Androgen receptor phosphorylation in prostate cancer

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Abstract

Prostate cancer is the most common male cancer in the UK. Although incidence is increasing, prostate cancer mortality is decreasing, mainly owing to the over diagnosis of disease that would not have become clinically apparent during the patient's lifetime. The gold-standard for prostate cancer diagnosis is transrectal ultrasound guided biopsy of the prostate. Whilst prostate biopsy can inform on diagnosis, it's prognostic ultiltiy is poor. Currently clinicians lack pathological biomarkers to differentiate between patients with prostate cancer who have indolent disease that can be safely managed with surveillance strategies, and those who will go onto develop aggressive disease which requires early radical curative treatment.

Phosphorylation of the androgen receptor has been extensively investigated in relation to prostate cancer development and progression. Androgen receptor phosphorylation has been shown to regulate cellular localisation, transcriptional activity, cell growth and sensitivity to androgens in prostate cancer. However, only a small number of studies have investigated the prognostic significance of androgen receptor phosphorylation, and only consider a limited number of serine residues in clinical specimens.

The research presented in this thesis sought to investigate the prognostic and predictive significance of AR phosphorylation at serine 578 in hormone-naïve prostate cancer. It was hypothesised that pAR^{S578} would be associated with poor outcomes in prostate cancer and may be utilised as a prognostic marker at diagnosis in prostate cancer and predict response to drug treatment with a PKC inhibitor. It was also hypothesised that PKC, the putative kinase for phosphorylation at serine 578, would be associated with poor outcomes and may offer a potential therapeutic target in prostate cancer.

In the current study, the phosphorylation site of primary interest was serine 578. Scansite 2.0, an online kinase search tool, predicted that PKC is the putative kinase mediating phosphorylation at serine 578 on the androgen receptor. Phosphorylation of the androgen receptor at serine 578 has been linked with increased AR transcriptional activity, cell growth, nuclear cytoplasmic shuttling,

modulation of other AR phosphorylation sites and DNA-repair mechanisms. The prognostic significance of androgen receptor phosphorylation at serine 81 was also investigated in this study. Serine 81 is phosphorylated in response to DHT via an alternative pathway to that of serine 578. Serine 81 phosphorylation is associated with increased androgen receptor transcriptional activity and increased cell growth in prostate cancer. It was therefore hypothesised that androgen receptor phosphorylation at serine 578 and serine 81 would be associated with poor outcome measures in prostate cancer.

Immunohistochemical analysis was performed in a cohort of 105 hormone-naïve prostate cancer patients undergoing active surveillance, representing a cohort of patients with low-risk disease, as defined by current clinical markers such as PSA and Gleason score at diagnosis. Nuclear PKC expression was significantly associated with pAR^{S578} expression in the clinical specimens, supporting the prediction of Scnasite 2.0 that PKC is the kinase responsible for phosphorylation of the AR at this site. High cytoplasmic expression of pAR^{S81} was associated with decreased time to intervention (HR 2.76 (95% CI 1.1-7.3), p=0.032). There was no association between pAR^{S578} and time to intervention in this cohort. Analysis of combined expression of both phosphorylation sites revealed an association between high dual expression of cytoplasmic pAR^{S81} and cytoplasmic pAR^{S578} and decreased time to treatment intervention (HR 2.35 (95% CI 1.2-4.6), p=0.031). These results suggest a synergistic prognostic effect when these two phosphorylation sites are combined and identifies a sub-population of low-risk prostate cancer patients who are at increased risk of disease progression.

A second study was conducted to investigate if these results could be replicated in a cohort of prostate cancer patients with all stages of disease at diagnosis. Immunohistochemical analysis in 90 hormone-naïve prostate cancer patients found that high expression of nuclear pAR^{S81} (HR 2.1 (95% CI 1.1 – 4.2), p=0.030), nuclear pAR^{S578} (HR 2.24 (95% CI 1.0-4.9), p=0.036) and cytoplasmic pAR^{S578} (HR 4.54 (95% CI 2.0-10.4), p=<0.001) was associated with decreased disease survival. Furthermore, high expression of cytoplasmic pAR^{S578} was associated with decreased time to biochemical relapse (HR 2.1 (95% CI 1.0-4.2), p=0.034) and decreased disease-specific survival following biochemical relapse (HR 3.2 (95% CI 1.0-9.9), p=0.034). Dual expression of nuclear, cytoplasmic and total pAR^{S81} and

pAR^{S578} were all associated with decreased-disease specific survival, suggesting that there is a sub-population of prostate cancer patients who may benefit from dual targeted therapy with androgen deprivation therapy and PKC inhibitors.

A validation cohort of 243 hormone-naïve prostate cancer patients with all stages of disease was utilised to verify the results of the second cohort. Unfortunately, due to technical issues and time constraints, IHC could not be completed for the phosphorylation sites of interest in all patients. Despite this, high expression of cytoplasmic pAR^{S578} was significantly associated with decreased time to biochemical relapse (HR 2.9 (95% CI 1.0-8.2), p=0.037) and trended towards an association with decreased overall survival (p=0.076). Interestingly, dual expression of high cytoplasmic pAR^{S81} and cytoplasmic pAR^{S578} was associated with decreased overall survival (HR 2.1 (95% CI 1.3-3.3) p=0.001) despite neither phosphorylation site independently predicting decreased overall survival.

Lastly, a study to develop a technique for isolation, propagation and characterisation of primary prostate cancer cells from TRUS biopsy specimens was undertaken. Two primary prostate cell cultures were developed which were confirmed to have a malignant luminal epithelial cell phenotype with a functional AR using flow cytometry, RT-PCR and immunofluorescence. This technique is of high translational relevance, as it provides a model with potential to identify biomarkers to predict individual patient's response to prostate cancer therapies.

Overall these results suggest that androgen receptor phosphorylated at serine 81 and serine 578 are associated with poor outcomes in prostate cancer and are potential targets for new drug therapies. Additional studies are required to validate these results in a larger multi-centre cohort of prostate cancer patients before either of these phosphorylation sites can be utilised as a biomarker in clinical practice.

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Publications and presentations

Publications relating to this thesis

Patek, S.C. et al. Androgen receptor phosphorylation status at serine 578 predicts poor outcome in prostate cancer patients. Oncotarget 2017; 8:4875-4887. PMID 27902483

Abstracts relating to this thesis

Patek, S.C. et al. Isolation, propagation and characterisation of primary prostate cancer epithelial cell lines from prostate specimens. Endocrine Abstracts 2016; 42:35

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Authors declaration

The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Samantha Patek

Dedication

This thesis is dedicated to my husband Mark and our beautiful daughter.

List of abbreviations

ADT	Androgen deprivation therapy
AF-1	Activation function 1
AF-2	Activation function 2
AR	Androgen receptor
ARE	Androgen response elements
AS	Active surveillance
ASAP	Atypical small acinar proliferation
BPH	Benign prostatic hyperplasia
BRCA	Breast cancer gene
Cdk	Cyclin-dependent kinase
CRPC	Castration-resistant prostate cancer
CT	Computed tomography
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DRE	Digital rectal examination
EBRT	External beam radiotherapy
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases
FSH	Follicle-stimulating hormone
GnRH	Gonadotrophin-releasing hormone
GWAS	Genome wide association studies

H&E	Haematoxylin and eosin
HGPIN	High-grade prostatic intraepithelial neoplasia
HIFU	High-intensity focused ultrasound
HRP	Horseradish peroxidase
HSP	Heat-shock proteins
IF	Immunofluorescence
IHC	Immunohistochemistry
ICCC	Interclass correlation coefficient
ISUP	International Society of Urological Pathology
KLK3	Kallikrein-3 gene
LBD	Ligand-binding domain
LH	Luteinising hormone
LNCaP	Lymph node carcinoma of prostate
LHRH	Luteinising hormone-releasing hormone
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
MP-MRI	Multi-parametric magnetic resonance imaging
MRI	Magnetic resonance imaging
NICE	National Institute for Clinical Excellence
NTD	N-terminal domain
PAK	p21-activated kinase
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PDPK1	3-phosphoinositide-dependent protein kinase 1
PFA	Paraformaldehyde
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PSA	Prostate specific antigen
SUMO	Small ubiquitin-like modifier
TBS	Tris-buffered saline
TMA	Tissue microarray
TNM	Tumour node metastasis
TRUS	Trans rectal ultrasound
TURP	Transurethral resection of the prostate
UTI	Urinary tract infection
VCaP	Vertebral carcinoma of prostate
WHO	World Health Organisation
WHS	Weighted histoscore

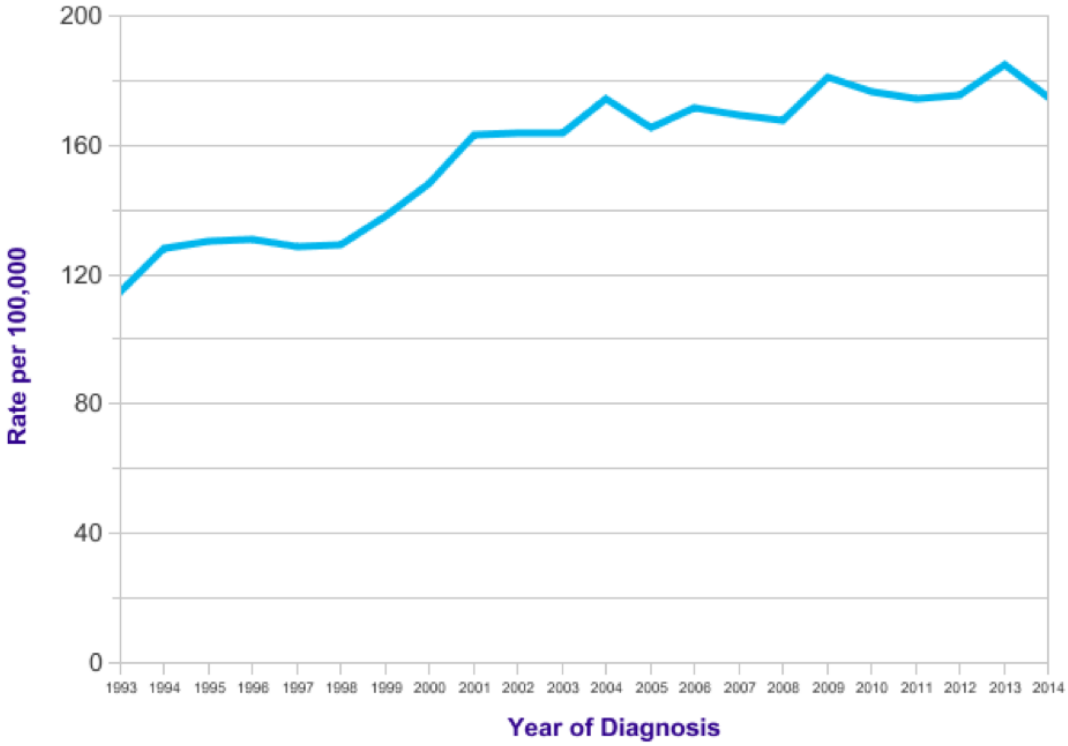
Chapter 1 Introduction

1.1 Prostate cancer epidemiology, pathology and prognostic factors

1.1.1 Prostate cancer incidence, mortality and survival

Prostate cancer is the most common male cancer in the UK, with 46,690 new cases diagnosed in 2014. (1) The incidence of prostate cancer has been increasing in the UK for the past 40 years (Figure 1.1). Although currently the second most common cancer overall, it is predicted that by 2035 there will be more than 75,000 new cases of prostate cancer each year, making it the most common cancer overall. (2) The increased incidence of prostate cancer is mainly due to the introduction of the prostate specific antigen (PSA) blood test in the late 1970s and the increased use of transurethral resection of the prostate (TURP) as a treatment for benign prostatic hyperplasia (BPH). This has resulted not only in the earlier detection of prostate cancer but also diagnosis of disease that may not have become clinically apparent during the patient's life time. This over-diagnosis has resulted in over-treatment, with significant associated healthcare costs and unnecessary side effects related to treatment. (3)

Figure 1.1 Incidence of prostate cancer in the UK over time



Age-standardised incidence rates per 100,000 men from 1993-2014. (1)

In the UK, prostate cancer-associated mortality has increased since the 1970s. Mortality rates have increased by more than 50% between the early 1970s and early 1990s. In the last decade, however, prostate cancer mortality has fallen by 13% and is projected to decrease by a further 16% in the next twenty years. (1) There is ongoing debate as to why mortality rates have decreased in the UK but it is thought that this is mainly due to increased use of the PSA test resulting in over-detection of low risk, localised prostate cancer and more aggressive treatment in prostate cancer patients. (4-7)

1.1.2 Prostate cancer risk factors

No definite modifiable risk factors have been identified for the development of prostate cancer. Whilst obesity appears to be linked to aggressiveness of prostate cancer, further evidence is required before it can be determined that obesity is a definite risk factor. (8, 9)

A number of non-modifiable risk factors have been identified that are thought to increase the probability of developing prostate cancer.

1.1.2.1 Age

Advancing age is the most common risk factor associated with prostate cancer development. Greater than half of all cases diagnosed are in those aged 70 years and over. The peak rate of incidence of prostate cancer is in the 90 years and over age group. (1)

1.1.2.2 Ethnicity

Whilst the overall life-time risk of prostate cancer in men in the UK is 1 in 8, there is significant variation amongst men of different ethnicity. (1) In England, it is estimated that 1 in 4 black men will be diagnosed with prostate cancer, double the estimated rate in white men. Asian men have the lowest life-time risk, estimated to occur in 1 in 13 men. (10) Similar trends are observed in the USA. (11)

1.1.2.3 Family History

Men with a first degree relative with prostate cancer are at least twice as likely to develop prostate cancer themselves. The risk increases further with two first degree relatives affected. (12, 13) Family history of a mother with breast cancer is associated with a 1.24-fold increased risk of prostate cancer. (14) A further study has shown that a family history of both prostate and breast cancer increases the risk of prostate cancer development by 89% than compared to a family history of prostate cancer alone. (15)

1.1.2.4 Genetic factors

Genome wide association studies have identified more than 70 genetic variations associated with an increased risk of prostate cancer. Although the majority of these variations have low prevalence, combined they account for approximately 30% of hereditary prostate cancer risk. (16)

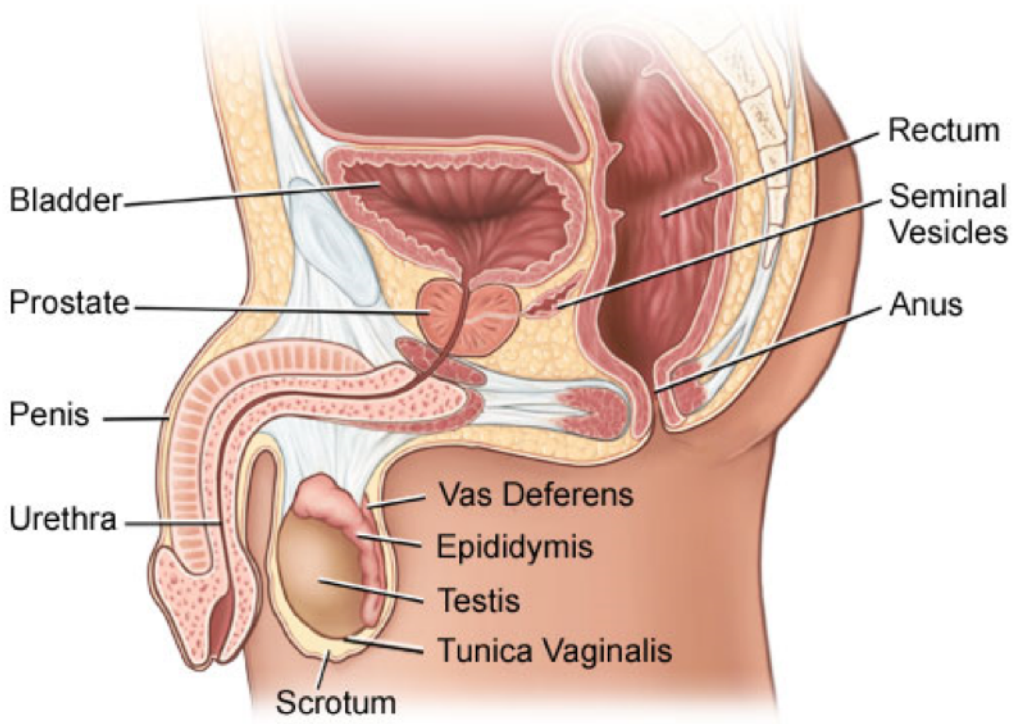
The BRCA1 and BRCA2 genes, well recognised for their role in the increased risk of breast cancer, are also associated with increased incidence of prostate cancer. It is estimated that 2% of men diagnosed with early onset prostate cancer have a BRCA2 mutation, compared to 1 in 300 in the general population. (17, 18) Germline BRCA2 mutations are associated with an 8.6-fold increased risk of prostate cancer development at or before 65 years of age, whilst germline BRCA1 mutation is associated with a more modest increased risk of 3.75-fold. (19, 20) In men with BRCA2 mutation and an elevated PSA, 48% were found to have prostate cancer on subsequent biopsy and were more likely to have intermediate or high-risk disease, suggesting that targeted PSA screening in this population is likely to be of benefit. (21)

1.1.3 Normal Prostate Anatomy and Histology

1.1.3.1 Prostate Anatomy

The prostate is a tubulo-alveolar exocrine gland that forms part of the male reproductive system. It surrounds the prostatic urethra, sitting inferior to the bladder neck and anterior to the rectum and is of similar size to a walnut (Figure 1.2).

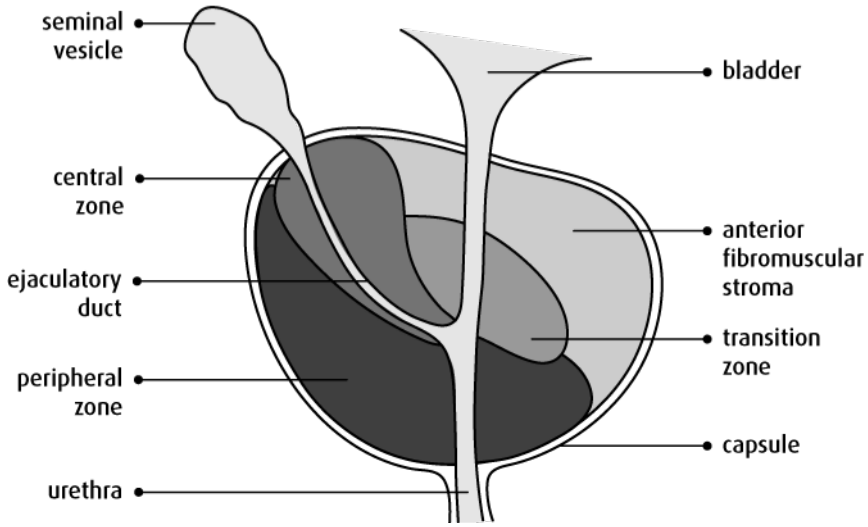
Figure 1.2 The anatomical relations of the prostate gland (22)



The prostate gland is approximately the size of a walnut and sits inferior to the bladder and anterior to the rectum

McNeal described four histologically distinct zones within the prostate (Figure 1.3). (23) The peripheral zone is found postero-laterally and forms 70% of the prostate. Approximately 70-80% of prostate cancers arise in the peripheral zone. The central zone forms 25% of the prostate and is positioned anterior to the peripheral zone. Fewer than 5% of prostate cancers arise in the central zone. The transitional zone surrounds the prostatic urethra and is the exclusive site of origin of BPH. It forms only 5% of the prostate but approximately 10% of prostate cancers arise here. Finally, the anterior fibro-muscular zone is devoid of glandular components, and forms the anterior surface of the prostate.

Figure 1.3 Sagittal image of the zonal anatomy of the prostate (24)



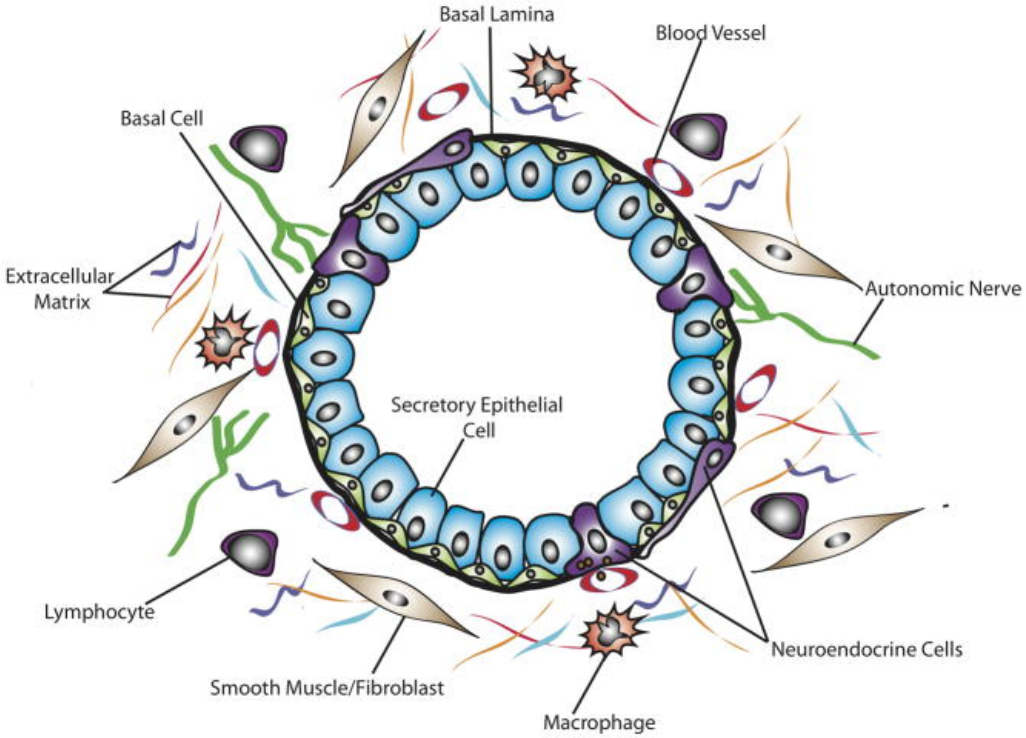
The prostate is made up of four histologically distinct zones. The peripheral zone, located postero-laterally, is the largest zone and most common site of prostate cancer, with 70-80% of prostate cancers arising here.

1.1.3.2 Prostate Histology

The normal prostate is composed of glands surrounded by stroma (Figure 1.4). The glandular lumen is lined by secretory luminal epithelial cells which express the androgen receptor (AR). These tall, columnar epithelial cells secrete prostatic acid phosphatase (PAP), PSA and human kallikrein-2 into the lumen of the gland to form seminal fluid. Basal epithelial cells separate luminal epithelial cells from the basement membrane and are thought to secrete components of the basement membrane. These low, cuboidal epithelial cells have low expression of AR. Amongst the basal cell population, it is proposed that progenitor stem cells produce prostatic epithelial cells via intermediate cell stages. (25) Neuroendocrine cells, the third epithelial cell type found within the prostate, are irregularly distributed throughout the glands. They do not express AR and their role is not fully understood.

The fibromuscular stroma of the prostate surrounds the glands and is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerve cells and inflammatory infiltrates.

Figure 1.4 Normal histology of the prostate (26)



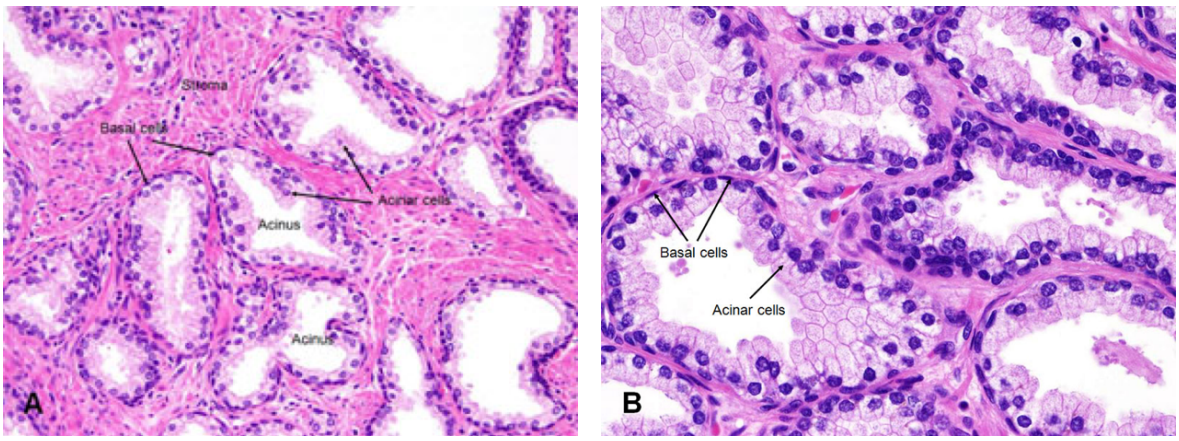
Glandular lumens within the prostate are lined with secretory epithelial cells on a basement membrane/ basal lamina. Basal cells separate the secretory epithelial cells from the basement membrane and secrete components of the basement membrane. Neuroendocrine cells are irregularly dispersed throughout the gland.

1.1.4 Prostate Pathology

1.1.4.1 Benign Prostatic Hyperplasia

The prostate is the only internal organ in men that continues to grow throughout adulthood. In BPH, there is benign proliferation of both stromal and epithelial components of the prostate, occurring exclusively in the transitional zone (Figure 1.5).

Figure 1.5 Histopathology of normal prostate tissue and BPH (27)



(A) Normal prostate tissue and (B) BPH characterised by epithelial and stromal hyperplasia.

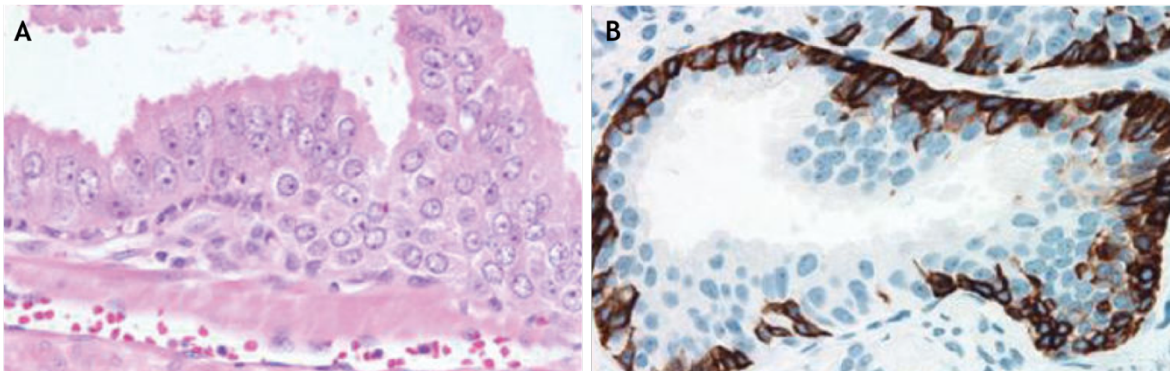
As the transitional zone enlarges, there is compression of the peripheral zone. Androgens are known to play a role in BPH; castrated pre-pubescent males do not develop BPH in later life. BPH is so common in men, that it is viewed by many as a normal part of the ageing process. It is estimated that 20% of men in their 40s will have BPH rising to 80-90% of men in their 70s and 80s. (28) The clinical manifestation of BPH is with bothersome lower urinary tract symptoms (LUTS) comprised of urinary frequency, urinary urgency, hesitancy and incomplete bladder emptying. Complications of BPH include recurrent urinary tract infection (UTI), bladder calculi and acute urinary retention. There is no association between BPH and the later development of prostate cancer. (29) Treatment for BPH includes conservative management with lifestyle advice initially where appropriate, before progressing to medical treatment and/or surgery if required. Medical management is with 5-alpha reductase inhibitors, which inhibits the conversion of testosterone to the more potent dihydrotestosterone (DHT), and alpha-blockers, which cause prostatic smooth muscle relaxation. The mainstay of surgical management in BPH is TURP, however this is becoming less common with the success of medical management.

1.1.4.2 High-grade Prostatic Intraepithelial Neoplasia

High-grade prostatic intraepithelial neoplasia (HGPIN) is an asymptomatic, pathological entity that is a premalignant lesion of prostate adenocarcinoma. It is characterised by the presence of atypical epithelial cells with prominent

nucleoli, increased nuclear-to-cytoplasmic ratio and increased nuclear size. In addition, there is increased proliferation of luminal epithelial cells, with complete or partial preservation of the basal cell layer but no invasion into the surrounding stroma (Figure 1.6). As with prostate cancer, HGPIN is most commonly identified in the peripheral zone and can be multifocal. HGPIN is more common with advancing age. (30) There is no evidence that HGPIN causes elevation of serum PSA. HGPIN has been found to be independently associated with increased risk of developing prostate cancer, particularly if multifocal. (31) As such, patients with HGPIN in ≥3 biopsy sites on an otherwise negative prostate biopsy are recommended to have repeat biopsy. (32)

Figure 1.6 Histopathology of high-grade prostatic intraepithelial neoplasia (30)



(A) Haematoxylin and eosin (H&E) stained section of HGPIN with luminal epithelial cell proliferation and prominent nuclei and nucleoli (B) Prostate biopsy section stained for high molecular weight keratin, a basal cell marker, showing HGPIN with disruption of the basal cell layer

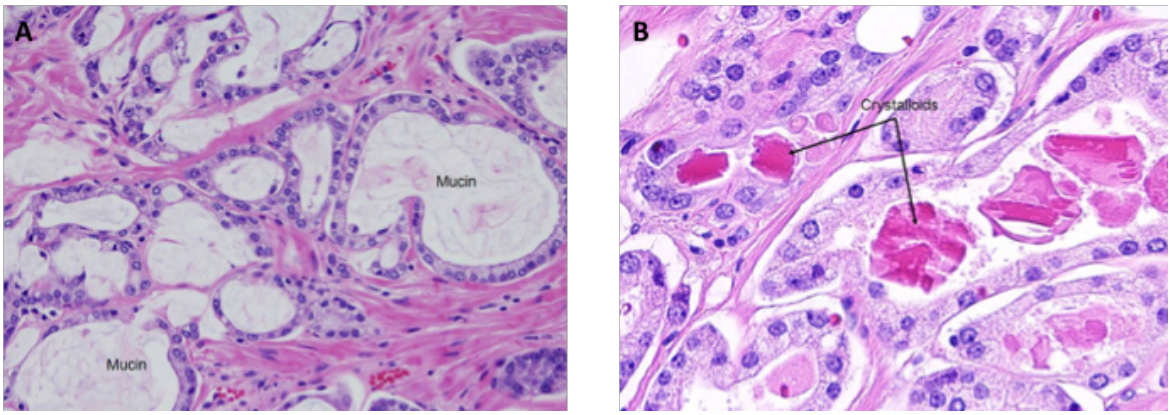
1.1.4.3 Atypical small acinar proliferation

Atypical small acinar proliferation (ASAP) is a histological entity by which prostate needle biopsy specimens contain suspicious foci with features of malignancy but is insufficient for definitive diagnosis or exclusion of prostate cancer. Approximately 5% of prostate needle biopsies performed are found to contain a focus of ASAP. (33) The presence of ASAP on an initial prostate biopsy is associated with a 40% risk of prostate adenocarcinoma on subsequent biopsy. (33) It is therefore recommended that patients with ASAP on an otherwise negative biopsy should have a repeat biopsy performed. (32)

1.1.4.4 Prostate Cancer

Acinar adenocarcinoma of the prostate is the most common malignancy of the prostate, accounting for more than 90% of prostate cancers. As earlier described, it most commonly occurs in the peripheral zone of the prostate but can also arise in the transitional and central zones to a lesser extent. Acinar adenocarcinoma is characterised by the presence of large nuclei with prominent nucleoli, luminal mucin, crystalloids and amorphous eosinophilic secretions and most importantly the absence of basal cells. Further pathognomonic features include glomerulation, mucinous fibroplasia and perineural invasion (Figure 1.7).

Figure 1.7 Histopathology of acinar adenocarcinoma of the prostate (27)



H&E stained sections of acinar adenocarcinoma of the prostate with (A) luminal mucin and (B) crystalloids, features which are pathognomonic for prostate cancer.

Non-acinar prostate cancer forms the remaining 5-10% of prostate cancers. Subtypes include ductal adenocarcinoma, the most common non-acinar prostate cancer, sarcomatoid carcinoma, squamous cell and adenosquamous carcinoma, transitional cell carcinoma, small cell carcinoma, basal cell carcinoma and clear cell carcinoma. Each subtype has specific histological and clinical features, with varying incidence and prognosis. (34)

The main focus of this thesis will be acinar adenocarcinoma of the prostate.

1.2 Management principles of prostate cancer

1.2.1 Presentation

Currently, there are no recognised signs or symptoms that are reliable predictors of prostate cancer. (35) Localised prostate cancer is often asymptomatic, whereas locally advanced prostate cancer may produce LUTS, and are difficult to distinguish between those secondary to BPH. Less frequently, and only with metastatic disease, patients may present with bone pain, fatigue and unintentional weight loss.

Most patients are diagnosed with prostate cancer in the UK following investigation for LUTS in primary care. (36) Patients who present with LUTS are frequently offered a serum PSA test and clinical examination, including digital rectal examination (DRE) of the prostate. An elevated PSA and/or abnormal DRE should prompt referral for further investigation.

A further small subpopulation of patients will be diagnosed with prostate cancer following an incidental finding of malignancy in prostate tissue resected for other diseases, such as BPH and bladder cancer.

1.2.2 Diagnosis

Definitive diagnosis of prostate cancer is made following histological examination of prostate tissue, most commonly obtained via trans rectal ultrasound (TRUS) guided biopsy of the prostate. The decision to perform prostate biopsy should be made based on PSA level, DRE findings, risk factors and co-morbidities. Biopsy is not routinely recommended in patients with a high clinical suspicion of prostate cancer and evidence of bone metastases unless entering a clinical trial. The Royal College of Pathologists has set out recommendations for the reporting of TRUS biopsies to ensure standardisation in practice and includes the number and location of the cores obtained, histological tumour type, histological grading using both the Gleason grading system and grade group, the number of cores involved, an estimate of tumour

extent, the presence of perineural invasion and evidence of extra-prostatic extension. (37)

Traditionally, TRUS biopsy was offered before cross-sectional imaging had confirmed the presence of tumour, however with increasing availability of MRI many patients now have an MRI prior to TRUS biopsy. TRUS biopsies are not without complication – side effects include discomfort, bleeding, transient impotence, urinary retention and rarely, life-threatening sepsis. Despite the use of MRI many biopsies are still non-targeted. It is estimated that in non-targeted TRUS biopsy 2-10% of patients will have a malignancy that is not detected on the initial biopsy. Furthermore, non-targeted TRUS biopsy has been found to detect a higher rate of clinically insignificant prostate cancers compared to targeted techniques with the use of multi-parametric magnetic resonance imaging (MP-MRI). The Prostate Imaging-Reporting and Data System version 2 (PI-RADS v2) is used to score findings on prostate mpMRI and assign a risk category for the likelihood of the presence of clinically significant prostate cancer. Patients are assigned a score of 1 to 5, where a score of PI-RADS 1 is associated with a very low risk of clinically significant prostate cancer and a score of PI-RADS 5 is associated with a very high risk of clinically significant prostate cancer. (38) The use of MP-MRI to guide biopsy has been shown to be more specific and less sensitive than non-targeted TRUS biopsy in the detection of clinically significant prostate cancer. It is estimated that MP-MRI performed before prostate biopsy could therefore reduce the number of TRUS biopsies performed by up to 25%. (39, 40) Inter-observer variability is a major limitation of mpMRI, (41) which PI-RADS v2 aims to overcome. Whilst recent studies of the reproducibility of PI-RADS v2 has demonstrated ongoing moderate inter-observer variability(42, 43), a meta-analysis has shown this scoring system to perform well in terms of sensitivity and specificity for prostate cancer detection. (44) As such, the National Institute for Clinical Excellence (NICE) is currently considering the introduction of MP-MRI before TRUS biopsy.

Following histological confirmation of prostate cancer, clinical staging should be completed if the patient is suitable for radical treatment. MP-MRI, or computed tomography (CT) if MRI is contraindicated, is recommended to obtain a radiological tumour node metastasis (TNM) stage. Once staging is complete,

patients should be discussed by a urological cancer multi-disciplinary team (MDT) to decide further management.

1.2.3 Current prognostic markers

1.2.3.1 PSA

PSA is a serine protease produced by luminal epithelial cells in the prostate in response to androgens. It is encoded by the kallikrein-3 (KLK3) gene in an androgen-dependent manner in the normal prostate. PSA forms a major component of seminal fluid. It is the protease responsible for cleavage of semenogelin I and II, resulting in liquefaction of ejaculate, thus increasing sperm motility and aiding fertilisation. (45, 46)

In the normal prostate, the basal cell layer and basement membrane surrounding luminal epithelial cells forms a barrier preventing PSA from entering the circulation. Prostate cancer causes disruption of this barrier with associated loss of the normal glandular architecture, allowing PSA to enter the systemic circulation, resulting in an elevated concentration of serum PSA.

PSA is a useful prognostic biomarker in prostate cancer. Increased pre-operative PSA levels in patients treated with radical prostatectomy are associated with higher Gleason scores, increased risk of extracapsular extension, positive surgical margins, seminal vesicle invasion and decreased time to biochemical progression. (47) Furthermore, PSA has been shown to predict pathological stage in prostate cancer, even when allowing for the increased diagnosis of low-risk, localised prostate cancers. (48) PSA is currently used in the NICE guidelines for risk stratification for men with localised prostate cancer (Table 1.1). PSA has also been used in assessing response to treatment and disease progression.

The 4K panel, a blood test that measures total PSA, free PSA, intact PSA and human kallikrein 2 in combination with patient age, can improve prediction of reclassification at the first surveillance biopsy in men entering active surveillance program. (49)

The role of PSA as a screening tool in prostate cancer remains highly controversial. Conflicting results have been reported from large population studies in the USA and Europe with regards to PSA screening. (6, 50) The Cluster Randomized Trial of PSA Testing for Prostate Cancer (CAP) recently reported on the effectiveness of a single PSA measurement as a screening tool in the UK. There was an increased diagnosis of low-risk prostate cancer in the intervention group with no significant difference in prostate cancer mortality at 10 years compared to the control group. These findings do not support single PSA testing as a screening program for prostate cancer in the UK. (7) Currently, there is no screening programme for prostate cancer in the UK.

Table 1.1 Risk stratification of men with localised prostate cancer (51)

Risk category	Serum PSA		Gleason score		Clinical stage
Low	<10 ng/ml	and	≤6	and	T1-T2a
Intermediate	10-20 ng/ml	or	7	or	T2b
High	>20 ng/ml	or	8-10	or	≥T2c

NICE recommends using serum PSA, Gleason score and clinical stage at diagnosis to assign men with localised prostate cancer a risk category. This can aid clinicians in deciding the most appropriate management for the patient.

1.2.3.2 Gleason

Gleason grading has been well established as a strong predictor of prostate cancer prognosis. Along with PSA, Gleason score is used in the NICE guidelines for risk stratification for men with localised prostate cancer (Table 1.1). Gleason grading was developed in the 1960s and 1970s as a histological grading system based on the architectural patterns of malignant cells on H&E stained prostate tissue specimens. (52) Traditionally, a score was assigned based on five basic grade patterns ranging from 1 to 5, with Gleason Pattern 1 showing very well differentiated prostate cancer and Gleason Pattern 5 being the most poorly differentiated pattern of prostate cancer. The two predominant Gleason patterns were added together to give a score of 2-10. (53) Since its inception, several modifications have been made to Gleason grading. Notably, in 2005 the International Society of Urological Pathology (ISUP) made a number of recommendations for changes to the Gleason grading system. They recommended discarding the use of patterns 1-2 and only reporting Gleason score 6 and above on needle biopsy specimens. Furthermore, they also recommend that should a tertiary pattern be identified on needle biopsy, that is the presence of patterns 3, 4 and 5, the tumour should be considered high grade. In this instance, the primary pattern and highest grade should be used to formulate the overall Gleason score. (54) Since the introduction of the modified criteria, there has been an overall upgrading of Gleason score with improvement in prognostic value of Gleason grading. (55, 56) In 2014, the ISUP made further recommendations to assign new grade groups based on Gleason score, as outlined in Table 1.2, and has been approved for use by the World Health Organisation (WHO). A recent study of more than 10,500 prostate cancer patients enrolled in the CaPSURE registry found the new prognostsic Gleason grade groups were associated with prostate cancer-specific mortality and development of metastases. (57) It is anticipated that the new grade groups will allow more accurate risk stratification, simplify the grading system and hopefully reduce the overtreatment of indolent prostate cancers. (58)

Table 1.2 Gleason Score Prognostic Grade Groups (58)

Prognostic Grade Group	Gleason score
1	≤6
2	3+4 = 7
3	4+3 = 7
4	8
5	9-10

In 2014, the ISUP made recommendations for the new Gleason Score Prognostic Grade groups, made up of five prognostically distinct grade groups, and is based on the 2005 Gleason score grading criteria.

1.2.3.3 TNM stage

The TNM classification is used in the staging of prostate cancer (Table 1.3). The T stage can be estimated pre-operatively based on clinical examination, needle biopsy results and MRI findings, whilst distant nodal and metastatic disease can be detected using CT and bone scans. Clinical T stage has consistently been shown to be associated with pathological stage at radical prostatectomy. (59) Based on the T stage, patients can be classified as having localised, locally advanced or advanced prostate cancer. Furthermore, T stage is an important component of the risk stratification system in localised prostate cancer (Table 1.1). T stage therefore has important implications for the management of patients with prostate cancer. TNM stage at diagnosis is associated with survival in prostate cancer, with the presence of nodal or metastatic disease associated with poor survival outcomes. (51, 60)

Table 1.3 The TNM classification system in Prostate Cancer (51)

Stage	Sub-stage	Definition
Tumour		**Primary Tumour**
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy, e.g., because of elevated PSA
T2		Tumour confined within prostate
	T2a	Tumour involves one-half of one lobe or less
	T2b	Tumour involves more than one-half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3		Tumour extends through the prostatic capsule
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck improvement
	T3b	Tumour invades seminal vesicle(s)
T4		Tumour is fixed or invades adjacent structures other than seminal vesicles: 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)

Prostate cancer staging is based on the extent of the primary tumour (T), lymph node involvement (N) and the absence or presence of metastases (M).

1.2.4 Management of prostate cancer

The management principles of prostate cancer are based predominantly on whether the disease is localised, locally advanced or advanced. Localised prostate cancer is defined as prostate cancer that is confined to the prostate. Locally advanced disease includes T3 and T4 tumours and any tumour that has metastasised to local lymph nodes. Advanced prostate cancer denotes distant metastatic spread of disease. Radical treatment options should be considered in all patients with localised and locally advanced disease whilst taking into consideration co-morbidities and patient age. Radical treatment options include

active surveillance (AS), radical prostatectomy and radical radiotherapy. Non-radical treatment options including watchful waiting, hormonal treatment and chemotherapy are reserved for those with advanced disease or those who are not suitable for radical treatment.

1.2.4.1 Active Surveillance

AS is an observational treatment strategy with curative intent by which patients with low to intermediate risk localised prostate cancer are intensively followed up and offered radical treatment if there is evidence of disease progression. Studies in both the USA and UK comparing surveillance strategies against radical treatment in localised prostate cancer have found no significant difference in prostate cancer-specific and all cause mortality. (61, 62) AS is a useful strategy to avoid overtreatment of indolent disease, thus minimising side-effects of radical treatment and the associated health care costs. Only patients who would tolerate radical treatment should be offered AS. The recommended protocol for AS as per the current NICE guidelines is outlined in Table 1.4.

Table 1.4 Suggested AS protocol (51)

Timing	Tests
Enrolment	MP-MRI (if not already performed)
Year 1	Serum PSA every 3-4 months
	DRE every 6-12 months
	Prostate re-biopsy at 12 months
Year 2-4	PSA every 3-6 months
	DRE every 6-12 months
Year 5 and every subsequent year until AS ends	PSA every 6 months
	DRE every 12 months

Active surveillance involves intensive follow-up of patients with low to intermediate risk prostate cancer with regular serum PSA testing, clinical examination and repeat biopsy for histological examination. DRE = digitial rectal examination

Patients should be offered radical treatment if there is any biochemical, histological or clinical evidence of disease progression. No set criteria have been recommended to indicate disease progression but it is generally accepted that a

rapidly rising PSA, high-grade disease on repeat biopsy or an increase in tumour clinical stage should warrant consideration for radical treatment. Again, the decision to proceed to radical treatment should take patient wishes, co-morbidities and life expectancy into account. The main risks associated with AS are development of advanced disease whilst on AS, psychological impact of non-treatment of a known cancer and developing a co-morbidity during AS which would make the patient ineligible for radical treatment.

1.2.4.2 Radical Prostatectomy

Radical prostatectomy is a major surgical procedure that was traditionally performed as an open surgical procedure but is increasingly performed using robotically assisted techniques. It involves the excision of the entire prostate gland and seminal vesicles. Radical prostatectomy should be offered to men with intermediate risk disease without co-morbidities and to those patients with low-risk disease who do not wish to delay treatment by entering AS. In high-risk disease and locally advanced prostate cancer, radical prostatectomy with extended lymph node dissection can be used as part of a multi-modal approach so long as the tumour is not fixed nor invading the urethral sphincter. (32) Furthermore, recent studies have found a survival benefit to offering radical prostatectomy and lymph node dissection to patients with a low burden of metastatic disease. (63-65) The TRoMbone trial, a pilot study to investigate the survival benefit of radical prostatectomy in patients with oligometastatic disease (1-3 skeletal metastatses without visceral metastases) is currently recruiting patients and will soon report their findings with a view to conducting a full study. (66) The findings of this study has potential to significantly change the current management of early metastatic prostate cancer. Significant risks associated with radical prostatectomy include urinary incontinence, erectile dysfunction, involved surgical margins and, in rare cases, death.

1.2.4.3 Radical radiotherapy

Radical radiotherapy can be delivered to the prostate by external beam x-rays (external beam radiotherapy, EBRT) or via radiation sources implanted directly into the prostate gland (brachytherapy).

Brachytherapy should only be offered to patients with low to intermediate risk localised disease. Low-dose rate brachytherapy with permanent radioactive seeds is the most commonly used protocol for brachytherapy. High-dose rate brachytherapy monotherapy has been shown to provide good local control and progression-free survival in low to intermediate risk prostate cancer with low toxicity and few side effects, however an optimal treatment schedule has not been agreed as yet.(67) A recent study has suggested that low-dose rate brachytherapy can be effective at controlling even high-risk localised prostate cancer and could benefit patients unable to tolerate radical prostatectomy or EBRT. (68)

EBRT can be offered to all patients with localised and locally advanced prostate cancer. Androgen deprivation therapy (ADT) is offered to patients with intermediate risk disease for 6 months prior to, during or after EBRT and for up to 3 years in patients with high-risk disease. Patients with high-risk disease unsuitable for ADT should be given EBRT at an increased dose or in combination with brachytherapy.(32) Risks associated with radical radiotherapy include urinary incontinence, erectile dysfunction, radiation induced enteropathy and radiation-induced bowel cancer.

1.2.4.4 High-intensity focused ultrasound and cryotherapy

Focal therapies have been developed for potential radical treatment of patients with localised prostate cancer.

High-intensity focused ultrasound (HIFU) is a technique that induces coagulative necrosis in malignant tissue by mechanical and thermal damage through the use of focused ultrasound waves. Cryotherapy involves the delivery of argon gas via cryoprobes inserted directly into malignant tissue under image guidance. The aim of cryotherapy is to induce local temperatures of -40 °C resulting in destruction of malignant cells.

Whilst HIFU and cryotherapy have shown promising results in the management of localised prostate cancer (69, 70), they are not currently recommended as a

radical treatment in prostate cancer unless enrolled in a clinical trial comparing their use to standard radical therapies. (71)

1.2.4.5 Watchful waiting

Watchful waiting is a conservative management strategy by which patients who are unsuitable for radical treatment either due to disease stage, life expectancy or co-morbidities, are offered treatment for symptomatic relief rather than curative intent. This approach is most commonly used in patients who are thought unlikely to have significant disease burden during their lifetime. Treatments may include hormonal therapy and palliative radiotherapy.

1.2.4.6 Hormonal therapy

The aim of hormonal therapy in prostate cancer is prevention of the androgen-dependent growth of prostate cancer. This can be achieved by androgen deprivation and/or AR blockade.

Androgen deprivation was first described in the management of prostate cancer by Huggins et al in 1941, who discovered disease activity in prostate cancer decreased following surgical or medical castration. (72) Bilateral orchidectomy is still offered today as an alternative to medical androgen deprivation. Bilateral orchidectomy achieves lower mean testosterone levels than compared to luteinising hormone-releasing hormone (LHRH) agonists . (73, 74) The advantage of orchidectomy over LHRH agonists is the avoidance of medical treatment with its associated side effects whilst being more cost-effective. (75) Orchidectomy is an irreversible procedure however, and patients should be counselled appropriately before proceeding.

LHRH agonists are recommended as monotherapy in the first line treatment of advanced prostate cancer. LHRH agonists downregulate the gonadotrophin-releasing hormone (GnRH) receptors in the pituitary, producing a hypogonadal effect by reducing the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH), and subsequently reducing androgen levels. The use of LHRH agonists are associated with an ‘androgen-flare’ by initially stimulating

release of LH and FSH when it binds to the GnRH receptors, and therefore many patients are offered a short course of anti-androgens to overcome this. Side effects of LHRH agonists include cardiovascular morbidity, hot flushes, sexual dysfunction, osteoporosis and fatigue.

Anti-androgens act peripherally to reduce the effect of endogenous androgens by competitively binding to the AR. Anti-androgens are not as effective as LHRH agonists in terms of overall survival, however, as the levels of circulating testosterone are not diminished, they have a more acceptable side effect profile than LHRH agonists. Side effects include gynaecomastia, liver dysfunction and diarrhoea and vomiting. As a result, anti-androgens may be offered as monotherapy to men with advanced prostate cancer who are unable to tolerate the side effects of LHRH agonists and are willing to accept the adverse impact on overall survival. Anti-androgens and LHRH agonists may be used in combination to produce maximum androgen blockade in patients who show early signs of biochemical relapse after initial monotherapy with LHRH agonists or bilateral orchidectomy.

Unfortunately, all men with advanced prostate cancer will eventually have disease progression despite hormonal therapy, signalling the development of castration-resistant prostate cancer (CRPC). This usually occurs after 2-3 years of ADT. CRPC has a poor prognosis, with a median survival time of 9-30 months. (76)

1.2.4.7 Chemotherapy

Traditionally, chemotherapy was reserved for patients with castrate-resistant prostate cancer. The primary chemotherapy agent used in CRPC is docetaxel, an anti-neoplastic taxane agent and is used in combination with prednisolone. Docetaxel treatment is associated with increased median survival of 2.4 months compared to the previous standard chemotherapeutic agent. (77) Side effects of docetaxel include neutropenia, anaemia, fatigue, gastro-intestinal upset and peripheral sensory neuropathy. As such, docetaxel is restricted for use in patients with good performance status.

Several recent studies have found that earlier treatment with docetaxel in combination with ADT in patients with hormone-sensitive metastatic prostate cancer is associated with decreased time to disease progression and improved overall survival. (78, 79) It is now recommended that men with metastatic disease at presentation should be offered combined ADT and docetaxel as first line treatment, provided they are fit enough to receive this treatment. (80)

1.2.4.8 Abiraterone acetate

Abiraterone acetate is an androgen biosynthesis inhibitor that prevents the production of androgens by the adrenals and testes. Two recent studies have shown addition of abiraterone and prednisolone to ADT provides a significant overall survival and progression-free survival benefit for newly diagnosed hormone-sensitive metastatic prostate cancer compared to treatment with ADT alone. (81, 82) Treatment with the addition of either docetaxel or abiraterone was compared in the STAMPEDE trial for patients with hormone-sensitive metatstatic prostate cancer. No significant overall or disease-specific survival benefit was identified between the two treatment arms. Whilst neither treatment arm had significantly worse toxicity, each treatment was associated with different toxicities. (83) It has therefore been suggested that both treatment arms should be considered in patients with newly diagnosed hormone sensitive metastatic prostate cancer taking treatment-spceific side effects and patient preference into account.

Abiraterone is also recommended for use as a first-line treatment in metastatic CRPC and in patients with disease progression whilst on docetaxel therapy. (80)

1.2.4.9 Enzalutamide

Enzalutamide is a new AR antagonist that is licensed for use in patients with metastatic CRPC who are asymptomatic and docetaxel is not yet clinically indicated. In this patient cohort, it has been shown to decrease the risk of radiological progression and improve overall survival. (84) The AFFIRM study found improved overall survival when using enzalutamide for treatment of

disease progression following docetaxel treatment in patients with metastatic CRPC. (85) As such, enzalutamide is recommended as a second-line treatment for metastatic CRPC in patients treated with docetaxel. (80)

1.2.4.10 Immunotherapy

Immunotherapy is a treatment that aims to assist the patients immune system to identify and destroy malignant cells. Sipuleucel-T, an autologous vaccine, was approved by the US Food and Drug Administration to treat asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer in 2010. The vaccine consists of autologous peripheral-blood mononuclear cells activated ex vivo with a recombinant fusion protein consisting of prostatic acid phosphatase antigen fused to granulocyte-macrophage colony-stimulating factor.(86)The IMPACT study found a 4.1 month median overall survival benefit in metastatic CRPC patients treated with Sipuleucel-T compared to the control group. (87) It is not currently licensed for use in Europe.

1.2.4.11 Pharmacoeconomics of prostate cancer treatment

Prostate cancer treatment is expensive. The diagnosis, treatment and 5 year follow-up cost of prostate cancer in the UK was estimated at £136, 278, 237 in 2010. (3) Since then, new treatments have been introduced for the treatment of advanced and castrate-resistant prostate cancer, including abiraterone and enzalutamide, with high associated costs. In the UK, abiraterone was initially deemed to not be cost-effective and was only recommended for use once the manufacturer agreed to a significant cost-reduction. This economic burden is expected to continue to rise as prostate cancer incidence increases, potentially making prostate cancer treatment in UK unsustainable. For example, NICE does not recommend the use of Sipuleucel-T for use in patients with metastatic CRPC due to its high incremental cost-effectiveness ratio, despite the findings of the IMPACT study as discussed above. As such, it is crucial that new tools such as predictive biomarkers are developed to assist clinicians in directing prostate cancer treatment to those patients who would gain most benefit.

1.3 Androgen Receptor

The AR is a class I nuclear receptor and forms part of the nuclear receptor superfamily. It is encoded by the AR gene, located on the X chromosome at position Xq11-12, producing a 110 kDa protein. AR is activated in response to androgens, causing translocation of the AR from the cytoplasm to the nucleus, where it acts as an intracellular ligand-dependent transcription factor capable of regulating the expression of many thousands of androgen-regulated genes.

In humans, AR is expressed in all organs with the exception of the spleen and bone marrow. It is essential for the development of male reproductive organs, puberty, male fertility and male sexual function. In the prostate, androgens are essential for normal development and function. AR is expressed in both epithelial cells and stromal cells. In epithelial cells, AR has a role in cellular differentiation, survival and the expression of secretory proteins. (88, 89) Stromal AR has a role in embryonic prostate development, epithelial differentiation and determining secretory protein expression. (90)

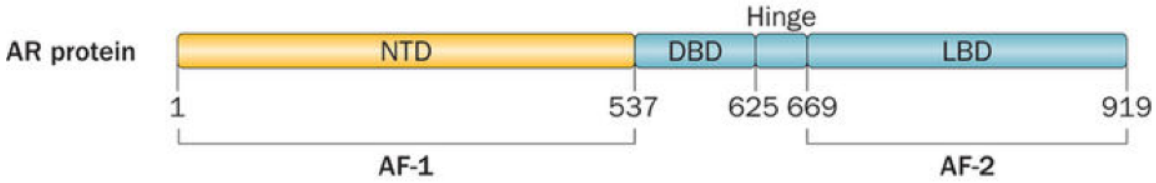
Prostate cancer development is androgen-dependent in approximately 90% of cases at diagnosis. (91) Increased AR expression in hormone-naïve prostate cancer is associated with markers of aggressive disease and poor prognosis. (92, 93)

1.3.1 AR structure

The AR is a 919-amino acid protein with three major functional domains, as demonstrated in Figure 1.8. The largest domain, the N-terminal domain (NTD) has a transcriptional modulatory function, controlled by the constitutively active activation function 1 (AF-1), independent of the interaction with ligands. (94) The DNA-binding domain (DBD) is adjacent to the NTD and is the smallest domain. It consists of two zinc fingers, the first containing the P-box, a recognition helix that binds to DNA, and the second containing the D-box which allows AR dimerization. The DBD therefore has a role in nuclear localisation, receptor dimerization and DNA binding to target genes. (95, 96) The ligand-binding domain (LBD) varies in size and is separated from the DBD by a hinge

region. It contains the ligand dependent C-terminal activation function 2 (AF-2), a surface hydrophobic groove, thought to be critical to co-activator recruitment. (97) Binding of ligand to the LBD leads to a conformational change allowing exposure of AF-2 to AR co-activators and co-repressors. (98)

Figure 1.8 Androgen receptor structure with functional domains (99)

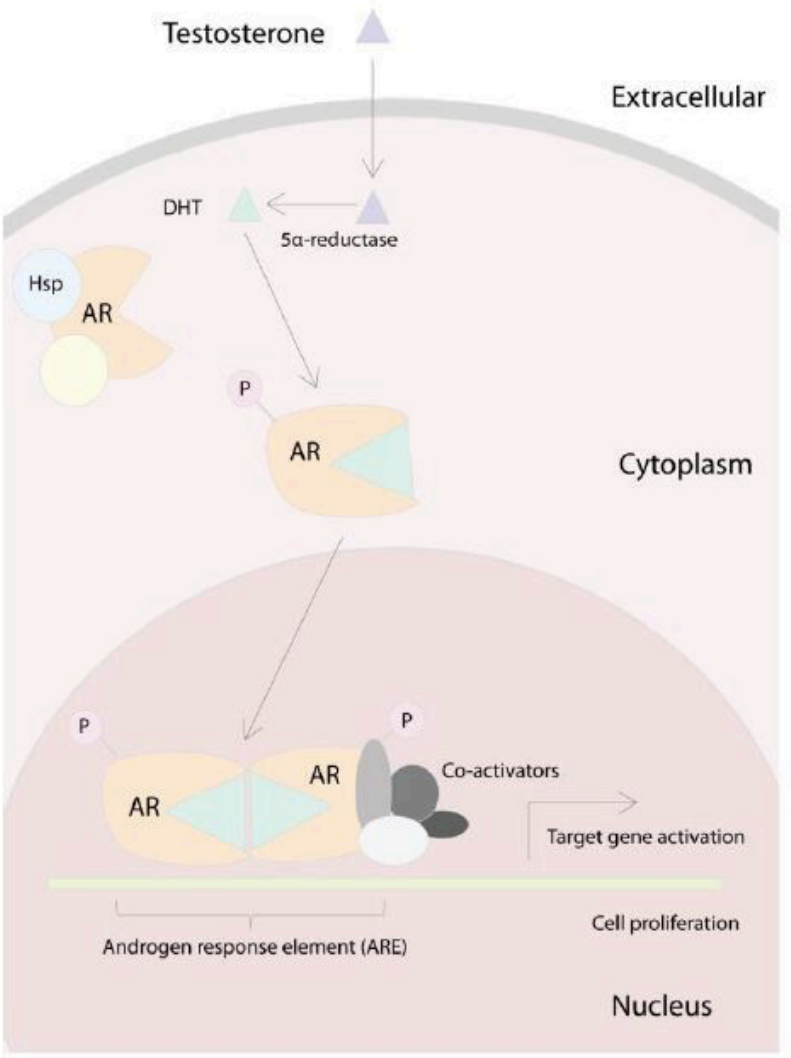


The AR contains three major functional domains and a hinge region. Each domain has a specific function. The NTD modulates transcriptional activity, controlled by AF-1. The DBD binds to target genes and is the site of AR dimerization. The LBD contains AF-2, which is activated by binding of ligand. Serine 81 is located within the NTD. Serine 578 is located in the DBD. NTD = N-terminal domain, DBD = DNA-binding domain, LBD = ligand binding domain, AF-1 = activation function 1, AF-2 = activation function 2.

1.3.2 Genomic AR signalling

In the absence of androgens, the AR is located in the cytoplasm bound to heat-shock proteins (HSP), cytoskeletal proteins and co-chaperones. Upon androgen binding to the LBD, AR undergoes conformational change, dissociates from HSPs and becomes phosphorylated. The AR subsequently interacts with co-regulators that facilitate translocation of the AR/androgen complex to the nucleus. Within the nucleus, AR dimerises and binds to androgen response elements (ARE) in promoter regions of its target genes. Binding of AR homodimers to AREs results in transcription of androgen-dependent genes including PSA (Figure 1.9). This process is modulated by co-regulators that can either enhance (co-activate) or repress AR transcription through chromatin remodelling and histone modifications. When the ligand dissociates from AR, the AR translocates back to the cytoplasm where it is again bound to HSPs awaiting further ligand binding.

Figure 1.9 Genomic androgen receptor signalling in the prostate (100)



Schematic diagram illustrating genomic AR signalling. In the absence of ligand, AR is in its inactive state within the cytoplasm, bound to HSPs, cytoskeletal proteins and co-chaperones. Testosterone enters the prostate cell and is converted to DHT by 5α-reductase. DHT binds to AR in the cytoplasm causing conformational changes resulting in the dissocation of AR from HSPs and co-chaperones, allowing AR to enter the nucleus. Within the nucleus, AR dimerises and binds to AREs of target genes in the presence of co-activators, resulting in gene transcription. AR = androgen receptor, HSP = heat shock protein, DHT = dihydrotestosterone, ARE = androgen response element

1.3.3 Non-genomic AR signalling

Non-genomic AR signalling is the interaction of activated AR with intracellular signalling molecules within the cytoplasm, independent of genomic AR signalling. Activated AR can bind to non-receptor tyrosine kinase Src, activating the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) cascade and enhances cell proliferation. (101) AR can also interact with

the phosphoinositide 3-kinase (PI3K)/Akt pathway by binding of the AR NTD to the SH2 domain of p85α regulatory subunit of PI3K. (102) This results in activation of Akt, with resultant inhibition of apoptosis and increased cell survival through regulation of transcription factors.

Both MAPK/ERK cascade and PI3K/Akt pathway have been implicated in prostate carcinogenesis and development of CRPC.

1.3.4 Post-translational modification of AR

The AR can undergo post-translational modification by phosphorylation, acetylation, SUMOylation, methylation and ubiquitination at 23 known sites on the receptor and is associated with regulation of AR structure, activity and stability.

Phosphorylation of the AR was the first reported, and most common, post-translational modification of AR. To date, 18 phosphorylation sites have been identified that include serine, threonine and tyrosine residues, each with differing biological effects. AR phosphorylation will be discussed in more depth in the following section.

Acetylation occurs at three known lysine residues on the AR – Lys 630, Lys 632 and Lys 633 located in the hinge region. (103) Deacetylation downregulates AR transcriptional activity, whilst acetylation enhances transcription and increases cell growth. (103-105)

In addition to acetylation, methylation can also occur at Lys 630 and Lys 632. SET9 is the methyltransferase responsible for methylation at these sites, and studies have shown it is responsible for AR regulation of androgen-target genes and enhances cell growth and survival in prostate cancer cells. (106, 107)

SUMOylation occurs at Lys 386 and Lys 520 within the NTD. SUMOylation is the attachment of the small ubiquitin-like modifier (SUMO) to lysine side chains. This has been shown to occur in response to the presence of androgens in the AR, and

is associated with enhancement of androgen-dependent gene transcription and cell proliferation in prostate cancer cells. (108, 109)

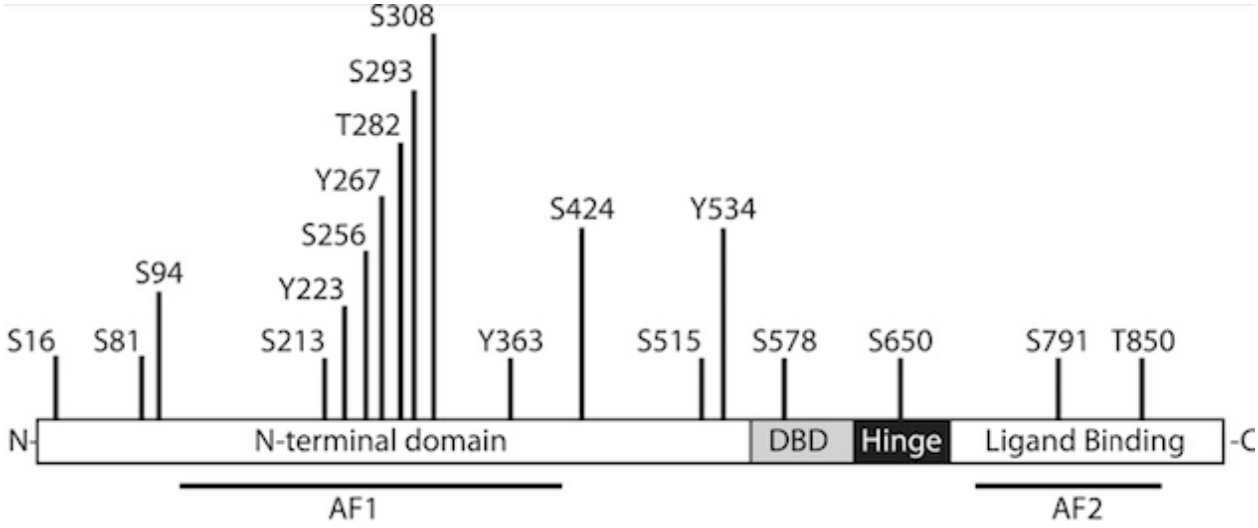
Finally, ubiquitination occurs at Lys 845 and Lys 847 in LBD of AR, (110) which is the attachment of ubiquitin chains to lysine residues. Ubiquitination of the AR has been linked with both the degradation of the receptor and enhanced transcription. (110, 111)

1.3.5 Post-translational serine phosphorylation of AR

AR phosphorylation was first reported in 1984 by Goueli et al who discovered that AR was phosphorylated in the presence of nuclear cAMP-independent protein kinase in rat prostates. (112) Since then, there has been extensive research in relation to AR phosphorylation.

AR is phosphorylated at 18 known serine, threonine and tyrosine residues, with phosphorylation occurring in all the three major domains and hinge region. The vast majority of phospho-sites are identified in the NTD, which includes the constitutively active AF1 domain (Figure 1.10). AR phosphorylation regulates AR cellular localisation, transcriptional activity, cell growth and sensitivity to androgens. Furthermore, AR can be phosphorylated in both the presence and absence of androgen, implicating AR phosphorylation in prostate cancer progression and development of CRPC. Serine phosphorylation is the most extensively researched area of post-translational phosphorylation, and will be the focus of this section going forwards.

Figure 1.10 Distribution of phospho-sites within AR (113)



Eighteen phospho-sites have been identified on the AR and include serine (S), tyrosine (T) and threonine (Y) residues, with the majority located within NTD of AR. Serine 81 is located within the NTD. Serine 578 is located in the DBD. DBD = DNA-binding domain, NTD = N-terminal domain

Serine 81 (Ser-81) is the phospho-site most phosphorylated on the AR in the presence of androgens. Gioeli et al investigated the phosphorylation status at several serine residues on the AR in prostate cancer cells in response to androgens. Mass spectrometry results identified that Ser-81 had the highest stoichiometric phosphorylation in response to androgens, whilst other serine residues including serines 16, 256, 308, 424, and 650 had varying increased levels of androgen-dependent phosphorylation also. (114). Chen et al identified that Ser-81 phosphorylation was reduced in LNCaP cells cultured in androgen-depleted medium, whilst DHT stimulated phosphorylation at Ser-81. In addition, expression of PSA, an androgen-regulated protein, increased in line with increased phosphorylation at Ser-81, further supporting Ser-81 phosphorylation to be androgen-dependent. (115) Several kinases from the cyclin-dependent kinase family have been linked to phosphorylation at this site, including Cdk 1, Cdk 5 and Cdk 9. (115-117) Ser-81 phosphorylation is associated with increased AR transcriptional activity and expression of androgen-dependent proteins such as PSA, chromatin-binding of AR to AREs, nuclear localisation of AR and enhanced cell growth in prostate cancer cells. (116-118)

Serine 16 (Ser-16) phosphorylation can occur in both an androgen-dependent and independent manner. Phosphorylation increases at Ser-16 on the AR upon treatment with androgens, however, it remains phosphorylated in the absence of the LBD. It has therefore been suggested that Ser-16 may have a role in androgen-independent prostate cancer (114, 119)

Serine 94 (Ser-94), located in the NTD, is constitutively active. Phosphorylation at Ser-94 was not altered by the deletion of the LBD, confirming phosphorylation status at this serine residue is independent of androgen. (114, 119) No biological function of Ser-94 phosphorylation on the AR has been identified.

Serine 213 (Ser-213), is phosphorylated by a number of different kinases, with Akt and PIM-1 the most studied. Effects of phosphorylation at Ser-213 in prostate cancer are kinase dependent. Phosphorylation by Akt at Ser-213 is associated with increased transcriptional activity, protein stability and nuclear localisation of AR and promotes cell survival in prostate cancer cells, (120, 121) whilst phosphorylation by PIM-1 is associated with destabilisation of AR, varying effects on AR transcriptional activity in different prostate cancer cell lines and increased prostate cancer cell growth in low androgen conditions.(122, 123) In clinical specimens, increased pAR^{S213} expression is associated with a shorter disease-free interval and decreased disease-specific survival in CRPC. Furthermore, increased expression of pAR^{S213} is seen as prostate cancers progress from hormone-sensitive to castrate-resistant disease. (123, 124)

As discussed above, serine 256 and serine 424 are phosphorylated in an androgen-dependent manner. Serine 293 phosphorylation occurs in an androgen-independent manner. The biological function of AR phosphorylated at serines 256, 424 and 293 is currently unknown.

AR phosphorylated at Serine 308 (pAR^{S308}) by cyclin D3/$CDK11^{p58}$ negatively regulates AR transcriptional activity in prostate cancer cells, resulting in decreased cellular proliferation in androgen-dependent prostate cancer cells. (125) Increased expression of pAR^{S308} confers a survival advantage in patients with CRPC, further supporting the role of pAR^{S308} in decreased transcriptional activity. (126)

Serine 515 (Ser-515) is phosphorylated by a number of kinases including Cdk1, Cdk7 and MAPK. (127-129) Whilst Ser-515 does not appear to have a role in AR localisation, it does have a role in AR transactivation. Upon ligand binding, pAR^{S515} recruits components required for transactivation of AR target genes through signalling cascades.(127) Epidermal growth factor (EGF) stimulated MAPK-dependent phosphorylation of Ser-515 resulted in increased transcriptional activity in both the presence and absence of androgens in CRPC cells. (129) In hormone-naïve prostate cancer patients, increased pAR^{S515} is associated with poor outcome measures including decreased time to biochemical relapse, decreased survival following biochemical relapse and decreased disease-specific survival. (128)

Serine 578, located in the DBD on AR, is predicted to be phosphorylated by protein kinase C (PKC). (130) EGF signalling via PKC-dependent phosphorylation at Ser-578 increases AR transcriptional activity, cell growth, nuclear-cytoplasmic shuttling, modulates phosphorylation at serine 515 and regulates AR interaction with Ku-70/80, a protein complex involved with DNA-repair in castrate resistant prostate cancer cells.(129) PAK6, a member of the p21-activated kinase (PAK) family and known tumour suppressor, also phosphorylates AR at serine 578. Phosphorylation by PAK6 at serine 578 is associated with inhibition of nuclear translocation and increased AR degradation. (131)

AR phosphorylation at serine 650, located in the hinge region, occurs through both androgen-dependent and independent processes. The stress kinases, protein kinase A and PKC are all linked to phosphorylation at this site. (114, 132) Serine 650 phosphorylation is associated with decreased transcriptional activity in prostate cancer cells by exporting AR from the nucleus. (132)

Finally, phosphorylation of the AR at serine 791 (Ser-791) is Akt-dependent. Ser-791 is located in the LBD of AR. Phosphorylation at this serine residue is associated with increased ligand-binding, ligand-dependent nuclear translocation and decreased AR stability.(133) In hormone naïve prostate cancer clinical specimens, increased pAR^{S791} is associated with improved outcomes, with patients with high expression having longer time to disease recurrence.

Increased pAR^{S791} expression in CRPC clinical specimens is also associated with a good prognosis, with increased disease-specific survival following relapse. (126) Overall, high expression of pAR^{S791} seems to have a protective role in prostate cancer.

It can be concluded that AR phosphorylation has an important role not only in prostate cancer progression but in the development of castrate-resistance. Interestingly, individual phosphorylation sites have differing effects with phosphorylation at certain sites being associated with poor prognosis whilst being protective at others. Furthermore, the individual kinases responsible for phosphorylation also appears to play a role in the effect of phosphorylation at the same serine residue in some cases. Phosphorylation status may be utilised in future as a prognostic marker in prostate cancer.

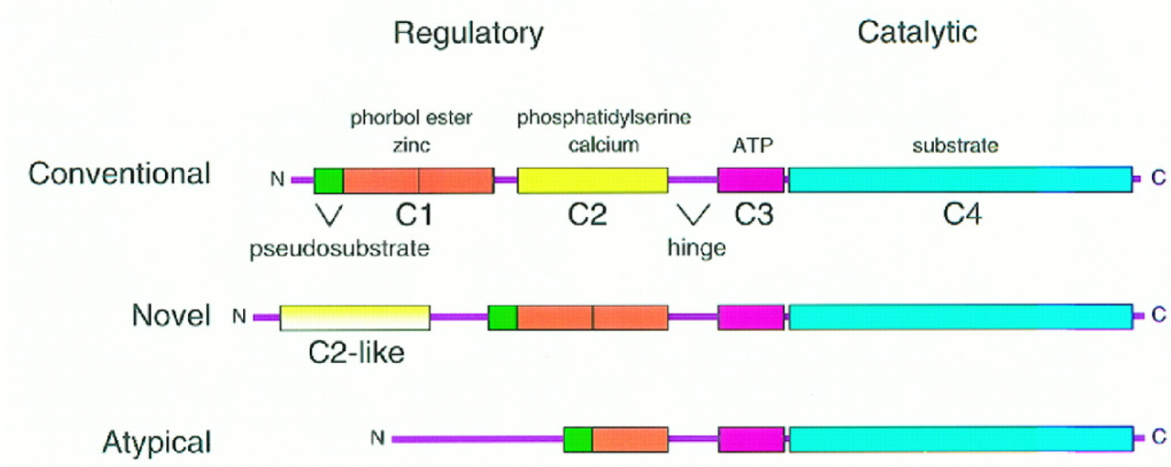
1.3.6 PKC and serine phosphorylation of AR

As described above, several kinases are known to phosphorylate serine residues on the AR in androgen-dependent and independent processes, including Akt, MAPK, Cdks and PKC. Some kinases phosphorylate the AR at more than one serine residue, resulting in differing biological effects depending on the serine site phosphorylated and are the target of new drug therapies in prostate cancer. The focus of this thesis is the role of AR phosphorylation at serine 578 in prostate cancer. PKC is the predicted kinase to phosphorylate the AR at this site (130), and will therefore be discussed in more detail below.

Protein kinase C is a family of serine/threonine kinases, comprised of at least 12 isozymes categorised into three sub groups according to their biochemical and structural properties. PKC isozymes are single polypeptides containing an N-terminal regulatory region and a catalytic C-terminal region, separated by a hinge region (Figure 1.11). (134) Conventional PKCs are calcium-dependent and are activated by phorbol esters or diacylglycerol (DAG) and includes PKC α, ß1, ß2 and γ. Novel PKCs (PKC δ, ε, η, and θ) lack a calcium-binding region and are therefore calcium-independent but require phorbol esters or DAG for activation.

Finally, the atypical PKCs (PKC ζ, λ, μ and ι) are calcium-independent and are activated independently of phorbol esters and DAG. (135)

Figure 1.11 Structure of conventional, novel and atypical PKC isozymes (134)



Schematic diagram illustrating the primary structure of conventional, novel and atypical PKC isozymes. Conventional PKCs are composed of four domains (C1-4), are calcium-dependent and are activated by phorbol esters or diacylglycerol. Novel PKCs lack a calcium binding region in the C2 domain but still require activation with phorbol esters or diacylglycerol. Atypical PKCs are calcium-independent and are activated independently of phorbol esters and DAG.

Newly synthesised PKC is dephosphorylated and in an inactive state. (136) The first step in PKC activation is phosphorylation by 3-phosphoinositide-dependent protein kinase 1 (PDPK1), a master kinase that phosphorylates and activates a number of protein kinases. This initial phosphorylation step is followed by two auto-phosphorylation steps, completing PKC activation. Activated PKC is released into cytosol where it can respond to lipid secondary messengers. (137)

Activated PKC isozymes have a variety of roles in both normal cell function and disease. The intracellular effects of PKC include proliferation, differentiation, cell to cell interaction, secretion, cytoskeletal functions, gene transcription and apoptosis. PKC is implicated in a number of disease processes including cardiovascular disease, metabolic disorders and carcinogenesis.

PKC was first linked to tumorigenesis in 1982 when Castagna and colleagues identified that PKC was directly activated by tumour-promoting phorbol esters. (138) Since then, the role of PKC in carcinogenesis has been extensively

investigated in many cancer types. Total PKC expression is elevated in both breast and lung cancer and reduced in colon cancer. (139-141) It has been found that individual PKC isozymes have varying levels of expression and biological effects in different cancer diseases. For example, PKCα enhances cell proliferation in lung, bladder, breast and gastric cancers, but has an anti-proliferative effect in colon cancer. (142-146)

PKC has previously been linked to prostate cancer development and progression. Expression of total PKC is elevated in CRPC compared to hormone-sensitive disease. Furthermore, patients who had increased expression of PKC with the development of CRPC had shorter survival following biochemical relapse. (147) Increased PKC expression secondary to phorbol 12-myristate 13-acetate (PMA) treatment, a potent PKC activator, in androgen-dependent LNCaP prostate cancer cells promotes PKC induced apoptosis through activation of down-stream signalling cascades. (148) Previous studies have demonstrated that PMA-induced apoptosis in LNCaP cells is mediated by PKC α and PKCδ. (149, 150) Conversely, PKCε has been linked to development of castrate-resistance with ongoing proliferation of LNCaP cells in androgen-deficient conditions, suggesting PKCε may have a role in tumour progression. (151) This suggests that in common with other malignancies, different PKC isozymes have varying biological effects in prostate cancer.

PKC is known to phosphorylate the AR in prostate cancer. In LNCaP cells, PMA increases phosphorylation of the AR at Serine 650. (114, 129) Scansite 2.0, an online kinase search tool, predicts that PKC is the putative kinase mediating phosphorylation at serine 578. (130) Phosphorylation at serine 578 has been shown to be PKC-dependent in CWR-R1 cells, a CRPC cell line. Introduction of a serine 578 mutation on the AR in CWR-R1 cells resulted in a 50% decrease in PKC-dependent AR phosphorylation compared to wild-type cells. (129)

1.4 Statement of research aims

The research presented in this thesis is a proof of concept study that sought to investigate the prognostic and predictive significance of AR phosphorylation at serine 578 and serine 81 in patients with hormone-naïve prostate cancer. This work was conducted with a view to identify new prognostic and predicitive biomarkers that can assist clinicians in the management of patients with prostate cancer.

Phosphorylation at Ser-578 is associated with increased AR transcriptional activity, cell growth, nuclear-cytoplasmic shuttling, modulates phosphorylation at serine 515 and regulates AR interaction with Ku-70/80, a protein complex involved with DNA-repair in castrate resistant prostate cancer cells in in vitro studies. As such, it was speculated that pAR^{S578} expression in clinical specimens would be increased in advanced disease and would be associated with poor outcomes in prostate cancer. It was therefore hypothesised that pAR^{S578} may be utilised as a prognostic biomarker at diagnosis in prostate cancer. Furthermore PKC, the putative kinase for phosphorylation at serine 578, would be associated with poor outcomes and may offer a potential therapeutic target in prostate cancer.

Ser-81 is the serine residue most commonly phosphorylated in response to DHT. Ser-81 phosphorylation is associated with increased AR transcriptional activity and expression of androgen-dependent proteins such as PSA, chromatin-binding of AR to AREs, nuclear localisation of AR and enhanced cell growth in prostate cancer cells. It was hypothesised that pAR^{S81} expression in clinical specimens would also be associated with poor outcomes in prostate cancer.

As serine 81 and serine 578 are predicted to be phosphorylated via alternative pathways, it was hypothesised that expression of AR phosphorylated at these sites may have a cumulative prognostic effect. Therefore, the prognostic significance of dual expression of AR phosphorylated at Ser-578 and Ser-81 was investigated in relation to outcome measures.

Using archival tissue from prostate cancer patients, the expression of androgen receptor phosphorylated at serine 578 and serine 81 was assessed in three clinical cohorts in relations to clinical outcome measures. Firstly a cohort of patients with 'low-risk' prostate cancer, managed with active surveillance was utilised to assess the significance of androgen receptor phosphorylation in a patients with early prostate cancer. It was anticipated that phosphorylation status of the androgen receptor may identify patients that may require early definitive treatment. A second 'discovery' cohort of hormone-naïve prostate cancer patients with all stages of disease was utilised to assess the significance of androgen receptor phosphorylation in relation to clinical outcome measures with a view to using phosphorylation status in clinical specimens as a prognostic biomarker. A third, larger cohort of hormone naïve prostate cancer patients was collated and assessed for androgen receptor phosphorylation to validate the findings of the second cohort of patients.

The main objectives of this study were to:

1. Assess the clinical significance of AR phosphorylation at serine 81,serine 578 and PKC expression in a cohort of early prostate cancer patients treated with AS
2. Assess the clinical significance of AR phosphorylation at serine 81, serine 578 and PKC expression in a discovery cohort of hormone-naïve prostate cancer patients
3. Verify the clinical significance of AR phosphorylation at serine 81, serine 578 and PKC expression in the discovery cohort in a larger, consecutive cohort of hormone-naïve prostate cancer patients
4. Develop a technique for the culture and characterisation of patient-derived cells from prostate needle biopsy specimens
5. Assess the impact of PKC inhibitors on expression of pAR^{S578} in patient derived cells to establish if pAR^{S578} may be utilised as a predictive biomarker for response to treatment

Chapter 2 Materials and Methods

2.1 Patients

2.1.1 Active surveillance prostate cancer cohort

A prospective cohort of one hundred and five consecutive prostate cancer patients treated with AS were recruited in NHS Ayrshire and Arran between 13/11/1998 and 17/03/2011. Clinical information was available for all patients, of which 84 patients had diagnostic tissue for use in this study. An anonymised database containing clinical, biochemical and pathological data for patients included in the cohort was created using electronic and paper medical records. Ethical approval was obtained from the West of Scotland Research and Ethics Committee (ref. 12/WS/0087). The clinical outcome measure for this cohort was time to intervention.

2.1.2 Discovery cohort

Ninety patients with hormone naïve prostate cancer were recruited from Glasgow Royal Infirmary between 1992 and 2000. Patients were included if they had tissue appropriate for use in a tissue micro-array (TMA) available. An anonymised database containing clinical, biochemical and pathological data for patients included in the cohort was created using electronic and paper medical records. Written consent was obtained for participation in the study. Ethical approval was obtained from the West of Scotland Research and Ethics Committee (ref. 05/S0704/94). Clinical outcome measures for this cohort was time to biochemical relapse, disease-specific survival following biochemical relapse and disease-specific survival. Biochemical relapse is treatment dependent. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment.

2.1.3 Validation cohort

A retrospective cohort of consecutive prostate cancer patients diagnosed in 2009 was identified using the NHS Greater Glasgow and Clyde Urology MDT records. 423 patients were identified. Patients were included if they had clinical information available on Clinical Portal. Clinical information was available for 361 patients, of which 243 had diagnostic tissue suitable for use in this study. An anonymised database containing clinical, biochemical and pathological data for patients included in the cohort was created using electronic medical records. While date of death was recorded in the electronic medical records, cause of death was not. Paper records could not be obtained for these patients. As a consequence, disease-specific survival could not be used as a clinical outcome measure in this cohort. Overall survival was therefore used as a clinical end point in this study. Ethical approval for tissue collection was obtained from Biorepository. Data collection was under NHS Greater Glasgow and Clyde Safe Haven ethical approval (GSH/16/ON/001). Clinical outcome measures for this cohort was time to biochemical relapse, overall survival following biochemical relapse and overall survival.

2.1.4 Primary prostate cell culture cohort

Thirty-six patients undergoing investigation for prostate cancer were prospectively recruited from a single institution in 2016. Patients were eligible for the study if they were undergoing investigation for prostate cancer. Patients were ineligible if they had previously received treatment for prostate cancer. Written consent was obtained for participation in the study. Primary prostate cell cultures were successfully grown from only two patients. Clinical information was available for all patients. An anonymised database containing clinical, biochemical and pathological data for patients included in the cohort was created using electronic medical records. Ethical approval was obtained from West of Scotland Research and Ethics Committee (ref. 16/WS/0015).

2.2 Tissue preparation

2.2.1 Tissue microarray construction

A TMA allows analysis of tissue from multiple patients on one slide. A TMA is constructed using cores of tissue from multiple patients. Cores 0.6 mm in diameter are obtained from tissue from a paraffin embedded block from individual patients. Each core is seeded into a second paraffin block along with cores from numerous other patients obtained in the same manner. This allows comparative analysis of multiple patients under the same conditions.

A TMA was constructed for the discovery study cohort. A uropathologist identified and marked tumour rich areas on the H&E slide. Cores from the corresponding area in the paraffin embedded tissue block were obtained for each patient. Three cores were obtained to allow the TMA to be constructed in triplicate. Three micrometer sections were cut and placed onto Superfrost Plus microscope slides (Fischer Scientific, Loughborough, UK). Sections were stored at 4°C until use.

2.2.2 Tissue section preparation

Both the AS cohort and validation cancer cohort utilise diagnostic prostate tissue for experimentation. The majority of patients have histological diagnosis of prostate cancer confirmed by TRUS-guided biopsy of the prostate. TRUS biopsy takes several cores from each side of the prostate. Each core measures less than 1mm and would not be sufficient tissue for construction of a TMA. A minority of patients are diagnosed with tissue obtained from other routes. TURP is a treatment for bladder outflow obstruction (BOO) secondary to BPH. Multiple chips of tissue are resected to widen the prostatic urethra and relieve bladder obstruction. Prostate cancer may be identified on histological examination of these prostatic chips. The AS cohorts and validation cohort did not have enough patients diagnosed via TURP to warrant construction of a TMA and therefore full tissue sections were used.

TRUS biopsy cores from the diagnostic specimen for each patient were obtained. Three micrometre sections were cut from the corresponding paraffin embedded tissue block and placed onto Superfrost Plus microscope slides. Sections were stored at 4°C until use. In the active surveillance cohort, tumour area was marked on a H&E section for each patient by a uropathologist, and the tumour area was then marked on the corresponding slides for each antibody. In the validation cohort, a uropathologist was not available to identify tumour area on the H&E sections. Therefore, three different areas suspicious for malignancy were identified in each slide and analysed. The median score was then calculated as a representative score for each patient's sample.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) is a technique that is employed to allow visualisation of a specific antigen within a tissue section or fixed cells using antibodies directed against the antigen of interest. When IHC was introduced in the 1930s, a direct method of visualisation was used. The direct method of IHC uses a primary antibody pre-labelled with a fluorophore or enzyme that allows visualisation of the antigen. The indirect method of IHC was first described in 1970 (152), with the introduction of a secondary antibody labelled with peroxidase antiperoxidase complex. The indirect method of IHC uses a primary antibody directed against the antigen of interest, and a labelled secondary antibody that binds to the primary antibody. The indirect method is employed in this study. EnVision (Dako) is a secondary antibody directed against rabbit or mouse immunoglobulins present on the primary antibody of interest. The secondary antibody is conjugated to horseradish peroxidase (HRP). HRP is only visible on oxidation, producing an insoluble brown precipitate. 3,3'-diaminobenzidine (DAB) was used to oxidise HRP, thus allowing visualisation of the antigen of interest.

2.3.1 Tissue preparation

IHC was performed on tissue sections described in sections 2.2.1 and 2.2.2. Tissue sections were baked at 56°C for twenty minutes and cooled for twenty minutes prior to use.

2.3.2 Tissue dewaxing and rehydration

Sections were dewaxed in xylene (2 x 3 min). The sections were rehydrated by immersion in serial graded alcohol solution washes; 100% ethanol (2 x 2 min), 90% ethanol (2 min) and 70% ethanol (2 min). Finally, the sections were rinsed in running water.

2.3.3 Antigen retrieval

Antigen retrieval is a process necessary to allow binding of the primary antibody to the antigen within the tissue. Formalin fixation of tissues is used almost universally in histopathology. During the fixation process, cross-links form between formalin and proteins (antigens). This adversely affects the availability of the antigen for binding of the primary antibody. A number of different techniques have been described for antigen retrieval in IHC, the majority of which involve heating the specimens in various solutions.

Two different methods of heat-based antigen retrieval were employed in this study. Antigen retrieval buffer for detection of AR was DakoCytomation Target Retrieval Solution 10x (DAKO). The retrieval solution was diluted 1:10. The buffer was preheated to 96°C in a water bath. Sections were incubated in the buffer for twenty minutes at 96°C before being cooled for twenty minutes. Antigen retrieval buffer for the remaining antigens was either tris-EDTA buffer (1mM EDTA and 5mM Tris) at pH 8 or sodium citrate buffer (8mM tri-sodium citrate and 2mM anhydrous citric acid) at pH 6. One litre of the specified buffer (Table 2.1) was pre-heated to 96°C in a microwave for 13.5 minutes. The sections were added to the pre-heated buffer and heated under pressure for 5 minutes before being cooled for twenty minutes. All sections were rinsed in distilled water after antigen retrieval.

2.3.4 Blocking of non-specific staining

The main cause of non-specific staining in IHC is thought to be due to binding of the Fc region of the antibody (both primary and secondary) to endogenous Fc

receptors present on cells within the tissue. Another cause of non-specific staining is the presence of endogenous peroxidases within the tissue section. DAB can react with endogenous peroxidases producing non-specific brown staining. (153) To overcome this, endogenous peroxidase activity was inactivated by treating sections with 3% hydrogen peroxide for ten minutes. Sections were rinsed in distilled water before being incubated in 5% horse serum (Vector) in tris buffered saline (TBS) (0.1M Tris/HCl, 1.5M NaCl) for twenty minutes to prevent non-specific binding of the antibody.

2.3.5 Incubation with primary antibody

Sections were incubated in the primary antibody as listed in Table 2.1. Antibody specificity was previously validated in the host laboratory.(154) Each antibody was optimised prior to use to ensure the correct antibody dilution, incubation time and conditions were used to give the best staining results. Antibodies were diluted to the desired concentration in antibody diluent (DAKO). A positive and negative control was included in each run of IHC to ensure consistency across the stained sections. The positive control ensured that the IHC method had worked and the negative control ensured there was no non-specific staining. Once the incubation period was complete, the sections were washed in TBS buffer (2 x 5 minutes).

Table 2.1 Antibody conditions for IHC

Protein	Antigen Retrieval Method	Antibody	Antibody dilution		Antibody incubation conditions
AR	High pH antigen	Mouse	AS	1:100	Overnight
	retrieval solution	Dako	Discovery	1:100	4°C
	20 min at 96°C	AR441	Validation	1:100	
pAR^{S81}	EDTA pH 8	Rabbit	AS	1:4000	Overnight
	5 min under	Millipore	Discovery	1:4000	4°C
	pressure	#07-1375	Validation	1:1000	
pAR^{S578}	EDTA pH 8	Rabbit	AS	1:1000	Overnight
	5 min under	Eurogentec	Discovery	1:1000	4°C
	pressure		Validation	1:400	
PKC	Citrate pH 6	Rabbit	AS	1:500	Overnight
	5 min under	Abcam	Discovery	1:500	4°C
	pressure	ab59363	Validation	1:500	

The details of the antigen retrieval method, antibody name, antibody dilution and incubation conditions for each protein of interest.

2.3.6 Incubation with secondary antibody

Sections were incubated in secondary antibody (EnVision+ detection system) for thirty minutes at room temperature. Once the incubation period was complete, the sections were washed in TBS buffer (2 x 5 minutes).

2.3.7 Detection of secondary antibody

As described above, DAB oxidises HRP bound to the secondary antibody, producing a brown insoluble precipitate. DAB peroxidase substrate kit (Vector Laboratories) was used to detect the secondary antibody in this study. Four drops of DAB stock, two drops of buffer stock and two drops of hydrogen peroxide were added to 5ml distilled water and mixed well. Sections were incubated with the DAB substrate at room temperature until a brown colour developed (up to ten minutes). The sections were then rinsed in running water for ten minutes.

2.3.8 Counterstaining

Counterstaining allows better visualisation of the antigen-antibody complex by staining the remaining tissue a contrasting colour to the principal stain. This makes the principal stain stand out more against the rest of the tissue. In this study, haematoxylin and Scott's tap water substitute were used to counterstain the sections. Haematoxylin is a cationic basic dye that binds to negatively charged nucleic acids within the nucleus, resulting in blue staining of the nucleus. The sections were soaked in haematoxylin for thirty seconds, before being quickly dipped in acid alcohol to remove excess haematoxylin stain. Scott's tap water substitute is a blueing counterstain solution that blues haematoxylin stained tissue. The sections were bathed in Scott's tap water substitute for thirty seconds before being washed in running water for one minute.

2.3.9 Dehydration and mounting

The sections were dehydrated by immersion in several graded alcohol solutions; 70% ethanol for one minute, 90% ethanol for one minute and 100% ethanol for 2 x one minute. The sections are submerged in xylene, an organic solvent, for 2 x 1 minute to further dehydrate the specimens. Finally, the slides were mounted using DPX and glass coverslips.

2.3.10 Analysis of protein expression

2.3.10.1 Slide visualisation

After IHC, slides were digitally scanned and uploaded to Slidepath Digital Image Hub (Leica Biosystems). Slidepath is a virtual microscopy system that allows visualisation and analysis of tissue sections on a computer.

2.3.10.2 Histoscoring

The weighted histoscore (WHS) method (155) was employed to assess tissue staining intensity in the sections. It relies on the subjective scoring of the intensity of staining in cells and an estimate of the percentage of cells staining at a particular intensity. The final histoscore is calculated using the following formula: (0 x % negative cells) + (1 x %weakly stained cells) + (2 x % moderately stained cells) + (3 x strongly stained cells). The total histoscore can range for 0 to 300. Where staining was conducted in triplicate, a mean histoscore was used for analysis.

In this study, the staining of the nuclei and cytoplasm of tumour epithelial cells was scored by two independent blinded observers. Sections were viewed on Slidepath at 20x magnification. Interclass correlation coefficients (ICCC) were calculated to ensure there was no significant variation between the two observers. Prior to calculating the ICCC, any sections with scores discordant by more than 50 were re-evaluated. Both observers reviewed the section in question on the same computer screen and a new score assigned that both observers agreed on. An ICCC score of >0.74 was considered excellent (155). For the purposes of statistical analysis, protein expression levels were grouped into low (≤median) and high (>median) expression.

2.4 In-vitro Studies

2.4.1 Culture of established prostate cell lines

Three established prostate cell lines were utilised in this study. Two prostate cancer cell lines, LNCaP and VCaP, and one benign prostate epithelial cell line, PNT2 were used.

LNCaP cells are a metastatic androgen-sensitive prostate cancer cell line that is epithelial in origin. LNCaP cells were first isolated in 1979 from a supraclavicular lymph node containing metastatic prostate cancer in a 50 year old Caucasian male (156). The LNCaP cell line is one of the most commonly used in prostate cancer research owing to its expression of the AR and its sensitivity to

androgens. LNCaP cells were cultured in RPMI 1640 (Invitrogen, UK) supplemented with 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK) and penicillin/ streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)). Cells were cultured in T-75 flasks and incubated in 5% CO_2 at 37°C. Media was exchanged twice weekly. Cells were split when they reached 70% confluency.

VCaP cells are a metastatic prostate cancer cell line, epithelial in origin and derived from a vertebral metastasis. The cells were originally obtained from a 59-year-old Caucasian male with castrate resistant metastatic prostate cancer. VCaP cells express wild-type AR, CK-8 and 18 and PSA. VCaP cells can grow in androgen-independent conditions. (157) VCaP cells were cultured in DMEM/F12 (Invitrogen, UK) supplemented with 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK) and penicillin/ streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)). Cells were cultured in T-75 flasks and incubated in 5% CO_2 at 37°C. Media was exchanged twice weekly. Cells were split when they reached 70% confluency.

PNT2 cells originate from benign prostate tissue obtained from a cadaver of a 33-year old male. The cells are epithelial in origin and express CK-8 and 18, indicating a luminal epithelial type. PNT2 cells were cultured in RPMI 1640 (Invitrogen, UK) supplemented with 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK) and penicillin/ streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)). Cells were cultured in T-75 flasks and incubated in 5% CO_2 at 37°C. Media was exchanged twice weekly. Cells were split when they reached 70% confluency.

All cell lines were passaged at a ratio of 1:4 by trypsinisation to maintain the monolayer growth pattern and prevent overcrowding. Media was removed from the flasks and the cells were washed twice with phosphate-buffered saline (PBS) (Invitrogen, UK) heated to 37°C. This step is performed to remove traces of foetal calf serum that inactivates trypsin. Cells were incubated in 3 ml of trypsin (Invitrogen, UK) for 5 minutes in 5% CO_2 at 37°C. Once cells were detached from the flask, 3 ml of cell line specific media was added to inactivate trypsin. Cells were gently pipetted against the side of the flask to disaggregate any clusters of

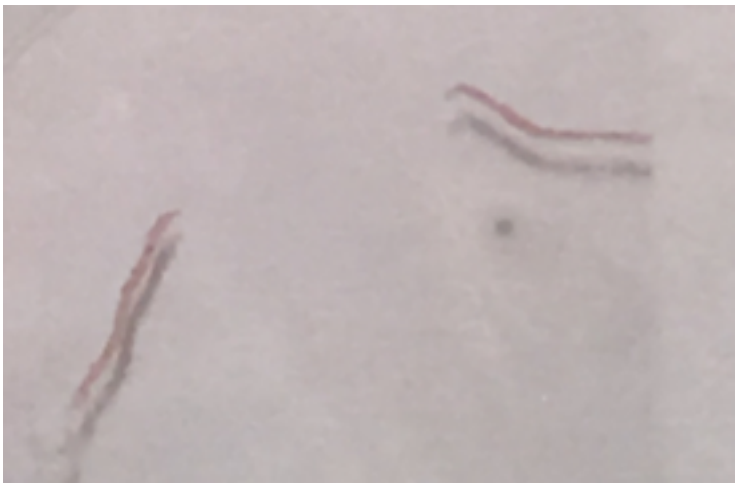
cells before seeding into new T-75 flasks containing 10 ml of fresh cell line specific media. Cells were incubated in 5% CO_2 at 37°C undisturbed for 48 hours to allow reattachment of cells to the flask.

2.4.2 Culture of primary prostate cells

2.4.2.1 Collection of human prostate tissue

Human prostate samples were obtained from patients undergoing TRUS biopsy for investigation of prostate cancer (Figure 1). Samples were stored in a serum-free RPMI (Invitrogen, UK) at 4°C overnight.

Figure 2.1 TRUS guided core biopsy of the prostate



Two trans rectal ultrasound guided prostate core biopsies were obtained for each patient.

2.4.2.2 Processing of prostatic tissue obtained at TRUS guided core biopsy

Biopsies were minced and suspended in serum-free RPMI (Invitrogen, UK) and incubated at 37°C in 5% CO_2 overnight. A cell strainer was used to remove fibroblasts from the cell suspension. The cell suspension was washed three times in 10ml of PBS (Invitrogen, UK). The remaining tissue clumps and cells were re-suspended in 5 ml of primary prostate cell media (Advanced DMEM/F12 (Invitrogen, UK) supplemented with Amphotericin B 2.5μg/ml (Invitrogen, UK),

Penicillin/Streptomycin (50 units/ml, 50 μg/ml (Invitrogen, UK)), 2mM L-glutamine (Invitrogen, UK) and B-27 (50X) supplement (20ml/L) (Invitrogen, UK) and the following growth factors: EGF 1μM (Sigma), N-acetyl-cysteine-L 1.25mM (Sigma), Human R-spondin-1 10 ng/ml (PeproTech), Human Noggin 10 ng/ml (PeproTech), DHT 1nM (Sigma), FGF-10 1 ng/ml (BioVision), FGF-2 0.1 ng/ml (BioVision), SB202190 10 μM (Sigma), Y27632 10 μM (USBiological Life Sciences) and Cholera Toxin 1 μg/ml (Sigma)).

The cell suspension was cultured in a T-25 Matrigel-coated flask at 37°C in 5% CO_2 undisturbed for 7 days to allow attachment of the cells/tissue clumps to the flask. Media was renewed after 7 days, and every 2-3 days thereafter.

Cells were passaged at approximately 70% confluency to maintain a monolayer and prevent overcrowding. Cells were washed twice in PBS heated to 37°C to remove any traces of media. Cells were incubated in 3 ml of trypsin (Invitrogen, UK) for 5 minutes in 5% CO_2 at 37°C. Once cells were detached from the flask, cells were washed three times in PBS. Finally, the cells were re-suspended in 10ml of primary prostate cell media and cultured in a T-75 flask at 37°C in 5% CO2. Again, cells were cultured to approximately 70% confluency before being passaged 1:4 into four T75 flasks. All experiments were conducted at passage 2 when the cells had reached approximately 70% confluency.

2.4.3 Cell treatments

Expression of AR and pAR^{S578} in the cell lines and one primary prostate cell culture was assessed in response to treatment with PMA and BIM-1.

2.4.3.1 PMA

PMA is a potent PKC activator. Cells were treated with 10nM PMA for one hour. A stock solution of 3μM PMA was prepared in dimethyl sulfoxide (DMSO).

2.4.3.2 BIM-1

Bisindolylmaleimide-1 (BIM-1) is a highly selective, reversible inhibitor of PKC. Cells were treated with 10μM BIM-1 for one hour. A stock solution of 6mM BIM-1 was prepared in DMSO.

2.4.4 Inhibition of PKC using BIM-1

Cells were trypsinised as described above at passage 2. Cells were seeded at 1 x 10^4 in 8 well chamber slides. Each cell line was incubated in the cell-specific media for 72 hours. Cells were incubated in serum/additive free media overnight. The media was removed and the cells were washed in warmed PBS. Cells were incubated in the presence or absence of 10μM BIM-1 for one hour, followed by stimulation with 10 nM PMA for one hour. At the end of the treatment, cells were washed twice using ice-cold PBS and fixed using 4% paraformaldehyde (PFA) as described in the immunofluorescence (IF) section below.

2.5 Immunofluorescence

IF is an imaging technique that utilises antibodies labelled with a fluorophore directed against a specific antigen. A fluorophore is a fluorescent marker that emits light on excitation with light. This technique can be used in both tissue sections and cells to visualise specific proteins or antigens of interest using a fluorescent microscope.

There are two methods of IF: direct and indirect. The direct method uses a primary antibody labelled with a fluorophore. The primary antibody binds to the antigen and the attached fluorophore can then be visualised under the microscope. The indirect method uses a primary antibody directed against the antigen of interest, and a secondary antibody labelled with a fluorophore that binds to the primary antibody. Although the indirect method is more complex than direct IF, it is preferable as signal intensity is higher as multiple secondary antibodies can bind to the primary antibody. The indirect method of IF was used in this study to characterise LNCaP, VCaP, and PNT2 cell lines as well as two

primary prostate cell cultures. IF was also performed on the cell lines treated as described above.

2.5.1 Cell culture for characterisation

Cells were seeded at 2.5 x 10^4 cells per well in 6 well chamber slides. Each cell line was incubated in the cell-specific media for 72 hours. Media was aspirated and cells were washed twice in ice-cold PBS.

2.5.2 Fixation of cells

Cells were fixed using 200µl of 4% PFA per well in a fume hood and incubated at 4°C for thirty minutes. PFA was removed and PBS was added to each well. The cells were stored in PBS in the chamber slides at 4°C until use.

2.5.3 Permeabilisation of cells

Intracellular antigens are inaccessible to the antibody if the cell membrane remains intact. There are two reagent types that can be used to permeabilise the cell membrane. Organic solvents such as methanol or acetone dissolve lipids within the cell membrane, and have the additional benefit of also being a fixative agent. A disadvantage of using organic solvents is that lipid antigens may be leached from the cell during this process. Detergents, such as Tween20 or Triton X-100, are uncharged hydrophilic molecules that interact with membrane proteins and create pores in the membrane, thus rendering the cell membrane permeable.

Excess PFA was removed by washing the cells twice in PBS at room temperature for five minutes on an orbital shaker. Cell membranes were permeabilised with 200µl of permeabilisation buffer (0.1% TritonX-100/PBS), applied twice for ten minutes at room temperature on an orbital shaker.

2.5.4 Blocking of non-specific staining

Non-specific staining can result in a false positive result. In order to reduce non-specific staining, cells were incubated in 200µl of blocking buffer (1.5% horse serum (Vector Laboratories) in 0.1% TBS-tween) for thirty minutes at room temperature on an orbital shaker.

2.5.5 Incubation with primary antibody

Various primary antibodies were used in this study as listed in Table 2.2 The primary antibody was diluted in blocking buffer described above. Cells were incubated in 100µl of the appropriate antibody per well for one hour at room temperature on an orbital shaker.

Cells were then washed three times in TBS for ten minutes at room temperature on an orbital shaker.

Table 2.2 Antibody conditions for IF

Protein	Primary antibody	Primary Antibody concentration	Secondary antibody	Secondary antibody concentration
AR	Mouse Dako AR441	1:200	Goat anti-mouse IgG Alexa-Fluor 488	1:500
PSA	Rabbit Dako	1:100	Goat anti-rabbit IgG Alexa-Fluor 488	1:500
pAR^{S578}	Rabbit Eurogentec	1:100	Goat anti-rabbit IgG Alexa-Fluor 488	1:500

The primary antibody name and concentration and its associated secondary antibody name and concentration are listed for each protein of interest.

2.5.6 Incubation with secondary antibody

Cells were incubated in 200µl of the appropriate secondary antibody labelled with Alexa Fluor-488 fluorophore at a concentration on 1:500 for one hour at room temperature protected from light. All subsequent steps were performed in semi-darkness to avoid excitation, and subsequent bleaching of the fluorophore. Cells were washed three times in TBS for ten minutes at room temperature.

2.5.7 Counterstaining and mounting

4',6-diamidino-2-phenylindole (DAPI) is a blue fluorescent stain that binds to double stranded DNA and is therefore a useful nuclear counterstain in IF. Vectorshield mounting media with DAPI (Vector Laboratories) was used to mount the chamber slides onto coverslips. Coverslips were sealed with clear nail varnish to prevent movement of the coverslips whilst viewing cells with the microscope and also to prevent drying out of the cells. The slides were then stored protected from light at 4°C.

2.5.8 Visualisation of immunofluorescent staining

Zeiss Confocal microscope was used to visualise the protein of interest. Cells were viewed and images obtained at x63 magnification. Zen software was used to process the images.

2.6 Gene expression profiling

2.6.1 RNA extraction

RNA was extracted from LNCaP, VCaP, PNT2, and the two primary prostate cell cultures described above using the RNeasy Mini kit (Qiagen, Manchester, UK). Cells were cultured in appropriate growth medium in a T75 flask to approximately 70% confluency.

2.6.1.1 Harvesting cells

Cells were trypsinised and neutralised with appropriate growth media. Cells were transferred to a corning tube, and centrifuged at 1200rpm for 3 mins. The supernatant was aspirated. Cells were re-suspended in 1ml of PBS and transferred to a nuclease free tube. Cells were centrifuged again at 1200rpm for 3 minutes and supernatant aspirated.

2.6.1.2 Lysing cells

To allow all RNA to be made available, cell membranes were disrupted and the cells homogenised. Cells were lysed using 350µl of RLT lysis buffer. Cells were homogenised by repeatedly aspirating the cell suspension through a 26G needle and 1ml syringe. 350µl of 70% ethanol was added before immediately transferring the homogenised cell sample to a spin column and centrifuged for 15 seconds at 8000 x g. Flow through was discarded.

2.6.1.3 Washing cells

Firstly, the cell sample was washed in RW1 buffer to remove large biomolecules that were not attached to the spin column membrane. RW1(700µl) was applied to the spin column membrane and centrifuged for 15 seconds at 8000 x g. Flow through was discarded. The cell sample was washed again with a mild washing buffer (RPE buffer) that removes any residual salts from the RNA extraction process. Two washes with 500µl RPE buffer were performed, centrifuging for 15 seconds at 8000 x g after the first wash and two minutes at 8000 x g. Flow through was discarded after each wash. Finally, the spin column was centrifuged for one minute at 8000 x g to dry the membrane.

2.6.1.4 Eluting RNA

The spin column was placed into a new collecting tube. RNase free water (30 µl) was added directly to the spin column and centrifuged for one minute at 8000 x g to elute RNA. The spin column was discarded and the RNA was stored at -80°C until use.

2.6.2 cDNA Synthesis

2.6.2.1 DNA elimination

DNA should be removed from the sample prior to cDNA synthesis as even small amounts of residual DNA can be amplified in quantitative real time-PCR (RT-qPCR). Firstly, the quantity and purity of the RNA before the DNA extraction step was quantified using the Nanodrop. To 2µg RNA, 2µl 10x DNase I reaction buffer (Promega, Southampton, UK), 1ul DNase I (Promega, Southampton, UK), 1ul RNase OUT (Life Technologies, Paisley, UK) and nuclease free water to a volume of 20ul was added. The mixture was incubated at room temperature for 15 minutes, before inactivating the DNase solution by the addition of 1µl of Stop solution (Promega, Southampton, UK). The solution was then heated to 65°C for ten minutes. After heating, the solution was stored on ice and transferred to the Nanodrop to determine the quantity and purity of the RNA post-DNA elimination.

2.6.2.2 cDNA synthesis

For a 100 µl cDNA synthesis reaction, 1 µg RNA from the cell line of interest was added to 5 µl random primers (Life Technologies, UK) and topped up with nuclease-free water to a volume of 62 µl. The RNA solution was heated to 65°C for ten minutes. After heating, 20 µl of 5x FS buffer,
2.5 µl of RNase OUT, 2.5 µl of Superscript II reverse transcriptase, 10 µl of 10nM dNTP and 3.5 µl of DMSO was added to the RNA solution to make a final volume of 100 µl. The RNA solution was heated for 10 minutes at 25°C, then 30 minutes at 50°C before heating to 85°C for 5 minutes to inactivate the transcriptor. cDNA was stored at -20°C until use.

2.6.3 Quantitative Real Time-PCR

RT-qPCR was performed to allow comparison between gene expression in the control sample (PNT2 benign cell line) and the prostate cancer cell lines and primary prostate cells. A 96 well optical fast PCR plate was used. To each well 40 ng cDNA from the cell line of interest, 10 µl of master mix (Life Technologies), 5 µl nuclease-free water and 1 µl of gene expression assay was added. The gene expression assays used in this study were all Taqman Gene Expression Assays and included ActB (housekeeping gene), AR, FASN, KLK-3, GOLM1 and AMACR. Blank control wells containing only the mixture and no cDNA were included in each plate to exclude contamination. Plates were sealed and centrifuged at 1200 rpm for 3 minutes. Air bubbles were removed using a Microlance needle. RT-qPCR was performed using an ABI 7500 real time PCR machine (Applied Biosystems). Samples were heated at 50°C for two minutes, 95°C for 10 minutes then 40 cycles of 95°C for 15 seconds and 60°C for one minute. Gene expression was normalised to the ActB housekeeping gene. The comparative cycle threshold ($\Delta\Delta$Ct) method was used to quantify relative gene expression.

2.7 Flow cytometry

Flow cytometry is a method for characterising mixed cell populations based on the expression of cell surface and intracellular molecules detected on individual cells by fluorescent-labelled antibodies. A cell suspension is incubated with fluorochrome-labelled antibodies and analysed using a flow cytometer.

2.7.1 Harvesting Cells

Cells were trypsinised and neutralised with appropriate growth media. Cells were counted and 5 x 10^5 cells were placed in a 10 ml falcon tube per test. Cells were washed twice in 3 ml of warmed PBS for 3 minutes. Cells were treated with 750 g/ml DAPI for 10 minutes as a live/dead stain.

2.7.2 Fixation of cells

Cells were fixed in a mixture of 125 µl of cold 2% PFA and 875 µl of cold PBS. Cells were vortexed and incubated in the fridge for one hour. Cells were washed in twice in PBS for five minutes. Cells can be stored in PBS at 4°C until use.

2.7.3 Permeabilisation of cells

Cells were washed twice in permeabilisation buffer (0.1% TritonX-100/PBS), applied twice for ten minutes at room temperature.

2.7.4 Incubation in primary antibody

Cells were stained using both CK-18-FITC conjugated antibody (1:100) and CD-90-APC conjugated antibody (1:100) for one hour at 4°C protected from light. Antibodies were diluted in a flow cytometry buffer (PBS, 1% BSA) to a volume of 100 µl. A negative control and APC and FITC control cells were included for each cell line. Cells were washed twice in 3 ml of PBS-T for three minutes and re-suspended in 1ml of flow cytometry buffer. Cells were analysed using the BD FACSVerse flow cytometer (BD Biosciences).

Chapter 3 Active Surveillance Prostate Cancer Cohort

In 2013, the majority of patients diagnosed with prostate cancer presented with Stage I disease, and more than half had localised prostate cancer. (158) NICE recommends that patients with low risk, localised prostate cancer should be offered AS as an alternative to immediate radical intervention. (51) The PIVOT trial comparing observation startegies against radical prostatectomy found no significant difference in all-cause or prostate cancer mortality in patients with localised prostate cancer. (61) AS is a delayed treatment strategy that aims to avoid over-treatment of low-risk prostate cancer, and thus unnecessary side effects, arising from over-diagnosis of indolent tumours. The PRIAS study found pathological evidence of progression in 28% of men with low-risk, localised prostate cancer undergoing AS. (159) There is a risk that the delay in treatment in these patients may result in prostate cancer progressing to an advanced stage, resulting in them being ineligible for radical treatment. Clinicians currently lack the tools to predict which patients that fulfil low risk criteria at diagnosis will progress to more advanced disease or remain with indolent disease. Therefore, biomarkers that can predict which patients will progress, and require radical treatment, and those patients whose disease will remain indolent and can therefore safely remain on AS, would be a useful tool to the urologist.

The aim of the current study was to determine whether AR phosphorylation at Ser-578 and Ser-81 and PKC expression is associated with clinico-pathological factors and time to intervention in an AS cohort. In addition, as phosphorylation of AR at Ser-578 and Ser-81 has been predicted to occur via different pathways, it was hypothesised that expression of AR phosphorylated at these sites may have a cumulative prognostic effect. Therefore, the prognostic significance of dual expression of AR phosphorylated at Ser-578 and Ser-81 was investigated in relation to time to intervention.

3.1 Cohort Demographics

Analysis was based on one hundred and five prospectively identified, consecutive prostate cancer patients under AS. Patient characteristics recorded included age, Gleason score at diagnosis, serum PSA at diagnosis, perineural invasion (PNI) and treatment for prostate cancer whilst on AS (Table 3.1). Median age at

diagnosis was 67.5 years (IQR 63.3- 72.0 years). Median PSA at diagnosis was 7.0ng/mL (IQR 4.5-10.2ng/mL). Median follow-up was 30.0 months (IQR 19.1-49.6 months).

Table 3.1 Clinico-pathological characteristics of the cohort

Clinical Parameter	Patients, n (%)
Age (<70/ ≥70 years)	63 (60)/ 42 (40)
Diagnosis PSA (<10/ ≥10 ng/ml)	77 (73.3)/ 27 (25.7)
Diagnostic procedure (TRUS/TURP)	81 (77.1)/ 24 (22.9)
Gleason score (6/>6)	98 (93.3)/ 7 (6.7)
Peri neural invasion (no/yes)	80 (76.2)/ 8 (7.6)
Prostatic intraepithelial neoplasia (no/yes)	77 (73.3)/ 12 (11.4)
Percentage tissue containing cancer (<50/ ≥50)	87 (82.9)/ 1 (1)
Intervention (no/yes)	78 (74.3)/ 27 (25.7)

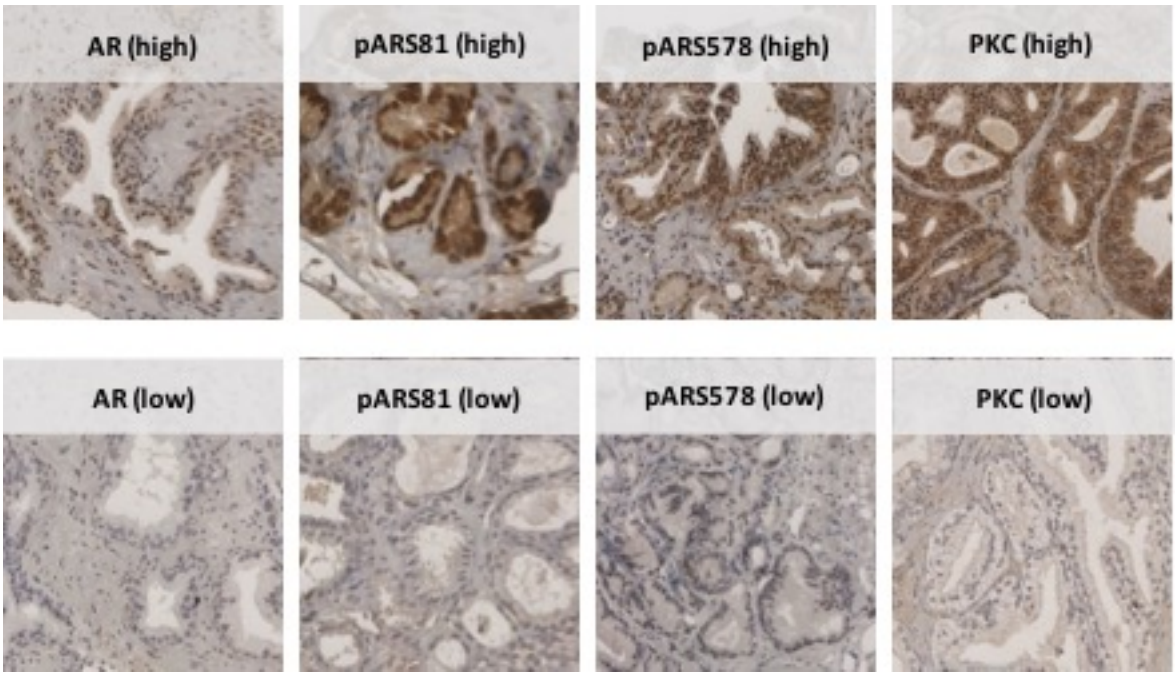
Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

TRUS biopsy was the most common procedure at diagnosis with eighty-one (77.1%) patients having diagnostic tissue obtained at TRUS biopsy. The remaining twenty-four (22.9%) patients had diagnostic tissue available from TURP. Ninety-eight (93.3%) patients had a Gleason 6 tumour and seven (7%) patients had Gleason 7. Eight (7.6%) patients had perineural invasion at diagnosis. Twelve (11.4%) patients had high grade PIN in their diagnostic specimen. Only one patient had greater than 50% of the diagnostic tissue positive for cancer. Twenty-seven (25.7%) patients required treatment for prostate cancer whilst on AS. Eighteen patients (17.1%) required treatment due to biochemical progression. The remaining nine (8.6%) patients had treatment for other reasons such as clinical or histological progression. Twenty-five patients received radical therapy, and two patients received hormonal therapy. The median time to treatment intervention was 21.9 months (IQR 18.7-35.8).

3.2 Protein expression analysis

Eighty-one patients had diagnostic prostate cancer specimens available for IHC. Protein expression in the nucleus and cytoplasm was recorded in epithelial tumour cells only. Expression of all proteins was heterogeneous throughout the cells. Representative specimens of high and low expression for each protein are displayed in Figure 3.1.

Figure 3.1 IHC of prostate cancer tissue for AR, pAR^{S81}, pAR^{S578} and PKC



Representative tissue specimens that were categorised as high and low expression of AR, pARS81, pARS578 and PKC. Expression of all proteins was heterogeneous throughout the cells.

Protein expression in the specimens was divided into low (≤median) and high (>median) for purposes of analysis (Table 3.2). All ICCC values were >0.90.

Table 3.2 Protein expression in patients with tissue available

	Patients, n(%)	Median Histoscore (Histoscore units)	Interquartile range (Histoscore units)
AR Nuclear (low/high)	41 (39)/ 40 (38.1)	120	95 – 165
AR Cytoplasmic (low/high)	42 (40)/ 39 (37.1)	80	50 – 100
pAR^{S81} Nuclear (low/high)	43 (41)/ 35 (33.3)	185	140 – 205
pAR^{S81} cytoplasmic (low/high)	40 (38.1)/ 38 (36.2)	100	80 – 125
pAR^{S578} Nuclear (low/high)	29 (27.6)/ 28 (26.7)	95	56 – 120
pAR ^{S578} Cytoplasmic (low/high)	34 (32.4)/ 23 (21.9)	90	110 - 135
PKC Nuclear (low/high)	35 (33.3)/ 33 (31.4)	70	35 – 95
PKC Cytoplasmic (low/high)	39(37.1)/29 (27.6)	110	92.5 – 120

The median histoscore for each protein of interest was calculated. Patients were grouped into low (≤median) and high expression (>median). The number of patients in each group is displayed for each protein.
Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

3.3 Association between PKC expression and expression of pAR^{S578} in clinical specimens

In the clinical specimens, nuclear PKC expression was significantly associated with pAR^{S578} expression both in the nucleus (c.c. 0.452, p=0.001) and cytoplasm (c.c. 0.442, p=0.001). In addition, cytoplasmic PKC expression was significantly associated with pAR^{S578} expression in the nucleus (c.c. 0.36, p=0.007) and cytoplasm (c.c. 0.492, p=<0.001). Table 3.3 demonstrates the association between PKC expression and pAR^{S578}.

Table 3.3 Association between PKC expression and expression of pAR^{S578} at both cellular locations in the clinical specimens

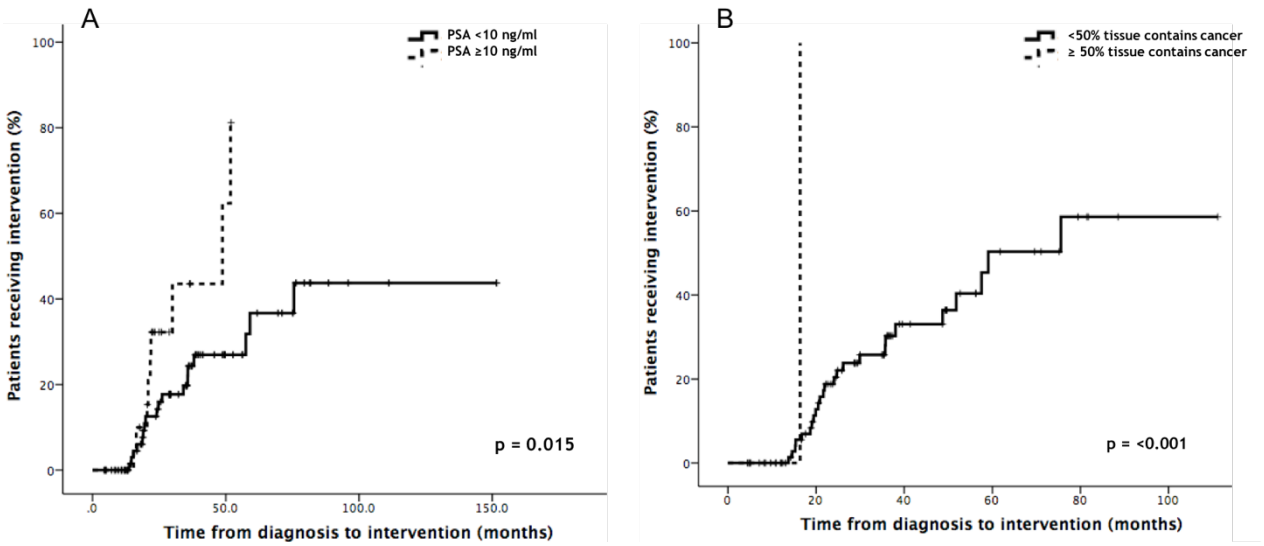
	PKC	
	Nuclear	**Cytoplasmic**
	p value, C.C	**p value, C.C**
pAR578 Nuclear	***0.001, 0.452***	***0.007, 0.36***
pAR578 Cytoplasmic	***0.001, 0.442***	***<0.001, 0.492***

Pearson's correlation coefficient was used to assess the association between PKC expression and expression of pAR^{S578} in the nucleus and cytoplasm. Expression of PKC was significantly associated with pAR^{S578} expression at both locations.
C.C. denotes Pearson's correlation co-efficient. Values highlighted in red denotes associations with a p value < 0.05.

3.4 Clinico-pathological parameters related to intervention

Serum PSA at diagnosis was associated with decreased time to intervention (proportion of patients receiving intervention at 2 years, PSA ≥10 ng/ml 32% vs PSA < 10 ng/ml 12%, HR 2.7 (95% CI 1.2-6.3) p=0.015) (Figure 3.2 A). The percentage of tissue containing cancer was associated with decreased time to intervention (proportion of patients receiving intervention at 2 years, ≥ 50% tissue contains cancer 100% vs <50% tissue contains cancer 12%, HR 17.5 (95% CI 2.0-157.0) p=<0.001) (Figure 3.2 B).

Figure 3.2 Kaplan Meier Graph showing PSA at diagnosis (A) and percentage of tissue positive for cancer (B) as related to time to intervention



Kaplan Meier plots showing High PSA (dashed line) (A) and high tumour volume (dashed line) (B) at diagnosis are significantly associated with decreased time to intervention

No other clinico-pathological parameters were associated with time to intervention. The univariate analysis of clinico-pathological parameters related to time to intervention are displayed in Table 3.4.

Table 3.4 Univariate analysis of clinico-pathological parameters related to time to intervention

Univariate analysis	
Clinico-pathological characteristic	**Time to intervention** **P value Hazard Ratio (95% CI)**
Age (<70/ ≥70 years)	0.823, 0.91, 0.4-2.0
Diagnosis PSA (<10/ ≥10 ng/ml)	***0.015, 2.72, 1.8-6.3***
Gleason score (6/>6)	0.100, 2.69, 0.8-9.2
Peri neural invasion (no/yes)	0.443, 1.60, 0.5-5.4
Prostatic intraepithelial neoplasia (no/yes)	0.361, 0.57, 0.2-1.9
Percentage tissue containing cancer (<50/≥50)	***<0.001, 17.5, 2.0-157.0***

The clinico-pathological variables were grouped and analysed by Kaplan-Meier methods and Cox regression with reference to time to treatment for prostate cancer. High PSA and high tumour volume are significantly associated with decreased time to intervention. Significant results (p = ≤0.05) are highlighted in red.

3.5 Expression of phosphorylated AR related to clinico-pathological parameters

High expression of nuclear pAR^{S578} was associated with increased PSA level at diagnosis (p=0.014). High nuclear and cytoplasmic pAR^{S578} expression was associated with presence of perineural invasion (PNI) (p=0.034 and p=0.008 respectively). Expression of pAR^{S81} or PKC in the clinical specimens was not associated with clinico-pathological parameters. Table 3.5 shows the univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC at both cellular locations related to clinico-pathological parameters.

Table 3.5 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinico-pathological parameters

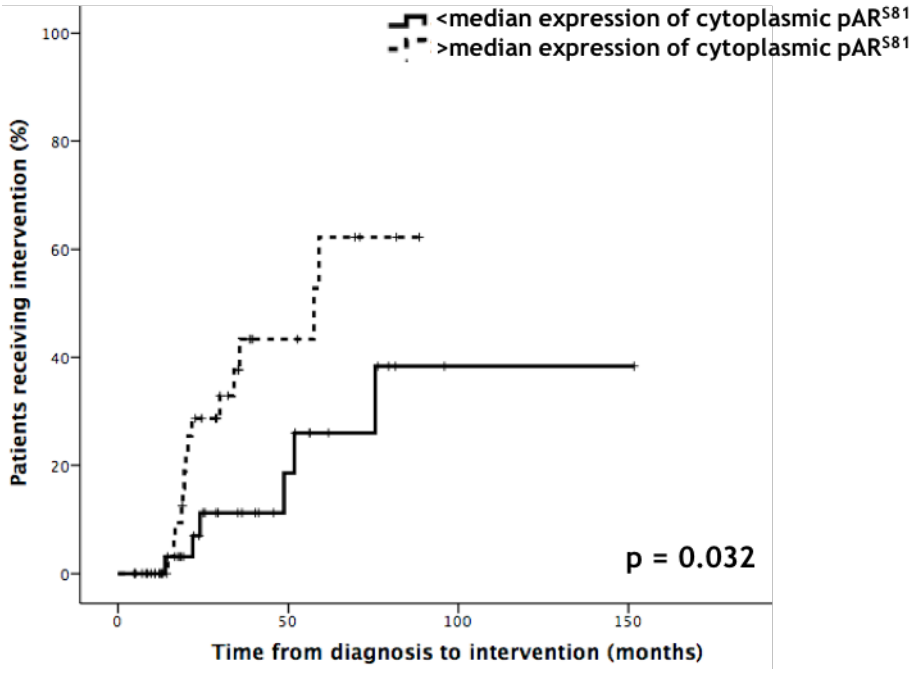
	Nuclear AR ^{S81}			Cytoplasmic AR ^{S81}			Nuclear AR ^{S578}			Cytoplasmic AR ^{S578}			Nuclear PKC			Cytoplasmic PKC		
	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value
Age (<70/>70)	23/20	23/12	0.278	22/18	24/14	0.467	16/13	15/13	0.904	17/17	14/9	0.423	22/13	17/16	0.348	23/16	16/13	0.756
Gleason (6/>6)	39/4	34/1	0.251	38/2	35/3	0.604	28/1	26/2	0.536	32/2	22/1	0.801	32/3	31/2	0.694	35/4	28/1	0.291
PSA at diagnosis (<10/>10)	31/12	25/9	0.889	28/12	28/9	0.579	***25/3***	***17/11***	***0.014***	27/6	15/8	0.162	25/9	23/10	0.730	28/10	20/9	0.673
Perineural invasion (no/yes)	29/3	29/2	0.670	28/1	30/4	0.227	***18/0***	***21/6***	***0.034***	***23/0***	***16/6***	***0.008***	24/2	25/4	0.473	30/2	19/4	0.195

Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was examined for significant relationships with clinical variables as shown. Protein expression was divided into high and low groups. Clinical variables were divided into groups and the Mann-Whitney U test was performed for statistical analysis. High PSA at diagnosis and presence of perineural invasion in pathological specimens are significantly associated with high expression of pAR^{S578}. Statistically significant results are highlighted in red.

3.6 Expression of pAR^{S81}, pAR^{S578} and PKC related to time to intervention

High cytoplasmic pAR^{S81} expression was associated with decreased time to treatment intervention (proportion of patients receiving treatment intervention at 2 years, >median expression 28.5% vs <median expression 7.1%, HR 2.76 (95% CI 1.1-7.3)), p=0.032 (Figure 3.3).

Figure 3.3 Kaplan Meier Graph showing expression of cytoplasmic pAR^{S81}related to time to intervention



Kaplan Meier plot showing time to intervention in patients with high expression of cytoplasmic pAR^{S81} (dashed line) and low expression of cytoplasmic pAR^{S81} (solid line). High expression of cytoplasmic pAR^{S81} was associated with decreased time to intervention.

Nuclear pAR^{S81} was not associated with time to treatment intervention. There was no significant association between pAR^{S578} or PKC at either cellular location and time to intervention. The univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC at both cellular locations related to time to intervention is outlined in Table 3.6.

Table 3.6 Univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC related to time to intervention

Univariate analysis	
Protein expression (<median/>median)	**Time to intervention P value Hazard Ratio (95% CI)**
pAR^{S81} Nuclear	0.748, 1.16, 0.5-2.8
pAR^{S81} cytoplasmic	***0.032, 2.76, 1.1-7.3***
pAR^{S578} Nuclear	0.419, 0.42, 0.5-4.2
pAR ^{S578} Cytoplasmic	0.242, 1.82, 0.7-5.1
PKC Nuclear	0.987, 0.99, 0.3-2.9
PKC Cytoplasmic	0.704, 1.23, 0.4-3.5

Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was analysed by Kaplan-Meier methods and Cox regression in relation to time to intervention from diagnosis. Protein expression was divided into high and low groups. High expression of cytoplasmic pAR^{S81} expression was associated with decreased time to intervention. Significant results (p = ≤0.05) are highlighted in red.

3.7 Expression of dual phosphorylation sites on the AR related to outcome measures

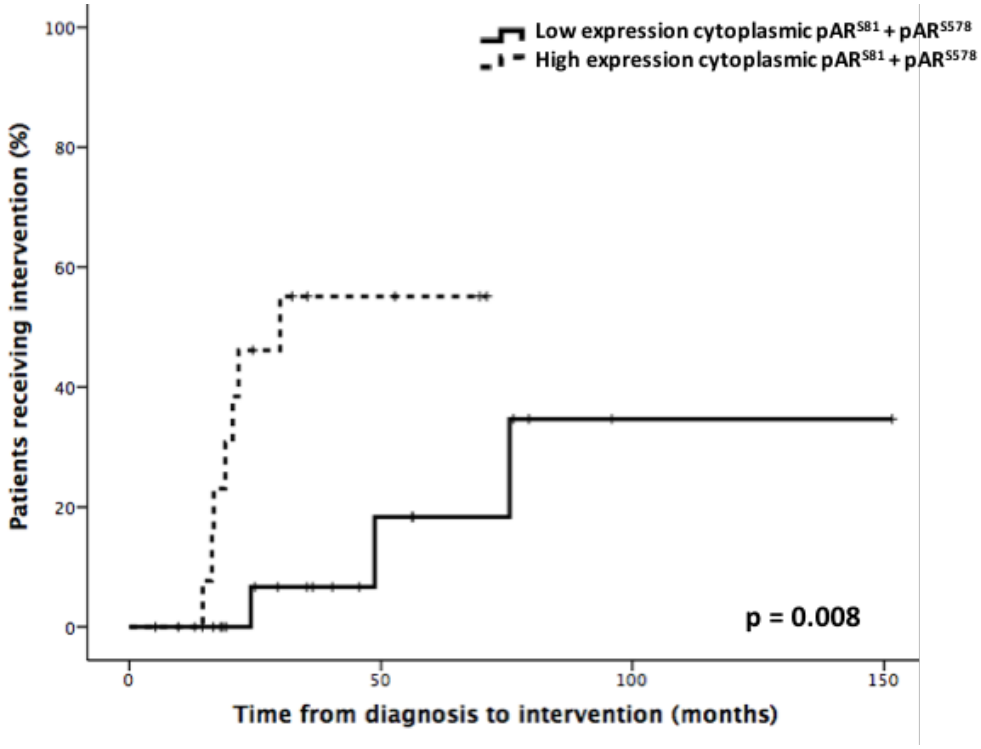
Phosphorylation of AR at Ser-578 and Ser-81 is predicted to occur in response to different kinases via separate pathways. It was therefore investigated if there is a cumulative predictive effect when expression at these sites are combined in relation to time to intervention. The two phosphorylation sites were combined as follows: (i) High expression (high pAR^{S81} and high pAR^{S578}) and (ii) Low expression (low pAR^{S81} and low pAR^{S578} expression).

High dual expression of cytoplasmic pAR^{S81} and cytoplasmic pAR^{S578} was associated with decreased time to treatment intervention (proportion of patients receiving treatment intervention at 2 years, high expression of both proteins 46% vs low expression of both proteins 0%, HR 6.4 (95% CI 1.3-31.0),

p=0.008) (Figure 3.4). There was no significant association between dual expression of nuclear pAR^{S81} and pAR^{S578} or total expression of pAR^{S81} and pAR^{S578} and time to treatment intervention.

Table 3.7 demonstrates the univariate analysis of dual expression of pAR^{S81} and pAR^{S578} as related to time to intervention.

Figure 3.4 Kaplan Meier Graph showing dual expression of cytoplasmic pAR^{S81} and pAR^{S578} related to time to intervention



Kaplan Meier plot showing time to intervention in patients with low expression of both cytoplasmic pAR^{S81} and pAR^{S578} (solid line) and high expression of both cytoplasmic pAR^{S81} and pAR^{S578} (dashed line). High expression of both cytoplasmic pAR^{S81} and pAR^{S578} is associated with decreased time to intervention than compared to low expression of both phosphosites.

Table 3.7 Univariate analysis of dual expression of pAR^{S81} and pAR^{S578} as related to time to intervention

Univariate analysis	
Protein expression	**Time to intervention** **P value Hazard Ratio (95% CI)**
pAR^{S578} cytoplasmic + pAR^{S81} cytoplasmic	***0.008, 6.4, 1.3-31.0***
pAR^{S578} nuclear + pAR^{S81} nuclear	0.233, 2.2, 0.6-8.4
Total cytoplasmic pAR^{S81} and pAR^{S578} + total nuclear pAR^{S81} and pAR^{S578}	0.089, 3.9, 0.7-21.9

Dual expression of pAR^{S81} and pAR^{S578} was analysed by Kaplan-Meier methods and Cox regression in relation to time to intervention. Protein expression was divided into high and low groups. High expression of both cytoplasmic pAR^{S81} and pAR^{S578} is associated with decreased time to intervention than compared to low expression of both phosphosites. Significant results (p = ≤0.05) are highlighted in red.

3.8 Discussion

This study investigated the role of AR phosphorylation at Ser-81 and Ser-578 in an AS cohort. The cohort was a prospectively identified group of consecutive patients who were undergoing AS. NICE recommends that patients selected for AS should have features of low risk localised prostate cancer, as indicated by PSA <10 ng/ml, a Gleason score ≤6 and a clinical stage of T1-2a (51). As would be expected, the cohort had features suggestive of low risk prostate cancer, with a median PSA at diagnosis of 7.0 ng/ml and >90% of the patients having Gleason 6 tumour. Clinical stage was poorly recorded and therefore was not used in this study. Some patients in this cohort, however, did have features indicative of moderate risk disease. Although it is not common in European countries to treat patients with features of moderate risk disease with AS, entry into AS programmes in the UK is less stringent. A survey of UK urologists found that 75% of respondents would consider patients with Gleason 7 disease and 53% would consider patients with a PSA >10 ng/ml for AS (160). This cohort is therefore reflective of common UK practice.

During the follow-up period (median follow-up 30.0 months), 25.7% patients had treatment whilst on AS for either biochemical, histological or radiological progression. This is in keeping with findings of previous studies. A study of 238 patients in the US who met AS inclusion criteria found 27% of patients progressed within 2 years (161). A further study of pathological specimens from 7,333 patients who met the low risk active criteria for AS but went on to have radical prostatectomy rather than enter an AS programme found that 21.8% of patients had an upgraded Gleason score and 23.1% had non-organ confined disease on final pathology (162). These findings suggest that whilst AS can be used as a tool to reduce over treatment in low risk prostate cancer, there is still a sub-cohort of patients that are at risk of disease progression. Biomarkers that are able to predict those at risk of progression are desperately needed.

In the current study, serum PSA ≥10 ng/ml at diagnosis was significantly associated with decreased time to intervention. This is to be expected, as PSA ≥10 ng/ml is included in the intermediate risk criteria for localised prostate cancer (51). Surprisingly, Gleason score was not significantly associated with

time to intervention. Diagnostic specimens with ≥ 50% of tissue containing cancer was also significantly associated with decreased time to intervention. This is in keeping with another study of AS patients, who found that patients with more than 3 prostate biopsy cores positive for cancer at diagnosis is significantly associated with decreased time to disease progression (161), thus supporting tumour volume at diagnosis as a predictor of disease progression amongst an otherwise relatively homogeneous group of patients.

PKC is predicted to be the kinase responsible for phosphorylation of AR at Ser-578 by Scansite 2.0. (130) Ponguta et al demonstrated that PKC mediated phosphorylation was approximately 50% less in mutant S578A recurrent prostate cancer cells than compared to wild-type, suggesting that Ser-578 is the consensus site for PKC. (129) In the current study, it was observed that PKC expression significantly correlates with phosphorylation of AR at Ser-578 in hormone-naïve low risk prostate cancer specimens. This supports the role of PKC as the kinase responsible for phosphorylation of AR at Ser-578 in both hormone naïve prostate cancer and castrate resistant prostate cancer.

The relationship between clinico-pathological parameters and expression of pAR^{S81}, pAR^{S578} and PKC was investigated. High expression of nuclear pAR^{S578} was associated with increased PSA level at diagnosis. High serum levels of PSA at diagnosis has been shown to be associated with other markers of high risk disease including high Gleason score, increased disease stage, and disease specific survival (163, 164). High expression of both nuclear and cytoplasmic pAR^{S578} is associated with the presence of perineural invasion in diagnostic specimens. PNI is a recognised mechanism for the development of extra-prostatic extension in prostate cancer (163). PNI has been associated with poorer outcomes following radical surgery (165, 166). This suggests that pAR^{S578} may have a role in the development of PNI, and thus may be a marker for high risk disease. These findings support the research hypothesis that high expression of pAR^{S578} is associated with advanced disease. Phosphorylation status at serine 578 could therefore be a surrogate marker of disease severity.

AR is known to be pivotal in growth and progression of prostate cancer and is therefore the main target in medical management of prostate cancer. It is well

established that the AR is activated in response to DHT. (167, 168) Phosphorylation of the AR occurs at a number of phosphosites in response to DHT, with Ser-81 being the most commonly phosphorylated site. (114) We found that high expression of pAR^{S81} was associated with shorter time to treatment intervention. This is in spite of pAR^{S81} expression not being associated with any of the clinical markers currently used to assess risk in localised disease. This supports the research hypothesis that pAR^{S81} expression would be associated with poor outcomes in prostate cancer. pAR^{S81} may be a useful independent marker of overall poorer prognosis in low-risk localised prostate cancer.

Phosphorylation of the AR can also occur at other serine residues and has been shown to regulate AR activity and cellular localisation. Our group has previously investigated the effect of AR phosphorylation at a number of serine residues on the AR on clinical outcome measures. Phosphorylation at serine 213 is associated with decreased disease specific survival in castrate resistant prostate cancer and phosphorylation at serine 515 is associated with decreased time to biochemical relapse and decreased disease specific survival in hormone naïve prostate cancer (124, 128). Phosphorylation of the AR is not always associated with poorer outcome however. Phosphorylation of the AR at serine 791 is associated with increased time to biochemical relapse and increased time to death in castrate resistant prostate cancers (126). It was hypothesised that pAR^{S578} may also be of prognostic significance in prostate cancer. It has previously been demonstrated that the PKC inhibitor, Calphostin, reduced both EGF-dependent and independent cell growth in a recurrent prostate cancer cell line. (129) Phosphorylation of the AR at Ser-578 has been linked to nuclear-cytoplasmic shuttling, DNA binding and modulation of other phosphosites on the AR. In the present study, there was no association between phosphorylation of the AR at Ser-578 and time to treatment intervention, despite a significant association with high PSA at diagnosis, an established marker of increased disease severity. pAR^{S578} expression is unable to predict which patients will require earlier intervention amongst a relatively homogeneous group of low risk prostate cancer patients.

Classical AR phosphorylation occurs via DHT binding, resulting in phosphorylation of AR at Ser-81. As we demonstrated earlier, Ser-578 is likely to be

phosphorylated in response to PKC. Phosphorylation of the AR in response to PKC occurs via an alternative pathway to that of classical AR phosphorylation secondary to DHT. We hypothesised that when combined, the phosphorylation status on the AR at these two serine residues may have a cumulative adverse prognostic effect. In this study, we demonstrated a cumulative prognostic effect when expression of cytoplasmic pAR^{S81} and pAR^{S578} were combined in relation to time to treatment intervention. This is in spite of pAR^{S578} having no independent significant association with time to intervention in this cohort. Patients who had high expression of both pAR^{S81} and pAR^{S578} required treatment sooner than those with low expression of both. The observed cumulative effect had greater prognostic power than expression of pAR^{S81} alone. These striking results suggest that there is a sub-population of patients who despite fulfilling the traditional criteria of low risk prostate cancer at diagnosis are more likely to progress and require treatment at an earlier stage.

A limitation of this study was the small cohort sample size. However, despite the small sample size, this study has demonstrated that pAR^{S81} alone and in combination with pAR^{S578} can predict earlier treatment intervention in low risk prostate cancer patients. This may provide clinicians with a much-needed prognostic biomarker to identify those patients who are more likely to require early intervention, and those patients that can safely remain on AS and avoid the harms of unnecessary treatment. These results should be validated in a larger, independent cohort of low-risk prostate cancer patients.

A pilot study will now be conducted in a group of consecutive hormone-naïve prostate cancer patients with all stages of disease at diagnosis to investigate if these findings can be replicated in a more heterogeneous group of patients.

Chapter 4 Discovery Prostate Cancer Cohort

Prostate cancer is the most common male cancer in the UK. (1) Following the introduction of the PSA test in the early 1990s, the incidence of prostate cancer has increased by 44% and is projected to rise by a further 12% by 2035. (1, 2) Many patients are diagnosed with low risk, indolent prostate cancer which would otherwise remain undetected in their lifetime. The reported rates of over-diagnosis vary widely (169), however, it remains clear that over-treatment of low-risk disease should be avoided to not only reduce unnecessary side effects but also to reduce the associated economic burden. Current methods for risk stratification are poor and rely on basic parameters such as PSA at diagnosis and Gleason score obtained from TRUS biopsy, which does not always represent the true pathological nature of the tumour. Recently, the Genomic Prostate Score has been developed, which utilises a panel of 17 genes grouped into four categories (AR signalling, stromal response, cellular organisation and proliferation) to predict prostate cancer aggressiveness using the initial diagnostic pathological specimen. (170) Whilst this score can assist clinicians in treatment recommendations, the test is expensive and there is dubiety over it's cost-effectiveness in clinical practice. Prostate cancer management is certainly moving towards individualised medicine and there is much need for new prognostic biomarkers that can easily translate into the clinic, be cost effective and ultimately aid clinicians in deciding which patients will gain most benefit from treatment or indeed if they require treatment at all.

In this study, we investigate the use of IHC to assess pAR^{S578} and pAR^{S81} status both as individual and combined biomarkers for prognosis in a cohort of patients with hormone-naïve prostate cancer of all stages of disease.

4.1 Patient Demographics

Ninety hormone-naïve prostate cancer patients, recruited between 1992 and 2002, were included in this study. Median follow up period was 11.7 years (IQR 9.9 – 14.0 years). Patient demographics including age at diagnosis, Gleason score, PSA at diagnosis and PSA at relapse were recorded and are summarised in Table 4.1.

Table 4.1 Clinico-pathological characteristics of the cohort

Clinical Parameter	Patients, n (%)
Age (<70/ ≥70 years)	34 (37.0)/ 56 (60.9)
Diagnosis PSA (<10/ 10 – 20/ ≥20 ng/ml)	19 (20.7)/ 14 (15.2)/ 36 (39.1)
Gleason score (<7/7/>7)	24 (26.1)/ 25 (27.2)/ 28 (30.4)
Lymphovascular invasion (no/yes)	84 (91.3)/ 6 (6.5)
Perineural invasion (no/yes)	72 (78.3)/ 18 (19.6)
Prostatic intraepithelial neoplasia (no/yes)	79 (85.9)/ 11 (12.0)

Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

Median age at diagnosis was 70.1 years (IQR 63.3 – 75.4 years). Median PSA at diagnosis was 22 ng/ml (IQR 7.7 – 60.5 ng/ml). Median PSA at biochemical relapse was 2.2 ng/ml (IQR 1.0 – 7.3 ng/ml). At diagnosis, twenty-three patients had metastatic disease to local lymph nodes (3), bone (13) or both sites (7). Forty-seven patients developed biochemical relapse following treatment. The median time to biochemical relapse was 2.7 years (IQR 1.5-3.8 years). Sixty-six patients died during the follow-up period. Forty-six patients died from prostate cancer (median time to death 4 years, IQR 1.9-7.2 years). Twenty patients died due to non-prostate cancer causes (median time to death 4.1 years, IQR 0.9-5.5 years).

4.2 Protein expression analysis

Eighty-nine patients had hormone-naïve prostate cancer specimens available for IHC. Protein expression in the nucleus and cytoplasm was recorded in epithelial tumour cells only. Expression of all proteins was heterogeneous throughout the cells.

Protein expression in the specimens was divided into low (≤median) and high (>median) for purposes of analysis. (Table 4.2) All ICCC values were >0.80.

Table 4.2 Protein expression in patients with tissue available

	Patients, n (%)	Median Histoscore (Histoscore units)	Interquartile range (Histoscore units)
AR Nuclear (low/high)	44 (47.8)/ 45 (48.9)	68	44 – 83
AR Cytoplasmic (low/high)	45 (48.9)/44 (47.8)	63	50 – 81
pAR^{S81} Nuclear (low/high)	34 (37.0)/ 31 (33.7)	140	74 – 207
pAR^{S81} cytoplasmic (low/high)	34 (37.0)/ 31 (33.7)	50	20 – 100
pAR^{S578} Nuclear (low/high)	30 (32.6)/ 30 (32.6)	169	130 – 206
pAR ^{S578} Cytoplasmic (low/high)	32 (34.8)/ 28 (30.4)	50	31 – 100
PKC Nuclear (low/high)	30 (32.6)/ 28 (30.4)	205	174 – 223
PKC Cytoplasmic (low/high)	29 (31.5)/ 29 (31.5)	138	100 – 163

The median histoscore for each protein of interest was calculated. Patients were grouped into low (≤median) and high expression (>median). The number of patients in each group is displayed for each protein.
Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

4.3 Association between PKC expression and expression of pAR^{S578} in clinical specimens

In the clinical specimens, nuclear PKC expression was significantly associated with pAR^{S578} expression both in the nucleus (c.c. 0.426, p=0.002) and cytoplasm (c.c. 0.469, p=0.001). Cytoplasmic PKC expression was associated with nuclear pAR^{S578} expression (c.c. 0.284, p=0.044). Table 4.3 demonstrates the association between PKC expression and pAR^{S578}.

Table 4.3 Association between PKC expression and expression of pAR^{S578} at both cellular locations in the clinical specimens

	PKC	
	Nuclear p value, C.C	**Cytoplasmic p value, C.C**
pAR578 Nuclear	***0.001, 0.469***	***0.044, 0.284***
pAR578 Cytoplasmic	***0.002, 0.426***	0.894, 0.019

Pearson's correlation coefficient was used to assess the association between PKC expression and expression of pAR^{S578} in the nucleus and cytoplasm. Expression of nuclear PKC was significantly associated with pAR^{S578} expression at both locations. Expression of cytoplasmic PKC was significantly associated with nuclear pAR^{S578} expression only.
C.C. denotes Pearson's correlation co-efficient. Values highlighted in red denotes associations with a p value < 0.05

4.4 Clinico-pathological parameters related to outcome measures

Time to biochemical relapse was calculated from diagnosis to biochemical relapse. Patients were considered to have biochemical relapse if serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. High Gleason score at diagnosis (proportion of patients relapsed at 5 years, <7 44% vs 7 74% vs >7 88%, HR 1.9, (95% CI 1.3-2.9) p=0.004) increased PSA at diagnosis (proportion of patients relapsed at 5 years, <10 ng/ml 44% vs 10 – 20 ng/ml 65% vs ≥20 ng/ml 81%), HR 2.0, (95% CI 1.3-2.9) p=0.002) presence of metastases (proportion of patients relapsed at 5 years, absence of metastases 37% vs presence of metastases 79%, HR 3.7, (95% CI 1.7-8.0) p=0.001) and LVI (proportion of patients relapsed at 5 years, absence of LVI 61% vs presence of LVI 0%, HR 4.6, (95% CI 1.7-12.0) p=0.001) are significantly associated with decreased time to biochemical relapse.

Survival following disease recurrence was calculated from biochemical relapse till death or last follow-up using cancer-specific deaths. Presence of metastases (10-year survival, absence of metastases 66% vs presence of metastases 17%, HR 4.8, (95% CI 1.4-17.4) p=0.008) and increased PSA at biochemical relapse (10-year survival, <20 ng/ml 41% vs ≥20 ng/ml 0%, HR 5.9, (95% CI 2.8-12.2) p=<0.001) are associated with shorter time to death following biochemical relapse. Presence of PNI is associated with increased survival following biochemical relapse (10-year survival, absence of PNI 19% vs presence of PNI 61%, HR 0.3, (95% CI 0.1-0.9) p=0.030).

Disease-specific survival was calculated from diagnosis till death or last follow-up using cancer-specific deaths. High Gleason score at diagnosis (10 year survival, <7 81% vs 7 41% vs >7 21%, HR 1.9, (95% CI 1.3-2.9) p=0.007) high PSA at diagnosis (10 year survival, <10 ng/ml 70% vs 10-20 ng/ml 79% vs >20 ng/ml 25%, HR 2.0, (95% CI 1.3-3.3) p=0.001), increased age at diagnosis (10 year survival, <70 years 58% vs >70 years 31%, HR 2.1, (95% CI 1.1-4.0) p=0.020), high PSA at biochemical relapse (10 year survival, <10 ng/ml 50% vs 10-20 ng/ml 0% vs >20 ng/ml 0%, HR 2.8, (95% CI 1.9-4.2) p=<0.001) and presence of metastases (10

year survival, absence of metastases 70% vs presence of metastases 30%, HR 5.0, (95% CI 2.0-12.4) p=<0.001) are associated with decreased disease-specific survival. Presence of PNI is associated with increased disease specific survival (10-year survival, absence of PNI 36% vs presence of PNI 62%, HR 0.4, (95% CI 0.2 – 1.0) p=0.036).

Clinico-pathological parameters as related to clinical outcome measures are summarised in Table 4.4.

Table 4.4 Univariate analysis of clinico-pathological parameters as related to clinical outcome measures

	Univariate analysis		
Clinico-pathological parameter	**Time to biochemical relapse**	**Disease-specific survival from biochemical relapse**	**Disease-specific survival**
	P value Hazard Ratio (95% CI)	**P value Hazard Ratio (95% CI)**	**P value Hazard Ratio (95% CI)**
Age (<70/ ≥70 years)	0.260, 1.4, 0.8 - 2.5	0.385, 1.4, 0.6 - 3.3	***0.020, 2.1, 1.1 - 4.0***
Diagnosis PSA (<10/ 10 - 20/ ≥20 ng/ml)	***0.002, 2.0, 1.3 - 2.9***	0.078, 1.5, 0.8 - 2.7	***0.001, 2.0, 1.3 - 3.3***
Gleason score (<7/7/>7)	***0.004, 1.9, 1.3 - 2.9***	0.060, 1.5, 0.8 - 2.6	***0.007, 1.9, 1.3 - 2.9***
Lymphovascular invasion (no/yes)	***0.001, 4.6, 1.7 - 12.0***	0.612, 1.3, 0.5 - 3.9	0.114, 2.1, 0.8 - 5.3
Presence of PIN (no/yes)	0.720, 0.8, 0.4 - 1.9	***0.026, 0.2, 0.0 - 0.9***	***0.014, 0.2, 0.1 - 0.8***
Presence of PNI (no/yes)	0.561, 1.2, 0.6 - 2.4	***0.030, 0.3, 0.1 - 0.9***	***0.036, 0.4, 0.2 - 1.0***
Presence of metastases (no/yes)	***0.001, 3.7, 1.7 - 8.0***	***0.008, 4.8, 1.4 - 17.4***	***<0.001, 5.0, 2.0 - 12.4***
PSA at biochemical relapse (<10/ 10 - 20/ ≥20 ng/ml)	N/a	***<0.001, 5.9, 2.8 - 12.2***	***<0.001, 2.8, 1.9 - 4.2***

The clinico-pathological variables were grouped and analysed by Kaplan-Meier methods and Cox regression with reference to time to biochemical relapse from diagnosis, disease-specific survival from biochemical relapse and disease-specific survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Significant results (p = ≤0.05) *are highlighted in red.*

4.5 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinico-pathological parameters

High expression of both nuclear and cytoplasmic pAR^{S81} was associated with the presence of perineural invasion (p = 0.028 and p = 0.028). High nuclear pAR^{S81} expression was associated with Ki67 expression (p = 0.027). High expression of nuclear pAR^{S578} was associated with increased PSA level at diagnosis (p = 0.015). High expression of cytoplasmic pAR^{S578} was associated with high Gleason score (p = 0.008). High expression of both nuclear and cytoplasmic PKC was associated with increased age (p = 0.032 and p = 0.018). High nuclear PKC expression was associated with increased PSA at diagnosis (p = 0.009). Table 4.5 demonstrates the expression of pAR^{S81}, pAR^{S578} and PKC as related to clinico-pathological parameters.

Table 4.5 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinico-pathological parameters

	Nuclear AR ^{S81}			Cytoplasmic AR ^{S81}			Nuclear AR ^{S578}			Cytoplasmic AR ^{S578}			Nuclear PKC			Cytoplasmic PKC		
	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value
Age (<70/>70)	15/19	12/19	0.661	18/16	9/22	0.053	14/16	10/20	0.296	16/16	8/20	0.94	*17/13*	*8/20*	***0.032***	*17/12*	*8/21*	***0.018***
Gleason (<7/7/>7)	13/8/9	6/8/12	0.111	10/10/11	9/6/10	0.973	9/10/7	8/7/12	0.324	*13/9/6*	*4/8/13*	***0.008***	10/10/7	10/3/12	0.431	10/7/8	10/6/11	0.630
Diagnosis PSA (<10/10-20/>20)	8/7/15	4/5/13	0.450	9/6/16	3/6/12	0.387	*9/4/11*	*2/5/19*	***0.015***	8/5/13	3/4/17	0.096	*9/8/9*	*3/3/18*	***0.009***	7/7/13	5/4/14	0.478
LVI (no/yes)	32/2	27/4	0.332	32/2	27/4	0.332	27/3	27/3	1.000	30/2	24/4	0.305	27/3	26/2	0.701	26/3	27/2	0.643
PNI (no/yes)	*23/11*	*28/3*	***0.028***	*23/11*	*28/3*	***0.028***	21/9	23/7	0.563	25/7	19/9	0.374	21/9	24/4	0.155	22/7	23/6	0.755
PIN (no/yes)	27/7	28/3	0.227	26/8	29/2	0.059	25/5	26/4	0.720	26/6	25/3	0.388	24/6	25/3	0.333	23/6	26/3	0.281
Metastases (no/yes)	13/8	8/8	0.475	15/9	6/7	0.345	11/6	8/10	0.236	12/9	7/7	0.682	9/7	11/6	0.625	13/5	7/8	0.141
Ki67 (≤median/>median)	*22/8*	*14/17*	***0.027***	19/12	17/13	0.716	17/10	18/10	0.920	16/12	19/8	0.312	17/9	16/12	0.539	17/9	16/12	0.539

Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was examined for significant relationships with clinical variables as shown. Protein expression was divided into high and low groups. Clinical variables were divided into groups and the Mann-Whitney U test was performed for statistical analysis. Statistically significant results (p = ≤0.05) are highlighted in red.

4.6 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures

Table 4.6 shows the univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures.

Table 4.6 Univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures

	Univariate analysis		
Protein expression (<median/>median)	**Time to biochemical relapse**	**Disease-specific survival from biochemical relapse**	**Disease-specific survival**
	P value Hazard Ratio (95% CI)	**P value Hazard Ratio (95% CI)**	**P value Hazard Ratio (95% CI)**
pAR^{S81} Nuclear	0.594, 0.8, (0.4-1.6)	0.407, 1.5, (0.6-3.5)	***0.030, 2.1, (1.1-4.2)***
pAR^{S81} Cytoplasmic	0.166, 1.6, (0.8-3.1)	0.578, 1.3, (0.5-3.1)	0.057, 1.9, (1.0-3.8)
pAR^{S578} Nuclear	0.461, 1.3, (0.6-2.6)	0.347, 1.6, (0.6-4.5)	***0.036, 2.2, (1.0-4.9)***
pAR ^{S578} Cytoplasmic	***0.034, 2.1, (1.0-4.2)***	***0.034, 3.2, (1.0-9.9)***	***<0.001, 4.5, (2.0-10.4)***
PKC Nuclear	0.712, 0.9, (0.4-1.8)	0.450, 1.5, (0.5-3.9)	0.203, 1.7, (0.8-3.7)
PKC Cytoplasmic	0.938, 1.0, (0.5-2.1)	0.799, 1.1, (0.4-3.1)	0.269, 1.6, (0.7-3.5)

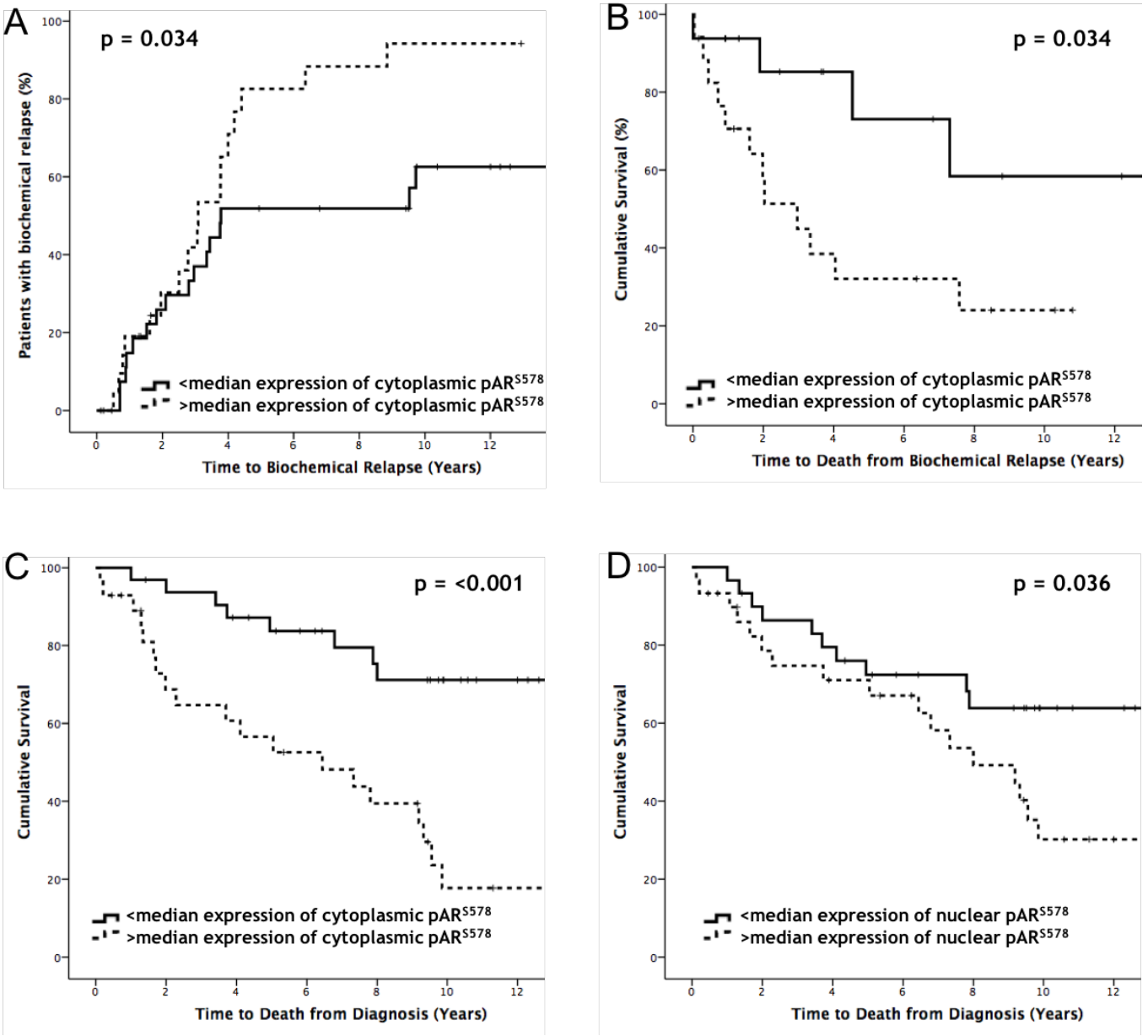
Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was analysed by Kaplan-Meier methods and Cox regression in relation to time to biochemical relapse from diagnosis, disease-specific survival from biochemical relapse and disease-specific survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Protein expression was divided into high and low groups by the median value. High expression of cytoplasmic pAR^{S578} was associated with all three outcome measures. High expression of nuclear pAR^{S81} and pAR^{S578} were both associated with decreased disease-specific survival. Significant results (p = ≤0.05) are highlighted in red.

High expression of cytoplasmic pAR^{S578} was associated with shorter time to biochemical relapse (proportion of patients relapsed at 5 years < median expression 51.9% vs > median expression 82.1%, HR 2.1 (95% CI 1.0-4.2), p=0.034) (Figure 4.1 A). No association was observed between PKC or pAR^{S81} and time to biochemical relapse.

High expression of cytoplasmic pARS578 was associated with decreased survival following biochemical relapse (10-year survival, < median expression 58.3% vs > median expression 24.3% HR 3.2 (95% CI 1.0-9.9), p=0.034) (Figure 4.1 B). No association was observed between pARS81 or PKC and survival from biochemical relapse.

Expression of both pAR^{S81} and pAR^{S578} were found to be significantly associated with disease-specific survival. High expression of nuclear pAR^{S81} was associated with decreased disease-specific survival (10-year survival, <median expression 55% vs > median expression 24%, HR 2.1 (95% CI 1.1 – 4.2), p=0.030. High cytoplasmic pAR^{S578} was associated with decreased disease-specific survival (10-year survival <median expression 71.3% vs >median expression 19.7% HR 4.54 (95% CI 2.0-10.4), p=<0.001) (Figure 4.1 C). High nuclear pAR^{S578} was associated with decreased disease-specific survival (10-year survival, <median expression 63.8% vs >median expression 30.5%, HR 2.24 (95% CI 1.0-4.9), p=0.036) (Figure 4.1 D).

Figure 4.1 Kaplan Meier Graph showing expression of pAR^{S578} related to clinical outcome measures



A. *Kaplan-Meier survival plot showing expression of cytoplasmic pAR^{S578} as related to time to biochemical relapse. High cytoplasmic pAR^{S578} (dashed line) is significantly associated with decreased time to biochemical relapse.*
B. *Kaplan-Meier survival plot showing expression of cytoplasmic pAR^{S578} as related to disease-specific survival following biochemical relapse. High cytoplasmic pAR^{S578} (dashed line) is significantly associated with decreased disease-specific survival following biochemical relapse.*
C. *Kaplan-Meier survival plot showing expression of cytoplasmic pAR^{S578} as related to disease-specific survival. High cytoplasmic pAR^{S578} (dashed line) is significantly associated with decreased disease-specific survival.*
D. *Kaplan-Meier survival plot showing expression of nuclear pAR^{S578} as related to disease-specific survival. High nuclear pAR^{S578} (dashed line) is significantly associated with decreased disease-specific survival.*

4.7 Expression of dual phosphorylation sites on the AR related to outcome measures

The two phosphorylation sites were combined as follows: (i) high pAR^{S81} and high pAR^{S578} and (ii) low pAR^{S81} and low pAR^{S578} expression.

High cytoplasmic pAR^{S81} and cytoplasmic pAR^{S578} was associated with decreased disease-specific survival (10-year survival, high expression of both proteins 15% vs low expression of both proteins 88%, HR 10.0, (95% CI 2.2-46.0) p=<0.001). (Figure 4.2 A) High nuclear pAR^{S81} and nuclear pAR^{S578} was associated with decreased disease-specific survival (10-year survival, high expression of both proteins 20% vs low expression of both proteins 73%, HR 3.9, (95% CI 1.3-11.2) p=0.007) (Figure 4.2 B).

Lastly, the expression of total cytoplasmic pAR^{S81} and pAR^{S578} and total nuclear pAR^{S81} and pAR^{S578} was investigated in relation to clinical outcome measures. Patients were grouped as follows: (i) high total cytoplasmic expression of pAR^{S81} and pAR^{S578} and high total nuclear expression of pAR^{S81} and pAR^{S578} and (ii) low total cytoplasmic expression of pAR^{S81} and pAR^{S578} and low total nuclear expression of pAR^{S81} and pAR^{S578}. Combined high expression of total cytoplasmic pAR^{S81} and pAR^{S578} and high total nuclear expression of pAR^{S81} and pAR^{S578} was associated with decreased disease-specific survival (10-year survival, high total expression of both proteins 0% vs low total expression of both proteins 86%, HR 10.6, (95% CI 2.0-54.4) p=0.001) (Figure 4.2 C).

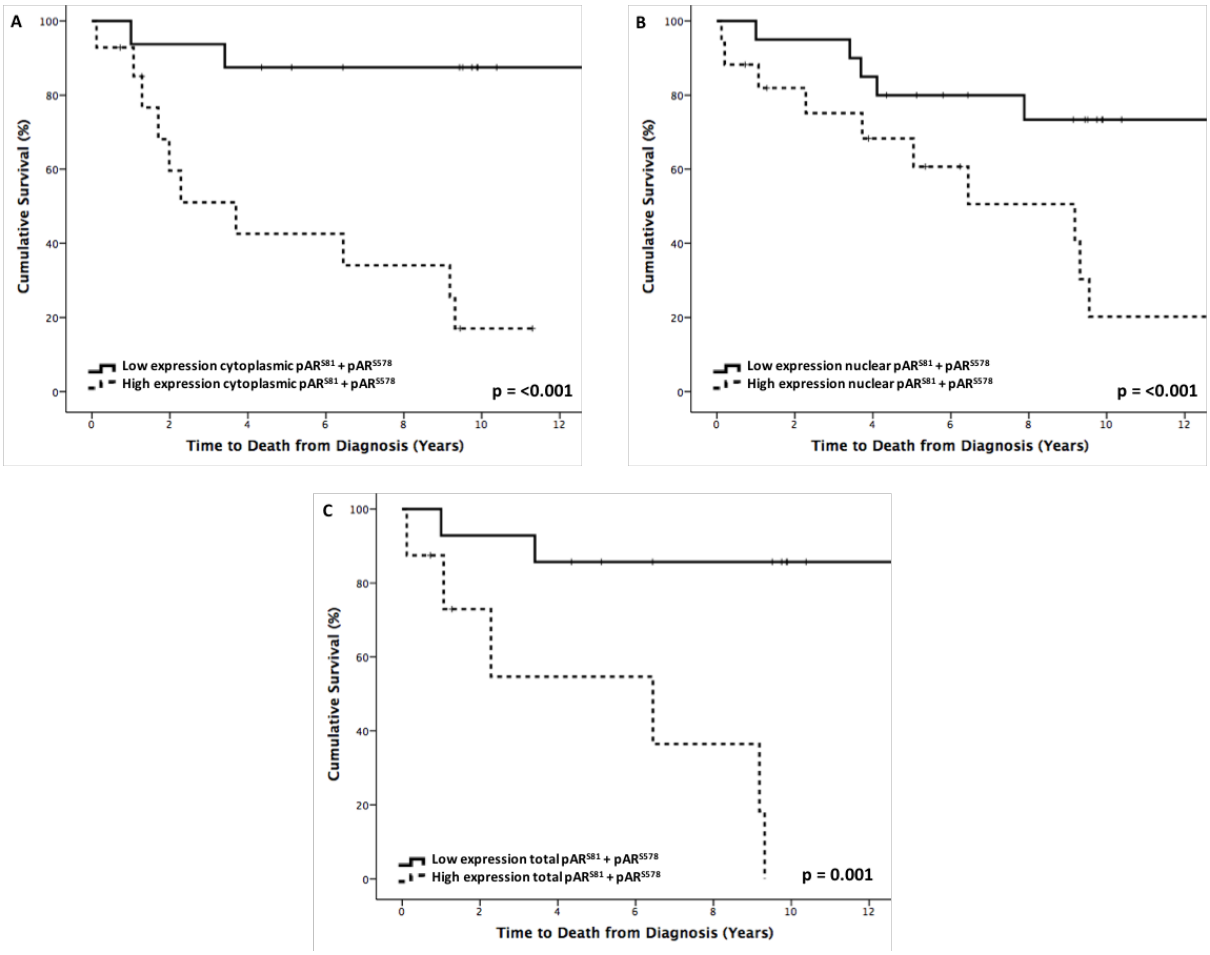
Dual expression of pAR^{S81} and pAR^{S578} was not associated with time to biochemical relapse or disease-specific survival from biochemical relapse.

Table 4.7 Univariate analysis of dual expression of pAR^{S81} and pAR^{S578} related to clinical outcome measures

	Univariate analysis		
	Time to biochemical relapse	**Disease-specific survival from biochemical relapse**	**Disease-specific survival**
	P value Hazard Ratio (95% CI)	**P value Hazard Ratio (95% CI)**	**P value Hazard Ratio (95% CI)**
pAR^{S578} cytoplasmic + pAR^{S81} cytoplasmic	0.091, 2.3, 0.9-5.9	0.069, .3.9, 0.8-19.0	***<0.001, 10.0, 2.2-46.0***
pAR^{S578} nuclear + pAR^{S81} nuclear	0.909, 1.0, 0.4-2.2	0.250, 2.0, 0.6-6.6	***0.007, 3.9, 1.3-11.2***
Total cytoplasmic pAR^{S81} and pAR^{S578} + total nuclear pAR^{S81} and pAR^{S578}	0.480, 1.5, 0.5-5.4	0.106, 3.7, 0.7-20.7	***0.001, 10.6, 2.0-54.4***

Dual expression of pAR^{S81}and pAR^{S578} was analysed by Kaplan-Meier methods and Cox regression in relation to time to biochemical relapse from diagnosis, disease-specific survival from biochemical relapse and disease-specific survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Protein expression was divided into high and low groups. Dual expression of pAR^{S81}and pAR^{S578}in all three groupings was significantly associated with decreased disease-specific survival. Significant results (p = ≤0.05) are highlighted in red.

Figure 4.2 Kaplan Meier Graph showing dual expression of pARS81 and pARS578 related to disease-specific survival



A. *Kaplan Meier survival plot showing disease-specific survival in patients with low expression of both cytoplasmic pARS81 and pARS578 (solid line) and high expression of both cytoplasmic pARS81 and pARS578 (dashed line). High expression of both cytoplasmic pARS81 and pARS578 is associated with decreased disease-specific survival than compared low expression of both phosphosites*

B. *Kaplan Meier survival plot showing disease-specific survival in patients with low expression of both nuclear pAR^{S81} and pAR^{S578} (solid line) and high expression of both nuclear pAR^{S81} and pAR^{S578} (dashed line). High expression of both nuclear pAR^{S81} and pAR^{S578} is associated with decreased disease-specific survival than compared low expression of both phosphosites*

C. *Kaplan Meier survival plot showing disease-specific survival in patients with low expression of total pAR^{S81} and pAR^{S578} (solid line) and high expression of total pAR^{S81} and pAR^{S578} (dashed line). High total expression of pAR^{S81} and pAR^{S578} is associated with decreased disease-specific survival than compared to low total expression of pAR^{S81} and pAR^{S578}*

4.8 Discussion

The current study investigates the use of the AR phosphorylated at Ser-81 and Ser-578 in a cohort of patients with hormone-naïve prostate cancer with a heterogeneous mix of disease severity at presentation. The clinico-pathological characteristics of this cohort reflect urological practice at the time when these patients were recruited. This is an elderly cohort, with 60.9% of patients aged >70 years. The PSA at diagnosis was >20 ng/ml in 39.1% of patients and 30.4% of patients had a Gleason score of >7, indicating that the majority of patients had at least one marker of high risk disease as defined by the current NICE guidelines (51).

As is expected, clinico-pathological markers of high risk disease were found to be significantly associated with poor clinical outcome measures. High Gleason score and increased PSA at diagnosis were found to be significantly associated with both shorter time to biochemical relapse and decreased disease-specific survival. This is in keeping with previous reports. (47, 171-173) Surprisingly, the presence of PNI was found to be associated increased disease-specific survival and longer survival following biochemical relapse. PNI is associated with other adverse clinico-pathological markers of prostate cancer including extra-prostatic extension, higher PSA at diagnosis and higher Gleason grade, and there has been increasing evidence regarding its role as a negative prognostic marker in prostate cancer. (165, 174) Our results should be interpreted with caution, as this is a small cohort from a single institution and differences in pathological interpretation of PNI may account for our findings. (175)

Protein expression in the discovery prostate cancer cohort differs to that of the AS cohort. The median expression of almost all of the AR proteins was less in the discovery prostate cancer cohort than compared to the AS. An exception was that of nuclear pAR^{S578} which was found to have higher median expression in the discovery prostate cancer cohort. Kinase expression was more intense in the discovery prostate cancer cohort, with the median expression of PKC higher than that observed in the AS cohort. This variation in staining intensity observed

between the two cohorts likely reflects the differences in the clinico-pathological parameters of these two cohorts and is in keeping with studies that demonstrate differences in the molecular profile of low and high-risk disease. (176, 177)

PKC expression strongly correlated with pAR^{S578} expression in this cohort. This is in agreement with the findings in the AS cohort and with that of previous castrate resistant prostate cancer cell line work and Scansite prediction(129, 130). The current study adds to this by suggesting that the link between PKC and AR phosphorylation at Ser-578 is also present in hormone naïve prostate cancer tissue and therefore may have important implications in not only high and low risk disease but also early and late stages of the disease too. Similar site directed mutagenesis studies are necessary in prostate cancer cell lines established from localised disease in order to confirm this finding.

AR phosphorylation status has previously been associated with markers of high risk disease in hormone-naïve prostate cancer. Willder et al found that cytoplasmic pAR^{S515} expression was associated with the presence of LVI and high expression of nuclear pAR^{S308} was associated with high PSA levels at diagnosis in hormone-naïve prostate cancer (128). In the current study, it was again shown that AR phosphorylation status is associated with established markers of high risk disease. Expression of pAR^{S578} was associated with both increased PSA level (nuclear) and high Gleason score (cytoplasmic) at diagnosis. PKC, the predicted kinase responsible for phosphorylation of the AR at Ser-578, was also associated with increased PSA at diagnosis with high expression in the nucleus. High expression of pAR^{S81} is associated the presence of PNI. These findings support those of the AS cohort, in which pAR^{S578} was associated with increased PSA at diagnosis and presence of PNI, suggesting that pAR^{S578} may be a useful surrogate biomarker of high risk clinico-pathological features.

The role of the AR expression in clinical specimens in relation to clinical outcome measures has been extensively investigated, with varying results reported. High nuclear expression of AR has been associated with decreased disease-specific survival in patients with castrate resistant prostate cancer(93). Furthermore, high

AR levels were associated with shorter time to biochemical relapse in patients undergoing radical prostatectomy (92, 178). However, conflicting results were reported by Sweat et al, who found no association between nuclear AR expression in tissue obtained from radical prostatectomy samples from hormone naïve prostate cancer patients in relation to clinical progression and survival(179). There are a number of factors that are likely to have a role in the differing reported findings such as age of specimens, antibody and methods used for detection of the AR protein and varying thresholds for stratification into high and low expression groups. In addition, the use of total AR quantifies both activated and inactive AR. In this study, we investigate the prognostic significance of AR phosphorylated at Ser-81 and Ser-578 in addition to total AR.

The prognostic significance of AR serine phosphorylation has been investigated previously by ourselves and others (123, 124, 126, 128). We again have demonstrated that pAR^{S578} is associated with poor outcome measures, as was found in the AS cohort, further supporting the research hypothesis that pAR^{S578} is associated with advanced disease. The expression of cytoplasmic pAR^{S578} was found to be the marker most commonly associated with poor outcome measures, predicting shorter time to biochemical relapse, decreased disease specific survival following biochemical relapse and decreased disease specific survival from diagnosis. This work will now be validated in a larger cohort of hormone-naïve prostate cancer patients to further assess the suitability of pAR^{S578} as a prognostic biomarker in prostate cancer.

Nuclear expression of pAR^{S81} is associated with shorter disease-specific survival from diagnosis. Cell line work has shown that the AR phosphorylated at Ser-81 has a role in AR transcriptional activity, modulation of AR phosphorylation and cell proliferation. (115, 117, 118, 180) We have again demonstrated that in prostate tissue specimens, pAR^{S81} is associated with poor outcome measures. This supports the findings in the AS cohort that demonstrated that cytoplasmic expression of pAR^{S81} was associated with decreased time to treatment intervention.

As phosphorylation of the AR at Ser-81 and Ser-578 is predicted to occur in response to different kinases, we investigated if there was any increased prognostic significance when the androgen is phosphorylated at both serine residues. Combined high expression of pAR^{S81} and pAR^{S578} is associated with shorter disease-specific survival than compared to high expression of pAR^{S81} and pAR^{S578} independently. These results are potentially of great importance clinically, as it highlights a sub-population of patients who may benefit from dual targeted therapy with ADT and PKC inhibitors.

A limitation of this study is the small sample number and therefore will now be validated in a larger cohort of hormone-naïve prostate cancer patients.

Chapter 5 Clinico-pathological parameters of validation prostate cancer cohort

Prostate cancer management in the UK is currently based primarily on a limited range of tumour factors, namely PSA at diagnosis, Gleason score and clinical stage. (51) Whilst risk classification systems are available to clinicians, they are not without problem. There remains heterogeneity amongst patients who fulfil the criteria of specific risk classification groups. As discussed in the AS chapter and in work by other groups, there are patients that despite being defined as low risk can experience disease progression, and conversely those with high risk criteria at diagnosis may still have indolent disease. (181) Differentiating between those patients who can be managed conservatively and those that require radical treatment presents a unique challenge in prostate cancer management. (182) A clinician's first priority should be to do no harm. Prostate cancer therefore presents clinicians with a difficult decision in deciding the most appropriate treatment, with the aim being to minimise overtreatment in indolent disease and under-treatment in high risk disease. Clinicians therefore desperately need additional tools to allow better risk stratification of patients at diagnosis.

A number of criteria in addition to those used in the NICE guidelines have been suggested to optimise risk stratification at diagnosis. The primary Gleason pattern, percentage of cancer containing biopsy cores at diagnosis and PNI have been shown to independently predict adverse outcomes(165, 183-185). The inclusion of these criteria into current risk stratification systems may aid clinicians in directing treatment towards those who would receive most benefit.

In addition to tumour factors, systemic inflammation has been increasingly recognised as having a role in disease progression in prostate cancer. CRP, a marker of systemic inflammation, has been shown to be an independent predictor of disease-specific survival in localised prostate cancer. (186) The mGPS, a score of systemic inflammation that combines CRP and albumin levels at diagnosis, has been shown to be independently associated with adverse outcomes in prostate cancer.(187)

The aim of the current study is to investigate the role of tumour factors in predicting prostate cancer outcomes and validate the findings of the discovery cohort in a larger, modern cohort of prostate cancer patients that is more reflective of today's clinical practice. It is anticipated that identification of additional risk factors than can be utilised in conjunction with existing risk stratification systems could aid in identification of those patients at increased risk of adverse outcomes and can therefore direct more aggressive treatment towards these patients. In addition, the role of systemic inflammation was investigated in this cohort to determine its prognostic value in prostate cancer.

5.1 Patient Demographics

361 patients diagnosed with prostate cancer in 2009 in NHS Greater Glasgow and Clyde were included in this study. 243 patients had tissue available for analysis. Median follow-up period was 5.2 years (IQR 4.7 – 5.9 years). Patient demographics including age at diagnosis, Gleason score, PSA at diagnosis and PSA at relapse were recorded and are summarised in Table 5.1.

Table 5.1 Clinico-pathological characteristics of the cohort

Clinical Parameter	Patients, n (%)
Age (<70/ ≥70 years)	186 (51.5)/ 161 (44.6)
Diagnosis PSA (<10/ 10 – 20/ ≥20 ng/ml)	140 (38.8)/ 75 (20.8)/128 (35.5)
Gleason score (<7/7/>7)	136 (37.7)/114 (31.6)/ 81 (22.4)
Perineural invasion (no/yes)	201 (55.7)/ 121 (33.5)
Metastases at diagnosis (no/yes)	275 (76.2)/ 37 (10.2)
% cores positive for malignancy (<50/≥50)	88 (24.4)/ 128 (35.5)

Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

Median age at diagnosis was 69.3 years (IQR 62.8 – 74.7 years). Median PSA at diagnosis was 12.6 ng/ml (IQR 6.9 – 35.4 ng/ml). 136 patients had a Gleason score of <7 at diagnosis. 37 patients had metastatic disease at the time of diagnosis.

Most patients (291 patients) had histological diagnosis confirmed by TRUS biopsy, whilst 35 patients were diagnosed following TURP. An incidental finding of prostate cancer following prostatectomy as part of radical surgery for non-prostatic disease was made in a further 6 patients. Two patients were diagnosed from biopsy of non-prostate tissue with local invasion of prostate cancer.

51 patients experienced biochemical relapse during follow-up. Median time to biochemical relapse was 1.8 years (IQR 1.0 – 2.9). Median PSA at relapse was 8.4 ng/ml (IQR 3.4 – 21.4 ng/ml).

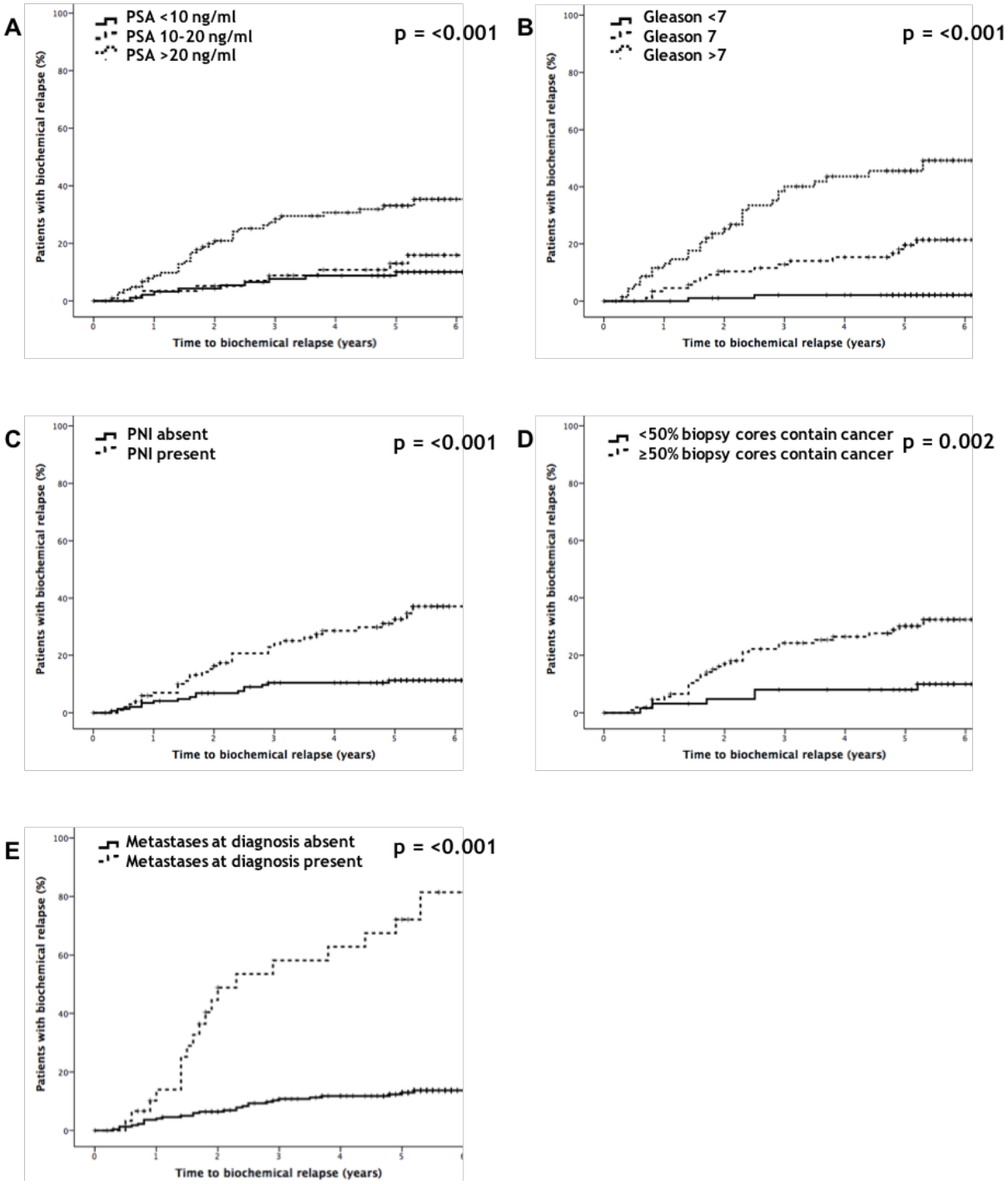
111 patients died during follow-up. Cause of death was not available for this cohort therefore overall survival was used as a clinical outcome measure. Median time to death from diagnosis was 3.2 years (IQR 1.9 – 4.6 years). Median time to death from biochemical relapse was 1.8 years (IQR 0.7 – 2.9 years). 53 patients were treated with AS. Patients treated with AS had a median PSA at diagnosis of 6.9 ng/ml (IQR 5.3 – 9.9). 44 patients had a Gleason score of 6, the remaining patients had a Gleason score of 7. 4 (7.5%) patients died during follow-up. Median time to death was 4.0 years. (IQR could not be calculated due to small number of deaths).

5.2 Clinico-pathological parameters related to outcome measures

Time to biochemical relapse was calculated from diagnosis to biochemical relapse. High PSA at diagnosis was associated with decreased time to biochemical relapse (proportion of patients with biochemical relapse at 5 years, <10 ng/ml 9% vs 10-20 ng/ml 13% vs >20 ng/ml 33%, HR 2.2, (95% CI 1.5 – 3.1) p=<0.001) (Figure 5.1 A). High Gleason score at diagnosis was associated with decreased time to biochemical relapse (proportion of patients with biochemical relapse at 5 years, <7 2% vs 7 18% vs >7 46%, HR 4.1, (95% CI 2.6 – 6.3) p=<0.001) (Figure 5.1 B). Perineural invasion was associated with decreased time to biochemical relapse (proportion of patients with biochemical relapse at 5 years, absence of PNI 11% vs presence of PNI 31%, HR 3.4, (95% CI 1.9 – 6.2) p=<0.001) (Figure 5.1 C). Increased percentage of biopsy cores containing cancer was associated with decreased time to biochemical relapse

(proportion of patients with biochemical relapse at 5 years, <50% of biopsy cores contain cancer 8% vs ≥50% biopsy cores contain cancer 30%, HR 3.7, (95% CI 15 – 8.9) p=0.002) (Figure 5.1 D). Metastases at diagnosis was associated with decreased time to biochemical relapse (proportion of patients with biochemical relapse at 5 years, absence of metastases 12% vs presence of metastases 73%, HR 8.6, (95% CI 4.7 – 15.6) p=<0.001) (Figure 5.1 E).

Figure 5.1 Kaplan-Meier Graphs showing clinico-pathological parameters as related to time to biochemical relapse



A. *Kaplan-Meier survival plot showing PSA at diagnosis as related to time to biochemical relapse. PSA >10 ng/ml at diagnosis (dotted line) is significantly associated with decreased time to biochemical relapse*

B. *Kaplan-Meier survival plot showing Gleason score at diagnosis as related to time to biochemical relapse. Gleason score >7 at diagnosis (dotted line) is significantly associated with decreased time to biochemical relapse*

C. *Kaplan-Meier survival plot showing PNI as related to time to biochemical relapse. The presence of PNI at diagnosis (dashed line) is significantly associated with decreased time to biochemical relapse*

D. *Kaplan-Meier survival plot showing percentage of biopsy cores positive for cancer as related to time to biochemical relapse. ≥50% of biopsy cores containing cancer at diagnosis (dotted line) is significantly associated with decreased time to biochemical relapse*

E. *Kaplan-Meier survival plot showing the presence or absence of metastases at diagnosis as related to time to biochemical relapse. The presence of metastases at diagnosis (dashed line) is significantly associated with decreased time to biochemical relapse*

Time to death from biochemical relapse was calculated from biochemical relapse to time to death from any cause. Increased age at diagnosis was associated with decreased time to death from biochemical relapse (5-year overall survival from biochemical relapse, age <70 years 55% vs age ≥70 years 15%, HR 3.6, (95% CI 1.5-8.3) p=<0.001). No other clinico-pathological parameters were related to time to death from biochemical relapse.

Unfortunately, specific cause of death was not available for this cohort and therefore disease-specific survival could not be calculated. Overall survival has therefore been used as an end-point in this study. Overall survival was calculated from diagnosis to time of death from any cause. Increased age at diagnosis was associated with decreased overall survival (5-year survival, age <70 years 84% vs age ≥70 years 66%, HR 3.0, (95% CI 2.0-4.6) p=<0.001) (F**igu**re 5.2 A).

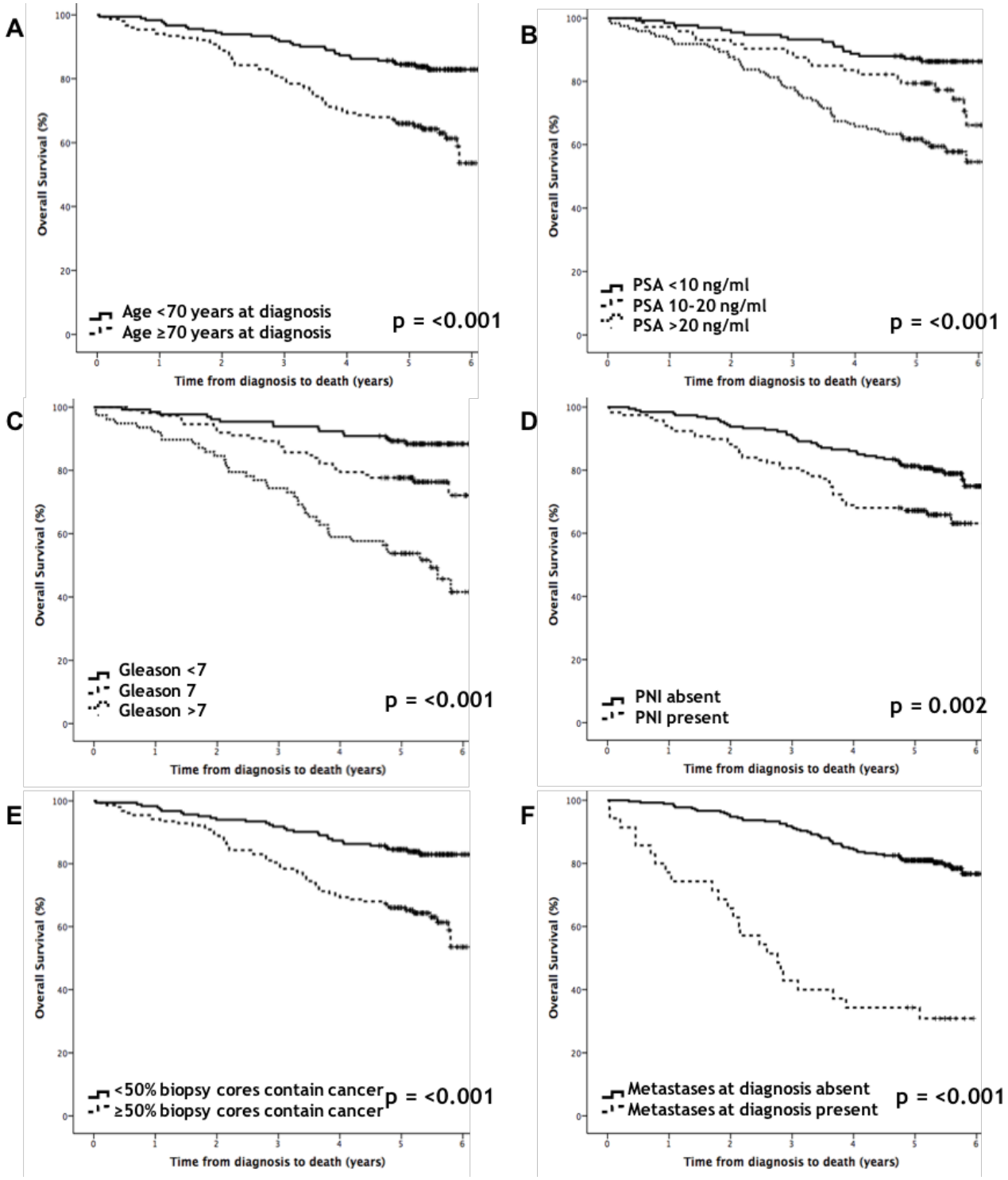
High PSA at diagnosis was associated with decreased overall survival (5-year survival, <10 ng/ml 87% vs 10-20 ng/ml 79% vs >20 ng/ml 61%, HR 2.0, (95% CI 1.5-2.6) p=<0.001) (Figure 5.2 B). High Gleason score at diagnosis was associated with decreased overall survival (5-year survival, <7 89% vs 7 77% vs >7 53%, HR 2.5, (95% CI 1.9-3.2) p=<0.001) (Figure 5.2 C). Perineural invasion was associated with decreased overall survival (5-year survival, absence of PNI 81% vs presence of PNI 67%, HR 1.9, (95% CI 1.2-2.9) p=0.002) (Figure 5.2 D). Increased percentage of biopsy cores containing cancer was associated with decreased overall survival (5-year survival, <50% of biopsy cores contain cancer 87% vs ≥50% biopsy cores contain cancer 64%, HR 2.7, (95% CI 1.6 – 5.1) p=<0.001) (Figure 5.2 E). Metastases at diagnosis was associated with decreased overall survival (5-year overall survival, absence of metastases 81% vs presence of metastases 34%, HR 5.7, (95% CI 3.6-9.5) p=<0.001) (Figure 5.2 F).

Table 5.2 Univariate analysis of clinico-pathological parameters as related to clinical outcome measures

	Univariate analysis		
Clinico-pathological parameter	**Time to biochemical relapse** **P value Hazard Ratio (95% CI)**	**Overall survival from biochemical relapse** **P value Hazard Ratio (95% CI)**	**Overall survival** **P value Hazard Ratio (95% CI)**
Age (<70/ ≥70 years)	0.113, 1.6, 0.9-2.7	***0.001, 3.6, 1.5-8.3***	***<0.001, 3.0, 2.0-4.6***
Diagnosis PSA (<10/ 10 – 20/ ≥20 ng/ml)	***<0.001, 2.2, 1.5-3.1***	0.088, 1.9, 1.0-3.5	***<0.001, 2.0, 1.5-2.6***
Gleason score (<7/7/>7)	***<0.001, 4.1, 2.6-6.3***	0.597, 1.3, 0.6-2.8	***<0.001, 2.5, 1.9-3.2***
Presence of PNI (no/yes)	***<0.001, 3.4, 1.9-6.2***	0.645, 1.2, 0.5-2.7	***0.002, 1.9, 1.2-2.9***
% of cores containing cancer (<50/≥50)	***0.002, 3.7, 1.5-8.9***	0.240, 2.3, 0.5-10.2	***<0.001, 2.7, 1.6-5.1***
Presence of metastases at diagnosis (no/yes)	***<0.001, 8.6, 4.7-15.6***	0.204, 1.7, 0.7-3.7	***<0.001, 5.7, 3.6-9.5***

The clinico-pathological variables were grouped and analysed by Kaplan-Meier methods and Cox regression with reference to time to biochemical relapse from diagnosis, overall survival from biochemical relapse and overall survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Significant results (p = ≤0.05) are highlighted in red.

Figure 5.2 Kaplan-Meier Graphs showing clinico-pathological parameters as related to overall survival



A. *Kaplan-Meier survival plot showing age at diagnosis as related to overall survival. Age ≥70 years (dotted line) is significantly associated with decreased overall survival*

B. *Kaplan-Meier survival plot showing PSA at diagnosis as related to overall survival. PSA >10 ng/ml at diagnosis (dotted line) is significantly associated with decreased overall survival*

C. *Kaplan-Meier survival plot showing Gleason score at diagnosis as related to overall survival. Gleason score >7 at diagnosis (dotted line) is significantly associated with decreased overall survival*

D. *Kaplan-Meier survival plot showing PNI as related to overall survival. The presence of PNI at diagnosis (dashed line) is significantly associated with decreased overall survival*

E. *Kaplan-Meier survival plot showing percentage of biopsy cores positive for cancer as related to overall survival. ≥50% of biopsy cores containing cancer at diagnosis (dotted line) is significantly associated with decreased overall survival*

F. *Kaplan-Meier survival plot showing the presence or absence of metastases at diagnosis as related to overall survival. The presence of metastases at diagnosis (dashed line) is significantly associated with decreased overall survival*

5.4 The role of systemic inflammation in prostate cancer

163 patients had a documented CRP level at diagnosis. Median CRP at diagnosis was 6 mg/L (IQR 2.3 – 23 mg/L). 282 patients had an albumin level documented at diagnosis. Median albumin at diagnosis was 39.5 g/L (IQR 37.0 – 42.0 g/L). The modified Glasgow Prognostic Score (mGPS) is a systemic inflammatory prognostic scoring system that makes use of serum CRP and albumin at the time of diagnosis to inform on prognosis. Patients with both a low CRP (≤ 10 mg/l) and normal albumin (≥35 g/l) are assigned a score of 0, patients with only an elevated CRP (>10 mg/l) are assigned a score of 1, and patients with both elevated CRP and hypoalbuminaemia (albumin <35 g/l) are given a score of 2. Table 5.3 outlines the mGPS scoring system.

Table 5.3 The modified Glasgow Prognostic Score

Markers of inflammation	Points allocated
CRP ⩽10 mg/l and albumin ≥ 35g/l	0
CRP >10 mg/l	1
CRP >10 mg/l and albumin <35 g/l	2

The modified Glasgow Prognostic Score is a systemic inflammatory prognostic scoring system the utilises serum CRP and albumin measurements to assign a prognostic group.

The mGPS could be calculated in 160 patients. Twenty-four patients (6.6%) had a mGPS of 2 (Table 5.4).

Table 5.4 Markers of systemic inflammation in the cohort

Marker of inflammation	Patients, n (%)
CRP (≤10 mg/L/>10 mg/L)	98 (27.1)/ 65 (18.0)
Albumin (<35 g/L/≥35 g/L)	38 (10.5)/ 244 (67.6)
mGPS (0/1/2)	96 (26.6)/40 (11.1)/ 24 (6.6)

Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to data being unavailable

5.4.1 The mGPS related to clinico-pathological parameters

Table 5.5 demonstrates the mGPS as related to clinico-pathological parameters as assessed by the Chi square test. High mGPS was associated with high Gleason score at diagnosis (p = 0.006), increased PSA at diagnosis (p = 0.015) and the presence of metastases at diagnosis (p = 0.001).

Table 5.5 The mGPS as related to clinico-pathological parameters

	The mGPS score			
Clinico-pathological parameter	**0**	**1**	**2**	**p-value**
Age (<70/ ≥70 years)	57/38	24/16	9/15	0.930
Diagnosis PSA (<10/ 10 – 20/ ≥20 ng/ml)	43/22/28	18/7/15	5/5/14	***0.015***
Gleason score (<7/7/>7)	40/31/19	17/12/10	4/6/12	***0.006***
Presence of PNI (no/yes)	51/37	25/14	12/10	0.996
% of cores containing cancer (<50/≥50)	19/34	11/15	2/11	0.355
Presence of metastases at diagnosis (no/yes)	80/7	27/7	14/8	***0.001***

The mGPS was examined for significant relationships with clinical variables using the Chi square test. High mGPS was associated with high Gleason score at diagnosis, increased PSA at diagnosis and the presence of metastases at diagnosis. Statistically significant (p = ≤0.05) results are highlighted in red.

5.4.2 Markers of systemic inflammation related to clinical outcome measures

High CRP at diagnosis was associated with decreased overall survival (5-year overall survival, CRP ≤10 mg/L 74% vs CRP >10 mg/L 58%, HR 1.9, (95% CI 1.1-3.2) p=0.015) (Figure 5.3 A). Low albumin at diagnosis was associated with decreased overall survival (5-year overall survival, albumin <35 49% vs albumin ≥35 77%, HR 0.4, (95% CI 0.2-0.7) p=0.001) (Figure 5.3 B). High mGPS score was associated with

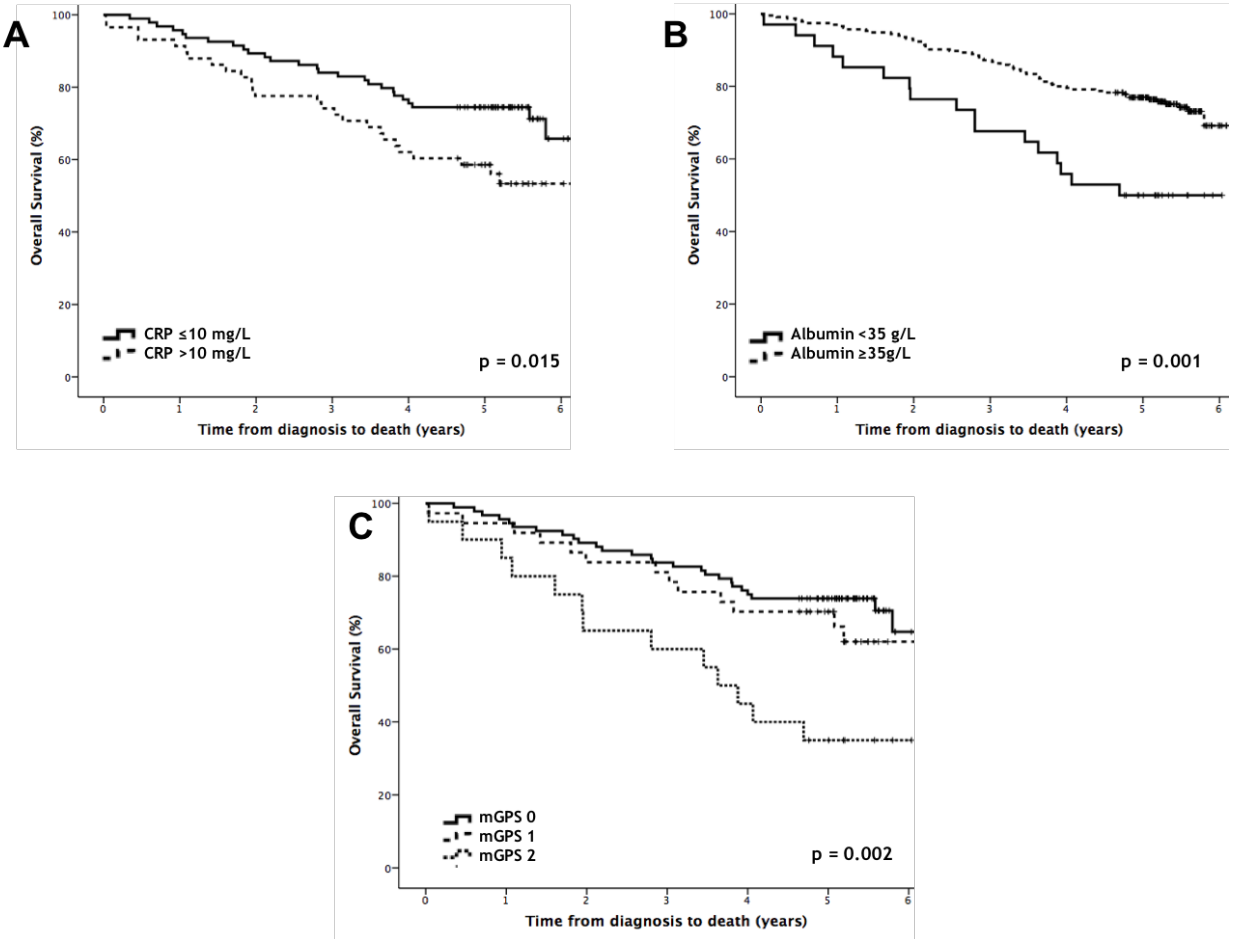
decreased overall survival (5-year overall survival, score 0 74% vs score 1 70% vs score 2 34%) HR 1.8, (95% CI 1.3-2.5) p=0.002) (Figure 5.3 C). Table 5.5 summarises the univariate analysis of inflammatory markers as related to clinical outcome measures.

Table 5.5 Univariate analysis of inflammatory markers as related to clinical outcome measures

	Univariate analysis		
Clinico-pathological parameter	**Time to biochemical relapse**	**Overall survival from biochemical relapse**	**Overall survival**
	P value Hazard Ratio (95% CI)	**P value Hazard Ratio (95% CI)**	**P value Hazard Ratio (95% CI)**
CRP (≤10 mg/L/>10 mg/L)	0.990, 1.0, 0.5-2.1	0.758, 1.2, 0.4-3.4	***0.015, 1.9, 1.1-3.2***
Albumin (<35 g/L/≥35 g/L)	0.155, 0.6, 0.3-1.2	0.940, 1.0, 0.3-3.2	***0.001, 0.4, 0.2-0.7***
mGPS (0/1/2)	0.224, 0.4, 0.8-2.1	0.833, 1.0, 0.5-1.9	***0.002, 1.8, 1.3-2.5***

The clinico-pathological variables were grouped and analysed by Kaplan-Meier methods and Cox regression with reference to time to biochemical relapse from diagnosis, overall survival from biochemical relapse and overall survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Elevated CRP, low albumin and high mGPS are all significantly associated with decreased overall survival. Significant results (p = ≤0.05) are highlighted in red.

Figure 5.3 Kaplan-Meier Graphs showing clinico-pathological parameters as related to clinical outcome measures



A. Kaplan-Meier survival plot showing CRP level at diagnosis as related to overall survival. CRP >10 mg/L at diagnosis (dashed line) is significantly associated with decreased overall survival

B. Kaplan-Meier survival plot showing albumin level at diagnosis as related to overall survival. Albumin <35 g/L at diagnosis (solid line) is significantly associated with decreased overall survival

C. Kaplan-Meier survival plot showing mGPS at diagnosis as related to overall survival. mGPS of 2 at diagnosis (dotted line) is significantly associated with decreased overall survival

5.4.3 Multivariate Analysis of Clinico-pathological factors and mGPS related to overall survival

The mGPS was combined with age at diagnosis, PSA at diagnosis, Gleason score, PNI, percentage of cores positive for cancer and presence of metastases at diagnosis in a multivariate cox regression analysis in relation to overall survival. Gleason score was independently associated with decreased overall survival (p = 0.001, HR 2.59, (95% CI 1.4 – 4.6). The remainder of the clinico-pathological parameters were not found to be independent with regards to overall survival.

5.5 Discussion

This cohort of patients diagnosed with prostate cancer in 2009 represents a modern cohort of prostate cancer patients. As a result, the median follow-up for this cohort is only five years. Due to the short follow-up period, there has been a relatively small number of events. In addition, the cause of death for patients was not available for this cohort. As a result, overall survival as opposed to disease-specific survival has been used as a clinical outcome measure. This makes direct comparison between the discovery cohort difficult, however, it is anticipated that cause of death will be made available in the coming years, and the cohort can be reassessed at that time, when the follow-up period will be longer also.

The clinico-pathological characteristics of this cohort reflects the increased use of the PSA test in general practice. A survey of UK GPs found that 76% of GPs reported performing a PSA test in an asymptomatic patient at least once in the preceding three months.(188) The median age of this cohort was 69.3 years, compared to 70.1 years in the older cohort. PSA at diagnosis is less and most patients are now diagnosed with a Gleason 6 prostate cancer, compared to the older cohort, in which median PSA at diagnosis is higher and most patients had Gleason 8 or higher disease at the time of diagnosis. These findings most likely reflect the increased use of the PSA test in asymptomatic men in general practice in the time period between the two cohorts. A study comparing the use of PSA as a screening tool to opportunistic testing found that men diagnosed with prostate

cancer in the screening cohort were younger and had lower PSA level at diagnosis than compared to those men in the opportunistic cohort. (189) This is in line with other studies, which have shown that age and PSA level at diagnosis has been falling. (190)

Age at diagnosis has been associated with poor outcome in prostate cancer. Several studies have shown that men with advancing age are not only more likely to be diagnosed with high risk disease but also to have decreased overall and disease-specific survival.(191) A possible reason for this is that elderly patients are treated more conservatively. A study of patients in the CaPSURE trial found that older men were more likely to receive ADT as first line management. (192) The current study supports these findings, with increased age at diagnosis associated with decreased overall survival from both diagnosis and biochemical relapse.

Increased PSA and Gleason score have consistently been associated with poor outcome measures in prostate cancer.(193-196) High PSA, Gleason score at diagnosis and the presence of metastases at diagnosis are all associated with decreased time to biochemical relapse and decreased overall survival. In addition, Gleason score was found to be an independent marker of overall survival. These results validate this cohort as a representative cohort of prostate cancer patients that can be utilised for further evaluation of biomarkers.

In addition to the established pathological criteria for risk stratification in localised prostate cancer, a number of other pathological parameters have been suggested to further determine risk. The percentage of positive biopsy cores has been associated with tumour stage and volume, increased risk of disease recurrence following radical treatment and decreased disease-specific survival and overall survival in localised prostate cancer. (197-199) In the current study, patients who were found to have prostate cancer in >50% of the biopsy cores obtained had significantly shorter time to biochemical relapse and decreased overall survival. This adds to the increasing body of evidence suggesting that percentage of positive biopsy cores may have a role in further risk stratification alongside established clinical risk factors.

As discussed in previous chapters, the role of PNI as a negative prognostic marker in prostate cancer has been extensively investigated. Perineural invasion is a pathological finding of cancer cells tracking along a nerve within the perineural space and is thought to aid the development of extra-prostatic extension. A systematic review of the prognostic significant of perineural invasion in prostate cancer biopsies concluded that perineural invasion is an important risk factor for poor outcome in localised disease, but does not inform on outcome in advanced disease. (166) Despite these findings, a survey in 2005 revealed that only 13% of urologists thought that the presence of perineural invasion would alter their management of patients with prostate cancer. (200) In the present study, perineural invasion is significantly associated with increased biochemical relapse and decreased overall survival. This is in contrast to the findings of the discovery cohort. This may reflect the difference in tissue used in each cohort. Tissue in the discovery cohort was obtained from men undergoing TURP or radical prostatectomy, whereas the current cohort consists predominantly of tissue obtained from TRUS biopsy. A study of 105 radical prostatectomies found that there was significant variation in reporting of PNI between pathologists. (175) As such, the Royal College of Pathologists now include reporting on the presence or absence of perineural invasion as a core data item for pathology reports of prostate biopsy. (201)

Systemic inflammation has been suggested as an alternative adjunct to existing risk stratification systems in prostate cancer. Much work has been done investigating the role of systemic inflammation in a variety of cancers, however, there are limited studies of the role of systemic inflammation in prostate cancer. McArdle et al found that elevated CRP was an independent predictor of decreased disease-specific survival in both localised and metastatic prostate cancer. (186, 202) The mGPS score has also been shown to predict survival in prostate cancer. (203) Markers of inflammation were available in the current cohort. It was found that increased CRP and decreased albumin at diagnosis were associated with decreased overall survival. Interestingly, there was no association between time to biochemical relapse and markers of systemic inflammation. CRP and albumin are

cheap and readily available clinical tests that we have shown can inform on outcome in prostate cancer from diagnosis, suggesting that mGPS is a simple, accessible tool that can be used in existing clinical practice.

As discussed earlier, a limitation of this cohort is the relatively short period of follow-up, due to the recent recruitment of patients. Despite limited follow-up, we have demonstrated that PNI and percentage of cores positive can inform on survival. Once data is available for disease-specific survival, it is anticipated that these findings can be further confirmed and support the increasing body of evidence to suggest that these parameters should be included in risk stratification tools at diagnosis.

Whilst we have identified a number of alternative clinico-pathological parameters that can be utilised alongside existing risk stratification systems, we will now go on to investigate the role of molecular markers that could assist in further stratifying patients within existing risk categories.

Chapter 6 Clinical significance of protein expression in validation prostate cancer cohort

One of the major challenges in the management of prostate cancer is identification of patients at diagnosis with aggressive disease that require earlier treatment and those that have indolent disease and can be managed conservatively. With increasing incidence of prostate cancer and rising costs of prostate cancer management, it is imperative that treatment is directed to those patients who will gain the most benefit. As previously discussed, current methods of stratifying patients into risk categories at diagnosis are inadequate and some patients fulfilling low or intermediate-risk criteria at diagnosis will develop aggressive disease.

In the discovery cohort, it was found that cytoplasmic pAR^{S578} was associated with poor outcome measures, predicting shorter time to biochemical relapse, decreased disease-specific survival following biochemical relapse and decreased disease-specific survival from diagnosis. Furthermore, combined high expression of pAR^{S81} and pAR^{S578} was associated with shorter disease-specific survival than compared to high expression of the individual phosphosites alone.

The aim of the current study was to validate the results of the discovery cohort in a larger, modern cohort of prostate cancer patients that is more reflective of today's clinical practice. Disease-specific survival was not available for this cohort, therefore study outcome measures in this study were time to biochemical relapse and overall survival.

6.1 Protein expression analysis

243 patients had tissue available for IHC. Protein expression in the nucleus and cytoplasm was recorded in epithelial tumour cells only. Unfortunately, due to technical difficulties and time constraints, protein expression of each antibody could not be performed for all patients in the cohort. Expression of all proteins was heterogeneous throughout the cells.

Protein expression in the specimens was divided into low (≤median) and high (>median) for purposes of analysis (Table 6.1). All ICCC values were ≥0.80.

Table 6.1 Protein expression in patients with tissue available

	Patients, n (%)	Median Histoscore (Histoscore units)	IQR (Histoscore units)
AR Nuclear (low/high)	109 (44.9)/ 106 (43.6)	153	120 – 185
AR Cytoplasmic (low/high)	111 (45.7)/ 104 (42.8)	83	40 - 100
pAR^{S81} Nuclear (low/high)	98 (40.3)/ 87 (35.8)	170	131 – 200
pAR^{S81} cytoplasmic (low/high)	95 (39.1)/ 90 (37.0)	100	100 – 120
pAR^{S578} Nuclear (low/high)	61 (15.9)/ 57 (14.8)	127	102 – 154
pAR ^{S578} Cytoplasmic (low/high)	61 (15.9)/ 57 (14.8)	130	107 – 163
PKC Nuclear (low/high)	41 (10.7)/ 41 (10.7)	66	16 – 114
PKC Cytoplasmic (low/high)	45 (11.7)/ 37 (9.6)	103	55 - 124

The median histoscore for each protein of interest was calculated. Patients were grouped into low (≤median) and high expression (>median). The number of patients in each group is displayed for each protein.
Number of patients with missing data is not displayed. Values that do not give a sum of 100% is due to tissue being unavailable

6.2 Association between PKC expression and expression of pAR^{S578} in clinical specimens

In the clinical specimens, nuclear PKC expression was significantly associated with pAR^{S578} expression in both the nucleus (c.c. 0.377, p=0.013) and cytoplasm (c.c. 0.378, p=0.012). There was no association between cytoplasmic PKC and pAR^{S578} expression. Table 6.2 demonstrates the associations between PKC and pAR^{S578}.

Table 6.2 Association between PKC expression and expression of pAR^{S578} at both cellular locations in the clinical specimen

	PKC	
	Nuclear	**Cytoplasmic**
	p value, C.C	**p value, C.C**
pAR578 Nuclear	***0.013, 0.377***	0.154, 0.221
pAR578 Cytoplasmic	***0.012, 0.378***	0.069, 0.280

Pearson's correlation coefficient was used to assess the association between PKC expression and expression of pAR^{S578} in the nucleus and cytoplasm. Expression of nuclear PKC was significantly associated with pAR^{S578} expression at both locations. C.C. denotes Pearson's correlation co-efficient. Values highlighted in red denotes associations with a p value < 0.05

6.3 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinico-pathological parameters

Expression of nuclear pAR^{S578} is significantly associated with age at diagnosis (p = 0.034). The expression of cytoplasmic pAR^{S578}, pAR^{S81} and PKC did not correlate with any clinico-pathological parameters. Table 6.3 demonstrates the expression of pAR^{S81}, pAR^{S578} and PKC as related to clinico-pathological parameters.

Table 6.3 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinico-pathological parameters

	Nuclear AR ^{S81}			Cytoplasmic AR ^{S81}			Nuclear AR ^{S578}			Cytoplasmic AR ^{S578}			Nuclear PKC			Cytoplasmic PKC		
	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value	Low expression	High expression	p-value
Age (<70/>70)	43/50	44/35	0.218	43/46	44/39	0.539	***22/38***	***29/22***	***0.034***	22/35	29/25	0.112	18/21	13/24	0.332	16/27	15/18	0.471
Gleason (<7/7/>7)	33/34	21/33	0.708	34/33/19	20/34/24	0.060	20/24/14	12/21/17	0.169	16/24/14	16/21/17	0.707	8/18/10	9/17/11	0.993	11/18/12	6/17/9	0.685
Diagnosis PSA (<10/10-20/>20)	31/19/43	24/18/37	0.791	33/17/38	22/20/42	0.175	13/15/31	17/8/27	0.489	14/13/29	16/10/29	0.847	10/9/20	4/10/23	0.152	8/9/26	6/10/17	0.638
PNI (no/yes)	50/41	33/35	0.424	49/36	34/40	0.142	34/23	27/21	0.726	30/23	31/21	0.756	13/23	20/17	0.126	18/23	15/17	0.801
% cores positive for malignancy (<50/≥50)	26/42	20/36	0.773	27/37	19/41	0.227	18/31	13/27	0.678	17/28	14/30	0.557	7/21	6/21	0.810	6/26	7/16	0.319
Metastases (no/yes)	71/14	58/10	0.766	67/13	62/11	0.841	49/6	38/7	0.494	46/5	41/8	0.335	27/6	30/5	0.665	31/7	26/4	0.574

Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was examined for significant relationships with clinical variables as shown. Protein expression was divided into high and low groups. Clinical variables were divided into groups and the Mann-Whitney U test was performed for statistical analysis. Expression of nuclear pAR^{S578} is significantly associated with age at diagnosis. Statistically significant (p = ≤0.05) results are highlighted in red.

6.4 Expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures

Table 6.4 shows the univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures.

Table 6.4 Univariate analysis of expression of pAR^{S81}, pAR^{S578} and PKC related to clinical outcome measures

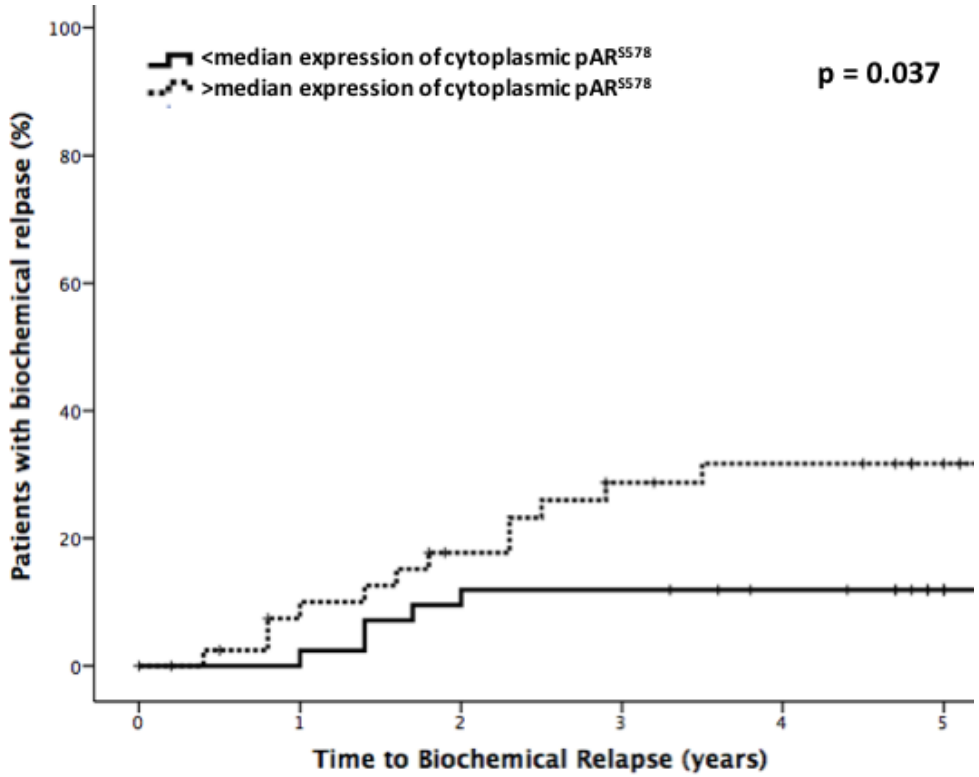
	Univariate analysis	
Protein expression (<median/>median)	**Time to biochemical relapse** **P value Hazard Ratio (95% CI)**	**Overall survival** **P value Hazard Ratio (95% CI)**
pAR^{S81} Nuclear	0.756, 1.1, 0.5-2.3	0.261, 1.3, 0.8-2.2
pAR^{S81} Cytoplasmic	0.647, 1.2, 0.6-2.4	0.136, 1.5, 0.9-2.4
pAR^{S578} Nuclear	0.135, 2.0, 0.8-5.4	0.689, 1.1, 0.6-2.1
pAR ^{S578} Cytoplasmic	***0.037, 2.9, 1.0-8.2***	0.076, 1.8, 0.9-3.2
PKC Nuclear	0.910, 1.1, 0.4-3.2	0.285, 1.4, 0.7-2.7
PKC Cytoplasmic	0.412, 1.6, 0.5-4.7	0.966, 1.0, 0.5-1.9

Expression of pAR^{S81}, pAR^{S578} and PKC in the nucleus and cytoplasm was analysed by Kaplan-Meier methods and Cox regression in relation to time to biochemical relapse from diagnosis, overall survival from biochemical relapse and overall survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Protein expression was divided into high and low groups by the median value. High expression of cytoplasmic pAR^{S578} was associated with shorter time to biochemical relapse. Significant results (p = ≤0.05) are highlighted in red.

High expression of cytoplasmic pAR^{S578} was associated with shorter time to biochemical relapse (proportion of patients relapsed at 5 years, <median expression 12% vs >median expression 32%, HR 2.9 (95% CI 1.0-8.2), p=0.037)

(Figure 6.1). No association was observed between pAR^{S81} or PKC and time to biochemical relapse.

Figure 6.1 Kaplan Meier Graph showing expression of pAR^{S578} related to time to biochemical relapse



Kaplan-Meier survival plot showing expression of cytoplasmic pAR^{S578} as related to time to biochemical relapse. High cytoplasmic pAR^{S578} (dashed line) is significantly associated with decreased time to biochemical relapse

High expression of cytoplasmic pAR^{S578} trended towards an association with decreased overall survival but did not reach clinical significance (p = 0.076). No association was observed between pAR^{S81} or PKC and overall survival.

Due to the short follow-up period and limited number of patients with IHC performed for pAR^{S81} and pAR^{S578}, overall survival following biochemical relapse as related to protein expression could not be calculated in this cohort.

6.5 Expression of dual phosphorylation sites on the androgen receptor related to outcome measures

The two phosphorylation sites were combined as follows: (i) high pAR^{S81} and high pAR^{S578}, (ii) high pAR^{S81} or high pAR^{S578} and (iii) low pAR^{S81} and low pAR^{S578} expression.

High cytoplasmic pAR^{S81} and cytoplasmic pAR^{S578} was associated with decreased overall survival (5-year survival, high expression of both proteins 48% vs low expression of both proteins 84%, HR 3.5, (95% CI 1.5-8.6) p=0.003) (Figure 6.2 A). High nuclear pAR^{S81} and nuclear pAR^{S578} was associated with decreased overall survival (5-year survival, high expression of both proteins 58% vs low expression of both proteins 89%, HR 3.6, (95% CI 1.2-11.4) p=0.016) (Figure 6.2 B).

Lastly, the expression of total cytoplasmic pAR^{S81} and pAR^{S578} and total nuclear pAR^{S81} and pAR^{S578} was investigated in relation to clinical outcome measures. Patients were grouped as follows: (i) high total cytoplasmic expression of pAR^{S81} and pAR^{S578} and high total nuclear expression of pAR^{S81} and pAR^{S578}, and (ii) low total cytoplasmic expression of pAR^{S81} and pAR^{S578} and low total nuclear expression of pAR^{S81} and pAR^{S578}. Combined high expression of total cytoplasmic pAR^{S81} and pAR^{S578} and high total nuclear expression of pAR^{S81} and pAR^{S578} was associated with overall survival (5-year survival, high total expression of both proteins 53% vs low total expression of both proteins 88%, HR 4.0, (95% CI 1.0-15.3) p=0.027) (Figure 6.2 C).

There was no association between combined expression of pAR^{S81} and pAR^{S578} in relation to time to biochemical relapse.

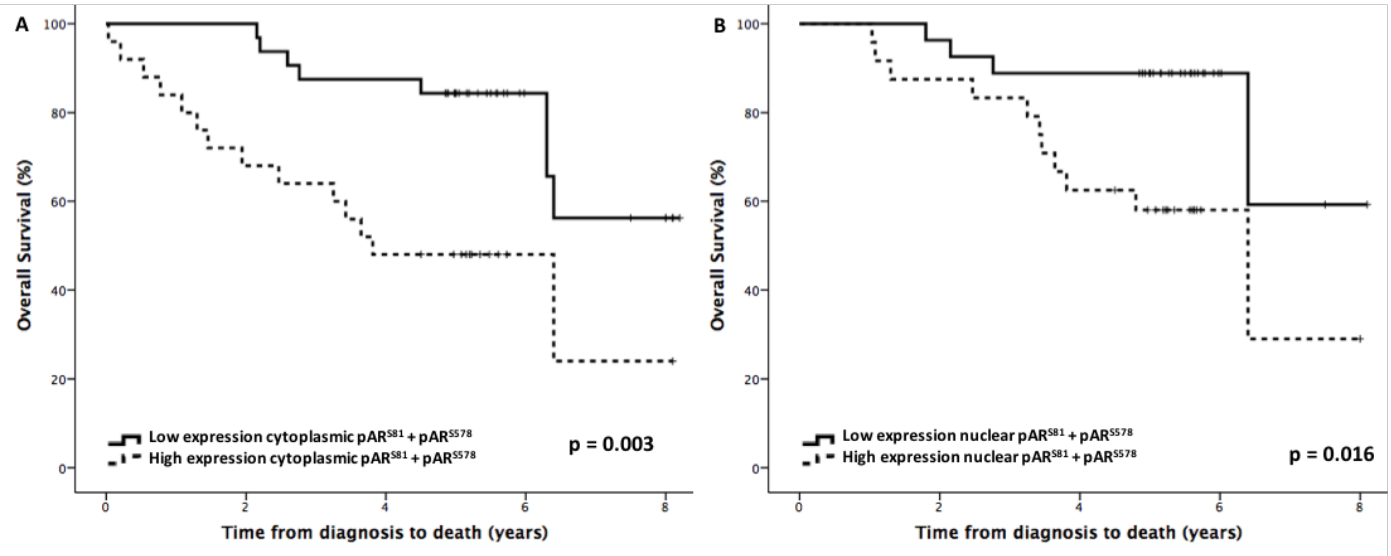
Table 6.5 shows the univariate analysis of dual phosphorylation sites as related to clinical outcome measures.

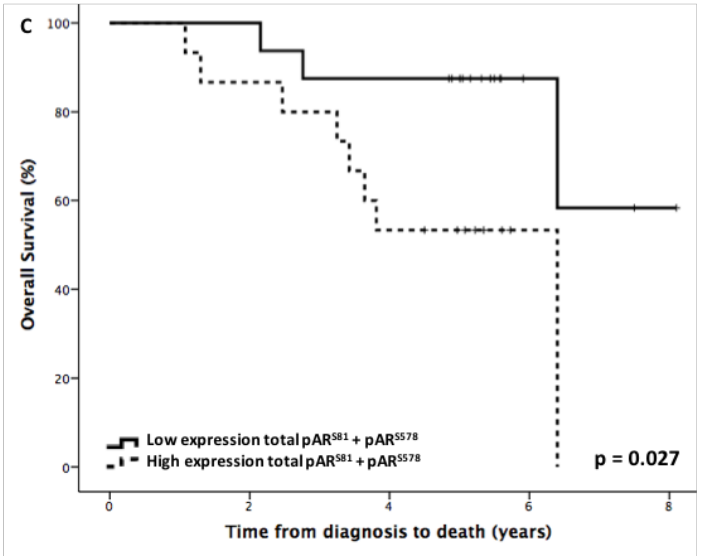
Table 6.5 Univariate analysis of expression of dual expression of pAR^{S81} and pAR^{S578} related to clinical outcome measures

	Univariate analysis	
	Time to biochemical relapse **P value Hazard Ratio (95% CI)**	**Overall survival** **P value Hazard Ratio (95% CI)**
pAR^{S578} cytoplasmic + pAR^{S81} cytoplasmic	0.624, 1.4, 0.4-5.7	***0.003, 3.5, 1.5-8.6***
pAR^{S578} nuclear + pAR^{S81} nuclear	0.654, 1.4, 0.3-5.5	***0.016, 3.6, 1.2-11.4***
Total cytoplasmic pAR^{S81} and pAR^{S578} + total nuclear pAR^{S81} and pAR^{S578}	0.607, 1.5, 0.3-6.6	***0.027, 4.0, 1.0-15.3***

Dual expression of pAR^{S81} and pAR^{S578} was analysed by Kaplan-Meier methods and Cox regression in relation to time to biochemical relapse from diagnosis, overall survival from biochemical relapse and overall survival. Patients were considered to have biochemical relapse with serum PSA >0.2 ng/ml following radical prostatectomy, serum PSA of 2.0 ng/ml above the post treatment nadir level following radical radiotherapy or 2-3 consecutive elevations of serum PSA above the nadir over intervals greater than 2 weeks following hormone treatment. Protein expression was divided into high and low groups. High dual expression of pAR^{S81} and pAR^{S578} is associated with decreased overall survival than compared to low expression of both phosphosites in all three groupings. Significant results ($p = \leq 0.05$) are highlighted in red.

Figure 6.2 Kaplan Meier Graphs showing dual expression of pAR^{S81} and pAR^{S578} as related to overall survival





A. *Kaplan Meier survival plot showing overall survival in patients with low expression of both cytoplasmic pARS81 and pARS578 (solid line) and high expression of both cytoplasmic pARS81 and pARS578 (dashed line). High expression of both cytoplasmic pARS81 and pARS578 is associated with decreased overall survival than compared low expression of both phosphosites*

B. *Kaplan Meier survival plot showing overall survival in patients with low expression of both nuclear pAR^{S81} and pAR^{S578} (solid line) and high expression of both nuclear pAR^{S81} and pAR^{S578} (dashed line). High expression of both nuclear pAR^{S81} and pAR^{S578} is associated with decreased overall survival than compared low expression of both phosphosites*

C. *Kaplan Meier survival plot showing overall survival in patients with low expression of total pAR^{S81} and pAR^{S578} (solid line) and high expression of total pAR^{S81} and pAR^{S578} (dashed line). High total expression of pAR^{S81} and pAR^{S578} is associated with decreased overall survival than compared to low total expression of pAR^{S81} and pAR^{S578}*

6.6 Discussion

Androgen receptor phosphorylation status has been investigated in this study in relation to clinico-pathological parameters and clinical outcome measures with a view to verification of the results of the discovery cohort in Chapter 4.

Protein expression in the validation cohort differs to that observed in the discovery cohort. The median expression of cytoplasmic pAR^{S578} and PKC was slightly higher than nuclear expression for both proteins, which is the inverse pattern to that observed in the discovery cohort but similar to that observed in the active surveillance cohort. Furthermore, median expression of all proteins observed in the validation cohort was higher than that in the discovery cohort, and similar to that in the active surveillance cohort. A possible reason for this may be the age of the tissue utilised in this study. The TMA for the discovery cohort was constructed several years ago and cut slides had been stored for a couple of years prior to use, whereas the tissue utilised in the AS and validation cohorts had been cut and utilised within a much shorter time period. Despite optimum storage conditions, pre-cut slides are exposed to ambient changes in humidity and temperature, which can lead to protein degradation through oxidation. (204) A recent study of archival breast tissue found the average antigenicity signal from detected biomarkers decreased with increasing age of the tissue specimen. (205) The varying expression patterns observed between the cohorts are in keeping with this study. Together these results suggest that an adjustment for tissue age may be required when comparing results from studies using archival tissue of varying age.

Nuclear PKC expression was significantly associated with pAR^{S578} expression in this cohort. These results taken with those of the AS and discovery cohorts supports PKC being the putative kinase for AR phosphorylation at Ser-578.

Androgen receptor phosphorylation at Ser-578 in the AS and discovery cohorts was associated with markers of high-risk disease. These findings could not be verified in the current cohort and may be due to the small number of patients available for analysis in the validation cohort. Surprisingly, in the current cohort there was a

significant association high expression of pAR^{S578} and younger age at diagnosis. This finding was not observed in the AS or validation cohort and the relevance of this is unclear. Further analysis should be performed once staining is complete for this cohort to verify the results of the earlier cohorts.

As discussed earlier, phosphorylation status of the AR at a number of serine residues has been associated with clinical outcome measures. (124, 126, 128) High expression of cytoplasmic pAR^{S578} was associated with shorter time to biochemical relapse and trended towards an association with decreased overall survival, supporting the findings of the discovery cohort. It may be that the use of overall survival as an outcome measure in this cohort as opposed to disease-specific survival may be masking the true significance of pAR^{S578} expression as a prognostic biomarker in prostate cancer. This is particularly relevant in prostate cancer patients, who are generally an older population in whom death is more likely from all causes. This may reduce the sensitivity of pAR^{S578} expression in identifying those patients with high-risk prostate cancer. Despite this, these findings clearly demonstrate that phosphorylation status of AR at Ser-578 is of clinical relevance in prostate cancer patients at diagnosis. Completion of IHC staining in this cohort should be conducted to confirm these results and should be validated in a multi-centre prospective cohort before recommending this biomarker for use in clinical practice.

The prognostic significance of dual expression of pAR^{S81} and pAR^{S578} was investigated in the validation cohort. High dual expression of pAR^{S81} and pAR^{S578} was significantly associated with decreased overall survival. This result is particularly striking when neither phosphorylation site was associated with overall survival when assessed independently. These results lend further support to the findings of the AS and discovery cohorts, suggesting a synergistic effect when these two phosphorylation sites are combined in relation to predicting poor outcome in prostate cancer patients. Furthermore, these findings suggest that there is a sub-population of prostate cancer patients who may benefit from dual targeted therapy with ADT and PKC inhibitors. Future work should include cell line studies

to assess the response of patients to ADT and PKC inhibitors in relation to pAR^{S81} and pAR^{S578} expression.

A number of limitations have been identified in this study. Unfortunately, due to time constraints, only a limited number of patients had complete staining for all antibodies of interest. This work should be completed and reanalysed to verify the preliminary results in this cohort. This cohort has a relatively short follow-up period and therefore only a small number of events occurred, which is unsurprising given the long natural history of prostate cancer. Despite these confounding factors, it has still been possible to demonstrate that androgen receptor phosphorylation status is relevant to clinical outcomes in prostate cancer patients.

Chapter 7 Isolation, propagation and characterisation of primary prostate cancer epithelial cell cultures from prostate specimens

Currently, the majority of *in vitro* prostate cancer research utilises established prostate cancer cell lines, the most common of which are LNCaP, PC-3 and DU145 cells. (206) Each cell line demonstrates specific characteristics such as AR expression and responsiveness to androgens, which corresponds to the clinical characteristics of the patient from whom these cell lines were originally developed. Many cell lines are derived from metastatic sites, indicating an advanced phenotype. (207) Furthermore, it has been suggested that prostate cancer cell lines are often genetically altered by their non-physiological *in vitro* conditions and thus are not truly representative of prostate cancer patients.

Primary prostate cell culture allows the development of a wide phenotypic range of prostate cancer cells. Due to the short culture period in many described techniques, these primary cells are thought to more accurately reflect the characteristics of the patients from whom the cells were derived. Not only can primary prostate cell cultures provide models for development of new drug treatments, but may also be utilised in personalised medicine to aid in the identification of biomarkers to predict which patients will respond best to specific treatments.

In this study, we develop a technique for isolation, propagation and characterisation of primary prostate cancer cells in 2-D culture from prostate specimens.

7.1 Establishing method for isolation and propagation of primary prostate cells

Over a one year period, 36 clinical specimens were collected via TRUS biopsy of the prostate and prepared for culture. Several problems were encountered with culturing primary prostate epithelial cells including infection, no/poor growth of epithelial cells and overgrowth of fibroblasts. A number of changes were made to the culture method including the removal of fetal bovine serum from the growth medium, minimising the use of Matrigel in cell culture flasks and refining

the quantities of growth factors included in the culture medium before successfully culturing two patient samples, T288 and T290.

7.2 Patient Characteristics

Two cell cultures were propagated from two patients attending a TRUS biopsy list at Glasgow Royal Infirmary in 2015. The patient characteristics are summarised in Table 7.1.

The T288 cell culture was obtained from a 56-year-old patient undergoing investigation from prostate cancer due to an elevated PSA of 12.3 ng/ml. Pathological examination of the other sampled cores obtained at the same time revealed a Gleason 3+3 adenocarcinoma of the prostate in 20% of the cores sampled, with less than 2% of the cores positive for malignancy. The patient went on to have a radical prostatectomy and was confirmed to have a Gleason 3+3 tumour.

The T290 cell culture was obtained from a 64 year-old patient with a known prostate cancer, having being diagnosed with a Gleason 3+3 adenocarcinoma of the prostate on a TURP in 2009. The patient was on AS and previous biopsy in 2012 was negative. Pathological examination of the biopsy cores revealed a benign result.

Table 7.1 Patient characteristics of primary prostate cell cultures

	Patients	
Clinical Parameter	**T288**	**T290**
Age at biopsy	56	64
PSA at biopsy (ng/ml)	12.3	0.6
Pathology	Adenocarcinoma	Benign
Gleason	3+3=6	-
% cores positive for malignancy	20%	-
Total tissue malignant	<2%	-
HGPIN	Yes	No
PNI	No	-
Extra-prostatic extension	No	-

Comparison of the clinico-pathological characteristics of the two patients included in this study.

7.3 Flow Cytometry

CK-18, a luminal epithelial cell marker, was used to confirm the presence of luminal epithelial cells in the cell culture. CD-90, is a cell surface glycoprotein expressed on stem cells and non-lymphoid tissues including fibroblasts, neurons and activated epithelial cells but not luminal epithelial cells.

Flow cytometry was performed for T288 and T290 cell cultures. DAPI confirmed that 97% of cells in T288 cell culture and 94% of cells in the T290 cell culture were alive at time of analysis. Only live cells were analysed for expression of CK-18 and CD-90. In both cell cultures, the majority of the primary prostate cells expressed CK-18 and did not express CD-90. This suggests that most cells cultured by this method were luminal prostate epithelial cells. In each cell culture, a small number of cells expressed only CD-90, suggesting there was a small sub-population of fibroblasts. Table 7.2 demonstrates the expression pattern in both cell lines.

Table 7.2 Flow cytometry results for primary prostate cell cultures

	T288	T290
Total cells analysed	32254	32103
Alive (%)	97.0	93.6
CK18+/ CD90- (%)	84.3	58
CK18-/ CD90+ (%)	0.3	1.9
CK18+/ CD90+ (%)	9.7	29.5
CK18-/ CD90- (%)	5.6	10.6

Flow cytometry was used to investigate the expression pattern on CK-18 and CD90 in the primary prostate cell cultures. Only live cells were analysed for expression of the proteins of interest.

7.4 Gene Expression in Primary Prostate Cell cultures

A panel of genes were selected in order to confirm the presence of prostate epithelial cancer cells in the primary cultures. The panel of genes tested include AR (androgen receptor), FASN (fatty acid synthase), KLK-3 (kallikrein related peptidase 3), GOLM1 (golgi membrane protein 1) and AMACR (alpha-methylacyl-CoA racemase). Table 7.3 summarises the functions of the gene and typical expression pattern in prostate cancer.

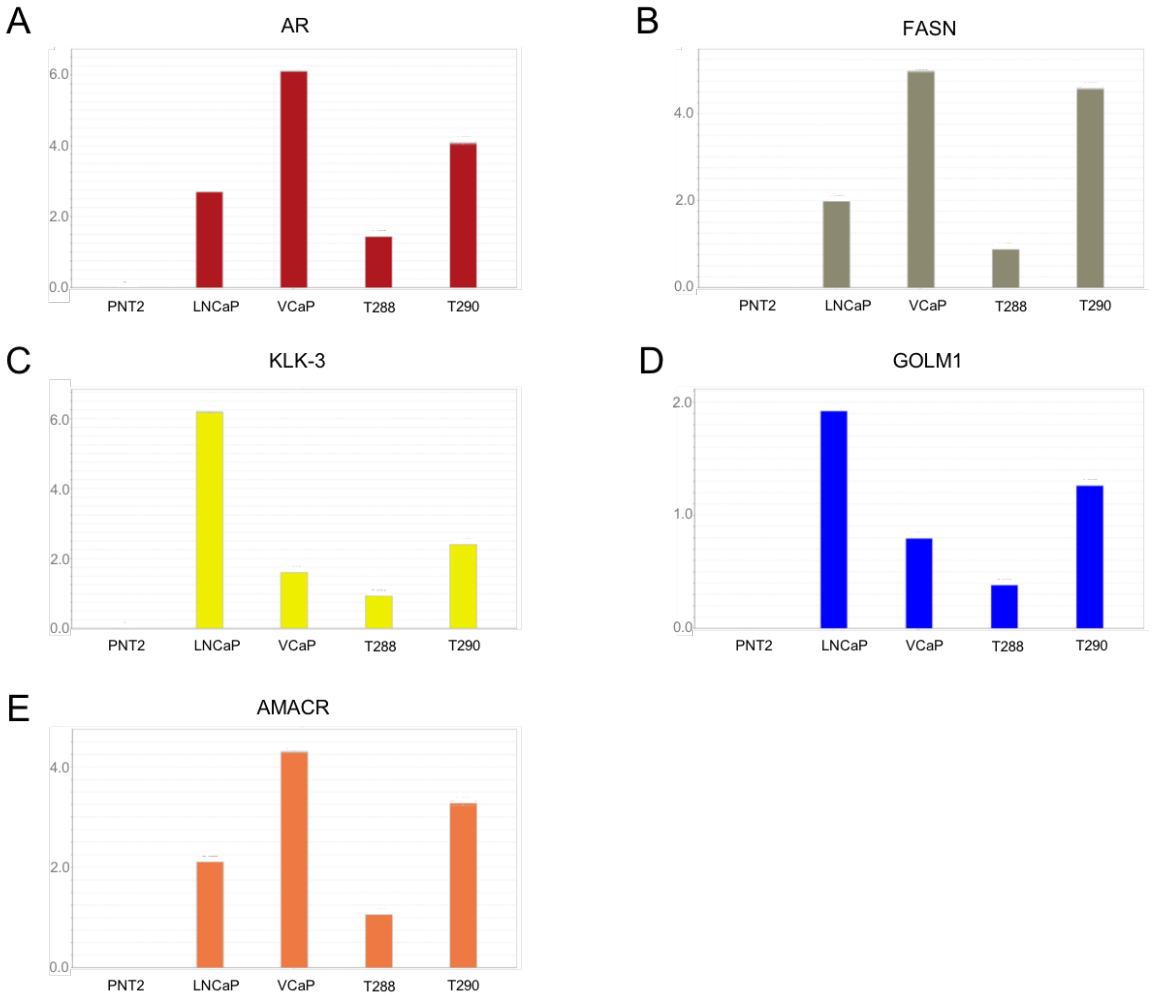
Table 7.3 Gene expression in prostate cancer

Gene	Function	Expression in prostate cancer
AR	Produces AR protein	Increased
FASN	Produces fatty acid synthase	Increased
KLK-3	Produces PSA	No significant correlation
GOLM1	Produces golgi membrane protein 1	Increased
AMACR	Produces alpha-methylacyl-CoA racemase	Increased

The function and expression pattern of the genes of interest in prostate cancer are detailed.

Levels of mRNA were normalised to those obtained for the housekeeping gene ActB. The comparative (ΔΔCt) method was used to quantify relative gene expression in two established prostate cancer cell lines (LNCaP and VCaP) and the two primary prostate cell cultures compared to a control sample of an established benign prostate cell line, PNT2. Results are presented in fold change in the target gene relative to ActB endogenous control. A fold change of 1.5 compared to the control was considered a relevant change in expression. Expression of mRNA for all genes of interest from both the prostate cancer cell lines and primary prostate cell cultures was increased compared to the benign control cell line (Figure 7.2).

Figure 7.2 Expression of mRNA in malignant cell lines compared to benign control cell line



The comparative (ΔΔCt) method was used to quantify relative gene expression in two established prostate cancer cell lines (LNCaP and VCaP) and the two primary prostate cell cultures *compared to a control sample of an established benign prostate cell line, PNT2. Results are presented in fold change in the target gene relative to ActB endogenous control. A 1.5-fold increase in gene expression was considered to be relevant.*

AR mRNA expression was highest in the VCaP cell line, where expression was increased by 6.1 fold compared to the benign cell line. In the LNCaP cell line, expression was increased by 2.7 fold. Expression was increased by 4.1 fold in the T290 cell culture and there was a non-relevant increase of 1.4 fold in the T288 cell culture (Figure 7.2 A).

FASN mRNA expression was highest in the VCaP cell line, where expression was increased by 5.0 fold compared to PNT2. Expression was increased by 4.6 fold in the T290 cell culture and there was a non-relevant increase in expression of 0.9 fold in the T288 cell culture (Figure 7.2 B).

KLK-3 mRNA expression was highest in the LNCaP cell line, where expression was increased by 6.2 fold. Expression was increased by 2.3 fold in the T290 cell culture. There was an increase in expression of 1.5 fold in the VCaP cell line and a non-relevant increase of 0.9 fold in the T288 cell culture (Figure 7.2 C).

GOLM1 mRNA expression was increased in all cell lines compared to control, however, only LNCaP cells showed a relevant increased fold change (1.9) compared to control (Figure 7.2 D).

AMACR mRNA expression was highest in the VCaP cell line, where expression was increased by 4.3 fold compared to the benign cell line. In the LNCaP cell line, expression was increased by 2.1 fold. Expression was increased by 3.3 fold in the T290 cell culture and there was a non-relevant increase of 1.1 fold in the T288 cell culture (Figure 7.2 E).

Table 7.4 summarises the fold change in mRNA expression in each cell line compared to the benign control cell line.

Table 7.4 Fold change in expression of mRNA in malignant cell lines compared to benign control cell line

	Gene				
Cell line	**AR**	**FASN**	**KLK-3**	**GOLM1**	**AMACR**
LNCaP	***2.7***	***2.0***	***6.2***	***1.9***	***2.1***
VCaP	***6.1***	***5.0***	***1.5***	0.8	***4.3***
T288	1.4	0.9	0.9	0.4	1.1
T290	***4.1***	***4.6***	***2.3***	1.3	***3.3***

The comparative (ΔΔCt) method was used to quantify relative gene expression in two established prostate cancer cell lines (LNCaP and VCaP) and the two primary prostate cell cultures compared to a control sample of an established benign prostate cell line, PNT2. Results are presented in fold change in the target gene relative to ActB endogenous control. A 1.5-fold increase in gene expression was considered to be relevant. Relevant results are highlighted in red.

7.5 Protein expression in primary prostate cell cultures

Protein expression of AR, PSA and pAR^{S578} was assessed in the primary prostate cell cultures, T288 and T290, using IF as shown in Figure 7.3.

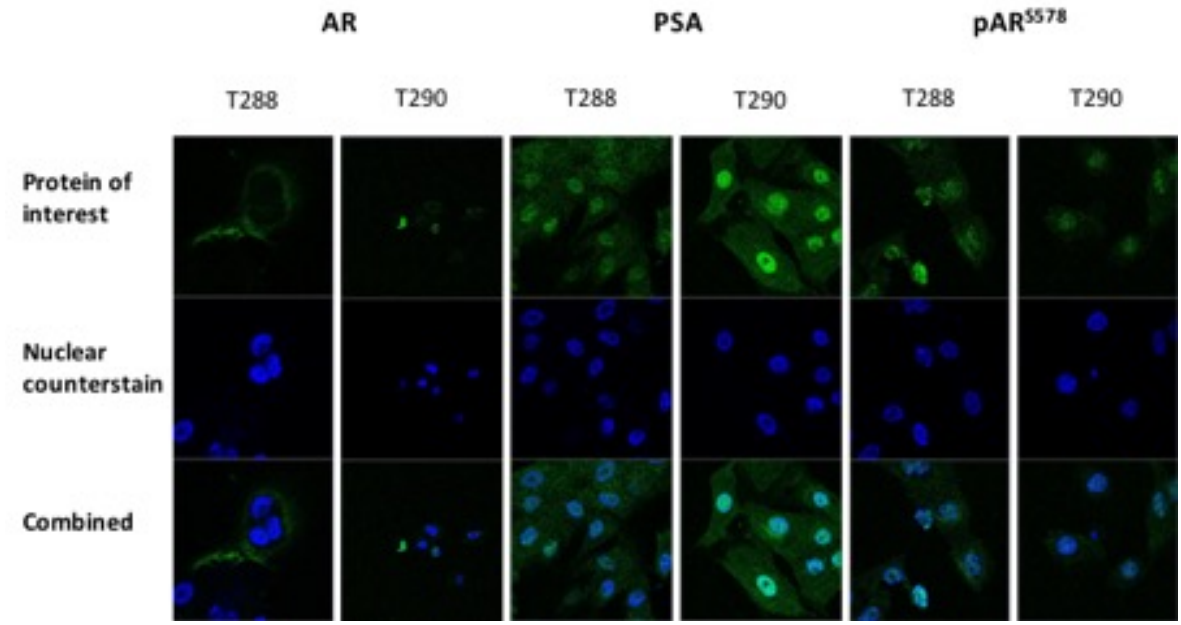
AR was expressed in both T288 and T290 cell cultures. In the T288 cell culture, expression was predominantly cytoplasmic, whereas in the T290 cell culture, expression was predominantly nuclear.

PSA is a serine protease expressed by both benign and malignant epithelial cells in the prostate. Expression of PSA is regulated by the AR, and is expressed in response to androgens activating the AR. PSA expression was demonstrated in both the nucleus and cytoplasm of both primary prostate cell cultures, thus confirming that AR is functionally active in both cell cultures.

As the main phosphorylation site of interest on the AR in this body of research has been Ser-578, the expression of pAR^{S578} was assessed in the two primary prostate cell cultures. Expression of pAR^{S578} was identified in both the nucleus

and cytoplasm in both cell cultures. Intensity of staining was greatest in the T288 cell culture compared to T290.

Figure 7.3 Protein expression of AR, PSA and pAR^{S578} in primary prostate cell cultures



IF confirms the presence of AR, which is demonstrated to be functional by the presence of PSA expression. The protein of interest, pAR^{S578}, is also shown to be expressed by both primary cell cultures.

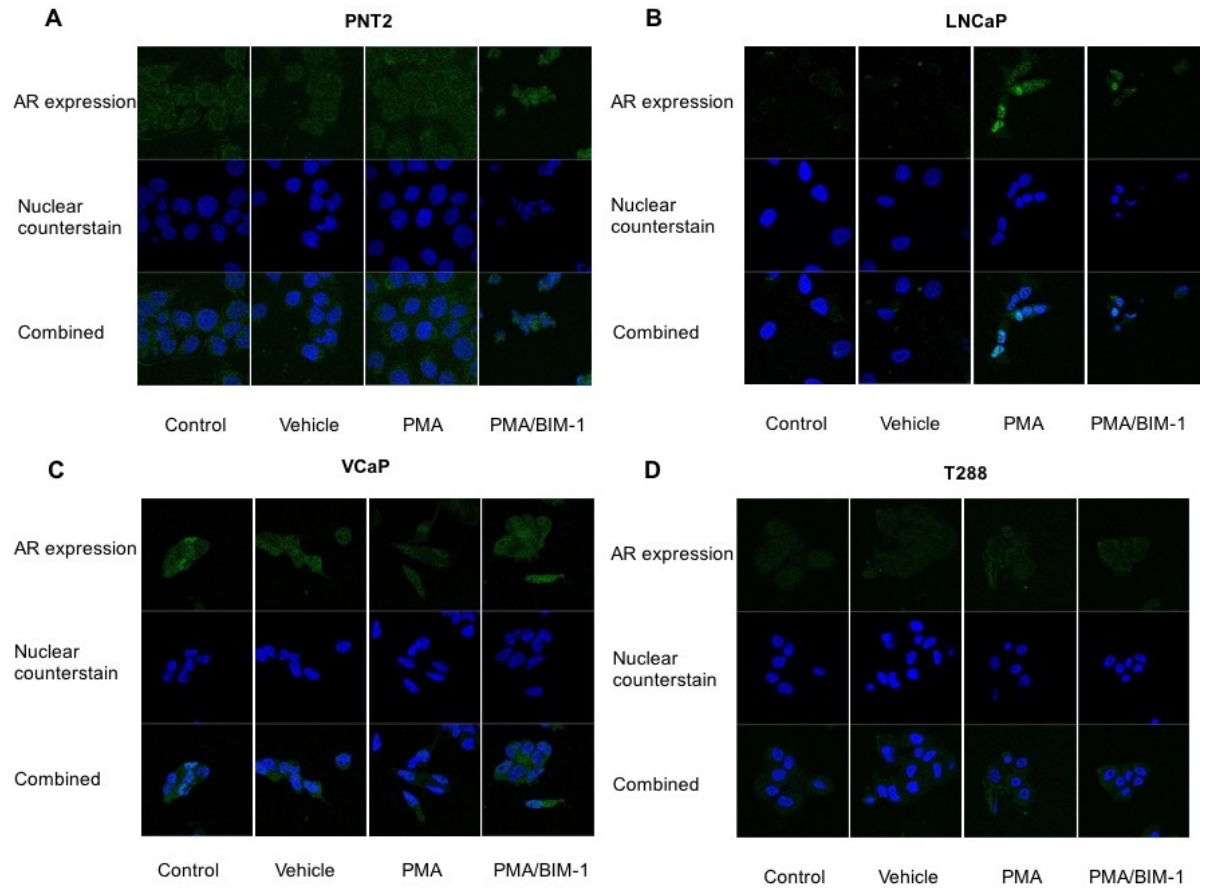
7.6 Effect of PKC inhibitors on AR protein expression in prostate cell cultures

As shown in Chapter 4, high expression of pAR^{S578} is associated with poor outcome measures in hormone-naïve prostate cancer (Figure 4.2). PKC is the predicted kinase responsible for phosphorylation of the AR as serine 578. Furthermore, PKC expression is significantly associated with pAR^{S578} expression in prostate cancer tissue (Table 3.3, 4.3 and 6.2). The effect of PMA, a PKC activator, and BIM-1, a highly selective, reversible inhibitor of PKC, on expression of AR and pAR^{S578} was therefore investigated.

Expression of AR was increased in the nucleus and cytoplasm of LNCaP cells and in the cytoplasm of T288 cells upon treatment with PMA (Figure 7.4B and 7.4D). No discernible difference was noted in the PNT2 and VCaP cells (Figure 7.4A and

7.4C). On treatment with BIM-1 following stimulation with PMA, there was increased nuclear localisation of the AR in the PNT2 and VCAP cells (Figure 7.4A and 7.4C). There was decreased expression of AR in LNCaP cells when treated with PMA and BIM-1 than compared to PMA alone (Figure 7.4B). No difference was observed in the T288 cells treated with PMA and BIM-1 than compared to PMA alone (Figure 7.4D).

Figure 7.4 Expression of AR in prostate cell cultures following treatment with PMA and BIM-1



Cells were incubated in the presence or absence of 10μM BIM-1 for one hour, followed by stimulation with 10 nM PMA for one hour. IF was perfomed to assess expression of AR in each cell type.

A. *PNT2 cells showed no change in expression of AR upon treatment with PMA. On treatment with BIM-1 following stimulation with PMA, there was increased nuclear localisation of the AR.*

B. *LNCaP cells showed increased expression of AR in both the nucleus and cytoplasm upon treatment with PMA. On treatment with BIM-1 following stimulation with PMA, there was decreased expression of AR than compared to PMA alone.*

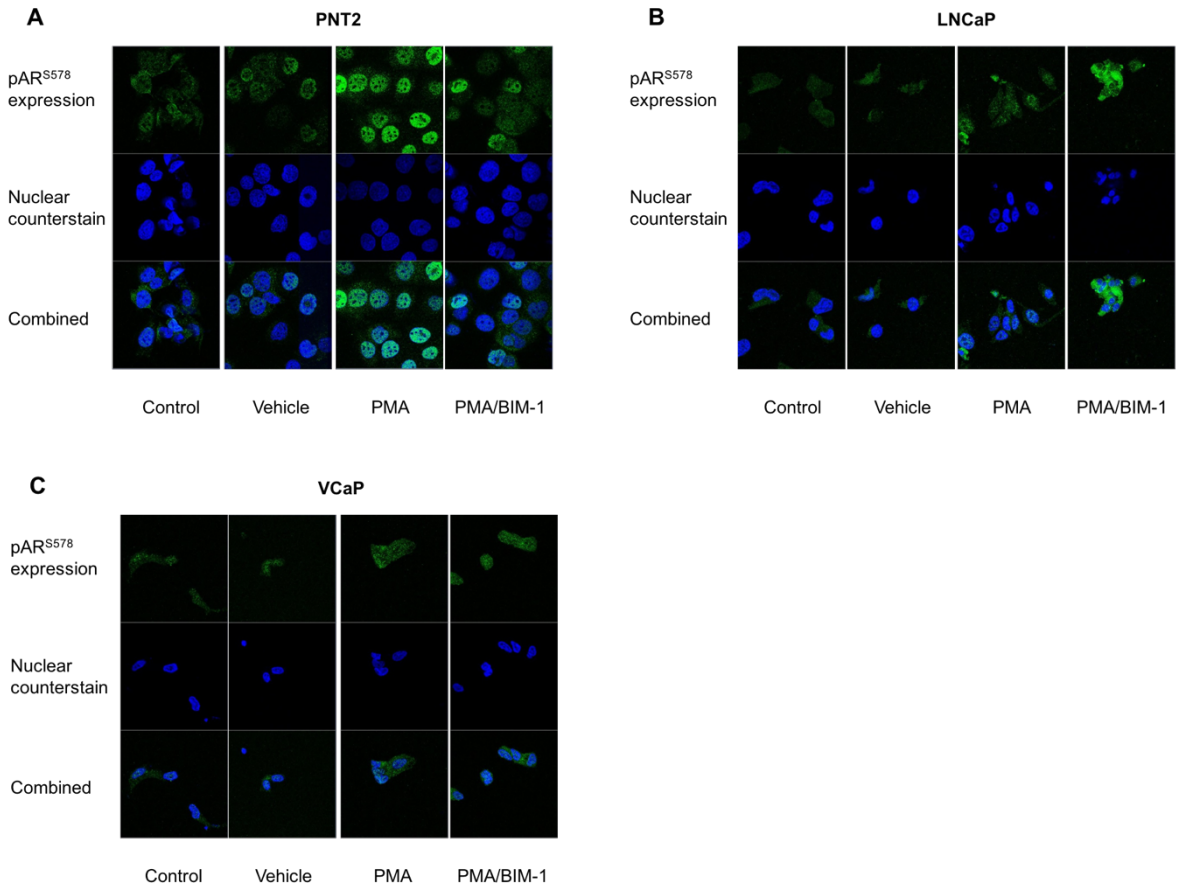
C. *VCaP cells showed no change in expression of AR upon treatment with PMA. On treatment with BIM-1 following stimulation with PMA, there was increased nuclear localisation of the AR.*

D. *T288 cells showed increased cytoplasmic expression of AR upon treatment with PMA. No difference was observed in the cells treated with PMA and BIM-1 than compared to PMA alone.*

Expression of pAR^{S578} was assessed in the established cell lines only. Unfortunately, the T288 cell culture stopped growing prior to completion of this experiment and therefore it was not possible to study the change in expression of pAR^{S578} with drug treatment.

In the PNT2 cells, expression of pAR^{S578} was markedly increased and demonstrated increased nuclear localisation upon treatment with PMA (Figure 7.5A). Expression was also increased in both the LNCaP and VCaP cells in both the cytoplasm and nucleus, although with less intensity to that observed in the PNT2 cells (Figure 7.5B and 7.5C). On treatment with BIM-1 following stimulation with PMA, there was increased cytoplasmic expression of pAR^{S578} than compared to nuclear in both the PNT2 and LNCaP cells than compared to PMA alone. No discernible difference was demonstrated in the VCaP cells treated with PMA and BIM-1 than compared to PMA alone.

Figure 7.5 Expression of pAR^{S578} in prostate cell lines following treatment with PMA and BIM-1



Cells were incubated in the presence or absence of 10μM BIM-1 for one hour, followed by stimulation with 10 nM PMA for one hour. IF was perfomed to assess expression of pAR^{S578} in each cell line.

A. *PNT2 cells showed increased expression of pAR^{S578} and increased nuclear localisation upon treatment with PMA. On treatment with BIM-1 following stimulation with PMA, there was increased cytoplasmic expression of pAR^{S578} and decreased nuclear expression of pAR^{S578}than compared to cells treated with PMA alone*

B. *LNCaP cells showed increased expression of nuclear and cytoplasmic pAR^{S578} upon treatment with PMA. On treatment with BIM-1 following stimulation with PMA, there was increased cytoplasmic expression of pAR^{S578} and decreased nuclear expression of pAR^{S578}than compared to cells treated with PMA alone*

C. *VCaP cells showed increased expression of nuclear and cytoplasmic pAR^{S578} upon treatment with PMA. There was no difference in expression pattern upon treatment with BIM-1 following stimulation with PMA than compared to cells treated with PMA alone*

Due to time constraints, the experiments above were only completed once and therefore no conclusions can be elucidated.

7.6 Discussion

In this study, a method for propagation of primary prostate cells from TRUS biopsy tissue was developed. A number of techniques were employed to confirm the propagation of a luminal epithelial cell culture and to investigate the genotype and phenotype of those cells isolated and propagated.

Flow cytometry has confirmed that this technique allows the isolation and propagation of luminal epithelial cells. This is confirmed by the presence of CK-18, a luminal epithelial cell marker that is absent in basal epithelial cells. (208) A small population of cells did not express CK-18, instead expressing CD-90. CD-90 is a cell adhesion molecule expressed by fibroblasts and has previously been shown to be overexpressed by prostate cancer-associated fibroblasts. (209, 210) This suggests that the majority of cells cultured by this method are luminal epithelial cells, with a small sub-population of fibroblasts or non-luminal epithelial cells.

The expression pattern of mRNA from five biomarkers in two prostate cancer cell lines and two primary prostate cancer cell cultures compared to a benign prostate cell line was investigated to confirm a luminal epithelial prostate cancer genotype in the primary prostate cancer cell cultures.

mRNA expression of AR was found to be elevated in all cell lines compared to the benign control. It is well recognised that both LNCaP and VCaP cells express AR mRNA.(211-213) In keeping with the literature, our results show that AR mRNA in VCaP is overexpressed compared to LNCaP. (214) mRNA expression of AR was also demonstrated in both the primary cell cultures. Of particular note, the expression of AR mRNA in the T290 cell culture was higher than that observed in the LNCaP cell line. Early studies have shown low or no expression of AR mRNA in primary prostate cell cultures, which was thought to be due to the growth of basal epithelial cells over luminal epithelial cells, which do not express AR mRNA (215). More recent studies have developed primary prostate cells cultures with AR expression. (216, 217) The expression of AR in the primary cell cultures in this study therefore supports the results of the flow cytometry suggesting differentiated luminal epithelial primary prostate cells have been

grown in this study. This is further supported by the expression of KLK3 mRNA, an AR target gene responsible for the production of PSA. The expression of KLK3 in all four cell lines indicates a luminal, secretory epithelial cell line, as it is known that basal epithelial cells do not express the KLK3 gene or PSA. (89, 218) Again, it is interesting to note that the T290 cell culture had higher expression of KLK3 than VCaP cell line.

Expression of mRNA of FASN, GOLM1 and AMACR was increased in both the immortalised prostate cancer cell lines and primary prostate cancer cell cultures compared to the beingn prostate control cell line. The increased expression in the immortalised prostate cancer cell lines supports the existing body of evidence that all three genes have been shown to be overexpressed with a malignant genotype. (219-223) Furthermore, the increased expression in the primary prostate cancer cell cultures, comparable to that of the immortalised prostate cancer cell lines, supports a malignant genotype in these cells. This is of particular note in the T290, which despite being benign on pathological examination, the prostate epithelial cells expressed a malignant genotype.

It is well recognised that expression levels of mRNA in biological samples do not necessarily correlate with protein expression. (224) We therefore sought to confirm the presence of AR and PSA protein in the primary prostate cells. Both the T288 and T290 were confirmed to express a functional AR, as indicated by the expression of PSA. The primary cell cultures were also confirmed to express the protein of interest in this study, pAR^{S578}, thus indicating that these cells can be utilised in future studies to evaluate the use of pAR^{S578} as a biomarker in prostate cancer.

The final element of this study investigated the effect of BIM-1, a PKC inhibitor on the expression of AR and pAR^{S578} in this study. As we have shown in earlier chapters, PKC expression is correlated with pAR^{S578} expression in tissue samples. It was hypothesised that treatment of established prostate cell lines with BIM-1 would decrease expression of pAR^{S578} through inhibition of PKC. As was expected, the expression of pAR^{S578} was increased in all three established prostate cell lines on treatment with PMA, an upstream activator of PKC. Treatment with BIM-1, a PKC inhibitor, resulted in increased expression of pAR^{S578} in the cytoplasm and

decreased expression in the nucleus in PNT2 and LNCaP cells. This suggests inhibition of nuclear translocation of the AR through inhibition of PKC-dependent phosphorylation of the AR. Surprisingly, no difference was observed in the VCaP cell line. Whilst these experiments were only conducted once, and therefore no definite conclusions can be drawn, these results lend support to the existing body of literature that pAR^{S578} plays a role in nuclear-cytoplasmic shuttling. (129)

An obvious limitation of this study is the small sample size. This was due to time constraints preventing the collection of further patient tissue for culture. Furthermore, as the primary cell cultures stopped growing it was not possible to complete all experiments of each patient and those that were conducted were only performed once for the same reason. However, despite the small sample size, it has been demonstrated that using the techniques described in this thesis primary cells can be isolated and propagated from TRUS biopsy samples and utilised for future experimentation.

This study describes a technique for the isolation and propagation of primary prostate cells from TRUS biopsy. The majority of primary prostate cells developed in other studies have originated from radical prostatectomy or metastatic tissue, thus producing a bias in the cell cultures developed. TRUS biopsy is the gold-standard investigation for the diagnosis of prostate cancer, and almost every patient diagnosed with prostate cancer will undergo biopsy. This technique allows culture of primary prostate cells from all stages of prostate cancer, including those who ordinarily would not undergo surgical treatment, such as AS patients and those with metastatic spread at the time of diagnosis. The ability to cultivate primary prostate cancer cells in vitro from an initial prostate biopsy, opens the door to further translational research for individualised medicine, in the hope that these models will be able to predict which patients will have aggressive disease and require earlier treatment and response to treatment through the use of biomarkers. Further work needs to be conducted to confirm this technique in a larger cohort of patients, including correlation with biomarkers expressed in prostate tissue and long-term follow-up of patients.

Chapter 8 General discussion

Prostate cancer incidence is rising in the UK, with new cases predicted to reach 75,000 per year by 2035. (1) This is mainly due to the increasing use of the PSA blood test in general practice, resulting in the over diagnosis of low risk localised prostate cancer that may not have been clinically relevant within the patient's life time. (225) Whilst there are effective treatments available for localised disease, there are significant side effects and escalating healthcare costs associated with radical treatment. Treatment must therefore be reserved for those patients who will gain most benefit. Clinical parameters utilised currently are inadequate for differentiating between indolent and aggressive disease leading to overtreatment of prostate cancer. Identification of reliable prognostic biomarkers that can be utilised at the time of diagnosis to differentiate between indolent and aggressive prostate cancer are therefore urgently required.

Androgen receptor phosphorylation has been extensively associated with prostate cancer development, progression and development of castrate-resistant disease. AR phosphorylation status has therefore emerged as a potential biomarker in assessment of disease severity. Our group has previously investigated several phosphorylation sites on the androgen receptor in relation to clinical outcome measures with promising results. (124, 126, 128) The aim of this thesis was to assess androgen receptor phosphorylation at serine 578 as a potential biomarker that can be used in clinical practice.

PKC is the putative kinase for phosphorylation of the androgen receptor at Ser-578, as predicted by Scansite 2.0.(130) Previous studies have shown PKC to phosphorylate the AR. Site-directed mutagenesis of Ser-578 in a CRPC cell line resulted in a 50% decrease in PKC-dependent AR phosphorylation compared to wild-type cells. (129) In all cohorts, PKC was significantly associated with expression of pAR^{S578} in the clinical specimens. Furthermore, in Chapter 7 it was observed that expression of pAR^{S578} in prostate cells appeared to increase on treatment with PMA, a potent PKC activator. These results must be interpreted with caution as they were only performed once and therefore no definite conclusions can be drawn. However, taken together with the results of the

clinical cohorts, these findings add to the existing body of evidence that PKC is the kinase responsible for androgen receptor phosphorylation at Ser-578.

Androgen receptor phosphorylation at Ser-578 has been linked to increased AR transcriptional activity, cell growth, nuclear cytoplasmic shuttling, modulation of other AR phosphorylation sites and DNA-repair mechanisms.(129) High expression of pAR^{S578} was significantly associated with several clinical markers of disease severity including increased PSA, Gleason score at diagnosis and the presence of PNI in the AS and/or discovery cohort but was not identified in the validation cohort. This suggests that there is potential for pAR^{S578} to be a marker of high risk-disease but requires further validation is a larger, multi-centre cohort of prostate cancer patients.

The relationship between pAR^{S578} and clinical outcome measures was the investigated in three clinical cohorts. There was no association between expression of pAR^{S578} and time to intervention in the AS cohort. In the discovery cohort however, high expression of cytoplasmic pAR^{S578} was found to be significantly associated with time to biochemical relapse, disease-specific survival following biochemical relapse and disease-specific survival. High cytoplasmic expression of pAR^{S578} was significantly associated with shorter time to biochemical relapse and trended towards significance in relation to overall survival in the validation cohort. It is possible that due to the short period of follow-up in the validation cohort, there are insufficient events to reach clinical significance. Survival data will therefore continue to be collected and re-assessed when 10 year follow-up is available. Overall, these findings support the hypothesis that pAR^{S578} expression is increased in advanced disease and is associated with poor outcomes in prostate cancer.

Classical androgen receptor phosphorylation in response to DHT occurs at Ser-81. (114, 115) Ser-81 phosphorylation is associated with increased AR transcriptional activity and expression of androgen-dependent proteins such as PSA, chromatin-binding of AR to AREs, nuclear localisation of AR and enhanced cell growth in prostate cancer cells. (116-118) As androgen receptor phosphorylation at Ser-578 occurs via an alternative pathway to that of Ser-81 it was hypothesised that dual phosphorylation at these sites may have a synergistic prognostic effect in

relation to clinical outcome measures. In all three clinical cohorts, high dual expression of pAR^{S81} and pAR^{S578} was associated with poor outcome measures and had greater prognostic effect than expression of each phosphosite alone. In some cases, this was observed even when the phosphosite was not independently associated with poor outcome measures, supporting the research hypothesis that expression of AR phosphorylated at these sites may have a cumulative prognostic effect. These striking results suggest that there is a sub-population of patients who are at significantly increased risk of disease progression and poor outcomes. Furthermore, due to the differing mechanisms of phosphorylation at Ser-81 and Ser-578, this study identifies a cohort of patients may benefit from dual targeted therapy with ADT and PKC inhibitors.

In addition to the above studies, a technique for isolation, propagation and characterisation of primary prostate cancer cells from TRUS biopsy specimens was developed with a view to providing a potential model for investigation of clinical biomarkers and drug discovery. The use of TRUS biopsy to culture primary prostate cells is particularly exciting, as most previous techniques use tissue obtained from radical prostatectomy or metastatic deposits, which can create bias. This technique allows prostate cancer of all disease stages to be investigated as TRUS biopsy is performed at diagnosis for virtually every prostate cancer patient. This work was only conducted in two patients and therefore requires validation in a much larger cohort of patients.

One primary cell culture was used to investigate the effect of PMA and BIM-1 on the expression pattern of AR. Whilst this experiment was only conducted once and no conclusions can be drawn, it does demonstrate the potential of this model for identification of biomarkers to predict response to drug treatments.

Future studies to continue the research conducted in this thesis should include validation of the results presented in the clinical cohorts in relation to expression of pAR^{S81} and pAR^{S578} and clinical outcome measures. Firstly, the IHC staining in the validation cohort should be completed and the results re-analysed to fully validate the results of the discovery cohort. In addition, the validation cohort should be re-assessed when 10-year survival data is available. Following on from this, the prognostic significance of pAR^{S578} alone and in combination

with pAR^{S81} should be validated by an independent laboratory with a multi-centre prospectively collected cohort of prostate cancer patients. As phosphoantibodies are known to be unstable, downstream markers should be identified using gene expression analysis that could be utilised at surrogate markers of pAR^{S81} and pAR^{S578} expression for use in clinical practice.

Future in vitro work should include validation of the primary cell culture technique in a larger cohort of prostate cancer patients. Primary cell cultures could then be utilised for a number of different research purposes including drug discovery, assessing for further biomarkers and 3-d culture.

In summary, the evidence presented in this thesis supports the research hypothesis that pAR^{S578} expression on it's own and in combination with pAR^{S81} are markers of high-risk disease and are potential biomarkers for poor prognosis in prostate cancer. IHC, the technique employed in the evaluation of expression of the proteins of interest in the clinical cohorts in this study, is widely used in current clinical diagnostic pathology and therefore is deemed to have potential for translation into clinical practice.

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