Androgen receptor phosphorylation in prostate diseases

Dr Jennifer Mary Willder

BSc Med Sci (Hons), MBChB

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Dedication

For Keith, my family and friends who have supported and endured me through this.

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Declaration

The work presented in this thesis was undertaken during a period of research between 2010 and 2013 in the Institute of Cancer Sciences at the University of Glasgow.

I declare that the work presented in this thesis was undertaken by myself except where indicated below:
Recruitment of patients and collection of clinical information in the prospective active surveillance cohort was performed by Dr Jawaher Ansari Consultant Oncologist at the Beatson West of Scotland Cancer Centre.

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List of abbreviations

AR	Androgen receptor
AUR	Acute urinary retention
CI	Confidence interval
BPH	Benign prostatic hyperplasia
Cdk	Cyclin dependent kinase
CRP	C-reactive protein
CRPC	Castrate resistant prostate cancer
CT	Computed tomography
CVD	Cardiovascular disease
DAB	Diaminobenzidine
DBD	DNA binding domain
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
DM2	Diabetes mellitus 2
ECL	Enhanced chemiluminesence
EGF	Epidermal growth factor
EGF	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases 1/2
FISH	Fluorescent in situ hybridisation
FOXO	Forkhead transcription factors

HR	Hazard ratio
H&E	Haematoxylin and eosin
LBD	Ligand binding domain
LHRH	Leutinising hormone releasing hormone
LUTS	Lower urinary tract symptoms
MAPK	Mitogen activated protein kinase
mGPS	Modified Glasgow prognostic score
MRI	Magnetic resonance imaging
NICE	National Institute for Clinical Excellence
PBS	Phosphobuffered saline
PI3K	Phosphatidylinositol 3 Kinase
PKC	Protein Kinase C
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinlylidene Difluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis
TBS	Tris buffered saline
TMA	Tissue microarray
TNM	Tumour node metastasis

TTBS	Tris buffered saline-tween
TRUS	Transrectal ultrasound
TUR	Transurethral resection

Summary

Prostatic diseases are common; benign prostate hyperplasia (BPH) is almost ubiquitous in elderly men (1) and 899,000 men were diagnosed with prostate cancer worldwide in 2008.(2) The incidence of both is increasing and expected to continue to rise.(3,4) Therefore, prostatic diseases represent a considerable economic burden, but there are currently no reliable markers available to accurately differentiate indolent from aggressive disease nor to predict who will benefit from treatment for either BPH or prostate cancer. This results in over and under-treatment of both diseases with consequent patient related morbidity and mortality.

The molecular mechanisms underlying the natural history of prostatic diseases remain elusive. It is accepted that prostate cell growth and survival are exquisitely dependent upon activation of the androgen receptor (AR) by androgens. Following ligand binding, AR undergoes further phosphorylation at serine residues, which inhibit proteolytic degradation, stabilise AR and influence AR transactivation. It is therefore plausible that alterations in AR phosphorylation may drive prostatic disease progression. However, few studies have explored the significance of AR phosphorylation, or the kinases driving AR serine phosphorylation in the clinical setting.

The over-riding objective of this study was to establish the clinical relevance of AR serine phosphorylation status in prostate tissue in both BPH and prostate cancer. The specific aims of the current study were:

- To firstly establish and validate a panel of AR phosphospecific antibodies.

- To evaluate site specific AR serine phosphorylation expression levels in prostate cancer and BPH patient cohorts, with full clinical data and follow-up.

- To investigate the expression of candidate kinases mediating such phosphorylation.

This involved establishing tissue banks with linked comprehensive clinical databases, and utilising this tissue to establish AR phosphorylation expression profiles for each patient.

Six AR phosphospecific antibodies (pAR^{S81}, pAR^{S94}, pAR^{S213}, pAR^{S515}, pAR^{S578}, pAR^{S650}) were verified using peptide competition assays and western blotting. Cdk1, ERK1/2, Akt and PKC were identified as putative kinases mediating AR phosphorylation using the online kinase search tool Scansite 2.0.

Immunohistochemistry was performed on hormone naïve diagnostic prostate cancer tissue relating to 90 patients. High expression levels of AR phosphorylation at serine sites 81, 515 and 578 were each associated with a poorer clinical outcome. Following cox regression analysis, cytoplasmic pAR^{S515} expression (p=0.038, HR 4.5 (95% CI 1.1–20.6)) and pAR^{S81} nuclear expression (p=0.030, HR 0.033 95% CI 0.002-0.721) were independently associated with shorter time to biochemical relapse and shorter disease specific survival respectively. Cdk1 and/or pCdk1^{161} were significantly associated with pAR^{S81} and pAR^{S515} as predicted by Scansite 2.0. Similarly, nuclear PKC expression was significantly associated with pAR^{S578} expression both in the cytoplasm and the nucleus. In patients with

PSA at diagnosis ≤20ng/ml, high cytoplasmic pAR^{S515} expression was associated with significantly shorter time to biochemical relapse (p=0.019). This translated into significantly shorter disease-specific survival (p<0.001, 10y survival 38.1% vs 100%).

Prostate cancer patients with a low serum PSA level at diagnosis may be suitable for delayed radical treatment via active surveillance. An investigation was therefore undertaken in 51 prostate cancer patients treated by active surveillance. Active surveillance is a deferred radical treatment approach which provides a potential solution to the problem of over treatment as a result of over-diagnosis. However some patients harbour occult aggressive disease and delay in treatment may result in disease progression and failure of radical therapy. Although none of the individual AR serine phosphorylation sites were associated with clinical outcome measures on univariate analysis, high expression of total AR in the cytoplasm (p=0.021, HR 4.6 (95% CI 1.3-16.8)) and presence of perineural invasion in the tumour specimen (p=0.003, HR 8.6 (95% CI 2.1-35.7)) were deemed independent with regards to shorter time to treatment intervention in a cox regression analysis.

Validation of the results seen in the first active surveillance prostate cancer cohort was undertaken in a second prospectively collected cohort consisting of 84 active surveillance patients. The results in the first cohort were not replicated in the second. Although cytoplasmic pAR^{S81} was associated with time to intervention (p=0.032) and pAR^{S515} expression trended towards an association (p=0.072), an increase in patient numbers in both cohorts may have provided more reliable results. However even with the numbers available in contrast to the first active

surveillance cohort, but in line with the pilot prostate cancer cohort, Cdk1 was associated with pAR^{S515} expression, and $pCdk1^{161}$ trended towards an association.

BPH is also an androgen driven disease dependent upon the AR. Previous research into predictive and prognostic markers in BPH is scant. Therefore a comprehensive analysis of clinical and novel pathological factors, including markers of inflammation, was performed in 336 BPH patients. Following this a complete panel of AR serine phosphorylation sites, and associated kinases, was analysed with reference to clinical outcome measures in the BPH cohort. Low expression levels of total AR and AR phosphorylated at Ser-81, 515 and 650 were associated with poorer clinical outcomes. Low expression of smooth muscle pARS515 (p=0.029, HR 0.31 (95% CI 0.10-0.94)) and older age (p=0.004, HR 5.13 (95% CI 1.43-18.41)) were deemed independent on cox regression analysis with regards to shorter time to postoperative acute urinary retention (AUR). Furthermore, low expression of pAR^{S515} in the smooth muscle was associated with increased incidence of postoperative AUR in patients over 70 years old (25.1% vs 2.8% at 10 years following transurethral resection of prostate (TUR)), (p=0.002, HR 0.20 (95% CI 0.06-0.62)). This may have important clinical implications in postoperative counselling of these patients. In addition it may influence the decision to commence early postoperative medical treatment (with 5-alpha-reductase inhibitors and/or alpha blockers) on a prophylactic basis in these patients. Cytoplasmic pAR^{S650} expression (p=0.010, HR 0.50 (95% CI 0.29-0.86)) and PSA at diagnosis (p=0.018, HR 1.89 (95% CI 1.11-3.16)) were independently associated with time to failure of surgical intervention. Furthermore, low expression of pAR^{S650} in the cytoplasm was associated with increased failure of surgical

intervention in patients with PSA ≥4ng/ml at diagnosis (45.5% vs 13% at 5 years post TUR), (p=0.026, HR 0.52 (95% CI 0.29-0.93)).

This comprehensive study on immunohistochemical expression of site specific AR serine phosphorylation and associated kinases fills a gap in the current literature. It has demonstrated the clinical significance of AR serine phosphorylation in prostate cancer and BPH and uncovered potentially exciting new avenues for future investigation. Site specific serine phosphorylation of the AR may serve as a prognostic and predictive biomarker in prostatic disease and has potential as a future target for therapeutic intervention.

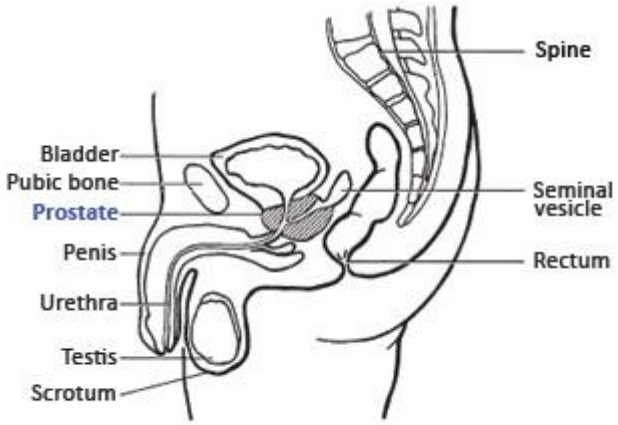
1 Introduction

Benign prostate hyperplasia (BPH) is the most common urological disease in elderly men and prostate cancer is the most common male malignancy in the UK. Both of these diseases are increasing in incidence and currently there is no way to accurately differentiate indolent from aggressive disease nor decipher who will respond favourably to treatment.

1.1 Normal prostate

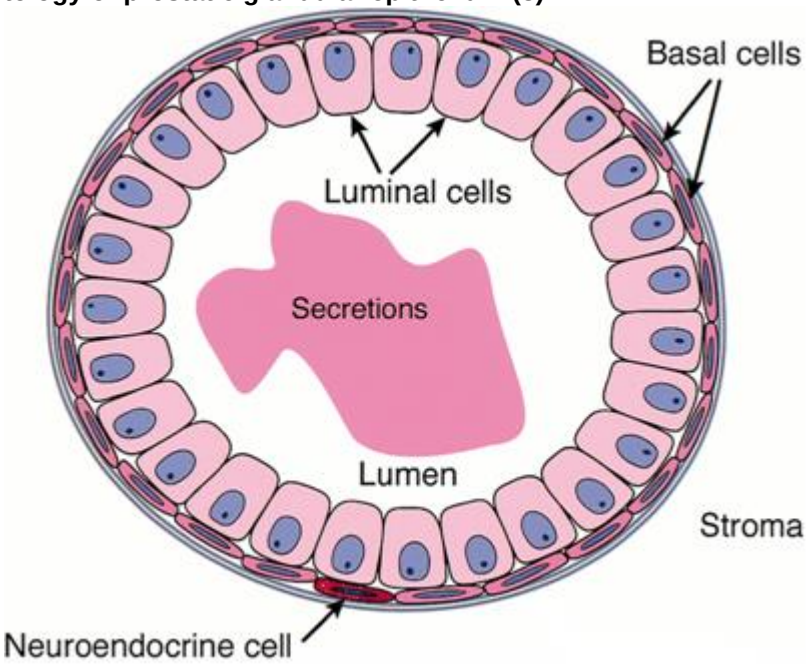
The prostate is a tubulo-alveolar gland which forms part of the male reproductive system. The human prostate is located inferior to the bladder neck and anterior to the rectum (Figure 1.1). The normal prostate weighs approximately 20g and is about the size of a walnut. It is enclosed by a capsule composed of collagen, elastin and large amounts of smooth muscle. In addition three distinct layers of fascia cover the prostate on the anterior, lateral, and posterior aspects. The gland is supported anteriorly by the puboprostatic ligaments and inferiorly by the external urethral sphincter and perineal membrane.(5)

Figure 1.1 Anatomical location of the prostate gland in humans (6)



The development of the human prostate begins in approximately the 10th week of gestation. It is dependent upon the production of testosterone by the foetal testis at around the 8th week of gestation. Following this, the growth of prostatic buds from the urogenital sinus is dependent upon binding of the potent androgen 5α-dihydrotestosterone to androgen receptors (AR) located in the surrounding mesenchymal tissue.(7) Postnatally, under the influence of androgens, the ducts form a patent lumen and the acini within the epithelial lining differentiate and begin the production of a number of secretory products.

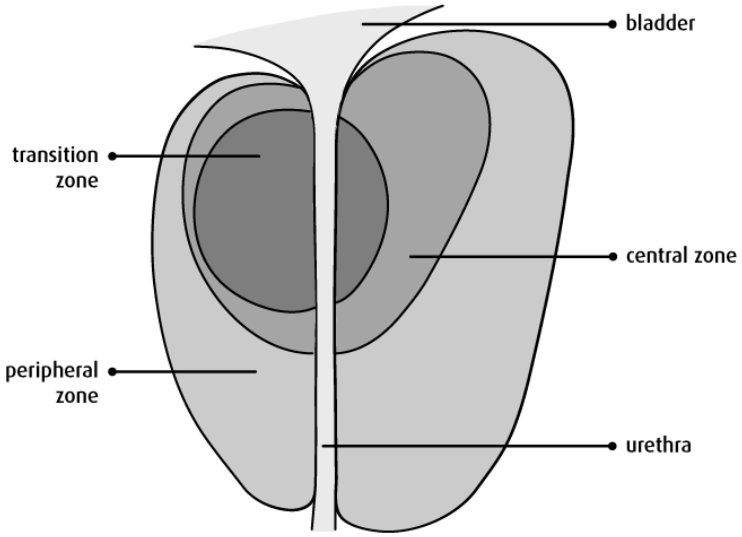
Figure 1.2 Histology of prostatic glandular epithelium (8)



Histologically the prostate glandular epithelium consists of three cell types; basal, luminal secretory and neuroendocrine (Figure 1.2). Basal cells are few in number and although their function is not completely understood they are known to secrete components of the basement membrane. Luminal cells secrete components of prostatic fluid, including prostate specific antigen (PSA) and express the AR. The prostatic stroma is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerve cells and inflammatory infiltrates. Some stromal cells are also androgen responsive and produce growth factors which act in a paracrine fashion on the epithelial cells.

The principal function of the prostate is as a secretory gland. The prostatic luminal cells produce a fluid containing; citric acid, acid phosphatase, PSA and zinc. Prostatic fluid combines with fluid from the seminal vesicles (containing fructose and prostaglandins) and spermatozoa from the testes to form the ejaculate.(9) This precise combination of secretions provides a mildly alkaline fluid in order to deposit viable sperm into the acidic female reproductive tract.

Figure 1.3 Crossectional image of prostatic zonal anatomy (10)



Anatomically the prostate can be divided into four zones; 1. peripheral zone, 2. central zone 3. preprostatic zone and 4. anterior fibromuscular zone (Figure 1.3). The peripheral zone surrounds the central and transitional zones posteriorly, adjacent to the rectum. It comprises approximately 70% of prostatic volume (11) and is the site of approximately 80% of prostatic cancers.

The central zone surrounds the transitional zone from the angle of urethra to the bladder base. It comprises approximately 25% of prostatic volume and is the site of approximately 5% of prostatic cancers. Cancers arising from the central zone tend to be more aggressive and more commonly invade the seminal vesicles.(12)

The preprostatic zone consists of periurethral glands and a transitional zone. Periurethral glands comprise less than 1% of the mass of the glandular prostate and do not possess their own periglandular muscularis and therefore are limited to the immediate periurethral stroma and grow proximally towards the bladder neck.(13) A small out-pouching of these periurethral glands is known as the transitional zone and is the innermost zone located adjacent to the prostatic urethra. It shows more duct branching and acinar proliferation than the periurethral glands. The transitional zone comprises approximately 5% of prostatic volume and is the site of approximately 10% of prostatic cancers. More commonly enlargement of the transitional zone and periurethral glands with increasing age via BPH can impinge upon the prostatic urethra leading to symptoms of bladder outflow obstruction.

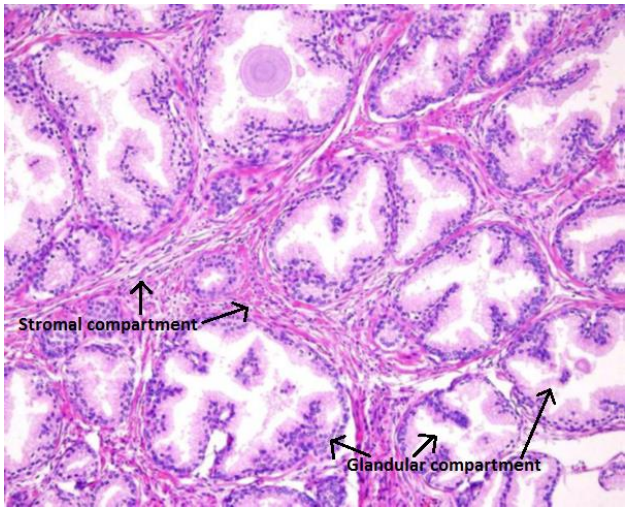
The anterior fibromuscular zone comprises the entire anterior surface of the prostate, is completely non-glandular and equates to approximately 33% of the

intracapsular prostatic tissue. It acts as a shield for the other three glandular zones.(13)

1.2 Benign prostatic hyperplasia

BPH is one of the most common diseases in elderly men. Autopsy studies have demonstrated a prevalence of 50% in men aged 50-60 years and 90% over 80 years.(1,14) BPH is a histological diagnosis associated with unregulated proliferation of connective tissue, smooth muscle and glandular epithelium within the prostatic transition zone (Figure 1.4). This may lead to compression of the prostatic urethra via an increase in prostatic volume and increased smooth muscle tone. Clinically BPH may cause significant morbidity in the form of bothersome lower urinary tract symptoms (LUTS) and complications such as acute urinary retention (AUR). Interestingly, in studies of large numbers of men, prostate volume does not correlate directly with symptom severity or urine flow rates (15), suggesting other factors are at play. Similarly not all men with histological BPH will experience LUTS and not all men with LUTS will have histological BPH.

Figure 1.4 Haematoxylin and eosin stained BPH section (16)



Haematoxylin and eosin stained section of BPH demonstrating both glandular and stromal hyperplasia.

1.2.1 Androgens and the androgen receptor in BPH

The molecular mechanisms underlying BPH development and progression are largely unclear. Many theories of aetiology have been proposed including; embryonic reawakening, aging, androgens, estrogens, oxidoreductase and inflammation theories. However, the presence of androgens in both foetal and adult life is known to a pre-requisite for the development and progression of BPH. Men castrated prior to puberty and those with genetic diseases affecting androgen production or action do not develop BPH.(17) Androgens act via the AR, a type 1 nuclear transcription factor. Higher serum concentrations of the potent androgen dihydrotestosterone (DHT) have been associated with larger prostate volume and higher prevalence of BPH in a cross-sectional study of 505 men.(18) In other work, DHT concentration was found to be highest in the periurethral area of the transtitional zone in BPH.(19) Similarly primitive BPH nodules in the periurethral transitional zone have been shown to exhibit higher nuclear AR expression than in other areas of the prostate.(20) These results indicate that cells in the transitional zone of the prostate may play an important role in development of BPH via modulation of androgen/AR signalling.

1.2.2 Stromal epithelial interactions in BPH

Continuous stromal-epithelial interactions are critical in prostatic development, homeostasis and disease as demonstrated by conditional knockout murine models. Previous work has demonstrated the importance of cell-specific AR in smooth muscle AR knockout mice that exhibit smaller prostates, histological abnormalities, altered gene expression, inflammation and fibrosis.(21) Stromal

fibroblastic selective AR knockout mice exhibit decreased proliferation of epithelial cells with increased apoptosis.(22) Double stromal knockout mouse models (deletion of AR in stromal fibroblasts and smooth muscle cells) demonstrated a reduction in the size of the anterior lobes of the prostate, decreased proliferation and increased apoptosis of epithelial cells in the anterior prostate.(23) Conditional knockout mice lacking AR in prostate epithelial cells however, developed larger, less differentiated prostates with increased cell death when compared to wild type.(14) The importance of stromal-epithelial cross talk has been further demonstrated in coculture experiments of primary human BPH stromal fibroblasts and epithelial cells whereby cell growth was significantly increased when compared to separate cell culture.(24) Although the individual roles of cell-specific AR are appreciated to be important, the clinical relevance of cell-specific AR expression has not been previously studied.

1.2.3 Age and BPH

AR expression remains high in elderly men despite falling levels of circulating androgens, suggesting that other regulatory mechanisms may be at play.(25) Alterations in the endocrine microenvironment leading to deregulation of prostatic growth have been postulated to account for the age related increase in BPH.(25,26)

1.2.4 Inflammation in BPH

Inflammation is thought to influence the development and progression of BPH although, again, its precise role is uncertain.(27) Inflammatory infiltrates are routinely found in prostate tissue specimens from men with BPH (28,29) and have been associated with increased prostate volume and international prostate symptom score.(29,30) In addition systemic measures of inflammation, such as

c-reactive protein (CRP), have been associated with development of BPH, increased LUTS and residual LUTS following medical treatment.(31-33) There is emerging evidence linking inflammation to AR expression in BPH.(34,35) Wang and colleagues demonstrated that stromal AR could enhance macrophage migration and macrophage-mediated stromal cell proliferation in BPH via its downstream target the inflammatory chemokine-chemokine (C-C motif) ligand 3.(34) Conversely, activation of prostatic smooth muscle cell AR via DHT was found to suppress the inflammatory response and reduce secretion of growth factors.(35) The relationship between inflammation and cell-specific AR remains unclear and therefore requires further investigation.

1.2.5 Medical treatment of BPH

The androgen axis is targeted in the medical treatment of BPH with 5-alpha reductase inhibitors which block the conversion of testosterone to DHT. The use of 5-alpha reductase inhibitors has repeatedly been shown to reduce prostate volume and symptomatic LUTS.(36) 5-alpha reductase inhibitors are often used in combination with α-blockers, which decrease urethral smooth muscle tone in the bladder neck, to achieve the best symptomatic response.(37)

1.2.6 Surgical treatment of BPH

Unfortunately, medical therapy for BPH often fails and surgical intervention via transurethral resection (TUR) of prostate is required, however this is associated with complications such as bleeding, urethral stricture and incontinence. In addition, a subset of men will have on-going symptoms despite surgical intervention and require further treatment with medication or repeat TUR operation.

1.2.7 Predictive and prognostic markers in BPH

1.2.7.1 Age

As discussed above prevalence of BPH and clinical progression is known to increase with increasing age. Prostate volume also increases with age and data suggests a prostate growth rate of 2.0-2.5% per year in older men.(38) Although prostate volume does not directly correlate with symptoms, prostate growth is a risk factor for LUTS progression and larger volume prostates are associated with increased risks of clinical progression of BPH, AUR and need for prostate surgery.(39)

1.2.7.2 Geographical location

Studies have shown geographic differences in prostate volume and LUTS. South east Asian and Indian men have been shown to have significantly lower volume prostates than western men.(40,41) Although Indian men had increased symptom severity scores than western men.(41)

1.2.7.3 Genetics

Studies have shown genetic influences of BPH and LUTS. Male relatives of men aged less than 64 years undergoing surgery for LUTS were four times more likely to undergo BPH surgery themselves.(42) In particular, brothers of the cases were six times more likely to undergo BPH surgery.(42) Monozygotic twin studies have shown concordance rates of 62% for LUTS and 26% for BPH.(43,44) Inherited forms of BPH tend to have a larger volume prostates and earlier age of onset of clinical symptoms than men with sporadic BPH.(45)

1.2.7.4 PSA

Although more commonly associated with prostate cancer, PSA has repeatedly been correlated with increased age in parallel with increased incidence of BPH.(46-48) Despite this association PSA has not been used routinely to diagnose or monitor treatment of BPH. It may be that the controversy surrounding PSA in prostate cancer has also limited its use in BPH.

1.3 Prostate cancer

An estimated 899,000 men were diagnosed with prostate cancer worldwide in 2008 with most cases in developed countries.(2) It is the most common cancer in Scottish men with 14,819 cases diagnosed and 4,212 deaths between 2008-2012.(49) At the end of 2011 there were 21,259 men living with prostate cancer in Scotland and this figure is expected to rise 80% by 2022.(49,50) Prostate cancer therefore represents a considerable burden on the health service and significant disease-related morbidity and mortality is suffered by patients. Treatment of prostate cancer is also costly; men are treated with radiation, surgery or hormones, but even surveillance strategies are expensive. The diagnosis, treatment and 5 year follow-up cost of prostate cancer in the UK was estimated at £136, 278, 237 in 2010.(51)

The vast majority of prostate cancers are acinar adenocarcinomas occurring within the peripheral zone of the prostate as discussed previously. Non acinar carcinoma variants of prostate cancer comprise 5-10% and include; sarcomatoid carcinoma, ductal adenocarcinoma, squamous and adenosquamous carcinoma, urothelial carcinoma, small-cell carcinoma, basal-cell carcinoma and clear cell adenocarcinoma.(52) The histological subtype has important implications for prognosis and treatment decision making.

Early prostate cancer is unlikely to produce any symptoms and men who are ultimately diagnosed with prostate cancer usually present in primary care. Further investigation may be prompted by an abnormal rectal examination or serum PSA testing in at risk individuals. Patients with progressive prostate cancer may present with LUTS similar to those seen in BPH and those with metastatic disease may present with bone pain. However, since the advent of serum PSA testing in the 1980s the number of men presenting with metastatic disease has significantly reduced.(53)

Prostate cancer may also be diagnosed secondary to the investigation or treatment of BPH. BPH is associated with a higher serum PSA level which may lead to a suspicion of prostate cancer, and pathological analysis of tissue resected during a TUR of prostate may result in a diagnosis of prostate cancer.

The diagnosis of prostate cancer involves investigation most commonly with a combination of biochemical, pathological and radiological measures. Following serum PSA testing and rectal examination the diagnosis of prostate cancer is usually confirmed via a transrectal ultrasound (TRUS) guided needle biopsy of the prostate gland. The aim of prostate biopsy is to confirm the presence or absence of prostate cancer in men suspected of having the disease. Currently approximately 25% of men undergoing TRUS biopsy with abnormal aged-based serum PSA levels will have a positive diagnosis for prostate cancer.(54) Interestingly detection rates vary dependent upon the number of previous biopsies; 14-22% for initial biopsy, 10-15% for second biopsy and 5-10% for third biopsy.(55-57) Radiological imaging may be undertaken following or preceeding TRUS guided prostate biopsy. This may take the form of MRI/CT/bone scan

dependent upon the clinical variables of each individual case. Low risk cancers deemed unlikely to have spread may not require any imaging following diagnosis.

1.3.1 Management challenges of 21st century prostate cancer

The weight of the economic burden of prostate cancer stems from the inability to accurately differentiate indolent cancer, which will never incur clinical sequale, from aggressive disease which is invasive and spreads ultimately causing death. Consequently many clinically insignificant prostate cancers are over-treated and some aggressive cancers are under-treated resulting in high morbidity to individual patients and cost to the health services. This arises largely from the inadequacy of the current tools used to risk stratify patients at diagnosis. Prostate biopsy is subject to a significant amount of sampling error with one study demonstrating 27% of low grade cancers upstaged or upgraded on repeat sample taken at 3 months.(58) Clearly the current tools available to guide treatment of prostate cancer are inadequate and translate into the significant morbidity and mortality of patients in Scotland. Further progress must be made in order to accurately identify those men at risk of developing the aggressive life shortening form of this disease.

1.3.2 Current predictive and prognostic markers

Clinical, pathological, biochemical and radiological markers are used to risk stratify patients at diagnosis.

1.3.2.1 Age

Advancing age is one of the strongest risk factors for prostate cancer. Incidence is estimated at 0.1% in those men aged under 50 and approximately 85% of all cases are diagnosed in those aged over 65 years. Age at diagnosis has also been

shown to be a significant predictor of overall survival in men with prostate cancer. This is likely to be a reflection on the age-related increase in comorbidities, susceptibility to major illness, and blunting of the immune response.(59)

1.3.2.2 Genetics

Family history has been shown to be a risk factor for prostate cancer.(60) In patients diagnosed under 55 years of age predisposing genes are thought to account for up to 40% of cases.(61,62) With each increase in number of first degree relatives affected by prostate cancer the relative risk to patient increases.(63) In addition familial prostate cancer has been linked to familial breast cancer, thought to be related to the BRCA1 and BRCA2 genes.

1.3.2.3 Ethnicity

Ethnicity has repeatedly been shown to be a risk factor for prostate cancer. Asian men have the lowest incidence rates of prostate cancer particularly in India, China and Japan. Furthermore south Asian men living in the UK have a lower incidence of prostate cancer than Caucasian UK men.(64) Black men have higher rates of prostate cancer, compared to Caucasian men; African-Americans have 1.3-2.0 times increased risk and black men have 3-times higher risk of developing prostate cancer.(65) Ethnic origin is also associated with disease specific mortality. Black men have a 30% higher mortality rate than White men. The mortality rate in South Asian men was found to be significantly less than in Caucasian men.(66)

1.3.2.4 PSA

Prostate specific antigen (PSA) is also known as 'kallikrein-3'. It is a glycoprotein produced exclusively by the prostate gland and has a role in promoting sperm motility and the dissolution of cervical mucus. Normally the basement membrane acts as a barrier preventing circulatory escape of PSA. Disruption of the basement membrane by prostatic disease (cancer, BPH, prostatitis) and/or manipulation (massage, rectal exam, biopsy) can allow leakage of PSA into the systemic circulation which can be measured by a simple blood test. PSA has poor specificity for prostate cancer with 15.2% of men with a "normal" level (<4.0ng/ml) demonstrating cancer on biopsy.(67) PSA screening was common place in the USA until relatively recently. Large population based studies have clearly demonstrated that prostate cancer screening using PSA increases prostate cancer detection but does not reduce prostate cancer deaths.(68) In addition the European Randomised Study of Screening for Prostate Cancer (ERSPC) trial suggested that in order to prevent one death from prostate cancer, 1055 men would require screening and 37 men would need treated for prostate cancer.(69)

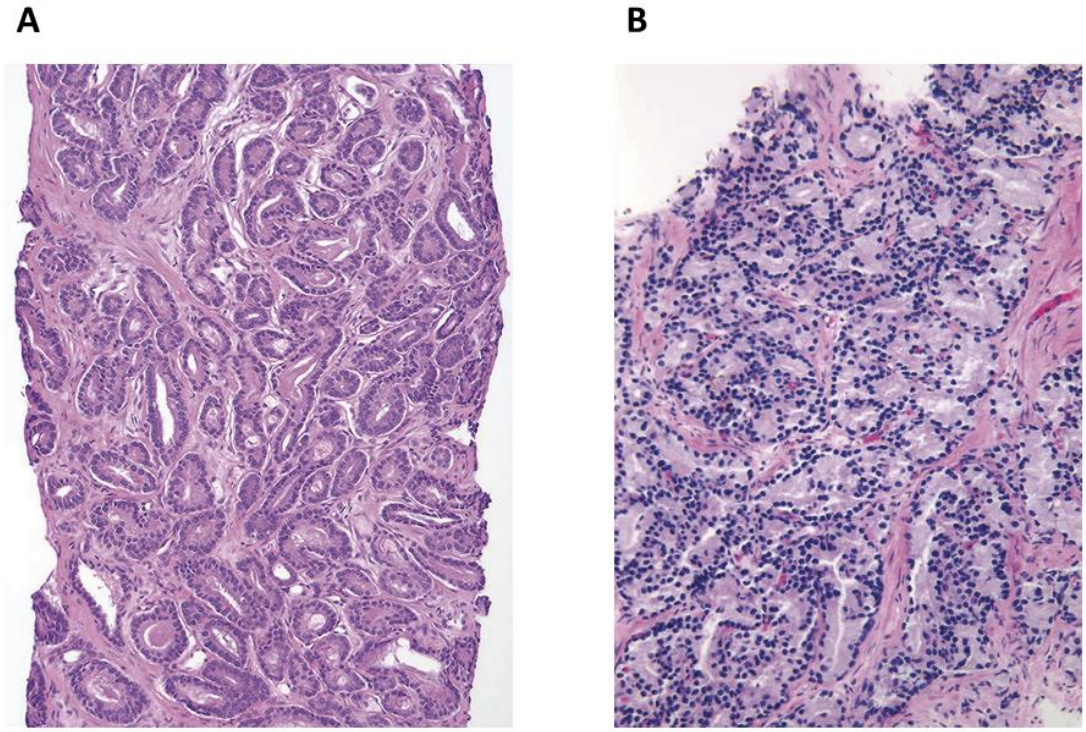
Serum PSA testing is often used as a marker of response to treatment for prostate cancer. It is utilised to determine and monitor the success of radical treatment. In addition PSA is also used to identify patients who require treatment intervention (both radical and non-radical). In addition it is frequently used to monitor patients in whom prostate cancer has been diagnosed but treatment has been purposefully delayed either via active surveillance or watchful waiting.

The introduction of PSA testing in the 1980s has really changed the face of prostate cancer and has led to significant stage and grade migration of the disease with more clinically insignificant cancers being detected. The current challenge centres around the differentiation of indolent from aggressive prostate cancer.

1.3.2.5 Gleason

The Gleason grading system for prostate cancer, developed in the 1960s and most recently modified in 2005, is still the strongest predictor of outcome in men diagnosed with the disease.(70,71) Gleason grading for prostate cancer is a histological score based upon differentiation. It ranges from 2-5 (well-poorly differentiated) and a score is calculated based on the two most predominant tumour patterns. Haematoxylin and eosin staining of high and low Gleason prostate cancer tissue is shown in Figure 1.5.

Figure 1.5 Haematoxylin and eosin staining of low (A) and high (B) Gleason grade prostate cancer (71)



Haematoxylin and eosin staining of prostate biopsy sections. A: Gleason score 6 (3+3) prostatic carcinoma composed of small discrete glands, variable in size and organisation. B: Gleason score 8 (4+4) prostatic carcinoma, composed of ill-defined glands with poorly formed glandular lumina and accompanied by gland fusion.

Gleason 6 (3+3) is recommended as the lowest score on biopsy material (71) and is considered low risk. Gleason 7 (3+4 and 4+3) is considered moderate risk and Gleason 8-10 is considered high risk disease. Gleason score has implications for both prognosis and treatment options and timing.

1.3.2.6 T stage

The TNM classification is used to stage prostate cancer (Table 1.1). It combines the extent of the primary tumour (T stage), the absence or presence of spread

to lymph nodes (N stage) and the absence or presence of metastasis (M stage). Increased clinical stage is associated with increased disease specific mortality.

Table 1.1 TNM staging for prostate cancer (72)

Stage	Sub-stage	Definition
Tumour		**Primary tumour**
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1		**Clinically inapparent tumour, neither palpable nor visible by imaging**
	T1a	Tumour incidental histological finding in <5% resected tissue
	T1b	Tumour incidental histological finding in >5% resected tissue
	T1c	Tumour identified by needle biopsy
T2		**Tumour confined within prostate**
	T2a	Tumour involves ≤50% of one lobe or less
	T2b	Tumour involves >50% of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3		**Tumour extends through the prostatic capsule**
	T3a	Extracapsular extension including microscopic bladder neck involvement
	T3b	Tumour invades seminal vesicle(s)
T4		**Tumour is fixed or invades adjacent structures other than seminal vesicles e.g. external sphincter, rectum, levator muscles, and/or pelvic wall**
Node		**Regional lymph nodes**
	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph nodes metastasis
	N1	Regional lymph node metastasis
Metastasis		**Distant metastasis**
	M0	No distant metastasis
	M1	Distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Metastasis at other site(s)

The factors above are combined in order to risk stratify patients at diagnosis as per the National Institute for Clinical Excellence (NICE) guidelines (Table 1.2).(72) This has implications for treatment decision making.

Table 1.2 Risk stratification for prostate cancer

	Serum PSA		Gleason		Clinical Stage
Low	<10ng/ml	**and**	≤6	**and**	T1-2a
Intermediate	10-20ng/ml	**or**	7	**or**	T2b
High	>20ng/ml	**or**	8-10	**or**	≥T2c

1.3.3 Management of prostate cancer

The management of prostate cancer depends upon whether it is clinically localised, locally advanced or metastatic at diagnosis. Other factors such as life expectancy and co-morbidities are taken into account when undertaking treatment decision making.

1.3.3.1 Radical treatment options

Radical treatment is available for all risk groups except patients with metastatic disease at diagnosis.

Active surveillance

Active surveillance for prostate cancer is a delayed treatment approach whereby low or intermediate risk patients suitable for radical therapy undergo intervention only when biochemical, histological or clinical progression is demonstrated.(72) Active surveillance avoids the side effects of radical treatment such as urinary incontinence, erectile dysfunction and a small risk of death. The recent stage and grade migration of prostate cancer secondary to serum PSA testing has led to the over diagnosis and over treatment of clinically insignificant disease. Active surveillance provides a potential solution to this problem and NICE recommend it as the preferred treatment option for low risk patients.(72) Intermediate risk patients can also be considered for active surveillance if they do not wish to undergo immediate radical therapy.

Radical prostatectomy

Radical prostatectomy is suitable for low and intermediate risk patients. In cases of high risk prostate cancer radical prostatectomy may be offered if there is a realistic chance of long term disease control. Surgery is performed in order to remove the prostate and seminal vesicles. This can be done via an open, laparoscopic or robotically assisted laparoscopic approach dependent upon geographical service provision. However, both laparoscopic and robotically assisted laparoscopic surgery have reduced length of patient stay in hospital and surgical blood loss. Risks of radical prostatecomy include incontinence, erectile dysfunction and a small risk of mortality. Radical prostatectomy is a major operation that is usually only offered to fit men without significant co-morbidities.

Radiotherapy

Radical radiotherapy can take the form of external beam or by placement of radiation sources directly into the prostate gland (brachytherapy). External beam radiotherapy is the most common treatment in the UK for men with localised prostate cancer. It can be used for all risk groups and is usually performed in conjunction with neoadjuvant hormone treatment, and is given in daily fractions over 4–8 weeks as an outpatient. Low and intermediate risk prostate cancer patients may be offered brachytherapy. Brachytherapy involves the insertion of small radioactive pellets directly into the prostate gland. These then deliver local radiotherapy over a period of weeks-months which minimises the damage to surrounding ‘normal’ tissue. Intermediate and high risk patients may be offered brachytherapy in combination with external beam radiotherapy.

The side effects of both forms of radiotherapy treatment can include alteration in urinary and bowel function and erectile dysfunction.

1.3.3.2 Non radical treatment options

Non radical treatment options are suitable for all risk groups and in particular patients with metastatic disease at diagnosis. These treatment options may be selected in preference to radical treatment in the context of significant comorbidities and reduced life expectancy.

Watchful waiting

Watchful waiting is a conservative approach without curative intent by which treatment is only initiated when symptoms develop. If and when symptoms develop treatment is usually with hormonal therapy. This approach is usually offered to older men or those with significant comorbidities, in whom prostate cancer is unlikely to be of clinical significance within their predicted lifetime.

Hormonal treatment

Hormonal treatment is the mainstay of treatment for metastatic prostate cancer.

Androgen deprivation

Androgen deprivation slows metastatic prostate cancer progression by 18 months on average. Castration can be surgical (bilateral orchidectomy) or medical via luteinising hormone releasing hormone (LHRH) agonists which downregulate the hypothalamic-pituitary axis inhibiting androgen production. Side effects include hot flushes, headaches, erectile dysfunction, loss of libido, gynaecomastia and osteoporosis.

Androgen blockade

Androgen action can be blocked peripherally by the use of anti androgens. Anti androgens bind competitively to the AR. Anti androgens can be used in place of or in combination with LHRH agonists. They are often used during the initiation of LHRH agonist therapy in order to prevent tumour flare. The use of both LHRH agonists and anti androgens is known as combined androgen blockade. Anti androgens have a similar but less severe sexual side effect profile to LHRH agonists. Men treated with anti androgens are more likely to develop gynaecomastia.

Although initial response rates to hormonal therapy are high eventually all prostate cancers will relapse developing castrate resistant disease within 18-24 months. Castrate resistant prostate cancer (CRPC) is characterised by the re-emergence of serum PSA expression following hormonal treatment. Once established CRPC is an ultimately fatal disease with limited treatment options. Median survival of patients with CRPC without treatment is 9-22 months.

Chemotherapy

Chemotherapy using a combination of docetaxel and prednisolone is used in CRPC and can provide a modest increase in survival. The side effects of this combination can be overwhelming and it may prohibit its use.

Current treatment is not optimal due to a lack of understanding of the underlying molecular mechanisms associated with development and progression of prostate cancer.

1.3.4 Molecular mechanisms of prostate cancer development and progression.

Dysregulation of many genes has been linked to the development and progression of prostate cancer.

1.3.4.1 PTEN

The phosphatase and tensin homolog (PTEN) is a tumour suppressor gene located on chromosome 10q23. The PTEN gene encodes a phospholipid phosphatase which is active against both protein and lipid substrates negatively regulating the PI3K/Akt/mTOR signalling pathway which is essential for cell cycle progression and cell survival. PTEN antagonises the action of PI3K by de-phosphorylating PIP3 (a PI3K product) at the D3 position. This removes the membrane-localisation factor from the Akt signalling pathway and thereby significantly inhibits the downstream activation of Akt.

In prostate cancer the most common somatic PTEN mutation is via copy number loss rather than point mutation.(73) PTEN loss is proposed as a late event in prostate carcinogenesis as loss of PTEN heterozygosity has been seen in up to 60% of advanced prostate cancer tumours (74), however germline PTEN mutations have not previously been associated with prostate cancer.(75,76) Furthermore, commonly utilised prostate cancer cell lines cultured from metastatic deposits in lymph node (LNCaP) and brain (PC3) are known to have PTEN deletion and highly active PI3K/Akt signalling.(77,78) Loss of PTEN has been associated with progression from hormone sensitive to CRPC in human tissue specimens. In a cohort of 57 prostate cancer patients with matched hormone sensitive and CRPC prostatic tissue PTEN gene deletion, identified via fluorescent in situ hybridisation (FISH), significantly increased from 23% to 52% in

CRPC tumours.(79) In addition loss of PTEN expression was an independent predictor of disease specific survival.(79)

1.3.4.2 c-Myc

c-Myc is a proto-oncogene of the helix–loop–helix leucine zipper protein family of transcription factors. It is located on chromosome 8q24 and encodes the c-Myc protein which dimerizes with its protein partner Max in order to transactivate gene expression which promotes cell proliferation and transformation. c-Myc is believed to regulate up to 15% of all genes involved in almost every important cellular function.(80)

The discovery that c-Myc is translocated to one of the immunoglobulin loci in virtually all Burkitt's lymphomas highlighted c-Myc as a human oncogene.(81) It is now known that c-Myc is overexpressed in the majority of human cancers. The amplification and overexpression of c-Myc has been observed in 8% of primary prostate cancers and in approximately 30% of metastatic deposits.(82,83) Over the past three decades c-Myc mRNA has been consistently shown to be overexpressed in the majority of prostate adenocarcinomas as compared to BPH and benign prostatic tissue.(84,85) More recently c-Myc overexpression has been demonstrated at the protein level in prostatic intraepithelial neoplasia (PIN), localised and metastatic prostate cancer.(86) A significant correlation with increasing Gleason grade and worsening prognosis has been described with c-Myc expression in advanced prostate cancer.(83) The molecular mechanisms underlying c-Myc overexpression in prostate cancer remain largely unclear however, gene amplification and rearrangement and the influence of other signalling cascades have all been shown to contribute.(87)

1.3.4.3 TMPRSS2-ERG

The transmembrane serine protease 2 (TMPRSS2) gene and members of the ETS transcription family (ETV1, ETV4, ETV5, ERG and FLI1) are responsible for genetic rearrangement and creation of the fusion gene TMPRSS2-ETS.(88) The most common ETS family member to undergo fusion with TMPRSS2 is ERG. Over 50% of all prostate cancers are thought to harbour the TMPRSS2-ERG fusion gene, and as such it is the most common genomic alteration in prostate cancer.(89)

TMPRSS2-ERG fusion results in the overexpression of the ERG oncogene. The precise function of ERG in prostate cancer is unclear, and it is known that ERG overexpression alone is not sufficient for prostatic carcinogenesis. ERG has been shown to cooperate with several different genes e.g. PTEN in the development of murine prostate cancer.(90) It has been postulated to have a role in prostatic epithelial cell migration and invasion.

TMPRSS2-ERG fusion has never been detected in BPH or the benign prostate. Therefore TMPRSS2-ERG fusion is a very specific prostate cancer biomarker. ERG overexpression has been seen in PIN lesions and as such is generally considered an early event in prostate carcinogenesis.(91) However, it remains to be established whether TMPRSS2-ERG fusion plays a role in PIN to cancer progression. Of all the genes linked to prostate cancer AR is the most widely studied.

1.4 Androgen receptor

The AR is a type one nuclear receptor which is activated by androgens and acts as a ligand dependent transcription factor. The gene for the AR is located on the X chromosome at Xq11-12 and the protein is comprised of 919 amino acids

with a molecular mass of approximately 110kDa. In the normal prostate AR is essential for prostate development, cellular proliferation, survival, apoptosis and secretion.

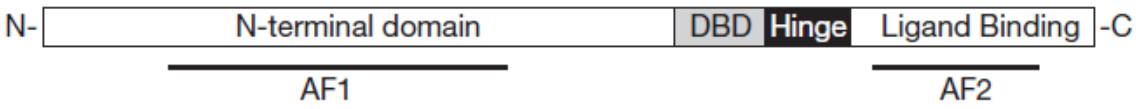
The AR is expressed in both stromal and epithelial prostatic cells (92) and is thought to mediate different effects within each cellular compartment. For example functional AR in prostatic stroma is necessary during prostate development while prostatic epithelium has been shown to induce smooth muscle differentiation of a subset of the stroma.(93) AR is required in both cellular compartments for the development of functional secretory prostatic epithelia.(93) Furthermore, cell-specific AR is reported to have differing effects on proliferation, with stromal AR shown to promote proliferation (94) while epithelial AR inhibits epithelial cell proliferation.(95) However these roles are complex with studies showing overexpression of epithelial AR increases epithelial proliferation.(96)

1.4.1 AR Structure

The AR is comprised of three major functional domains (Figure 1.6). The N-terminal domain is the largest and comprises over half of the receptor. The N terminal exhibits an activation domain called activation function 1 (AF1). AF1 is constitutively active in truncated receptors that do not contain the ligand binding domain (LBD). The DNA binding domain (DBD) is an independently folded protein domain which contains two zinc fingers. The first zinc finger contains a P-box and interacts with the half-site of the androgen-response element (ARE).(97-99) The second zinc finger contains a D-box and facilitates dimerization of the receptor on AREs. A short flexible peptide sequence known as the hinge region links the DBD and LBD. It is responsible for the regulation of AR nuclear localisation, DNA binding, and coactivator recruitment.(100) The LBD

contains the C terminal and the ligand dependent transcriptional activation function 2 (AF2). In the case of most nuclear receptors AF2 is more potent and more important for ligand activation than AF1. However, in AR transactivation AF2 appears to have less impact than AF1. Interestingly, a ligand-dependent functional interaction between AF1 and AF2 suggests that the two regions work in synergy in order to maximise AR transactivation.(101)

Figure 1.6 Structure of the androgen receptor

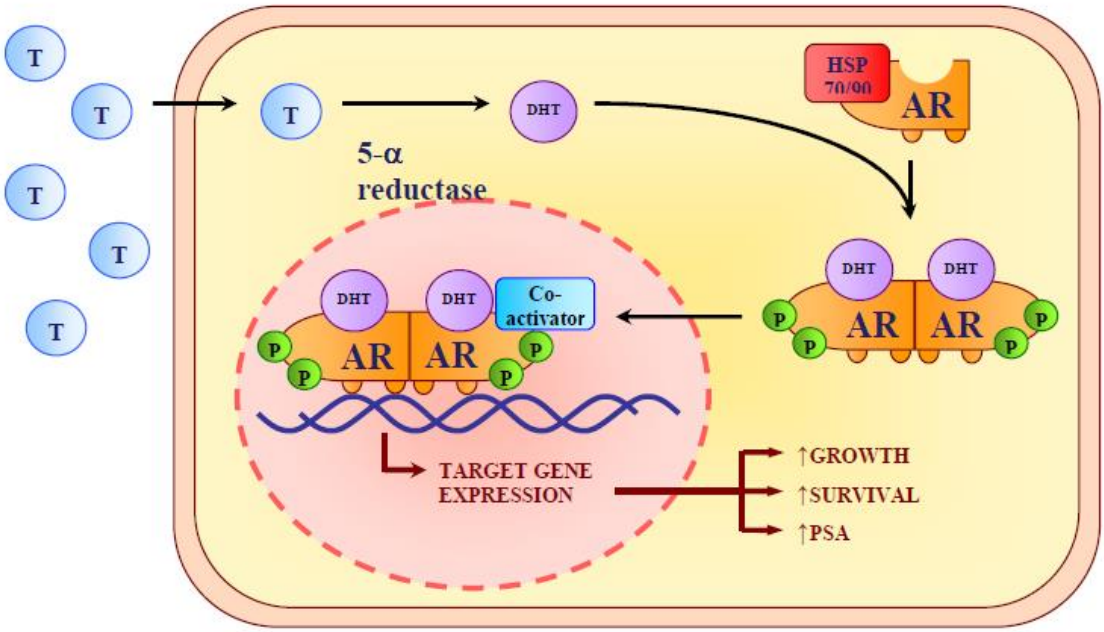


The location of the activation function (AF) sites 1 and 2 are shown. DBD = DNA binding domain.

1.4.2 AR activation

In the absence of androgens the AR is located in the cytoplasm in an inactive state bound to heat shock proteins (HSPs), which prevent it from entering the nucleus. The activation of AR occurs via androgen binding as already stated. Testosterone is primarily produced by the Leydig cells of the testes and is converted to the more potent androgen, dihydrotestosterone (DHT) by the enzyme type II 5 alpha reductase. DHT then binds to inactive AR in the cytoplasm which causes a conformational change and dissociation of HSPs, activation of AR cofactors, formation of homodimers and translocation of AR to the nucleus. AR then binds to AREs which results in gene transcription (Figure 1.7). Coactivators, corepressors and chromatin remodelling complexes are recruited to the promoter in order to facilitate transcription of AR target genes. PSA is one of the best known genes regulated by AR, however AR also regulates many other genes that are involved in proliferation and apoptosis.

Figure 1.7 Androgen receptor activation in prostatic cells by androgens

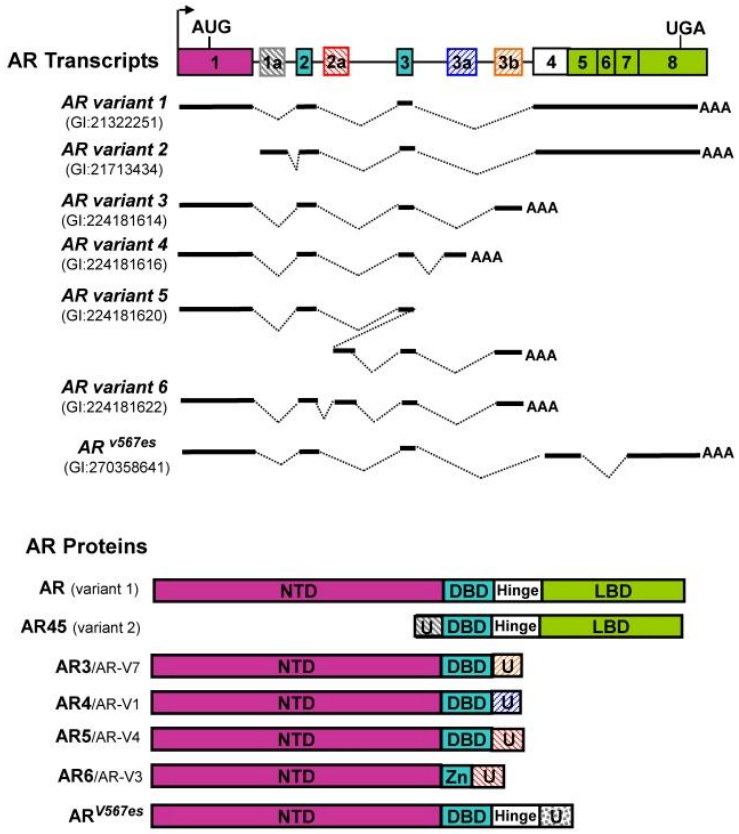


Testosterone (T) enters the prostatic cells and is converted to dihydrotestosterone (DHT) in the cytoplasm by 5-α-reductase enzyme. DHT then binds to the androgen receptor (AR) which causes dissociation of heat shock proteins (HSP 70/90), and transactivation by dimerization and phosphorylation (p). AR homodimers then translocate to the nucleus where, in the presence of co-activators, AR binds to androgen response elements resulting in gene transcription.

1.4.2.1 AR splice variants

In addition to classical activation of the AR via ligands it may also be activated when truncated. Truncated versions of AR are known as AR splice variants and co-exist with full length AR in clinical specimens. The expression levels of individual AR variants almost always comprise a small fraction of the expression level of full length AR.(102,103) The most common AR splice variant is AR-V7/AR3, this receptor lacks the LBD (Figure 1.8) and as a consequence is constitutively active. However many different splice variants have been observed (Figure 1.8), most of which lack the LBD.

Figure 1.8 AR splice variants (104)



Constitutive AR activation is commonly observed in CRPC, in particular AR-V7/AR3 was detected at approximately 20x higher levels in CRPC than in hormone naïve clinical specimens, driving expression of androgen-responsive genes in an androgen independent manner.(102) Overexpression of AR-V7/AR3 in cell lines activated AR gene transcription in the presence and in the absence of full length AR. However the transcriptional activity of other AR variants has been shown to be cell type specific. AR-V1 and AR-V9 demonstrated transcriptional activity in LNCaP cells (full length AR positive) but not in PC3 cells (full length AR negative).(105)

Truncated forms of the AR result in variability of molecular weight of the receptor as detected by western blot. This is readily observed in prostatic cell lines, including LNCaP, where molecular weight bands in the range of 70–90 kDa

are commonly observed by western blot in addition to bands at 110-112 kDa due to full length AR.

1.4.3 Post translational serine phosphorylation of the AR

Modulation of AR functional activity can be achieved by post translational modifications, which may include phosphorylation, acetylation, ubiquitination, sumoylation and methylation. Post translational modification of AR at a total of 23 sites can result in changes to transcriptional activity, protein stability, cellular localisation and protein structure.

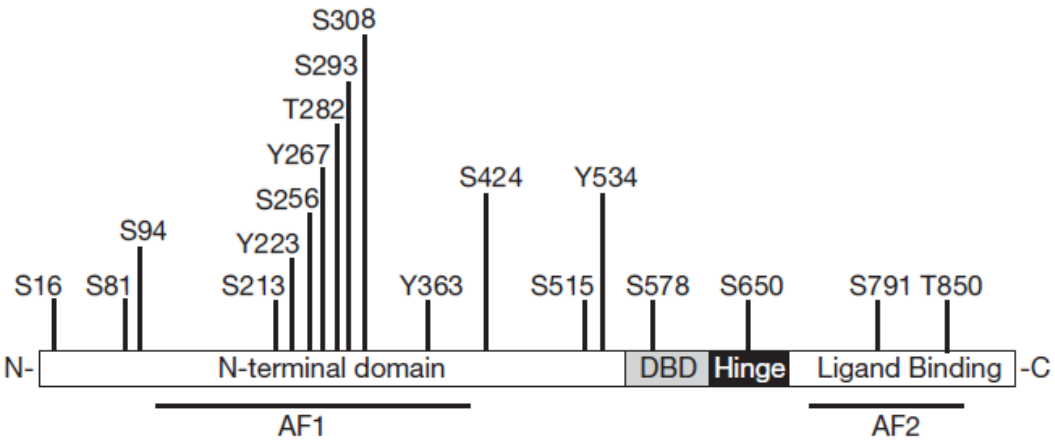
Phosphorylation of the AR influences receptor stability, DNA binding, subcellular localisation, transcription and interactions with coregulators. It is by these methods that AR phosphorylation regulates the activity of the receptor. Therefore alterations in AR may be involved in the progression and development of prostatic disease.

It has previously been shown that mutations of the AR have been linked to disease states. For example germline missense mutations resulting in an amino acid substitution in the LBD or DBD results in abnormal receptor function and have been implicated in androgen insensitivity disorders. Non-sense mutations and others resulting in premature splicing of AR mRNA have also been identified in relation to disorders of androgen insensitivity.(106) Somatic mutations of the AR have been observed in prostate cancer specimens. Increased frequency of mutations is observed in CRPC compared to early prostate cancer, implicating that they may have a role in tumour progression.(107) As discussed above, truncated forms of the androgen receptor have been identified lacking the LBD. These variants have been shown to be constitutively active and highly expressed

in CRPC.(108) Amplification of AR expression at the gene and protein level has been previously shown in CRPC, and is postulated as a mechanism for androgen escape.(109) As it has influences on AR activation, AR phosphorylation status may play a role in prostatic disease.

Phosphorylation of the AR was first reported in 1984 by Goueli and colleagues (110) and has been extensively studied since. The AR is phosphorylated in 18 serine, threonine and tyrosine residues and each of the major functional domains contains at least one phosphorylation site (Figure 1.9). The NTD contains the majority of the phosphorylation sites whilst the DBD and hinge region harbour only one site each (Ser-578 and Ser-650 respectively). The LBD contains two phosphorylation sites. Each phosphorylation site is reported to have individual functional consequences by increasing or decreasing protein interactions that occur proximal to the phosphosite.(100)

Figure 1.9 Location of androgen receptor phosphorylation sites (111)



The location of serine (S), tyrosine (T) and threonine (Y) phosphorylation sites in relation to the major structural domains of the androgen receptor are shown. DBD = DNA binding domain, AF = activating function.

The majority of the phosphorylation sites on the AR correspond to serine residues (Figure 1.9). Dependent upon the phosphosite concerned the AR can be phosphorylated in the presence and/or absence of androgens. Phosphorylation of AR at serine residues influences receptor stabilisation and proteasomal degradation.(112) AR phosphorylation may also influence transactivation of the AR since AR transcriptional activity correlates strongly with phosphorylation of specific serine residues.(113) Therefore AR serine phosphorylation may influence the development and progression of AR-dependent prostatic diseases such as prostate cancer and BPH.

Serine site 81 is the most commonly phosphorylated site in response to androgens on the AR.(114) It has been postulated to have a role in AR transactivation. When LHS nontumorigenic immortalized human prostate epithelial cells were transfected with wild type and S81A mutant AR, cell growth rate was increased 15% in the wild type cells.(115) The effect of Ser-81 phosphorylation on AR transcriptional activity was studied in the same transfected LHS cells. From 11 AR target genes TMPRSS2 and ORM1 showed reduced transcription in S81A mutants when compared to wild type AR cells.(115) However, the remainder of the AR target genes either showed no change or increased expression in S81A mutants compared to wild type AR. In support of this finding loss of Ser-81 phosphorylation led to a reduction in AR transactivation of PSA and TMPRSS2 in prostate cancer LNCaP cells using a ligand switch model.(116) This data suggests that AR Ser-81 phosphorylation may regulate AR promoter selectivity. In addition the reduction in growth of S81A mutants in comparison to the wild type AR LHS cells may be related to overall changes in the AR transcriptional program. Multiple studies have also linked AR phosphorylation at Ser-81 to nuclear localisation of AR.(116-119)

Evidence suggests that Ser-94 is constitutively phosphorylated; Ser-94 phosphorylation was present in Human Embryonic Kidney 293 cells (HEK 293) when the LBD truncated AR was expressed.(118) Ser-94 phosphorylation has been shown to increase with cytoplasmic localisation.(120) The function of Ser-94 phosphorylation has not been previously investigated, however when both S81 and S94 alanine mutants were employed in HEK 293FT cells AR transcriptional activity was not altered.(121,122)

The functional consequences of AR Ser-213 phosphorylation are postulated to be dependent upon the activating kinase. Interestingly Ser-213 phosphorylation by Akt has been shown to have differing effects on AR transcriptional activity in different prostate cancer cell lines corresponding to different stages of disease. When AR was transfected into the castrate resistant prostate cancer cell line DU145, AR transactivation was repressed by Akt treatment.(123) However in androgen dependent prostate cancer LNCaP cells, Akt mediated AR phosphorylation increased AR transcriptional activity and promoted cell survival.(123,124) Negative regulation of AR signalling has been demonstrated by the AR regulated gene hematological and neurological expressed 1 (HN1). In LNCaP cells HN1 overexpression resulted in a reduction of AR Ser-213 phosphorylation corresponding to an overall decrease in AR expression levels.(125) The reverse was also observed in HN1 knockdown cells resulting in heightened Ser-213 phosphorylation and increased AR expression levels.(125) PIM-1 kinase mediated phosphorylation at Ser-213 has been shown to enhance AR interaction with Mdm2 and promote protein destabilisation and proteolysis.(126) Furthermore in AR knockdown LNCaP cells re-expression of S213A mutant increased cellular stability when compared to wild type AR.(123) PIM-1L isoform has been shown to increase expression of AR target genes under low androgen

conditions via Ser-213 phosphorylation.(127) Conversely PIM-1S mediated AR Ser-213 phosphorylation has been shown to decrease AR target gene expression under high androgen conditions.(128) In 68 matched hormone sensitive and CRPC clinical specimens a significant increase in AR phosphorylation at Ser-213 was seen with the development of CRPC.(129) In addition high expression of Ser-213 phosphorylation in the CRPC cohort was associated with decreased survival from biochemical relapse and a three year reduction in disease specific survival.(129)

Ser-515 phosphorylation is associated with AR transactivation as demonstrated by the S515A mutant which had reduced transcriptional activity in assays using a PSA reporter.(130,131) In addition AR Ser-515 phosphorylation may regulate protein turnover as wild type AR was seen to recruit the E3 ligase Mdm2 to the PSA promotor whilst S515A mutants recruited the E3 ligase CHIP. Both ligases result in ubiquitination, however Ser-515 phosphorylation status appears to direct which E3 ligase is utilised to regulate AR activity.(131)

Previous mutagenesis studies investigated the effect on subcellular localization of AR in a fibroblast-like cell line derived from monkey kidney (COS cells). This work demonstrated that in wild type cells AR is distributed between the nucleus and cytoplasm indicative of nuclear-cytoplasmic shuttling.(130) However in S578A mutant cells AR expression was found exclusively in the nucleus and associated with Ku70/80 regulatory subunits of DNA-PK which may have implications for AR transactivation.(130)

AR phosphorylation at Ser-515 and Ser-578 has been proposed to be linked. The S578A and S515A mutants both reduced AR transactivation, with the greatest effect seen with S578A.(130) The double S515/S578A mutant had almost no

transcriptional activity.(130) It is therefore postulated that AR phosphorylation at Ser-578 is dominant to that at Ser-515.

Ser-650 is located in the hinge region in close proximity to the nuclear export signal contained within the DBD. Phosphorylation at Ser-650 has been shown to be required for nuclear export of the AR via mutant studies.(132,133) Ser-650 phosphorylation is therefore a negative regulator of AR gene transcription.

As discussed above AR serine phosphorylation may have exciting implications for the development and progression of AR-dependent prostatic diseases. Although extensively studied in cell lines the significance of AR serine phosphorylation in prostatic disease has not been previously investigated in clinical specimens relating to hormone naïve prostate cancer and BPH.

1.5 Kinases associated with AR serine phosphorylation

As discussed above AR serine phosphorylation is influenced by kinases in the presence/absence of androgens. Kinases may be mutated or deregulated in disease resulting in alterations in phosphorylation status and downstream gene transcription. The kinases driving AR serine phosphorylation are therefore of functional importance in both prostate cancer and BPH and may harbour opportunities for therapeutic intervention.

Candidate kinases of AR serine phosphorylation include members of the cyclin dependent kinase (Cdk) family, extracellular signal-regulated kinases (ERK), protein kinase C (PKC) and Akt.

1.5.1 Cdk1

There are 11 classical Cdks that regulate the cell cycle. Cdk1-6 are involved in cell cycle progression and Cdk7-11 act as transcriptional regulators. Cdk1 is essential for progression from G2 phase into the mitotic phase of the cell cycle. Cdk1 binds to cyclin B1 which activates the kinase forming a complex upon which progression into mitosis is critically dependent. Further activation of the Cdk1/Cyclin B1 complex is via phosphorylation of Cdk1 threonine 161 site by the CDK activating kinase.

Cdk1 has been linked with prostate cancer previously.(134,135) Cdk1 has been shown to inhibit the tumour suppressor forkhead transcription factor FOXO1, promoting cellular proliferation and survival in prostate cancer LNCaP cells.(135) In addition Cdk1 has been shown to phosphorylate the AR at Ser-81.(136) Co-transfection of AR and Cdk1 into HEK 293T cells resulted in increased Ser-81 phosphorylation of the AR and total AR protein expression levels.(136) The use of the pan-Cdk inhibitor roscovitine blocked DHT-stimulated Ser-81 phosphorylation and decreased AR expression in both LNCaP cells and AR transfected HeLa cells.(136) Interestingly when HEK 293T cells were co-transfected with wild type or S81A mutant AR alongside active or inactive Cdk1, Cdk1 enhanced AR expression in both wild type and S81A mutants.(136) This suggests that Cdk1 can influence AR protein expression by a mechanism independent of Ser-81 phosphorylation. Ser-81 phosphorylation has also shown to be influenced by Cdk5 and Cdk9.(115,117)

Cdk1 has been shown to increase AR stability by phosphorylation at other serine sites.(136) Ser-515 phosphorylation, although not previously linked with Cdk1 has been associated with Cdk7. Purified recombinant TFIIH (general

transcription factor), of which CDK7 is an essential component, phosphorylated the AR in vitro and phosphorylation was reduced when wild type AR was replaced with a S515A mutant.(131) This suggests that CDK7 may play a role in phosphorylation of the AR on Ser-515.

1.5.2 ERK1/2

ERKs form part of the classical mitogen-activated protein kinases (MAPKs) group and are widely expressed protein kinase intracellular signalling molecules. Signalling generally follows a three-tiered kinase cascade, members of the Raf family are activated by the Ras family of GTPases. These activated Raf members then translocate to the cell membrane where they further phosphorylate and activate MEK1/2. MEK1/2 then continues to phosphorylate and activate ERK1/2. Activation of ERK1/2 has been shown to increase the transcription of AR target genes via phosphorylation of AR or its cofactors.(137-140)

ERK1/2 has previously been shown to be associated with poor prognosis in prostate cancer patients. A rise in total ERK1/2 expression in CRPC compared to hormone sensitive clinical specimens was associated with a decrease in survival from biochemical relapse and translated to an overall reduction in disease specific survival.(141) In the same previous study ERK1/2 expression strongly correlated with pERK1/2 expression.(141) With regards to AR phosphorylation, ERK1/2 has been shown previously to phosphorylate AR at Ser-515.(130) Following alanine/phenylalanine mutational study of candidate AR serine, threonine and tyrosine phosphorylation sites Ser-515 was identified as the site of AR phosphorylation in response to EGF treatment.(130) Furthermore the treatment with a MEK1/2/5 inhibitor (U0126) was seen to reduce Ser-515

phosphorylation after treatment with EGF.(130) Ser-650 phosphorylation has previously been observed in response to EGF treatment (114), suggesting that MAPKs may also be involved in regulating this phosphosite. However, further investigation demonstrated that Ser-650 phosphorylation is likely primarily influenced by stress kinases; MKK4/JNK and MKK6/p38 and not the MEK/ERK pathway.(132) Moreover, treatment of LNCaP cells with U0126 had no effect on Ser-650 phosphorylation.(132)

1.5.3 PKC

PKC isozymes are a family approximately 15 serine/threonine kinase enzymes. They are divided into 3 subgroups based on their structural and biochemical properties; conventional, novel and atypical. Conventional PKCs require calcium, diacylglycerol and a phospholipid for activation. Novel PKCs require diacylglycerol but do not require calcium and atypical PKCs require neither diacylglycerol nor calcium. PKCs then undergo further activation by phosphorylation upon which PKC proteins are translocated to the plasma membrane by receptors for activated C kinase (RACK) proteins. Following membrane translocation the opening of the isozyme allows binding of ATP and phosphorylation of various substrates. PKCs have a central role in cellular signaling transduction involved in cell proliferation, differentiation, apoptosis and angiogenesis. PKC isoforms have been detected in normal, BPH and neoplastic prostate tissue.(142-144) PKC has been previously shown to promote the proliferation of human prostatic stromal cells, which may play a role in BPH development and/or progression.(145) Deregulation of PKC signalling has been reported in many cancers including prostate and is a target of therapeutic intervention within the context of clinical trials.(146) Expression of PKC, along with its transcriptional target c-Jun, has previously been associated with

decreased survival from biochemical relapse in CRPC clinical specimens.(144) In the same cohort an increase in PKC expression between hormone sensitive and CRPC was associated with decreased survival from biochemical relapse.(144)

PKC is the putative kinase for AR phosphorylation at Ser-578. Site directed mutagenesis of Ser-578 in castrate resistant prostate cancer cell lines demonstrated that PKC-dependent AR phosphorylation was reduced on average by 50% when compared to wild type cells.(130) In addition ligands such as epidermal growth factor have been shown to increase AR transcriptional activity and cell growth via PKC dependent Ser-578 phosphorylation.(130)

1.5.4 Akt

Akt comprises three family members (Akt 1, 2 and 3) and are activated by the PI3K cascade which recruits Akt to the plasma membrane inducing a conformational change which results in its activation. Phosphorylation of Akt at threonine and serine residues increases its stability and level of activation. Activated Akt translocates to the cytoplasm and the nucleus where it acts as a critical mediator of downstream signal transduction cascades. Akt has been previously shown to regulate cellular proliferation, survival, angiogenesis and invasion. Akt1 dysregulation has been frequently implicated in prostate cancer via loss of PTEN and mutations in the catalytic subunit of PI3K, p110α.(79,147,148) High expression levels of activated Akt (phosphorylated at serine 473) have previously been associated with low expression of PTEN in clinical prostate cancer specimens.(79) Although not quite reaching significance ($p=0.058$), previous work has shown that an increase in activated Akt expression in primary prostate cancer tumour samples trended towards an association with shorter overall survival.(149) In addition an increase in activated Akt expression

between matched hormone sensitive and CRPC clinical specimens was associated with a significant reduction in survival from biochemical relapse and disease specific survival.(129,149)

Akt has been shown to phosphorylate the AR at Ser-213. Transfection of wild type and S213A AR mutants into DU145 cells showed a significant increase of phosphorylation at Ser-213 in response to Akt treatment in the wild type cells compared to the mutants.(123) In addition the use of immunocomplex kinase assays showed that phosphorylation of AR was significantly increased in response to Akt in wild type versus S213A mutants.(124) Furthermore, co-immunoprecipitation revealed that activated Akt specifically associated with endogenous AR in LNCaP cells.(123) In human prostate cancer tissue immunohistochemical analysis of activated Akt was associated with poor prognosis (149) and AR phosphorylation at Ser-213 expression showed a strong correlation in expression levels in hormone refractory prostate cancer tissue.(129)

The candidate kinases influencing AR serine phosphorylation have been previously identified and investigated in mutagenesis cell line studies, however this has not been translated into prostatic tissue specimens. In addition the kinases mediating AR serine phosphorylation in BPH have not been previously studied. The kinases driving clinically significant AR serine phosphorylation in prostatic disease therefore require further investigation within BPH and prostate cancer clinical specimens and may have implications for therapeutic intervention.

1.6 Statement of research aims

The overarching aim of this thesis was to determine the prognostic and predictive significance of AR serine phosphorylation, and the candidate kinases driving such phosphorylation, in patients with BPH and prostate cancer. It was hypothesised that AR phosphorylation by candidate kinases could prognosticate and predict response to treatment for these diseases. Therefore to investigate this hypothesis we aimed to:

1. Establish and verify the specificity of a panel of phosphospecific AR antibodies.

2. Determine the clinical significance of AR serine phosphorylation sites, and the candidate kinases driving such phosphorylation, in a pilot cohort of hormone naïve prostate cancer.

3. Further investigate clinically significant AR phosphorylation sites, and associated kinases, identified in the pilot prostate cancer cohort in a cohort of prostate cancer patients treated by active surveillance.

4. Verify the results of the AR phosphorylation sites and candidate kinases in the first active surveillance cohort in a second prospectively collected cohort of prostate cancer patients treated by active surveillance.

5. Determine the clinical significance of AR serine phosphorylation sites, and the candidate kinases driving such phosphorylation, in a cohort of benign prostatic hyperplasia patients.

2 Materials and Methods

2.1 Patients

Four patient cohorts were established as described below as part of this proof of concept study.

2.1.1 Pilot prostate cancer cohort

Ninety patients with hormone-naïve prostate cancer and tissue samples available for analysis were recruited from Glasgow Royal Infirmary between 1992 and 2002. Last date of follow up was 11/01/2012. Paper and electronic medical records were reviewed for each patient and an anonymised database was created comprising of clinical, biochemical and pathological information. Patients gave written consent for the use of their clinical information and tissue and West of Scotland Research Ethics Committee approved the study (reference: 05/S0704/94).

2.1.2 Clinical outcome measures pilot prostate cancer cohort

The clinical outcome measures and rational for the pilot prostate cancer cohort were:

1. Time to biochemical relapse

   This was measured from the time of diagnosis to the time of biochemical relapse as evidenced by rising serum PSA level dependent on treatment; radical prostatectomy serum PSA >0.2ng/ml, radical radiotherapy serum PSA of 2.0ng/ml above the post treatment nadir level, hormone treatment 2-3 consecutive rises in serum PSA levels above the nadir

obtained at intervals of >2 weeks.(150,151) This end point is equivalent to progression/disease free survival. This is important in prostate cancer for two reasons; 1. for the majority of patients who have undergone primary treatment with the aim of cure this is the point at which their disease becomes incurable, 2. in patients who have undergone hormone treatment this is the point at which their disease becomes castrate resistant, with a mean life expectancy of 18-24 months. Therefore patients who experience biochemical relapse are more likely to die from prostate cancer and therefore may harbour more aggressive disease at diagnosis.

2. Disease specific survival.

   This was measured from the time of diagnosis to the time of death where "prostate cancer" was recorded as a primary or secondary cause of death on the official death certificate. This is an important end point as it is a major goal of cancer treatment and is of direct benefit to patients. If patients can be identified at diagnosis as being high risk for death from prostate cancer any delay in potentially curative treatment, e.g. via active surveillance, can be avoided.

2.1.3 Retrospective active surveillance prostate cancer cohort

One hundred and twelve consecutive active surveillance patients were identified from the Greater Glasgow and Clyde urology multidisciplinary team (MDT) records between May 2005 and March 2010. Ninety patients had clinical information available and of these 51 had diagnostic tissue available and were thus eligible for inclusion in the study. Paper and electronic medical records

were reviewed for each patient and an anonymised database was created comprising of clinical, biochemical and pathological information.
West of Scotland Research Ethics Committee approved the study (reference: 12/WS/0087).

2.1.4 Prospective active surveillance prostate cancer cohort

One hundred and four consecutive active surveillance patients were recruited prospectively, following tissue diagnosis, in NHS Ayrshire and Arran between 13/11/1998 and 17/03/2011. All patients had clinical information available and 84 had diagnostic tissue available and were thus eligible for inclusion in the study. Paper and electronic medical records were reviewed for each patient and an anonymised database was created comprising of clinical, biochemical and pathological information.

West of Scotland Research Ethics Committee approved the study (reference: 12/WS/0087).

2.1.5 Clinical outcome measures active surveillance cohorts

The clinical outcome measures and rational for the retrospective and prospective active surveillance cohorts were:

1. Time to treatment intervention

   This was measured from the time of diagnosis to the time of treatment intervention. This end point is deemed important in active surveillance prostate cancer patients as it likely represents disease progression (biochemical, clinical, radiological).

2. Time to development of metastases.

   This was measured from the time of diagnosis to the time of detection of local or distant metastases usually via radiological imaging. This end point relies on clinical follow up and detection, therefore accuracy in relation to precise timing is lost. However this end point is deemed important in prostate cancer patients treated by active surveillance as it is a marker of disease progression and occult aggressive disease. If these patients with occult aggressive disease can be identified at diagnosis they can undergo immediate radical treatment and avoid delay via active surveillance.

2.1.6 Benign prostatic hyperplasia cohort

Six hundred and seventy eight consecutive patients with histological evidence of BPH diagnosed on transurethral resection (TUR) of prostate specimens between 01/01/1996 and 31/12/2005 were identified from the north Glasgow pathology archives. Of these patients 336 had clinical information and diagnostic tissue available and were thus eligible for inclusion in the study. Paper and electronic medical records were reviewed for each patient and an anonymised database was created comprising of clinical, biochemical and pathological information. West of Scotland Research Ethics Committee approved the study (reference: 11/AL/0214).

2.1.7 Clinical outcome measures benign prostatic hyperplasia cohort

The clinical outcome measures and rational for the benign prostatic hyperplasia cohorts were:

1. Time to postoperative acute urinary retention (AUR) >30 days post TUR

   This was measured from 31 days post TUR of prostate operation to time of AUR. AUR was defined as a sudden and painful inability to void requiring a urinary catheter to be inserted. This was an important end point as if high risk patients were identified immediately following TUR then prophylactic medical treatment could be instituted and emergency hospital attendances could be avoided as well as the risks to patients and costs to the health service.

2. Time to failure of surgical management

   This was measured from the time of TUR of prostate operation to the time of prescription of an alpha blocker and/or 5-alpha reductase inhibitor postoperatively. This was also deemed an important end point as if AR serine phosphorylation was found to be significant it could provide a rational for undertaking a prostate biopsy at first assessment in all BPH patients in order to identify those who would most benefit from operative management. It would allow better patient selection for TUR and avoid the anaesthetic and surgical risks in those patients who are unlikely to benefit symptomatically from such a procedure.

3. Time to reoperation.

   This was measured from the time of original TUR operation to the time of second TUR operation if it occurred. This was deemed important because those patients at risk of reoperation may have prophylactic medical therapy instituted immediately postoperatively. In addition if a diagnostic prostate biopsy was undertaken then it may inform the surgical technique, e.g. more tissue may be resected than in a patient who was not high risk for reoperation, or a total prostatectomy may be performed.

2.2 Prostatic tissue preparation

Areas of prostate cancer or BPH were identified and marked by a consultant uropathologist on haematoxylin and eosin (H&E) stained sections of the diagnostic prostatic tissue specimens for all 4 cohorts.

2.2.1 Tissue micro array construction

Tissue micro arrays (TMAs) were constructed for the pilot prostate cancer cohort and the BPH cohort. TMAs allow rapid tissue processing of large numbers of samples under standardised conditions. Following marking of the H&E stained sections the corresponding paraffin embedded tissue blocks were retrieved from the pathology archives. Three 0.6mm^2 cores of tissue were then removed from the areas of interest identified (marked by a uropathologist) in each block. Recipient array blocks were constructed in triplicate in order to account for heterogeneity of the prostatic tissue. Three micrometer thick sections were then cut from the TMA blocks using a Leica RM 2135 microtome. The sections were then floated in a water bath heated to 45^{o}C in order to allow the tissue to

flatten out. The sections were then applied to Superfrost Plus microscope slides (Fischer Scientific, Loughborough, UK). After drying in an oven overnight at 56^{o}C the slides were stored at 4^{o}C.

2.2.2 Tissue section preparation

The diagnostic tissue for the active surveillance cohorts was obtained via TUR of prostate operations and trans rectal ultrasound (TRUS) guided prostate biopsies. The tissue obtained from TRUS biopsy has an average diameter of 0.866mm and therefore was insufficient for TMA construction. Following marking of the H&E stained sections the corresponding paraffin embedded tissue blocks were retrieved from the pathology archives. Three micrometer thick sections were cut from each block and prepared as per section 2.2.1. The marked H&E section was retained for reference in order to perform the analysis.

2.3 Inflammatory scoring systems

Assessment of the local and systemic inflammatory response was undertaken on original H&E stained tissue sections. The sections were reviewed by a pathologist and the best representative areas of BPH were marked. Two independent observers graded the local inflammatory response and extent of tissue necrosis and any differences in results were settled by discussion.

2.3.1 Systemic inflammatory response

The modified Glasgow prognostic score (mGPS) is an assessment of the preoperative systemic inflammatory response. The mGPS comprises a combination of serum albumin and C-reactive protein (CRP).(152) Routine laboratory measurements of albumin and CRP were recorded preoperatively.

Patients with both a raised CRP level (more than 10 mg/l) and hypoalbuminaemia (below 35g/l) were allocated a score of 2. Those in whom neither of these abnormalities was present were allocated a score of 0. Patients with a raised level of CRP alone were scored 1, whereas those with hypoalbuminaemia alone were scored 0.

2.3.2 Local inflammatory response

The Klintrup-Makinen criteria is a well-established measure of the inflammatory cell infiltrate at a local level.(153) The Klintrup-Makinen criteria involved the margin of each marked area of BPH being scored on a four point scale. A score of 0 indicated absence of inflammatory cells; score 1 denoted a mild or patchy presence of inflammatory cells, score 2 a prominent inflammatory reaction, and score 3 a florid 'cup-like' inflammatory infiltrate.

2.3.3 Tissue necrosis

The extent of tissue necrosis was graded with 0 when no necrosis was present; a score of 1 when there was <25% necrosis, a score of 2 when necrosis was 25–50%, and 3 for necrosis of >50%. Necrosis related to haemorrhage and foci of hyalinization were not considered.

2.4 Identification of candidate kinases mediating AR phosphorylation

Scansite 2.0 (http://scansite.mit.edu/) was utilised to identify the candidate kinases mediating AR phosphorylation.(154) Scansite searches for motifs within proteins that are likely to be phosphorylated by specific protein kinases. Optimal phosphorylation sites for each individual kinase are predicted using the matrix of selectivity values for amino acids at each position relative to the

phosphorylation site as determined from the orientated peptide library technique.(155,156) The search was conducted using the protein ID "ANDR_HUMAN" (Accession number: P10275).

2.5 In-vitro studies

An androgen sensitive human prostate adenocarcinoma cell line was utilised (LNCaP). LNCaP cells were originally derived from the left supraclavicular lymph node metastasis from a 50-year-old caucasian male in 1977.(157) They are the most clinically relevant prostate cancer cell line consisting of adherent epithelial cells which grown both in aggregates and as single cells. LNCaP cells are also known to express AR and respond to androgen stimulation.(157) This was key to their selection for use within this study.

2.5.1 Culturing prostate cancer cells

LNCaP cells (ATCC) were routinely maintained in RPMI 1640 (Invitrogen) containing phenol red and supplemented with 10% foetal calf serum (Invitrogen, UK) and 2mM L-glutamine. LNCaP cells were grown in T-75 flasks in an incubator with conditions set to 5% CO_2 and 37^{o}C. The medium was changed twice per week and the flasks were split when the cells reached 70-80% confluence.

2.5.2 Trypsinisation of cells

LNCaP cells were routinely spilt 1:6 by trypsinisation when around 70-80% confluent. This was in order to maintain the monolayer growth pattern and prevent formation of cell towers due to overcrowding. The medium was removed from the flasks and the cells were washed twice in warmed PBS (Invitrogen) in order to remove all traces of medium. The cells were then

incubated in 3ml of trypsin (Invitrogen) for 5min in 5% CO_2 at 37°C. Once the cells had detached from the flask a further 3ml of RPMI was added in order to neutralise the trypsin. The cells were disaggregated from their clusters by gentle pipetting and then reseeded 1:6 into new flasks containing 10ml of RPMI. Cells were then incubated in 5% CO_2 at 37°C without disruption for up to 48h in order to allow reattachment to the bottom of the flask.

2.5.3 Cell treatments

The response of protein phosphorylation status in LNCaP cells to extracellular stimuli and inhibitors was measured.

2.5.3.1 Dihydrotestosterone

Dihydrotestosterone (DHT) is a potent androgen which binds to the androgen receptor resulting in post translational modifications, including phosphorylation, ultimately leading to gene transcription. Cells were treated with 10nM DHT diluted in dimethyl sulphoxide (DMSO) for 5, 10, 15 and 30min.

2.5.3.2 Roscovitine

Roscovitine (Cell Signaling) is a selective inhibitor of Cdks (Cdk1, 2 and 5). Cells were treated with 20μM Roscovitine diluted in PBS for 24h.

2.5.4 Time course stimulation and inhibitor treatments of LNCaP cells

Following trypsinisation cells were seeded at $4x10^5$ cells/ml/well in 6 well plates. Each well was made up to a total volume of 2ml with RPMI. After 72

hours the cells were incubated in serum free RPMI overnight. The appropriate treatments were added to each well along with a vehicle (containing diluent only) and an untreated control well. The treatments were applied for the appropriate times in 5% CO_2 and at 37^{o}C as documented above. All treatments were applied identically and in triplicate in three different passages of the cell line. All plates were put on ice and the cells were removed using a cell scraper. Care was taken to ensure the cell scraper was washed well between wells to avoid contamination. The cells were transferred to labelled 15ml tubes, kept on ice, using disposable pastettes. Each well was then washed in 1-2ml of ice cold PBS and the wash was transferred to the appropriate 15ml tube. The 15ml tubes were then centrifuged at 1400rpm for 10min until a pellet was formed. The supernatant was carefully removed and the pellet was resuspended in 1ml ice cold PBS and then vortexed thoroughly in order to wash the cells. The cell suspension was then transferred to a labelled eppendorf. The eppendorfs were centrifuged at 1600rpm for 10min until a pellet was formed. The supernatant was carefully removed and the cells were lysed in 150µl of Radio Immuno Precipitation Assay (RIPA) lysis buffer. The RIPA lysis buffer contained:

150 mM sodium chloride

1.0% NP-40

0.5% sodium deoxycholate

0.1% SDS (sodium dodecyl sulphate)

50 mM Tris, pH 8.0

1:100 protease inhibitor

The cells were mechanically sheared by passing them repeatedly through a needle syinge tip. The lysates were then stored short term at -20^{o}C and long term at -80^{o}C.

2.5.5 Time course treatments of LNCaP cells cell pellet formation

Cell lines were grown in T-75 flasks until 70-80% confluent, the medium was removed and the cells washed in warmed PBS. The cells were then incubated in serum free RPMI overnight. The following day, medium was removed and the cells washed in warmed PBS in preparation for the appropriate drug treatment. LNCaP cells were treated with Roscovitine (20μM) for 24h or DHT (10nM) for 3h. A vector flask (diluent only) and an untreated control were included in each experiment. Each flask was treated with 3mls inhibitor, stimulator or control, ensuring that all cells were completely covered, and incubated at 37°C in 5% CO2 for the required time. The cells were trypsinised and collected by centrifugation at 1200rpm. The supernatant was discarded and pellets were washed in HBSS. Cells were fixed in 4% formalin, briefly vortexed and rested at room temperature for 15 min. The cells were then centrifuged at 2500rpm for 3min, the supernatant discarded and then washed in HBSS. The cells were set in 1% agarose at 4^{o}C for 1h. Cell pellets were dehydrated through graded alcohol and xylene and embedded into paraffin blocks. Immunohistochemistry was performed as described (antigen retrieval reduced to 2.5 min) for protein expression.

2.6 Antibody Validation

Cross-reactivity of phospho-specific antibodies is a known issue. Therefore validation of the specificity of these antibodies is required by more than one method. In addition to the manufacturer's validation techniques, western blotting and peptide competition assays were performed on each phospho-specific antibody.

2.6.1 Western blotting

Western blotting was performed to confirm antibody specificity before their usage in immunohistochemical staining of the TMAs and tissue sections. Western or immunoblotting is a method by which a specific protein can be detected and quantified in tissue or cells. In brief this technique includes extracting protein samples from cultivated tissue or cells, separating the denaturated proteins by gel electrophoresis and transferring them to a PVDF membrane. The membrane is then exposed to a primary antibody specific to the protein of interest, followed by a secondary antibody recognising the antibody-antigen complex. To detect the proteins chemi-luminescent and chemi-fluorescent method is used. Western blotting was used to verify the specificity of all antibodies and to measure the quantity of protein in samples from time course and inhibitor studies.

2.6.1.1 Preparation of protein samples

Determination of concentration of protein samples

In order to maintain consistency the same amount of protein from each sample tested must be used. Therefore the protein concentration of each sample must be known before undertaking western blotting. The method utilised to achieve this is the Bio-Rad protein assay, based on the Bradford dye-binding procedure.(158) A colorimetric assay is used to measure the total protein concentration.

The protein samples were prepared as a low-concentration assay in disposable cuvettes (Gibo). A standard solution (200µl of Bio-Rad Reagent and 795µl dH_2O) was pipette into one cuvette, followed by 5µl of protein sample. The solution was mixed thoroughly with a pipette in order to ensure an accurate

concentration reading. Protein standards were prepared using Bovine Serum Albumin (BSA). Stock BSA (2mg/ml) was diluted with dH_2O to 1mg/ml. One reference sample (dH_20 only and Bio-Rad Dye) and seven protein standards (serial dilutions from 1-50µg/ml) were prepared in disposable cuvettes. The reference sample and protein standards were used to calibrate the spectrophotometer (Bio-Rad) applying the Protein 595 Assay program.
Optical density for the reference and the protein standards were measured at 595 nm. Optical density at 595 nm (O.D. 595) was then read for all other protein samples. The spectrophotometer calculated at that time the amount of protein (µg/ml) present in the sample, plotting a graph of absorbance at 595 nm against the protein concentration of the standard samples. This is the standard curve used to determine the protein concentration of the measured sample from its O.D. 595 value.

The initial protein concentration (µl/ml) was calculated from a diluted protein sample (1:200). The final protein concentration in mg/ml was calculated using the formula 'Protein reading (µl/ml) x 0.2 = Final protein concentration (mg/ml)'.
Proteins were aliquoted and stored at -80^{o}C until required. A standard 50µg of protein was used for western blotting, the volume of each sample required (µl) was calculated from the final concentration.

Protein denaturisation

Proteins were denatured to unfold them in order to allow the antibody access to the epitope. Denaturisation also improves the efficiency at which the proteins run through the gels. Having determined the protein concentration in each

sample an appropriate volume was removed from each sample and placed on ice in a new Eppendorf tube. 2x Laemmli's buffer at a ratio of 1:1 was added to each protein sample. 2x Laemmli's buffer contains:

4% SDS

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris HCl.

The ingredients in 2x Laemmli's buffer each have a specific purpose. SDS denatures proteins by 'wrapping round' the polypeptide backbone. SDS then provides a negative charge to the proteins by their attachment to SDS anions. SDS attaches to proteins at a mass ratio of 1.4:1. The unfolded proteins therefore become negatively charged rods with a charge consistently proportional to its length. The samples were then boiled at 100^{o}C on a preheated hotplate for 5min. 2-mercaptoethanol reduces disulphide bridges in proteins and helps to maintain the protein in its denatured state. Glycerol increases the density of the sample in order to maintain the loaded sample at the bottom of the well and prevent well overflowing and uneven gel loading. Bromophenol blue is a small ionic dye which migrates to the front of the protein mixture to be separated. It allows visualisation of the separation process. After mixing with Laemmli's buffer the samples were boiled for 5min at 100^{o}C on a preheated hotplate. This heat treatment contributes to the protein denaturisation process. The molecular weight marker (Biotinylated Protein Ladder –Cell Signaling Technology) that was used to determine the size of the detected protein was also boiled at 100°C for 5min. Following heating all samples were vortexed well and then immediately stored back on ice.

2.6.1.2 Preparation of SDS-PAGE Gels

The separation of proteins according to their molecular weight is routinely done by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Polyacrylamide gels are formed from the polymerization acrylamide and N,N-methylenebis-acrylamide (Bis). Bis is a cross-linking agent for the gels. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by the total amount of acrylamide (%T) and the amount of cross-linker (%C) present. As the total amount of acrylamide increases, the pore size decreases. In general a higher percentage of resolving gels are used for smaller proteins, and lower percentage gels are more effective for separating larger proteins. The percentage of the gel utilised must be carefully selected as this determines the rate of migration and degree of separation between proteins.
Throughout this study 1.0mm 4-12% Bis-Tris gels from NuPAGE® (Invitrogen) were utilised to maintain consistency.

2.6.1.3 Electrophoresis

As previously described SDS confers a negative charge onto the proteins. This negative charge allows the proteins to move through the gel as they are attracted to the positive anode. Proteins are separated by gel electrophoresis by their molecular weight. Small proteins travel much quicker through the acrylamide pores than large proteins. Therefore small proteins can be found much further down the gel than larger ones.

Gels were placed in the electrode assembly in a mini buffer tank and combs were removed. The tank was then filled with running buffer (Novamix 10x running buffer (Invitrogen, Life Technologies Ltd, Paisley, UK) diluted to 1x with

dH_20) until the whole gel complex was completely submerged. Denatured protein samples (50µg) and the molecular weight marker (10µl) were then carefully loaded into the wells using special gel loading tips. Tubulin (MW 55kDa) was used as a loading control to check that each lane was equally loaded with sample in each gel. Care was taken not to poke the well bottom with the tip as this can create a distorted band. In addition it was paramount that the wells were not overfilled as this could lead to spillage into adjacent wells and inaccurate data. Once all samples were loaded the gel was run at approximately 140V for approximately 90min.

2.6.1.4 Protein transfer

The proteins require to be transferred from the polyacrylamide gel to a PVDF (polyvinylidene difluoride) membrane. This process uses the same principle of electrical charge in order to induce the proteins to travel via an electrical field from a gel to a membrane that 'blots' the proteins from the gel. Due to the larger size of the proteins of interest (110-112kDa) wet transfer method was used in this study. PVDF membranes are soaked for 5min in 100% methanol prior to transfer. The gel was carefully separated from its plastic cover and the wells were cut away. The gel was equilibrated in transfer buffer in order to prevent shrinkage during the transfer process. The gel, membrane, sponges and 3M Whatmann paper (VWR) are then soaked in transfer buffer and assembled as a 'transfer sandwich'; sponge/ paper/ gel/ membrane/ paper/ sponge. All air bubbles were eradicated as they can prevent adequate transfer of protein to the membrane. Once this was complete the sandwich was clamped tightly together and locked in a cassette which was placed in the electrode assembly and positioned into the Mini-Trans Blot Cell tank (Bio-Rad Laboratories) which was

prefilled with transfer buffer. A Bio-Ice pack was added to the tank in order to keep the temperature down during transfer. A magnetic stirrer also maintained an even temperature and ion distribution within the tanks. An electrical charge was applied to transfer the proteins from gel (negative/cathode) to membrane (positive/anode) over 90min at 240mAmp.

2.6.1.5 Blocking membranes

Blocking the membrane is necessary in order to prevent non-specific background binding of the primary and/or secondary antibodies to the membrane. The membrane has a high capacity for binding proteins and therefore the same is true for antibodies. This was achieved by incubating the membrane in 5% non-fat dry milk (Marvel) for 1 hour on an orbital shaker. The orbital shaker was utilised for all future steps.

2.6.1.6 Incubation with primary antibody

The membrane was incubated with the primary antibodies diluted in 5% non-fat dry milk (Marvel) in order to reduce further non-specific binding. Membranes were incubated with 20ml antibody overnight at 4^{o}C in 50ml universal containers on a roller. The membrane was then washed thoroughly (6x 15min) in TBST (0.001% Tween-20 in TBS) at room temperature to remove residual primary antibody.

2.6.1.7 Incubation with secondary antibody

The membrane was incubated with the secondary antibody diluted in the appropriate blocking agent. Detection of the protein of interest required a secondary antibody bound to either biotin or an enzyme conjugate, such as

horseradish peroxidase (HRP), which was species-specific to the primary antibody. The secondary antibodies used were therefore HRP-linked anti-mouse IgG or anti-rabbit IgG (both Cell Signaling Technology). Each was diluted 1:10,000 in the appropriate blocking agent and incubated with the membrane on an orbital shaker for 90min at room temperature. The membrane was then washed thoroughly (6x 15min) in TBST (0.001% Tween-20 in TBS) at room temperature to remove residual secondary antibody.

2.6.1.8 Visualisation

Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film. Horse-radish peroxidase catalyzes oxidation of luminol, a chemiluminescent substrate, in alkaline conditions. Oxidation results in the luminol being in an excited state which then decays to ground state via a light emitting pathway. In order to perform this method ECL plus (Amersham, GE Healthcare Life Sciences, Little Chalfont, UK) was used. The following steps were performed in a dark room as ECL reagents are photo sensitive. The reagents, stored at 4^{o}C, were first warmed to room temperature. ECL reagent A was then mixed with reagent B at 1:1 ratio to a final minimum volume of 0.1ml/cm^{2} of membrane. The mixed detection reagent was then pipetted onto the membrane (protein side up) and incubated for 5min at room temperature. Excess detection reagent was blotted from the membrane and they were transferred to a fresh piece of Saran wrap, which it was then enveloped in. Finally the membrane was transferred to a film cassette where it was exposed to autoradiography film for various times. Generally the incubation times were 30 seconds and 1, 5, 15 minutes. The film

was developed using a Kodak X-OMAT x-ray processor and both the marker and protein bands visualised.

2.6.1.9 Stripping membranes

In order to confirm equal sample loading, the primary antibody was removed from probed membranes using Re-Blot Stripping buffer (Chemicon). The membrane was then washed in TBST (3x 15min) to remove excess antibody. Membranes were incubated in 20mls of stripping buffer (diluted 1:10 in dH_2O) at room temperature for 20min. The membranes were then blocked again in 5% Non-Fat Dry Milk/TTBS and re-probed with anti-αTubulin HRP linked antibody (1:1000 AbCam) to confirm equal protein loading.

2.6.2 Peptide competition assays

Peptide competition assays were performed to confirm antibody specificity for each AR serine phosphorylation site. pAR^{S213} (Protein sequence GRAREA(pS)GAPTSSKD raised in rabbit by EZbiolab Inc., Carmel, IN, USA) peptide was incubated at a ratio 1:1 for 1h with each antibody. pAR^{S81} (Protein sequence QQQQQQET(pS)PRQQ raised in rabbit by EZbiolab Inc., Carmel, IN, USA), pAR^{S94} (Protein sequence QQQQQGEDG(pS)PQAH raised in rabbit by EZbiolab Inc.), pAR^{S515} (Protein sequence MVSRVPYP-S(pS)-PTCV raised in rabbit by Eurogentec Ltd.), pAR^{S578} (Protein sequence ALTCG-S*(pS)*-CKVFFKR raised in rabbit by Eurogentec Ltd., Seraing, Belgium) and pAR^{S650} (Protein sequence EEGEASSTT(pS)PTEE raised in rabbit by EZbiolab Inc.) peptides were incubated at ratios of 1:1, 2:1, 500:1, 200:1 and 100:1, respectively, with each antibody overnight at 4°C. Immunohistochemistry was then performed as described in section 2.7.

2.7 Immunohistochemistry

Immunohistochemistry is a method by which a particular antigen within cells and/or tissue can be detected using a specific antibody. In general there are two methods by which immunohistochemistry can be conducted; direct and indirect. The direct method is the more simplistic, 'one step' staining method by which a marker attaches directly onto the antigen of interest. Indirect immunohistochemistry uses a secondary antibody which binds to the primary antibody which, in turn, is bound to the antigen of interest. Indirect immunohistochemistry is more sensitive than the direct method as it generates signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The method used in this study was the DAKO Envision+ dual link system-HRP. This is a two step staining procedure based on dextran polymer technology. Primary rabbit or mouse antibodies recognise and bind to the antigens of interest. The DAKO Envision detection reagent is a peroxidase-conjugated polymer which also carries antibodies to mouse and rabbit immunoglobulins. Following incubation with the Envision detection reagent 3,3, diaminobenzidine (DAB) is applied. The peroxidase molecules then react with the DAB which produces an insoluble brown precipitate which can be readily viewed via light microscopy.

2.7.1 Tissue preparation

Immunohistochemistry was conducted on 3μm sections taken from formalin fixed, paraffin embedded prostatic tissue blocks as described in sections 2.2.1 and 2.2.2. Sections were baked at 56^{o}C for 1h prior to use. Sections were then

placed in a slide rack and dewaxed in xylene (2 x 3min) and rehydrated through graded alcohol solutions; 100% (2 x 2min), 90% (1 x 2min) and 70% (1x 2min).

2.7.2 Antigen retrieval

Antigen retrieval is a necessary step in immunohistochemistry in order to unmask epitopes and allow antibody binding. Masking of epitopes often occurs during tissue fixation and can be caused by crosslinking of amino acids within the epitope, crosslinking of peptides near the epitope resulting in conformational change at the epitope or a change in the electrostatic charge of the antigen. Antigen retrieval is any method which reverses the masking process.

Two heat-based methods of antigen retrieval were utilised in this study. The first involved preheating 1l of buffer solution tris-EDTA pH8 (1mM EDTA (Sigma) and 5mM Tris (VWR)) or sodium citrate pH6 (10mM tri-sodium citrate and 2mM anhydrous citric acid), for 13.5min to 96^{o}C in a microwave. The tissue sections were then added to the preheated solution and cooked under pressure for 5min before being cooled for 20min. The second method involved incubating the tissue sections in approximately 50ml of DAKO high pH target retrieval solution (1:10 dilution) or tris-EDTA pH9 (0.25mM EDTA (Sigma) and 10mM Tris (VWR)) for 20min at 96^{o}C in a waterbath (Table 2.1).

Table 2.1 Antibody information

Protein	Antibody	Antibody Dilution	Antibody Incubation Conditions	Retrieval Method	Blocking Agent
Cdk1	Mouse Abcam ab18	1:400	1h room temperature	EDTA pH 8 5min under pressure	5% Horse Serum
pCdk1^{161}	Rabbit Abcam ab47329	1:500	Overnight 4°C	EDTA pH 8 5min under pressure	5% Horse Serum
pERK1/2	Rabbit Cell Signaling #9101L	1:8000	Overnight 4°C	EDTA pH 9 20min at 96°C	10% Casein
PKC	Rabbit Abcam ab59363	1:500	Overnight 4°C	Citrate pH 6 5min under pressure	5% Horse Serum
Ki67	Mouse Dako F0788	1:150	1h room temperature	Citrate pH 6 5min under pressure	5% Horse Serum
AR	Mouse Dako AR441	1:100	Overnight 4°C	High pH antigen retrieval solution 20min at 96°C	5% Horse Serum
pAR^{S81}	Rabbit Millipore # 07-1375	1:4000	Overnight 4°C	EDTA pH 8 5min under pressure	5% Horse Serum
pAR^{S94}	Rabbit Abcam ab62205	1:200	Overnight 4°C	EDTA pH 8 5min under pressure	5% Horse Serum
pAR^{S213}	Mouse Imgenex IMG-561	1:100	1h room temperature	EDTA pH 8 5min under pressure	5% Horse Serum
pAR^{S515}	Rabbit Eurogentec	1:500	1h room temperature	EDTA pH 8 5min under pressure	5% Horse Serum
pAR^{S578}	Rabbit Eurogentec	1:1000	Overnight 4°C	EDTA pH 8 5min under pressure	5% Horse Serum
pAR^{S650}	Rabbit Abcam ab47563	1:200	Overnight 4°C	EDTA pH 8 5min under pressure	5% Horse Serum

2.7.3 Blocking of non-specific staining

Peroxidase interacts with 3,3, diaminobenzidine and is therefore a potential source of background staining. Background staining was prevented by quenching endogenous peroxidase activity using 3% hydrogen peroxide for 10min followed by a wash in water. The formation of hydrophobic bonds between tissue proteins and immunoglobulins can result in non-specific binding of the primary/secondary antibody to the tissue. This also has the potential to result in background staining, so in order to counteract this the sections were incubated with 5% horse serum (vector) in TBS buffer (0.1M Tris/HCl, 1.5M NaCl, pH 7.5) or 10% casein in TBS buffer as per Table 2.1.

2.7.4 Incubation with primary antibody

Antibodies for the following proteins were used; Cdk1, pCdk1^{161}, pERK1/2, PKC, Ki67 (proliferation index), AR and AR phosphorylated at serine 81, 94, 213, 515, 578 and 650. The quality of staining produced was first optimised by serial alterations to antigen retrieval methods, blocking agents, antibody dilutions and incubation times and temperatures. The antibodies were diluted to the optimal concentrations in DAKO Antibody Diluent and incubated as shown in Table 2.1. A positive and negative isotype matched control was included in each immunohistochemistry run in order to ensure no false positive staining. The positive control confirmed that the antibody and method was working whilst the negative control checked for any non-specific antibody binding.

2.7.5 Incubation with secondary antibody

Following completion of incubation with the primary antibody the sections were washed in TBS buffer (2x 5min) and then incubated with the secondary antibody; DAKO Envision+ detection system. As previously described this uses a secondary antibody raised in goat attached to a dextran backbone upon which peroxidase molecules are also attached. The tissue was incubated with the secondary antibody for 30min at room temperature. Following this the sections were thoroughly washed in TBS (2x 5min).

2.7.6 Detection

The substrate chromogen used to detect the secondary antibody is DAB (Vector). A combination of 4 drops DAB, 2 drops hydrogen peroxide and 2 drops pH buffer were added to 5ml distilled water. The slides were then incubated for 10min at room temperature with the DAB solution until a brown coloured appeared, before being thoroughly washed in running water for a further 10min.

2.7.7 Counterstaining

The slides were counterstained with haematoxylin and Scott's tap water substitute. The slides were submerged in haematoxylin for approximately 30s before being dipped in acid alcohol in order to remove excess staining. The slides were then immersed for 30s in Scott's tap water substitute in order to produce a blue counter stain. The slides were then rinsed for 1min in running water.

2.7.8 Dehydration and mounting

The slides were then dehydrated through graded alcohol solutions; 70% (1x 1min), 90% (1x 1min) and 100% (2x 1min). Finally the slides were submerged in xylene (2x 1min) and mounted using DPX and glass coverslips.

2.8 TUNEL Assay

Apoptosis is a form of cell death which eliminates compromised or superfluous cells. Overall, apoptosis results in a well characterised process of change in cellular morphology including; shrinkage, chromatin margination, membrane blebbing, nuclear condensation, segmentation and then division into apoptotic bodies which may be phagocytosed. These characteristic apoptotic bodies are short-lived and minute, and can resemble other cellular constituents when viewed under the light microscope. In addition, DNA fragmentation in apoptotic cells is followed by cell death and removal from the tissue, usually within several hours. A rate of tissue regression of 25% per day can result from apparent apoptosis in only 2-3% of the cells at any one time. Therefore, the quantitative measurement of an apoptotic index by morphology alone can be difficult. DNA fragmentation is usually associated with ultrastructural changes in cellular morphology in apoptosis. The Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay is a method by which to examine DNA fragmentation during apoptosis. The ApopTag® In Situ Apoptosis Detection Kit (Millipore) was used to detect DNA strand breaks by enzymatically labeling the free 3'-OH termini with modified nucleotides. These new DNA ends that are generated upon DNA fragmentation are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative

nuclei, which have relatively insignificant numbers of DNA 3'-OH ends, usually do not stain with the kit. Apoptosis is distinct from accidental cell death (necrosis). The ApopTag® kit distinguishes apoptosis from necrosis by specifically detecting DNA cleavage and chromatin condensation associated with apoptosis. However, it is accepted that there may be some instances where cells exhibiting necrotic morphology may stain lightly or, rarely, DNA fragmentation can be absent or incomplete in induced apoptosis.

2.8.1 Rehydration of tissue

Briefly, tissue sections were dewaxed in xylene (2x 3min) and rehydrated in graded alcohol solutons; 100% (2x 2min), 90% (1x 2min) and 70% (1x 2min). The tissue sections were then washed in tap water for 2min.

2.8.2 Pre-treatment of tissue

Proteinase K solution was prepared to a concentration of 20µg/ml in PBS. The solution was then incubated with slides for 25min at room temperature. Slides were then washed twice in dH_2O for 2min.

2.8.3 Quenching of endogenous peroxidase activity

Endogenous peroxidase activity was quenched with 3% H_2O_2 5min at room temperature. The slides were then washed twice in dH_2O for 5min.

2.8.4 Application of equilibriation buffer

Excess liquid was tapped off and the slides were blotted dry. The slides were then incubated with equilibration buffer at 25^{o}C for 1h.

2.8.5 Application of terminal deoxynucleotidyl transferase enzyme

Terminal deoxynucleotidyl transferase (TdT) enzyme was diluted to working strength in reaction buffer (70% reaction buffer: 30% TdT) and mixed well by vortexing. The slides were incubated in a humidified chamber at 37°C for 1h.

2.8.6 Application of stop/wash buffer

Working strength stop/wash buffer was prepared by diluting the stop/wash buffer 1:35 with dH_2O. Slides were then washed with working strength stop/wash buffer for 10min at room temperature. Slides were then washed in TBS (3x 1min) at room temperature.

2.8.7 Application of anti-digoxigenin conjugate

An aliquot of anti-digoxigenin conjugate was removed from the stock vial and brought to room temperature. Excess liquid was tapped and then blotted from the slides before incubating with antidigoxigenin conjugate for 30min at 25°C in a humidified chamber. Slides were washed in PBS at room temperature (4x 2min).

2.8.8 Detection

The substrate chromogen used is DAB (Vector). A combination of 4 drops DAB, 2 drops hydrogen peroxide and 2 drops pH buffer were added to 5ml distilled water. The slides were then incubated for 10min at room temperature with the DAB solution until a brown coloured appeared, before being thoroughly washed in running water for a further 10min.

2.8.9 Counterstaining

The slides were counterstained with haematoxylin and Scott's tap water substitute. The slides were submerged in haematoxylin for approximately 30s before being dipped in acid alcohol in order to remove excess staining. The slides were then immersed for 30s in Scott's tap water substitute in order to produce a blue counter stain. The slides were then rinsed for 1min in running water.

2.8.10 Dehydration and mounting

The slides were then dehydrated through graded alcohol solutions; 70% (1x 1min), 90% (1x 1min) and 100% (2x 1min). Finally the slides were submerged in xylene (2x 1min) and mounted using DPX and glass coverslips.

2.9 Analysis of protein expression

Following immunohistochemical staining of prostatic tissue it was necessary to analyse the staining level using a semi-quantitive scoring method.

2.9.1 Digitalisation of slides

Slides stained by immunohistochemistry were scanned using a Hamamatsu NanoZoomer (Hertfordshire, UK). Visualisation of slides on a computer monitor was carried out using the Slidepath Tissue IA system version 3.0 (SlidePath's Tissue IA system, Dublin, Ireland).

2.9.2 Histoscoring

Tissue staining intensity was scored visually on a computer monitor by two blinded independent observers using a semi-quantitative weighted histo-score (H-score) method.(159,160) Staining in the nucleus and cytoplasm of epithelial cells was evaluated. In the stromal compartment nuclear staining of smooth muscle cells and stroma (fibroblastic) cells was evaluated. The intensity of staining was assessed and graded as negative (0), weak (1), moderate (2) and strong (3) staining. The percentage of tumour/BPH cells within each category was then estimated and a histoscore was calculated using the following formula: 0x (% negative tumour cells) + 1x (% of cells staining weakly positive) + 2x (% of cells staining moderately positive) + 3x (% of cells staining strongly positive). Therefore the histoscore ranged from zero (minimum) to 300 (maximum). Results were considered discordant if scores differed by more than 50 between observers. These cases were re-evaluated by both observers and settled by discussion. In addition both intra-(variation in individual scoring) and inter-(variation between two observers) class correlation coefficients were calculated. Agreement between observers was considered excellent if the ICCC value was ≥0.80 (an ICCC of 1 indicates identical scoring). Bland Altman plots were constructed to ensure there was no bias between scorers. The mean histoscore from staining conducted in triplicate was used for analysis. Protein expression levels were subsequently divided into low (≤median) and high expression (>median) for further statistical analysis.

2.9.3 Automated Ki67 and TUNEL Scoring

The Ki-67 antigen is a nuclear protein which, during interphase, can be exclusively detected within the nucleus, whereas in mitosis most of the protein

is relocated to the surface of the chromosomes.(161) The Ki-67 protein is expressed in all proliferating cells during late G1, S, M and G2 phases of the cell cycle while cells in the G0 (non-cycling cells) phase consistently lack the Ki-67 antigen. In diagnostic histopathology and cell biology numerous studies have shown the use of measuring the Ki-67 labelling index or growth fraction in various solid tumours and proliferative disorders using immunohistochemistry.(161)

Digitised slides were accessed via the Slidepath Image Analysis system and further evaluated for Ki67 and TUNEL cell counts using the program's nuclear scoring algorithm. This quantifies nuclear staining within individual cores and derives a counting score for each target area. Nuclei stained brown with DAB and/or blue with haematoxylin are identified. These nuclei are then separated by a thresholding and segmentation algorithm. Using the Slidepath software, specific cell populations within a heterogeneous sample can be selected for analysis according to an operator adjusted cell nuclear area. Staining intensity thresholds (positive/negative) can also be specified by the observer. Thresholds for staining intensity and nuclear area were chosen based on a sample of prostate tissue and once set they were used for analysis over all patient cohorts without adjustment. This method has been validated within our laboratory previously.(162) In order to ensure consistency 10% of each patient cohort was also scored visually on a computer monitor. The percentage of Ki-67 and TUNEL positive prostatic cells was evaluated at x400 magnification by scoring a minimum of 1000 prostatic cells of each core of the tissue microarray/tissue section. The number of positive cells was then divided by 1000 and multiplied by 100 in order to gain a percentage score. ICCC scores were undertaken in order to establish concurrence of scoring.

2.10 Statistics

Statistical analysis was performed using IBM SPSS Statistics version 19.0 for Windows. Inter-class correlation coefficients (ICCCs) confirmed histo-scoring consistency between observers. Pearson's rank correlation coefficients (c.c.) assessed associations between protein expression. Chi-squared test assessed relationships between protein expression and clinico-pathologic characteristics. Kaplan-Meier methods, using the log-rank test compared clinical outcome measures between patients according to clinico-pathologic parameters and high/low protein expression. Significant univariate results were included in a cox-regression model to determine independence from current clinical parameters. A <0.05 significance level was utilised. The Bonferroni correction was applied when >10 variables were being studied. It is acknowledged that if a lower global significance level was used then some of the results would become insignificant.

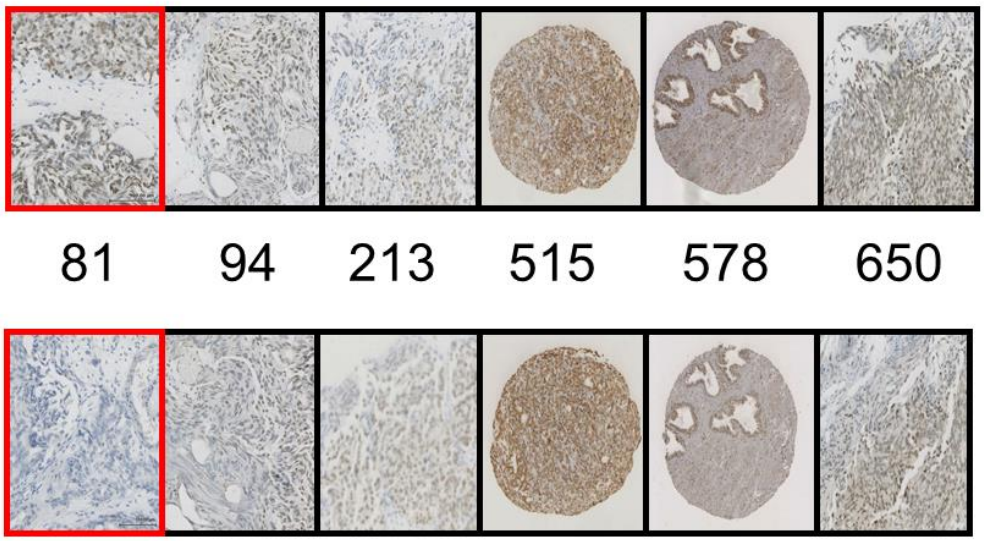
3 Antibody Validation Results

One of the major challenges in the investigation of protein phosphorylation is the acquisition of antibodies of high specificity and sensitivity. Cross reactivity of phospho specific antibodies is a recognised problem. This is complicated by the transient nature of phosphorylation and the potential of degradation during the tissue fixation process and over time, leading to issues with archival stored specimens. For this reason it was essential to further validate the specificity of the antibodies utilised in this study in addition to the manufacturer's own specificity checks.

3.1 Peptide competition assays

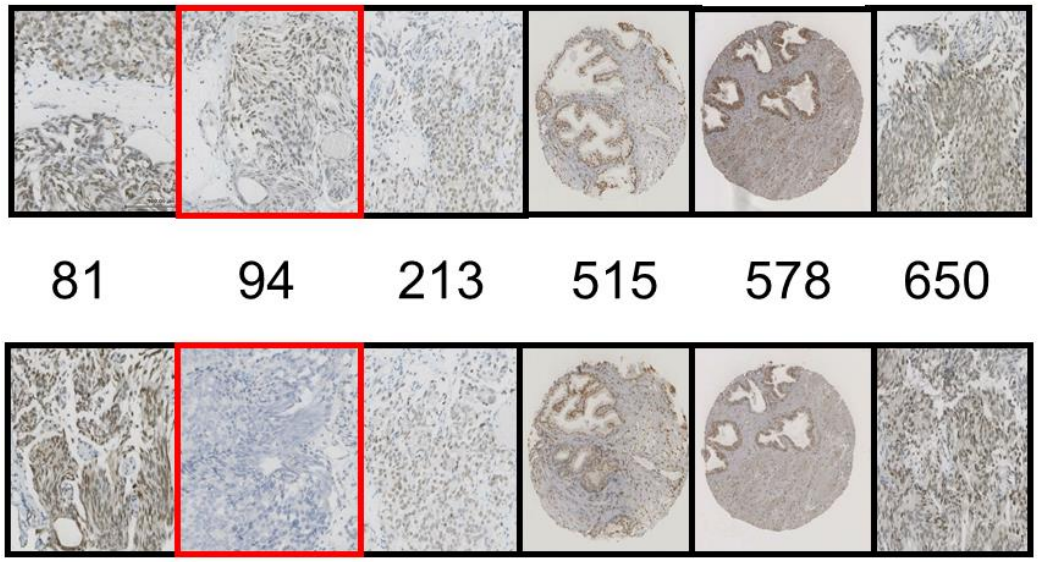
Peptide competition assays were performed for each AR serine phosphorylation site studied Figures 3.1-3.6.

Figure 3.1 pAR^{S81} peptide competition assay



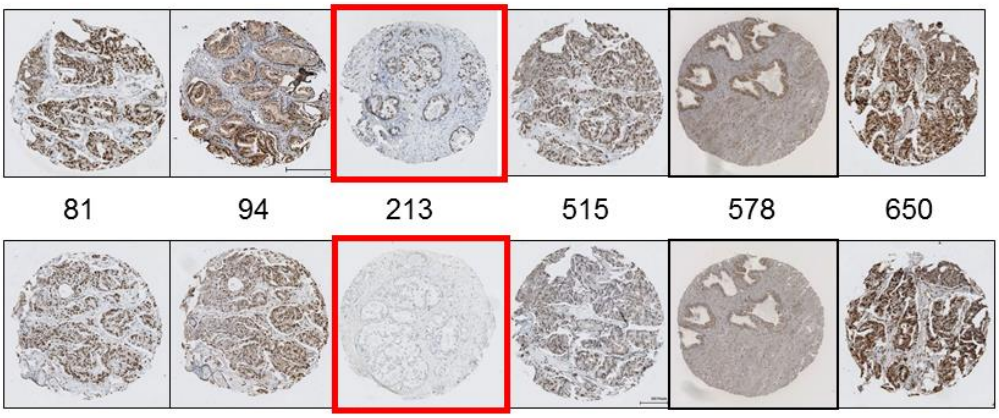
Each phospho-AR antibody is pre-incubated with pAR^{S81} peptide at a 1:1 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S81} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S81} antibody is specific.

Figure 3.2 pAR^{S94} peptide competition assay



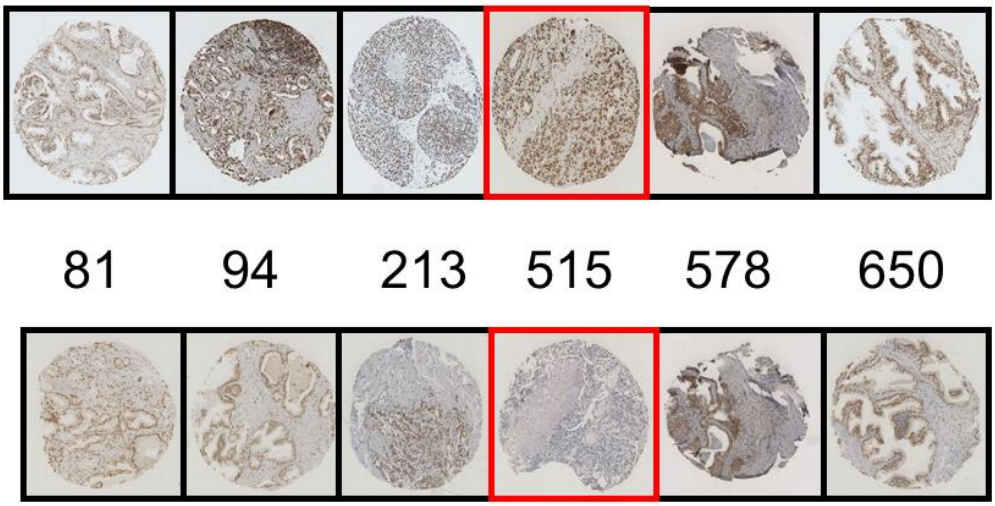
Each phospho-AR antibody is pre-incubated with pAR^{S94} peptide at a 1:2 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S94} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S94} antibody is specific.

Figure 3.3 pAR^{S213} peptide competition assay



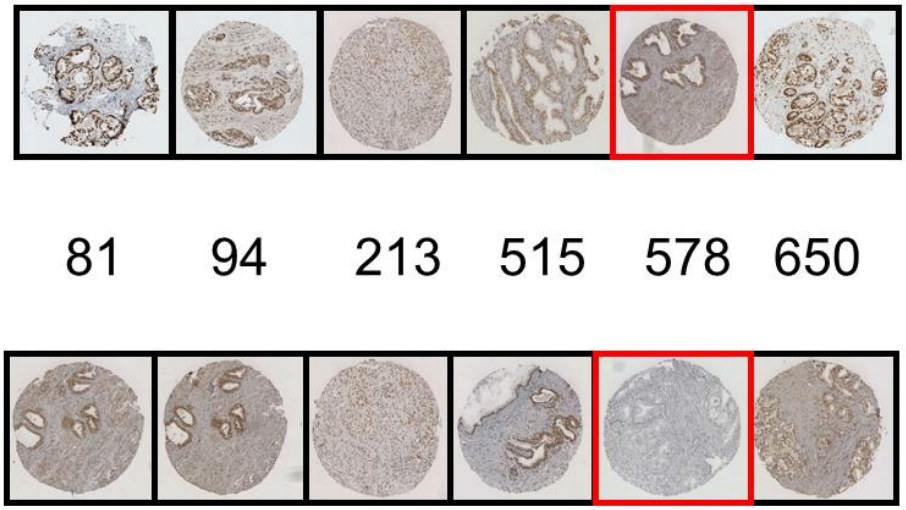
Each phospho-AR antibody is pre-incubated with pAR^{S213} peptide at a 1:1 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S213} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S213} antibody is specific.

Figure 3.4 pAR^{S515} peptide competition assay



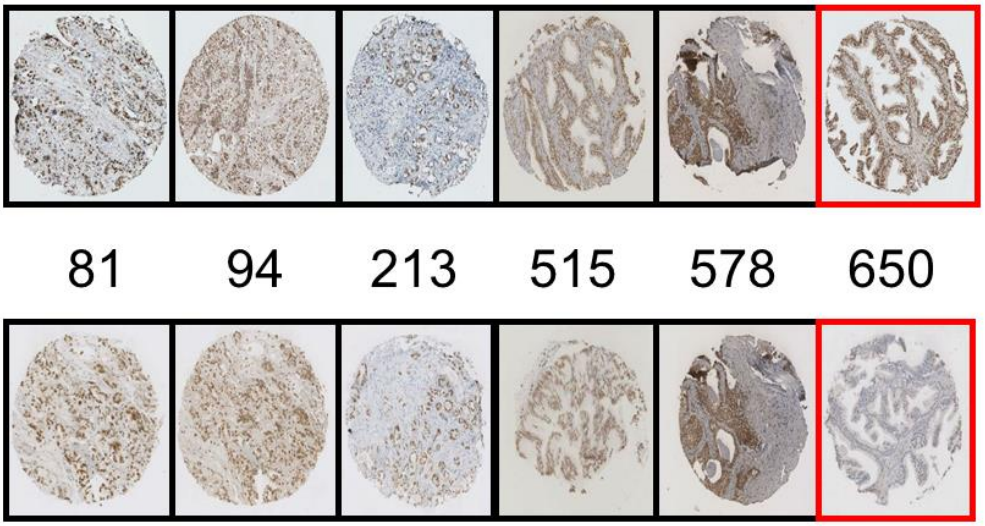
Each phospho-AR antibody is pre-incubated with pAR^{S515} peptide at a 1:500 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S515} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S515} antibody is specific.

Figure 3.5 pAR^{S578} peptide competition assay



Each phospho-AR antibody is pre-incubated with pAR^{S578} peptide at a 1:200 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S578} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S578} antibody is specific.

Figure 3.6 pAR^{S650} peptide competition assay



Each phospho-AR antibody is pre-incubated with pAR^{S650} peptide at a 1:100 ratio and subsequent immunohistochemical staining is displayed on the bottom row. The top row represents the positive control for each phospho-AR antibody. Immunohistochemical staining is absent when pAR^{S650} antibody is combined with its corresponding peptide (highlighted in red), however staining is maintained across the other phosphorylation sites. Therefore pAR^{S650} antibody is specific.

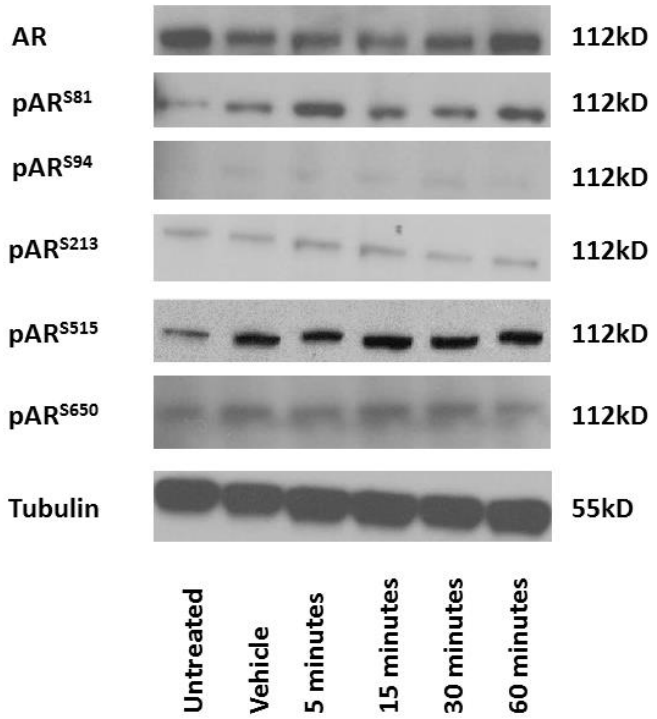
3.2 Western blotting

Western blots were performed to support antibody specificity experiments and were successful for each AR serine phosphorylation site studied with the exception of Ser-578, unfortunately this antibody was not suitable for use in western blots. In all experiments the dominant band observed was for full length AR at 112 kDa (Figure 3.7), known splice variants for the AR were also observed at 45 kDa and between 70-90 kDa for antibodies to AR Ser-213, 515 and 650, these are sizes associated with truncation of the ligand binding domain. No

bands at 90 kDa were observed for AR Ser-81 or 94, this is believed to be due to truncation in the NTD region, this splice variant is known as AR-A and is truncated from amino acid 1 to 188 (Figure 3.8).

The western blots achieved are shown in relation to treatment with DHT (10nM) (Figure 3.7)

Figure 3.7 Western blot AR and AR phosphorylated at serine sites following DHT treatment



Western blot was performed on 50ug of extracts from LNCaP cells treated with 10nM DHT for various lengths of time as indicated.

Figure 3.8 Splice variant of the androgen receptor (AR-A)

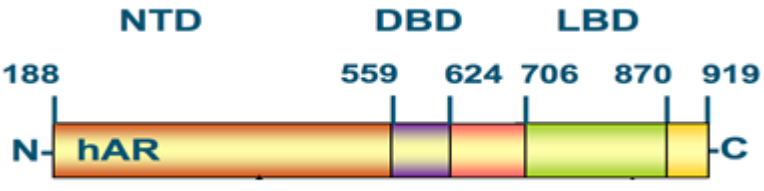


Illustration of splice variant AR-A of the androgen receptor. The N-terminal is truncated from amino acid 1-188. Abbreviations: NTD = N-terminal domain; DBD = DNA binding domain, LBD = ligand binding domain.

3.3 Discussion

Phosphorylation is a key post translational modification which effects a conformational change altering the activity, binding properties and subcellular localisation of proteins. Phosphorylation is both rapid and reversible occurring on serine, tyrosine and threonine residues. Generally only a small fraction of the available target protein undergoes phosphorylation and following signal activation this process is then quickly arrested via phosphatases or degradation resulting in de-phosphorylation. This transient nature of phosphorylation presents the biggest challenge for its investigation, which is the production of antibodies of high enough specificity and sensitivity in order to capture an intracellular event of low frequency and abundance.(163)

Phosphospecific antibodies are the most commonly used method for investigating protein phosphorylation status. Phosphosite specific antibodies not only recognise the phosphorylation site (serine/ tyrosine/ threonine) but also the amino acid sequence surrounding the phosphorylation site and therefore a particular target site within a particular protein can be identified. However, target sites can be conserved or similar between several proteins which is a potential confounder. To strike a balance between the identification of the phosphosite within a particular protein and restriction of antibodies to the non-phosphorylated portions of said protein, during antibody synthesis the phosphorylated amino acid is placed in the centre of a 10-14 amino acid long peptide.

Verification of antibody specificity is imperative to ensure that none of the other phosphorylation sites within the same protein are recognised by the same antibody. This is of particular importance on a protein such as AR with multiple phosphorylation sites. It is generally recommended that a variety of methods are used for verification and these may include ELISA, western blotting, siRNA and knockout mice, phosphatase and competition assays, kinases and phosphatase inhibitors, cell transfectants and site specific mutagenesis, immunohistochemistry and flow cytometry.(164)

The accepted initial step for antibody validation is via western blot in order to demonstrate that it detects a single band (or multiple bands if family members share the same motifs) of appropriate molecular weight. However, validation of the specificity of an antibody is partly dependent on the type of immunogen e.g. synthetic peptide or purified protein. In the case of synthetic peptides the amino acid sequence to which the antibody binds is known, however, the 3-D structure of the native protein is often not taken into account.(165) Therefore antibodies generated against a synthetic peptide may produce suboptimal results when a protein is in its native form and may not be useful in techniques such as immunohistochemistry where this is the case. However such antibodies may work well in western blot following protein denaturisation. Similarly antibodies raised against the purified protein may work well in immunohistochemistry when the protein is in its native form, however may not work well on denaturised proteins in western blot. This has been shown in the current study with some of the phosphospecific antibodies displaying higher quality results in western blot than others. In addition pAR^{S578} antibody did not produce any results in western blot. Therefore western blot cannot be considered the standard for verification of antibody specificity and other methods such as immunohistochemistry must be employed. This is of particular relevance if, as in the current study, the aim

is to use the antibody for another application. The antibody must be demonstrated to specifically recognise its target in the application concerned, in this case immunohistochemistry. Although pAR^{S578} antibody demonstrated good results in the peptide competition assay experiment, the results should be interpreted with caution and further validation work is required.

In addition to western blot, peptide competition experiments were utilised to evaluate antibody specificity in immunohistochemistry.(166) As demonstrated above the peptide sequences used to generate the antibodies are pre-incubated with the antibody. Immunohistochemistry is then performed on the tissue of interest with the antibody alone and the antibody pre-incubated with the peptide. Specificity is determined if the addition of the peptide results in loss of staining on the tissue, as seen for each phosphospecific antibody in the current study. Peptide competition experiments demonstrate antibody specificity for the peptide from which it was generated. However, as in the case of G protein –coupled receptor antibodies (167), it does not demonstrate selectivity, as pre-incubation with the peptide will also inhibit off-target binding activity of the antibody. Future work would include the immunohistochemical staining of cell pellets corresponding to the cell lines and treatments utilised for western blot. In addition target expression can be quantified both in western blot and in immunohistochemistry via the use of software programs such as Image J, AQUA and Slidepath.(168-170) The expression levels of the target proteins between the two methods should correlate to demonstrate antibody specificity. Unfortunately there was insufficient laboratory time to undertake this analysis within the scope of the present project and the work will be carried out by future lab members.

Reproducibility of antibodies over time using different lots on different days is another criterion for validation. Over the course of the current study the

reproducibility of the antibodies concerned was consistently demonstrated. Each new lot was considered as a new antibody and validation was undertaken using the methods described above before proceeding with the immunohistochemical staining of experimental tissue.

Further issues regarding the use of phosphospecific antibodies in human tissue are largely related to tissue collection and fixation. The method of tissue collection and the speed of fixation is therefore critical to the attainment of accurate results upon further analysis. Some of the tissue utilised in this study was obtained by diathermy resection and it is acknowledged that this may affect tissue phosphorylation status. Heat treatment of tissues is known to induce a number of biological processes including gene activation, cell cycle arrest and apoptosis. Following heat treatment in prostate cancer cells via high-intensity focused ultrasound the expression level of phosphorylated human checkpoint kinase2 was increased.(171) Diathermy resection of tissues, as performed during TUR of prostate surgery, may also alter tissue phosphorylation status. Tissue ischaemia times can vary hugely in clinical practise from a few minutes to several hours. Tissue ischaemia time can affect the phosphorylation status of a surgical specimen. The opening of tissues can alone result in rapid phosphatase activation and resultant de-phosphorylation. Recent studies have demonstrated a significant influence of tissue ischaemia time on immunohistochemical staining of breast, colorectal and lung tumour tissue specimens with phosphospecific antibodies.(172,173) The prostatic tissue utilised in the current study is generally fixed in the operating theatre within minutes following extraction. However this process is not standardised and the potential confounding effect of tissue ischaemia cannot be ignored.

The type of fixative used has been related to the quality of immunohistochemical staining. A recent study found that standard formalin fixation was best for immunohistochemical detectability for a range of proteins including phosphorylated HER2 when compared to five other fixation methods.(174) In particular, when using alcohol fixation HER2 protein was detected however the phosphorylated form of the receptor was not.(174) This reinforces the importance on the type of fixative used especially in the investigation of phosphorylated proteins in human tissue. All the samples in the current study were fixed in standard formalin.

Fixative penetration has been acknowledged as a potential issue regarding the interpretation of protein phosphorylation status in human tissue specimens. All prostatic tissue specimens utilised in the current study were fixed in formalin and embedded in paraffin. Fixative penetrates tissue at an approximate rate of 1mm/hour in formalin. Therefore the innermost areas of larger pathological specimens may suffer from progressive ischaemia until the fixative has fully penetrated and crosslinked the cellular structures within the specimen. However in small tissue specimens of 1mm diameter phosphorylated protein expression has been shown to be conserved between formalin fixed paraffin embedded and fresh frozen samples from the same patient.(172) Prostatic biopsies and TUR chips are both small and submerged in fixative minutes following extraction from the patient so the effect of tissue ischaemia is minimised. However, this process is not standardised and this limitation is acknowledged.

The inherent limitations surrounding the investigation of phosphorylated proteins in human tissue specimens have been described. However, the study of phosphorylated target proteins may well be more precise in evaluating deregulated intracellular signalling in disease states, as presence of

phosphorylation suggests an actively signalling pathway. With regards to the current study, the further investigation of AR serine phosphorylation status is deemed necessary in order to provide the preliminary assessment of its clinical relevance, and potential as a therapeutic target, in prostatic disease. This study is certainly not conclusive but will provide a foundation for future work.

4 Pilot Prostate Cancer Cohort Results

4.1 Cohort demographics

Analysis was based on 90 hormone-naïve prostate cancer patients. Patient characteristics are shown in Table 4.1. Patients were treated by surgery (18), radiotherapy (21) and hormones (37). Treatment information was missing for 14 patients. Twenty three patients had metastases; local lymph nodes (3), bone (13) and at both sites (7).

Forty seven patients had biochemical relapse (median time to biochemical relapse 2.7y, (interquartile range (IQR) 1.5–3.8). Twenty four patients were alive at time of analysis, median follow-up 11.7y (IQR 9.9–14.0). Forty six died of their disease (median time to death 4y, IQR 1.9–7.2) and 20 deaths were attributed to intercurrent disease (median time to death of 4.1y, IQR 0.9–5.5).

Table 4.1 Pilot prostate cancer cohort demographics

Clinical Parameter		Percentage of patients (%)	Number of patients
Age	<70yr	37.8	34
	≥70 yr	62.2	56
Gleason	<7	31.2	28
	=7	32.5	29
	>7	36.4	33
PSA at diagnosis	<10ng/ml	27.5	25
	10-20ng/ml	20.3	18
	>20ng/ml	52.2	47
Lymphovascular Invasion	absence	93.3	84
	presence	6.7	6
Recurrence PSA	<10ng/ml	77.6	70
	10-20ng/ml	2.0	2
	>20ng/ml	20.4	18

4.2 Clinicopathological factors related to outcome measures

Table 4.2 shows associations with clinical parameters (grouped data) and outcome measures using Kaplan-Meier methods.

Table 4.2 Pilot prostate cancer cohort clinicopathological factors related to outcome

	Time to biochemical relapse	Disease Specific Survival
Age (<70 vs ≥70 yrs)	0.260	**0.020**
Gleason (<7 vs =7 vs >7)	**0.013**	**0.007**
Diagnosis PSA (<10 vs 10-20 vs >20ng/ml)	**0.002**	**0.001**
Recurrence PSA (<10 vs 10-20 vs >20ng/ml)		**<0.001**
Lymphovascular Invasion (presence vs absence)	**0.001**	0.114
Presence of metastases (presence vs absence)	**0.001**	**<0.001**
Proliferation Index (Ki67) (low vs high)	0.730	**0.033**

Well known features of aggressive disease were associated with shorter time to biochemical relapse; higher Gleason score, higher PSA level at diagnosis, presence of lymphovascular invasion and presence of metastases. Interestingly older patients had shorter disease specific survival than younger patients. Clinicopathological factors associated with aggressive disease were associated with shorter disease specific survival as expected; higher Gleason score, diagnostic PSA ≥20ng/ml, relapse PSA ≥10ng/ml, presence of metastases and higher proliferation index.

4.3 Androgen receptor expression

Expression of all proteins was observed at varying levels in the cytoplasm and nucleus of both stromal and epithelial cells (Figure 4.1). Protein expression was found to be heterogeneous throughout and less intense in the stromal cells. There was presence of PIN and benign tissue, adjacent to the neoplastic tissue,

in some of the TMA cores. Expression of proteins in the interspersed PIN and benign tissue and the normal prostate control core was heterogeneous and less intense than the neoplastic tissue. Only protein expression observed in the tumour cells was scored. ICCCs were performed to verify consistency between scorers and all values were >0.80. Scatter plots for each antibody were used to display this data and to confirm there was no bias between scorers Bland Altman plots were constructed. Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis. Median AR and pAR histoscore expression levels are shown in Table 4.3. Median cell counts for Ki67 protein was 1.0% IQR 0.0-3.0%.

Figure 4.1 Example high/low AR and pAR immunohistochemical staining

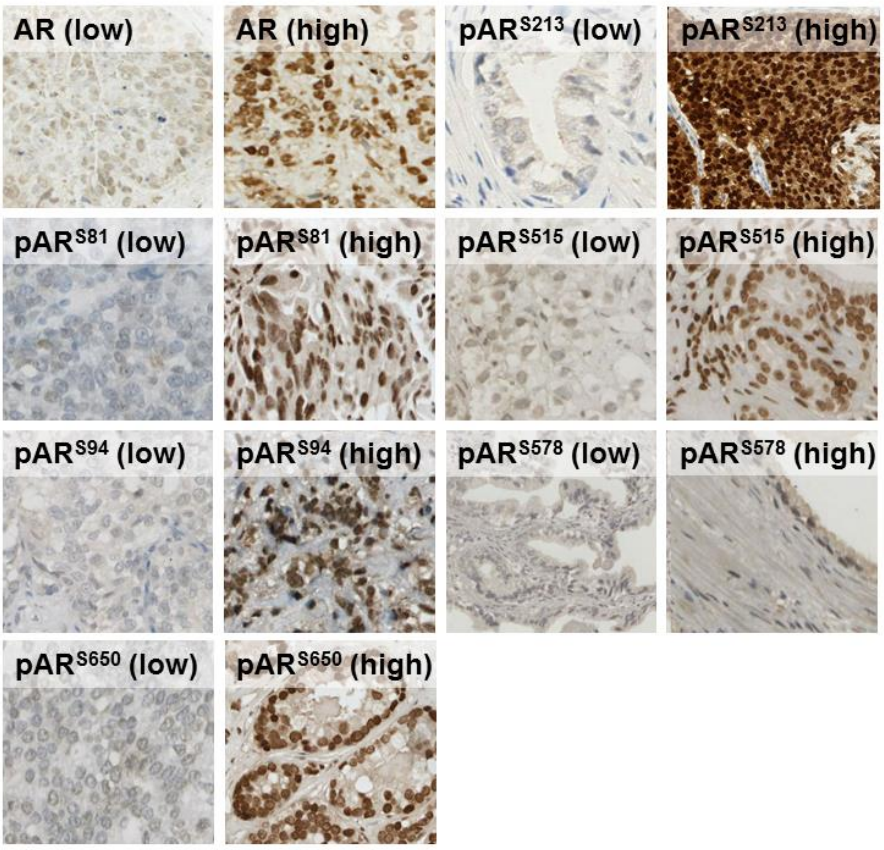


Table 4.3 Protein expression levels for AR and pAR sites

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
AR	Cytoplasm	62.5	50.0-80.5
	Nucleus	67.7	43.8-82.9
pAR^{S81}	Cytoplasm	50.0	20.0-100.0
	Nucleus	140.0	73.8-206.3
pAR^{S94}	Cytoplasm	30.0	10.0-51.3
	Nucleus	60.0	33.8-105.0
pAR^{S213}	Cytoplasm	5.0	2.5-11.7
	Nucleus	70.0	50.0-113.3
pAR^{S515}	Cytoplasm	5.0	1.3-10.0
	Nucleus	130.0	82.5-165.0
pAR^{S578}	Cytoplasm	50.0	31.3-100.0
	Nucleus	169.2	130.0-206.3
pAR^{S650}	Cytoplasm	52.5	20.0-100.0
	Nucleus	120.0	68.3-187.5

4.4 Phosphorylated androgen receptor related to clinicopathological factors

AR and phosphorylated AR is related to clinicopathological factors as shown in Table 4.4. High expression of pAR^{S81} (cytoplasmic), pAR^{S515} (nuclear and total) and pAR^{S650} (cytoplasmic, nuclear and total) were associated with increased age. High expression of cytoplasmic pAR^{S213} and pAR^{S578} was associated with increased Gleason score. High expression of nuclear pAR^{S578} was associated with increased PSA level at diagnosis. Presence of LVI was associated with high AR (nuclear, cytoplasmic and total), pAR^{S213} (nuclear) and pAR^{S515} (cytoplasmic) expression. Presence of metastases was associated with high AR (total) and high pAR^{S515}

(cytoplasmic) expression. High expression of pAR^{S81} (nuclear and total) and low expression of pAR^{S94} (cytoplasmic) were associated with high Ki67 score.

Table 4.4 Clinicopathological factors as related to high/low AR and pAR expression

Proteins		Clinical Variables						
		Age (<70 vs ≥70yrs)	**Gleason** (<7 vs =7 vs >7)	**Diagnosis PSA** (<10 vs 10-20 vs >20ng/ml)	**Recurrence PSA** (<10 vs 10-20 vs >20ng/ml)	**Lymphovascular Invasion** (presence vs absence)	**Presence of metastases** (presence vs absence)	**Ki67** (≤median vs >median)
AR	Cytoplasm	0.909	0.841	0.979	0.496	**0.028**	0.061	0.230
	Nucleus	0.284	0.450	0.301	0.380	**0.006**	0.107	0.154
	Total	0.422	0.464	0.493	0.800	**0.002**	**0.027**	0.627
pAR^{S81}	Cytoplasm	**0.035**	0.811	0.269	0.566	0.175	0.660	0.498
	Nucleus	0.651	0.401	0.462	0.601	0.666	0.229	**0.039**
	Total	0.220	0.425	0.389	0.632	0.290	0.195	**0.041**
pAR^{S94}	Cytoplasm	0.447	0.714	0.437	0.820	0.234	0.165	**0.040**
	Nucleus	0.651	0.069	0.726	0.386	0.234	0.892	0.361
	Total	0.754	0.417	0.964	0.496	0.864	0.554	0.191
pAR^{S213}	Cytoplasm	0.189	**0.011**	0.306	0.860	0.100	0.277	0.145
	Nucleus	0.546	0.062	0.282	0.167	**0.019**	0.407	0.919
	Total	0.543	0.087	0.160	0.167	0.115	0.277	0.676

Table 4.4 continued Clinicopathological factors as related to high/low AR and pAR expression

Proteins		Clinical Variables						
		Age (<70 vs ≥70yrs)	**Gleason** (<7 vs =7 vs >7)	**Diagnosis PSA** (<10 vs 10-20 vs >20ng/ml)	**Recurrence PSA** (<10 vs 10-20 vs >20ng/ml)	**Lymphovascular Invasion** (presence vs absence)	**Presence of metastases** (presence vs absence)	**Ki67** (≤median vs >median)
pAR^{S515}	Cytoplasm	0.221	0.109	0.181	0.273	**0.018**	**0.040**	0.233
	Nucleus	**0.042**	0.336	0.052	0.116	0.673	0.948	0.156
	Total	**0.048**	0.120	0.287	0.120	0.463	0.528	0.229
pAR^{S578}	Cytoplasm	0.094	**0.008**	0.096	0.071	0.305	0.682	0.312
	Nucleus	0.296	0.324	**0.015**	0.394	1.000	0.236	0.920
	Total	0.117	0.341	0.061	0.296	1.000	0.923	0.762
pAR^{S650}	Cytoplasm	**0.046**	0.446	0.976	0.761	0.773	0.750	0.260
	Nucleus	**0.018**	0.531	0.169	0.935	0.279	0.216	0.756
	Total	**0.020**	0.465	0.216	0.827	0.516	0.447	0.504

4.5 Phosphorylated androgen receptor related to outcomes

Univariate analysis of AR and pAR protein expression was carried out using Kaplan Meier methods with reference to the clinical outcome measures. The results are shown in Table 4.5.

Table 4.5 Univariate analysis of phosphorylated AR expression and clinical outcome measures

Protein		Time to biochemical relapse	Disease specific survival
AR	Cytoplasm	0.466	0.517
	Nucleus	**0.001**	0.233
	Total	**<0.001**	0.580
pAR^{S81}	Cytoplasm	0.166	0.057
	Nucleus	0.594	**0.031**
	Total	0.925	**0.039**
pAR^{S94}	Cytoplasm	0.927	0.864
	Nucleus	0.375	0.991
	Total	0.178	0.884
pAR^{S213}	Cytoplasm	0.987	0.308
	Nucleus	0.548	0.069
	Total	0.596	**0.026**
pAR^{S515}	Cytoplasm	**0.020**	**<0.001**
	Nucleus	0.877	0.072
	Total	0.708	**0.034**
pAR^{S578}	Cytoplasm	**0.034**	**<0.001**
	Nucleus	0.461	**0.036**
	Total	0.496	**0.004**
pAR^{S650}	Cytoplasm	0.977	0.113
	Nucleus	0.909	0.177
	Total	0.530	0.059

4.5.1 Biochemical Relapse

As shown in Table 4.5 several proteins were associated with time to biochemical relapse. Specifically high nuclear AR was associated with shorter time to biochemical relapse (proportion of patients relapsed at 5y 79.2% vs 46.9%) HR 2.8 (95% CI 1.5–5.3). High total AR was associated with shorter time to biochemical relapse (proportion of patients relapsed at 5yr 85.1% vs 40.2%) HR 3.03 (95%CI 1.6-5.6). High cytoplasmic pAR^{S515} was also associated with shorter time to biochemical relapse (proportion of patients relapsed at 5y 78.6% vs 56%) HR 2.2 (95% CI 1.1–4.2). High cytoplasmic pAR^{S578} was also associated with time to biochemical relapse (proportion of patients relapsed at 5y 82.6% vs 51.9%) HR 2.1 (95% CI 1.0-4.2).

4.5.2 Disease specific survival

Several proteins were found to have a significant relationship with disease specific survival. High nuclear pAR^{S81} was associated with reduced disease-specific survival (10y survival 24.4% vs 54.5%), HR 2.1 (95% CI 1.1–4.2). High total pAR^{S81} was associated with reduced disease-specific survival (10yr survival 25.6% vs 56.5%) HR 2.0 (95%CI 1.0-4.1). High total pAR^{S213} was associated with shorter disease specific survival (10y survival 33.7% vs 64.3%) HR 2.7 (95% CI 1.1-4.7). High cytoplasmic pAR^{S515} was associated with disease-specific survival (10y survival 14.4% vs 65.2%) HR 4.4 (95% CI 2.1–9.1). High total pAR^{S515} was associated with reduced disease-specific survival (10yr survival 35.8% vs 57.5%) HR 2.1 (95%CI 1.0-4.2). High cytoplasmic and total pAR^{S578} was also associated with disease specific survival (10y survival 17.7% vs 71.1%) HR 4.5 (95% CI 2.0-10.4) and (10y survival 26.1% vs 66.9%) HR 3.0 (95% CI 1.4-6.7) respectively.

High nuclear pAR^{S578} was associated with disease specific survival (10y survival 30.2% vs 63.9%) HR 2.2 (95% CI 1.0-4.9).

4.6 Kinase protein expression

Scansite 2.0 predicted Cdk1 as a strong candidate mediating phosphorylation of Ser-81 and Ser-515. ERK1/2 was a strong candidate mediating Ser-515 phosphorylation. Similarly PKC was a strong candidate mediating Ser-578 phosphorylation.

Expression of all proteins was observed at varying levels in the cytoplasm and nucleus of both stromal and epithelial cells (Figure 4.2). Protein expression was found to be heterogeneous throughout and less intense in the stromal cells. There was presence of PIN and benign tissue, adjacent to the neoplastic tissue, in some of the TMA cores. Expression of proteins in the interspersed PIN and benign tissue and the normal prostate control core was heterogeneous and less intense than the neoplastic tissue. Only protein expression observed in the tumour cells was scored. ICCCs were performed to verify consistency between scorers and all values were >0.80. Scatter plots for each antibody were used to display this data and to confirm there was no bias between scorers Bland-Altman plots were constructed. Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis. Median kinase histoscore expression levels are shown in Table 4.6.

Figure 4.2 Example high/low immunohistochemical staining of candidate kinases

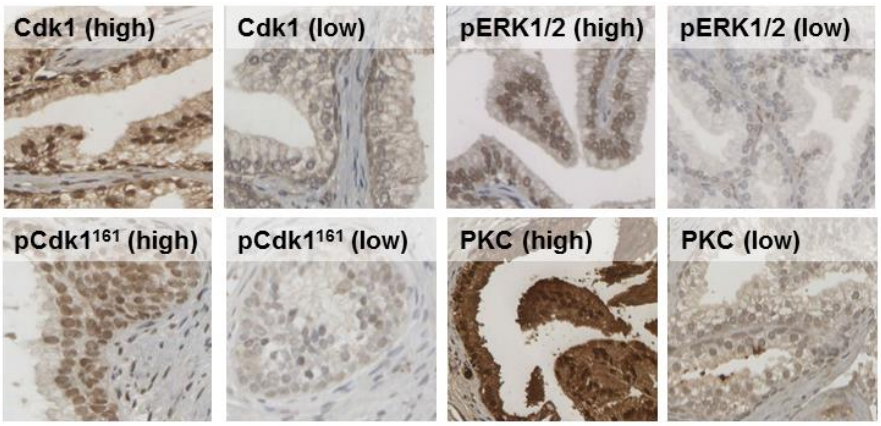


Table 4.6 Protein expression levels for kinases

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
Cdk1	Cytoplasm	40.0	20.0-76.3
	Nucleus	46.3	17.5-80.0
pCdk1^{161}	Cytoplasm	30.0	17.5-43.8
	Nucleus	82.5	42.5-128.8
pERK1/2	Cytoplasm	150.0	129.2-183.8
	Nucleus	10.0	0.0-57.1
PKC	Cytoplasm	138.3	100.0-162.5
	Nucleus	205.0	174.4-222.5

4.7 Kinases related to clinicopathological factors

Candidate kinase expression as related to clinicopathological factors is shown in Table 4.7. High total pCdk1^{161} and high nuclear, cytoplasmic and total PKC protein expression were associated with increased age. High nuclear and total pCdk1^{161} and nuclear PKC expression were associated with increased PSA level at diagnosis. High nuclear pCdk1^{161} expression was also associated with increased PSA level at biochemical relapse.

Table 4.7 Clinicopathological factors as related to high/low candidate kinase expression

Proteins		Clinical Variables						
		Age (<70 vs ≥70yrs)	**Gleason** (<7 vs =7 vs >7)	**Diagnosis PSA** (<10 vs 10-20 vs >20ng/ml)	**Recurrence PSA** (<10 vs 10-20 vs >20ng/ml)	**LVI** (presence vs absence)	**Presence of metastases** (presence vs absence)	**Ki67** (≤median vs >median)
Cdk1	Cytoplasm	0.902	0.687	0.774	0.268	0.327	0.760	0.534
	Nucleus	0.815	0.431	0.133	0.227	0.398	1.000	0.957
	Total	0.187	0.831	0.277	0.130	0.583	0.432	0.879
pCdk1^{161}	Cytoplasm	0.928	0.271	0.148	0.120	0.262	0.281	1.000
	Nucleus	0.069	0.985	**0.028**	**0.011**	0.643	0.648	0.743
	Total	**0.044**	0.263	0.108	**0.017**	0.621	0.204	0.918
pERK1/2	Cytoplasm	0.481	0.630	0.903	0.304	0.463	0.365	0.175
	Nucleus	0.920	0.591	0.903	0.069	0.417	0.691	0.131
	Total	0.315	0.555	0.936	0.569	0.160	0.428	0.280
PKC	Cytoplasm	**0.018**	0.630	0.478	0.910	0.643	0.141	0.539
	Nucleus	**0.032**	0.431	**0.009**	0.683	0.701	0.625	0.539
	Total	**0.003**	0.621	0.135	0.683	0.217	0.414	0.296

4.8 Kinases related to outcomes

Univariate analysis of kinase protein expression was carried out using Kaplan Meier methods with reference to the clinical outcome measures. The results are shown in Table 4.8.

Table 4.8 Univariate analysis of candidate kinase expression and clinical outcome measures

Protein		Time to biochemical relapse	Disease specific survival
Cdk1	Cytoplasm	0.988	**0.007**
	Nucleus	0.791	0.434
	Total	0.827	0.134
pCdk1^{161}	Cytoplasm	0.396	**0.013**
	Nucleus	0.889	**0.001**
	Total	0.166	**0.007**
pERK1/2	Cytoplasm	0.353	0.668
	Nucleus	0.384	0.509
	Total	0.525	0.973
PKC	Cytoplasm	0.938	0.267
	Nucleus	0.712	0.203
	Total	0.691	0.055

4.8.1 Biochemical relapse

As shown in Table 4.8 high none of the candidate kinases were associated with time to biochemical relapse.

4.8.2 Disease specific survival

As shown in Table 4.8 several proteins were associated with disease specific survival. Specifically high cytoplasmic Cdk1 was associated with reduced disease specific survival (10y survival 26.8% vs 57.2%) HR 2.4 (95% CI 1.2–4.5). High

cytoplasmic, nuclear and total pCdk1[161] were also associated with reduced disease specific survival; 10y survival 29.8% vs 58.3%, HR 2.5 (95% CI 1.2-5.4), 10y survival 27.8% vs 67.9%, HR 3.7 (95% CI 1.6-8.5) and 10yr survival 30.6% vs 64.4%, HR 2.9 (95%CI 1.3-6.4) respectively.

4.9 Association of candidate kinases to phosphorylated AR sites

Once the clinical significance of the candidate kinases had been analysed, their association with AR phosphorylation was then investigated in order to establish whether the predicted kinase was observed to correlate with AR phosphorylation in the clinical samples (Table 4.9).

Table 4.9 Associations between candidate kinases and clinically relevant androgen receptor phosphorylation sites in pilot prostate cancer cohort

			Candidate Kinases											
AR phosphorylation sites			**Cdk1**			**pCdk1^{161}**			**pERK1/2**			**PKC**		
			Cytoplasmic	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**
Ser-81	**Cytoplasmic**	**C.C.**	**0.545**	0.208	**0.509**	**0.446**	0.211	0.346	-0.068	-0.158	0.218	0.321	0.361	**0.443**
		p value	**<0.001**	0.099	**<0.001**	**0.001**	0.134	0.012	0.61	0.231	0.113	0.020	0.008	**0.001**
	Nuclear	**C.C.**	0.057	**0.569**	**0.439**	0.185	0.278	0.305	0.019	0.003	0.014	0.101	**0.533**	**0.406**
		p value	0.657	**<0.001**	**<0.001**	0.190	0.046	0.028	0.886	0.979	0.918	0.476	**<0.001**	**0.003**
	Total	**C.C.**	0.300	**0.500**	**0.552**	0.345	0.297	0.382	-0.019	-0.072	0.218	0.224	**0.559**	**0.504**
		p value	0.016	**<0.001**	**<0.001**	0.012	0.032	0.005	0.888	0.586	0.113	0.110	**<0.001**	**<0.001**
Ser-515	**Cytoplasmic**	**C.C.**	0.273	-0.025	0.167	**0.455**	0.008	0.172	0.051	0.061	0.074	0.293	-0.033	0.189
		p value	0.025	0.843	0.178	**<0.001**	0.954	0.210	0.706	0.649	0.583	0.031	0.814	0.172
	Nuclear	**C.C.**	0.181	**0.532**	**0.507**	0.105	**0.558**	**0.531**	-0.098	0.038	-0.029	-0.037	**0.543**	0.337
		p value	0.143	**<0.001**	**<0.001**	0.444	**<0.001**	**<0.001**	0.464	0.780	0.831	0.793	**<0.001**	0.013
	Total	**C.C.**	0.275	**0.457**	0.167	0.297	**0.500**	**0.549**	-0.061	0.060	0.009	0.106	**0.472**	0.392
		p value	0.024	**<0.001**	<0.001	0.028	**<0.001**	**<0.001**	0.648	0.656	0.949	0.447	**<0.001**	0.003

Table 4.9 continued Associations between candidate kinases and clinically relevant androgen receptor phosphorylation sites in pilot prostate cancer cohort

			Candidate Kinases											
AR phosphorylation sites			**Cdk1**			**pCdk1^{161}**			**pERK1/2**			**PKC**		
			Cytoplasmic	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**	**Cytoplasmic**	**Nuclear**	**Total**
Ser-578	**Cytoplasmic**	**C.C.**	0.262	0.375	**0.445**	0.163	**0.430**	**0.434**	0.070	-0.081	-0.02	0.019	**0.426**	0.284
		p value	0.043	0.003	**<0.001**	0.257	**0.002**	**0.002**	0.614	0.559	0.887	0.894	**0.002**	0.044
	Nuclear	**C.C.**	0.137	**0.540**	**0.485**	0.364	**0.604**	**0.663**	0.030	-0.035	-0.009	0.284	**0.469**	**0.498**
		p value	0.298	**<0.001**	**<0.001**	0.009	**<0.001**	**<0.001**	0.831	0.804	0.951	0.044	**0.001**	**<0.001**
	Total	**C.C.**	0.231	**0.526**	**0.538**	0.304	**0.600**	**0.635**	0.058	-0.067	-0.016	0.172	**0.518**	**0.450**
		p value	0.075	**<0.001**	**<0.001**	0.032	**<0.001**	**<0.001**	0.678	0.629	0.906	0.227	**<0.001**	**0.001**

As shown in Table 4.9 pERK1/2 expression was not significantly associated with any of the clinically relevant AR phosphorylation sites, however Cdk1 and/or pCdk1^{161} were significantly associated with pAR^{S81} and pAR^{S515} as predicted by Scansite 2.0. Similarly, nuclear PKC expression was significantly associated with pAR^{S578} expression both in the cytoplasm and the nucleus. Interestingly nuclear pAR^{S81} and pAR^{S515} were both associated with nuclear PKC expression. Nuclear Cdk1 expression was significantly correlated with nuclear pAR^{S578} expression. Nuclear pCdk1^{161} expression was associated with both cytoplasmic and nuclear pAR^{S578} expression (Table 4.9). This suggests that there is cross talk between these kinases and AR serine phosphorylation sites.

In view of the correlation with Scansite 2.0 predictions further investigations were undertaken separating the phospho-AR sites by their kinases, i.e:

- PKC → pAR^{S578}
- Cdk1/pCdk1^{161} → pAR^{S81} and pAR^{S515}

4.10 Multivariate analysis

Significant univariate results were included in a backwards conditional cox-regression model to determine independence from current clinical parameters.

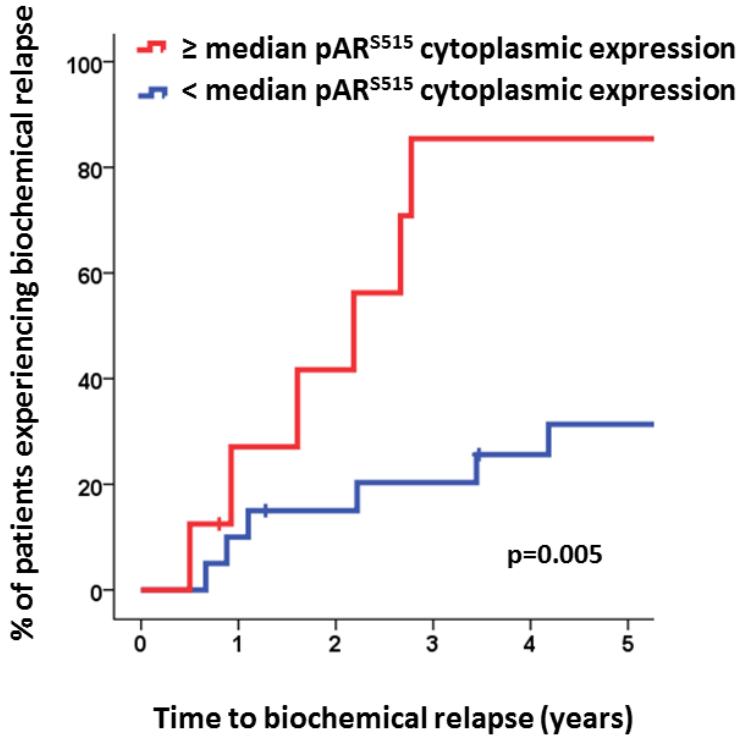
4.10.1 PKC predicted AR phosphorylation sites

AR and pAR^{S578} expression were combined with significant clinicopathological variables in a multivariate analysis for each of the three clinical outcome measures. Neither AR nor nuclear or cytoplasmic pAR^{S578} were deemed independent from current clinical parameters with regards to time to biochemical relapse or disease specific survival. Therefore further analysis of these parameters was not pursued

4.10.2 Cdk1/pCdk1[161] predicted AR phosphorylation sites

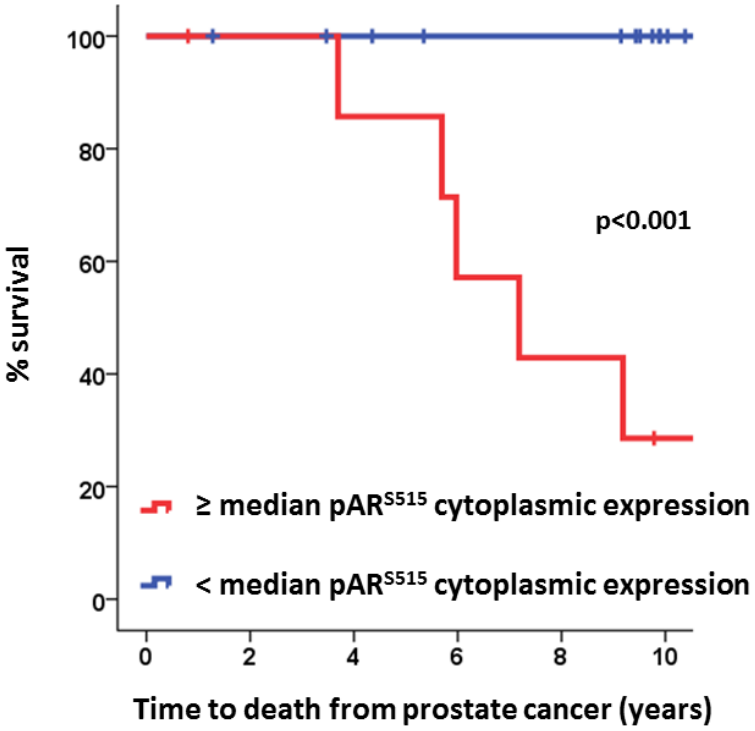
AR and pAR^{S515} expression were combined with Gleason score, PSA at diagnosis, LVI and presence of metastases in a multivariate cox regression analysis. Cytoplasmic pAR^{S515} expression (p=0.038, HR 4.5 (95% CI 1.1–20.6)) and PSA at diagnosis (p=0.003, HR 7.3 (95% CI 2.0–27.5)) were independently associated with time to biochemical relapse. As these variables were deemed independent it was investigated whether pAR^{S515} expression could inform on patients with PSA ≤20ng/ml at diagnosis. These patients, within each individual clinical context, are generally considered suitable for immediate or delayed radical treatment (via active surveillance). Many of these patients will have indolent disease that may never reach clinical consequence and therefore are at risk of overtreatment. In contrast, a subset have occult aggressive tumours that will progress if treatment is delayed. Therefore biomarkers to aid treatment decision-making in this group of patients would have important clinical implications. When patients with PSA at diagnosis ≤20ng/ml, were stratified by cytoplasmic pAR^{S515} expression, those tumours with high expression had significantly shorter time to biochemical relapse than those patients with low expression (p=0.005) (Figure 4.3). This translated into significantly shorter disease-specific survival (p<0.001, 10y survival 38.1% vs 100%) (Figure 4.4).

Figure 4.3 Kaplan Meier Graph illustrating pAR^{S515} cytoplasmic expression as related to time to biochemical relapse



Kaplan Meier survival plot showing time to biochemical relapse in patients with PSA ≤20ng/ml at diagnosis (n=28) stratified according to low and high cytoplasmic pAR^{S515} expression. Patients received a variety of treatments (surgery, radiotherapy and hormones) and, due to small numbers, it was not possible to unpick these groups.

Figure 4.4 Kaplan Meier Graph illustrating pAR^{S515} cytoplasmic expression as related to disease specific survival

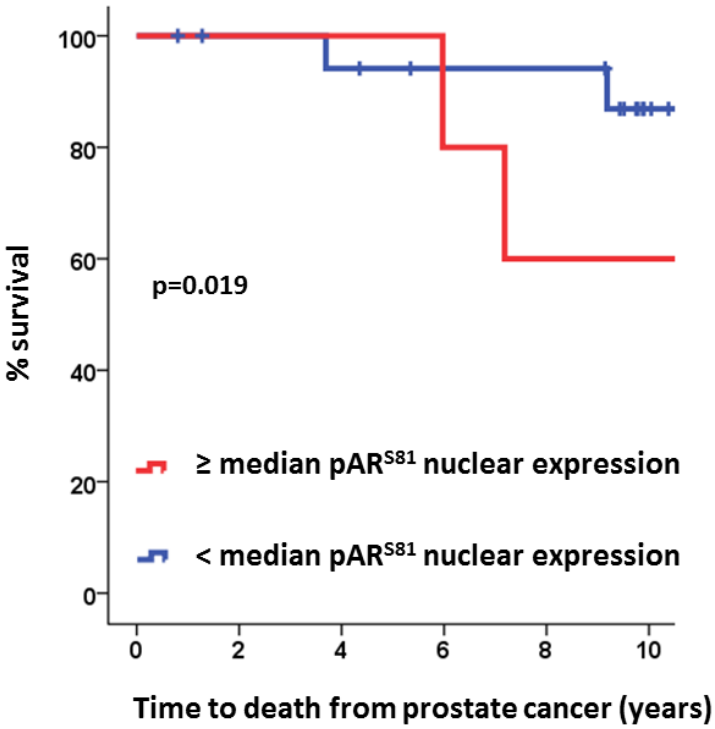


Kaplan-Meier survival plot showing disease-specific survival in patients with PSA ≤20ng/ml at diagnosis (n=28) stratified according to low and high cytoplasmic pAR^{S515} expression.

High/low pAR^{S81} nuclear expression was combined with age, Gleason score, PSA at diagnosis, PSA at biochemical relapse, presence of metastases and proliferation index in a multivariate cox regression analysis. pAR^{S81} nuclear expression (p=0.030, HR 0.033 95% CI 0.002-0.721), proliferation index (p=0.019, HR 47.20 95% CI 1.88-1184.95), PSA at diagnosis (p=0.029, HR 105.00 95% CI 1.63-6778.60) and Gleason score (p=0.049, HR 4.74 95% CI 1.01-22.24) were independently associated with disease specific survival. As these variables were deemed independent it was investigated whether pAR^{S81} nuclear expression could inform on patients with PSA ≤20ng/ml at diagnosis. When patients with PSA at diagnosis ≤20ng/ml, were stratified by nuclear pAR^{S81} expression, those tumours

with high expression had significantly shorter time to cancer specific death than those patients with low expression (p=0.014, 10y survival 50% vs 93.3%) (Figure 4.5).

Figure 4.5 Disease specific survival by pAR^{S81} expression in patients with PSA ≤20ng/ml



Kaplan-Meier survival plot showing disease specific survival in patients with PSA ≤20ng/ml at diagnosis (n=24) stratified according to low and high cytoplasmic pAR^{S81} expression.

These results therefore warranted further investigation in cell line studies.

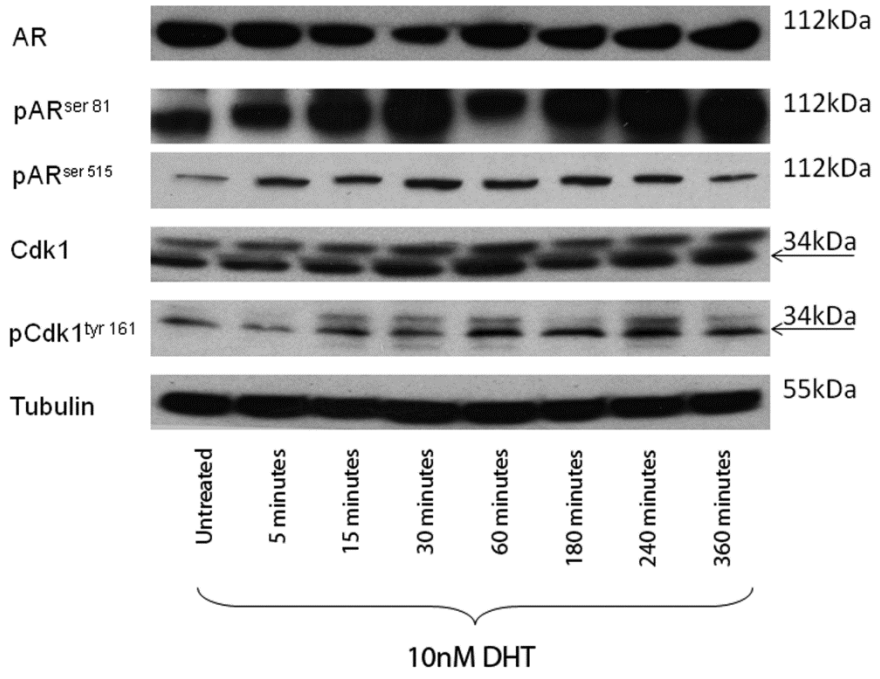
4.11 Cell stimulation / inhibition studies

4.11.1 AR agonists stimulate Cdk1 and AR phosphorylation

In the clinical specimens pAR^{S81} and pAR^{S515} were demonstrated to correlate with pCdk1^{161} (Table 4.9). In vitro, it was observed by western blot analysis that the androgen DHT could induce phosphorylation of Cdk1^{161}, AR^{S81} and AR^{S515}, with AR and Cdk1 expression levels remaining constant (Figure 4.6). Analysis of paraffin-

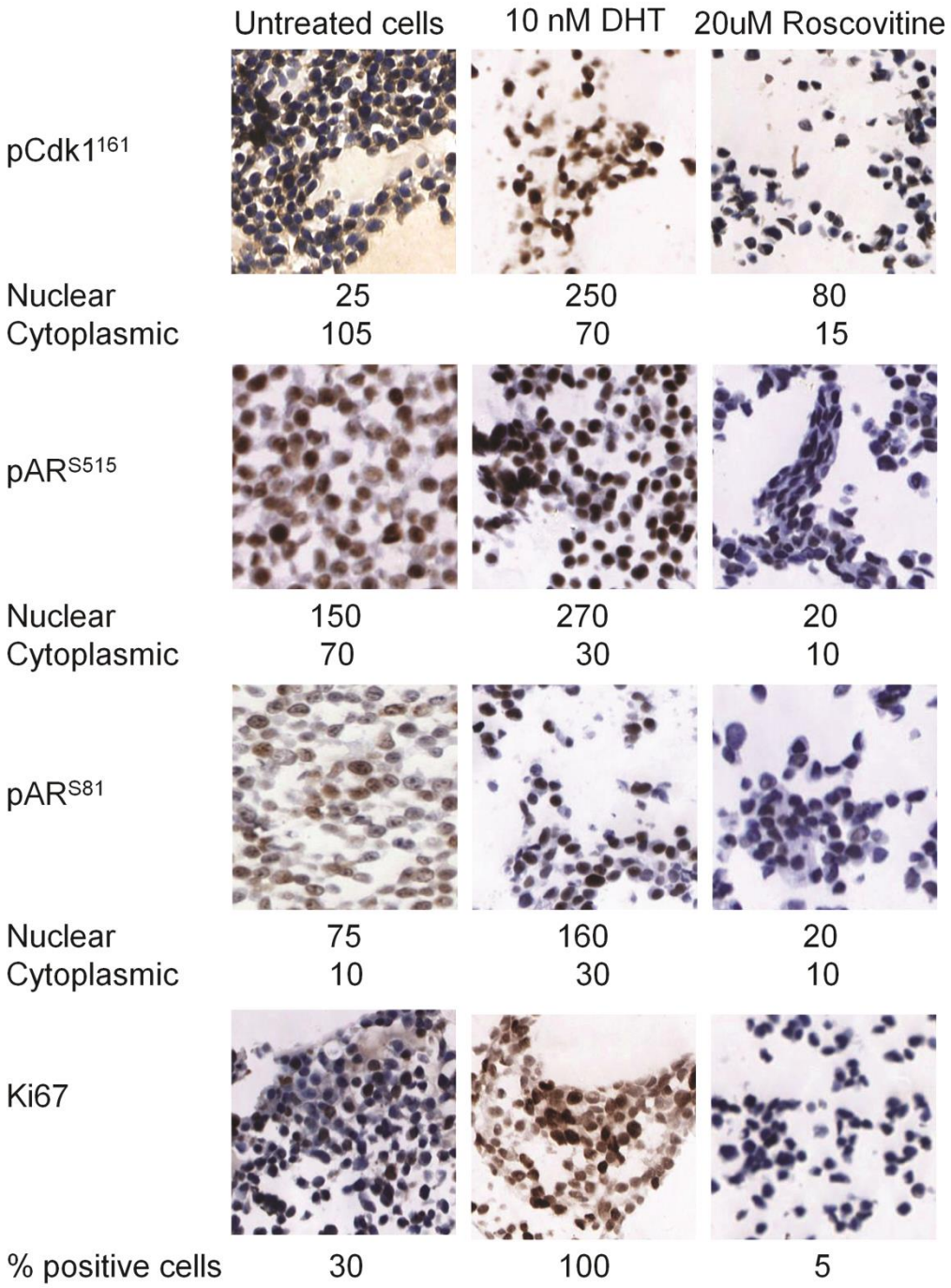
embedded cell pellets confirmed the observation that treatment with DHT stimulates phosphorylation at Cdk1^{161}, AR^{S81} and AR^{S515} (Figure 4.7). As shown in Figure 4.7 it was demonstrated that DHT stimulated cellular proliferation, as assessed by Ki67. In addition, treatment with a Cdk inhibitor, roscovitine, markedly decreased basal expression of pCdk1^{161}, pAR^{S81}, pAR^{S515} and cell proliferation (Figure 4.7).

Figure 4.6 DHT inducing protein phosphorylation on western blot



Western blot was performed on 50ug of extracts from LNCaP cells treated with 10nM dihydrotestosterone (DHT) for various lengths of time as shown. Cdk1 is known to have an isomer called CDC2deltaT that lacks 171 nucleotides corresponding to 57 amino acids, which compose most of the T-loop.(175) CDC2deltaT has been identified in breast cancer tissue, and currently there is no evidence for this in prostate cancer but it may account for the duplex band seen in Cdk1 and pCdk1^{161}.

Figure 4.7 Treated cell pellets stained for protein expression



LNCaP cells were grown in full media and treated with either 10nM dihydrotestosterone (DHT) (3hr) or 20uM Roscovitine (24hr). Pellets were stained by immunohistochemistry for expression of pCdk1^{161}, pAR^{S81}, pAR^{S515} and the nuclear marker of proliferation; Ki67. Weighted histoscores for cytoplasmic and nuclear expression are shown below each image for

pCdk1^{161}, pAR^{S81} and pAR^{S515}. Percentage of positive cells counted is shown below the images for Ki67.

4.12 Discussion

Previous work relating to total AR expression has demonstrated widely conflicting results with regards to its use as a prognostic marker.(176-179) These conflicting findings may depend on several factors such as tissue heterogeneity, timing of specimen dissection and methods to detect AR expression including the use of different antibodies. In addition, the simple expression of AR does not reflect its function or its activity, and therefore may account for the variations reported with regards to prognostic significance. AR phosphorylation, which is known to provide molecular stability, may therefore be a marker of activation. The current study lends support to this argument as it has demonstrated, for the first time *in vivo*, that those patients who have a high level of pAR^{S578} have reduced overall survival when compared to AR expression alone. This result should, however, be interpreted with caution as a single band western blot was not achieved for this antibody. Ser-81 is the most frequently phosphorylated site on AR in response to androgen binding.(136) However, pAR^{S515} was found to be independent of current clinical parameters ahead of pAR^{S81}. This is in line with previous work which demonstrated that abolishment of pAR^{S81} in cell lines did not alter androgen-dependent AR transcriptional activity, rapidly-induced AR-regulated genes or AR stabilization mediated by Cdk1.(136) In contrast, similar removal of pAR^{S515} resulted in reduction of AR transcriptional activity in response to androgens.(130) The current study provides additional evidence to support the hypothesis that site-specific AR phosphorylation is of clinical importance in prostate cancer.

It was interesting to observe that phosphorylated AR expression in the cytoplasm was a stronger prognostic factor than nuclear expression. However, presence of cytoplasmic AR is expected as the AR localizes to the cytoplasm in the absence of ligand-binding due to a ligand-regulated nuclear export signal.(124,180) It is suggested that immunohistochemical detection of cytoplasmic AR is an adverse prognostic feature as it may indicate high levels of nuclear receptor, as was the case with glucocorticoid receptors.(160) This is not the first study to observe that cytoplasmic AR expression is a stronger prognostic factor than nuclear expression, cytoplasmic AR expression in patients with negative surgical margins after radical prostatectomy was associated with worse prognosis.(181) Furthermore, the expression of cytoplasmic AR increased with the progression of prostate intraepithelial neoplasia to prostate cancer and from hormone-naïve to castrate-resistant cancer.(181) In addition the subcellular location and activity of AR is likely to be directly influenced by its phosphorylation status. Previous mutagenesis studies investigated the effect on subcellular localization of AR in COS cells. This work demonstrated that in wild type pAR^{S578} cells AR is distributed between the nucleus and cytoplasm indicative of nuclear-cytoplasmic shuttling.(130) However, in cells where the pAR^{S578} site was mutated, AR expression was found exclusively in the nucleus.(130) The current study is in agreement with this work and provides further evidence for the involvement of phosphorylated AR in nuclear-cytoplasmic shuttling in neoplastic prostate tissue.

With reference to the clinical outcome measures, total (nuclear + cytoplasmic) protein expression was observed to be of significance only when expression in at least one subcellular location (nucleus or cytoplasm) was significant. This highlights the importance of analysing protein expression within each subcellular compartment and also within each cell type. For this reason total protein

expression was not analysed in the remaining patient cohorts within the following chapters.

Contrary to previous reports, pERK1/2 did not correlate with any of the AR phosphorylation sites predicted by Scansite.(182) In addition, pERK1/2 was not associated with any clinical outcome measures. Work performed in the host laboratory has previously reported that ERK1/2 is a negative prognostic marker in castrate resistant prostate cancer, therefore activation of the ERK1/2 pathway may be a late event and not associated with hormone-naïve disease.(141) In support of this hypothesis it was previously reported that ERK1/2 expression was low or undetectable in the majority of prostate cancer specimens at diagnosis, however increased with stage, Gleason grade and progression to castrate resistant disease.(132) In the pilot prostate cancer cohort phosphorylated ERK1/2 was not associated with Gleason grade and it is postulated that disease progression may occur via other indirect mechanisms such as phosphorylation of the AR coactivator steroid receptor cofactor 1, and increasing cellular proliferation through AP-1, c-MYC, and NF-κB (139,183-185).

The results suggest that Cdk1 and PKC may phosphorylate serine sites on the AR and demonstrate that they are of clinical significance in prostate cancer. In broad agreement with predictions by Scansite (154), phosphorylation of all putative Ser-Pro target sites on the AR correlated significantly with the expression of Cdk1 or PKC, but not ERK1/2, in at least one cellular location. Not only did phosphorylation of the AR at serine sites correlate with the predicted kinases, both Cdk1 and pCdk1^{161} were also strongly associated with clinical outcome measures. The association of Cdk1 expression in radical prostatectomy specimens with prostate cancer recurrence has been reported previously.(186)

Our study has added to this by observing that high expression of Cdk1, pCdk1[161] and phosphorylation of the putative Cdk1-consensus sites pAR^{S81} and pAR^{S515} were associated reduced disease-specific survival. These results suggest that phosphorylation of the AR by Cdk1 may be of functional importance.

In agreement with previous reports PKC expression correlated strongly with pAR^{S578} expression. Previous site directed mutagenesis work in castrate resistant cell lines has demonstrated that PKC induced phosphorylation is reduced in pAR^{S578} knock down cells.(130) The current study adds to this by suggesting that the link between PKC and AR phosphorylation is also present in hormone naïve prostate cancer tissue and therefore may have important implications in both early and late stages of the disease. Similar site directed mutagenesis studies are necessary in early prostate cancer cell lines in order to confirm this finding.

Through inhibitor studies, it was observed that pAR^{S81} and pAR^{S515} expression is mediated, at least in part, by Cdk1. Previous work has suggested that increased Cdk1 activity is a mechanism for increasing AR expression, stability and cellular proliferation.(136) In the current study although an increase in Cdk1 phosphorylation was observed, AR expression remains stable, however change in expression might take place over a longer time frame than investigated in the current study. Another role of Cdk1, out-with cell cycle progression, may be AR phosphorylation (in particular at serine 515). Thus, Cdk1 may provide a mechanism for accelerating disease progression in hormone-naïve prostate cancer and a novel point for therapeutic intervention.

The phosphorylated AR antibodies were stringently validated using western blot and peptide competition assays. The possibility of cross-reactivity in the usage of phosphospecific antibodies is acknowledged, particularly on a protein such as

AR with multiple phosphorylation sites. Future work would include additional antibody validation via site-directed mutagenesis followed by immunohistochemistry of cell pellets to establish with absolute certainty that the results observed are due to the individual phosphorylation sites.

An obvious limitation of this study is the small sample size and as such the results should be interpreted with caution and future work should include validation in a large independent cohort. However, even with low patient numbers, it has been demonstrated that site specific AR phosphorylation by Cdk1 is of independent prognostic significance. These results are striking in particular when considered that this was a hormone-naïve cohort of patients who subsequently received a variety of treatments (surgery, radiotherapy and hormones) and that, due to small numbers, it was not possible to unpick these groups.

In combination with current diagnostic tools, site specific AR serine phosphorylation may provide a desperately needed prognostic marker to aid treatment decision-making in hormone-naïve prostate cancer patients. This finding has the potential to reduce over-treatment of clinically insignificant disease and prevent delay in treatment of occult aggressive disease. To this end this study was extended to ascertain the clinical significance of site specific AR phosphorylation in prostate cancer patients treated by active surveillance. These patients have low risk disease which is suitable for delayed radical treatment if clinical, biochemical or pathological progression is demonstrated.

As previously stated there are potential complications associated with the use of phosphorylated antibodies. It has previously been demonstrated that use of phosphorylated antibodies is unreliable when comparing the expression levels of

phosphorylated proteins in tumour core biopsies and tumour excision tissue. The limitations associated with phosphorylated antibodies are manageable in the research environment; however; this is not feasible in the routine diagnostic laboratory setting. Therefore it is appropriate to undertake further validation of AR phosphorylation as a maker in active surveillance prostate cancer patients as discussed in the following chapter. However if validated, the host laboratory plan to undertake a study that will enable the barrier of the use of phosphorylated antibodies to be overcome by identifying surrogate markers of pAR to ensure that findings from experimental studies remain suitable for translation into clinically useful biomarkers and drug targets. However this is out with the scope of the current study and funding will be applied for in order for the work to be performed by future team members.

5 Active surveillance prostate cancer cohort results of clinical audit and clinical relevance of clinicopathological variables

Active surveillance for prostate cancer is a deferred treatment approach whereby low risk patients suitable for radical therapy undergo intervention only when biochemical, histological or clinical progression is demonstrated.(72) The aim of active surveillance is to identify and cure those patients with progressive disease and to avoid the complications of radical treatment, such as urinary incontinence, erectile dysfunction and a small risk of death, in those who have clinically insignificant prostate cancer. The recent stage and grade migration of prostate cancer secondary to serum PSA testing has led to the over-diagnosis and over-treatment of clinically insignificant disease. Active surveillance provides a potential solution to this problem and the National Institute for Clinical Excellence (NICE) recommend it as the preferred treatment option for low risk patients in whom radical treatment is suitable.(72) An investigation into the adherence to these guidelines was undertaken in the form of a clinical audit.

5.1 Clinical Audit

A snapshot clinical audit was undertaken in order to evaluate adherence to clinical guidelines regarding the selection and management of active surveillance patients. NICE published clinical guidelines entitled “Prostate Cancer: diagnosis and treatment” in February 2008 and these were used for reference. The selection of patients was examined with regards to the NICE ‘low risk’ criteria illustrated in Figure 5.1. The subsequent management of active surveillance patients was examined with regards to the phase III randomised study of ‘active surveillance versus radical treatment in patients with favourable-risk prostate cancer’ (proSTART) protocol as recommended by

NICE; PSA every 3 months until 2 years from diagnosis and 6 monthly thereafter, TRUS guided biopsy years 1, 4, 7 and 10 following diagnosis and then every 5 years.

Figure 5.1 Selection Criteria for Active Surveillance

NICE LOW RISK CRITERIA FOR PROSTATE CANCER (NICE 2008)
1. Pathological stage T1c 2. ≥10 biopsy cores taken at diagnosis 3. Cancer in less than 50% of the total number of biopsy cores 4. Gleason score 3+3 5. PSA <10 ng/ml 6. PSA density <0.15ng/ml/ml

5.1.1 Selection of active surveillance patients

One hundred and twelve patients with non metastatic organ confined prostate adenocarcinoma at diagnosis, initially treated by AS, were identified. Data was available for all patients, unless otherwise stated. Table 5.1 shows the overall adherence to the NICE selection criteria. Overall just 10% of patients fulfilled all NICE selection criteria.

Table 5.1 Adherence to NICE selection criteria for patients treated by active surveillance

Criteria		Adherence (%)
Pathological Stage	T1c	89.3
Number of biopsy cores taken at TRUS	≥10	27.7
Percentage of cancer in biopsy cores	<50%	64.9
Gleason	3+3	77.7
PSA	<10ng/ml	71.4

83.9% were pathological stage T1c. Of the remainder 12.5% were T1a and 3.6% were T1b with no follow up diagnostic TRUS performed. Of those who had a diagnostic TRUS, 44.6% had <10 biopsy cores taken, 27.7% had ≥10 cores taken

and 27.7% had an undefined number of cores taken. 64.9% had cancer in <50% biopsy cores, 17% not documented, 18.1% had cancer in ≥50% biopsy cores. 77.7% of patients were Gleason 3+3, 17.9% were Gleason 3+4, 3.6% were 4+3 and 0.9% had a Gleason score of 4+4 at diagnosis. 71.4% had serum PSA <10ng/ml at diagnosis, 24.1% had PSA between 10-20ng/ml and in 4.5% of patients PSA was >20ng/ml. PSA density was unavailable for all patients.

5.1.2 Management of active surveillance patients

5.1.2.1 PSA checks

Data was missing for 1 patient. 100% of patients had PSA follow up after diagnosis. 49.5% had 3 monthly PSA checks in the first 2 years following diagnosis. 87.2% of those eligible (n=86) had 6 monthly PSA checks after 2 years.

5.1.2.2 Rebiopsy schedule

Overall 63.4% had a rebiopsy at any time following diagnosis. Two patients were offered but declined rebiopsy at 1 and 4 years respectively, these patients were excluded from the analysis. 10.8% had a rebiopsy at 1 year +/- 30 days after diagnosis. Median time till first rebiopsy was 18 months (IQR 12.4-26.9). Of those eligible (n=32), 3.1% had a rebiopsy at 4 years +/- 30days. Median time till second rebiopsy was 34.3 months (IQR 20.5-50.5).

5.2 Clinical investigation of active surveillance patients

Given the poor adherence to the NICE guidelines regarding the selection and management of active surveillance patients further investigation was conducted in the active surveillance cohort. The effect of clinical and pathological variables on clinical outcome measures was assessed.

5.2.1 Cohort demographics

Median age at diagnosis was 67y (range 48-78). Median follow up was 40.5 months (IQR 30.3–53.8). Median PSA at diagnosis was 7.4ng/ml (IQR 5.1–10.6). 10 (8.9%) patients were type 2 diabetic. 35 (31.3%) were on regular aspirin, 42 (37.5%) were ex-smokers and 15 (13.4%) were current smokers.

Eighteen (16.1%) patients had a trans-urethral resection (TUR) of prostate at diagnosis, the remainder had a trans-rectal ultrasound (TRUS) guided biopsy of prostate. Twenty one (18.8%) patients had evidence of high grade prostate intraepithelial neoplasia (HG PIN) in their diagnostic specimen. Eleven (9.8%) patients had perineural invasion (PNI). Breakdown of Gleason scores is shown in Table 5.2.

Twenty nine (25.9%) patients received or were offered intervention for clinical, biochemical or histological progression, one (0.9%) of whom declined treatment. Six (5.4%) patients were transferred to watchful waiting due to advancing age and/or change in performance status. Median time to intervention was 23.6 months (IQR 15.5–34.4).

Three (10.7%) patients who received treatment intervention experienced biochemical relapse.

Six (6.8%) patients developed metastases; 5 (5.7%) to lymph node and 1 (1.1%) to bone. Median time to development of metastases was 31.5 months (IQR 2.2–38.2).

Table 5.2 Patient Breakdown by Gleason Sum

Gleason Sum	Gleason Grade	No of patients (%)
6	3+3	87 (77.7)
7	3+4	20 (17.9)
	4+3	4 (3.6)
8	4+4	1 (0.9)

5.2.2 Clinicopathological variables related to outcome measures

Table 5.3 details the clinicopathological variables as related to clinical outcome measures.

Table 5.3 Univariate analysis of clinicopathological variables related to clinical outcome measures in first active surveillance cohort

Host/Tumour Variable	Time to Intervention	Time to development of Metastases
Age <70	0.177	0.959
Diabetes	0.881	0.312
Aspirin	0.417	**0.046**
Smoking Status	0.389	0.359
PSA <10ng/ml	**0.014**	0.326
Gleason >6	**0.029**	0.392
PIN	0.622	0.244
PNI	**0.005**	0.753
% of cores positive for cancer	**0.017**	0.708

5.2.2.1 Intervention

Serum PSA level <10ng/ml was associated with increased time to treatment intervention (60 month intervention free survival 71.4% vs 45.5% p=0.014) (Figure 5.2). Gleason >6 was associated with decreased time to treatment intervention (60 month intervention free survival 54.6% vs 66.1% p=0.029) (Figure 5.3).

Figure 5.2 Kaplan Meier plot illustrating PSA at diagnosis and time to treatment intervention

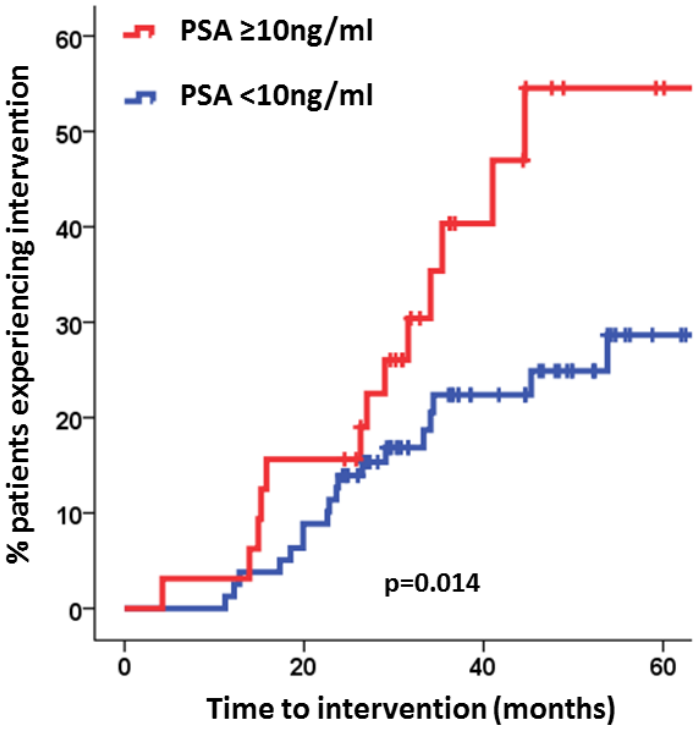
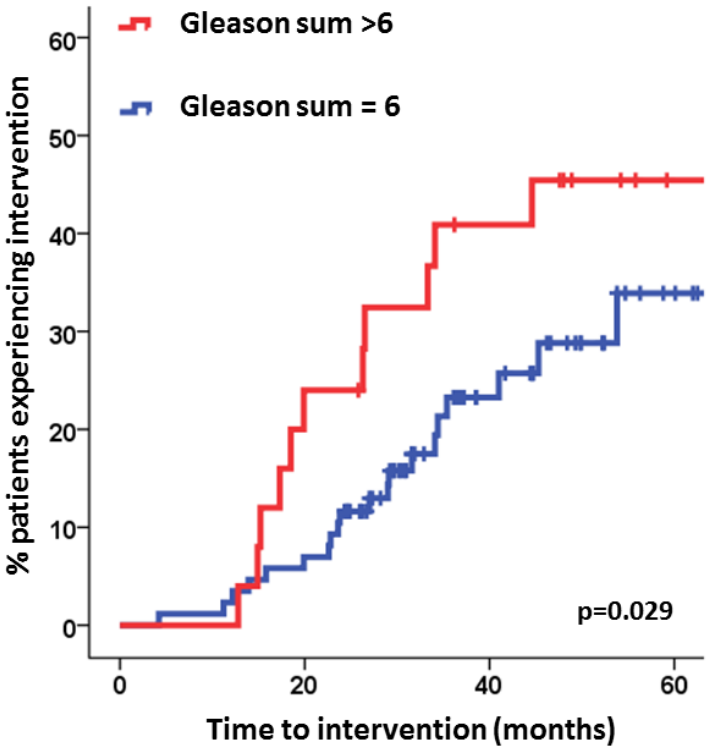
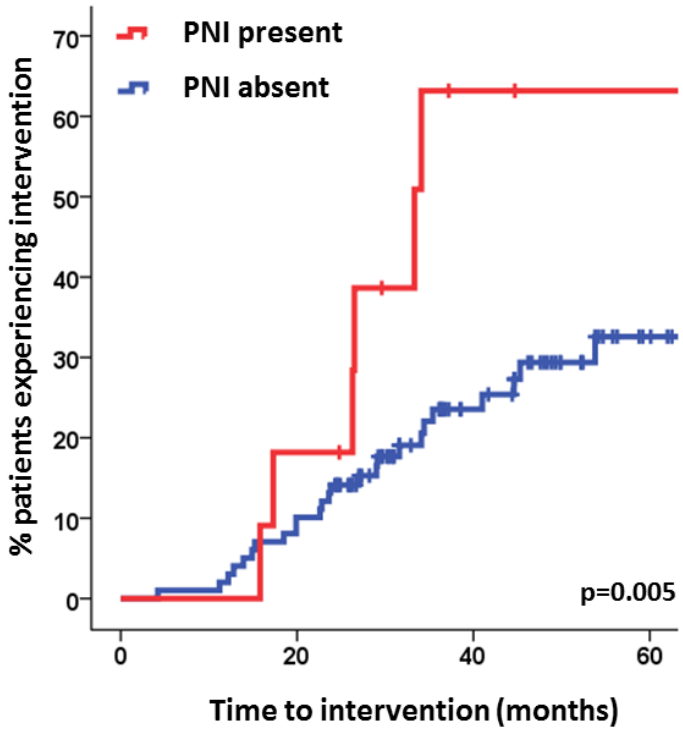


Figure 5.3 Kaplan Meier plot illustrating Gleason sum and time to treatment intervention



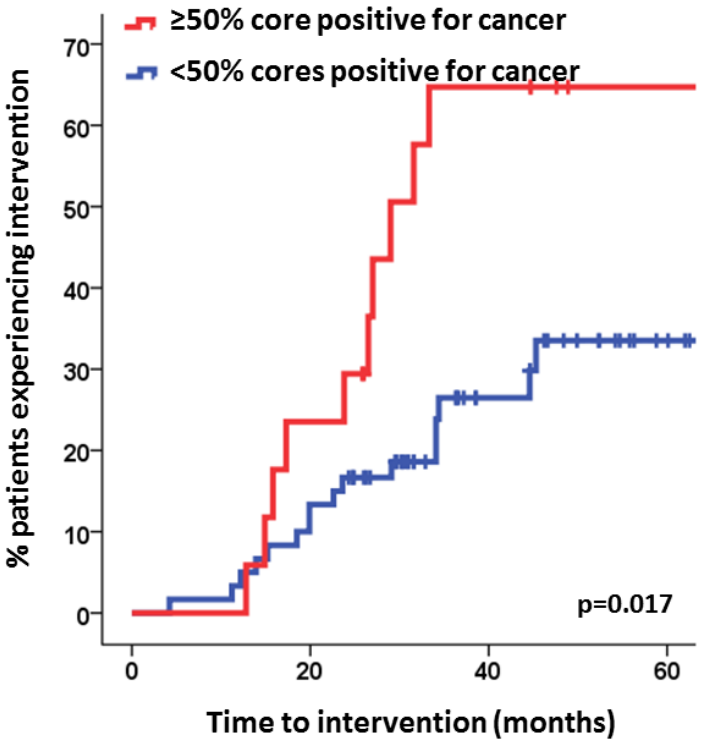
As shown in Figure 5.4 absence of PNI was associated with increased time to treatment intervention (60 month intervention free survival 67.4% vs 36.8% p=0.005).

Figure 5.4 Kaplan Meier plot illustrating PNI and time to treatment intervention



<50% total number of biopsy cores positive for cancer was associated with increased time to treatment intervention (60 month intervention free survival 66.5% vs 35.3% p=0.017) (Figure 5.5).

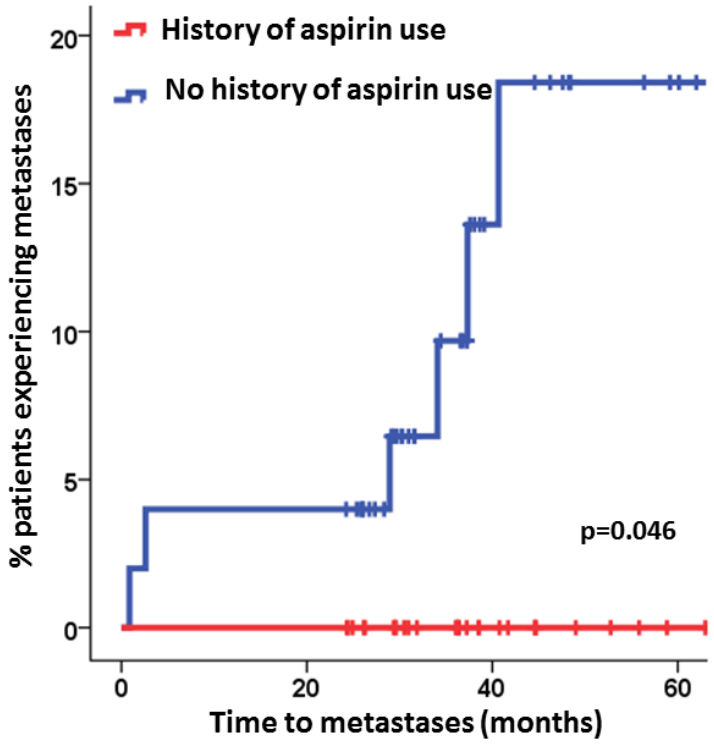
Figure 5.5 Kaplan Meier plot illustrating percentage of total number of biopsy cores positive for cancer and time to treatment intervention



5.2.2.2 Development of Metastases

Aspirin usage was associated with increased time to development of metastases, in fact all patients who developed metastases were not on aspirin (60 month metastases free survival 100% vs 81.6% p=0.046) (Figure 5.6).

Figure 5.6 Kaplan Meier plot illustrating aspirin usage and time to development of metastases



5.3 Discussion

The clinical audit revealed that although 100% of patients had PSA follow up, only 63.4% of patients had a rebiopsy at any time following diagnosis. Rebiopsy has been repeatedly demonstrated to be a strong prognostic indicator of disease progression.(58) Prostate biopsy remains one of the most objective measures of prostate cancer progression. Tumour grade and stage, as determined by prostate biopsy, direct the timing and type of treatment patients with prostate cancer receive. A study of 104 men with low risk prostate cancer treated by AS, who underwent repeat biopsy within 3 months of diagnosis showed 27% were upgraded or up staged. Those up graded/upstaged cases that subsequently underwent radical prostatectomy were significantly more likely to show higher final pathological stage and grade.(58) In addition a study of 186 patients on

active surveillance where 92 underwent at least 1 repeat prostate biopsy, 5-year progression-free probability was 82% for patients with a negative first repeat biopsy compared with 50% for patients with a positive first rebiopsy.(187) Although there is consensus on the importance of rebiopsy for patients on AS, the timing and frequency of such is highly debated and a recent systematic review failed to find sufficient evidence to support any one follow up regime.(188)

Poor adherence to the NICE low risk entry criteria for active surveillance was found. Established clinical parameters such as Gleason score were associated with time to treatment intervention. In addition higher volume disease, as represented by ≥50% of the total number biopsy cores containing tumour, was associated with shorter time to disease progression. PSA <10ng/ml at diagnosis was also found to be associated with longer time to treatment intervention. PSA has repeatedly been proven to have poor specificity for prostate cancer with 15.2% of men with a "normal" level (<4.0ng/ml) demonstrating cancer on biopsy.(67) This is further illustrated by the recent abandonment of the PSA screening programme in North America. The study cohort was from an unscreened population and comprised of patients with a high clinical suspicion for prostate cancer which may account for the findings of the importance of PSA in relation to clinical outcome.

Rate of treatment intervention was 25.9% this is similar to other recent larger studies.(189,190) The relatively high rate of biochemical recurrence and development of metastases may be accounted for by the inclusion of patients who did not conform to the NICE low risk criteria. In addition two of the patients who developed metastases did so less than 3 months post diagnosis. It

is therefore likely that the disease had already micro-metastasised at presentation. However the remainder of patients all underwent delayed treatment following a period of surveillance, thereby creating an opportunity for disease progression and spread. This is clearly the major problem with active surveillance and at present there are no consistently reliable diagnostic tools to differentiate aggressive cancers from those which are slow growing and clinically insignificant.

The host-tumour interaction is becoming recognised as one of the key influences on tumour progression and spread.(191) PNI is the invasion of nervous structures by tumour cells. It can be seen as one of the main "highways" by which the tumour-host interface is connected and thereby a recognised mechanism by which cancer cells spread beyond the prostate.(192) The prognostic significance of PNI in prostate cancer has been widely studied and debated.(193) The majority of these studies involve the use of PNI at diagnosis to predict outcome in the final pathological specimen at radical prostatectomy.(194-196) The current study presents a unique "real life" cohort of prostate cancer patients that have been undergone a MDT assessment and active surveillance has been selected as the initial treatment strategy. Time to treatment intervention has been used as a surrogate of disease progression. In addition development of metastases was utilised as a clinical end point and the results presented are similar to others.(197) The pathological specimens of those patients who underwent radical prostatectomy were not available for review. However, they are few in number and the overarching aim of this study was solely to assess the prognostic significance of the features of the diagnostic tumour specimen. An obvious limitation of the current patient cohort is the small size and relatively short follow up time in the context of the long natural history of prostate

cancer. However despite this short follow up length one quarter of patients underwent treatment intervention for disease progression and this is in agreement with previous work.(197)

Interestingly, no patients who took regular aspirin developed metastases. This correlates with current thinking that controlling the host's inflammatory response (e.g. via aspirin) can modulate the host-tumour interface likely via inhibition of cyclo-oxygenase (COX) enzymes in order to prevent tumour growth, angiogenesis and subsequent spread.(198)

Of course there are inherent difficulties with the usage of PNI as a prognostic marker. Firstly there is the problem of intra-observer variability between reviewing pathologists.(199) This can be reduced via the central review of all tissue specimens and the addition of immunohistochemical staining in order to enhance nerve fibres.(199) Secondly the small amount of tissue involved in TRUS guided prostate biopsies limits the amount of nerve tissue that is included. However the counterargument for this is that if there is sufficient volume of PNI invasion to be apparent on TRUS then it must be of clinical significance.

A recent study by Al-Hussain retrospectively analysed 313 biopsy specimens of prostate cancer patients who subsequently underwent radical prostatectomy but who would have met the criteria for AS on biopsy.(194) They found no difference in outcome at radical prostatectomy in patients with PNI on biopsy. The current study provides a clearer picture of how PNI can be utilised in a modern day real active surveillance cohort. Treatment intervention and development of metastases were utilised as clinical end points, both are clear markers of disease progression. The Al-Hussain study examined only

pathological markers at radical prostatectomy as end points (194) and as such the current study is deemed to be more clinically relevant and patient centred.

Clinical staging is crucial in order to select the most appropriate management strategy for patients with prostate cancer. This is particularly pertinent to those patients who are thought to have low risk curable disease in whom treatment is delayed during a period of active surveillance until evidence of progression is demonstrated. This strategy works well for most patients, avoiding the risks and side effects of radical therapy, however some patients experience progression from curable to incurable disease. The current clinical staging and risk stratification tools are therefore inadequate and the investigation into the use of additional host and/or tumour factors could yield rapid patient benefits.

Further work is necessary in order to consider how PNI can be optimally utilised in established prognostic algorithms for prostate cancer patients on active surveillance.

6 Active surveillance prostate cancer cohort clinical significance of protein expression

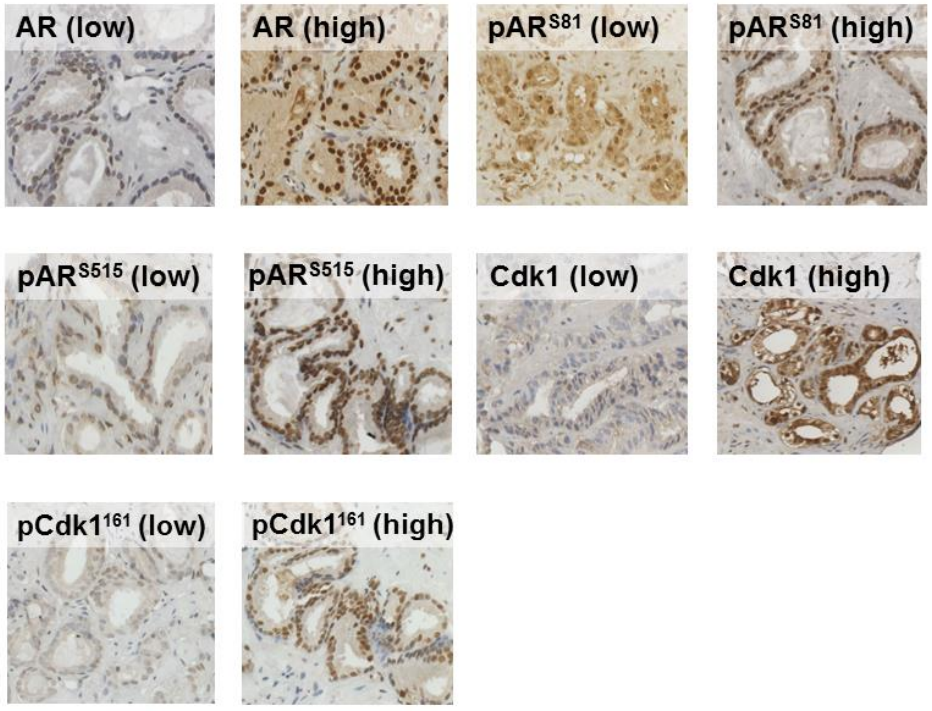
The aim of active surveillance is to identify and cure those patients with progressive disease and to avoid the risks of over treatment in those who have clinically insignificant prostate cancer. Active surveillance therefore provides a potential solution to the problem of over diagnosis and over treatment. However, although these patients are carefully selected at diagnosis and subjected to intense follow up consisting of regular repeat biopsies and serum PSA tests, some patients experience disease progression and require treatment intervention. The delay in treatment during the period of surveillance may result in disease progression after which the disease is no longer curable.

As described in the previous chapter patients are selected for active surveillance via strict criteria including host and tumour factors.(72) Despite this careful selection, currently there is no accurate way to differentiate indolent from occult aggressive disease at diagnosis. In the pilot prostate cancer cohort pAR^{S81} and pAR^{S515}, driven by Cdk1/$pCdk1^{161}$, were independent predictors of outcome in patients with PSA ≤20ng/ml at diagnosis. The aim of the current study was to determine the prognostic significance of AR, AR phosphorylated at serine 81 and 515 and Cdk1/$pCdk1^{161}$ expression for disease progression in a cohort of prostate cancer patients whom, following discussion at a multidisciplinary team meeting, were initially treated by active surveillance. Study outcome measures were treatment intervention (a surrogate for disease progression) and development of metastases.

6.1 Protein expression

AR, AR phosphorylated at Ser-81 and Ser-515, Cdk1 and pCdk1^{161} protein expression was analysed in the AS cohort. Tissue was available for 51 patients. Expression of all proteins was observed at varying levels in the cytoplasm and nucleus of epithelial cells (Figure 6.1) in both cancer and non-cancer areas.

Figure 6.1 Example high/low protein immunohistochemical staining in first active surveillance cohort



Protein expression was found to be heterogeneous throughout. Only protein expression observed in the tumour cells was scored. ICCCs were performed to verify consistency between scorers and all values were >0.80 and are shown in Table 6.1. Scatter plots for each antibody were used to display this data and to confirm there was no bias between scorers Bland Altman plots were constructed.

Table 6.1 ICCC scores for protein expression analysis in first active surveillance cohort

Protein	Nucleus	Cytoplasm
Cdk1	0.977	0.983
pCdk1^{161}	0.953	0.937
Ki67	0.998	-
AR	0.821	0.946
pAR^{S81}	0.951	0.882
pAR^{S515}	0.923	0.986

Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis. Median protein histoscore expression levels are shown in Table 6.2. Median cell counts for Ki67 protein were 0% range 0-0.5%.

Table 6.2 Protein expression levels in first active surveillance cohort

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
AR	Nucleus	150	117.5-201.3
	Cytoplasm	100	70-132.5
pAR^{S81}	Nucleus	180	135-218.3
	Cytoplasm	120	80.7-180
pAR^{S515}	Nucleus	200	155-217.5
	Cytoplasm	65	40-100
Cdk1	Nucleus	102.5	37.5-130
	Cytoplasm	120	85-140
pCdk1^{161}	Nucleus	143.8	83.8-183.8
	Cytoplasm	97.5	48.3-122.5

6.2 Protein expression related to clinicopathological factors

Table 6.3 displays protein expression as related to clinicopathological factors as assessed by the chi square test. High Cdk1 nuclear expression was associated with older age at diagnosis, p=0.004. High cytoplasmic Cdk1 expression was associated with a positive smoking history, p=0.021. High Ki67 expression was

associated with higher PSA at diagnosis and higher percentage of cores positive for cancer (p=0.033 and 0.019 respectively). High nuclear Cdk1 expression was associated with a greater volume of cores positive for cancer, p=0.017.

Table 6.3 Chi squared analysis of high/low protein expression as related to clinicopathological variables in first active surveillance cohort

Proteins		Clinicopathological Variables								
		Age (<70 v ≥70yrs)	**DM2** (presence v absence)	**Aspirin Usage** (yes v no)	**Smoking History** (non- v ex- v smoker)	**PSA at Diagnosis** (<10 v ≥10ng/ml)	**Gleason** (≤6 v >6)	**PIN** (yes v no)	**PNI** (yes v no)	**%Cores Positive for Cancer** (<50 v ≥50%)
AR	Nuclear	0.222	0.564	0.199	0.081	0.593	0.767	0.570	0.851	0.760
	Cytoplasmic	0.367	0.880	0.136	0.417	0.462	0.745	0.617	0.836	0.631
pAR^{S81}	Nuclear	0.980	0.564	0.590	0.820	0.792	0.980	0.570	0.870	0.888
	Cytoplasmic	0.689	0.716	0.237	0.514	0.632	0.689	0.304	0.666	0.574
pAR^{S515}	Nuclear	0.980	0.192	0.917	0.824	0.746	0.522	0.830	0.570	0.531
	Cytoplasmic	0.689	0.639	0.917	0.236	0.487	0.408	0.439	0.245	0.835
Cdk1	Nuclear	**0.004**	0.917	0.916	0.347	0.304	0.677	0.401	0.658	**0.017**
	Cytoplasmic	0.210	0.348	0.429	**0.021**	0.968	0.531	0.328	0.555	0.878
$pCdk1^{161}$	Nuclear	0.120	0.519	0.936	0.690	0.763	0.351	0.422	0.713	0.481
	Cytoplasmic	0.120	0.519	0.936	0.690	0.365	0.120	1.000	0.13	0.958
Ki67	Nuclear	0.488	0.452	0.142	0.052	**0.033**	0.601	0.152	0.284	**0.019**

6.3 Protein expression related to clinical outcome measures

Table 6.4 displays the univariate analysis using Kaplan meier curve and log rank test with regards to protein expression as related to the clinical outcome measures.

Table 6.4 Univariate analysis of protein expression as related to clinical outcome measures in first active surveillance cohort

Protein		Time to intervention	Time to development of metastases
AR	Nucleus	0.506	0.480
	Cytoplasm	**0.021**	0.480
pAR^{S81}	Nucleus	0.490	0.808
	Cytoplasm	0.488	0.092
pAR^{S515}	Nucleus	0.384	0.225
	Cytoplasm	0.806	0.225
Cdk1	Nucleus	0.872	0.808
	Cytoplasm	0.851	0.225
$pCdk1^{161}$	Nucleus	0.925	0.317
	Cytoplasm	0.490	0.317
Ki67	Nucleus	**0.043**	-

High total AR expression in the cytoplasm was associated with shorter time to treatment intervention, p=0.021 (Figure 6.2). In addition high proliferation index, represented by high Ki67 expression, was also associated with shorter time to treatment intervention, p=0.043 (Figure 6.3)

Figure 6.2 Kaplan Meier plot illustrating total AR cytoplasmic expression and time to treatment intervention in first active surveillance cohort

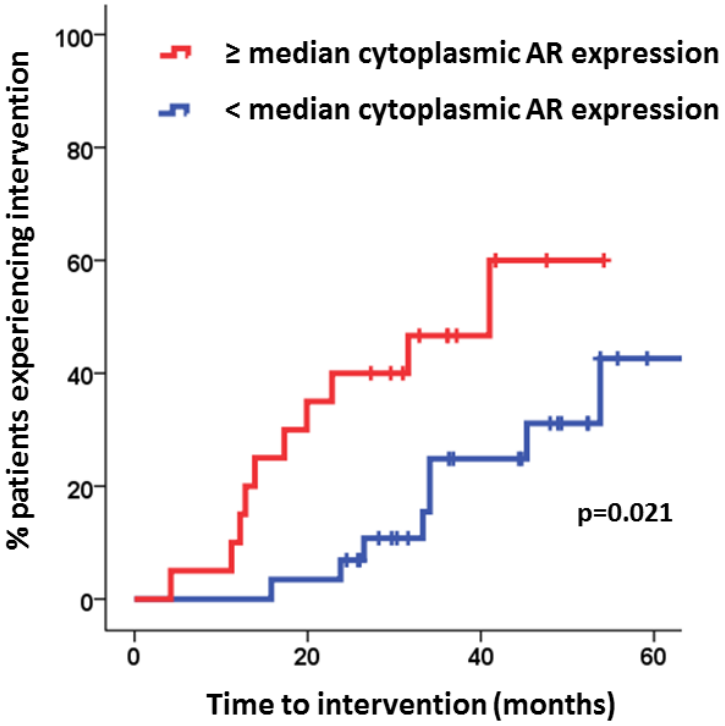
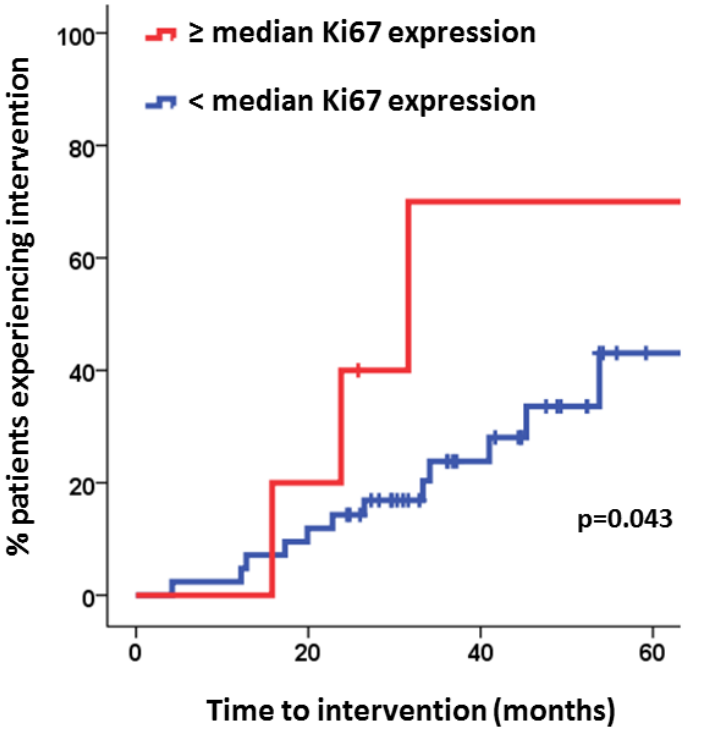


Figure 6.3 Kaplan Meier plot illustrating Ki67 expression and time to treatment intervention in first active surveillance cohort



6.4 Association of candidate kinases to phosphorylated AR sites

Once the clinical significance of protein expression had been analysed, the association between candidate kinases and AR phosphorylation was then investigated in order to establish whether the predicted kinase was observed to correlate with AR phosphorylation in the clinical samples (Table 6.5).

Table 6.5 Associations between candidate kinases and androgen receptor phosphorylation sites in active surveillance cohort

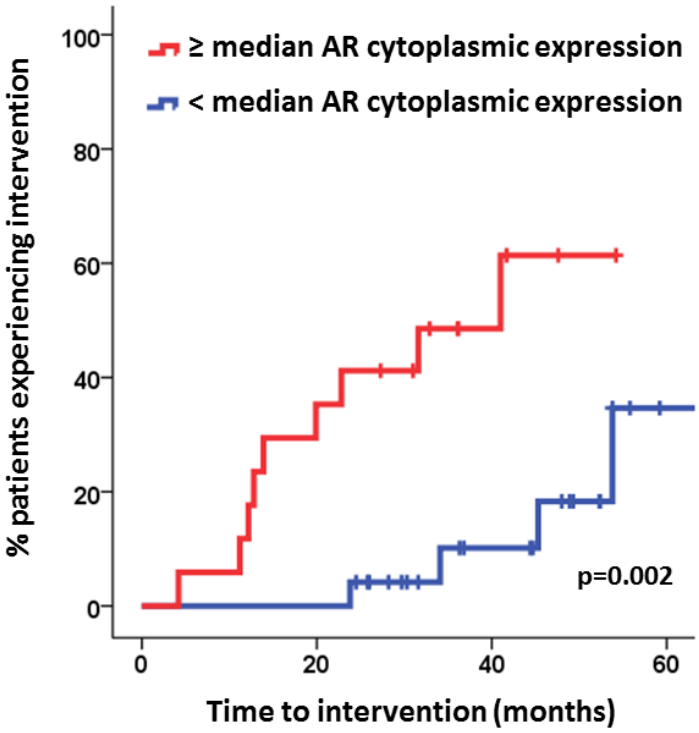
	Candidate Kinases					
AR phosphorylation sites			**Cdk1**		**pCdk1^{161}**	
			Nuclear	**Cytoplasmic**	**Nuclear**	**Cytoplasmic**
Ser-81	**Nuclear**	**C.C.**	0.192	-0.101	0.256	0.100
		p value	0.656	0.656	0.262	0.666
	Cytoplasmic	**C.C.**	0.371	**0.643**	0.429	**0.662**
		p value	0.090	**0.001**	0.052	**0.001**
Ser-515	**Nuclear**	**C.C.**	0.261	-0.030	0.100	0.061
		p value	0.229	0.892	0.659	0.786
	Cytoplasmic	**C.C.**	-0.032	-0.016	0.181	0.275
		p value	0.884	0.941	0.419	0.215

High cytoplasmic Cdk1 and pCdk1^{161} were associated with high expression of pAR^{S81}. High nuclear pCdk1^{161} expression trended towards an association with pAR^{S81} cytoplasmic expression. Neither Cdk1 or pCdk1^{161} were associated with pAR^{S515} expression in either the cytoplasm or the nucleus.

6.5 Multivariate analysis

Significant univariate results were included in a backwards conditional cox-regression model to determine which, if any, factors were independently significant with regards to time to treatment intervention. Both PNI (p=0.003, HR 8.6 (95% CI 2.1-35.7)) and total cytoplasmic AR expression (p=0.021, HR 4.6 (95% CI 1.3-16.8)) were independently associated with time to treatment intervention. As these variables were deemed independent it was investigated whether total AR expression in the cytoplasm could further inform on patients with or without PNI noted on diagnostic pathological specimen. In patients with presence of PNI total AR expression was not significant in predicting time to treatment intervention (p=0.494). However in patients without PNI high total cytoplasmic AR expression in the diagnostic specimen conferred a shorter time to treatment intervention than those with low expression (p=0.002, HR 5.4 (95% CI 1.6-17.9)) 60 month progression free survival 38.6% vs 65.3%) (Figure 6.4).

Figure 6.4 Kaplan Meier plot illustrating total AR cytoplasmic expression in patients without perineural invasion and time to treatment intervention in first active surveillance cohort



6.6 Verification of results in a second cohort

A second cohort of active surveillance patients was established prospectively in order to verify the results seen in the first retrospective active surveillance cohort. The second cohort was demographically comparable to the first; median age at diagnosis 68y (IQR 63-72), median PSA at diagnosis 7.0ng/ml (IQR 4.5-10.2). Median length of follow up was 30.0 months (IQR 19.1-49.6). 25.7% (n=27) patients underwent treatment intervention. Median time to treatment intervention was 21.9 months (IQR 18.7-35.8). Median expression levels for proteins of interest in the second cohort were also comparable to the first and are shown in Table 6.6. All ICCCs were >0.80.

Table 6.6 Median histoscores of protein expression in second active surveillance cohort

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
AR	Nucleus	120	92.5-165
	Cytoplasm	80	50-100
pAR^{S81}	Nucleus	185	138.8-205
	Cytoplasm	100	80-126.2
pAR^{S515}	Nucleus	220	200-250
	Cytoplasm	100	80-120
Cdk1	Nucleus	120	90-170
	Cytoplasm	145	120-175
$pCdk1^{161}$	Nucleus	180	150-215
	Cytoplasm	120	100-150
Ki67	Nuclear	1	1-2

6.6.1 Univariate analysis of clinical variables in second active surveillance cohort

The univariate analysis of clinical variables in the second active surveillance cohort, with regards to treatment intervention, is shown in Table 6.7.

Table 6.7 Univariate analysis of clinicopathological variables and time to intervention in second active surveillance cohort

Clinicopathological Variable	Time to intervention
Age <70	0.823
PSA <10ng/ml	**0.015**
Gleason >6	0.100
PIN	0.361
PNI	0.443
% of cores positive for cancer	**<0.001**

As demonstrated in the first active surveillance cohort PSA ≥10ng/ml at diagnosis was associated with shorter time to treatment intervention HR 2.7 (95% CI 1.2-6.3), p=0.015 (Figure 6.5) in the second cohort. In addition, ≥50% of cores positive for cancer was associated with shorter time to treatment intervention in the second cohort as previously demonstrated in the first HR 17.5 (95% CI 2.0-157.0), p<0.001 (Figure 6.6).

Figure 6.5 Kaplan Meier plot illustrating PSA at diagnosis and time to treatment intervention in second active surveillance cohort

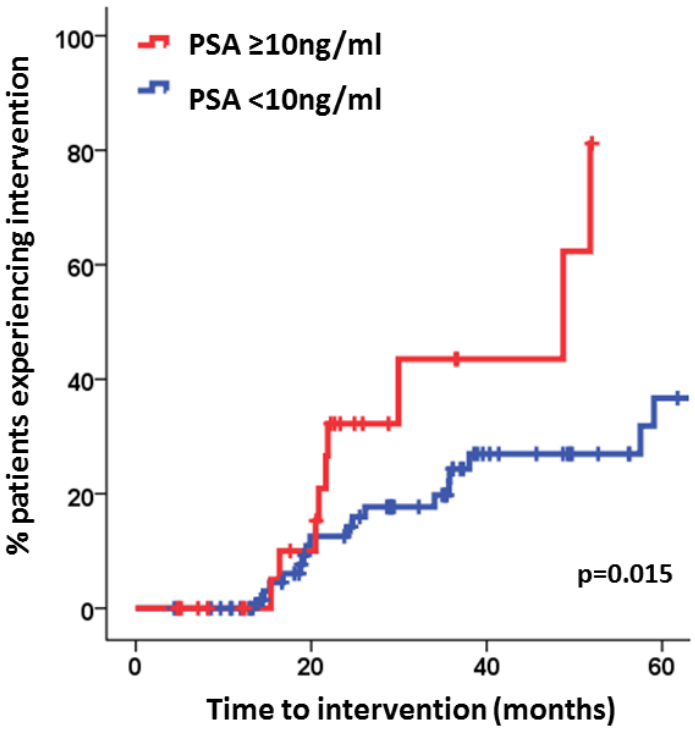
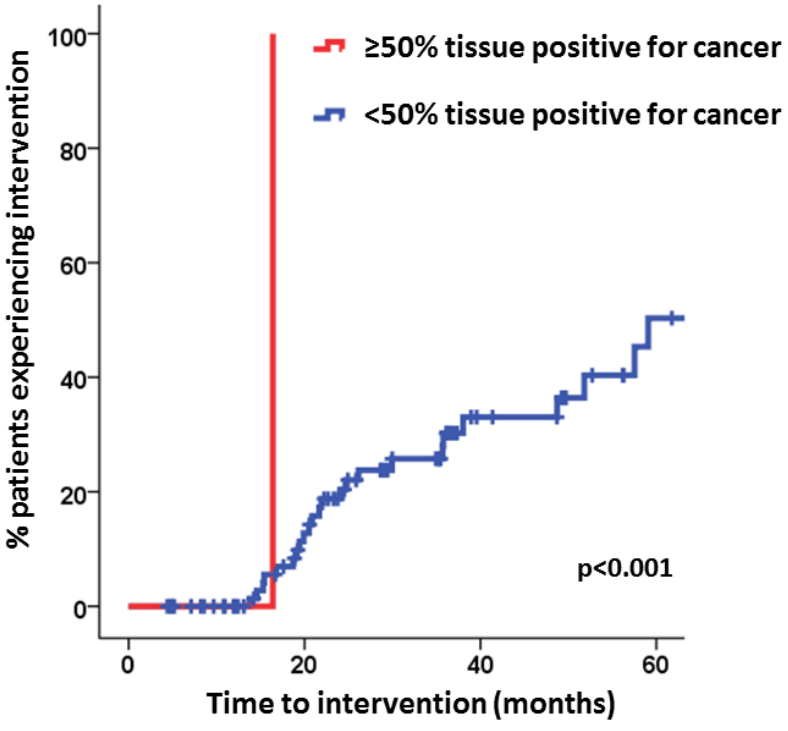


Figure 6.6 Kaplan Meier plot illustrating percentage of cores positive for cancer and time to treatment intervention in second active surveillance cohort



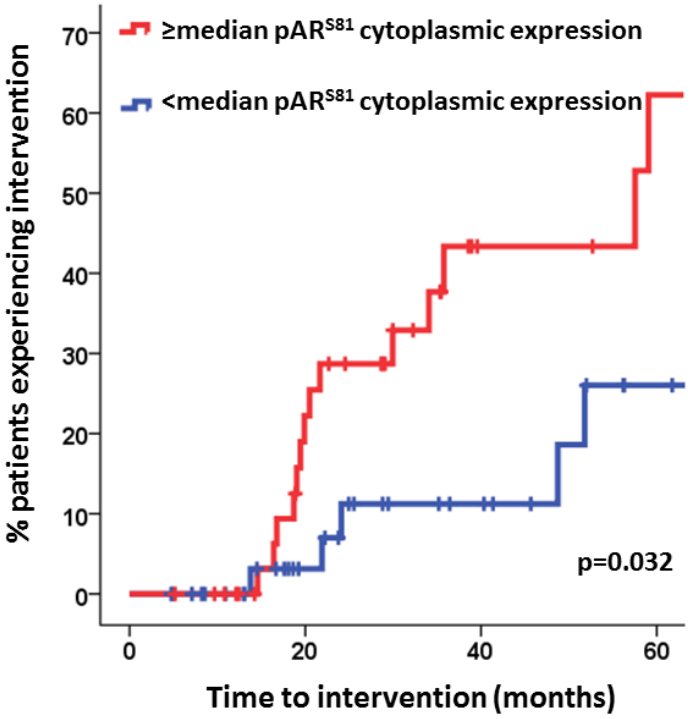
The results from univariate analysis of protein expression in the second active surveillance cohort are shown in Table 6.8.

Table 6.8 Univariate analysis of protein expression and time to intervention in second active surveillance cohort

Protein		Time to Intervention
AR	Nuclear	0.170
	Cytoplasm	0.654
pAR^{S81}	Nuclear	0.748
	Cytoplasm	**0.032**
pAR^{S515}	Nuclear	0.510
	Cytoplasm	0.079
Cdk1	Nuclear	0.443
	Cytoplasm	0.409
$pCdk1^{161}$	Nuclear	0.074
	Cytoplasm	0.178
Ki67	Nuclear	0.757

The relationships between high total cytoplasmic AR and high Ki67 expression and shorter time to treatment intervention were not replicated in this cohort. However, high cytoplasmic pAR^{S81} expression was associated with shorter time to treatment intervention, HR 2.8 (95% CI 1.1-7.3) (Figure 6.7).

Figure 6.7 Kaplan Meier plot illustrating total AR cytoplasmic expression and time to treatment intervention in second active surveillance cohort



Similar to results seen in the pilot prostate cancer cohort high cytoplasmic pAR^{S515} expression and high pCdk1^{161} expression in the nucleus both trended towards an association with shorter time to treatment intervention but did not reach clinical significance (p=0.079 and p=0.074 respectively).

Multivariate analysis of significant protein and clinicopathological variables did not yield any independently significant variables with regards to time to treatment intervention in the second active surveillance cohort.

The significant relationship of cytoplasmic total AR expression in patients without PNI in the first cohort was also reanalysed in the second. Unfortunately the relationship was not significant in the second active surveillance cohort for either cytoplasmic AR (p=0.552) or pAR^{S81} expression (p=0.378).

6.6.2 Kinases related to AR phosphorylation in the second active surveillance cohort

Table 6.9 displays the correlation between protein expression of pAR sites and candidate kinases. As in the first active surveillance cohort cytoplasmic pCdk1^{161} expression was associated with pAR^{S81} expression in the cytoplasm and nuclear pCdk1^{161} trended towards an association. In contrast to the first active surveillance cohort, but in line with the pilot prostate cancer cohort, cytoplasmic Cdk1 expression was associated with cytoplasmic pAR^{S515} expression and trended towards an association with nuclear pAR^{S515}. In addition pCdk1^{161} trended towards an association with both nuclear and cytoplasmic pAR^{S515} expression. These results are interesting and may reflect the larger size of the second active surveillance cohort, which is in line with the pilot prostate cancer cohort, enabling the relationships to be uncovered.

Table 6.9 Pearson's correlation coefficients between pAR sites and candidate kinases in the second active surveillance cohort

	Candidate Kinases					
AR phosphorylation sites			**Cdk1**		**pCdk1^{161}**	
			Nuclear	**Cytoplasmic**	**Nuclear**	**Cytoplasmic**
Ser-81	**Nuclear**	**C.C.**	0.285	0.058	0.215	0.118
		p value	0.012	0.617	0.062	0.310
	Cytoplasmic	**C.C.**	0.149	0.241	0.309	**0.451**
		p value	0.197	0.035	0.007	**<0.001**
Ser-515	**Nuclear**	**C.C.**	0.172	0.314	0.318	0.343
		p value	0.131	0.005	0.005	0.002
	Cytoplasmic	**C.C.**	0.185	**0.418**	0.301	0.397
		p value	0.103	**<0.001**	0.008	<0.001

6.7 Discussion

The results presented have highlighted the clinical significance of AR and AR serine phosphorylation in prostate cancer patients treated by active surveillance.

Firstly the median expression levels of the proteins analysed are similar in both the first and second cohort. This adds weight to the comparability of the two cohorts and the quality and consistency of the immunohistochemical staining. Interestingly the expression levels of almost all proteins are higher than those in the pilot prostate cancer cohort and the BPH cohort. The single exception is Cdk1 staining in the first active surveillance cohort is slightly less intense than that observed in the BPH cohort, although still comparable. The differences in staining between the pilot prostate cancer cohort and the active surveillance cohorts confirms the hypothesis that these groups are quite separate with a molecularly different profile and therefore should be managed as such in the clinical setting.

In the first cohort high Cdk1 nuclear expression was associated with older age at diagnosis. This is similar to Cdk1 stromal expression in the BPH cohort. The reasons for this relationship are unclear and there is no previous work regarding chronological variation in Cdk1 expression levels. It may be that the inverse relationship with Cdk inhibitor $p27^{(Kip1)}$ and age in BPH (200) also crosses over to early prostate cancer patients which would explain this finding in active surveillance patients but not in the pilot prostate cancer cohort.

High cytoplasmic Cdk1 expression was associated with a positive smoking history. A recent study observed a greater risk of prostate cancer in smokers with

homozygote variant genotype and heterozygous mutant genotype of the Cdk inhibitor p21.(201) This suggests that Cdk inhibitor genotype may have an impact on cell cycle control induced by DNA damage caused by carcinogens in tobacco smoke.(201) This may translate into deregulation of Cdk driven cell cycle progression and may explain the findings in the current study.

As expected high proliferation index was associated with higher PSA at diagnosis and higher percentage of cores positive for cancer. Cell cycle deregulation is common in human cancer and as a key regulator of cell cycle progression it was unsurprising to observe that high nuclear Cdk1 expression was associated with a greater volume of cores positive for cancer.

The Ki-67 protein is well known and widely used to assess the tumour proliferation rate. In fact in breast cancer Ki67 tumour labelling is recommended when choosing the appropriate treatment.(202) In the current study highly proliferating tumours were found to have a shorter time to disease progression in the first cohort. Ki67 has been previously demonstrated to be an independent predictor of response to surgical treatment of prostate cancer.(203,204) In particular Zellweger and colleagues analysed 279 pre-teatment prostate biopsies in which high Ki67 labelling index was an independently predictive factor of biochemical relapse in low-risk disease.(205) High Ki67 expression has also been shown to be a predictor of biochemical recurrence, development of metastases and disease specific survival in prostate cancer patients treated with radical radiotherapy.(206-209) With immediate relevance to the current study in 2009 Berney and colleagues (210) analysed the prognostic significance of 693 cases of conservatively managed localised prostate cancer diagnosed by TUR. They found Ki67 to be an independent prognostic

factor of disease specific and overall survival.(210) However when patients were grouped by Gleason score (<7 vs ≥7), the independent prognostic significance of Ki67 was not maintained for disease specific survival in those patients with Gleason <7.(210) When the same research group analysed similar cases diagnosed by needle biopsy in 2013 they found once again that Ki67 was an independent prognostic factor of disease specific survival, although assessment in low risk patients was not undertaken.(211) The current study utilised the diagnostic prostate specimen of which 89.3% were TRUS biopsies. This represents a contemporary prostate cancer cohort and may account for the differences seen in low risk patients in Berney's 2009 study.(210) In addition the current study includes patients only treated by active surveillance, however Berney's studies include localised prostate cancers some of which may be high risk and treated with watchful waiting. To add weight to this argument a previous study conducted in needle biopsies of 60 active surveillance patients observed that high Ki67 expression was an independent determinant of shorter time to treatment intervention.(212) These results were confirmed in the first active surveillance cohort. However, the significant results related to Ki67 seen in the first cohort were not replicated in the second. Differences in pre-analytical factors may explain these results as the two active surveillance cohorts were from two separate health boards utilising different pathology laboratories. Several studies have found that certain pre-analytical factors can decrease Ki67 expression; significant (overnight) delay in time to fixation, freezing of the specimen for frozen section analysis before fixation, any fixative other than neutral buffered formalin and prolonged storage of tissue on glass slides (rather than paraffin embedded blocks).(213-216) Regardless, further work is required in a large prospectively collected cohort in order to clarify the relationship between Ki67 expression and time to treatment intervention.

Both PNI and total AR expression in the cytoplasm were independent variables for time to treatment intervention. AR expression has been assessed previously with regards to treatment outcome in prostate cancer patients. In contrast to the current study work involving 62 hormone sensitive prostate cancer patients who underwent radical prostatectomy low cytoplasmic total AR expression was associated with increased risk of biochemical relapse.(181) However, in those patients with negative surgical margins high cytoplasmic total AR expression was associated with decreased recurrence free survival.(181) Similarly in 211 radical prostatectomy specimens low AR expression was associated with a worse prognosis for relapse free survival.(217) However, high AR nuclear expression in 551 prostate cancer patients treated by radical prostatectomy was an independent predictor of shorter time to biochemical relapse.(218) Similarly high nuclear AR expression was associated with decreased disease specific survival in 104 patients with castrate resistant disease.(219) The reasons for these varying results are unclear however it may relate to disease stage and treatment type. There are no previous studies examining expression levels of AR in active surveillance patients. As discussed in the previous chapter presence of PNI was associated with shorter time to treatment intervention. In patients without PNI total AR expression was found to be clinically significant in predicting time to treatment intervention. However these results were not replicated in the second active surveillance cohort. Verification in a large prospective clinical trial is required and if successful these results may be translated into clinical practice directing the early radical treatment of patients initially considered suitable for active surveillance.

In the second active surveillance cohort high cytoplasmic pAR^{S81} expression was associated with shorter time to treatment intervention. In the pilot prostate

cancer cohort cytoplasmic expression trended towards a relationship with disease specific survival. Ser-81 is the most frequently phosphorylated site on the AR in response to androgen binding.(136) Phosphorylation of AR at Ser 81 is thought to play important roles in AR transactivation, cellular localization and stability as well as cell proliferation.(117,136) Interestingly these results were not verified in the first active surveillance cohort and may reflect the larger number of cases in which tissue was available in the second cohort and/or the prospective nature of the second cohort. pCdk1^{161} expression was correlated with pAR^{S81} expression in the cytoplasm in both cohorts. This is consistent with both the pilot prostate cancer cohort and previous work which has shown that activation of Cdk1 by cyclin B increases AR phosphorylation.(136)

Interestingly, in the second active surveillance cohort high cytoplasmic pAR^{S515} expression trended towards significance with regards to shorter time to treatment intervention. This was not seen in the first active surveillance cohort. The relevance of this is unclear and further work is required in order to establish the clinical application of the significant results seen in the pilot prostate cancer cohort in active surveillance patients.

Similarly the relationship between Cdk1/pCdk1^{161} and AR phosphorylation at Ser-515 remains unclear. The positive results in the second cohort were not replicated in the first. A prospectively collected large cohort would unpick these results in combination with further mechanistic cell line work.

This work clearly demonstrates that AR and phosphorylation of AR at serine residues by Cdk1/pCdk1^{161} has some clinical relevance in prostate cancer patients treated by active surveillance, however these results require validation as there are discrepancies between both cohorts, therefore a study performed

on a large multi centre prospective cohort would conclusively demonstrate if these biomarkers are worth pursuing for clinical practice.

7 Benign prostate hyperplasia and clinicopathological factors

Benign prostatic hyperplasia (BPH) is one of the most common diseases in elderly men. Autopsy studies have demonstrated a prevalence of 50% in men aged 50-60 years and 90% over 80 years.(1) BPH can result in significant patient morbidity in the form of lower urinary tract symptoms (LUTS) and complications such as acute urinary retention (AUR). Medical therapy for BPH often fails and surgical intervention is required. A subset of men have ongoing symptoms despite surgical intervention and require further treatment with medication or repeat transurethral resection (TUR) of prostate operation. Currently there is no way to predict the best time to offer surgical intervention or identify which patients will benefit from such a procedure.

BPH has repeatedly been linked with clinicopathological factors such as type 2 diabetes (DM2), cardiovascular disease (CVD), obesity and inflammation. However the significance of these relationships is highly debated and the influence on clinical outcome or response to treatment has not been previously investigated. The aim of this study was to assess, in a cohort of patients with histologically proven BPH, the prognostic use of classical clinicopathological factors and both (novel) systemic and pathological factors not currently employed clinically in BPH, but demonstrated to have diagnostic utility in other diseases.

7.1.1 Cohort demographics

Six hundred and seventy eight patients were identified of which 336 had clinical data and pathological tissue available for analysis. Cohort demographics for the 336 patients are shown in Table 7.1. Median length of follow up for these

patients was 8.2y (IQR 6.2–11.9). Median time to failure of surgical management following primary TUR of prostate operation was 7.2y (IQR 5.4-10.1), experienced by 37.1% of patients. Median time to postoperative AUR occurring beyond 30 days following primary TUR was 7.8y (IQR 6.1-11.8), experienced by 10.2% of patients. Reoperation rate was almost a quarter (24.4%) and median time to reoperation was 7.3y (IQR 5.7-10.3).

Table 7.1 Demographic information BPH cohort

Classical clinicopathological variables		Patients (%)	Patients (n)
Age	<70 years	53.3	179
	≥70 years	46.7	157
CVD	Yes	49.1	165
	No	50.9	171
DM2	Yes	16.9	57
	No	83.1	279
BMI	Normal	32.1	108
	Overweight	44.4	149
	Obese	23.5	79
Aspirin usage	Yes	53.5	179
	No	46.5	157
Smoking history	Non smoker	43.5	146
	Ex smoker	41.6	140
	Smoker	14.9	50
PSA at diagnosis	<4ng/ml	61.3	206
	≥4ng/ml	38.7	130
Weight of chips resected at TUR	<11g	50.1	168
	≥11g	49.9	168
Urinary catheter in-situ preoperatively	Yes	31.5	106
	No	68.5	230
Novel systemic and pathological factors			
Systemic inflammatory response (mGPS)	Low	70.2	236
	Medium	27.5	93
	High	2.2	7
Local inflammatory infliltrate (Klintrup-Makinen)	None (0)	0.3	1
	Mild/patchy (1)	93.5	314
	Prominent (2)	5.9	20

	Florid (3)	0.3	1
Tissue necrosis	None	97.7	328
	<25%	2.3	8
	25-50%	0	0
	>50%	0	0
Proliferation Index (Ki67)	Low	61.6	207
	High	38.4	129
Apoptotic Index (TUNEL)	Low	50.9	171
	High	49.1	165

7.1.2 Clinicopathological factors related to outcome measures

Table 7.2 shows associations with clinical parameters (grouped data) and outcome measures using Kaplan-Meier methods.

Table 7.2 Univariate analysis of clinicopathological factors in the BPH cohort

Classical clinicopathological Variables	Postoperative AUR	Failure of surgical management	Reoperation
Age (<70 v ≥70 years)	**0.002**	0.849	0.810
BMI (normal v overweight v obese)	0.508	**0.008**	0.204
CVD (presence v absence)	**0.036**	0.584	0.326
DM2 (presence v absence)	0.414	0.331	0.933
Aspirin Usage (presence v absence)	0.577	0.211	0.232
Smoking History (Non-smoker v ex-smoker v smoker)	0.586	0.299	0.631
PSA at diagnosis (<4 v ≥4ng/ml)	0.693	**0.010**	0.435
Weight of chips (≤11 v >11g)	0.761	0.616	**0.043**
Preoperative urinary catheter in situ (presence v presence)	**0.024**	0.455	0.798
Novel systemic and pathological factors			
Local inflammation (Klintrup-Makinen) (none v mild v prominent v florid)	0.340	0.682	0.914
Tissue Necrosis (0 v <25 v 25-50 v >50%)	0.633	0.752	0.708
Systemic inflammation (mGPS) (mild v moderate v high)	0.214	0.086	0.364
Apoptotic index (TUNEL) (low v high)	0.086	0.264	0.093
Proliferation index (Ki67) (low v high)	0.215	0.746	**0.007**

7.1.2.1 Classical clinicopathological variables related to outcome measures

As shown in Table 7.2 several clinical variables were associated with clinical outcome measures. Increased age (p= 0.02, HR 3.2 (95% CI 1.4-7.3)), presence of CVD (p=0.036, HR 2.3 (95% CI 1.0-5.1)) and presence of a preoperative urinary catheter (p=0.024, HR 2.5 (95% CI 1.1-5.7)) were all associated with increased incidence of postoperative AUR >30 days after TUR (Figures 7.2, 7.3 and 7.4 respectively).

Figure 7.1 Kaplan Meier plot of age and time to postoperative AUR in BPH patients

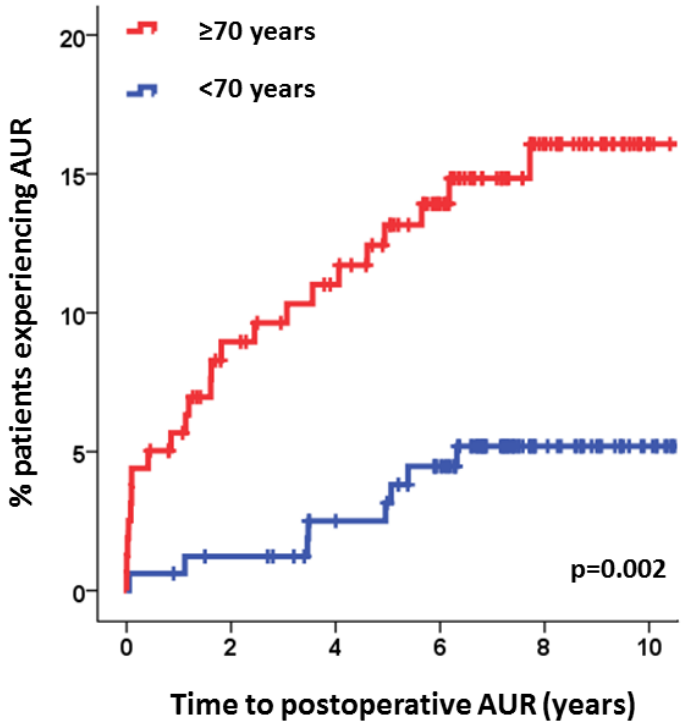


Figure 7.2 Kaplan Meier plot of CVD and time to postoperative AUR in BPH patients

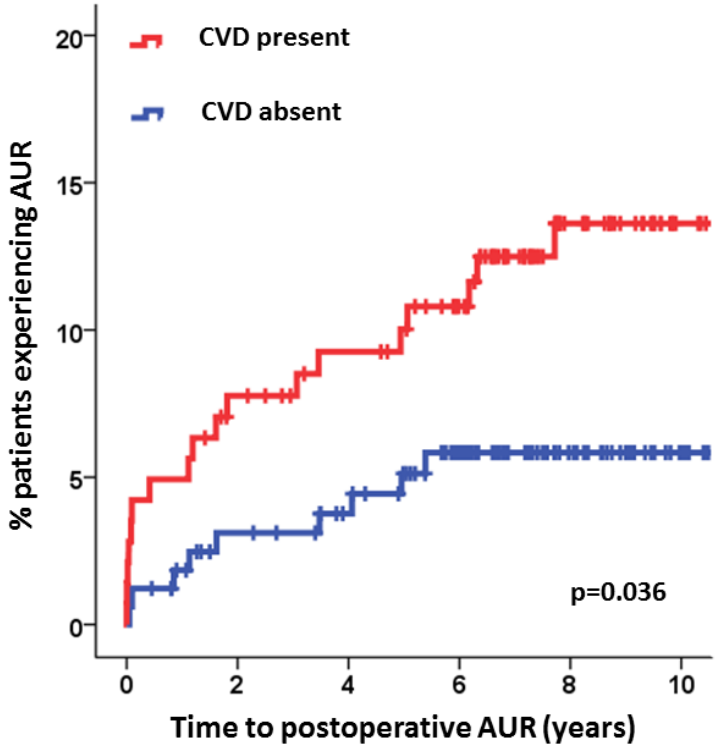
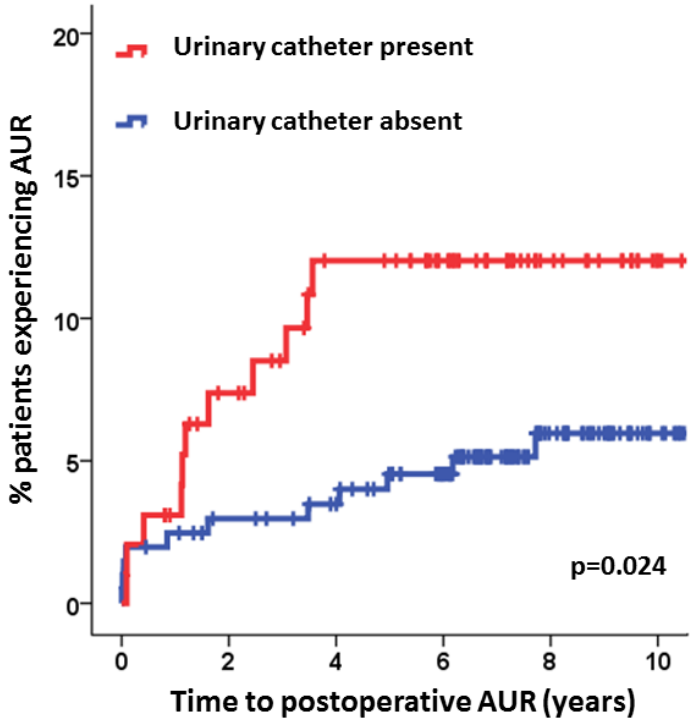


Figure 7.3 Kaplan Meier plot of presence of preoperative urinary catheter and time to postoperative AUR in BPH patients



As shown in Table 7.2 and Figure 7.4 higher BMI was found to be associated with shorter time to failure of surgical management p=0.008, HR 1.6 (95% CI 1.2-2.1). Successful surgical management of BPH at 15 years was 69% in normal weight, 56.6% in overweight and 24.6% in obese patients. High serum PSA at diagnosis was also associated with shorter time to failure of surgical management p=0.010, HR 1.7 (95% CI 1.1-2.6) (Figure 7.5).

Figure 7.4 Kaplan Meier plot of BMI and time to failure of surgical management in BPH patients

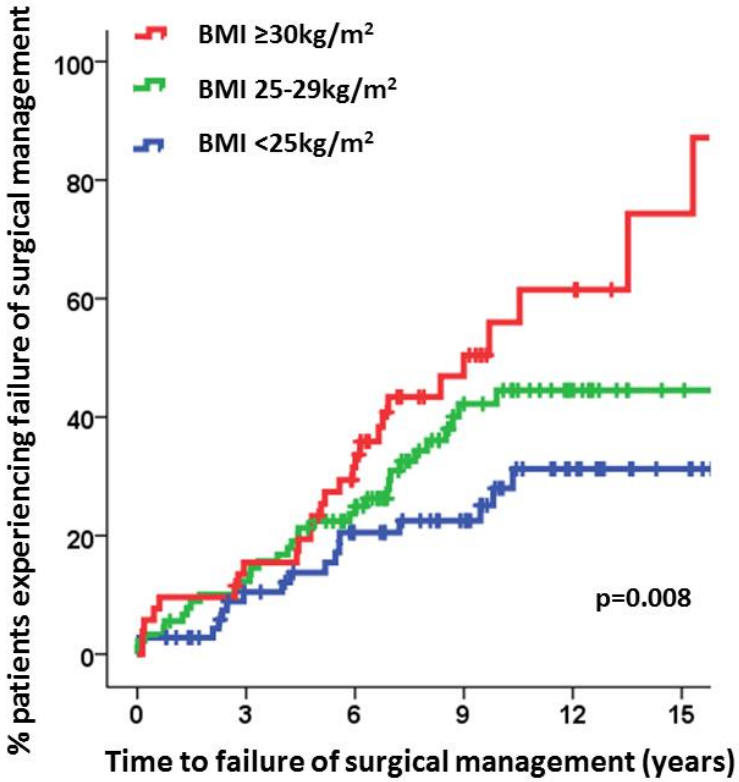
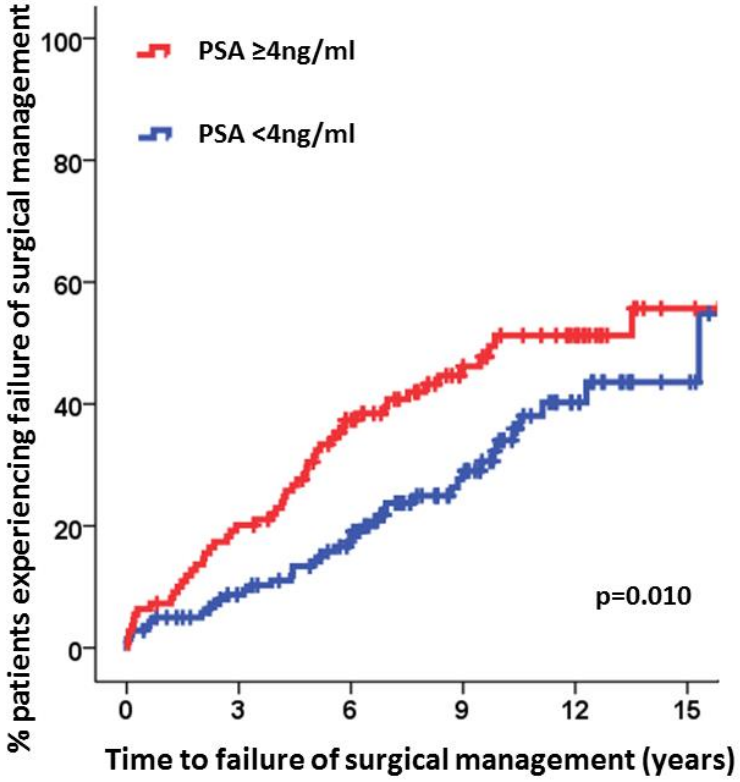
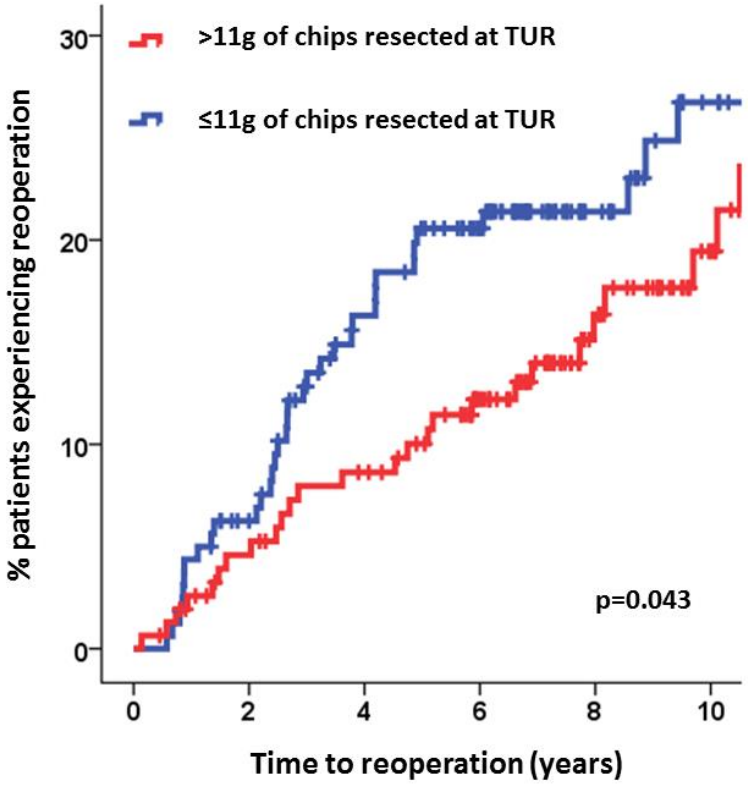


Figure 7.5 Kaplan Meier plot PSA at diagnosis and time to failure of surgical management in BPH patients



Lower weight of chips resected at TUR was associated with shorter time to reoperation p=0.043, HR 0.62 (95% CI 0.39-0.99) (Figure 7.6).

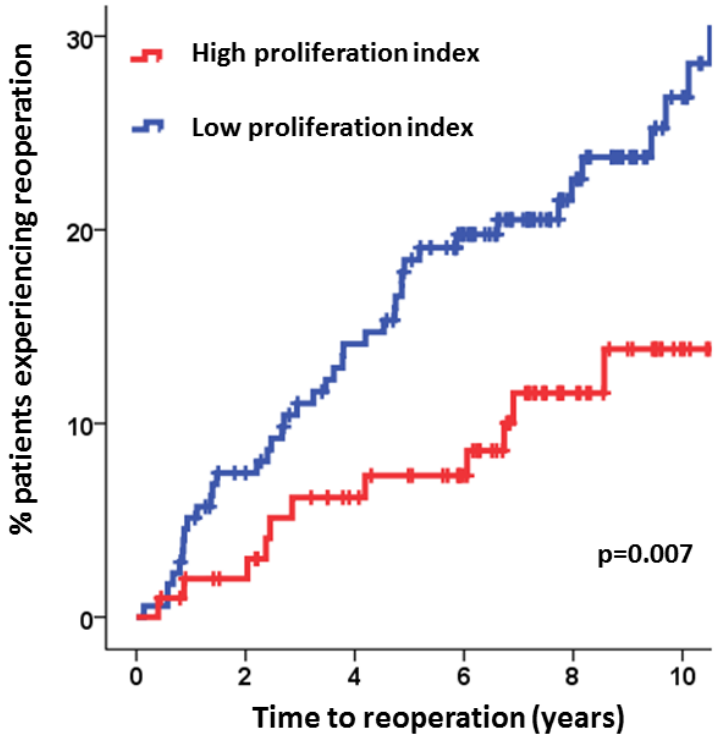
Figure 7.6 Kaplan Meier plot weight of chips resected at TUR and time to reoperation in BPH patients



7.1.2.2 Novel systemic and pathological variables related to outcome measures

When considering the non-classical factors or novel factors for BPH there was a trend for higher apoptotic index to be associated with increased incidence of postoperative AUR (Table 7.2). There was also a trend for higher levels of systemic inflammation to be associated with shorter time to failure of surgical management (Table 7.2). Low proliferation index, as assessed by Ki67, was associated with a shorter time to reoperation p=0.007, HR 0.44 (95% CI 0.24-0.81) (Figure 7.7) and a trend for low apoptotic index was observed to be associated with shorter time to reoperation but did not reach clinical significance (p=0.093, Table 7.2).

Figure 7.7 Kaplan Meier plot proliferation index and time to reoperation in BPH patients



7.2 Discussion

Several clinicopathological factors were associated with clinical outcome measures in BPH. Regarding the clinical factors, increased age was associated with increased incidence of postoperative AUR. It is likely that increased age corresponds to increased duration of symptoms and increased number of comorbidities, both of which may make postoperative AUR more likely. Presence of a preoperative indwelling urinary catheter was associated with increased incidence of postoperative AUR. Although the clinical indication for the urinary catheter is unknown it can be assumed that a large majority would have had preoperative AUR. Preoperative AUR has been demonstrated to be associated with a higher risk of complications following TUR in a large population study (220) and may explain the observed relationship.

As observed in the current study, CVD has been linked to BPH/LUTS previously. The Massachusetts Male Aging Study involving over 1000 men with a mean follow up of 9 years found that a history of heart disease at baseline more than doubled the odds of subsequent clinical BPH.(221) Similarly the Flint Men's Health Study found an increased risk of moderate to severe LUTS in patients with a history of heart disease.(222) This may explain our finding that presence of CVD was associated with shorter time to postoperative AUR. In addition patients with CVD are likely to be prescribed several medications that may worsen symptoms; the Massachusetts Male Aging Study found beta-blockers to be associated with an increased incidence of BPH, perhaps through effects on the sympathetic nervous system.(221) Risk factors for CVD have also repeatedly been linked to BPH. In a pre-clinical study where rats were fed high fat diets resulting in high serum concentrations of low density lipoprotein-cholesterol (a strong risk factor for cardiovascular disease), the animals were also noted to develop prostatic smooth muscle hypertrophy and bladder over-activity.(223) In a population based study of 780 men risk factors for CVD were linked to progression of symptoms of BPH.(224) Similarly factors that protect against CVD have been shown to protect against BPH/LUTS. A systematic review and meta-analysis described a risk reduction of up to 25% with moderate to vigorous physical exercise.(225) It is widely debated whether BPH is a risk factor for CVD or vice versa.(226) The current study reinforces the link between the two conditions but further work is required in order to unpick this complex relationship.

High serum PSA level at diagnosis was found to be an independent predictor on multivariate analysis with regards to failure of surgical management. It is well recognised that serum PSA level in benign prostatic disease correlates strongly with prostatic volume.(227-229) Increased prostate volume strongly predicts

adverse clinical outcomes associated with BPH.(46,230) The correlation between prostate volume and severity of LUTS is not clear cut, however patients with larger volume prostates (and higher serum PSA levels) undergoing surgical management may not have adequate tissue removed thus resulting in ongoing/recurrent LUTS requiring further prescription of postoperative medical therapy. This hypothesis is strengthened by the finding that a lower weight of chips resected at primary TUR is associated with a shorter time to reoperation. Data on grade of operating surgeon was not available for this cohort but it may be that junior trainees undertaking these procedures are more cautious with regards to the volume of tissue resected thus accounting for the observed relationships. BMI was strongly associated with time to failure of surgical management in BPH patients undergoing TUR of prostate. Few studies have examined the effect of obesity on response to treatment in BPH. The Asian multinational prospective observational registry of patients with benign prostatic hyperplasia recorded data on BMI, treatment for BPH and IPSS but unfortunately did not examine this relationship.(218) One study observed that high BMI and waist circumference were associated with greater response to medical treatment of LUTS as determined by IPSS.(231) These results are contradictory to the current study's findings that high BMI confers shorter time to failure of surgical management as it must be assumed that patients requiring treatment have worse symptoms. This may be explained by the low rates of preoperative IPSS (38.8%) and maximum urine flow rate (Qmax) (56.6%) (data not shown) which is in line with the findings of the national prostatectomy audit.(232) Taken together, Lee's work and the current study may suggest a strategy whereby medical treatment of BPH is instituted in preference to surgery in obese patients.(231) Clearly this hypothesis requires investigation within the context of a randomised controlled trial.

Interestingly BMI was found to be associated with failure of surgical management but not associated with reoperation. One explanation may be that high BMI patients are more likely to have significant comorbidities such as CVD and DM2. Significant comorbidities confer a higher perioperative risk and, on balance, repeat TUR may be evaluated as too hazardous to undertake in these patients.

Obesity is known to be associated with a systemic, low-grade inflammatory state. Pathological factors such as the role of inflammation in the development and progression of BPH is highly debated. It is postulated that a chronic local inflammatory cell infiltrate creates a cytokine rich environment which can support the process of fibromuscular growth in BPH.(233) Regarding the local inflammatory response previous work has demonstrated the presence of an inflammatory cell infiltrate in 98% of BPH specimens.(28) The current study supports this work with inflammatory cells present in 100% of cases examined. The clinical significance of the inflammatory cell infiltrate remains speculative. This study is the first example of application of the Klintrup-Makinen scoring system in prostate disease. Other studies have examined the local inflammatory cell infiltrate in BPH, with varying results. The expression of CD4+ and CD8+ T cell lymphocytes in BPH diagnosed on prostate biopsies was not associated with progression of LUTS.(234) Another histological study used a tissue microarray to review the inflammatory infiltrates on 275 BPH specimens. Higher levels of inflammation were found to be associated with larger prostate volume and higher international prostate symptom score.(30) Similarly Di Silverio examined 3942 prostate specimens and found larger prostates to be positively associated with inflammation.(29) Data was not available on prostate volume for this cohort however the relationship between IPSS and Klintrup-Makinen score was examined and demonstrated no association (data not shown).

The importance of tissue necrosis secondary to prostatic infarction in BPH is unclear. This study reported a small amount (2.3%) of tissue necrosis in routine TUR prostate specimens. These rates of necrosis are in line with previous studies, which showed rates of 0-9%.(235-237) However, the presence of necrosis in other urological conditions has been well established. In particular tissue necrosis in renal cancer specimens forms part of the risk stratification in these patients.(238,239) Recent renal cancer studies have demonstrated that quantification of necrosis is superior to a basic presence/absence assessment.(240,241) The prognostic significance of prostate tissue necrosis in BPH is largely unknown. Prostate infarction has previously been associated with AUR which is a common complication of BPH. Prostatic infarction was 85% and 3% respectively when patients with AUR were compared with those undergoing elective surgery for BPH.(242) However, the connection between AUR and prostate infarction has not been reproduced in recent studies.(235,236) It is likely that the relationship between BPH and prostatic infarction is complex and subject to many confounders as BPH patients are likely to have larger prostates and therefore suffer AUR (with or without concurrent infection) requiring urethral instrumentation. In addition BPH patients are likely to be older and therefore more at risk of atherosclerosis all of which may distort the intraglandular vascular supply leading to prostatic infarction.(243,244) Some studies have linked prostate infarction to smoking and pre-existing cardiovascular disease (245), although this was not found in the current study. General anaesthesia and perioperative hypotension have also been suggested to play a role in prostate infarction due to reduced blood supply. This has particularly been seen in those patients undergoing aortic aneurysm repair likely secondary to significant blood loss producing hypotension and the association with atherosclerosis of the iliac arteries further jeopardising blood flow to the

central part of the prostate gland.(246) Previous general anaesthetic data was unavailable but this may account for the small proportion of prostate tissue necrosis seen in this study.

Systemic inflammation as measured by circulating levels of CRP has been shown to be associated with development of BPH in a case matched prospective study.(247) CRP has also been linked with irritative LUTS and decreased peak urinary flow rates in a population based study of 2115 men with BPH/LUTS.(33) CRP has been further validated in 205 men with BPH, whereby higher serum levels were found to be an independent predictor of on-going LUTS following medical treatment.(248) Since the prognostic use of CRP has been previously studied it was assessed whether mGPS, an established systemic inflammatory scoring system utilising CRP in combination with albumin, could predict outcome in BPH. mGPS has been associated with outcome in prostate cancer where it has recently been shown to predict poorer 5 year overall and relative survival in prostate cancer patients independent of Gleason score.(249) Despite this significant relationship in neoplastic prostatic tissue mGPS was not found to be associated with clinical outcome measures in BPH. To our knowledge this is the first investigation of mGPS in BPH patients and clearly further work is required in this field to validate the current study's findings.

The relationship between proliferation and apoptotic indices and clinical outcome measures was perhaps contrary to expectations. We suggest that our results represent a shift in the normal balance of cells in BPH; a high proliferation index may result in more cells exhibiting mutations and therefore entering programmed cell death. This could account for our finding that there was a trend for high apoptotic index to be associated with a shorter time to AUR

in BPH patients, which might contribute to the uncontrolled progression of the disease. Both low proliferation index and low apoptotic index were associated with shorter time to reoperation. The mechanisms behind this relationship are unclear however this could suggest that following primary surgery a reverse relationship may manifest. It may also be true that too little tissue was removed to achieve symptomatic response or that the relationship is secondary to fast proliferation cells out growing their nutrient supply resulting in inflammation and necrosis causing a feedback pathway to shut down the overall growth of the gland. These explanations represent hypotheses only and clearly there is a need for further investigation using mechanistic models, which is out with the scope of this project.

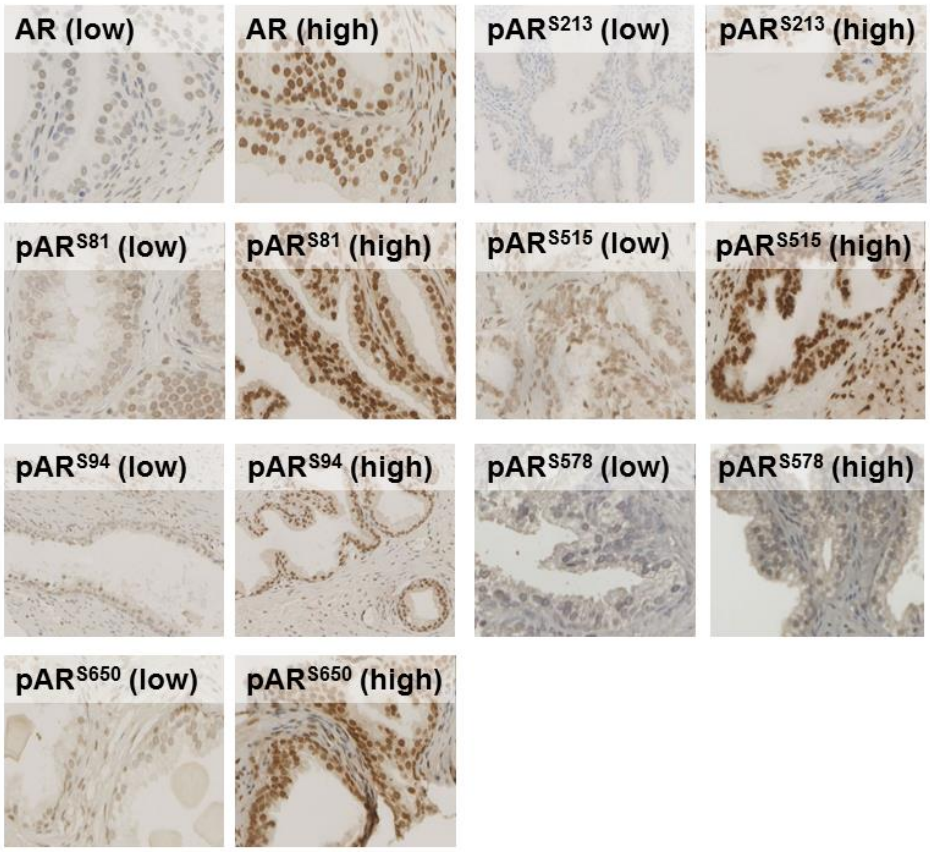
In conclusion clinicopathological factors are of prognostic importance in BPH. However the role of some factors, such as inflammation and tissue necrosis, in BPH remains unclear. Some of these factors provide an opportunity for intervention; high BMI was associated with worse outcome from primary TUR surgery therefore weight loss should form part of the routine management of BPH in obese patients. This study was conducted retrospectively therefore prospective, randomised trials with appropriate length of follow up are required in order to definitively establish the impact of clinicopathological factors on the clinical manifestations of BPH and their role as prognostic indicators of disease progression.

8 Benign prostate hyperplasia and serine phosphorylation of the androgen receptor

8.1 Androgen receptor expression

Expression of AR and all phosphorylated forms investigated were observed at varying levels in the cytoplasm and nucleus of both stromal and epithelial cells (Figure 8.1). Staining in epithelial cells was easily differentiated between the nucleus and the cytoplasm and thus both compartments were scored independently. However, staining in the stromal and smooth muscle cells could only be confidently scored within the nuclear compartments therefore cytoplasmic scoring was not undertaken. Protein expression was found to be heterogeneous throughout and, with the exception of pAR^{S515} staining, less intense in the smooth muscle, stromal and cytoplasmic component of the epithelial cells compared to epithelial nuclear expression. There was presence of benign prostatic tissue, adjacent to the BPH tissue, in some of the TMA cores. Only protein expression observed in the BPH tissue was scored.

Figure 8.1 Example high/low AR and pAR immunohistochemical staining in BPH



ICCCs were performed to verify consistency between observers and all values were >0.80 (Table 8.1).

Table 8.1 ICCC scores for AR and pAR protein expression analysis

Protein	Nucleus	Cytoplasm	Smooth muscle	Stroma
AR	0.804	0.826	0.808	0.934
pAR^{S81}	0.807	0.802	0.894	0.879
pAR^{S94}	0.806	0.812	0.870	0.864
pAR^{S213}	0.832	0.849	0.862	0.885
pAR^{S515}	0.818	0.804	0.837	0.871
pAR^{S578}	0.852	0.805	0.981	0.954
pAR^{S650}	0.802	0.801	0.983	0.814
Ki67	0.952	X	X	X
TUNEL	0.920	X	X	X

Scatter plots for each antibody were used to display correlations between individual observers' scores and to confirm there was no bias between observers Bland Altman plots were constructed. Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis. Median AR and pAR histoscore expression levels are shown in Table 8.2. Proliferation and apoptotic indices were observed in 85.7% and 98.8%, respectively, of the BPH tissue examined and median cell counts (IQR) were 1.0% (0.3-1.7) and 2.0% (1.0-4.0) respectively.

Table 8.2 Protein expression levels for AR and pAR sites

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
AR	Cytoplasm	5.0	1.7-10.0
	Nucleus	126.7	110-141.7
	Smooth muscle	38.3	18.3-58.3
	Stroma	63.3	38.3-91.7
pAR^{S81}	Cytoplasm	40.0	25.0-58.3
	Nucleus	175.0	155.0-195.0
	Smooth muscle	90.0	61.7-117.5
	Stroma	136.7	110.0-163.8
pAR^{S94}	Cytoplasm	60.0	43.3-75.0
	Nucleus	116.7	92.1-142.9
	Smooth muscle	40.0	21.7-63.3
	Stroma	65.0	40.0-86.3
pAR^{S213}	Cytoplasm	13.3	7.5-34.2
	Nucleus	125.0	106.7-141.7
	Smooth muscle	19.2	10.0-55.0
	Stroma	52.5	28.3-87.3
pAR^{S515}	Cytoplasm	16.7	10.0-26.7
	Nucleus	131.7	110.0-156.7
	Smooth muscle	133.3	110.0-160.0
	Stroma	156.7	136.7-184.6
pAR^{S578}	Cytoplasm	56.7	38.3-75.0
	Nucleus	35.0	15.0-60.0
	Smooth muscle	1.7	0-3.3
	Stroma	6.7	3.3-11.7
pAR^{S650}	Cytoplasm	43.3	30.0-56.7
	Nucleus	155.0	130.0-175.0
	Smooth muscle	66.7	38.3-90.0
	Stroma	103.3	73.3-130.0

8.2 Correlation of cell-specific phosphorylated androgen receptor expression

Continuous stromal-epithelial interactions are believed to be critical in prostatic development, homeostasis and disease. The importance of stromal-epithelial cross talk has been demonstrated in co-culture experiments of primary human BPH stromal fibroblasts and epithelial cells in which cell growth was significantly

increased when compared to culture of either cell type separately.(24) Given the wealth of data available the opportunity was taken to determine whether there was any relationship between cell-specific (stromal, smooth muscle or epithelial) expression of AR and AR phosphorylated at serine residues. Pearson's correlation coefficients were performed and the results are shown in Tables 8.3-8.9.

Table 8.3 Pearson's correlation coefficients total AR expression in epithelial, smooth muscle and stromal cells in BPH

AR			Total AR			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Total AR	Nuclear	C.C.		0.200	0.248	0.304
		p value		<0.001	<0.001	<0.001
	Cytoplasmic	C.C.	0.200		0.041	0.051
		p value	<0.001		0.450	0.351
	Smooth muscle	C.C.	0.248	0.041		**0.607**
		P value	<0.001	0.450		**<0.001**
	Stroma	C.C.	0.304	0.051	**0.607**	
		P value	<0.001	0.351	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.4 Pearson's correlation coefficients pAR^{S81} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation sites			Ser-81			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-81	Nuclear	C.C.		0.260	0.375	**0.480**
		p value		<0.001	<0.001	**<0.001**
	Cytoplasmic	C.C.	0.260		0.262	0.150
		p value	<0.001		<0.001	0.006
	Smooth muscle	C.C.	0.375	0.262		**0.582**
		P value	<0.001	<0.001		**<0.001**
	Stroma	C.C.	**0.480**	0.150	**0.582**	
		P value	**<0.001**	0.006	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.5 Pearson's correlation coefficients pAR^{S94} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation site			Ser-94			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-94	Nuclear	C.C.		**0.477**	**0.556**	**0.618**
		p value		**<0.001**	**<0.001**	**<0.001**
	Cytoplasmic	C.C.	**0.477**		0.333	0.331
		p value	**<0.001**		<0.001	<0.001
	Smooth muscle	C.C.	**0.556**	0.333		**0.656**
		p value	**<0.001**	<0.001		**<0.001**
	Stroma	C.C.	**0.618**	0.331	**0.656**	
		p value	**<0.001**	<0.001	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.6 Pearson's correlation coefficients pAR^{S213} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation site			Ser-213			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-213	Nuclear	C.C.		0.225	0.292	**0.405**
		p value		<0.001	<0.001	**<0.001**
	Cytoplasmic	C.C.	0.225		**0.439**	**0.407**
		p value	<0.001		**<0.001**	**<0.001**
	Smooth muscle	C.C.	0.292	**0.439**		**0.718**
		P value	<0.001	**<0.001**		**<0.001**
	Stroma	C.C.	**0.405**	**0.407**	**0.718**	
		P value	**<0.001**	**<0.001**	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.7 Pearson's correlation coefficients pAR^{S515} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation site			Ser-515			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-515	Nuclear	C.C.		0.136	0.360	**0.461**
		p value		0.013	<0.001	**<0.001**
	Cytoplasmic	C.C.	0.136		0.086	0.046
		p value	0.013		0.121	0.407
	Smooth muscle	C.C.	0.360	0.086		**0.654**
		P value	<0.001	0.121		**<0.001**
	Stroma	C.C.	**0.461**	0.046	**0.654**	
		P value	**<0.001**	0.407	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.8 Pearson's correlation coefficients pAR^{S578} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation sites			Ser-578			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-578	Nuclear	C.C.		**0.568**	**0.413**	**0.490**
		p value		**<0.001**	**<0.001**	**<0.001**
	Cytoplasmic	C.C.	**0.568**		0.296	0.345
		p value	**<0.001**		<0.001	<0.001
	Smooth muscle	C.C.	**0.413**	0.296		**0.665**
		P value	**<0.001**	<0.001		**<0.001**
	Stroma	C.C.	**0.490**	0.345	**0.665**	
		P value	**<0.001**	<0.001	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

Table 8.9 Pearson's correlation coefficients pAR^{S650} expression in epithelial, smooth muscle and stromal cells in BPH

AR phosphorylation sites			Ser-650			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-650	Nuclear	C.C.		0.357	**0.466**	**0.631**
		p value		<0.001	**<0.001**	**<0.001**
	Cytoplasmic	C.C.	0.357		0.216	0.182
		p value	<0.001		<0.001	0.001
	Smooth muscle	C.C.	**0.466**	0.216		**0.702**
		P value	**<0.001**	<0.001		**<0.001**
	Stroma	C.C.	**0.631**	0.182	**0.702**	
		P value	**<0.001**	0.001	**<0.001**	

Siginicant values (highlighted bold) are denoted by Pearson's correlation coefficient (C.C.) >0.400 and p value <0.05.

As shown in Table 8.3 there was a strong correlation between total AR stromal and smooth muscle cell expression. Tables 8.4-8.9 illustrate that in all AR serine phosphorylation sites studied, there was a strong correlation between both stromal and smooth muscle cell expression and stromal and nuclear epithelial cell expression.

8.3 Phosphorylated androgen receptor related to clinicopathological factors

Environmental, host and local disease factors have been implicated in the progression of BPH, therefore the relationship between clinicopathological

factors and AR and phosphorylated AR expression was investigated in the BPH cohort using the chi-squared test (Table 8.10 and 8.11).

Table 8.10 Chi squared analysis of clinical factors as related to high/low AR and pAR expression in BPH patients

Proteins		Clinical Variables								
		Age (<70 v ≥70yrs)	**CVD** (presence v absence)	**DM2** (presence v absence)	**Aspirin Usage** (yes vs no)	**Smoking History** (non- v ex- v smoker)	**PSA at diagnosis** (<4 v ≥4ng/ml)	**BMI** (<25 v 25-29 v ≥30kg/m^2)	**Weight of chips resected** (<11 v ≥11g)	**Catheter in situ preop** (yes v no)
AR	Cytoplasm	0.239	0.220	0.359	0.179	0.350	0.329	0.170	**0.017**	0.259
	Nucleus	0.965	0.253	**0.014**	0.449	0.549	0.777	0.881	**0.047**	0.566
	Smooth Muscle	0.992	0.272	0.141	0.240	0.360	0.092	**0.004**	0.193	0.843
	Stroma	0.742	0.681	0.150	0.432	0.337	0.969	0.478	0.067	0.181
pAR^{S81}	Cytoplasm	0.397	0.585	0.542	0.338	0.510	0.820	0.542	0.200	0.932
	Nucleus	0.864	0.597	0.535	0.311	0.188	0.081	0.464	0.095	0.557
	Smooth Muscle	0.181	0.102	0.585	0.582	0.712	0.355	0.088	0.521	0.941
	Stroma	0.199	0.670	0.982	0.334	0.906	0.816	0.544	0.941	0.774
pAR^{S94}	Cytoplasm	0.606	0.229	0.098	0.864	0.840	0.482	0.994	0.161	0.656
	Nucleus	0.303	0.953	0.064	0.985	0.648	0.713	0.065	0.190	0.192
	Smooth Muscle	0.113	0.859	0.871	0.606	0.484	0.629	0.155	0.227	0.596
	Stroma	0.082	0.857	0.876	0.963	0.712	0.732	0.670	0.280	0.623
pAR^{S213}	Cytoplasm	0.411	0.686	1.000	0.665	0.853	0.733	0.124	0.144	0.569
	Nucleus	0.053	0.444	0.247	0.990	0.432	0.106	0.811	0.282	0.569
	Smooth Muscle	0.124	**0.005**	0.574	0.089	0.371	0.694	0.226	0.765	0.531
	Stroma	0.156	0.941	0.097	0.988	0.181	0.470	0.881	0.958	0.419
pAR^{S515}	Cytoplasm	0.648	0.264	0.109	0.156	0.759	0.069	0.201	**0.037**	0.110
	Nucleus	0.071	0.935	0.768	0.595	0.343	0.340	0.885	0.178	0.526
	Smooth Muscle	0.171	0.485	0.438	0.275	0.273	0.926	0.150	0.510	0.181
	Stroma	0.234	0.186	0.189	0.468	0.597	0.920	0.144	0.794	0.650
pAR^{S578}	Cytoplasm	0.092	0.425	0.350	0.393	0.454	0.832	0.227	0.505	0.842
	Nucleus	0.220	0.064	0.610	0.213	0.845	0.507	0.632	0.883	0.320
	Smooth Muscle	0.784	0.526	0.538	0.890	0.108	0.661	0.419	0.928	0.173
	Stroma	0.345	0.915	0.865	0.746	0.619	0.815	0.808	0.918	0.527
pAR^{S650}	Cytoplasm	0.974	0.902	0.763	0.204	0.143	0.510	0.892	0.788	0.710
	Nucleus	0.708	0.730	**0.037**	0.412	0.131	0.971	0.556	0.830	0.894
	Smooth Muscle	0.175	0.129	0.374	0.275	0.701	0.326	0.183	**0.012**	0.922
	Stroma	0.066	0.452	0.890	0.963	0.746	0.270	0.710	**0.039**	0.814

8.3.1 Clinical factors related to androgen receptor expression

Low smooth muscle pAR^{S213} expression was associated with an increased incidence of cardiovascular disease. Low nuclear total AR and pAR^{S650} expression was associated with increased incidence of DM2. Low total AR expression in the smooth muscle cells was associated with higher BMI. Low cytoplasmic and nuclear total AR expression was associated with increased weight of chips resected at TUR. High expression of cytoplasmic pAR^{S515} and both smooth muscle and stromal pAR^{S650} were all associated with increased weight of chips resected at TUR (Table 8.10). Age, aspirin usage, smoking history, PSA at diagnosis and presence of a preoperative urinary catheter were not associated with protein expression at any of the phosphorylated AR sites.

Table 8.11 Chi squared analysis of pathological factors as related to high/low AR and pAR expression in BPH patients

Proteins		Pathological Variables				
		Proliferation Index (Ki67)	**Apoptotic Index (TUNEL)**	**Local inflammation** (Klintrup-Makinen	**Tissue Necrosis**	**Systemic inflammation** (mGPS)
AR	Cytoplasm	0.499	0.177	0.076	**0.009**	0.244
	Nucleus	0.480	0.410	0.351	0.661	0.995
	Smooth Muscle	0.119	**0.006**	0.463	0.280	0.192
	Stroma	**<0.001**	**0.002**	**0.007**	0.252	0.971
pAR^{S81}	Cytoplasm	**<0.001**	0.422	0.387	0.603	0.149
	Nucleus	**0.002**	0.301	0.161	0.263	0.312
	Smooth Muscle	**0.013**	0.181	0.921	0.506	0.154
	Stroma	**0.028**	0.267	0.246	0.500	0.761
pAR^{S94}	Cytoplasm	0.935	0.116	0.091	0.199	0.680
	Nucleus	0.679	**0.014**	0.337	**0.009**	0.167
	Smooth Muscle	0.292	0.702	0.526	0.256	0.267
	Stroma	0.611	0.916	0.344	0.063	0.968
pAR^{S213}	Cytoplasm	0.132	**0.014**	**0.006**	0.242	0.434
	Nucleus	0.496	**0.013**	0.962	0.291	**0.048**
	Smooth Muscle	0.572	0.124	**0.004**	0.259	0.938
	Stroma	0.252	**<0.001**	**0.002**	0.252	0.526
pAR^{S515}	Cytoplasm	**0.009**	0.894	**0.030**	0.833	0.678
	Nucleus	0.742	0.090	0.337	0.062	0.271
	Smooth Muscle	0.640	0.962	0.727	0.062	0.080
	Stroma	0.297	0.193	0.770	0.273	0.846
pAR^{S578}	Cytoplasm	0.138	0.847	0.458	0.879	0.580
	Nucleus	**0.024**	0.815	0.375	0.940	0.977
	Smooth Muscle	0.467	0.821	0.390	0.845	0.684
	Stroma	0.401	0.413	0.248	0.983	0.540
pAR^{S650}	Cytoplasm	**0.003**	0.860	0.652	**0.041**	0.543
	Nucleus	0.730	0.873	0.170	0.062	**0.026**
	Smooth Muscle	0.784	0.356	0.471	0.277	0.137
	Stroma	0.723	0.188	0.634	0.068	**0.013**

8.3.2 Pathological factors related to androgen receptor expression

High stromal total AR expression was associated with a low proliferation index. Low expression of pAR^{S81} in the epithelia cell nucleus and cytoplasm and the smooth muscle and stromal cells was associated with a low proliferation index. Low cytoplasmic pAR^{S650} expression was associated with a low proliferation index. High expression of cytoplasmic pAR^{S515} and nuclear pAR^{S578} were

associated with a low proliferation index (Table 8.11). Low smooth muscle and stromal total AR expression was associated with a high apoptotic index, p=0.006 and 0.002 respectively. High nuclear pAR^{S94} expression was associated with a high apoptotic index p=0.014. Low cytoplasmic, nuclear and stromal pAR^{S213} expression was associated with a high apoptotic index (Table 8.11). Therefore expression of total AR in the stroma was directly linked, with cell turnover and depending on the phosphorylation site investigated was either directly (serine 81 and 650) or inversely (serine 515 and 578) associated with proliferation. Suggesting that phosphorylation status of AR is important when considering its role in driving cellular proliferation. It was interesting to note the phosphorylation sites associated with phosphorylation in response to androgens serine 81 and 650 were directly associated with proliferation in contrast to those phosphorylated by growth factor signalling pathways e.g. 515 being inversely associated with proliferation.

Emerging evidence linking inflammation to AR expression in BPH (34,35) prompted the investigation of the relationship between the local (Klintrup-Makinen) and systemic (mGPS) inflammatory responses and AR and phosphorylated AR expression (Table 8.11). High stromal total AR expression was associated with increased levels of local inflammation, p=0.007. High cytoplasmic, smooth muscle and stromal pAR^{S213} expression was associated with increased levels of local inflammation (Table 8.11). High cytoplasmic pAR^{S515} expression was associated with increased levels of local inflammation, p=0.030. High levels of tissue necrosis were associated with high cytoplasmic expression of total AR and pAR^{S650}, p=0.009 and p=0.041 respectively. Low levels of tissue necrosis were associated with high nuclear expression of pAR^{S94}, p=0.009. High stromal pAR^{S650} expression was associated with increased levels of systemic

inflammation, p=0.013. High nuclear pAR^{S213} and pAR^{S650} expression were associated with increased levels of systemic inflammation, p=0.048 and p=0.026 respectively.

Once again this illustrates different roles for the different serine phosphorylation sites in BPH. In general high levels of AR and pAR were associated with increased inflammation and necrosis. This is with the exception of the constitutively phosphorylated site Ser-94 in which an inverse relationship was observed. It is likely that it is not only AR phosphorylation that is associated with inflammation and necrosis, but a concert of kinase and growth factor driven intracellular signalling pathways.

8.4 Phosphorylated androgen receptor related to outcomes

Univariate analysis of AR and pAR protein expression was carried out using Kaplan Meier methods with reference to the clinical outcome measures. The results are shown in Table 8.12.

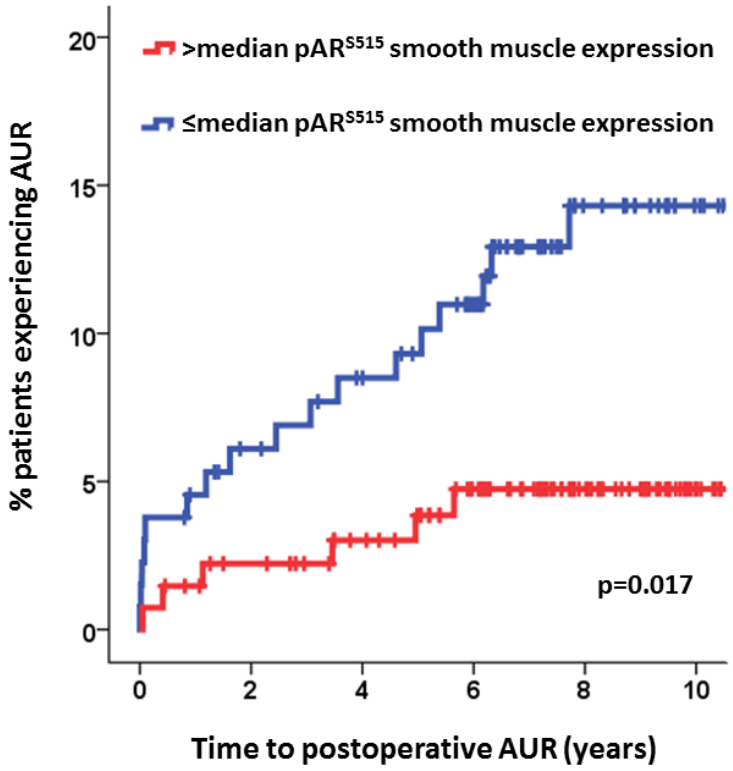
Table 8.12 Univariate analysis of AR and phosphorylated AR expression and clinical outcome measures in BPH

Proteins		Postoperative AUR	Failure of Surgical management	Reoperation
AR	Nucleus	0.290	0.929	0.746
	Cytoplasm	0.507	0.294	0.413
	Smooth Muscle	0.978	**0.010**	0.879
	Stroma	0.852	0.480	0.820
pAR^{S81}	Nucleus	0.148	0.290	**0.027**
	Cytoplasm	0.951	0.182	0.183
	Smooth Muscle	0.578	0.392	0.358
	Stroma	0.282	0.721	0.194
pAR^{S94}	Nucleus	0.494	0.600	0.230
	Cytoplasm	0.523	0.341	0.340
	Smooth Muscle	0.344	0.729	0.152
	Stroma	0.802	0.844	0.514
pAR^{S213}	Nucleus	0.494	0.205	0.306
	Cytoplasm	0.232	0.176	0.927
	Smooth Muscle	0.760	0.094	0.755
	Stroma	0.756	0.052	0.143
pAR^{S515}	Nucleus	0.216	**0.022**	0.373
	Cytoplasm	0.850	**0.010**	0.294
	Smooth Muscle	**0.017**	0.646	0.682
	Stroma	0.441	0.556	0.533
pAR^{S578}	Nucleus	0.267	0.861	0.293
	Cytoplasm	0.674	0.801	0.357
	Smooth Muscle	0.324	0.553	0.945
	Stroma	0.692	0.681	0.449
pAR^{S650}	Nucleus	0.780	**0.016**	**0.022**
	Cytoplasm	0.874	**0.023**	0.752
	Smooth Muscle	0.317	0.698	0.750
	Stroma	0.992	0.091	0.081

8.4.1 Postoperative acute urinary retention

Only low pAR^{S515} smooth muscle cell expression was associated with shorter time to postoperative AUR, HR 0.34 (95% CI 0.15-0.86) (Figure 8.2).

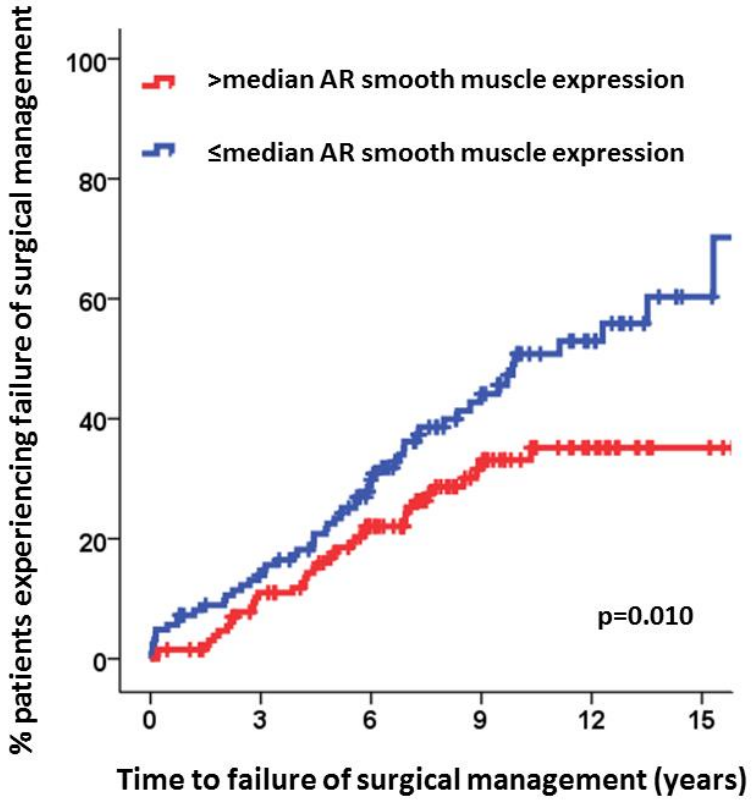
Figure 8.2 Kaplan Meier plot of smooth muscle pAR^{S515} expression and time to postoperative AUR in BPH patients



8.4.2 Failure of surgical management

Several proteins were associated with time to failure of surgical management of BPH. Specifically low smooth muscle total AR expression was associated with shorter time to failure of surgical management, HR 0.59 (95% CI 0.39-0.88) (Figure 8.3).

Figure 8.3 Kaplan Meier plot of smooth muscle total AR expression and time to failure of surgical management in BPH patients



Low cytoplasmic and nuclear pAR^{S515} expression (HR 0.58 (95% CI 0.38-0.88) and (HR 0.62 (95% CI 0.41-0.94) respectively) were associated with shorter time to failure of surgical management (Figure 8.4 and 8.5).

Figure 8.4 Kaplan Meier plot of cytoplasmic expression and time to failure of surgical management in BPH patients

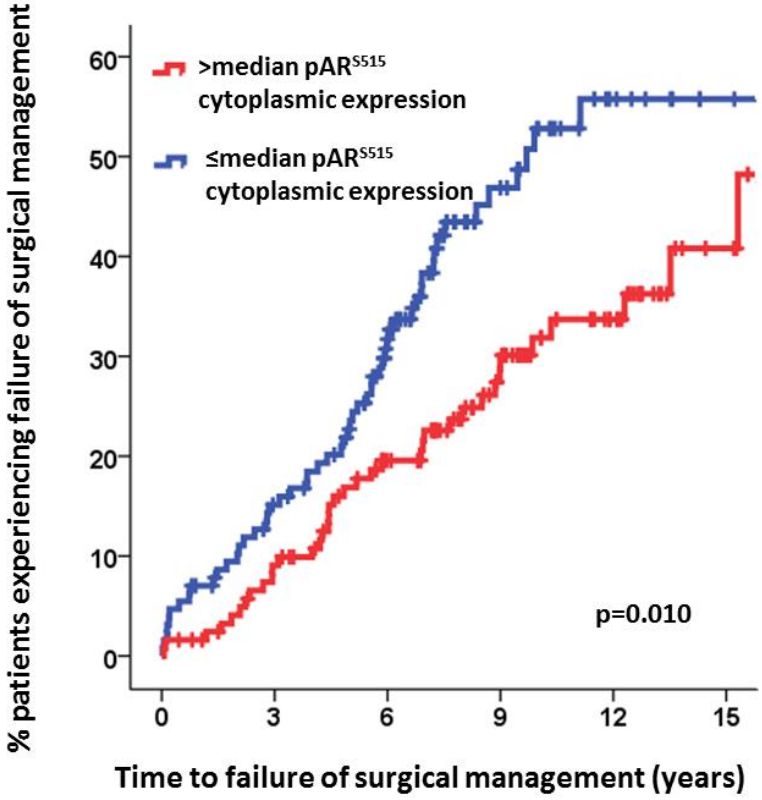
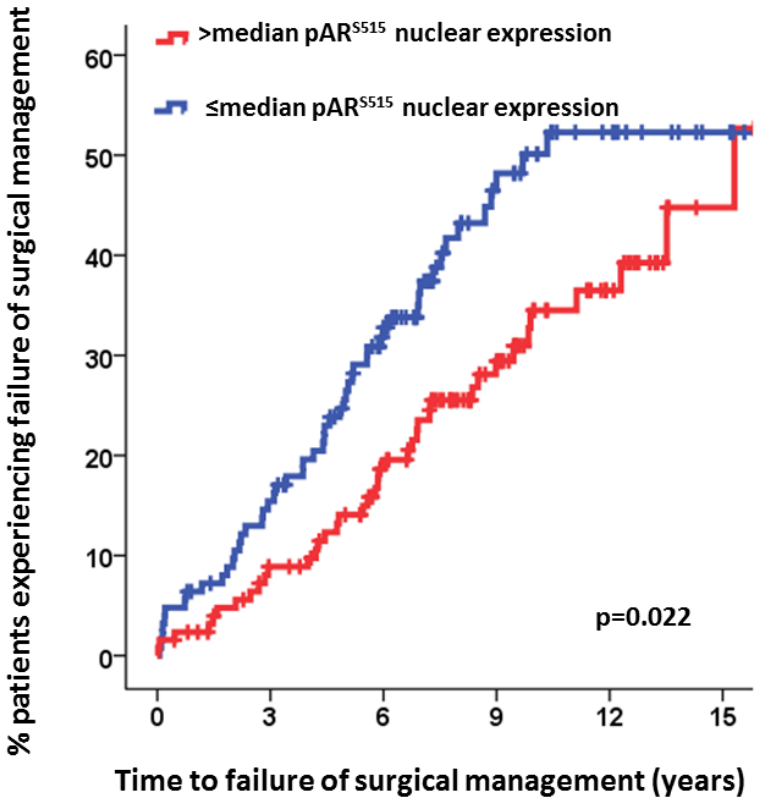


Figure 8.5 Kaplan Meier plot of nuclear pAR^{S515} expression and time to failure of surgical management in BPH patients



Similarly low cytoplasmic and nuclear pAR^{S650} expression (HR 0.62 (95% CI 0.41-0.94) and HR 0.61 (95% CI 0.40-0.92) respectively) were also associated with shorter time to failure of surgical management of BPH (Figure 8.6 and 8.7).

Figure 8.6 Kaplan Meier plot of cytoplasmic pAR^{S650} expression and time to failure of surgical management in BPH patients

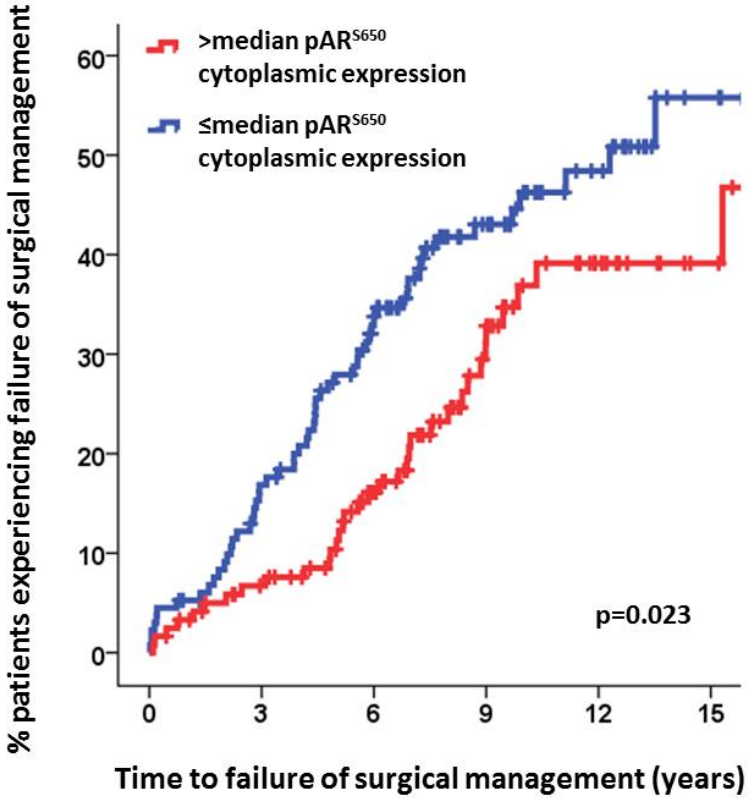
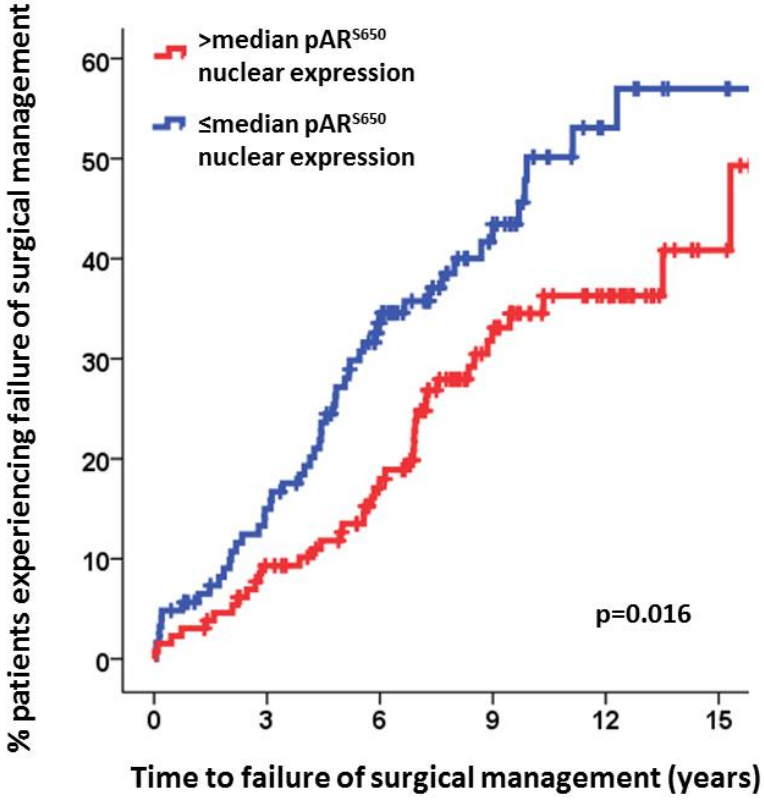


Figure 8.7 Kaplan Meier plot of nuclear pAR^{S650} expression and time to failure of surgical management in BPH patients



Given the inverse relationship with proliferation the relationships of total AR and pAR^{S515} are not wholly unexpected. However slowly proliferating prostates as demonstrated by low cytoplasmic pAR^{S650} expression are more difficult to explain and compounding influences are likely to be at play.

8.4.3 Reoperation

Both low nuclear pAR^{S81} and pAR^{S650} expression were associated with shorter time to reoperation, HR 0.57 (95% CI 0.34-0.94) and HR 0.56 (95% CI 0.34-0.93) respectively (Figure 8.8 and 8.9).

Figure 8.8 Kaplan Meier plots of nuclear pAR^{S81} expression and time to reoperation in BPH patients

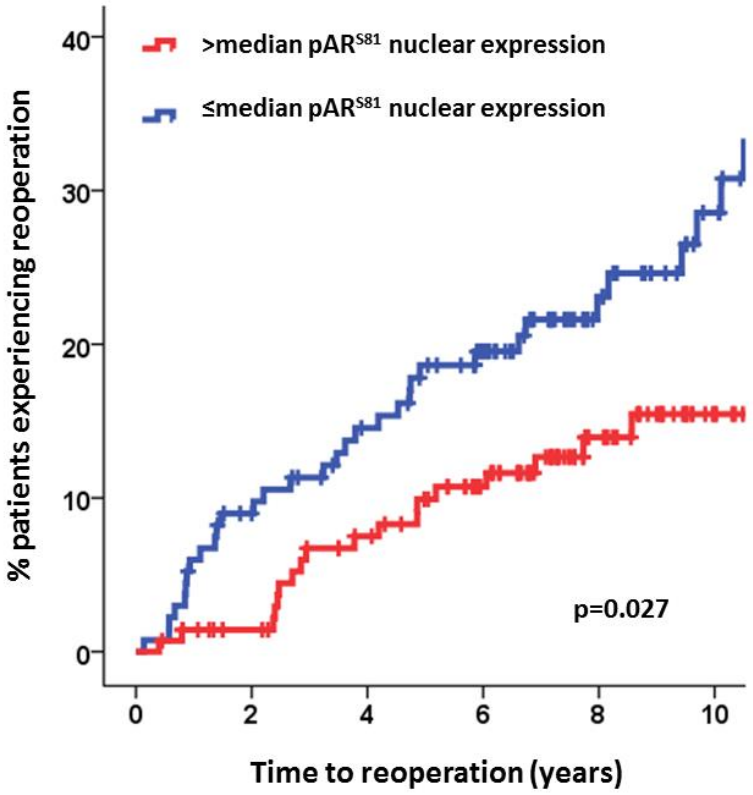
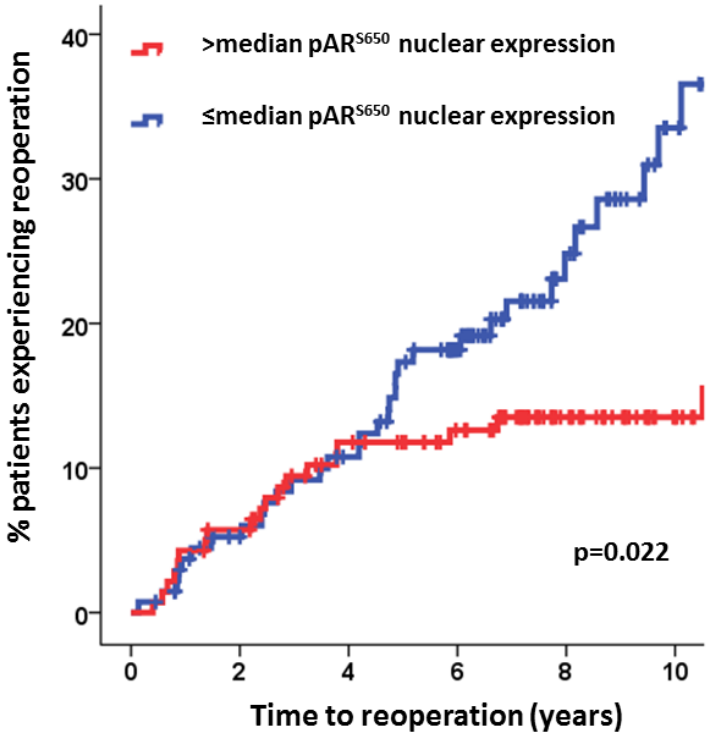


Figure 8.9 Kaplan Meier plot of nuclear pAR^{S650} expression and time to reoperation in BPH patients

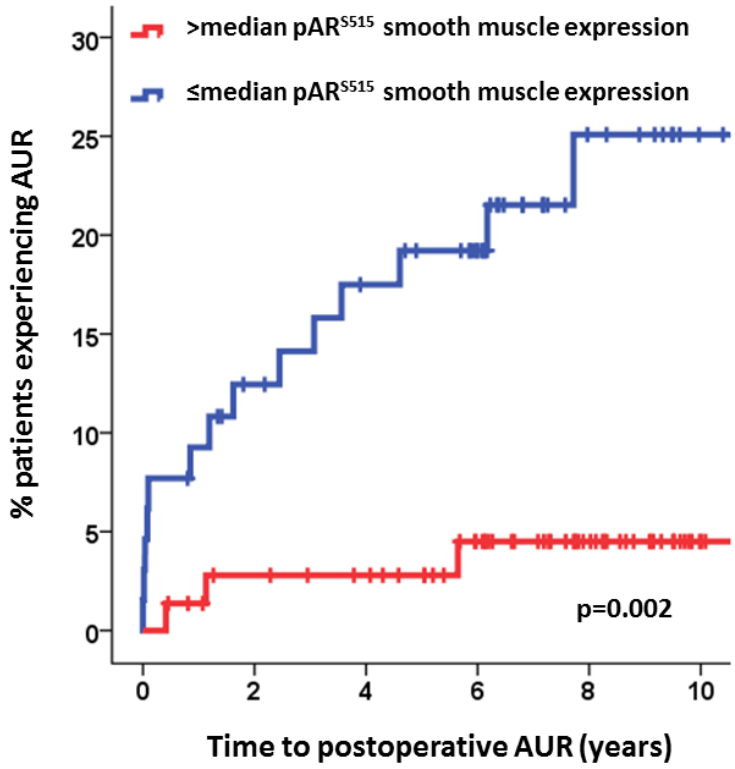


8.5 Multivariate analysis

Significant univariate results were included in a backwards conditional cox-regression model to determine independence from significant clinical parameters.

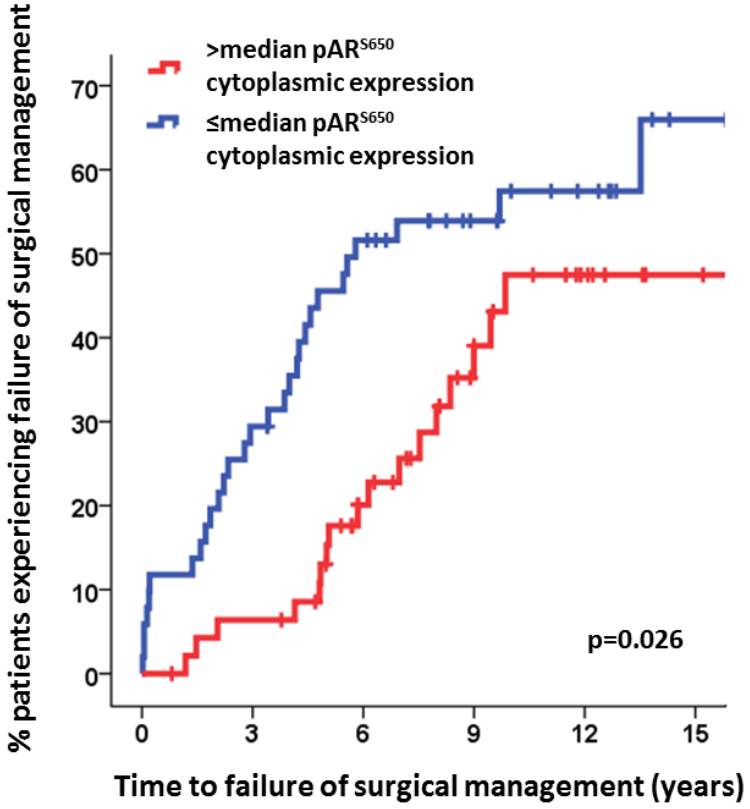
Smooth muscle pAR^{S515} expression was combined with age, CVD and presence of a preoperative catheter in a multivariate cox regression analysis. Both smooth muscle pAR^{S515} (p=0.029, HR 0.31 (95% CI 0.10-0.94)) and age (p=0.004, HR 5.13 (95% CI 1.43-18.41)) were independently associated with postoperative AUR. As these variables were deemed independent it was investigated whether smooth muscle pAR^{S515} expression could inform on the likelihood of postoperative AUR in patients older than 70 years at diagnosis. Low expression of pAR^{S515} in the smooth muscle was associated with increased incidence of postoperative AUR in patients over 70 years old (25.1% vs 2.8% at 10 years post TUR), (p=0.002, HR 0.20 (95% CI 0.06-0.62)) (Figure 8.10). This may have important clinical implications in postoperative counselling and prophylactic medical treatment of these patients.

Figure 8.10 Kaplan Meier plot of smooth muscle pAR^{S515} expression and time to postoperative AUR in BPH patients over 70 years old



Nuclear and cytoplasmic pAR^{S515} and pAR^{S650} expression were combined with smooth muscle AR expression, BMI and PSA at diagnosis in a multivariate cox regression analysis. BMI (p=0.038, HR 1.43 (95% CI 1.01-2.00)), PSA at diagnosis (p=0.018, HR 1.89 (95% CI 1.11-3.16)), smooth muscle AR (p=0.016, HR 0.51 (95% CI 0.29-0.89)) and cytoplasmic pAR^{S650} expression (p=0.010, HR 0.50 (95% CI 0.29-0.86)) were independently associated with time to failure of operative management. As these variables were deemed independent it was investigated whether cytoplasmic pAR^{S650} expression could inform on the likelihood of failure of operative management in patients with PSA ≥4ng/ml at diagnosis. Low expression of pAR^{S650} in the cytoplasm was associated with increased failure of operative management in patients with PSA ≥4ng/ml at diagnosis (45.5% vs 13% at 5 years post TUR), (p=0.026, HR 0.52 (95% CI 0.29-0.93)) (Figure 8.11).

Figure 8.11 Kaplan Meier plot of cytoplasmic pAR^{S650} expression and time to failure of surgical management in BPH patients with PSA ≥4ng/ml at diagnosis



When nuclear pAR^{S81} and pAR^{S650} expression were combined with proliferation index and weight of chips resected in a multivariate cox regression analysis, nuclear pAR^{S650} expression (p=0.020, HR 0.54 (95% CI 0.32-0.91)), proliferation index (p<0.001, HR 0.33 (95% CI 0.17-0.65)) and weight of chips resected (p=0.013, HR 0.52 (95% CI 0.31-0.87) were independently associated with time to reoperation.

8.6 Discussion

Serine phosphorylated AR expression has been shown to have relevance with regards to clinical outcome in BPH. Interestingly these results are both overlapping and subtly different to those seen in the pilot prostate cancer and active surveillance cohorts.

Protein expression was observed at all serine phosphorylation sites, in all cell types and subcellular locations in the BPH tissue which is consistent with previous work.(250) A strong correlation was observed between stromal and smooth muscle total AR and phosphorylated AR expression at all serine sites. Interestingly nuclear epithelial AR expression was associated with stromal AR in all serine phosphorylation sites but not with regards to total AR expression. BPH has been reported to comprise of 88.4% stromal cells and 9.0% epithelial cells (251) it is therefore logical that the stromal compartment may be the central communicating cell type perhaps influencing actions of the others. These results suggest that expression levels and site specific phosphorylation status of AR in one cell type could affect or be linked to AR expression in other cell type, adding weight to the growing body of evidence regarding androgen-driven stromal-epithelial cell interactions in prostate disease.(252)

In contrast to prostate cancer the expression levels of AR in human BPH tissue and their relationship to clinicopathological factors and clinical outcome has not been widely explored. This study comprises the most extensive investigation into the clinical relevance of AR and AR serine phosphorylation expression levels in BPH to date.

Regarding the clinical factors, low expression of AR and AR phosphorylated at serine residues were associated with CVD, DM2 and BMI. As discussed previously CVD has been linked to BPH. This study has observed, for the first time, that the androgen receptor may directly link these two diseases. From the current study it is unclear whether it is the underlying heart disease itself or the commonly used drugs to treat CVD that underpin this relationship. Work in prostate cancer cell lines has shown that statins (cholesterol-lowering drugs) decreased AR

protein levels by proteolysis resulting in a reduction in androgen sensitivity and cell proliferation (in AR positive cells).(253) The metabolic consequences of DM2 (hyperglycaemia/ hyperinsulinaemia/ insulin resistance) have been demonstrated to promote production of insulin growth factor (IGF) 1 by prostate stromal (fibroblast) cells and it is hypothesised that this might have an impact on other intracellular signalling pathways involving the AR.(254) Another element of the metabolic syndrome, high BMI, was shown to be associated with low smooth muscle AR expression; this is in agreement with the well-established observations that androgen insensitivity is associated with the number of CAG trinucleotide repeats in the AR, which in turn decreases AR-mediated gene transcription and results in elevated visceral fat.(255) In addition, male mice lacking AR develop late onset visceral obesity with increased lipogenesis in white adipose tissue and liver.(256,257) This result may be viewed alongside that described previously whereby high BMI was associated with shorter time to failure of surgical management in BPH patients. Low smooth muscle AR expression was also associated with shorter time to failure of surgical management of BPH in the current study. Selective smooth muscle AR ablation in mice leads to significant histological abnormalities such as hyperplasia, inflammation and fibrosis and it may be that even after surgery in patients with low smooth muscle AR expression these histological abnormalities translate into symptom recurrence requiring medical intervention at a faster rate.(21) The relationship between smooth muscle AR expression, BMI and failure of surgical management in BPH patients requires further investigation to discern whether low smooth muscle AR expression is the cause or result of high BMI and indeed whether failure of surgical management is due to high BMI and/or low smooth muscle AR expression. Taken together, these results suggest that BPH patients with low smooth muscle AR expression and/or high BMI may not respond

favourably to surgical management. These patients could represent an exciting opportunity for the development of novel cell-specific AR targeted drug therapy.

There was a variable relationship between AR and pAR expression and weight of chips resected at TUR. This may be explained by the differing roles of AR at different phosphorylation sites.(100) In general AR serine phosphorylation activates AR and is associated with increased gene transcription and cellular proliferation. However, phosphorylation at Ser-650 is associated with nuclear export and negative regulation of gene transcription, the results in the current study whereby cytoplasmic levels of Ser-650 were associated with proliferation and therefore difficult to explain especially as cytoplasmic expression trended towards a direct relationship with nuclear expression. Phosphorylation at Ser-515 is associated with transcriptional activity and receptor stability.(100) However an inverse relationship was seen with proliferation and it may be the case that in BPH cells the primary role of Ser-515 phosphorylation is receptor stabilisation. Increased receptor stability (Ser-515) may translate into larger prostates which have more tissue available for surgical resection and this may account for the results in this study. It is also worth noting that no data was available for grade of surgeon performing the surgery and this may have confounded the results.

In keeping with previous studies in prostate cancer cell lines pAR^{S81} expression was strongly associated with proliferation in all cellular locations.(100,116) In the current study high apoptotic index was associated with both low stromal and smooth muscle AR expression. This agrees with previous work in double stromal AR knockout mouse models which exhibited increased levels of apoptosis.(23) In addition, stromal fibroblast selective AR knockout mice showed increased

apoptosis (22) whilst smooth muscle selective AR knockout mice showed no change in apoptosis.(258) We suggest that our results represent a shift in the normal balance of cells in BPH; a high proliferation index may result in more cells exhibiting mutations and therefore entering programmed cell death. This could account for our finding that stromal AR expression was associated with both increased apoptosis and proliferation in BPH patients which might contribute to the uncontrolled progression of the disease. Ser-578 phosphorylation has been implicated in gene transcription and nuclear-cytoplasmic shuttling of the AR, however its role in non-neoplastic prostatic tissue has not been previously studied. Differing roles of AR serine phosphorylation sites in benign disease and neoplastic tissue may explain the relationship of Ser-578 phosphorylation with low levels of proliferation in BPH. Interestingly low expression levels of cytoplasmic, nuclear and stromal pAR^{S213} expression were associated with high levels of apoptosis. This novel finding in BPH tissue is in line with previous work by the host laboratory in matched hormone naïve and castrate resistant prostate cancer samples whereby an increase in pAR^{S213} expression was associated with decreased disease specific survival. Uncontrolled cellular proliferation with associated dysregulation/inhibition of apoptosis may be driven by pAR^{S213} in castrate resistant prostate cancer. The current study suggests that the role of pAR^{S213} may be similar in benign and neoplastic prostatic disease. Further work is required to substantiate this relationship.

In line with previous work, evidence of local inflammation was found in all samples.(28) The association of prostatic stromal cells with the immune response is supported by Penna and colleagues who demonstrated that human fibromuscular stromal BPH cells can behave as antigen presenting cells and

activate alloantigen-specific CD4+ T cells to produce IFN-γ and IL-17, thereby inducing and sustaining an autoimmune response.(259) Unfortunately, AR expression levels in Penna's work were not characterised and it is therefore difficult to draw direct comparisons. Phosphorylation at Ser-213 was also strongly associated with local inflammation in the cytoplasm (along with pAR^{S515}) and smooth muscle cells. This is in contrast to previous work whereby smooth muscle AR activation has been shown to mediate a direct anti-inflammatory effect via the use of the selective AR ligand DHT.(35) Furthermore selective ablation of AR in murine prostate smooth muscle cells results in diffuse stromal hyperplasia, characterised by an infiltration of leukocytes.(21) One explanation for these seemingly conflicting results may be AR phosphorylation status which augments its activation state and was not investigated in the previous studies.

Patchy areas of necrosis are not infrequently seen in routine prostate pathology specimens with concurrent BPH. In line with previous work our study reported a small amount of tissue necrosis in routine TUR prostate specimens.(236) High epithelial AR and pAR^{S650} expression in the cytoplasm were associated with tissue necrosis. This may be explained by an increase in the host inflammatory response, which is known to be influenced by the presence of tissue necrosis. If the local inflammatory response is increased this may lead to increased epithelial AR expression as supported by previous work.(260) This is supported by the trend for high cytoplasmic AR expression to be associated with high levels of local inflammation. Interestingly, low levels of pAR^{S94} were associated with high levels of tissue necrosis. These findings may illustrate the individual roles for serine phosphorylation sites in BPH. These results require verification in a large, prospectively collected, independent cohort.

Overall low protein expression was generally associated with worse outcome in BPH patients. Effects on clinical outcome measures were mediated by different phosphorylation sites of the AR in different cellular locations. This suggests a differing role for androgen signalling in each cellular location and also highlights the importance of considering these cellular compartments individually in the development of new treatments for BPH.

Low smooth muscle AR expression was associated with shorter time to failure of surgical management of BPH in the current study. Selective smooth muscle AR ablation in mice leads to significant histological abnormalities such as hyperplasia, inflammation and fibrosis and it may be that even after surgery in patients with low smooth muscle AR expression these histological abnormalities translate into symptom recurrence requiring medical intervention at a faster rate.(21) Low expression of Ser-515 and Ser-650 were also associated with shorter time to failure of surgical management in BPH patients. Phosphorylation of AR at Ser-650 by JNK1 and p38 is associated with nuclear export of the receptor to the cytoplasm and subsequently antagonises AR transcription.(132) Low levels of Ser-650 may equate to uninhibited AR transcription and disease progression. Ser-515 nuclear expression correlated strongly with Ser-650 nuclear expression (c.c. 0.419, $p<0.001$) and it may be that the expression of these two phosphorylation sites is linked. This is further substantiated by the results in this study that they are both associated with the candidate upstream kinase Cdk1/pCdk1. The low expression of Ser-515 as an adverse prognostic marker is in stark contrast to the findings in prostate cancer whereby high expression was associated with a poorer clinical outcome. This adds weight to the long argued point that despite often occurring concurrently, BPH behaves as a separate disease process from prostatic carcinogenesis.(261) Further functional cell line

studies coupled with prospective clinical trials would clarify the role and clinical application of these serine phosphorylation sites in BPH.

Low expression of smooth muscle pAR^{S515} expression was found to inform on the risk of AUR after primary TUR surgery in older patients with BPH. The role of Ser-515 phosphorylation in AR stability may explain this relationship. Low expression of pAR^{S515} may result in instability in the receptor making it more vulnerable to modulation by other growth factors. This may result in increased unregulated activity within the smooth muscle compartment of the prostate eventually leading to the clinical consequence of AUR. This has potential clinical relevance in counselling of patients postoperatively with regards to the increased risk of AUR and in addition may provide an opportunity for intervention with current medical treatment for BPH and/or a novel targeted drug following further investigative work as to the mechanistic consequences of pAR^{S515} smooth muscle expression with regards to disease progression. In addition low levels of cytoplasmic pAR^{S650} were found to inform on patients with high/abnormal PSA at diagnosis associated with increased failure of operative management in patients with PSA ≥4ng/ml at diagnosis. Once again this has potential clinical utility with regards to the pre and post operative counselling of these patients and the targeted use of prophylactic medical treatment.

9 Benign prostate hyperplasia and kinases mediating androgen receptor serine phosphorylation

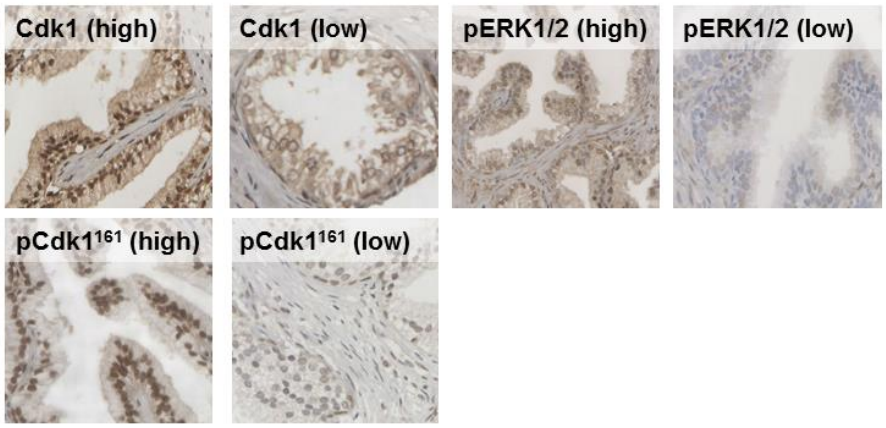
In the previous chapter it was demonstrated that phosphorylation of the AR at specific serine residues is associated with clinical outcome in BPH patients. As in prostate cancer AR serine phosphorylation in BPH is thought to be controlled by kinases. To our knowledge there are no previous studies investigating the kinases responsible for AR serine phosphorylation in BPH therefore this chapter is the first thorough exploration of kinases mediating AR phosphorylation at clinical relevant serine sites. The aim was to explore whether these candidate kinases may represent novel therapeutic targets for treatment of BPH.

9.1 Kinase protein expression

Scansite 2.0 was utilised to identify the candidate kinases mediating AR phosphorylation on the clinically relevant sites; Ser-81, Ser-515, and Ser-650.(154) Scansite 2.0 predicted Cdk1 and ERK1/2 as strong candidates mediating phosphorylation of Ser-81, 515 and 650.

Expression of all proteins was observed at varying levels in the cytoplasm and nucleus of both stromal and epithelial cells (Figure 9.1)

Figure 9.1 Example high/low candidate kinase immunohistochemical staining in BPH



Only protein expression observed in the BPH tissue was scored. ICCCs were performed to verify consistency between scorers, all values were >0.70 and are shown in Table 9.1. Scatter plots for each antibody were used to display this data and to confirm there was no bias between scorers Bland-Altman plots were constructed.

Table 9.1 ICCC scores for candidate kinase protein expression analysis in BPH

Protein	Nucleus	Cytoplasm	Smooth muscle	Stroma
Cdk1	0.809	0.871	0.818	0.820
pCdk1^{161}	0.812	0.804	0.979	0.951
pERK1/2	0.917	0.940	0.796	0.748

Protein expression levels were subdivided into low (≤median) and high expression (>median) for analysis. Median kinase histoscore expression levels are shown in Table 9.2.

Table 9.2 Protein expression levels for kinases in BPH cohort

Protein	Subcellular Location	Median Histoscore (Histoscore units)	Interquartile Range (Histoscore units)
Cdk1	Cytoplasm	125.0	102.5-150.0
	Nucleus	130.0	101.7-162.5
	Smooth muscle	38.3	20.0-65.0
	Stroma	71.7	45.0-100.0
pCdk1	Cytoplasm	30.0	20.0-50.0
	Nucleus	121.7	78.3-155.8
	Smooth muscle	95.0	86.7-98.3
	Stroma	96.7	87.7-100.0
pERK1/2	Cytoplasm	13.3	1.7-33.3
	Nucleus	14.2	2.2-35.0
	Smooth muscle	6.7	0-30.0
	Stroma	0	0-3.3

9.2 Candidate kinase expression related to clinicopathological factors

The relationship between clinicopathological factors and high/low expression levels of the candidate kinases phosphorylating AR at clinically significant serine residues was investigated in the BPH cohort using the chi-squared test (Table 9.3 and 9.4).

9.2.1 Clinical factors related to candidate kinase expression

As shown in Table 9.3 high expression of stromal Cdk1 was associated with increased age. Low expression of nuclear pERK1/2 was associated with increased incidence of DM2. Low expression of stromal pERK1/2 and smooth muscle Cdk1 was associated with a higher BMI.

Table 9.3 Clinical factors as related to high/low candidate kinase expression in BPH cohort

Proteins		Clinical Variables								
		Age (<70 v ≥70yrs)	**CVD** (presence v absence)	**DM2** (presence v absence)	**Aspirin Usage** (yes vs no)	**Smoking History** (non- v ex- v smoker)	**PSA at diagnosis** (<4 v ≥4ng/ml)	**BMI** (<25 v 25-29 v ≥30kg/m^2)	**Weight of chips resected** (<11 v ≥11g)	**Catheter in situ preop** (yes v no)
Cdk1	Cytoplasm	0.341	0.564	0.718	0.545	0.575	0.091	0.847	0.277	0.914
	Nucleus	0.874	0.738	0.524	0.728	0.222	0.193	0.690	0.220	0.881
	Smooth Muscle	0.855	0.587	0.237	0.246	0.822	0.676	**0.004**	0.423	0.233
	Stroma	**0.033**	0.105	0.789	0.885	0.688	0.319	0.080	0.184	0.328
$pCdk1^{161}$	Cytoplasm	0.629	0.481	0.442	0.871	0.623	0.058	0.753	0.438	0.507
	Nucleus	0.410	0.907	0.128	0.972	0.090	0.795	0.283	0.823	0.705
	Smooth Muscle	0.430	0.677	0.510	0.296	0.300	0.699	0.656	0.375	0.687
	Stroma	0.526	0.698	0.479	0.645	0.477	0.796	**0.015**	0.316	0.953
pERK1/2	Cytoplasm	0.191	0.235	0.217	0.290	0.729	0.349	0.896	0.870	0.757
	Nucleus	0.099	0.245	**0.042**	0.661	0.566	0.555	0.532	0.332	0.786
	Smooth Muscle	0.919	0.149	0.239	0.595	0.785	0.142	0.136	0.785	0.430
	Stroma	0.279	0.641	0.801	0.970	0.227	0.659	0.990	0.091	0.582

9.2.2 Pathological factors related to candidate kinase expression

As shown in Table 9.4 low expression of nuclear pCdk1^{161} and stromal pERK1/2 were associated with a high proliferation index. Low expression levels of nuclear Cdk1 and pERK1/2 were associated with a higher level of tissue necrosis. High expression of cytoplasmic and smooth muscle pERK1/2 were associated with increased levels of systemic inflammation. Low expression of smooth muscle pCdk1^{161} and stromal pCdk1^{161} and pERK1/2 were associated with a high apoptotic index.

Table 9.4 Pathological factors as related to high/low candidate kinase expression in BPH cohort

Proteins		Pathological Variables				
		Proliferation index (Ki67)	**Apoptotic index (TUNEL)**	**Local inflammation** (Klintrup-Makinen)	**Tissue Necrosis**	**Systemic inflammation** (mGPS)
Cdk1	Cytoplasm	0.285	0.631	0.232	0.460	0.402
	Nucleus	0.659	0.250	0.323	**0.014**	0.761
	Smooth Muscle	0.204	0.460	0.691	0.469	0.846
	Stroma	0.157	0.299	0.825	0.058	0.102
pCdk1^{161}	Cytoplasm	0.385	0.165	0.735	0.945	0.601
	Nucleus	**0.005**	0.137	0.309	0.994	0.060
	Smooth Muscle	0.722	**0.005**	0.181	0.580	0.284
	Stroma	0.223	**0.001**	0.207	0.800	0.112
pERK1/2	Cytoplasm	0.757	0.575	0.462	0.113	**0.018**
	Nucleus	0.428	0.509	0.536	**0.014**	0.124
	Smooth Muscle	0.330	0.189	0.499	0.266	**0.048**
	Stroma	**0.045**	**0.002**	0.913	0.930	0.092

9.3 Kinases related to outcomes

Univariate analysis of the candidate kinases (Cdk1, pCdk1^{161} and pERK1/2) with regards to the four clinical outcome measures is shown in Table 9.5.

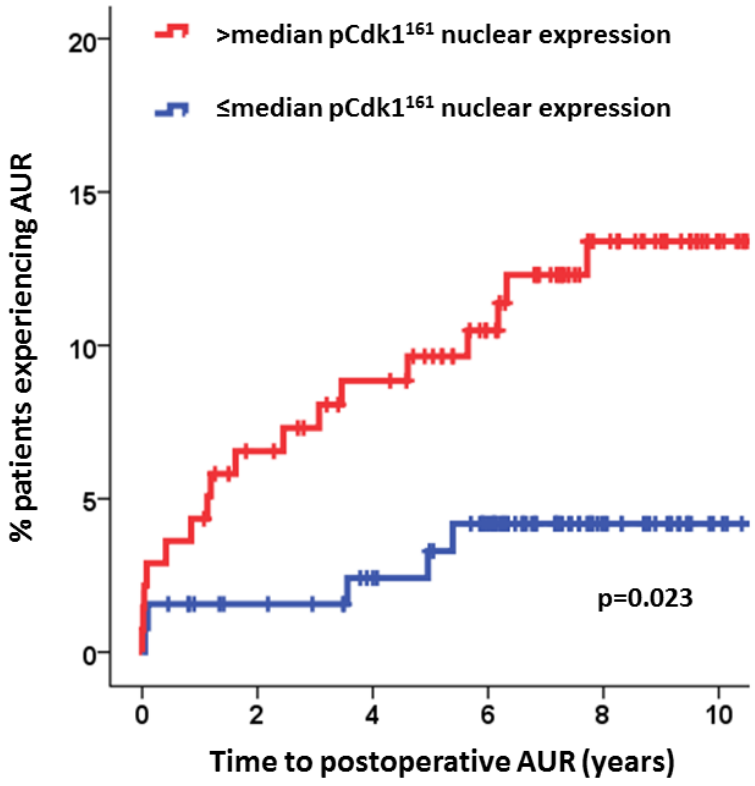
Table 9.5 Univariate analysis of candidate kinase expression and clinical outcome measures in BPH

Proteins		Postoperative AUR	Failure of Surgical Management	Reoperation
Cdk1	Nuclear	0.983	0.718	0.431
	Cytoplasm	0.272	0.102	**0.016**
	Smooth Muscle	0.081	0.511	0.558
	Stroma	0.780	0.244	0.742
pCdk1^{161}	Nuclear	**0.023**	0.597	0.668
	Cytoplasm	0.227	0.398	0.179
	Smooth Muscle	0.884	**0.027**	0.584
	Stroma	0.659	0.866	0.308
pERK1/2	Nuclear	0.637	0.492	0.773
	Cytoplasm	0.784	0.538	0.690
	Smooth Muscle	0.554	0.099	0.978
	Stroma	0.437	0.446	0.056

9.3.1 Postoperative acute urinary retention

High nuclear pCdk1^{161} was associated with shorter time to postoperative acute urinary retention HR 2.79 (95% CI 1.12-7.04) (Figure 9.2).

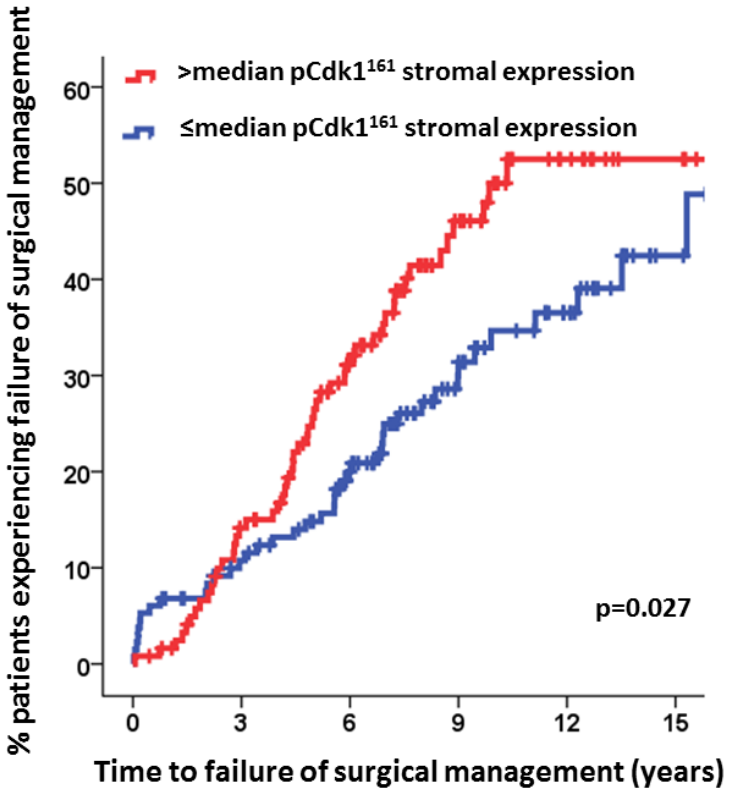
Figure 9.2 Kaplan Meier plot nuclear pCdk1^{161} expression and time to postoperative AUR in BPH patients



9.3.2 Failure of surgical management

High stromal pCdk1^{161} was associated with shorter time to failure of surgical management of BPH HR 1.58 (95% CI 1.05-2.39) (Figure 9.3).

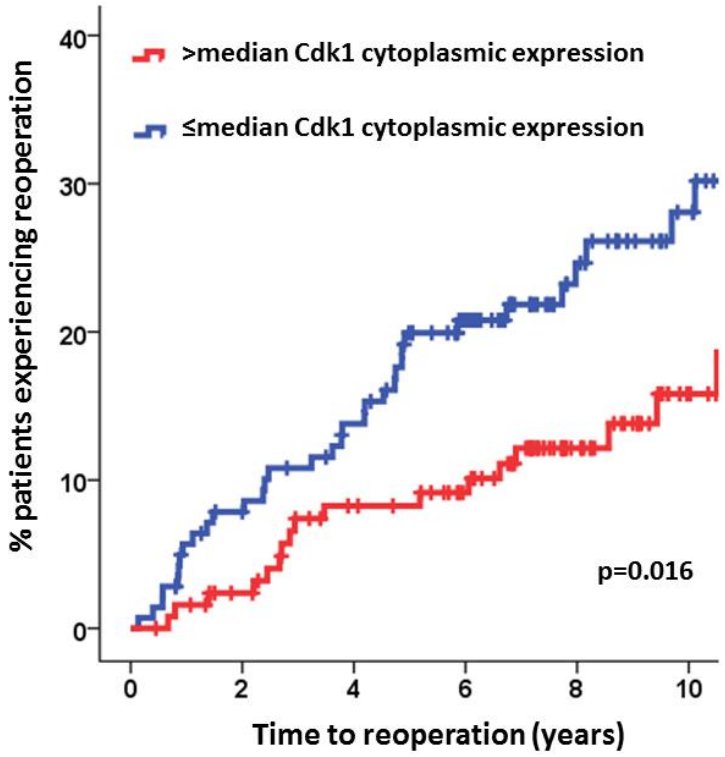
Figure 9.3 Kaplan Meier plot stromal pCdk1^{161} expression and time to failure of operative management in BPH patients



9.3.3 Reoperation

Low cytoplasmic Cdk1 expression was associated with shorter time to reoperation HR 0.53 (95% CI 0.31-0.90) (Figure 9.4).

Figure 9.4 Kaplan Meier plot cytoplasmic Cdk1 expression and time to reoperation in BPH patients



9.4 Multivariate analysis

As with AR and pAR expression significant univariate candidate kinase results were included in a backwards conditional cox-regression model to determine independence from significant clinical parameters.

When nuclear pCdk1^{161} expression was combined with age, CVD and presence of a preoperative catheter in a multivariate cox regression analysis, age (p=0.016, HR 20.51 (95% CI 1.13-372.41)) was independently associated with postoperative AUR.

No independent variables were found when significant clinicopathological factors were combined with protein expression in a cox regression analysis with regards to failure of surgical management and reoperation.

9.5 Association of candidate kinases to phosphorylated AR sites

Once the clinical significance of the candidate kinases had been established, their association with AR phosphorylation was then investigated in order to establish whether the predicted kinase was observed to correlate with clinically relevant AR phosphorylation sites in the clinical samples.

As shown in Table 9.6 pERK1/2 expression was not significantly associated with any of the clinically relevant AR phosphorylation sites, however Cdk1 and/or pCdk1^{161} were significantly associated with pAR^{S81}, pAR^{S515} and pAR^{S650} as predicted by Scansite 2.0 (Table 9.6).

Table 9.6 Pearson's correlation of significant AR serine phosphorylation sites and candidate kinases in BPH

AR phosphorylation sites			Cdk1				pCdk1^{161}				pERK1/2			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma	Nuclear	Cytoplasmic	Smooth muscle	Stroma	Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-81	Nuclear	c.c.	**0.517**	0.225	0.104	0.050	0.145	0.064	-0.65	-0.100	0.247	0.158	0.027	-0.180
		p value	**<0.001**	<0.001	0.061	0.361	0.008	0.247	0.240	0.069	<0.001	0.004	0.626	0.001
	Cytoplasmic	c.c.	0.060	0.385	-0.005	-0.083	-0.075	**0.431**	-0.97	-0.066	0.047	0.077	0.074	-0.104
		p value	0.279	<0.001	0.922	0.133	0.175	**<0.001**	0.077	0.233	0.390	0.162	0.176	0.057
	Smooth muscle	c.c.	0.223	0.240	0.267	0.189	0.114	0.360	0.178	0.146	0.144	0.173	0.020	-0.124
		P value	<0.001	<0.001	<0.001	0.001	0.038	<0.001	0.001	0.008	0.009	0.002	0.714	0.024
	Stroma	c.c.	0.224	0.227	0.157	0.183	0.121	0.162	0.029	0.087	0.147	0.128	0.077	-0.190
		P value	<0.001	<0.001	0.005	0.001	0.029	0.003	0.595	0.111	0.008	0.021	0.161	<0.001
Ser-515	Nuclear	c.c.	**0.452**	0.385	0.143	0.194	0.208	0.074	-0.123	-0.089	0.200	0.141	0.106	-0.059
		p value	**<0.001**	<0.001	0.010	<0.001	<0.001	0.183	0.026	0.108	<0.001	0.011	0.055	0.287
	Cytoplasmic	c.c.	0.005	0.218	0.108	0.237	0.291	0.266	0.025	0.103	0.255	0.217	0.214	0.189
		p value	0.932	<0.001	0.050	<0.001	<0.001	<0.001	0.649	0.062	<0.001	<0.001	<0.001	0.001
	Smooth Muscle	c.c.	0.344	0.283	0.370	0.353	0.193	0.204	0.082	0.106	0.090	0.090	0.021	-0.086
		p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.139	0.055	0.106	0.104	0.702	0.119
	Stroma	c.c.	**0.404**	0.279	0.297	0.385	0.182	0.093	-0.034	0.029	0.097	0.068	0.095	0.004
		p value	**<0.001**	<0.001	<0.001	<0.001	0.001	0.091	0.540	0.597	0.079	0.216	0.082	0.939

Table 9.6 continued Pearson's correlation of significant AR serine phosphorylation sites and candidate kinases in BPH

AR phosphorylation sites			Cdk1				pCdk1^{161}				pERK1/2			
			Nuclear	Cytoplasmic	Smooth muscle	Stroma	Nuclear	Cytoplasmic	Smooth muscle	Stroma	Nuclear	Cytoplasmic	Smooth muscle	Stroma
Ser-650	Nuclear	c.c.	0.343	0.119	0.138	0.283	**0.506**	0.128	0.011	-0.040	0.263	0.240	0.301	0.190
		p value	<0.001	0.031	0.012	<0.001	**<0.001**	0.020	0.839	0.470	<0.001	<0.001	<0.001	<0.001
	Cytoplasmic	c.c.	0.175	0.344	0.141	0.094	0.040	0.331	0.015	-0.095	0.097	0.087	0.056	0.048
		p value	0.001	<0.001	0.011	0.086	0.465	<0.001	0.784	0.082	0.079	0.113	0.308	0.386
	Smooth Muscle	c.c.	0.145	0.178	**0.430**	**0.434**	0.366	0.271	0.205	0.200	0.127	0.172	0.315	0.071
		p value	0.009	0.001	**<0.001**	**<0.001**	<0.001	<0.001	<0.001	<0.001	0.021	0.002	<0.001	0.199
	Stroma	c.c.	0.244	0.144	0.374	**0.521**	**0.495**	0.230	0.104	0.207	0.194	0.191	0.309	0.119
		p value	<0.001	0.009	<0.001	**<0.001**	**<0.001**	<0.001	0.059	<0.001	<0.001	<0.001	<0.001	0.030

9.6 Discussion

Kinases hypothesised to be responsible for AR phosphorylation were identified via Scansite. These were Cdk1 for Ser-81, Ser-515 and Ser-650 and ERK1/2 for Ser-515 and Ser-650. The expression of these candidate kinases was observed to be associated with clinicopathological factors in the BPH cohort. Specifically, high expression of Cdk1 in the stroma was associated with increased age. Previous work has demonstrated an inverse link between the Cdk inhibitor $p27^{(Kip1)}$ and age in BPH tissue (200), which supports the results of the current study.

Low expression of nuclear and stromal pERK1/2 was associated with increased incidence of DM2 and increased BMI respectively. DM2 and obesity are interlinked diseases forming part of the metabolic syndrome. These relationships are in contrast to previous work which has shown that activation of the ERK pathway is strongly associated with whole-body insulin resistance, and also indicated that ERK is likely a key modulator of the development of insulin resistance.(262) Furthermore the inhibition of ERK phosphorylation by administration of a MEK inhibitor (U0126) decreased the blood glucose levels of a mouse model for metabolic syndrome (db/db knockouts).(263) The explanation for these results is unclear and may reflect tissue specificity, however requires further investigation in a prospective cohort of BPH patients and mechanistic work in BPH cell lines.

Low proliferation index and low apoptotic index were associated with high expression of $pCdk1^{161}$ and pERK1/2. This relationship is perhaps contrary to expectations and could suggest that following primary surgery a reverse relationship may manifest. As previously discussed, fast proliferating cells that out grow their nutrient supply could result in inflammation and necrosis causing

a feedback pathway to shut down the overall growth of the gland. To add weight to this theory low expression levels of Cdk1 and pERK1/2 were also associated with increased levels of tissue necrosis and high cytoplasmic pERK1/2 (suggesting low nuclear expression) was associated with increased systemic inflammation. These explanations represent hypotheses only and clearly there is a need for further investigation using mechanistic models, which is out with the scope of this project.

Interestingly pERK1/2 expression was not found to be associated with any of the clinical outcome measures, as was the case in the pilot prostate cancer cohort. Previous studies comparing pERK1/2 expression in normal, hyperplastic and cancerous human prostate specimens has shown increased overall pERK1/2 expression in prostate cancer > BPH > normal prostate.(264) This relationship was replicated in the current study with regards to BPH and prostate cancer. pERK1/2 likely has downstream consequences in BPH however the current study infers that these may be operating primarily via different pathways than via AR. The clinical relevance of pERK1/2 expression and downstream signaling in BPH requires further investigation.

Similar to the pilot prostate cancer cohort, expression of both Cdk1 and pCdk1^{161} were associated with clinical outcome measures suggesting that Cdk1 may be of functional importance. This is reinforced by previous work in which p27$^{(Kip1)}$ knock out mice develop histological BPH (265). Studies have demonstrated the complete absence of p27(Kip1) expression in human BPH nodules.(266) In contrast, diffuse p27(Kip1) expression was observed in normal prostate tissue with reduced expression in prostate cancer.(266) This points towards a pivotal role of Cdk signalling in the development and progression of BPH.

pERK1/2 was not related to any of the AR serine phosphorylation sites observed to be clinically relevant in the previous chapter. However, Cdk1 and pCdk1^{161} were associated with pAR^{S81}, pAR^{S515} and pAR^{S650} in at least one subcellular location.

In the previous chapter low expression of smooth muscle pAR^{S515} expression was found to inform on the risk of AUR after primary TUR surgery in older patients with BPH. Although correlation of smooth muscle pAR^{S515} expression with Cdk1/pCdk1^{161} did not reach significance there was a trend for association of Cdk1 in all cell types/subcellular locations.

In addition low levels of cytoplasmic pAR^{S650}, which trended towards a correlation with expression of cytoplasmic Cdk1 and pCdk1^{161}, were found to inform on patients with high/abnormal PSA at diagnosis associated with increased failure of operative management in patients with PSA ≥4ng/ml at diagnosis. Once again this has potential clinical utility with regards to the pre and post operative counselling of these patients and the targeted use of prophylactic medical treatment. Over 20 Cdk inhibitors are already in use in clinical trials in monotherapy and in combination therapy for other diseases.(267-270) Inhibition of cell cycle Cdks is thought to inhibit cellular proliferation and therefore disease progression. Both broad Cdk inhibitors (e.g. Cdk 1, 2, 4, 5 and 9) and Cdk specific inhibitors (Cdk4 and 6) are available. Their potential use in BPH is as yet unexplored. In the context of the current study high pCdk1^{161} expression was associated with shorter time to failure of surgical management and postoperative AUR which may benefit from direct inhibition. However, low expression of pAR at serine residues was associated with poorer clinical outcome and these sites were directly associated with

Cdk1/pCdk1^{161} in this case a Cdk inhibitor may actually prove to be detrimental. Further work is required in order to unpick the modulation of pAR expression by Cdk1 in BPH prior to an investigation into the usefulness of Cdk inhibitors in this disease.

Serine phosphorylation of the androgen receptor has been shown to be associated with Cdk1/pCdk1^{161} in BPH patients. Future work will include the undertaking of mechanistic cell line studies in order to clarify these relationships, this is unfortunately out with the scope of the current study.

Conclusion

In conclusion the work presented comprises the first fully comprehensive examination of the relevance of AR serine phosphorylation, and associated kinases, in prostatic disease in the clinical setting.

Key findings

The key findings in study were:

1. AR serine phosphorylation may have clinical relevance in both BPH and prostate cancer.

2. High levels of AR phosphorylation at serine residues is generally associated with worse outcome in prostate cancer, whilst low expression is associated with worse outcome in BPH.

3. Upstream kinases mediating AR serine phosphorylation, in particular Cdk1, may also have clinical relevance in BPH and prostate cancer.

Key advancement in science

The current study has demonstrated for the first time the significance of AR serine phosphorylation in prostate cancer and BPH human tissue specimens in relation to clinical outcome. In addition, the Cdk1/pAR axis may have potential as a new target for therapeutic intervention. AR serine phosphorylation has been identified as a worthwhile avenue for future investigation as a prognostic and predictive biomarker in prostatic disease.

Potential clinical applications

AR serine phosphorylation has been demonstrated to have potential as a predictive and prognostic biomarker in both prostate cancer and BPH. Furthermore, the primary experimental technique utilised (immunohistochemistry) is currently employed in every diagnostic pathology lab in the UK and it is for this reason that this is considered a fully translational research study.

Areas of future research

Although the results presented are exciting they are far from conclusive and this study lays the foundation for further investigation. Future work will firstly include the additional validation of the phosphospecific antibodies utilised in this study via siRNA and knockout mice, phosphatase assays, kinase and phosphatase inhibitors, cell transfectants and site specific mutagenesis. Once further antibody specificity has been established immunohistochemistry on a large, multicentre, prospectively collected cohort of both BPH and prostate cancer patients should be undertaken. In parallel mechanistic cell line work via silencing and functional assays would further clarify the nature of the kinase/pAR axis.

AR serine phosphorylation in prostatic disease represents a potential diagnostic tool to aid the differentiation of indolent from aggressive disease. In the future this may translate as increased survival and reduction of potential harm to patients from overtreatment of both prostate cancer and BPH.

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Appendix Published Manuscripts