown by immunofluorescence. Red arrows indicate a possible expression of alpha enolase around the membrane of the cell in DU-145. *Objective magnification x40.*

4.3.1.2 Alpha enolase protein expression in other PCa cell lines demonstrated by flow cytometry

As the previous chapter indicated that there was no cell surface alpha enolase expression in DU-145 (figures 3.9 and 4.2), the expression of intracellular alpha enolase was investigated in LNCaP, PC-3 and OPCT-1 using single colour flow cytometry against a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK) and a goat anti-mouse secondary antibody conjugated to FITC. Using appropriate isotype controls, gated regions were defined in order to determine positivity. Figure 4.3 shows a histogram overlay of isotype control, secondary alone and cells alone compared to the test (PCa cells stained with alpha enolase antibody). Flow cytometry showed a right shift of cells positive for alpha enolase, compared to the controls (secondary alone, cells alone or isotype control) in PC-3, LNCaP and OPCT-1 (figure 4.3). DU-145 expression is also shown for comparison. Similar to results obtained with DU-145, no cell surface alpha enolase expression was observed in any of the PCa cell lines tested (appendix VI).

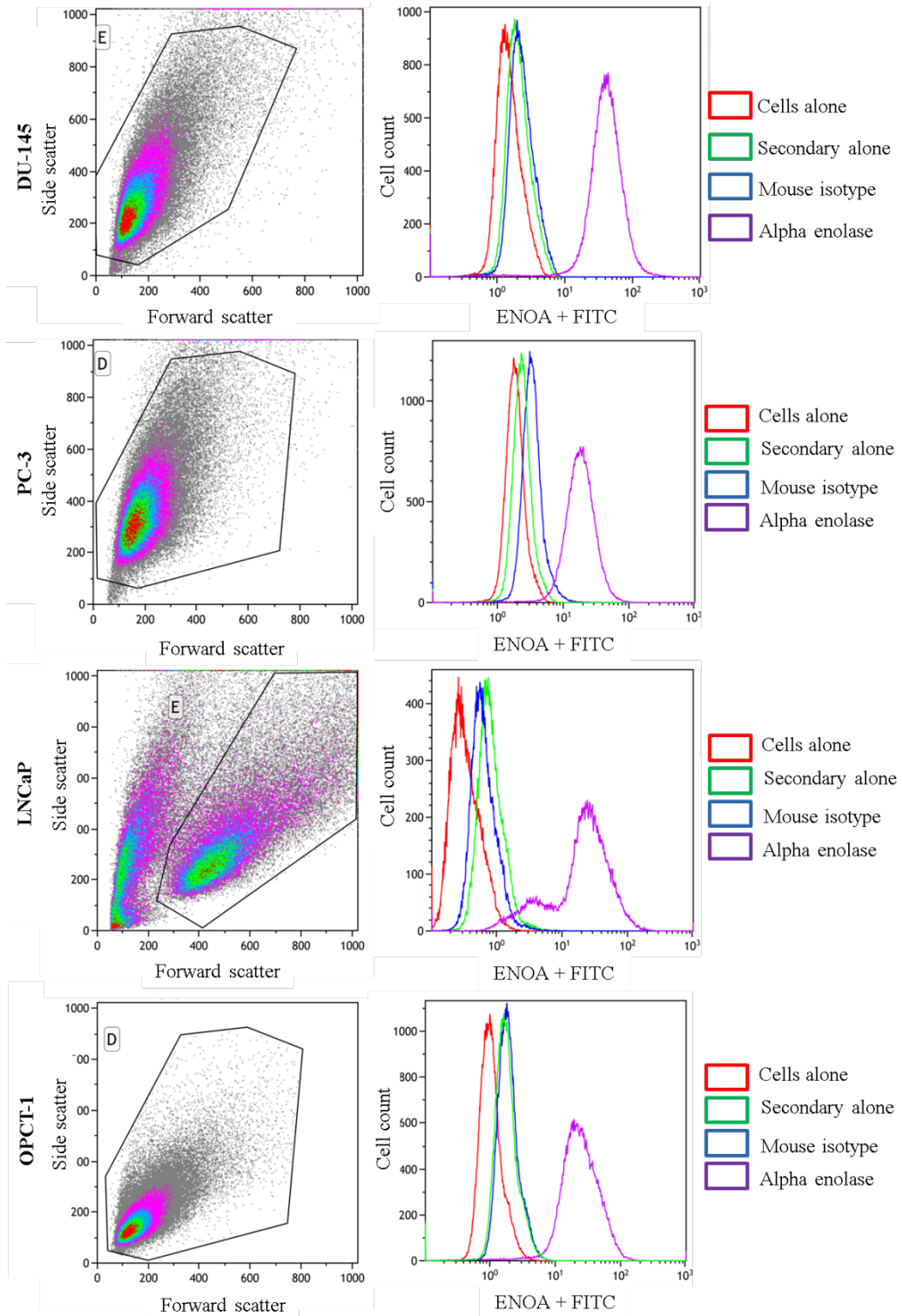


Figure 4.3: Intracellular alpha enolase expression in all PCa cell lines demonstrated by flow cytometry.

Flow cytometric data showing intracellular alpha enolase expression in 4 available PCa cell lines (DU-145, PC-3, LNCaP and OPCT-1). All cells were stained using a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK) or a goat anti-mouse secondary antibody conjugated to FITC. Histogram overlay shows a shift to the right for positive cells compared to isotype, secondary and cells alone.

4.3.1.3 Alpha enolase protein expression in other PCa cell lines demonstrated by Western blotting

In order to validate and confirm the expression of alpha enolase in all available PCa cell lines, Western blotting was performed on the cell lysates prepared from PC-3, LNCaP and OPCT-1. Cells lysates were separated on 10% SDS PAGE gels and transferred onto nitrocellulose membranes before immunoblotting with a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK). Correct expression of alpha enolase was demonstrated by recombinant alpha enolase loaded into separate wells on Western blots (figure 4.4). Figure 4.4 shows the presence of an alpha enolase specific band at 47 kDa in PC-3, LNCaP, and OPCT-1. No bands were identified in BSA which served as a negative control (figure 4.4).

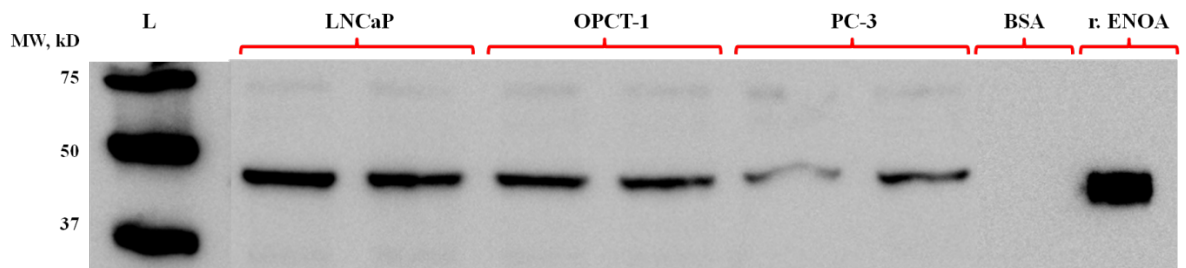


Figure 4.4: Alpha enolase expression in PCa cell lines demonstrated by Western blotting.

Western blot showing the expression of alpha enolase in 3 available PCa cell lines (LNCaP, OPCT-1 and PC-3). All cell lines were screened for alpha enolase expression using a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK). A specific band at 47 kDa was identified in all cell lines, where correct alpha enolase expression was demonstrated by a recombinant alpha enolase protein. A fainter band was detected at 75 kDa. No expression was detected in BSA (negative control). L; molecular weight ladder.

4.3.2 Alpha enolase expression in urine

To determine whether alpha enolase is secreted into the urine of the samples studied, PCa and healthy control samples that made up the discovery set were pooled into two main pools – a PCa (n=26 patients) and healthy pool (n=3 patients), due to the limited number of healthy control samples available. From each pool, the same amount of urinary proteins (50 µg) were separated on 10% SDS PAGE gels and run in duplicate before transferring onto nitrocellulose membranes for Western blot analyses. Transferred proteins were then

immunoprobed with a monoclonal mouse anti-alpha enolase antibody (clone L-27, Santa Cruz, UK) at a pre-optimised antibody dilution.

Correct expression of alpha enolase was demonstrated at 47 kDa by comparison to recombinant alpha enolase, which was loaded into a separate well on Western blots (figure 4.5). Figure 4.5 shows the presence of an intense band at 47 kDa in pooled PCa and healthy urine, where both bands were equally as intense. No bands were identified in BSA which served as a negative control (figure 4.5). It is also worth noting, that although alpha enolase is a 47 kDa protein, an intense band was observed with this monoclonal antibody at approximately 66 kDa in both PCa and healthy urine (figure 4.5). The band at 66 kDa was more intense in PCa urine compared to healthy controls, suggesting that there may be an increased expression of alpha enolase at 66 kDa in PCa (figure 4.5). To our knowledge, alpha enolase does not have a transcript variant at 66 kDa, which implies that this band may be due to a posttranslational modification or because it is bound to another (carrier) protein.

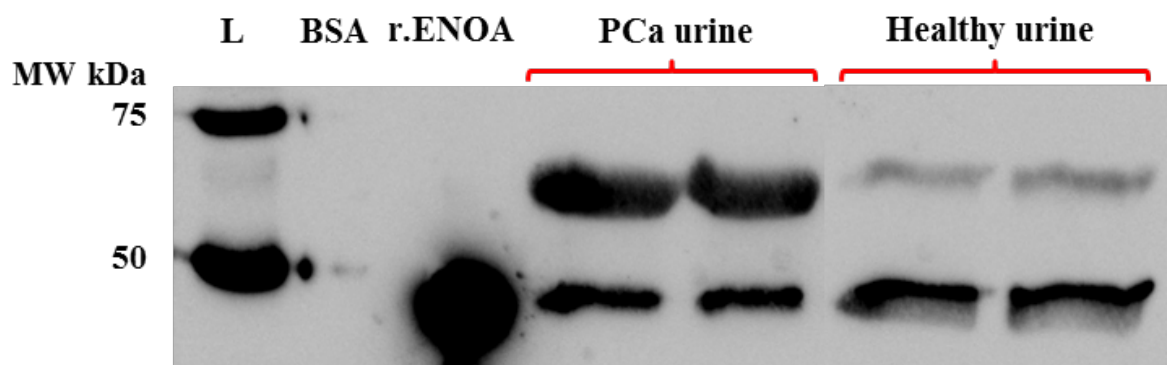


Figure 4.5: Alpha enolase expression in urine demonstrated by Western blotting.

Urine samples which made up the discovery pool were pooled into two separate pools (PCa n=26, healthy control n=3). Urinary proteins were separated by SDS PAGE and run in duplicate before screening with a monoclonal mouse anti-alpha enolase antibody. A band at 47 kDa was observed in healthy and PCa urine samples similar to the positive control using a recombinant alpha enolase protein. Differential alpha enolase expression was identified in PCa urine compared to healthy controls at approximately 66 kDa. No expression was detected in BSA (negative control).

4.3.3 Alpha enolase expression in tissue

Despite being useful tools to study the presence of alpha enolase, urine, PCa cell lines and serum autoantibody responses are not useful tools in demonstrating cellular protein localisation or protein abundance at the tissue level (Even-Desrumeaux *et al.*, 2011 and Alvarez-Chaver *et al.*, 2014). In addition, although our previous studies with cell lines have demonstrated the presence of alpha enolase in transformed PCa cell lines, it was necessary to show that this protein was also present in non-immortalised PCa tumour tissue, and not simply present in cell lines due to long term culture or as a result of immortalisation. Investigating alpha enolase in tissue, aimed to further evaluate the robustness of alpha enolase as a biomarker and confirm its subcellular localisation as shown by immunofluorescence.

4.3.3.1 Alpha enolase expression in normal organ tissues

To this end, commercially produced tissue microarrays (TMAs; purchased from US Biomax) from normal organ tissues (slide #BN234A) (patient details listed in appendix III) immunohistochemically stained for their alpha enolase protein expression using a monoclonal mouse-anti alpha enolase antibody (clone L-27, Santa Cruz, UK) alongside an isotype control. Before the study commenced, a series of optimisation experiments were performed on tissue core off-cuts collected from the Nottingham Prostate Research Group (NPRG) on formalin fixed paraffin embedded (FFPE) TMA blocks. Optimal conditions for alpha enolase tissue staining were determined to be citrate buffer for antigen retrieval, 1:3000 dilution of a 1 mg/mL antibody stock solution, and antibody incubation for 1 h at room temperature.

In collaboration with Nottingham University Hospitals, tissue scoring was carried out by Dr Desmond Powe (Histopathology Department at the Queens Medical Centre, Nottingham University Hospitals, Nottingham, UK) for the expression of alpha enolase in the stained tissue sections. In line with previous reports, alpha enolase expression was identified mostly in the cytoplasm and also in the nucleus in some cases. However, for this preliminary study, only alpha enolase cytoplasmic expression was scored, by designating alpha enolase tissue staining a value (score) between 0 – 3 (0 = negative; no staining, 1 = weak staining, 2 = moderate staining or 3 = strong staining), as depicted in figure 4.6.

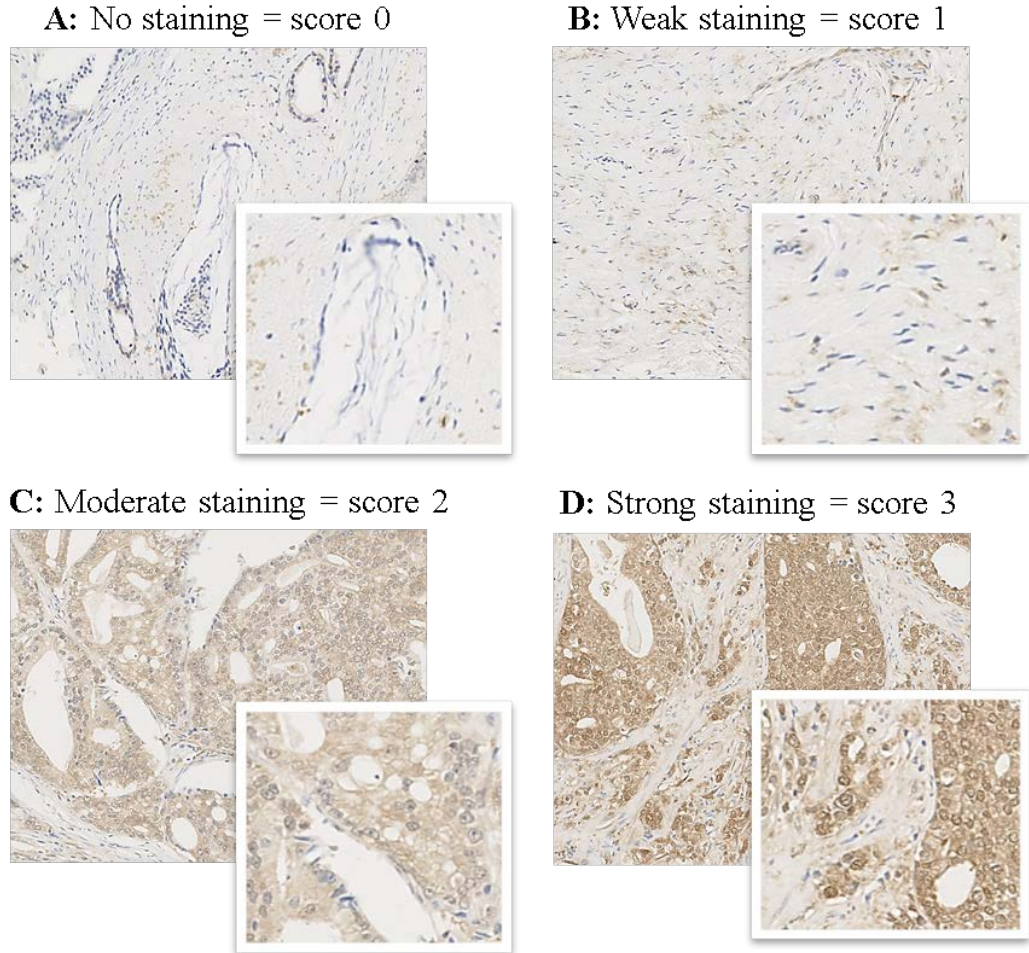


Figure 4.6: Semi-quantitative scoring method used to determine the immunohistochemical alpha enolase protein expression in PCa.

The figure illustrates the scoring method used for all TMA sections. *Objective magnification: x20 and x40 inset.*

From this IHC staining, it was observed that there was a diffuse immunostaining pattern localised predominantly in the cytoplasm and the nucleus of all tissues stained (figure 4.6). Figure 4.7 shows the alpha enolase expression in multiple organ normal tissues. Out of all the tissues stained, 42% of the tissues showed no alpha enolase staining (cerebrum, cardiac muscle, lung, ovaries and prostate), 25% showed weak staining (pancreas, uterine cervix, skin), while 33% showed moderate staining for alpha enolase (liver, kidney, breast and colon). It is important to note that in figure 4.7, uterine cervix (E) is a cancer adjacent normal cervix tissue. Staining for each tissue was carried out in duplicate and representative figures for each normal tissue type are shown in figure 4.7. A summary of the TMA scores demonstrating alpha enolase expression in multiple organ normal tissues is summarised in table 4.2.

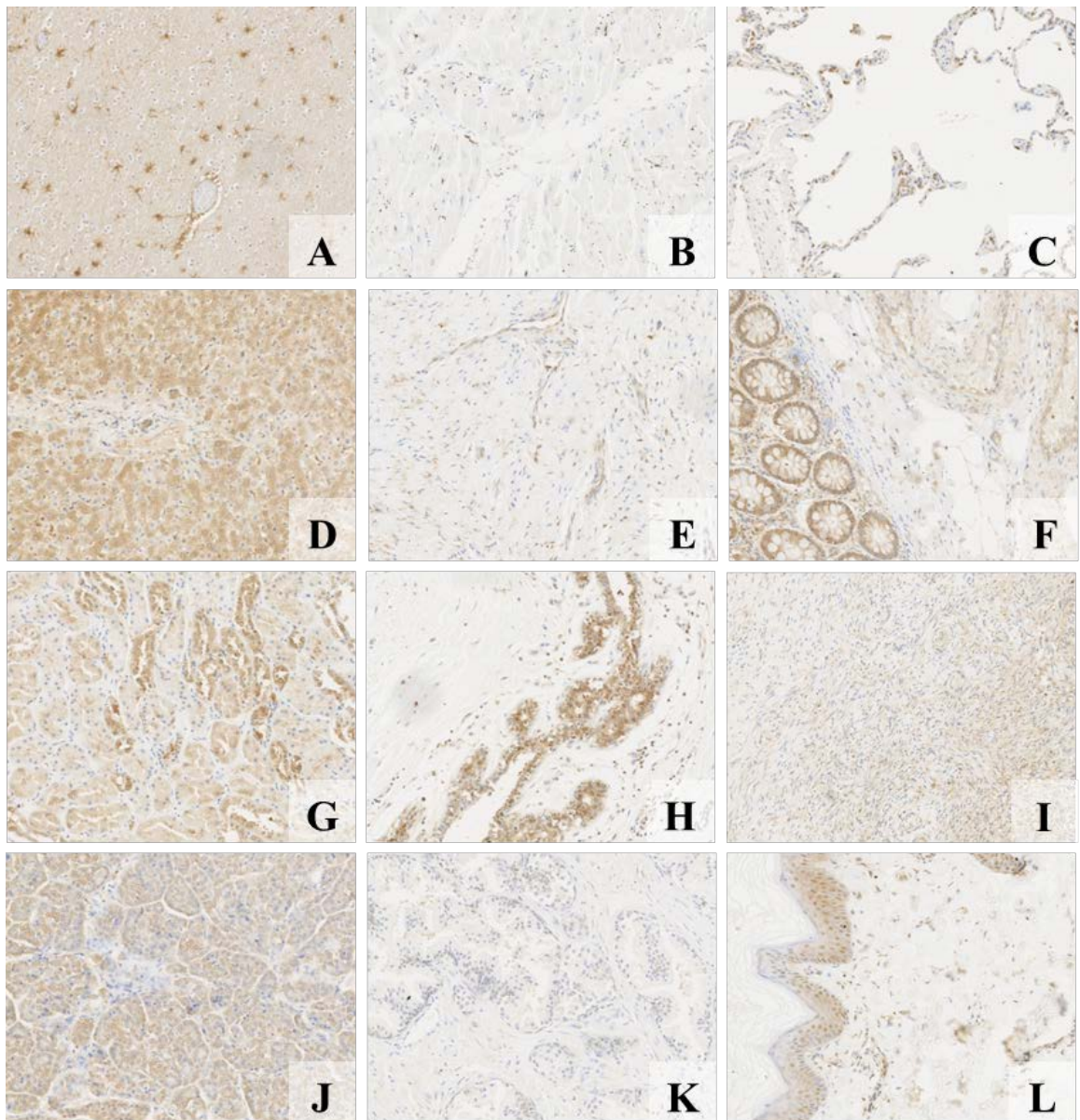


Figure 4.7: Immunohistochemical staining showing alpha enolase protein expression in multiple organ normal tissues.

Immunohistochemical staining demonstrates alpha enolase expression in normal cerebrum (A), cardiac muscle (B), lung (C), liver (D), cancer adjacent normal cervix tissue (E), colon (F), kidney (G), breast (H), ovary (I), pancreas (J), prostate (K) and skin (L). Duplicate cores stained per patient. Image shows a representative image from one of the duplicates. *Objective magnification: x20.*

Table 4.2: Alpha enolase protein expression in multiple organ normal tissues

Alpha enolase expression in multiple organ normal tissues					
		Stain intensity			
Tissue	Score	0	1	2	3
Cerebrum	0	0			
Cardiac	0	0			
Lung	0	0			
Liver	2			2	
Uterine cervix	1		1		
Colon	2			2	
Kidney	2			2	
Breast	2			2	
Ovary	0	0			
Pancreas	1		1		
Prostate	0	0			
Skin	1		1		

Tissues were purchased from US Biomax (Cat. BN243A), and stained in duplicate, where scores of each representative organ is shown. Cytoplasmic staining was performed by a pathologist using a numerical scoring system between 0 - 3 (0 = negative; no staining, 1 = weak staining, 2 = moderate staining or 3 = strong staining). Numbers in unshaded and shaded cells represent the score for each tissue using the score system.

4.3.3.2 Alpha enolase expression in multiple cancers and adjacent normal tissue

Alongside studying the alpha enolase expression in multiple organ normal tissues, it was also of interest to investigate the protein expression of alpha enolase in multiple cancers as well as corresponding adjacent normal tissues. As before, tissue cores were purchased from US Biomax which included tumours and adjacent normal tissues from 12 organs (slide #MC246, patient details listed in appendix IV). TMAs were stained using already optimised conditions for alpha enolase as before, and scored by the same pathologist.

Figures 4.8 and 4.9 depict alpha enolase protein expression in multiple cancer tissues alongside adjacent normal tissues. Interestingly, an increased cytoplasmic alpha enolase expression was observed in 50% of cancers compared to their corresponding adjacent

normal tissues (lung, colon, ovary, oesophagus, skin and cerebrum; summarised in table 4.2), while no change in expression was observed in 42% of cancers (prostate, uterine cervix, stomach, liver and kidney). A lower expression of alpha enolase in cancer compared to adjacent normal tissues was identified in breast cancer alone.

It is important to note that the highest alpha enolase score in cancer occurred in melanoma, which had a score of 3 in cancer and 1 in the adjacent normal tissue. As before, a diffuse immunostaining pattern localised predominantly in the cytoplasm and the nucleus was observed in cancer adjacent normal and in tumour tissues. There was a remarkable staining difference observed in normal skin tissue which exhibited a weak reactivity (score 1; figure 4.9J) compared to melanoma which exhibited an intense alpha enolase immunoreactivity (score 3; figure 4.9I). Alpha enolase tissue staining in cancer and adjacent normal tissues are summarised in table 4.3. A comparison between alpha enolase expression in normal organ tissues and cancer tissues in all organs studied are summarised in table 4.4.

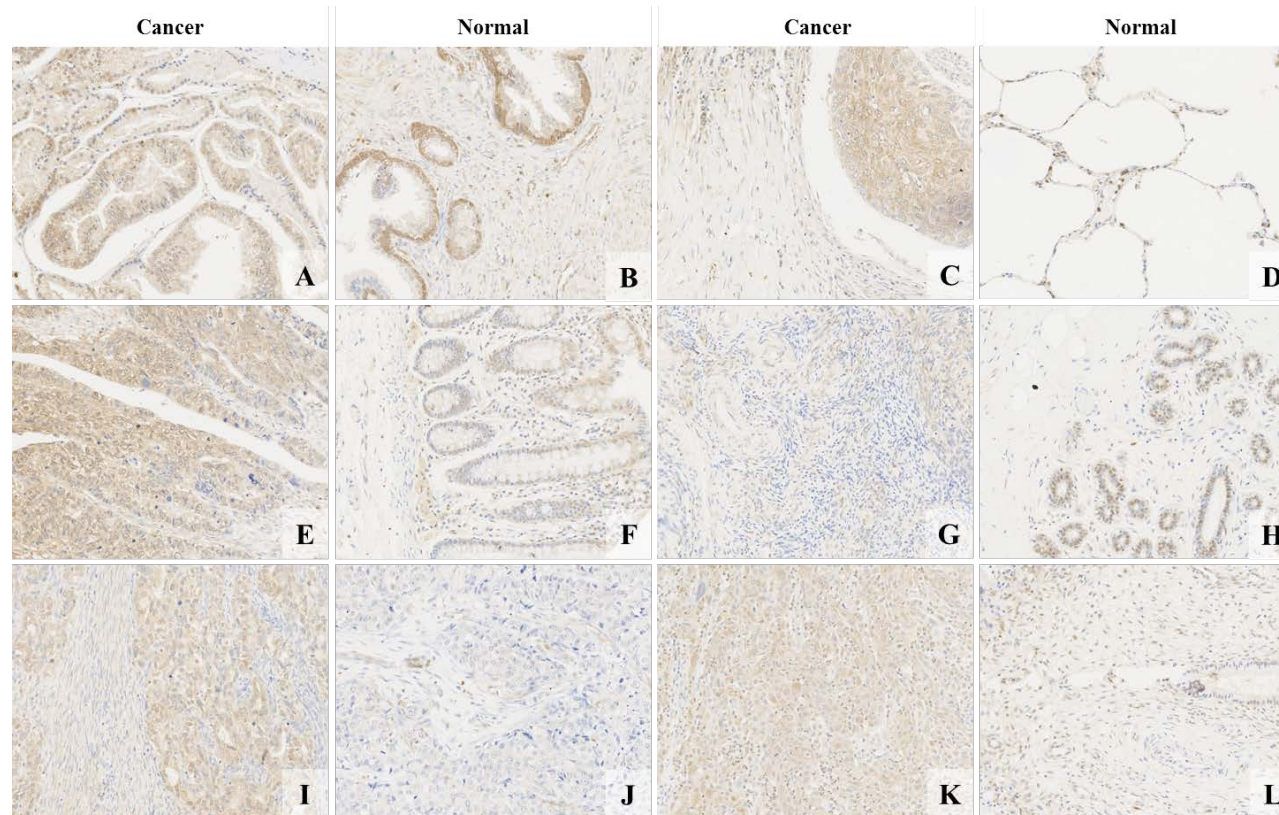


Figure 4.8: Immunohistochemical staining showing alpha enolase protein expression in multiple organ tumours with matched adjacent normal tissues I.

Immunohistochemical staining demonstrates alpha enolase expression in prostatic adenocarcinoma (A), adjacent normal prostate tissue (B), squamous cell carcinoma of the lungs (C), adjacent normal lung tissue (D), colon adenocarcinoma (E), adjacent normal colon tissue; fibrous tissue and blood vessel (F), invasive ductal carcinoma (G), adjacent normal breast tissue (H), serous ovarian adenocarcinoma (I), adjacent normal ovary tissue (J), squamous cell carcinoma of the uterine cervix (K) and adjacent normal cervical canals tissue (L). *Objective magnification: x20.*

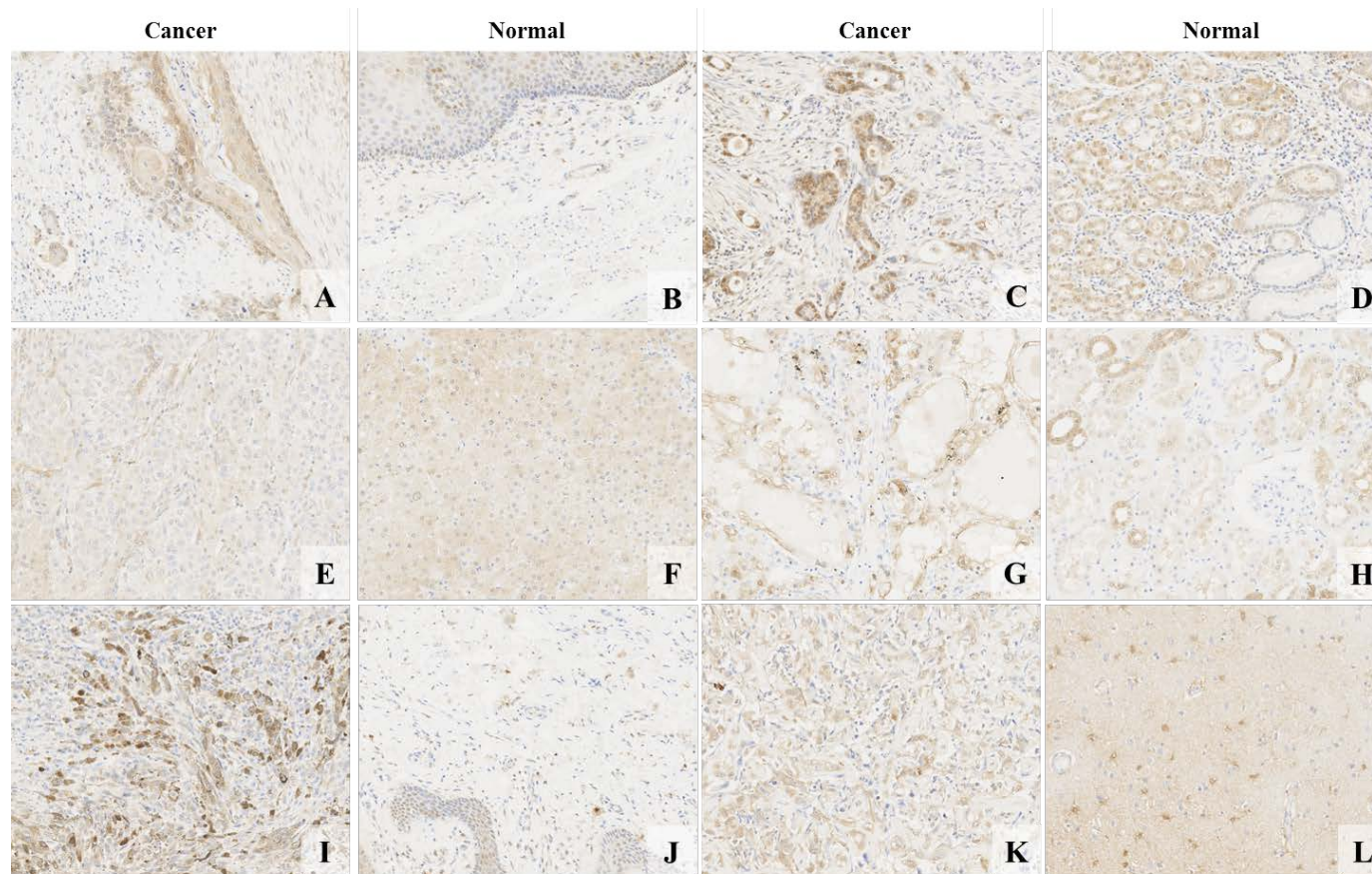


Figure 4.9: Immunohistochemical staining showing alpha enolase protein expression in multiple organ tumours with matched adjacent normal tissues II.

Immunohistochemical staining demonstrates alpha enolase expression in squamous cell carcinoma of the oesophagus (A), adjacent normal oesophagus tissue (B), stomach adenocarcinoma (C), adjacent normal stomach tissue (D), hepatocellular carcinoma (E), adjacent normal hepatic tissue (F), clear cell renal carcinoma (G), adjacent normal kidney tissue (H), malignant melanoma of the right heel (I), adjacent normal skin tissue (J), glioblastoma (K) and adjacent normal cerebrum tissue (L). *Objective magnification: x20.*

Table 4.3: A summary of alpha enolase protein expression in multiple cancer tissues and adjacent normal tissues

A						B						C
Alpha enolase expression in adjacent normal tissues						Alpha enolase expression in multiple cancer tissues						Change in expression
		Stain intensity						Stain intensity				
Tissue	Score	0	1	2	3*	Tissue	Score	0	1	2	3*	
Prostate	2			2		Prostate	2			2		0
Lung	0	0				Lung	2			2		+
Colon	0	0				Colon	2			2		+
Breast	2			2		Breast	0	0				-
Ovary	0	0				Ovary	2			2		+
Uterine cervix	0	0				Uterine cervix	1		1			0
Esophagus	0	0				Esophagus	2			2		+
Stomach	2			2		Stomach	2			2		0
Liver	2			2		Liver	1		1			0
Kidney	1		1			Kidney	1		1			0
Skin	1		1			Skin	3				3	+
Cerebrum	0	0				Cerebrum	2			2		+

*Alpha enolase expression observed in various tumours alongside their corresponding adjacent healthy tissues. Tissues were purchased from US Biomax (Cat. MC246), and cytoplasmic staining was performed by a pathologist using a numerical scoring system between 0 - 3 (0 = negative; no staining, 1 = weak staining, 2 = moderate staining or 3 = strong staining). * Numbers in unshaded and shaded cells represent the highest score for each tissue using the score system. Alpha enolase expression in adjacent normal tissues are shown in (A) and cancer tissues (B). Changes in protein expression for alpha enolase protein in cancer compared to control tissues is indicated in (C), where + = increased expression, 0 = no change, - = low expression in cancer relative to normal tissues.

Table 4.4: Alpha enolase protein expression in multiple organ normal tissue and cancer tissues

A Alpha enolase expression in multiple organ normal tissues						B Alpha enolase expression in multiple cancer tissues						C Change in expression
		Stain intensity						Stain intensity				
Tissue	Score	0	1	2	3	Tissue	Score	0	1	2	3	
Prostate	0	0				Prostate	2			2		+
Lung	0	0				Lung	2			2		+
Colon	2			2		Colon	2			2		0
Breast	2			2		Breast	0	0				-
Ovary	0	0				Ovary	2			2		+
Uterine cervix	1		1			Uterine cervix	1		1			0
Liver	2			2		Liver	1		1			-
Kidney	2			2		Kidney	1		1			-
Skin	1		1			Skin	3				3	+
Cerebrum	0	0				Cerebrum	2			2		+

Alpha enolase stain intensities in multiple organ normal controls compared to multiple cancer tissues. Tissues were purchased from US Biomax (Cat. MC246) and BN243A) and cytoplasmic alpha enolase staining was performed by a pathologist using a numerical scoring system between 0 - 3 (0 = negative; no staining, 1 = weak staining, 2 = moderate staining or 3 = strong staining). Numbers in unshaded and shaded cells represent the score for each tissue using the score system. Alpha enolase expression in multiple organ normal controls is depicted in (A) while expression in multiple cancer tissues is shown in (B). Changes in expression in cancer compared to control tissues is indicated in (C), where + = increased expression, 0 = no change, - = low expression in cancer relative to normal tissues.

4.3.3.3 Alpha enolase protein expression in prostate cancer

Previous tissue staining with alpha enolase (section 4.3.3.2) demonstrated a differential protein expression in 7 out of 12 cancer tissues studied, with the highest cytoplasmic expression occurring in melanoma. From table 4.2 and 4.3, alpha enolase expression in normal prostate tissue has a score 0 while adjacent normal tissues and prostatic adenocarcinoma scored a value of 2 and 2 respectively. Although there was no change in cytoplasmic alpha enolase score in PCa and its adjacent normal tissues (score 2 and 2), there was a lower expression of alpha enolase in normal prostate (score 0), indicating that further investigation into the expression of alpha enolase in PCa needed to be carried out. Moreover, each cancer and adjacent normal tissue studied in section 4.3.3.2 was collected from a single patient, making it difficult to decipher the general pattern of alpha enolase

expression in PCa. In addition, our group has an interest in PCa research and identifying biomarkers for PCa.

Thus, it was essential that more PCa patient tissues were studied to better understand alpha enolase expression in PCa.

To this end, TMAs were purchased from US Biomax (slide #T195B) containing 9 cases - patients from various Gleason grades and tumour stages (table 4.5 and appendix V). TMAs included tissue cores from two healthy males and a case of low grade malignant leiomyosarcoma which acted as a control (figure 4.10). Figure 4.10 shows the protein expression of alpha enolase in patients with various grades and stages of PCa where, as before, a diffuse immunostaining pattern localised predominantly in the cytoplasm and the nucleus was observed in all tissue sections (PCa and control tissues). Patient information and alpha enolase tissue scores are summarised in table 4.5.

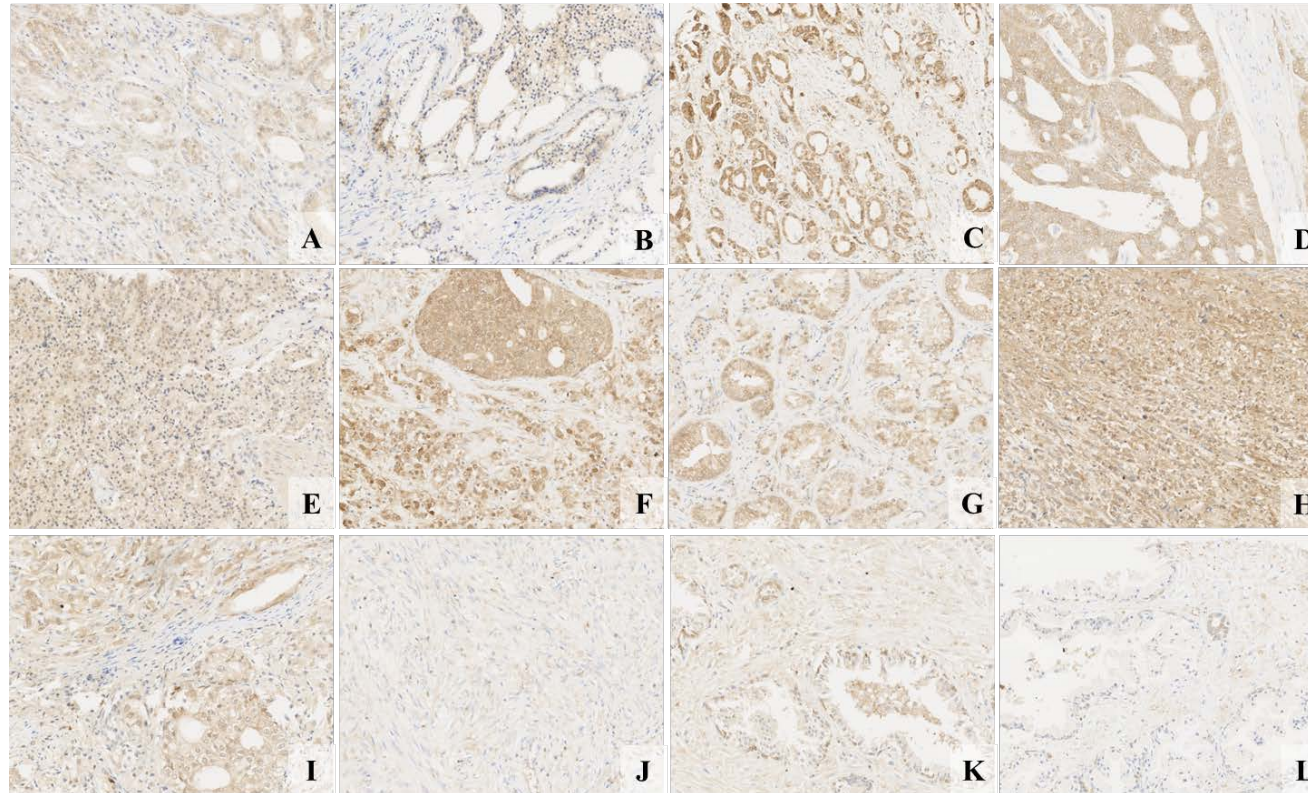


Figure 4.10: Immunohistochemical staining showing alpha enolase protein expression in multiple prostate cancer patients and normal prostate tissues.

Immunohistochemical staining demonstrates alpha enolase expression in prostatic adenocarcinomas of a 64 year old male diagnosed at grade 1, stage I cancer (A), 73 year old male diagnosed at grade 2, stage II cancer (B), 73 year old male diagnosed at grade 2-3, stage IV cancer (C), 61 year old male diagnosed at grade 1, stage IV cancer (D), 70 year old male diagnosed at grade 2, stage III cancer (E), 66 year old male diagnosed at grade 2-3, stage III cancer (F), 65 year old male diagnosed at grade 1-2, stage II cancer (G), 66 year old male diagnosed at grade 3, stage II cancer (H), 62 year old male diagnosed at grade 3, stage II cancer (I), 69 year old male diagnosed at low grade malignant leiomyosarcoma, stage Ia (J), normal prostate tissue of a 33 (K) and 43 year old male (L) respectively. Duplicate cores stained per patient. Image shows a representative image from one of the duplicates. *Objective magnification: x20.*

Table 4.5: A summary of patients studied for alpha enolase expression in PCa tissues

Pos.	Pathology diagnosis	Age	Grade	Stage	Gleason Grade	Gleason Score	TNM	Alpha enolase IHC score
A	Adenocarcinoma	64	1	I	1	1+2	T1N0M0	2
B	Adenocarcinoma	73	1	II	3	3+3	T2N0M0	0
C	Adenocarcinoma	73	2-3	IV	4	4+4	T3N0M1	2
D	Adenocarcinoma	61	1	IV	2	3+2	T3N1M0	2
E	Adenocarcinoma	70	2	III	3	3+4	T3N0M0	2
F	Adenocarcinoma	66	2-3	III	4	3+4	T3aN0M0	3
G	Adenocarcinoma	65	1-2	II	2-3	2+3	T2N0M0	2
H	Adenocarcinoma	66	3	II	5	5+5	T2N0M0	2
I	Adenocarcinoma	62	3	II	3	5+4	T2N0M0	2
J	Low grade malignant leiomyosarcoma	69	-	Ia	-	-	T1N0M0 G1	0
K	Normal prostate tissue	33	-	-	-	-	-	0
L	Normal prostate tissue	43	-	-	-	-	-	0

Alpha enolase protein expression in multiple prostate cancer, low grade malignant leiomyosarcoma and normal prostate tissues. Tissues were purchased from US Biomax (Cat. T195B), and cytoplasmic staining was performed by a pathologist using a numerical scoring system between 0 - 3 (0 = negative; no staining, 1 = weak staining, 2 = moderate staining or 3 = strong staining). The table shows the patient age, grade, stage, Gleason grade, Gleason sum, TNM status alongside alpha enolase tissue score for each patient studied. Pos = position; image reference on figure 4.10, IHC; immunohistochemistry score.

4.4 DISCUSSION

The previous chapter identified alpha enolase as a potential TAA that elicits a differential humoral immune response in some PCa patients, compared to healthy controls (section 3.3.2 and 3.3.3). Furthermore, its expression in the metastatic DU-145 PCa cell line was also verified (section 3.3.4). In order to demonstrate its suitability as a potential PCa biomarker, it was important to first determine its expression in other biological sample sources. Hence, this chapter aimed to demonstrate alpha enolase expression in other available PCa cell lines, urine and tissue.

4.4.1 Alpha enolase expression in other PCa cell lines

The expression of alpha enolase in other PCa cell lines was determined by immunofluorescence, flow cytometry and Western blotting (as these techniques had successfully verified alpha enolase in the previous chapter; section 3.3.4.1 and figure 3.9). Using immunofluorescence, figure 4.2 confirms the subcellular localisation of alpha enolase in PCa cell lines, showing a strong, diffuse cytoplasmic staining and a slightly weaker nuclear expression in PC-3, LNCaP and OPCT-1, similar to findings by Perconti *et al.* (2007). Furthermore, no membranous alpha enolase expression was identified in any of these three cell lines by immunofluorescence, unlike in DU-145 (red arrows in figure 4.2 and figure 3.9).

However, in order to confirm the possibility of a potential membranous localisation of alpha enolase in other PCa cell lines, each cell line was subject to cell surface staining using flow cytometry. Using this technique, no alpha enolase expression was observed in any cell line (appendix VI). Contrary to these findings, the membranous expression of alpha enolase has been well studied and reported in pancreatic (Capello *et al.*, 2009), lung (Chang *et al.*, 2006 and Hsiao *et al.*, 2013) and non-small cell lung cancers (He *et al.*, 2007). However, it is well documented that the surface expression of alpha enolase depends on the physiological status of the cell (Fontan *et al.*, 2000 and Arza *et al.*, 1997), potentially explaining the lack of cell membrane expression in these cell lines. It is also important to highlight that methods of harvesting cells (e.g. trypsin and versene) for

staining by flow cytometry may cleave off cell surface markers, thereby explaining the lack of alpha enolase expression observed in this study.

Flow cytometry, however, confirmed the intracellular expression of alpha enolase in PC-3, LNCaP and OPCT-1, showing a shift to the right in the histogram of all PCa cell lines screened (figure 4.3), while Western blotting confirmed the presence of a band at 47 kDa, similar to expression by recombinant alpha enolase. All three studies combined demonstrate that alpha enolase is present in all PCa cell lines tested.

As all four cell lines have different characteristics, further studies could compare the expression of alpha enolase in all PCa cell lines in reference to a housekeeping protein such as β -actin using Western blotting, facilitating the normalisation of protein expression levels in each cell line. This would determine the expression levels of alpha enolase in metastatic (PC-3 and DU-145) compared to non-metastatic (OPCT-1) and hormone sensitive (LNCaP) PCa in order to generate a better understanding of alpha enolase expression in PCa.

Furthermore, although it would have been of interest to compare the expression levels of alpha enolase in cell lines obtained from a healthy prostate with PCa cell lines, in order to determine if a differential expression exists in PCa, this was not feasible due to the lack of normal prostate cell lines available during the study. However, it is already well documented that alpha enolase protein and/or mRNA expression is increased in several tumours such as brain (Altenberg and Greulich, 2004), breast (Hennipman *et al.*, 1987), head & neck (Tsai *et al.*, 2010), liver (Takashima *et al.*, 2005), lung (Rubporn *et al.*, 2009), ovary (Cao *et al.*, 2010), pancreas (Shen *et al.*, 2004), prostate (Rehman *et al.*, 2004) and skin cancers (Suzuki *et al.*, 2010). A study investigating the expression levels of alpha enolase in cancer and normal prostate tissue was carried out subsequently in this chapter (section 4.3.3).

4.4.2 Alpha enolase expression in urine

The expression of urinary alpha enolase was also studied in the samples that made up the discovery cohort (samples detailed in table 2.11). Figure 4.5 shows a band at 47

kDa at the same intensity in PCa and healthy urine. This finding is similar to Rehman *et al.* (2004) who identified alpha enolase as one of 6 commonly expressed urinary proteins on 2-DE gel spots of PCa and benign samples after prostatic massage.

Using this specific monoclonal anti-alpha enolase antibody, a differential protein expression was observed between PCa and healthy urine towards a protein at 66 kDa, although the molecular weight of alpha enolase is 47 kDa (figure 4.5). One of the reasons for this observed band could be the occurrence of post translational modifications (PTMs) in alpha enolase, or the presence of carrier proteins bound to alpha enolase. Interestingly, Tomaino *et al.* (2011) identified that phosphorylation of alpha enolase on serine 419 induces the production of a specific autoantibody in patients with pancreatic ductal adenocarcinoma. Their study also found 95% diagnostic accuracy using autoantibodies produced towards phosphorylated alpha enolase and CA19.9 levels in both advanced and resectable pancreatic ductal adenocarcinoma. In addition, alpha enolase autoantibodies correlated with significantly better clinical outcome in patients treated with chemotherapy. The study by Tomaino *et al.* (2010) demonstrates the diagnostic value of PTMs associated with TAAs, in serving as potential biomarkers for cancer.

Similar to Tomaino *et al.* (2011), it would have been beneficial to demonstrate (using data dependent acquisition in LTQ Orbitrap MS) whether any PTMs were present in the urine samples screened, and if these PTMs were responsible for eliciting the immune responses observed. It would have also been useful to determine which PTMs occurred the most after screening a cohort of PCa (compared to normal volunteer) urine samples; and to confirm whether alpha enolase PTMs have any diagnostic utility. However, these studies could not be performed due to limitations in the technology utilised in this study and due to time and feasibility constraints.

A further limitation of this study is that pooled samples were utilised to determine alpha enolase urine expression (figure 4.5). As a result, it is not certain how many samples in the pool contain alpha enolase. It could have been only one sample that had alpha enolase or it could have been several or even all of the urine samples. Thus, the only way to definitively determine this would be to screen each sample that made up the pool individually. Although it would have been advantageous to screen patients individually, this was not carried out due to time and feasibility constraints. Further studies need to investigate the

expression of alpha enolase in the urine of patients with PCa compared to healthy controls (e.g. in an ELISA assay) in order to determine if a differential expression of alpha enolase exists in urine, facilitating its use as a potential PCa biomarker.

4.4.3 Alpha enolase expression in tissue

Although the expression of alpha enolase is well reviewed by Capello *et al.* (2011), alpha enolase tissue expression was carried out to confirm its expression in a range of normal organs, multiple cancers, and adjacent normal tissues. It also served as an avenue to confirm its subcellular localisation and optimise conditions to determine the expression of alpha enolase in a large PCa cohort which constitutes part of the next chapter – as our group has a focus on PCa biomarker discovery.

Using immunohistochemistry, multiple normal organ tissues demonstrated negative to moderate staining for alpha enolase (figure 4.7 and table 4.2). Out of all 12 organs stained, 42% (5 out of 12 organs) showed negative alpha enolase expression, while 25% (3 out of 12) and 33% (4 out of 12) showed weak and moderate staining, respectively. Although each organ is derived from a single patient, making it difficult to draw definite conclusions from these results, this study shows a low to moderate expression of alpha enolase in normal tissues (table 4.2). In contrast, alpha enolase expression in multiple cancer tissues demonstrated a range of negative to strong staining, where out of 12 organs stained, 8% (1 out of 12) showed negative alpha enolase expression, while 25% (3 out of 12), 58% (7 out of 12) and 8% showed weak, moderate and strong expression, respectively (table 4.2B). Table 4.3A also shows a similar expression pattern of alpha enolase in normal tissues and adjacent tissues (negative to moderate staining). These results imply a differential expression of alpha enolase between healthy tissues and cancer tissues, similar to findings in the literature and reviewed by Capello *et al.*, (2001) and table 4.1.

When the alpha enolase expression was compared between cancer and adjacent normal tissues, no changes were observed in 41% of the organs (prostate, uterine cervix, stomach, liver and kidney) (table 4.3). Furthermore, although each organ screened was derived from a single patient, it is interesting to note that a reduced expression of alpha enolase only occurred in invasive ductal carcinoma in breast cancer, out of all the tissues stained. This

finding is similar to work published by Shih *et al.* (2010) who found lower levels of autoantibodies to alpha enolase in patients with stage IV non-small cell lung cancer, small cell lung cancer and breast cancer compared with healthy controls. This study also showed an increased alpha enolase expression in lung, colon, ovary, oesophagus, skin and cerebrum, similar to work already published and reviewed by Capello *et al.* (2011).

It is also important to note that, while no change in expression between adjacent normal tissue and PCa tissue was identified from this small TMA cohort, there was a difference in the expression pattern between normal prostate tissue and PCa/adjacent prostate tissue (table 4.4). This indicates that there may be a differential expression of alpha enolase in PCa; however, because of the small sample size used in this TMA, it is not possible to make any firm conclusions about the expression of alpha enolase in PCa. This work then led to the final phase of the study which assessed the expression of alpha enolase in a small sample set of PCa patients (n=9 PCa patients; table 4.4). From table 4.5, 7 out of 9 patients (78% of cases) demonstrated a moderate alpha enolase expression, while 1 out of 9 (11%) showed a negative and high expression of alpha enolase in PCa, respectively (table 4.5). No expression was observed in healthy prostate tissue and low grade malignant leiomyosarcoma (table 4.5). This study again points to a differential expression of alpha enolase in PCa, but as previously mentioned, due to the small number of patients utilised in this study, firm conclusions cannot be drawn as to the association between alpha enolase and PCa.

The aim of this chapter was to demonstrate the expression of alpha enolase in different biological samples, in order to validate its potential utility as a PCa biomarker. Apart from PCa cell lines, where no healthy prostate cells were available to serve as a control, all other biological samples (urine and tissue) have shown a high expression of alpha enolase in PCa compared to healthy controls; further indicating that alpha enolase is a TAA of potential interest in PCa and its diagnostic value needs to be validated in a larger sample cohort.

CHAPTER 5: VALIDATION OF ALPHA ENOLASE AS A BIOMARKER FOR PROSTATE CANCER AND ITS CORRELATION WITH CLINICO-PATHOLOGICAL VARIABLES

5.1 INTRODUCTION

In the UK, PCa is the second leading cause of cancer-related deaths in men after lung cancer (Cancer Research UK, 2015b), and arises mainly from prostatic intraepithelial neoplasia (PIN). PIN is a precursor lesion that progresses to adenocarcinoma and eventually into metastatic disease (DeMarzo *et al.*, 2003). In addition, PCa is asymptomatic until it becomes advanced or metastatic disease, thus, patient screening to aid early PCa diagnosis is mandatory (Smith *et al.*, 2003).

Chapter 3 (section 3.3.2 and 3.3.3) identified alpha enolase in two well-characterised PCa cell lines as potential TAAs in PCa. In addition, using the same set of patient sera, a differential autoantibody expression in alpha enolase was identified in a small subset of patients with PCa compared to healthy controls (3.3.4.4). This initial findings warranted the evaluation and verification of alpha enolase in PCa as a potential PCa biomarker, which constituted part of the previous chapter. From that study, a differential alpha enolase expression was identified almost exclusively in a small cohort of PCa patients compared to healthy controls in tissues (section 4.3.3.3) and urine (section 4.3.2). These initial studies were the first to demonstrate an increased alpha enolase expression in a small cohort of PCa patients compared to healthy controls and warranted the validation of alpha enolase as a potential biomarker in PCa.

It is well documented that the existence of TAAs in cancer patients induce the production of IgG autoantibodies triggered by CD4⁺ T cells and B-cells. The release of these TAAs by shedding, secretion or cell lysis allow them to be taken up by antigen presenting cells where they are processed and presented by major histocompatibility complex (MHC) class I or MHC class II molecules. These antigenic fragments are primed for the activation of

CD8+ and CD4+ T cells respectively. Thus, MHC class II molecules present antigenic fragments to CD4+ T-cells, which via the secretion of cytokines, trigger the proliferation of B cells and the production of IgGs against the same TAAs (Sahin *et al.*, 1997). The immune response generated against TAAs suggests that autoantibodies can be used as a diagnostic tool for the detection of many cancers (Mojtahedi *et al.*, 2011).

Alpha enolase is overexpressed in many cancers (table 4.1) has been reported to induce the production of autoantibodies in many cancers such as pancreatic (Tomaino *et al.*, 2011), leukaemia (Cui *et al.*, 2005 and Zou *et al.*, 2005), melanoma (Suzuki *et al.*, 2010 and Forgber *et al.*, 2009), head and neck (Tsai *et al.*, 2010, Shukla *et al.*, 2007 and Shukla *et al.*, 2009), breast (Shih *et al.*, 2010 and Ejma *et al.*, 2008) and lung cancers (He *et al.*, 2007, Li *et al.*, 2006, Shih *et al.*, 2010 and Chang *et al.*, 2006, Jankowska *et al.*, 2004, Nakanishi *et al.*, 2006, Ueda, 2005 and Dot *et al.*, 2005). In addition, many studies have demonstrated the diagnostic and prognostic utility of alpha enolase autoantibodies. For example, Tu *et al.* (2010) identified that increased alpha enolase expression in breast cancer correlates with poor nodal status, tumour size, and a shorter disease free survival, while Shih *et al.*, 2010 identified a reduction in alpha enolase autoantibody expression in advanced stages of breast and lung cancer. In hepatocellular carcinoma, the increased expression of alpha enolase correlates positively with venous invasion and increased with tumour differentiation (Takashima *et al.*, 2005 and Hamaguchi *et al.*, 2008), while patients with high alpha enolase autoantibody expression have been shown to have significantly poorer clinical outcomes than patients with lower expression, including shorter overall and progression free survival in head and neck and non-small cell lung cancers (Tsai *et al.*, 2010 and Chang *et al.*, 2006).

Despite the utility of alpha enolase autoantibodies in cancer detection, no studies have investigated its expression in a cohort of PCa patients till date. As a result, no studies have determined whether or not there is any correlation between alpha enolase protein expression or autoantibody expression and clinical clinico-pathological variables. Thus, the primary objective of this chapter was to assess the expression profile of alpha enolase in tumour tissues of from a large cohort of patients with various grades and stages of PCa and to determine whether the alpha enolase expression correlates with important clinical variables. The second aim is to determine whether the autoantibody response to alpha

enolase in PCa patient sera correlates with any clinico-pathological variables in patients with various Gleason grades and stages of PCa.

5.1.1 Aims and objectives

The aim of this chapter is to investigate the diagnostic significance of alpha enolase in PCa. This will be carried out by:

1. Screening a prostate TMA cohort from Nottingham University Hospitals to determine levels of alpha enolase protein expression using immunohistochemistry.
2. Statistically determining whether alpha enolase expression correlates with any clinico-pathological variables.
3. Demonstrating that autoantibodies to alpha enolase are differentially expressed in PCa compared to healthy controls/benign diseases using an ELISA technique
4. Determining whether alpha enolase autoantibody expression correlates with important clinico-pathological variables in PCa.

5.2 METHODS

5.2.1 Patient tissue and sera samples

Tissue samples

Tissue microarray analysis of alpha enolase expression was carried out at the department of Histopathology, Queens Medical Centre, Nottingham University Hospitals, NHS Trust, Nottingham, UK in collaboration with Dr Desmond Powe. Ethical approval for collection and use of pathology specimen was granted by the North West 7 Research Ethics Committee – Greater Manchester Central Research Ethics Committee number 10/H1008/72. Samples were collected between 1999 and 2001 from a total of 235 diagnosed patients at the Nottingham City Hospital, as part of a wider study carried out by the Nottingham Prostate Research Group (NPRG).

From each patient, two 0.6 mm cores of TURP or radical prostatectomy tissue specimens were assembled using a Grand Master Arrayer (3DHitech Ltd, Hungary) to make tissue microarrays using formalin-fixed paraffin embedded benign and clinically confirmed PCa cores. Pathological variables such as age, histology, location of metastasis, PSA, Gleason score, time taken to metastasis and D’Amico risk were recorded.

Sera samples

Human sera was collected from patients at the Leicester General Hospital, Leicester, UK after ethical approval by the National Research Ethics Committee. Samples were collected from two separate patient cohorts – the LE sample series, consisting of patients who had a TRUS biopsy (ethical number NREC #09/H0401/92 and UHL 10858). This cohort was gathered under the title ‘*A pilot study to identify gene fusions in prostate cancer*’. The second sample cohort was collected under the title ‘*Define the role of the Transperineal Template guided prostate biopsy*’ (approval number NREC #11/EM/0312 and UHL 11068), making up the TP series. Males aged 51-89 years (for the LE cohort) or 50-84 years (for the TP cohort), referred by their GP for high PSA levels (>4 ng/mL) and/or having symptoms of PCa and attending the urology clinic under the supervision of Mr Masood Khan, were recruited after signed informed consent forms had been collected.

In addition, healthy control sera was obtained from volunteers at Nottingham Trent University (Ethical number 135) after informed consent forms had been signed and collected. As described in detail in section 2.2.1, samples were collected and processed using the protocol outlined in section 2.2.1 and stored at -80°C , thawing only prior to processing.

5.2.2 Immunohistochemical staining of alpha enolase

TMA sections were immunostained as described in section 4.2.4. In brief, TMA sections cut at the Queens Medical Centre, were melted at 60°C and deparaffinised and hydrated using an Autostainer. Slides were immersed twice in xylene baths and then in graded alcohol baths (100%, 100% and 70% ethanol) before a wash at 5 min. Antigen retrieval was carried out using a citrate buffer in a microwave and slides were rinsed and loaded onto sequenza reservoir. Tissue staining was carried out using the Novolink Polymer Detection System kit following the manufacturer's instructions. First, a peroxidase block step was carried out, followed by washes and the addition of a protein block to eliminate excess background staining. An optimised dilution (1:3000) of mouse monoclonal alpha enolase antibody (clone L-27, Santa Cruz, UK) was added for 60 min followed by washing and the addition of a post-primary antibody, polymer, DAB stain and counterstain using haematoxylin. Excess haematoxylin was rinsed and slides were placed in the Autostainer for dehydration using dH_2O and graded alcohol baths (70%, 100% and 100% ethanol) before two final immersions in a xylene bath. Coverslips were mounted onto each slide using DPX mounting medium.

5.2.3 ELISA to detect alpha enolase autoantibody responses

An ELISA assay was carried out to detect the presence of autoantibodies to alpha enolase in patients with PCa, and described in detail in section 3.2.3.3. In brief, 96 well plates were coated overnight with a recombinant alpha enolase protein (100 ng/mL) and washed. Each well was blocked to eliminate non-specific binding using a BSA blocking buffer before 3 wash steps with TBST. Sera from PCa, benign, healthy controls or monoclonal mouse anti-alpha enolase antibody (positive control) was allowed to bind for 90 min before a wash step and incubation with a rabbit anti-human IgG (for sera) or goat anti-mouse (alpha

enolase positive control) secondary antibody conjugated to HRP. Wells were washed and a TMB substrate was added, which was stopped with a sulphuric acid stop solution and an absorbance reading at 450 nm was taken.

5.2.4 Statistical analysis

Stained TMA sections were microscopically assessed by a pathologist (Dr Desmond Powe) and semi-quantitative tissue scoring was carried out using a scale ranging from 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The association between alpha enolase expression and clinical variables are calculated using univariate chi-square analysis and cumulative survival probabilities were modelled using Kaplan-Meier survival plots and a Cox regression hazards risk model (SPSS software version 20.0, SPSS Inc., USA). Statistical significance was considered as $p \leq 0.05$.

5.3 RESULTS

The validation of alpha enolase as a potential PCa biomarker and its correlation with clinico-pathological variables is subdivided into 2 sections:

- i. Alpha enolase expression in NPRG prostate TMAs (section 5.3.1)
- ii. Alpha enolase sera autoantibody expression (section 5.3.2).

5.3.1 Alpha enolase protein expression in NPRG prostate TMAs

As this study was based on the identification of markers for PCa, a large TMA consisting of 235 patients, in collaboration with the NPRG at the Queen's Medical Centre, Nottingham was utilised to verify alpha enolase expression. The same assay conditions used in the previous chapter were applied to this cohort (antibody dilution, incubation times outlined in section 4.2.4). This patient cohort consisted of 10 TMA FFPE blocks which were constructed using cores taken from patients with BPH and clinically confirmed PCa. As stated previously, a semi-quantitative scoring intensity scale ranging from 0 – 3 (0 = no staining, 1 = weak; 2 = moderate; 3 = strong staining) was utilised for cytoplasmic expression of alpha enolase or 0-2 for nuclear expression of alpha enolase. The association between alpha enolase and clinical variables are modelled using Kaplan-Meier plots and the Cox regression hazards risk model was used to calculate univariate chi-square analysis. A statistical significance at $p \leq 0.05$ was utilised in each case.

5.3.1.1 Cytoplasmic alpha enolase expression in PCa and benign prostatic disease

As demonstrated in the previous figure 4.6, alpha enolase expression was observed mainly in the cytoplasm of the prostatic epithelia. Table 5.1 shows the frequency of cytoplasmic alpha enolase expression in PCa and benign tissues within the NPRG cohort (n=235 patients). Of all cases that were negative for cytoplasmic alpha enolase (n=21), 71.4% of them occurred in PCa while 28.6% occurred in benign patients. However, of all cases with a positive alpha enolase expression (n=214), 58.9% occurred in PCa compared to 41.1% of benign controls patients (table 5.1).

Table 5.1: Frequency of cytoplasmic alpha enolase expression in NPRG cohort

Pathology	Frequency	Negative (n=21)	Positive (n=214)	Total (n=235)
		0	1, 2 and 3	
Benign disease	Number of cases	6	88	94
	Percentage (%)	28.6	41.1	40.0
Prostate cancer	Number of cases	15	126	141
	Percentage (%)	71.4	58.9	60.0

Benign and PCa tissues were scored for cytoplasmic alpha enolase expression using a semi-quantitative 3 point scoring system ranging from 0 – 3 (0 = negative expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression). Negative or positive staining in benign or cancer patients is depicted in the table 5.1.

Although from table 5.1, 41.1% of benign cases showed positive alpha enolase expression, further examination of positive staining distribution for each patient group showed that most of the benign tissues demonstrated weak (score 1; 54.5%) or moderate staining (score 2; 19.1%) compared to cancer patients with 45.5% and 80.9% (table 5.2). Similarly, strong alpha enolase tissue expression (score 3) was observed in 78.6% of cancer patients compared to 21.4% in benign cases (table 5.2). Statistical significance ($p=0.000003$) was observed when weak cytoplasmic alpha enolase expression (staining intensity score 0 and 1; 17%) was compared to strong expression (staining intensity score 2 and 3; 46.8%). These findings demonstrate that higher cytoplasmic alpha enolase expression is more frequent in PCa compared to benign prostatic tissue.

Table 5.2: Cytoplasmic alpha enolase positive staining intensity distribution

Pathology	Frequency	Positive intensity (stain score)			Total (n=214)
		1 (n=132)	2 (n=68)	3 (n=14)	
Benign disease	Number of cases	72	13	3	88
	Percentage (%)	54.5	19.1	21.4	41.1
Prostate cancer	Number of cases	60	55	11	126
	Percentage (%)	45.5	80.9	78.6	58.9

Benign and PCa tissues scored for cytoplasmic alpha enolase expression using a semi-quantitative 3 point scoring system ranging from 0 – 3 (0 = negative expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression). The table shows the positive staining distribution for cancer or benign controls.

To determine whether cytoplasmic alpha enolase expression correlated with any clinicopathological variables in PCa (Gleason grade, D'Amico risk prediction, bone metastasis or survival), a dichotomous categorisation of cytoplasmic alpha enolase expression in PCa patients was performed using Kaplan Meier survival modelling in order to assess these score categories. The first categorisation used was negative cytoplasmic alpha enolase expression (stain intensity 0) vs. positive expression (stain intensity 1, 2 and 3). This was compared to a low (stain intensity 0 and 1) vs. high expression (stain intensity 2 and 3). Figure 5.1 shows the Kaplan Meier survival time analysis of negative vs. positive cytoplasmic alpha enolase expression compared to low vs. high cytoplasmic alpha enolase expression.

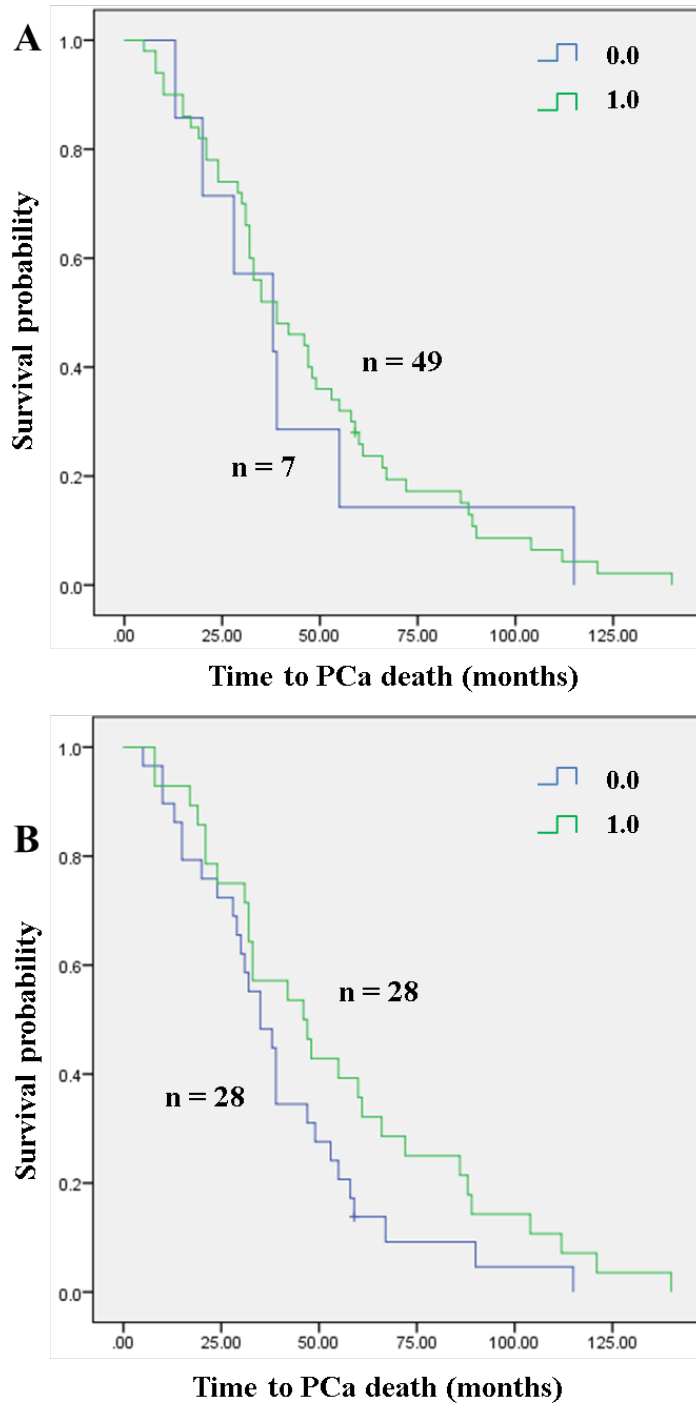


Figure 5.1: Dichotomous categorisation of cytoplasmic alpha enolase expression modelled by Kaplan-Meier survival graphs.

Immunohistochemical staining for cytoplasmic alpha enolase staining was carried out and scored using a 3 point scoring system ranging from 0 – 3 (0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression). Survival analysis of clinically confirmed PCa patients for different categories is shown between 0-125 months. A shows analysis for negative (score 0; 0.0 on survival graph) vs. positive cytoplasmic staining (score 1, 2 and 3; 1.0 on survival graph) ($p=0.759$), while B shows low (score 0 and 1; 0.0 on survival graph) vs. high alpha enolase expression (score 2 and 3; 1.0 on survival graph) ($p=0.101$).

Low vs. high cytoplasmic alpha enolase expression (staining score 0 and 1 vs. 2 and, 3; figure 5.1B) produced the best separation for predicting survival outcome in PCa ($p=0.101$). Figure 5.1B also shows that increased alpha enolase expression (staining intensity 2 or 3) is associated with an improved survival over 5 years, but this was not statistically significant. As a result, the low vs. high categorisation was applied to determine the association between alpha enolase expression and clinico-pathological variables in PCa.

5.3.1.2 Association between cytoplasmic alpha enolase expression and clinico-pathological features

Next, the association between cytoplasmic alpha enolase expression in PCa samples and known clinic-pathological features were determined using a chi-square test (table 5.3). To summarise, cytoplasmic alpha enolase expression was not associated with Gleason grade (Gleason grade <7 vs. ≥ 7 , $p=0.464$), initial PSA ($p=0.992$), D'Amico risk ($p=0.831$), proliferation (Ki67) ($p=0.13$), bone metastasis ($p=0.295$), or castrate treatment resistance ($p=0.116$) (figure 5.2 and table 5.3). Figure 5.2 shows a Kaplan-Meier survival graph for the association between cytoplasmic alpha enolase and survival, where no statistical significance was observed at $p<0.05$. There is some limited indication that patients with alpha enolase positive cancers are however more likely to live longer ($p=0.101$) (fig 5.2) and less likely to develop any metastasis ($p=0.149$) (table 5.3). However none of these findings are statistically significant at $p<0.05$ (table 5.3).

Table 5.3: Association between cytoplasmic alpha enolase staining and clinico-pathological variables in PCa

Characteristics		Frequency	Negative	Positive intensities	p-value
			0	1 2 3	
Gleason grade	Grade <7	120	11	12	0.464
	Grade ≥7		50	47	
Initial PSA	0 - 10 ng/mL	119	16	15	0.992
	10.1 - 20 ng/mL		10	10	
	>20 ng/mL		35	33	
D'Amico risk prediction	High	125	12	8	0.831
	Intermediate		48	43	
	Low		6	8	
Proliferation (Ki67)	0 - 10%	71	38	8	0.13
	11 - 100%		10	15	
Bone metastasis	No metastasis at presentation	120	48	52	0.295
	Prostate associated bone metastasis at presentation		12	7	
	Bone metastasis due to other cancer causes		1	0	
Any metastasis	Negative	99	24	33	0.149
	Positive		23	19	
Castrate treatment resistance		50	25	25	0.116
Survival	Months	56	28	28	0.101

The association between cytoplasmic alpha enolase expression and clinico-pathological variables. No statistical significance was found between cytoplasmic alpha enolase expression and clinical variables at $p < 0.05$.

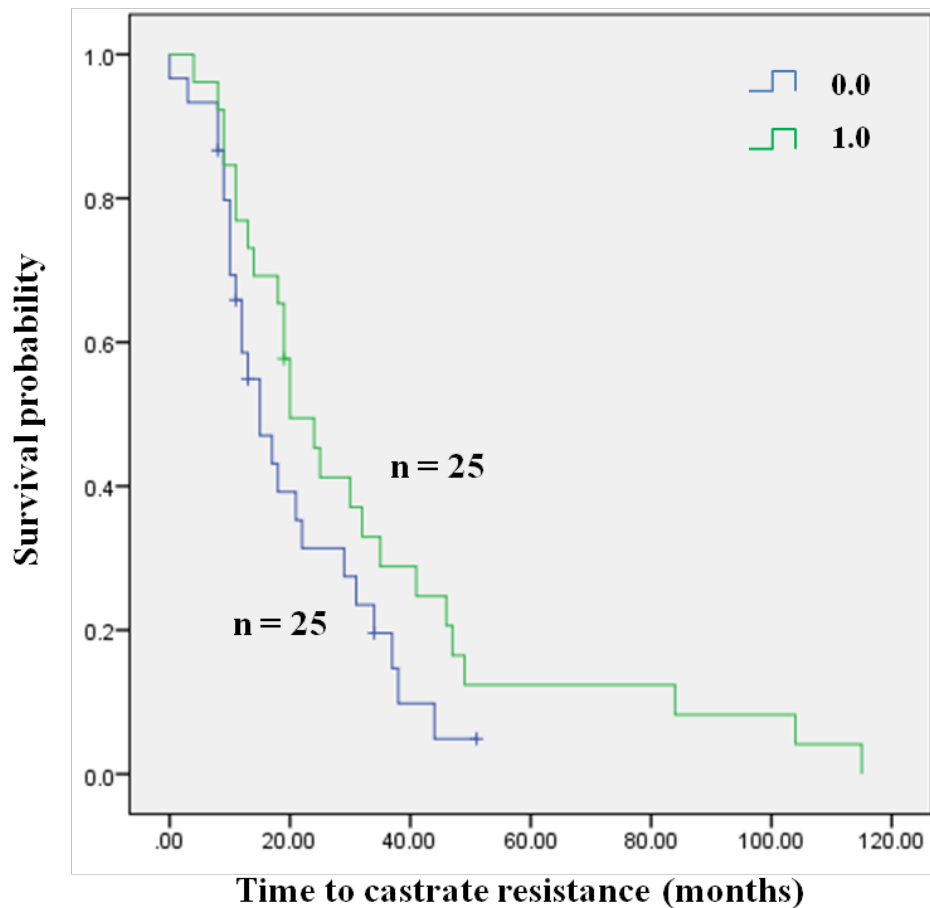


Figure 5.2: Kaplan-Meier survival analysis from diagnosis to castrate resistance using low vs. high cytoplasmic alpha enolase scoring intensities.

Survival analysis of clinically confirmed PCa patients showing time from initial diagnosis (0 months) to castrate resistance. A numerical scoring method assigning 0.0=low alpha enolase expression vs. high expression (1.0) is shown. Results show no statistical significance $p=0.116$.

5.3.1.3 Nuclear alpha enolase expression in PCa and benign prostatic diseases

Although alpha enolase expression is more abundant in the cytoplasm (section 4.3.3.1), protein expression was also identified in the nucleus of the prostatic epithelia in benign and PCa tissues. For nuclear expression, score intensities ranging from 0-2 were utilised (0 = no staining, 1 = weak, 2 = strong staining), as a weaker staining pattern was observed compared to cytoplasmic staining. Table 5.4 shows the frequency of nuclear alpha enolase expression in PCa and benign tissues within this cohort, whereby of all the patients who showed negative alpha enolase nuclear expression ($n=209$), 56.9% of them were PCa patients compared to 43.1% of benign patients. Similarly, of all patients who had a positive

alpha enolase expression (n=29), 13.8% of positive staining was identified in benign diseases compared to 86.2% of PCa patients; demonstrating that high alpha enolase score intensities occur more frequently in PCa (table 5.4).

Table 5.4: Frequency of nuclear alpha enolase expression in NPRG cohort

Pathology	Frequency	Negative (n=209)	Positive (n=29)	Total (n=238)
		0	1 and 2	
Benign disease	Number of cases	90	4	94
	Percentage (%)	43.1	13.8	39.5
Prostate cancer	Number of cases	119	25	144
	Percentage (%)	56.9	86.2	60.5

Benign and PCa tissues scored for nuclear alpha enolase expression using a semi-quantitative 2 point scoring system ranging from 0 – 2 (0 = negative expression, 1 = weak expression, 2 = strong expression). Negative or positive staining in benign or cancer patients is depicted in the table.

Positive nuclear staining distribution showed that 90% of all the strong staining intensities (staining score 2) occurred in PCa compared to benign disease (10%) (table 5.5). A similar pattern was also observed with weakly stained tissues (staining score 1) with 84.2% for PCa and 15.8% for benign patients, further indicating that high alpha enolase score intensities occur more frequently in PCa. Statistical significance (p=0.003) was observed when weak nuclear alpha enolase expression (staining intensity score 0; 4.3%) was compared to strong expression (staining intensity score 1 and 2; 17.4%). These findings demonstrate that higher nuclear alpha enolase expression is more frequent in PCa compared to benign prostatic tissue.

Table 5.5: Nuclear alpha enolase positive staining intensity distribution

Pathology	Frequency	Positive intensity		
		1 (n=19)	2 (n=10)	Total (n=29)
Benign disease	Number of cases	3	1	4
	Percentage (%)	15.8	10	13.8
Prostate cancer	Number of cases	16	9	25
	Percentage (%)	84.2	90	86.2

Benign and PCa tissues scored for nuclear alpha enolase expression using a semi-quantitative 2 point scoring system ranging from 0 – 2 (0 = negative expression, 1 = weak expression, 2 = strong expression). The table shows the positive staining distribution for cancer or benign tissues.

Similar to cytoplasmic expression, a dichotomous categorisation of nuclear alpha enolase expression in PCa was performed using Kaplan Meier survival modelling to determine whether nuclear alpha enolase expression correlated with any clinico-pathological variables. The first categorisation used was negative nuclear alpha enolase expression (stain intensity 0) vs positive expression (stain intensity 1 and 2), which was compared to a low (stain intensity 0 and 1) vs. high nuclear alpha enolase expression (stain intensity 2). Figure 5.3 shows the Kaplan Meier survival time analysis of negative vs. positive nuclear alpha enolase expression compared to low vs. high expression.

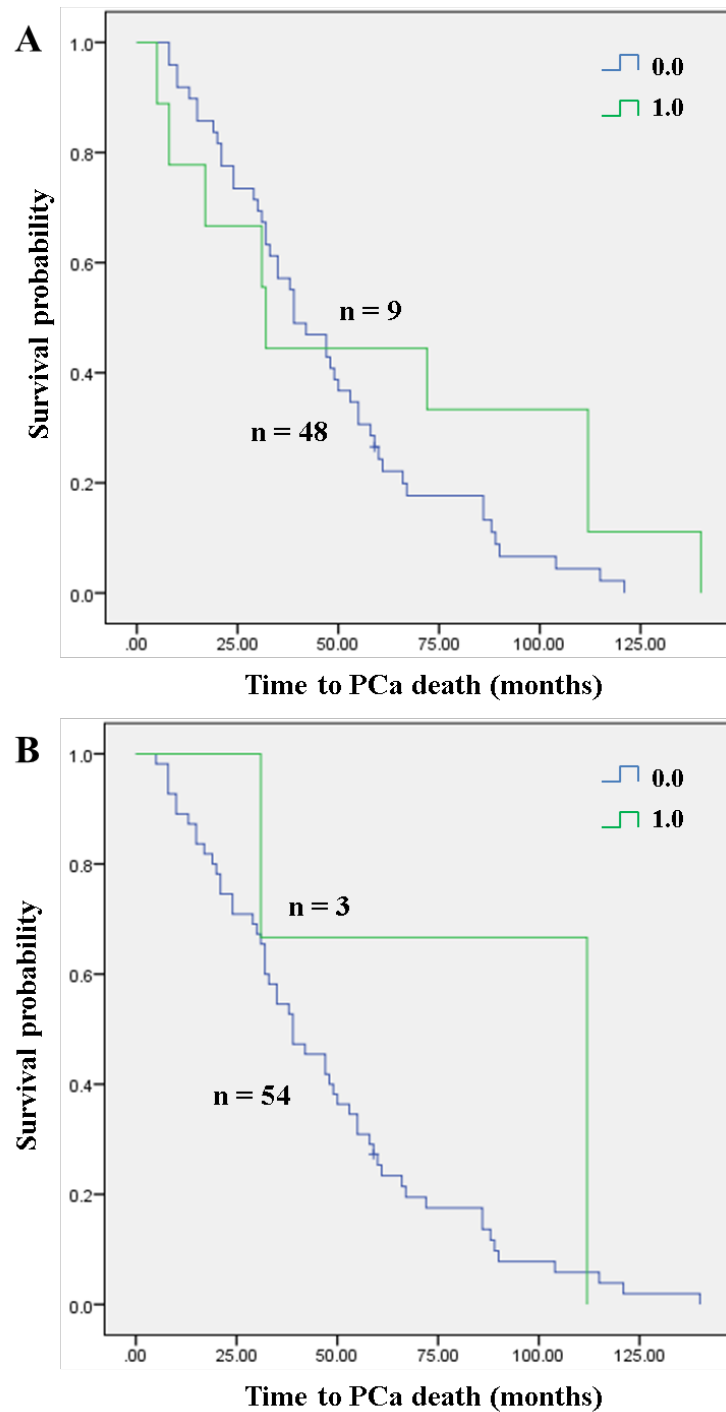


Figure 5.3: Dichotomous categorisation of nuclear alpha enolase expression modelled by Kaplan-Meier survival graphs.

Immunohistochemical staining for nuclear alpha enolase staining was carried out and scored using a 2 point scoring system ranging from 0 – 2 (0 = no expression, 1 = weak expression, 2 = strong expression). Survival analysis of clinically confirmed PCa patients for different categories are shown between 0-125 months. A shows analysis for negative (score 0; 0.0 on survival graph) vs. positive nuclear staining (score 1 and 2; 1.0 on survival graph) ($p=0.205$), while B shows low (score 0 and 1; 0.0 on survival graph) vs. high nuclear alpha enolase expression (score 2; 1.0 on survival graph) ($p=0.194$).

Although a low vs. high nuclear alpha enolase expression (figure 5.3B) produced the best separation for predicting survival outcome in PCa ($p=0.194$) compared to $p=0.205$ for negative vs. positive expression (figure 5.3A), there were not enough patients in the high stain intensity group (score 1.0 on the survival curve for figure 5.3B). As a result, the negative vs. positive intensity category (staining score 0 vs. 1 or 2; figure 5.3A) was applied to determine a correlation between nuclear alpha enolase staining and any clinico-pathological variables in PCa.

5.3.1.4 Association between nuclear alpha enolase expression and clinico-pathological features

The association between nuclear alpha enolase expression in PCa and known clinico-pathological features (Gleason grade, D'Amico risk prediction, bone metastasis, survival and initial PSA) were also determined using a chi-square test. Similar to results obtained in the cytoplasmic expression, nuclear alpha enolase was not associated with Gleason grade (Gleason grade <8 vs. ≥ 8 , $p=0.36$), initial PSA ($p=0.645$), D'Amico risk ($p=0.79$), proliferation (Ki67) ($p=0.365$), bone metastasis ($p=0.642$), metastasis (0.623), castrate treatment resistance ($p=0.219$) or survival ($p=0.205$) (table 5.6). Figure 5.4 shows a Kaplan-Meier survival graph for the association between nuclear alpha enolase and survival, where no statistical significance was observed at $p<0.05$.

Table 5.6: Association between nuclear alpha enolase staining and clinico-pathological variables in PCa

Characteristics		Frequency	Negative	Positive intensities			p-value
			0	1	2	3	
Gleason grade	Grade <8	123	39	9		0.36	
	Grade ≥8		64	11			
Initial PSA	0 - 10 ng/mL	122	27	6		0.645	
	10.1 - 20 ng/mL		19	2			
	>20 ng/mL		56	12			
D'Amico risk prediction	High	128	15	5		0.79	
	Intermediate		77	16			
	Low		12	3			
Proliferation (Ki67)	0 - 10%	96	55	20		0.365	
	11 - 100%		14	7			
Bone metastasis	No metastasis at presentation	123	97	7		0.642	
	Prostate associated bone metastasis at presentation		18	1			
Any metastasis	Negative	103	56	4		0.623	
	Positive		40	3			
Castrate treatment resistance		51	41	10		0.219	
Survival	Months	57	48	9		0.205	

The association between nuclear alpha enolase expression and clinico-pathological variables. No statistical significance was found between nuclear alpha enolase expression and any clinical variables at $p < 0.05$.

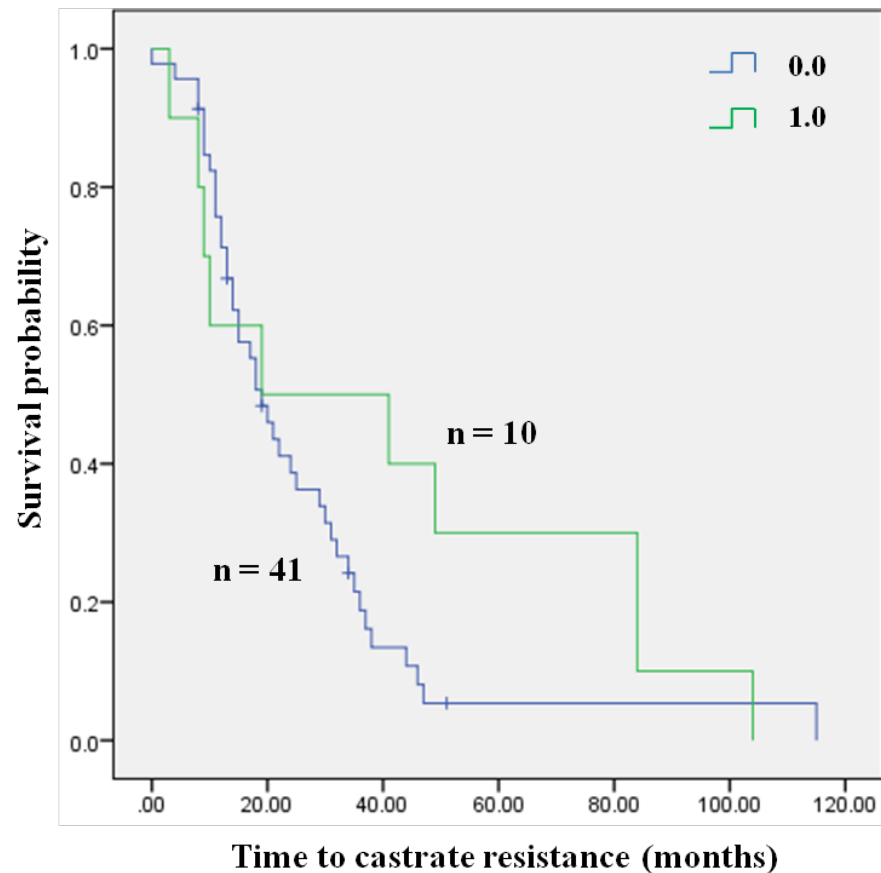


Figure 5.4: Kaplan-Meier survival analysis from diagnosis to castrate resistance using negative vs. positive nuclear alpha enolase scoring intensities.

Survival analysis of clinically confirmed PCa patients from initial diagnosis (0 months) to time to castrate resistance. A numerical scoring method assigning 0.0 = negative nuclear alpha enolase expression (stain intensity 0) vs. positive expression (1.0; stain intensity 1 and 2)) is shown. Results show no statistical significance $p=0.219$.

5.3.1.5 Membranous alpha enolase expression in PCa

Immunohistochemical staining of the NPRG cohort revealed membranous alpha enolase expression in a small subset of patients (8 patients out of 235), and as a result, no statistical tests were performed on this small number of cases. Score intensities of 0 for no staining and 1 for positive staining was allocated to all tissues analysed. Membranous alpha enolase expression was only identified in PCa patients and not in benign cases. Table 5.7 shows the frequency of membranous alpha enolase staining, where no follow up clinical information was available for 3 out of 8 membrane positive patients.

Table 5.7: Membranous expression of alpha enolase in PCa

Patient analysis no.	Gleason Grade	Any metastasis (Yes or No)	Membrane expression (0 or 1)	Cytoplasmic expression (0-3)	Nuclear expression (0-2)
48	9	No	1	2	1
85	9	Yes	1	2	0
97	Not available	Not available	1	2	1
105	5	No	1	3	0
214	8	No	1	3	2
233	Not available	Not available	1	3	0
241	Not available	Not available	1	2	0
365	6	No	1	1	0

Membrane positive alpha enolase expression was identified in 8 patients out of 235. Cytoplasmic and nuclear expression score intensities ranging from 0-3 (cytoplasmic expression) or 0-2 (nuclear expression) in the same patients is also shown.

In cases with available clinical information, alpha enolase membranous expression was identified in 3 out of 5 patients with high Gleason grade tumours (Gleason 8 & 9; 60% of cases) compared to 2 patients with low grade PCa (Gleason 5 and 6; 40% of cases) (table 5.7). Furthermore, 1 out of 5 patients positive for alpha enolase membrane expression had metastatic PCa (20%) compared to 80% with no metastasis. Figure 5.5 shows membranous alpha enolase expression in 2 patients out of 5 with available clinical information.

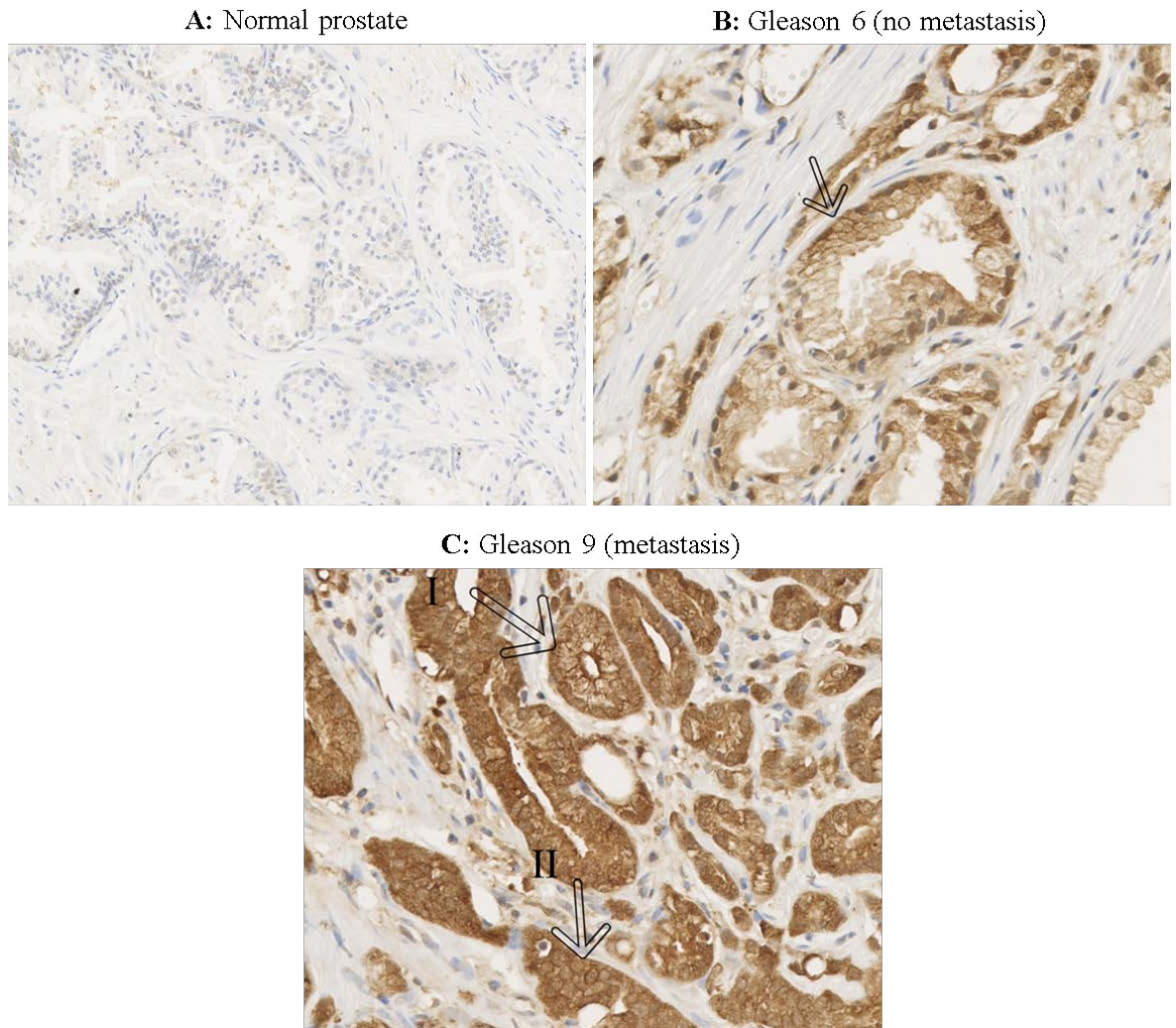


Figure 5.5: Membranous expression of alpha enolase in PCa.

Membrane expression of alpha enolase was observed in 8 out of 235 patients by immunohistochemistry. A shows alpha enolase expression in a normal prostate, while B shows alpha enolase expression in one membrane positive (arrow) patient with Gleason 6 PCa (patient no. 365). C shows alpha enolase expression in one patient with Gleason 9 metastatic adenocarcinoma (patient no. 85); where intense expression is observed in the lumen (I) and positive membrane expression on the basal cells of the prostatic epithelium (II). *Objective magnification x 20 (A, normal prostate), x 40 (B & C).*

5.3.2 Alpha enolase autoantibody expression in sera

Autoantibodies to alpha enolase were detected in the sera of patients with PCa (section 3.3.4.4 and figure 3.12). However, as not enough healthy control patients were used as a comparison, alpha enolase could not be validated as a potential biomarker for PCa. As a result, the aim of this study was to validate the frequencies of autoantibodies against alpha enolase using an ELISA assay against a full length recombinant alpha enolase protein. Sera from patients with various grades of PCa (n=69), various forms of benign diseases (n=26) including benign prostatic disease (n= 7), atypical glands suspicious of malignancy (n=10), high grade prostatic intraepithelial neoplasia (n=9) and healthy controls (n=25) were tested.

5.3.2.1 Alpha enolase serum autoreactivity

The mean OD \pm 1 SD for healthy sera (from 25 healthy patients) was 0.41 \pm 0.12. When '*mean OD 25 healthy control sera + 3 x SD*' was used as a cut-off point to determine positive reactivity (\geq OD 0.77; dashed red line in figure 5.6), the prevalence of autoantibodies against alpha enolase occurred in 0% of healthy controls and 12% in benign patients (3 out of 26) - 22% in PIN patients (2 out of 9), 10% in patients with atypical glands suspicious of malignancy (1 out of 10) and 13% in benign tumours (1 out of 8 patients). More significantly, alpha enolase expression also occurred in 22% in PCa patients (16 out of 70) (table 5.8 and figure 5.6).

Table 5.8: Frequency distribution of alpha enolase autoreactivity in sera

Patient group	Number of subjects	No. (%) of subjects positive for anti-alpha-enolase autoantibody
Healthy controls	25	0 (0%)
Benign diseases	26	3 (12%)
➤ High PIN	9	2 (22%)
➤ Atypia	10	1 (10%)
➤ Benign	7	0 (0%)
Prostate cancer	69	16 (22%)
➤ Gleason 6	25	5 (20%)
➤ Gleason 7 (4+3)	11	3 (27%)
➤ Gleason 7 (3+4)	18	2 (11%)
➤ Gleason 8	2	1 (50%)
➤ Gleason 9	13	5 (38%)

Reactivity of anti-alpha enolase autoantibodies from healthy controls, patients with various forms of benign prostatic disease and PCa.

Next, the study aimed to determine if there was any statistical significance between alpha enolase autoantibody expression in healthy controls (n=25) compared to benign (n=26) or PCa patients (n=69). Using a one way ANOVA, statistical significance was observed between healthy controls and PCa ($p=0.0038$) (figure 5.6). Statistical significance was not observed between healthy controls and benign diseases ($p=0.1451$) (figure 5.6) or between benign diseases and PCa ($p=0.8867$) at $p<0.05$.

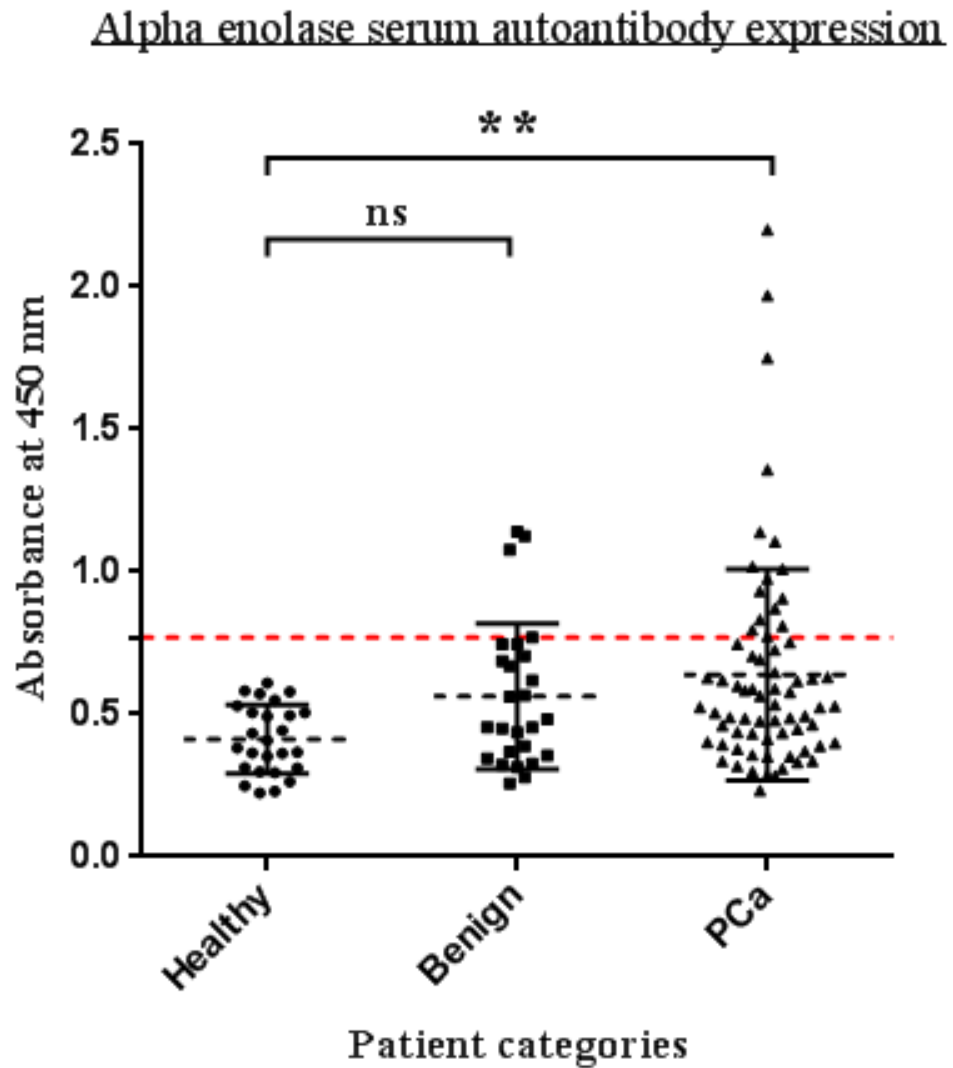


Figure 5.6: Alpha enolase sera autoantibody expression in healthy controls, benign prostatic diseases and PCa.

Mean optical density of ELISA for the detection of autoantibodies to alpha enolase in sera from healthy controls (n=25), benign prostatic diseases (n=26) and PCa patients (n=69). Vertical error bars indicate standard deviation, while middle dashed line indicates mean of each sample group. No statistical significance (ns) was observed between healthy controls and benign cases ($p=0.1451$), while statistical significance was observed between healthy control and PCa ($p=0.0038$) at $p<0.05$. The dashed red line indicates a positive alpha enolase cut off value (OD of 0.77), which is derived from mean OD healthy control sera + 3SD.

5.3.2.2 The association between alpha enolase autoantibody expression and Gleason grade

The correlation between the prevalence of autoantibodies against alpha enolase and Gleason grade in PCa was investigated. Table 5.8 outlines the frequency of alpha enolase autoantibody positivity in various Gleason grades for PCa, where positive reactivity (\geq OD 0.77; dashed red line in figure 5.7) was identified in 20% of Gleason 6 patients (5 out of 25), 27% in Gleason 7 (4+3) (3 out of 11), 11% in Gleason 7 (3+4) (2 out of 18), 50% in Gleason 8 (1 out of 2) and 38% in Gleason 9 PCa patients (5 out of 13). Using a one way ANOVA, there was no statistical significance between the occurrence of autoantibodies against alpha enolase in healthy controls and Gleason grade 6 (0.0990), Gleason grade 7 (3+4) ($p=0.4334$), Gleason 7 (4+3) ($p=0.2852$), or Gleason 8 ($p=0.3892$) (figure 5.7). Statistical significance was however observed between healthy controls and Gleason 9 PCa ($p=0.0043$) at $p<0.05$ (figure 5.7).

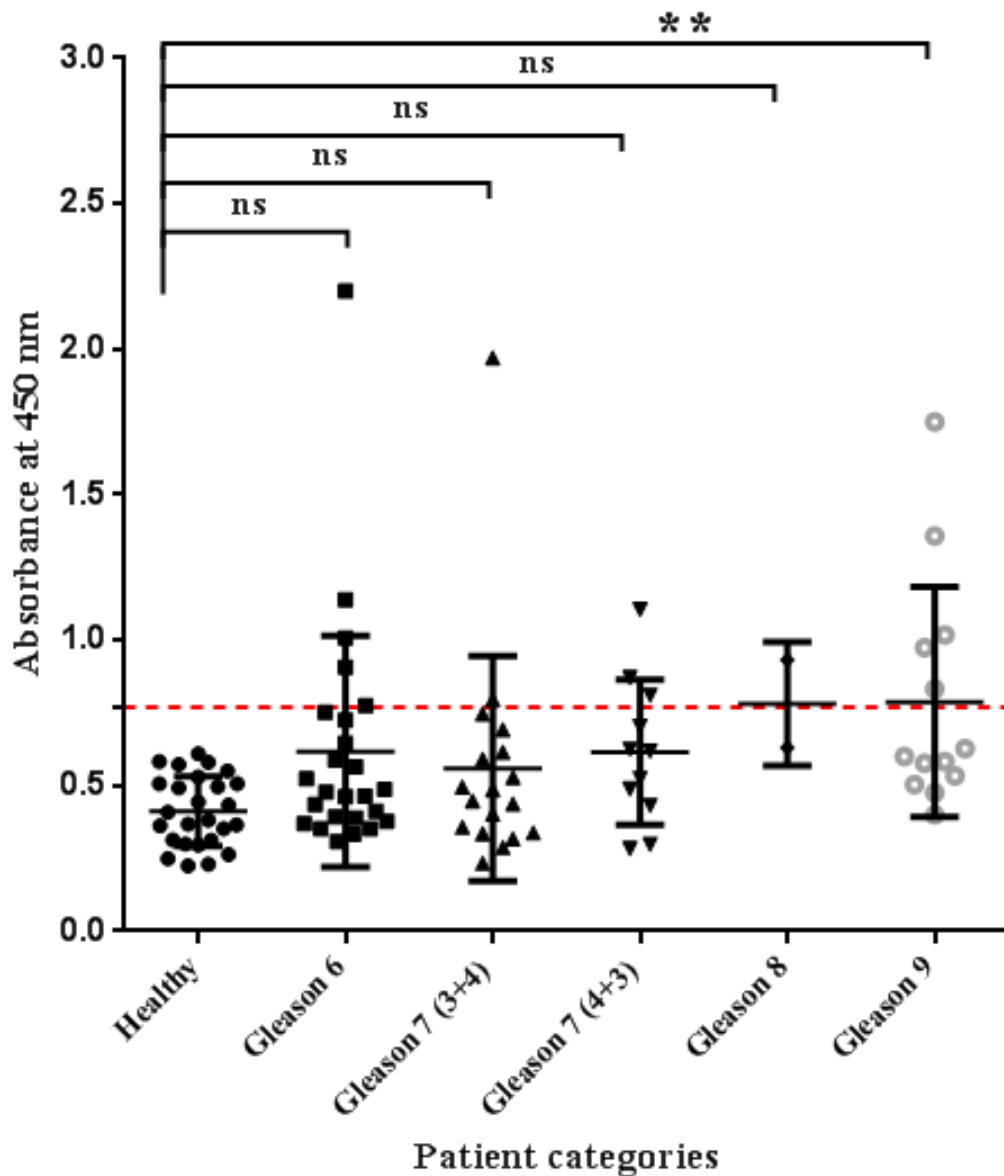
Alpha enolase serum autoantibody expression vs. Gleason grade

Figure 5.7: Alpha enolase serum autoantibody expression in various grades of PCa and healthy controls.

Mean optical density of ELISA for the detection of autoantibodies to alpha enolase in sera from healthy controls (n=25), Gleason 6 (n=25) Gleason 7 (3+4) (n=18), Gleason 7 (4+3) (n=11), Gleason 8 (n=2) and Gleason 9 (n=13). Vertical error bars indicate standard deviation, while middle line indicates mean of each sample group. No statistical significance (ns) was observed between healthy controls and Gleason 6 (p=0.0990), Gleason grade 7 (3+4) (p=0.4334), Gleason 7 (4+3) (p=0.2852) or Gleason 8 patients (p=0.3892). Statistical significance was observed between healthy control and Gleason 6 (p=0.0446) and healthy controls and Gleason 9 PCa (p=0.0043) at p<0.05. Dashed red line indicates a positive alpha enolase cut off value (OD of 0.77), which is derived from mean OD healthy control sera + 3SD.

5.3.2.3 The association between alpha enolase autoantibody expression and D'Amico risk classification

The correlation between alpha enolase and D'Amico risk classification was also investigated. Using a one way ANOVA at $p < 0.05$, statistical significance was not observed between healthy and low D'Amico risk ($p = 0.7927$), but observed between healthy and intermediate D'Amico risk ($p = 0.0125$) and healthy and high D'Amico risk classification ($p = 0.0059$) (figure 5.8). No statistical significance was observed between low and intermediate D'Amico ($p = 0.1292$) or intermediate and high D'Amico ($p = 0.7245$). Statistical significance was however observed between low and high D'Amico ($p = 0.0262$).

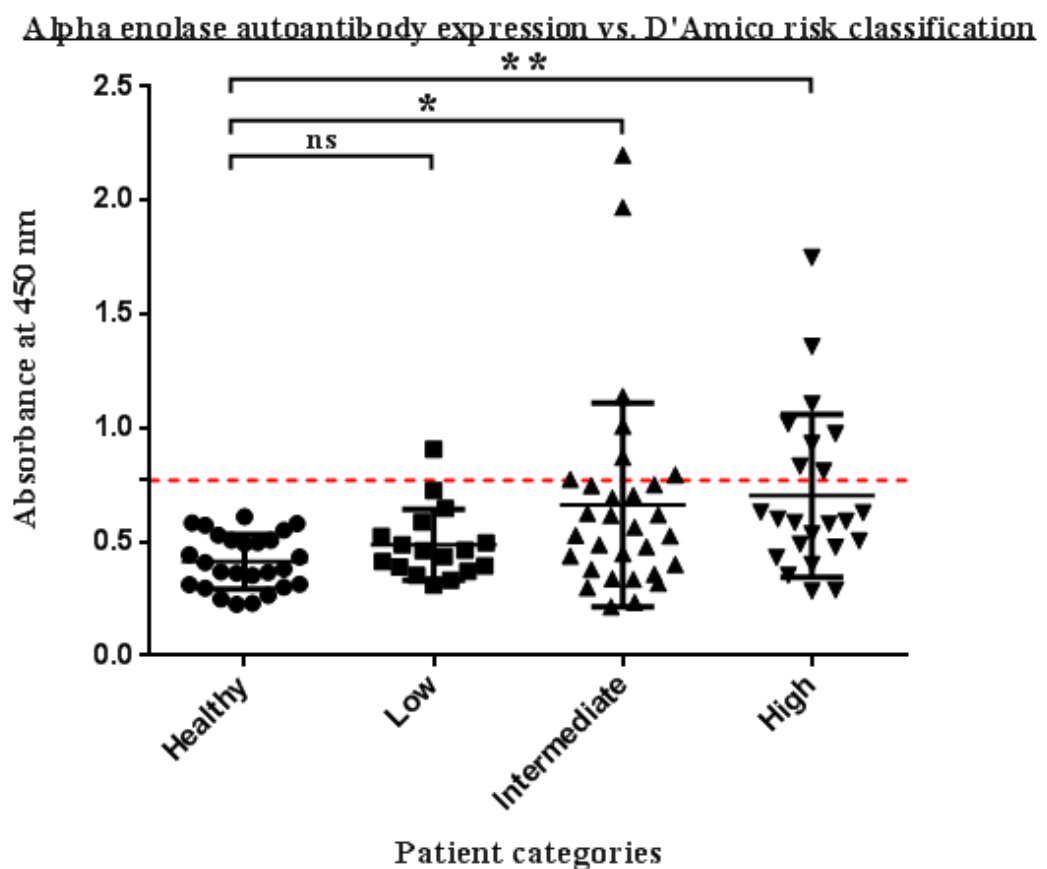


Figure 5.8: Alpha enolase autoantibody expression and D'Amico classification in PCa.

ELISA mean optical density for the detection of autoantibodies to alpha enolase in sera samples from healthy controls and PCa patients from various D'Amico risk categories. Vertical error bars indicate standard deviation, while middle line indicates mean of each sample group. No statistical significance (ns) was observed between healthy controls and low D'Amico risk ($p = 0.7927$), while statistical significance was observed between healthy control and intermediate risk ($p = 0.0125$) and healthy controls and high D'Amico risk in PCa ($p = 0.0059$) at $p < 0.05$. The dashed red line indicates a positive alpha enolase cut off value (OD of 0.77), which is derived from mean OD healthy control sera + 3SD.

5.4 DISCUSSION

The lack of specificity and sensitivity of PSA for the detection of PCa emphasises the need for early PCa detection. The previous chapter observed an increased expression of alpha enolase in urine and PCa tissue from a small sample cohort, compared to healthy controls (section 4.3.2 and 4.3.3). Thus, the aim of the present chapter was to utilise proteomic approaches such as immunohistochemistry and ELISA assays to validate the expression profile of alpha enolase protein and autoantibody responses in tumour tissues and sera from a larger sample cohort. Furthermore, the chapter aimed to determine whether alpha enolase protein or autoantibody expression correlates with important clinical variables. Due to the interest our group has in PCa and our access to a PCa TMA and sera, this study aimed to investigate the potential of alpha enolase as a biomarker for PCa.

5.4.1 Alpha enolase expression in NPRG TMAs

Similar to findings in the previous chapter (section 4.3.3.1) and reported by Perconti *et al.* (2007), alpha enolase expression was observed mainly in the cytoplasm of the patient tissues stained. Increased staining intensity occurred more frequently in PCa (121 patients) compared to benign diseases (88 patients) (table 5.1). A similar expression pattern was also observed when the positive intensity distribution was assessed, where the highest alpha enolase expression (staining intensity score 3) occurred more frequently in PCa (11 patients) compared to benign controls (3 patients) (table 5.2). Similarly, a weak alpha enolase expression (staining intensity score 1) occurred more frequently in benign cases (72 patients) compared to PCa patients (60 patients) (table 5.2).

When weak cytoplasmic alpha enolase expression (staining intensity score 0 and 1) was compared to strong expression (staining intensity 2 and 3), statistical significance was observed between PCa and benign diseases ($p=0.000003$). These results suggest that an increased cytoplasmic alpha enolase expression may be associated with PCa or malignancy, consistent with findings by Chu *et al.*, (2011), who found that cytoplasmic alpha enolase was overexpressed by 18% of all canine mammary carcinomas studied, where alpha enolase overexpression correlated with oestrogen receptor positivity. A list of reported cancers showing increased alpha enolase mRNA and/or protein expression has been

reviewed by Capello *et al.*, (2001) and can be found in table 4.1. Our results, combined with other studies showing an increased alpha enolase expression in a diverse range of tumours indicate that alpha enolase could serve as a potential diagnostic indicator for multiple cancers.

A similar, but weaker pattern of staining intensity was observed with the nuclear alpha enolase expression. Positive alpha enolase expression was observed more frequently in PCa (25 patients) compared to benign diseases (4 patients) (table 5.4). Furthermore, analysis of the distribution of positive staining demonstrated that more patients with PCa had an increased strong alpha enolase nuclear expression (9 patients) compared to patients with benign diseases (1 patient). As before, when weak alpha enolase expression (staining intensity score 0) was compared to strong expression (staining intensity score 1 and 2), statistical significance was observed ($p=0.003$). These findings complement the cytoplasmic alpha enolase expression pattern; reemphasising that higher nuclear alpha enolase expression is more frequent in PCa compared to benign prostatic tissue. This suggests that alpha enolase may be associated with PCa or malignancy.

The novel finding of this study was that statistically significant alpha enolase cytoplasmic and nuclear overexpression (demonstrated by immunohistochemistry) occurs in patients with PCa, compared to benign controls. These results indicate that alpha enolase levels increases in PCa and suggest that its expression should be evaluated as a potential biomarker for PCa.

5.4.2 The association between alpha enolase expression and clinico-pathological variables in PCa

The findings from this study showed no statistical significance between cytoplasmic or nuclear alpha enolase and any clinico-pathological variables in PCa such as Gleason grade, initial PSA, D'Amico risk, proliferation, bone metastasis, castrate treatment resistance, survival or metastasis (table 5.3 and table 5.6). On close inspection of cytoplasmic alpha enolase expression, some of the clinico-pathological variables analysed yielded p values that neared significance – proliferation ($p=0.13$), metastasis ($p=0.149$), castrate treatment resistance ($p=0.116$) and survival ($p=0.101$) (figure 4.2).

Furthermore, Kaplan-Meier survival analysis (figure 5.2) showed that although there was no statistical significance between castrate treatment resistance and alpha enolase cytoplasmic expression, patients with cytoplasmic alpha enolase positive tumours may be more likely to live longer ($p=0.101$), although this finding was not statistically significant. It is evident from the Kaplan-Meier survival graphs (especially the graph analysing the association between time to castrate treatment resistance, survival probability and nuclear alpha enolase expression; figure 5.4) that there are insufficient data points in each category, making it difficult to draw firm conclusions from the data. As a result, in order to fully validate the association between alpha enolase and clinico-pathological variables in PCa, it would be advantageous to screen another PCa TMA with a larger number of patients to get a definite association between alpha enolase expression and clinico-pathological variables in PCa.

To our knowledge, this is the first report evaluating the association between alpha enolase expression in PCa and important clinico-pathological variables. Although this study has not identified any significant associations between any clinico-pathological variables and alpha enolase cytoplasmic or nuclear expression, our study has identified some interesting findings that need to be repeated in a larger sample cohort.

5.4.3 The expression of membranous alpha enolase in PCa

Immunohistochemical staining of the NPRG PCa TMA revealed that out of 235 patients screened, only 8 patients demonstrated membranous alpha enolase expression (table 5.7). This highlights the possible frequency of alpha enolase membrane expression in PCa. However, the expression of alpha enolase in normal cells is well accepted and reviewed by Capello *et al.*, (2010), where it is reported to act as a plasminogen receptor on the surface of many prokaryotic and eukaryotic cells. Hsiao *et al.*, (2013) reported that surface alpha enolase promotes extracellular matrix degradation and tumour metastasis in lung cancer cells while Song *et al.*, (2014) reported similar findings in glioma. To our knowledge, this is the first study reporting the expression of membranous alpha enolase in PCa, albeit in a small number of cases. The presence of membranous alpha enolase in a small subset of PCa patients suggests that alpha enolase may be a potential target for antibody dependent cytotoxicity (ADCC).

Due to the small number of patients positive for alpha enolase expression and the limited follow up clinical information available for the patients screened, no correlation could be made between the presence of membranous alpha enolase and the staining intensity of cytoplasmic or nuclear alpha enolase. In addition, no correlation could be made between Gleason grade, stage or D'Amico risk classification in this study. However, this study identified intense staining around the cell membrane of one patient with Gleason 6 PCa (figure 5.5B) and in the lumen of another patient with metastatic Gleason 9 PCa (figure 5.5C).

It is interesting that in the only patient with tumour metastasis, a strong alpha enolase expression was identified in the lumen of the prostatic epithelia (figure 5.5C). This suggests that alpha enolase may be secreted by the secretory columnar cells of the prostatic epithelia (in this patient). Also of interest is the fact that these cells also secrete PSA, the current diagnostic biomarker for PCa (Cramer *et al.*, 2007). Although this finding was demonstrated in one patient, it would be interesting to determine whether urinary alpha enolase correlates with tissue alpha enolase expression, as urine is a less-invasive biomarker source for PCa. As demonstrated from the previous chapter, alpha enolase is normally present in urine (section 4.3.2), but studies investigating its expression in urine from a cohort of PCa/benign or healthy controls have not been carried out. Using a large sample cohort, it would be advantageous to screen patients with various grades of PCa for urinary alpha enolase expression, in order to determine whether alpha enolase urine expression can be utilised as a diagnostic or prognostic PCa biomarker.

5.4.4 Alpha enolase autoantibody expression in sera

Findings from the previous chapter (section 3.3.4.3 and 3.3.4.4) demonstrated that a differential alpha enolase autoantibody expression occurred in a small subset of patients with PCa. However, due to the small number of patients studied and the limited sample volumes available for comparison with healthy sera, the appropriate statistical test was not carried out. The aim of this follow up study was to evaluate the expression of a differential autoantibody expression in a larger PCa, benign and healthy sera cohort.

Using a cutoff of 0.77 (*mean OD 25 healthy control sera + 3 x SD*), positivity was defined as any OD value above 0.77. The results show that there was a higher occurrence of

positive autoantibody expression in PCa (16 patients) compared to benign disease (3 patients) or healthy controls (0 patients) (table 5.8). In addition, when PIN samples are eliminated (PIN is thought to be the precursor to PCa, Cramer *et al.*, 2007), alpha enolase autoantibody expression was only identified in 1 out of 17 patients with benign disease (5% of cases), compared to a 22% occurrence in PCa. The difference in autoantibody expression between healthy controls and PCa was statistically significant ($p=0.0038$), while no statistical significance was observed between healthy controls and benign diseases ($p=0.1451$) or benign diseases and PCa (0.8867) (figure 5.6).

These findings further demonstrate the high expression of alpha enolase autoantibodies in PCa. To our knowledge, this is the first report on the frequency of alpha enolase autoantibody occurrence in PCa; and indicates that alpha enolase autoantibodies may potentially be added to a panel of diagnostic biomarkers for PCa after additional validation. Other studies have supported these findings; Peng *et al.*, (2013) found a high incidence of autoantibody expression in liver cancer (14.3%; 12/84 cases) compared to healthy sera (4.1%; 3/74), while Shih *et al.*, (2010) found a significantly decreased expression of alpha enolase autoantibodies in stage IV non-small cell lung cancer, small cell lung cancer and breast cancer patients. These studies suggest that differential expression of alpha enolase in cancers may serve as a potentially useful biomarker to stage and monitor cancer patients in the future.

5.4.5 The association between alpha enolase autoantibody expression and Gleason grade

When the correlation between alpha enolase autoantibody expression and Gleason grade was investigated, this study found no statistical significance between healthy controls and Gleason grade 6 (0.0990), Gleason grade 7 (3+4) ($p=0.4334$), Gleason 7 (4+3) ($p=0.2852$), or Gleason 8 ($p=0.3892$) (figure 5.7). Statistical significance was however observed between healthy controls and Gleason 9 PCa ($p=0.0043$) at $p<0.05$ (figure 5.7). This suggests that alpha enolase autoantibody expression may correlate with more advanced PCa. As stated previously, these findings are similar to Shih *et al.*, (2010) who identified that the titre status of alpha enolase autoantibodies are highly associated with late stage of lung and breast cancer patients compared to healthy controls.

Although statistical significance was observed between healthy controls and Gleason 9 PCa ($p=0.0043$), there were not enough patients in this study diagnosed with Gleason 8 PCa ($n=2$ patients, compared to $n=11$ in Gleason 7, 4+3), to determine if a similar correlation could have been present in Gleason 8 compared to healthy controls, or if alpha enolase expression correlates with Gleason grade when more patients in each category. As a result, future studies could investigate the expression of autoantibodies to alpha enolase in more samples, ensuring that a minimum of 70 patients are in each category.

5.4.6 The association between alpha enolase autoantibody expression and D'Amico risk classification

The D'Amico risk classification stratifies patients into low, intermediate or high risk of biochemical recurrence after radical prostatectomy (Hernandez *et al.*, 2007). This chapter also investigated the association between D'Amico risk classification and sera autoantibody alpha enolase expression (section 5.3.2.3). Figure 5.8 shows that alpha enolase autoantibody expression correlates with D'Amico risk classification, where no statistical significance was observed between healthy controls and low D'Amico risk ($p=0.7927$) but between healthy controls and intermediate ($p=0.0125$) and high D'Amico risk (0.0059). These results suggest that autoantibodies to alpha enolase may be used as a prognostic indicator to determine PCa recurrence or treatment response. It further suggests that autoantibodies to alpha enolase may be added to a panel of prognostic PCa biomarkers for better patient management. To our knowledge, this is the first report on the association between alpha enolase autoantibody expression in sera and D'Amico risk classification.

To summarise, the aim of this chapter was to identify the association between alpha enolase protein and autoantibody expression in PCa and see whether there was any association between its expression and important clinico-pathological variables in PCa. This study has identified a high expression of cytoplasmic and nuclear alpha enolase protein in PCa compared to benign disease however there was no correlation to any important clinico-pathological variables, perhaps due to the limited number of samples screened in the NPRG TMA cohort. Our study is the first to report these findings in PCa, where statistical significance has been observed between autoantibodies to alpha enolase and PCa, Gleason 9 PCa and D'Amico risk classification. Although the NPRG TMA study showed no diagnostic/prognostic potential for alpha enolase, the high expression of

autoantibodies in sera and potential membranous expression in a small cohort of patients make alpha enolase an interesting candidate to study in a larger sample cohort to give validity to these results.

CHAPTER 6: SUMMARY OF DISCUSSION

6.1 INTRODUCTION

PCa is the most common cancer in men above 65 years (Drewa and Styczynski, 2008). Studies at autopsy have found that by 30-39 years of age, approximately 29% of men have microscopic evidence of disease, increasing to approximately 65% by age 70 (Sakr *et al.*, 1994). Approximately 41,700 new cases of PCa are diagnosed every year, where it is expected that 1 in 8 men will be diagnosed with PCa in their lifetime (Cancer Research UK, 2015b). Unfortunately, this figure is expected to rise, due to the lack of specificity and sensitivity of PCa screening and detection methods (Madu and Lu, 2010).

PCa risk is associated with age. Research has shown that only 1% of diagnoses occurred in patients under 50 (Cancer Research UK, 2015b), while patients of Caribbean and African origin have a greater risk of developing PCa (1 in 4 lifetime relative risk) (Prostate Cancer UK, 2015), and those with a family history (with more than one affected 1st degree relative) have 3.5 times relative risk of developing PCa (Johns and Houlston, 2003).

The nature of PCa is such that many patients with early stages of the disease are asymptomatic, while symptoms of locally advanced PCa (e.g. nocturia, terminal dribbling, increased urinary frequency and poor stream) may be difficult to differentiate from patients with BPH, as these lower urinary tract symptoms are common to both conditions (GP online, 2014). Furthermore, PCa screening and diagnosis relies on a PSA blood test alongside an invasive and subjective DRE and TRUS guided biopsy (Roth-Kauffmann, 2011). However, despite the utility of these tests, PCa diagnosis is associated with overdiagnosis, overtreatment, false positive results (PSA tests), bleeding, subjectivity (DRE), infection, and pain associated with biopsy (Cochrane, 2013). This causes unnecessary patient distress and increased clinical costs; which warrants the need for more sensitive and specific biomarkers to differentiate between PCa and other non-malignant conditions.

It is well documented that antibodies present in cancer sera towards TAAs may provide insights into the interrelationship between the immune system and cancer (Chen *et al.*,

1996, Rattner *et al.*, 1997 and Gure *et al.*, 2000). It may also provide a new understanding into, and guide the development of new treatment strategies for cancer and identify new biomarkers for the diagnosis and prognosis of cancer (Kellner *et al.*, 2002, Usener *et al.*, 2003, Neumann *et al.*, 2004, Ehlken *et al.*, 2004 and Zippelius *et al.*, 2007). As a result, the identification of these TAAs and autoantibodies has become the subject of much research.

One of the proteome based techniques to identify TAAs and autoantibodies is known as PROTEOMEX, which involves screening patient sera with the proteomes of tumour cell lysates separated by 2-DE electrophoresis and subsequent TAA identification with mass spectrometry (Kellner *et al.*, 2002). PROTEOMEX has successfully been utilised in the identification of many TAAs for various cancers such as HSP-60 and RS/DJ-1 in breast cancer (Hamrita *et al.*, 2008, Desmetz *et al.*, 2008 and Le Naour *et al.*, 2011), cytokeratin 8 and thioredoxin in renal cell carcinoma (Kellner *et al.*, 2002 and Lichtenfels *et al.*, 2003), eukaryotic elongation factor, alpha enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase and heterogeneous nuclear ribonucleoproteins A2B1 and galectin-3 in melanoma (Suzuki *et al.*, 2010 and Forger *et al.*, 2009) and alpha enolase in pancreatic adenocarcinoma (Tomaino *et al.*, 2007). Despite the utility of this method, no studies have utilised the PROTEOMEX technique for the identification of TAAs in PCa. Thus, the aim of this research was to identify TAAs and autoantibodies for PCa using the PROTEOMEX technique in combination with mass spectrometry.

6.2 Urine contains prostate relevant TAAs that can be isolated and identified using the PROTEOMEX technique

Urine is an excellent source for biomarker discovery for PCa due to the proximity of the bladder to the prostate gland (Hessels and Schalken, 2013). In patients who have PCa, the gentle massage of both sides of the prostate gland during DRE, stimulates the release of prostatic fluids and detached epithelial cells (expressed prostate secretions) into the urethra (Duijvesz *et al.*, 2011). As a result, urine obtained after DRE in patients with PCa may contain prostatic secretions and cells originating from the prostate tumour, which could serve as early PCa biomarkers (Downes *et al.*, 2006). Although the samples utilised from this study were not obtained from DRE massaged urine, chapter 2 aimed to determine if TAAs could be identified from non-massaged urine which could serve as PCa biomarkers.

In the pilot study, urine samples from PCa patients were separated by SDS PAGE, where differential bands were observed (between 37 – 75 kDa) in immunoblots screened with pooled allogenic PCa or healthy sera (figure 2.7) suggesting the specificity of the immune response for PCa. When the frequency and specificity patterns in autoreactivity were evaluated, section 2.3.2.1 shows that apart from one band that was common to all patient groups (50 kDa, figure 2.8), different autoreactivity patterns occurred in each PCa patient (2 bands in PC01, 4 bands in PC02, 2 bands in PC03), further emphasising the need to screen each patient individually, after a pooled PROTEOMEX study, similar to findings by Forgber *et al.*, (2009). Furthermore, this study demonstrated two bands common to two PCa patients – PC02 and PC03 (at 43 and 40 kDa). The band at 43 kDa was excised from a Coomassie stained replica gel and identified by LC MALDI TOF/TOF as 25 significant proteins (table 2.9), the most significant of which was a well-known PCa biomarker, prostatic acid phosphatase identified using 14 peptides (table 2.10). Although this study verified prostatic acid phosphatase expression by Western blotting, consistent with findings by (Gutman, *et al.*, 1936), the study highlighted the need to carry out more fractionation before TAA identification by PROTEOMEX.

The next study (section 2.3.3) demonstrated that 2-DE in combination with MALDI TOF/TOF is a more suitable technique for TAA identification using PROTEOMEX, as a larger number of spots were observed from the immunoblot compared to the SDS PAGE screen (figure 2.11). Zinc-alpha-2 glycoprotein, alpha-1-acid glycoprotein, and serum albumin were identified from gel spots (table 2.12), and interestingly these same proteins were also identified from the previous study, although majority of the proteins identified from this study were serum albumin. From the immunoblot, zinc-alpha-2 glycoprotein demonstrated intense differential expression in PCa compared to healthy controls, consistent with findings by Gagnon *et al.*, (1990) and Hassan *et al.*, (2007), suggesting that zinc-alpha-2 glycoprotein may serve as a potential PCa biomarker.

The final study aimed to determine if a combined OFFGEL and SDS PAGE approach could be utilised as a separation and screening method for PROTEOMEX prior to TAA identification by ESI MS. As before, a differential expression between PCa and healthy controls was identified in two bands (figure 2.15). Protein identification from one band identified serum albumin, zinc-alpha-2 glycoprotein and prostatic acid phosphatase (band #4; table 2.17). This study demonstrated that the best method for TAA identification was

2-DE in combination with MALDI TOF/TOF, where spots of interest yield one or two proteins, making it easy to narrow down proteins responsible for the immune response, similar to findings by Forgber *et al.*, (2007). It also highlighted, most importantly that urine contains prostate relevant TAAs that can be isolated and identified using the PROTEOMEX method.

6.3 Tumour cell lines provide a suitable source of TAAs for identifying autoantibodies associated with PCa

The lack of available tumour tissue lysates led to the investigation of immortalised PCa cell lines as an alternative source of TAAs for PROTEOMEX studies. Although immortalised cell lines have been used as a TAA source in renal cell carcinoma (Kellner *et al.*, 2002 and Lichtenfels *et al.*, 2003), breast cancer (Hamrita *et al.*, 2008, Desmetz *et al.*, 2008 and Le Naour *et al.*, 2011), pancreatic adenocarcinoma (Tomaino *et al.*, 2007) and melanoma (Suzuki *et al.*, 2010 and Forgber *et al.*, 2009), till date no studies have demonstrated the use of immortalised PCa cell lines for TAA identification using PROTEOMEX. Thus, the aim of this study was to use PROTEOMEX to identify TAAs and autoantibodies in two well-characterised PCa cell lines, which may serve as biomarkers for PCa.

Initial studies in the PC-3 cell line showed a differential autoantibody response between PCa, benign and healthy controls, where within PCa patients, more intense autoreactivity was observed in Gleason 7 (4+3) compared to Gleason 7 (3+4), suggesting that a higher alpha enolase autoantibody titre may occur in patients with a more aggressive form of PCa (figure 3.3). Interestingly, this finding was also observed in a small number of cases in the subsequent chapters (figure 5.7), although this finding was not statistically significant. As a result, further studies need to investigate the relationship between alpha enolase autoantibody expression and aggressive PCa in order to validate these findings.

In DU-145, patients from various grades and stages of PCa were utilised due to insufficient spot yielded from the PC-3 study. DU-145 immunoreactivity produced more spots (18 spots, where only 16 of them could be matched on the replica gel. The identified spots represent proteins responsible for the immune response (HSP-60), metabolic enzymes (alpha enolase), energy metabolism (ATP synthase subunit) and cell signalling (WNT7A).

This study identified for the first time that autoantibodies to alpha enolase are differentially expressed in PCa sera compared to healthy controls, warranting the need to carry out further studies investigating the possibility of alpha enolase as a potential biomarker for PCa.

Due to the possibility of false identification in biomarker studies, it was paramount that the TAA and autoantibody response was verified in the discovery sample cohort. As a result, the aim of the next section (section 3.3.4) was to confirm the alpha enolase protein identification in DU-145 alongside the presence of a differential alpha enolase autoantibody response in the discovery sample cohort of PCa patients compared to healthy sera. Studies confirming alpha enolase expression in PC-3 were carried out in the subsequent chapter (section 4.3.1). Using immunofluorescence, intracellular staining by flow cytometry, Western blotting and MALDI TOF MS, the presence of alpha enolase in DU-145 was verified (section 3.3.4). Although there was a suggested staining observed around the cell in DU-145 using immunofluorescence (figure 3.9), extracellular staining using flow cytometry failed to confirm any alpha enolase cell surface expression (appendix VI). The final study confirmed a differential alpha enolase autoantibody expression in pooled and individual samples that made up the discovery cohort.

Taken together, both studies show that although alpha enolase is normally present in cells (Diaz-Ramos *et al.*, 2012) and PCa cell lines, a differential autoantibody expression exists in sera, similar to findings in non-small cell lung cancer (He *et al.*, 2007) and pancreatic cancer (Tomaino *et al.*, 2011). This suggests that autoantibodies to alpha enolase can possibly be exploited as a potential PCa biomarker following additional investigation, which forms part of the penultimate chapter (section 5.3.2). Most importantly, chapter 3 demonstrated the utility of immortalised PCa cell lines as a suitable source of TAAs for identifying autoantibodies associated with PCa.

6.4 A differential alpha enolase expression in PCa occurs in other biological samples

In order to validate its potential utility as a PCa biomarker, it was important to evaluate alpha enolase expression in more than one biological sample. In so doing, the potential of alpha enolase as a PCa biomarker for invasive (serum, tissue) or non-invasive (urine)

biological samples can be assessed. To this end, alpha enolase expression in other PCa cell lines, urine and tissue were determined using immunofluorescence, flow cytometry, Western blotting and immunohistochemistry.

Chapter 4 identified alpha enolase expression in all PCa cell lines studied - PC-3, OPCT-1 and LNCaP by immunofluorescence, flow cytometry and Western blotting (figure 3.9). Furthermore, extracellular staining failed to demonstrate the presence of membranous alpha enolase in PCa cell lines, contrary to findings by Capello *et al.* (2009), Chang *et al.* (2006) and He *et al.* (2007) who identified membranous alpha in pancreatic, lung and small cell lung cancers respectively.

The next study evaluated alpha enolase expression in urine using Western blotting. Although urinary alpha enolase has previously been reported in one study investigating urine samples obtained after prostatic massage (Rahman *et al.*, 2004), this is the first study to demonstrate the presence of urinary alpha enolase in non-massaged urine in patients with PCa. Alpha enolase was identified at 47 kDa in healthy controls and PCa patients, while a higher (differential) expression was observed in PCa at approximately 66 kDa, possibly due to posttranslational modifications in alpha enolase, as observed in pancreatic adenocarcinoma by Tomaino *et al.* (2011).

Alpha enolase tissue expression was carried out to confirm its expression in a range of normal, tumour and adjacent normal tissue samples. From this study, a cytoplasmic and nuclear alpha enolase expression was observed in all TMAs screened, although higher cytoplasmic expression occurred in most cancers, compared to controls (section 4.3.3.1 and 4.3.3.2); similar to work reviewed by Capello *et al.* (2001). A reduced alpha enolase expression was only identified in breast cancer, contrary to reports by Tu *et al.* (2010), who found that increased mRNA expression occurred in patients with breast cancer, where increased expression correlated with tamoxifen resistance, suggesting that alpha enolase expression may correlate to therapeutic outcome in breast cancer.

Due to our interest in PCa, the expression of alpha enolase in a small cohort of patients (9 patients) was investigated in this chapter. The results indicate an increased alpha enolase expression in 8 out of 9 patients studied (score intensity >0, where score intensity 0 was observed for normal prostate; table 4.4A). This study combined with all other findings

warrant the investigation of the utility of alpha enolase as a potential PCa biomarker. This chapter demonstrated that differential expression of alpha enolase occurs in tissue and urine (at 66 kDa), two main sample sources for PCa biomarkers, indicating that more studies need to be carried out in a larger sample cohort to evaluate the possibility of alpha enolase as a diagnostic PCa biomarker.

6.5 High alpha enolase tissue expression occurs in PCa but does not correlate with any important clinico-pathological variables

The 3rd and 4th chapters identified alpha enolase as a potential PCa biomarker worth investigation. In order to validate the utility of alpha enolase as a PCa biomarker, it was important to carry out studies in a larger sample cohort in order to determine whether statistical significance could be attributed to the preliminary findings. The aim of chapter 5 was to screen a prostate TMA cohort for alpha enolase expression and determine if it correlates with any important clinico-pathological variables in PCa.

Increased alpha enolase cytoplasmic and nuclear expression occurred more frequently in PCa compared to benign diseases, while weaker alpha enolase expression occurred more frequently in benign diseases. When weak cytoplasmic alpha enolase expression was compared with stronger expression, statistical significance was observed between PCa and benign diseases ($p=0.000003$), suggesting an increased expression occurs in malignancy. These findings are similar to Chu *et al.* (2011). A similar association was also observed with nuclear staining ($p=0.003$). These findings suggest that alpha enolase may serve as a potential PCa diagnostic biomarker, being able to differentiate PCa from benign disease.

Chapter 5 failed to identify any association between cytoplasmic and nuclear alpha enolase and any important clinico-pathological variables in PCa, although in most cases, there were not enough patients in each dataset to make any firm conclusions about the possible associations between alpha enolase expression and clinico-pathological variables in PCa. Interestingly, it was observed that patients with cytoplasmic alpha enolase positive tumours may live longer ($p=0.101$), than those without. However, these findings were not significant but should be repeated in a larger sample cohort.

In tissue, alpha enolase was identified in 8 out of 235 patients screened, where it also occurred in the lumen of one patient with metastatic Gleason 9 PCa. Due to the low numbers of patients positive for alpha enolase membrane expression, no statistical test could be performed. This study however highlighted the association between cytoplasmic, membrane alpha enolase and clinico-pathological variables for the first time in PCa.

6.6 High serum alpha enolase autoantibody expression occurs in PCa and correlates with D'Amico risk classification

The 3rd and 4th chapters identified a differential autoantibody response to alpha enolase as a potential PCa biomarker in a small sample cohort. In order to validate these findings, alpha enolase autoantibody responses were screened in a larger sample cohort of patients with various stages and grades of PCa compared to benign and healthy controls.

Using a cutoff defined by the mean OD of healthy sera, a higher occurrence of autoantibodies to alpha enolase occurred in PCa (16 patients) compared to benign (3 patients) or healthy controls (0 patients), similar to work by Peng *et al.* (2013) who found a high incidence of autoantibody expression in liver cancer. The study also found statistical significance between the autoantibody expressions in healthy controls compared to PCa patients (0.0038). Our study is the first to identify a high expression of alpha enolase autoantibodies in PCa patients, suggesting that differential alpha enolase autoantibody expression may be added to a panel of diagnostic biomarker for PCa.

Our study also identified a significant difference between alpha enolase expression in healthy controls compared to Gleason grade 9 PCa, suggesting that there may be an association between autoantibody expression and advanced PCa. Similarly, this study reported for the first time a correlation between alpha enolase autoantibody expression and D'Amico risk classification (section 5.3.2.3). This implies that autoantibodies to alpha enolase may not only serve as a diagnostic biomarker for PCa, but also a means of identifying patients with a high risk of biochemical recurrence in PCa after radical prostatectomy.

CHAPTER 7: CONCLUSIONS & FUTURE WORK

7.1 CONCLUSIONS

Early detection of PCa is essential for patient management, thus patient screening to aid early PCa diagnosis is mandatory (Smith *et al.*, 2003). Using PROTEOMEX in combination with various mass spectrometry methods, this study identified prostate relevant TAAs (prostatic acid phosphatase, zinc-alpha-2 glycoprotein and serum albumin in urine, alpha enolase in PCa cell lines) and autoantibodies (alpha enolase) in various biological samples that have shown some differential expression in PCa compared to benign or healthy controls, suggesting that these identified biomarkers may have some diagnostic utility in PCa detection, staging and monitoring. Although the study failed to identify an association between alpha enolase tissue expression and any clinic-pathological variables in PCa, the study has yielded some interesting results that should be validated in a larger sample cohort.

7.2 FUTURE WORK

This study has paved the way for future projects to identify and verify/validate potential TAAs and biomarkers for PCa, using the already optimised PROTEOMEX method utilised throughout this study. Furthermore, this project has also enabled the identification of TAAs/autoantibodies in various biological samples with a view to demonstrating which sample source would serve as a better biomarker for PCa. Future work should most importantly demonstrate the diagnostic utility of alpha enolase as a biomarker in other sample sources alongside aim to identify new potential biomarkers for PCa.

7.2.1 Immunodepletion of urinary proteins for the identification of TAAs and biomarkers for PCa

One of the limitations from this study was that crude urinary lysates were utilised for biomarker discovery studies. It is well known that the presence of high abundant proteins in urine (e.g. albumin, IgG) mask lower abundant proteins which may serve as potential cancer biomarkers. Future work with urine samples could immunodeplete the top 20 most abundant urine proteins from PCa and healthy patient samples (e.g. using a Sigma

ProteoPrep 20 immunodepletion kit) to reduce sample complexity and improve the chances of finding potential PCa biomarkers. This kit will deplete the top 20 proteins which are: albumin, IgG, transferrin, fibrinogen, IgA, alpha-2-macroglobulin, IgM, alpha-1-antitrypsin, complement C3, haptoglobin, apolipoprotein A3, apolipoprotein A2, apolipoprotein B, acid-1-glycoprotein, ceruloplasmin, complement C4, complement C1q, IgD, prealbumin and plasminogen. Depletion of these 20 proteins removes 95% of the human plasma protein mass (as assayed in human serum). This kit has never been applied in the removal of abundant proteins in urine, which is a novelty of this approach for biomarker discovery. Depletion of these samples will reduce the complexity of before their analysis by 2-DE or OFFGEL in combination with PROTEOMEX.

7.2.2 Validation of identified biomarkers to investigate their diagnostic/prognostic value in PCa

Although some verification and validation (in a small cohort) was carried out in this study, all identified biomarkers (prostatic acid phosphatase, zinc alpha 2 glycoprotein, serum albumin and alpha enolase – protein and autoantibody responses) should be validated in a large sample cohort (minimum of 2,000 patients), in order to make firm conclusions about the potential of these candidates as PCa biomarkers. In such studies, each marker should be combined with PSA to determine whether a combination of both or a panel of these identified markers could increase the sensitivity and/or specificity of PCa detection.

This study showed a differential expression of alpha enolase in urine. Studies could investigate the expression of urinary alpha enolase in a large cohort of patients, perhaps using an ELISA assay. In addition the possible post translational modification of urinary alpha enolase in PCa should also be investigated in order to determine if alpha enolase PTMs can serve as a PCa biomarker, similar to findings by Tomaino *et al.* (2011).

7.2.3 Subcellular fractionation of PCa cell lines for TAA and biomarker identification

Tumour cell lysates from PCa cell lines can be subcellular fractionated into nuclear, cytoplasmic and membranous fractions in order to enrich TAA for biomarker discovery. Each enriched fraction could be separated by 2-DE before immunoprobing with a cohort of

PCa (n=20) vs. healthy sera (n=20). In order not to mask individual immunoreactivity to TAA, five patients from each sample set could be pooled to make 4 sample pools for PCa and healthy controls respectively. Each pool will be used to screen the nuclear, cytoplasmic and membranous fractions to identify TAA for subsequent mass spectrometry identification.

APPENDIX I

I Laboratory consumables and equipment

II Laboratory reagents

All chemicals and reagents were stored according to manufacturer's instructions and used within their expiry date.

Culture media	Supplier
Dulbecco's Modified Eagle Media (DMEM)	Lonza
Eagle's Minimal Essential Media (EMEM)	Lonza
Ham's F12	Lonza
Keratinocyte Serum Free Media (KSFM)	Gibco
RPMI 1640 media	Lonza
F-12 Kaighn's	Gibco
Supplements to cell structure	Supplier
Fetal calf serum (FCS)	Perbio Thermo Fisher
Glucose	Lonza
Hepes	Lonza
Hydrocortisone	Sigma Aldrich
L-glutamine	Lonza
Non-essential amino acids (NEAA's)	Lonza
Sodium pyruvate	Lonza
Testosterone	Sigma Aldrich
Other cell culture reagents	Supplier
Dimethyl Sulfoxide Hybri-Max (DMSO)	Lonza
Dulbecco's phosphate buffer saline (dPBS)	Lonza

Trypan blue 0.4% (v/v)	Sigma Aldrich
Trypsin/Versene (T&V)	Lonza
Chemical reagents	Supplier
Acetic acid	Fisher Scientific
Acetone (for HPLC)	Fluka Analytical
Acetonitrile	Sigma Aldrich
Ammonium bicarbonate	Sigma Aldrich
Ammonium persulphate (APS)	Geneflow National Diagnostics
Acrylamide (30%)	Geneflow National Diagnostics
Bovine serum albumin (BSA)	Calbiochem
Bovine serum albumin (BSA) (for protein assay standards)	Pierce
Bromophenol blue	Promega
Coomassie brilliant blue	PhiBio Systems
Deionising resin	Bio RAD
Dithiothreitol (DTT)	Apollo Scientific
Ethanol	Fisher Scientific
Glycerol	Sigma Aldrich
Hydrochloric acid (HCl)	Fisher Scientific
Iodoacetamide	Sigma Aldrich
Isopropanol	Fisher Scientific
Isoton	Beckman Coulter
Liquid nitrogen	BOC
Marvel skimmed milk powder	Premier Brands
Methanol	Fisher Scientific
N, N, N', N'-tetramethyl-ethylenediamine (TEMED)	Geneflow National
Diagnostics	
NANOpure diamond distilled water	Barnstead
Nitrocellulose membrane	Gilson Anachem
Octyl β -D-glucopyranoside (OGP)	Apollo Scientific
Paraformaldehyde	Sigma Aldrich
Ponceau S solution	Sigma Aldrich
Precept tablets	Johnson and Johnson

Precision plus protein WesternC standards	Bio-RAD
Propan-2-ol	Fisher Scientific
Protein assay BCA solution	Bio-RAD
Protogel running buffer	Geneflow National Diagnostics
Protogel stacking buffer	Geneflow National Diagnostics
RapidStep ECL reagent	Calbiochem
ReadyPrep overlay agarose	Bio-RAD
Sodium chloride (NaCl)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma Aldrich
StrepTactin HRP	Bio-RAD
Sulphuric acid	Fisher Scientific
Teepol	Johnson and Johnson
Tris hydrochloride	Sigma Aldrich
Trizma base	Sigma Aldrich
Tween-20	Sigma Aldrich
Ultrapure 10X Tris-Glycine (transfer buffer)	Geneflow National Diagnostics
Ultrapure 10X Tris-Glycine/SDS (running buffer)	Geneflow National Diagnostics
Urea	Melford
VectaShield (with DAPI)	Vector Labs

Proteomics reagents**Supplier**

2-DE sample rehydration buffer	Bio-RAD
AG 501-X8 Resin, biotech, Grade, 20-50 mesh (100g)	Bio-RAD
C ₁₈ ZipTip	Millipore
CHCA (α-Cyano-4-hydrocinnamic acid)	Bruker Daltonics
Dichloromethane (HPLC grade)	Fisher Scientific
Formic acid (HPLC grade)	Fluka Analytical
Methanol (HPLC grade)	Fisher Scientific
Mineral oil (for 2-DE)	Bio-RAD
Peptide calibrant standard II	Bruker Daltonics
ReadyStrip IPG Strips (pH 3-10), 7cm	Bio-RAD
Recombinant alpha enolase protein	Fitzgerald
Trifluoroacetic acid (TFA)	Fisher Scientific

Trypsin gold (MS grade)	Promega
Vivaspin 20 columns	Sartorius Stedim Biotech

Immunochemical reagents**Supplier**Primary antibody

Mouse anti-human alpha enolase (L-27)	Santa Cruz
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Secondary antibodies

Rabbit anti-human IgG (HRP)	Dako
Goat anti-mouse IgG (HRP)	Dako
Goat anti-mouse IgG (Alexa flour 488)	Life Technologies

III Buffers and stains

Buffers were prepared as follows:

Buffers and stains used for tissue culture

Trypan blue for cell counting:	01% (v/v) trypan blue solution
	DPBS

Buffers and stains for cell lysis and Western blotting

- Cell lysis buffer:
 - 9.5 M urea
 - 2% DTT
 - 1% OGP
- Sample reducing buffer:
 - 10mL 0.5 M Tris HCl 6.8 (stacking gel buffer)
 - 2.0 g SDS
 - 6.4 mL Glycerol
 - 0.05 g Bromophenol blue
 - Adjusted to 20 mL with ddH₂O
- Ammonium persulphate:
 - 0.1 mg Ammonium persulphate

- 1 mL ddH₂O
- Running buffer: 100 mL Geneflow 10X Tris/Glycine/SDS solution
900 mL dH₂O
- Transfer buffer: 100mL Geneflow 10X Tris/Glycine
900mL dH₂O
- TBS 0.1% Tween-20: 12.1 g Trizma base
87.6 g NaCl
Adjust to pH 7.5
- 3% marvel TBS 0.1% Tween-20 (blocking buffer): 0.3 g Marvel milk powder
10 mL TBS 0.1% Tween-20
- Coomassie blue stain: 1.5 g Coomassie blue
25 mL acetic acid
250 mL methanol
Adjust to 500 mL with dH₂O
- Coomassie blue destain: 500 mL methanol
50 mL acetic acid
Adjusted to 1 L with dH₂O

Buffers used for crude subcellular fractionation

- Subcellular fractionation buffer 150 mM Tris-HCl
0.05% Tween-20
Adjust to pH 7.4

Buffers used for 2-DE

- 8.5M Urea stock solution: 127.6 g Urea
250 mL ddH₂O

5 g deionising resin
Filter through Whatmann no.1 filter paper

- Equilibration buffer II: 177 mL 8.5 M urea (deionised)
5.0 g SDS
50 mL Glycerol
8.25 mL Tris-HCl (1.5 M) pH 8.8
Adjust to 250 mL with ddH₂O

Buffers used for immunofluorescence

- 4% paraformaldehyde: 4% (w/v) paraformaldehyde
1 x DPBS
- 10% BSA TBS 0.1% Tween-20 (blocking buffer): 0.3 g Bovine serum albumin powder
10 mL TBS 0.1% Tween-20

Buffers for ELISA

- Coating buffer: 15 mM Na₂CO₃
30 mM NaHCO₃
0.2% Azide
Adjust to pH 9.6
- Wash buffer (TTBS): 50 mM Tris HCl (pH 7.4)
150 mM NaCl
0.05% Tween-20
- Dilution buffer: 2% BSA in TTBS
- Blocking buffer: 4% BSA in TTBS
- Stop solution: H₂SO₄

Buffer for flow cytometry

- FACS buffer: 0.5% (w/v) BSA
0.02% (W/V) NaN₃
1 x PBS
- Wash buffer: 1% (w/v) BSA in 1X dPBS

Kits	Supplier
3100 OFFGEL Low Res Kit, pH 3-10	Agilent
Bradford protein assay kit	Bio-RAD
Mini format 1-D electrophoresis systems	Bio-RAD

IV Equipment

Laboratory consumables – glassware, plastics and sharps

All pyrex glassware used was washed using teepol, rinsed twice in distilled water before autoclaving.

Item	Supplier
0.2 µL filter tips	Starlabs
20 mL universal tubes	Starlabs
384 MALDI ground steel target plate	Bruker
Bijou tubes (7 mL)	Starlabs
Cell scraper	Helena Biosciences
Coverslips	Scientific Laboratory Supplies
Cryovials (1.2 mL)	Helena Biosciences
Disposable scalpel	Swann-Morton
Eppendorf tubes (0.5 mL, 1.5 mL)	Sarstedt
Falcon tubes (15 mL, 50 mL)	Sarstedt
Filter paper (for gel blotting)	Fisher Scientific

Filter tips (10 µL, 200 µL, 1 mL)	Sarstedt
Flat bottom culture dishes) 24 and 96-well)	Sarstedt
Flow cytometry (FACS tubes)	Elkay
Light microscope	Nikon/Olympus
Measuring cylinders (10 mL, 100 mL, 500 mL, 1 L, 2 L)	Sarstedt
MTP AnchorChip target plates	Bruker
Nitrocellulose membranes	Gilson Anachem
Plastic Pasteur pipettes	Sarstedt
Scalpel	Swann Morton
Serological pipettes (5 mL, 10 mL, 25 mL)	Sarstedt
Tissue culture flasks (T25, T75 and T175 cm ³)	Sarstedt

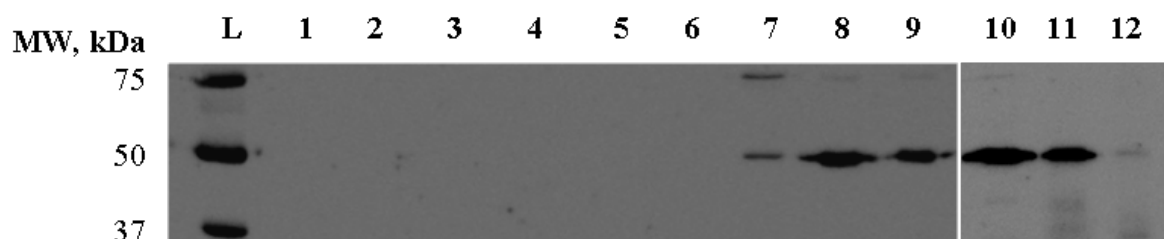
Laboratory equipment

Equipment	Supplier
-20°C Freezer	Lec
37°C Incubator; CO ₂ water jacked	Forma
4°C Refrigerator centrifuge	Eppendorf/Sanyo
4°C Refrigerator	Lec
-80°C Freezer	Revco/Sanyo
Autoclave	Rodwell
Benchtop vortex mixer	Scientific industries
CCD camera	Nikon
Centrifuge	Sanyo
Class II safety cabinets	Walker
Distilled water system	Barnstead
Electrophoresis running gel tanks	Bio-RAD
Electrophoresis transfer tanks	Geneflow
Flow cytometer	Beckman Coulter
Gallios flow cytometer	Beckman Coulter
Haemocytometer (counting chamber)	Weber
Leica TCS SP5 confocal microscope	Leica
Liquid chromatography (LC)	Bruker Daltonics

MALDI-TOF/TOF mass spectrometer	Bruker Daltonics
Microwave	Scientific Laboratory Supplies
Multichannel pipette	Starlabs
Nanodrop ND-1000 micro-spectrophotometer	Nanodrop Technologies
Nanopure diamond water reservoir	Barnstead
Nikon eclipse Ts100 light microscope	Nikon
Olympus BX51 fluorescence microscope	Olympus
Orbital shaker	Stuart
pH meter	Bio-RAD
Plate reader	Tecan
Power supply for electrophoresis	Bio-RAD
Protean IEF Cell	Bio-RAD
Sonicator	VWR
Spectrophotometer	Tecan Ultra
UNO-Thermoblock	Biometra
Wet transfer apparatus	Geneflow National Diagnostics
Software	Company
Biotoools	Bruker
ClinPro Tools	Bruker
FlexAnalysis	Bruker
FlexControl	Bruker
Kaluza	Beckman Coulter

APPENDIX II

Proof of principle of the OFFGEL SDS PAGE methodology in DU-145 PCa cell line




5 mg of protein from the DU-145 PCa cell line was lysed before fractionation by OFFGEL electrophoresis (pH 3-10, low resolution IPG strip). OFFGEL fractions were separated using a 10% SDS PAGE gel and probed with a monoclonal mouse anti-alpha enolase antibody (1:1000 dilution; L-27, SantaCruz, UK). Strong expression of alpha enolase at 47 kDa was observed in fractions 8, 9, 10 and 11, while a moderate expression was identified in fraction 7, and weak expression in fraction 12. Moderate alpha enolase expression was also identified in fraction 7 at 66 kDa, while weak expression was identified in fractions 8, 9 and 10.

APPENDIX III

TMA datasheet purchased from US BIOMAX: Slide #BN243a

BN243a
Multiple organ normal tissue array, 12 cases/24 cores, replaced by BN243b



Microarray Panel: Multiple organ normal tissue microarray, containing 12 types of normal organs (cerebrum, cardiac muscle, lung, liver, uterine cervix, colon, kidney, breast, ovary, pancreas, prostate and skin), each organ taken from 1 normal human individuals, duplicate cores per case

Cores: 24

Cases: 12

Layout: 6 cols x 4 rows

Core Diameter: 1.5mm

Thickness: 5µm

Quality Control: Anti-Actin confirmed

Applications: Routine histology procedures including Immunohistochemistry (IHC) and In Situ Hybridization (ISH), protocols which can be found on our support page.

Notes: Unless specified, all TMA slides are not coated with extra layer of paraffin (tissue cores can be easily seen on the glass), so there is no need to bake, can be directly put into xylene for de-paraffin procedure.

US Biomax, Inc. BN243a (serial)

	1	2	3	4	5	6
A	Cer	Cer	Cer	Cer	Lun	Lun
B	Liv	Liv	Liv	Liv	Col	Col
C	Kid	Kid	Bre	Bre	Ova	Ova
D	Pan	Pan	Pro	Pro	Ski	Ski

Legend: Bre - Breast, Cer - Cardiac muscle, Cer - Cerebrum, Col - Colon, Kid - Kidney, Liv - Liver, Lun - Lung, Ova - Ovary, Pan - Pancreas, Pro - Prostate, Ski - Skin, Liv - Uterine cervix
 ● - Malignant tumor, ● - NAT, ● - Normal tissue

Pos No.	Sex	Age	Organ	Pathology diagnosis	Type †
A1	F	38	Cerebrum	Normal cerebrum tissue	Normal
A2	F	38	Cerebrum	Normal cerebrum tissue	Normal
A3	F	42	Cardiac muscle	Normal cardiac muscle tissue	Normal
A4	F	42	Cardiac muscle	Normal cardiac muscle tissue	Normal
A5	F	21	Lung	Normal lung tissue	Normal
A6	F	21	Lung	Normal lung tissue	Normal
B1	M	40	Liver	Normal liver tissue	Normal
B2	M	40	Liver	Normal liver tissue	Normal
B3	F	50	Uterine cervix	Cancer adjacent normal cervix tissue (sparse)	NAT
B4	F	50	Uterine cervix	Cancer adjacent normal cervix tissue (sparse)	NAT
B5	M	35	Colon	Normal colon tissue	Normal
B6	M	35	Colon	Normal colon tissue	Normal
C1	M	40	Kidney	Normal kidney tissue	Normal
C2	M	40	Kidney	Normal kidney tissue	Normal
C3	F	21	Breast	Normal breast tissue	Normal
C4	F	21	Breast	Normal breast tissue	Normal
C5	F	18	Ovary	Normal ovary tissue	Normal
C6	F	18	Ovary	Normal ovary tissue	Normal
D1	M	50	Pancreas	Normal pancreas tissue	Normal
D2	M	50	Pancreas	Normal pancreas tissue	Normal
D3	M	43	Prostate	Normal prostate tissue	Normal
D4	M	43	Prostate	Normal prostate tissue	Normal
D5	M	25	Skin	Normal skin tissue	Normal
D6	M	25	Skin	Normal skin tissue	Normal
-	M	42	Adrenal gland	Pheochromocytoma (tissue marker)	Malignant

† For precise diagnosis, refer to pathology description.

APPENDIX IV

TMA datasheet purchased from US BIOMAX: Slide #MC246

MC246
Multiple organ tumor with matched adjacent normal tissue array, including TMA, clinical stage and pathology grade, 12 cores/24 cores, replacing MK 244

Microarray Panel: Multiple organ tumor and matched adjacent tissue microarray, containing 12 types of organs (prostate, lung, colon, breast, ovary, uterine cervix, esophagus, stomach, liver, kidney, skin, cerebnum), duplicate cores per case

Cores: 24

Cases: 12

Layout: 6 cols x 4 rows

Core Diameter: 1.5 mm

Thickness: 5 µm

Quality Control: Anti-Actin confirmed

Applications: Routine histology procedures including Immunohistochemistry (IHC) and In Situ Hybridization (ISH) protocols which can be found on our support page.

Notes: Unless specified, all TMA slides are not coated with extra layer of paraffin (tissue cores can be easily seen on the glass), so there is no need to bake, can be directly put into xylene for de-paraffin procedure.

US Biomax, Inc. MC246 (serial)

Microarray Panel Display

Legend: Bre - Breast, Cer - Cerebrum, Col - Colon, Eso - Esophagus, Kid - Kidney, Liv - Liver, Lun - Lung, Ova - Ovary, Pro - Prostate, Ski - Skin, Sto - Stomach, Ute - Uterine cervix

● - Malignant tumor, ○ - Malignant tumor (stage I), ○ - Malignant tumor (stage II), ○ - Malignant tumor (stage III), ○ - Malignant tumor (stage IV), ○ - Malignant tumor (stage V), ○ - NAT

Pos No./Serial	Age	Organ	Pathology diagnosis	Grade	Stage	TNM	Type †		
A1	1	M	71	Prostate	Adenocarcinoma (Gleason score 2-2)	I	II	T2N0M0	Malignant
A2	2	M	71	Prostate	Cancer adjacent normal prostate tissue	-	-	-	NAT
A3	3	M	55	Lung	Squamous cell carcinoma	2	II	T2N1M0	Malignant
A4	4	M	55	Lung	Cancer adjacent normal lung tissue	-	-	-	NAT
A5	5	M	54	Colon	Adenocarcinoma	2	I	T2N0M0	Malignant
A6	6	M	54	Colon	Cancer adjacent normal colon tissue (fibrous tissue and blood vessel)	-	-	-	NAT
B1	7	F	40	Breast	Invasive ductal carcinoma	2	Ib	T2N1M0	Malignant
B2	8	F	40	Breast	Cancer adjacent normal breast tissue	-	-	-	NAT
B3	9	F	68	Ovary	Serous adenocarcinoma (ovary tissue)	-	II	T2N0M0	Malignant
B4	10	F	68	Ovary	Cancer adjacent normal ovary tissue	-	-	-	NAT
B5	11	F	50	Uterine cervix	Squamous cell carcinoma	3	I	T1N0M0	Malignant
B6	12	F	50	Uterine cervix	Cancer adjacent normal cervical canal tissue	-	-	-	NAT
C1	13	F	49	Esophagus	Squamous cell carcinoma	1	Ib	T2N0M0	Malignant
C2	14	F	49	Esophagus	Cancer adjacent normal esophagus tissue	-	-	-	NAT
C3	15	F	78	Stomach	Adenocarcinoma	1	II	T2N0M0	Malignant
C4	16	F	78	Stomach	Cancer adjacent normal stomach tissue	-	-	-	NAT
C5	17	F	65	Liver	Hepatocellular carcinoma	2	II	T2N0M0	Malignant
C6	18	F	65	Liver	Cancer adjacent normal hepatic tissue	-	-	-	NAT
D1	19	M	41	Kidney	Clear cell carcinoma	2	I	T1N0M0	Malignant
D2	20	M	41	Kidney	Cancer adjacent normal kidney tissue	-	-	-	NAT
D3	21	M	52	Skin	Malignant melanoma of right heel	-	IV	T2N1M1	Malignant
D4	22	M	52	Skin	Cancer adjacent normal skin tissue	-	-	-	NAT
D5	23	M	51	Cerebrum	Glioblastoma	4	-	-	Malignant
D6	24	M	51	Cerebrum	Cancer adjacent normal cerebrum tissue	-	-	-	NAT
-	-	F	55	Liver	Hepatocellular liver cancer (tissue marker)	3	-	T2N0M0	Malignant

† For precise diagnosis, refer to pathology description.

**The grade I-3 (or I-III) in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.

Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.

Grade 2 or moderately-differentiated: Cells appear slightly different than normal.

Grade 3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

Grade 4 or undifferentiated: (For certain tumors), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

APPENDIX V

TMA datasheet purchased from US BIOMAX: Slide #T195b

T195b
Prostate cancer test tissue array, including TMA, clinical stage and pathology grade, 12 cases/24 cores, replacing T195a

Microarray
Prostate adenocarcinoma test tissue microarray, including 9 cases of prostate adenocarcinoma and 1 prostate low grade malignant leiomyosarcoma, plus 2 normal prostate tissue, duplicate cores per case, divided into two identical 12 core arrays for testing different antibody dilutions

Panel:
1

Cores: 24

Cases: 12

Layout: 8 cols x 3 rows


Core: 1.5 mm

Diameter: 1.5 mm

Thickness: 5 µm

Applications:
Routine histology procedures including Immunohistochemistry (IHC) and In Situ Hybridization (ISH), protocols which can be found on our support page.

Notes:
Unless specified, all TMA slides are not coated with extra layer of paraffin (tissue cores can be easily seen on the glass) so there is no need to bake, can be directly put into xylene for de-paraffin procedure.



US Biomax, Inc. T195b (serial)

A	1	2	3	4	5	6	7	8
B	1	2	3	4	5	6	7	8
C	1	2	3	4	5	6	7	8

Legend: Pro - Prostate
● - Malignant tumor (stage I), ● - Malignant tumor (stage II), ● - Malignant tumor (stage III), ● - Malignant tumor (stage IV), ● - Malignant tumor (stage Ia), ● - Normal tissue

Pos	No./Sex	Age	Organ	Pathology diagnosis	Grade	Stage	Grade	Gleason Score	TNM	Type †
A1	1	M	64	Prostate	Adenocarcinoma	1	I	1-2	T1N0M0	Malignant
A2	2	M	73	Prostate	Adenocarcinoma	2	II	3-3	T2N0M0	Malignant
A3	3	M	73	Prostate	Adenocarcinoma	2-3	IV	4-4	T3N0M1	Malignant
A4	4	M	61	Prostate	Adenocarcinoma	1	IV	3-2	T3N1M0	Malignant
A5	5	M	64	Prostate	Adenocarcinoma	1	I	1-2	T1N0M0	Malignant
A6	6	M	73	Prostate	Adenocarcinoma	2	II	3-3	T2N0M0	Malignant
A7	7	M	73	Prostate	Adenocarcinoma	2-3	IV	4-4	T3N0M1	Malignant
A8	8	M	61	Prostate	Adenocarcinoma	1	IV	2	T3N1M0	Malignant
B1	9	M	70	Prostate	Adenocarcinoma	2	III	3-4	T3N0M0	Malignant
B2	10	M	66	Prostate	Adenocarcinoma	2-3	III	4	T3aN0M0	Malignant
B3	11	M	65	Prostate	Adenocarcinoma	1-2	II	2-3	T2N0M0	Malignant
B4	12	M	66	Prostate	Adenocarcinoma	3	II	5	T2N0M0	Malignant
B5	13	M	70	Prostate	Adenocarcinoma	2	III	3-4	T3N0M0	Malignant
B6	14	M	66	Prostate	Adenocarcinoma	2-3	III	4	T3aN0M0	Malignant
B7	15	M	65	Prostate	Adenocarcinoma	1-2	II	2-3	T2N0M0	Malignant
B8	16	M	66	Prostate	Adenocarcinoma	3	II	5	T2N0M0	Malignant
C1	17	M	62	Prostate	Adenocarcinoma	3	II	5	T2N0M0	Malignant
C2	18	M	69	Prostate	Low grade malignant leiomyosarcoma	-	Ia	-	T1N0M0	Malignant
C3	19	M	33	Prostate	Normal prostate tissue	-	-	-	-	Normal
C4	20	M	43	Prostate	Normal prostate tissue	-	-	-	-	Normal
C5	21	M	62	Prostate	Adenocarcinoma	2	II	3	T2N0M0	Malignant
C6	22	M	69	Prostate	Low grade malignant leiomyosarcoma	-	Ia	-	T1N0M0	Malignant
C7	23	M	43	Prostate	Normal prostate tissue	-	-	-	-	Normal
C8	24	M	43	Prostate	Normal prostate tissue	-	-	-	-	Normal
-	-	M	58	Skin	Malignant melanoma (tissue marker)	-	-	-	-	Malignant

† For precise diagnosis, refer to pathology description.

**The grade 1-3 (or I-III) in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.

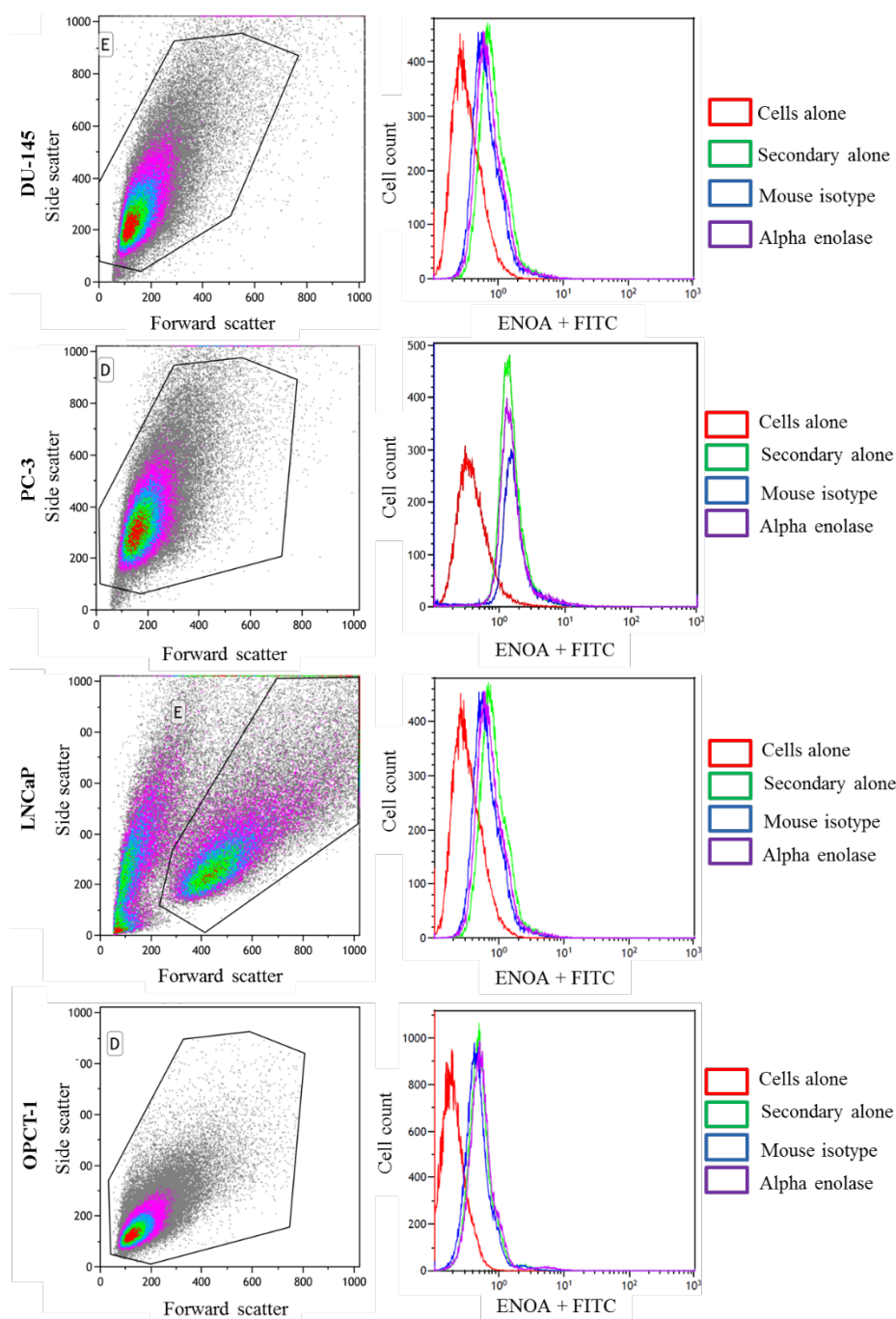
Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.

Grade 2 or moderately-differentiated: Cells appear slightly different than normal.

Grade 3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

Grade 4 or undifferentiated: *(for certain tumors) features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

APPENDIX VI



Appendix VI: Extracellular alpha enolase expression in all PCa cell lines demonstrated by flow cytometry

Flow cytometric data showing alpha enolase expression in 4 available PCa cell lines (DU-145, PC-3, LNCaP and OPCT-1). All cells were stained using a monoclonal mouse anti-human alpha enolase antibody (clone L-27, Santa Cruz, UK) or a goat anti-mouse secondary antibody conjugated to FITC. Histogram overlay shows no right shift in alpha enolase expression for DU-145, PC-3, LNCaP or OPCT-1 cell lines compared to the isotype or secondary controls, indicating that these PCa cell lines are negative for alpha enolase membrane expression.

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SCIENTIFIC COMMUNICATION

Research publications

Research article in preparation: Alpha enolase expression in prostate cancer.

Oral presentations

Dede, AY ‘The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers’. Final year PhD seminar at Nottingham Trent University, UK. 13th March, 2015.

Dede, AY ‘The Identification of Tumour Associated Antigens and Biomarkers for Prostate Cancer’ 2nd year oral presentation at the Science & Technology Research Conference, Nottingham Trent University. 2nd May, 2012.

Dede, AY ‘The Identification of Tumour Associated Antigens and Biomarkers for Prostate Cancer’. Applied Biomedical Science Conference, East Midlands, UK. 23rd June, 2011.

Magazine articles

Dede AY, ‘A Day In The Life of A Cancer Researcher’ published in the IBMS Gazette – October 2013.

Magazine article in preparation for the IBMS Gazette: Prostate cancer biomakers in clinical research.

Conference posters

Dede AY, Boocock DJB, Miles, AK and Rees RC. ‘*The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers*’ Voluntary poster presentation at the Science & Technology Research Conference, Nottingham Trent University. 1st May, 2014.

Dede AY, Boocock DJB, Miles, AK and Rees RC. ‘*The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers*’ Poster presentation at the Biomedical Science Congress, Birmingham, UK. 25th September, 2013.

Dede AY, Boocock DJB, Miles, AK and Rees RC. ‘*The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers*’ Poster presentation at the East Midlands Proteomics Workshop, Loughbrough University. 31st October, 2012.

Dede AY, Boocock DJB, Miles, AK and Rees RC. '*The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers*' Poster presentation at the Progress In Vaccinations Against Cancer, Nottingham Trent University. 11th September, 2012.

Dede AY, Boocock DJB, Miles, AK and Rees RC. '*The Identification of Prostate Cancer Associated Proteins as Biomarkers of Disease*' Poster presentation at the Midlands Vitae Poster Conference, Coventry, UK. 6th June, 2012.

Dede AY, Boocock DJB, Miles, AK and Rees RC. '*The Identification of Prostate Cancer Associated Tumour Antigens & Biomarkers*' Poster presentation at the Science & Technology Research Conference, Nottingham Trent University. 2nd May, 2012.