Prostate Cancer-Associated Protein Biomarkers in African Heritage Populations

Mr Philip Goodall

BSc BMBS MRCS

A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Master of Philosophy

March 2021

Copyright Statement

Acknowledgements

I would like to thank my supervisors Dr Des Powe and Prof Graham Ball for their invaluable expertise and dedicated support and guidance throughout my MPhil thesis.

I would also like to acknowledge the financial support from the Nottingham University Hospitals Charity who funded the TMA production and IHC costs and Birmingham Biobank and associated team for their help and assistance in the new TMA production.

I am also very grateful to the John van Geest Cancer Research Centre for funding my MPhil degree registration fees, it is greatly appreciated.

Abstract

Background

African Heritage (AH) men have a higher overall incidence, earlier age of onset, more clinically advanced and aggressive disease, higher incidence of metastases and mortality from prostate cancer (CaP) compared to Caucasian men. Biological and genetic factors relating to tumour biological pathways are thought to account for this disparity. Pathways associated with apoptosis and proliferation, cell adhesion and epithelial-mesenchymal-transition (EMT), inflammation and host immune response have all been implicated.

Aims and Objectives

We aimed to develop a panel of biomarkers related to these pathways likely to account for the disparity of aggressive disease between AH and Caucasian men with CaP. Specifically we aimed to identify potential candidate biomarkers that could potentially be used to predict clinical outcomes in these ethnic groups, review if they displayed differential expression and to confirm or negate relevant important biological pathways for possible therapeutic targeting.

Methods

A panel of candidate biomarkers was selected from an extensive literature review of biological tumour pathways in AH men with prostate cancer and a bioinformatic approach using an artificial neural network (ANN) analysis

to identify biomarkers conferring increased risk of metastasis in AH men. Immunohistochemical analysis was performed on a Caucasian predominant historical tissue microarray (TMA) constructed from transurethral resection of prostate (TURP) and transrectal ultrasound-guided (TRUS) prostate biopsy CaP samples. A new AH TMA was produced from radical prostatectomy and TURP samples. Univariate and multivariate analysis was performed to establish the association of protein biomarker expression with various clinical prognostic endpoints, specifically time to disease specific death and metastasis development.

Results

We demonstrated the utility of several biomarkers, Lambda FLC, TGF-β EPB41L4A and PD-L1, in predicting disease specific survival, time to metastasis development and castrate resistance. In particular increased protein expression of Immunoglobulin Free light chain (FLC) lambda FLC, which is a completely novel biomarker in CaP, was statistically significant in predicting disease specific death and time to metastases. Greater expression was seen in AH men.

Conclusions

We have identified a completely novel biomarker, Lambda FLC, in CaP with the prognostic ability to predict disease specific death and development of metastases with increased protein expression seen in AH men. Our study supports the hypothesis and findings from previous studies that suggest the regulation of inflammation and modulation of the immune system and

resultant change in extracellular matrix (ECM), of the tumour microenvironment, appears to be an important biological process and might account for the aggressive nature of CaP in AH men.

Abbreviations

Prostate Cancer – CaP

United Kingdom – UK

African Heritage – AH

Genome-wide sequencing studies – GWAS

Single nucleotide polymorphisms – SNPs

Transurethral Resection of the Prostate – TURP

High-Grade Prostatic Intraepithelial Neoplasia - HGPIN

Prostatic Intraepithelial Neoplasia – PIN

Prostate specific Antigen – PSA

Epithelial –to-mesenchymal transition – EMT

Digital Rectal examination – DRE

General Practitioner – GP

Benign Prostatic hyperplasia – BPH

Prostate Cancer Antigen 3 – PCA3

Polymerase Chain Reaction – PCR

Transmembrane Protease, Serine 2-ETS fusion – TMPRSS2-ERG

Tumour, Nodal, Metastases – TNM

External Beam Radiotherapy – EBRT

European Association of Urology – EAU

Transrectal Ultrasound – TRUS

Magnetic Resonance Imaging – MRI

United States – US

Tumour Necrosis Factor – α – TNF- α

Nuclear Transcription Factor – kB – NFkB

Androgen Receptor – AR

Extracellular Matrix – ECM

Immunohistochemistry – IHC

Cytotoxic T Lymphocyte Antigen-4 – CTLA-4

Programmed Cell Death 1 – PD-1

Programmed Cell Death Ligand 1 – PDL-1

Artificial Neural Network – ANN

Immunoglobulin free light chains – FLCs

Androgen deprivation Therapy – ADT

Table of Contents

List Of Tables

List of Figures

1.0 Introduction

1.1 Prostate cancer

Prostate cancer (CaP) is the commonest cancer amongst men in the United Kingdom (UK) accounting for 13% of all new cases and the second most common cancer in men worldwide (1,2). Incidence rates for CaP in the UK have risen by 44% since the early 1990s and are projected to continue to rise by 12% between 2014 and 2035, to 233 cases per 100,000 males by 2035. Currently 1 in 8 Caucasian men will be diagnosed with CaP during their lifetime (3).

Although CaP remains the second most common cause of cancer death in UK males, mortality rates have decreased by more than a tenth (13%) over the last decade (3). CaP survivorship has tripled in the last 40 years in the UK; currently more than 8 in 10 (84%) men diagnosed with CaP in the UK survive their disease for 10 years or more. Widespread prostate specific antigen (PSA) testing is largely responsible for rising incidence and rising survivorship with detection of latent, earlier, slow growing CaP which may not require treatment (4).

The rising disparity between incidence and mortality with rising survivorship has led to an increasing need for risk stratifying techniques to determine risk of CaP progression and CaP related death. Risk stratification would identify men that would benefit from radical treatment and those that

could be safely monitored with a surveillance protocol, avoiding the morbidity and side effects from potentially unnecessary intervention.

1.2 Aetiology

1.2.1 Age

Prostate cancer is strongly linked with age. Age-specific incidence rates rise sharply from around age 50-54, peak in 75-79 group, drop in 80-84 group before increasing again to the 90+ age group. This age distribution of CaP detection is reflected largely by the time periods PSA testing and transurethral resection of the Prostate is carried out.(5–7)(Figure 1.)

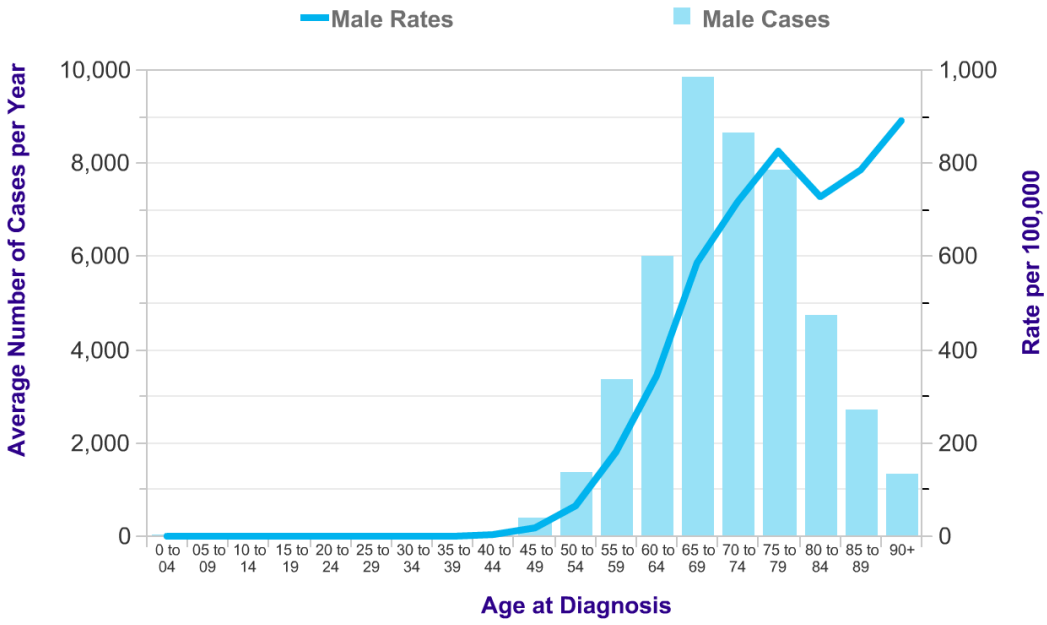


Figure 1: UK CaP incidence by age (reused with permission from Cancer, Research UK) (7)

1.2.2 Ethnicity

In the UK African heritage (AH) men are twice as likely to be diagnosed with CaP and twice as likely to die from the disease compared to their

counterparts of Caucasian heritage (CH) (8). Similar findings have been shown in North American populations, with the incidence of CaP 60% greater and the mortality rate 2-3 times higher in AH men compared with Caucasian men (9). The PROCESS study in the UK (10) demonstrated that AH men in UK have substantially greater risk of developing CaP being 3 times more likely to be diagnosed with CaP compared with Caucasian men, although this risk is lower than that of black men in the United States. It also showed that on average AH men are diagnosed 5 years earlier than Caucasian men and present with a higher PSA (11,12). Large scale genome-wide sequencing studies (GWAS) have led to the discovery of 170 single nucleotide polymorphisms (SNPs) associated with increased CaP risk (13–18). SNPs of chromosome 8q24 and 17q21 have been shown to confer increased risk of CaP and found more commonly in AH men (13,19,20). Currently the PROFILE study (NCT02543905) is recruiting patients investigating the role of targeted screening in men with a genetic susceptibility to CaP. The study aims to use SNP profiling in men with a family history of CaP or AH to determine the association of genetic profiling with MRI/prostate biopsy result in men with a genetic susceptibility to CaP.

1.2.3 Geographical distribution

CaP is more common in western nations particularly western and Northern Europe, North America, Australia and New Zealand. The disease is rare in Asia and Far East countries (21). Interestingly it appears that incidence of CaP in black African men is lower than among their African American counterparts (22). (Figure 2.)

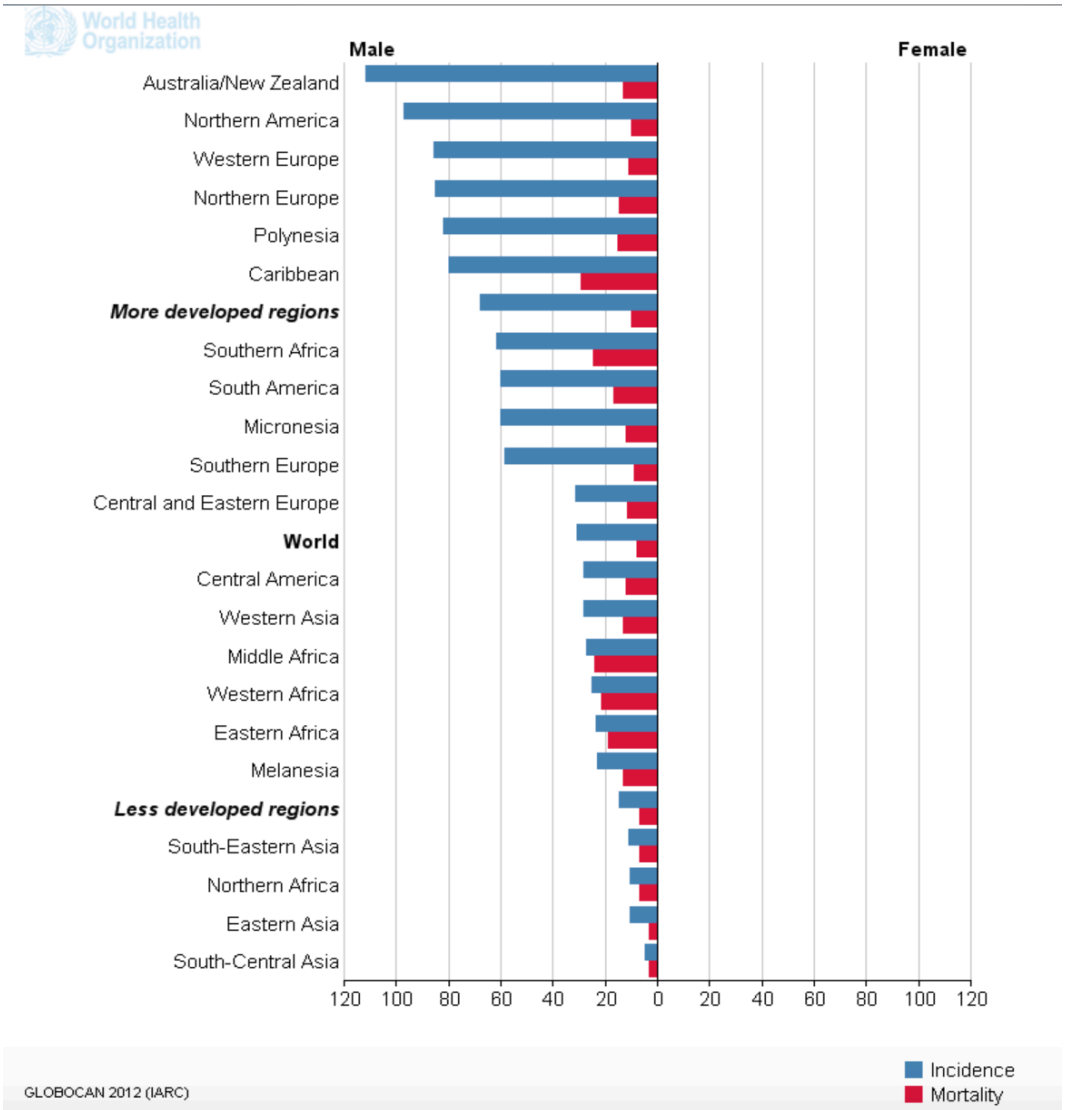


Figure 2: Geographical Distribution of Prostate Cancer worldwide (reused with permission from Cancer, Research UK)(21)

1.2.4 Genetic and familial factors

It is estimated that inherited factors account for 5-9% of prostate cancers (23). Studies in Caucasian men have shown a familial clustering within families; with evidence suggesting that men with two and three first-degree relatives have a 5 and 11-fold increased risk of developing prostate cancer respectively (24). Prostate cancer is 2.1-2.4 times higher in men whose father has had the disease, 2.9-3.3 times higher in men whose brother has

had the disease and 1.9 times higher in men with a second-degree relative affected (25).

Hereditary prostate cancer refers to specific subtype of familial prostate cancer marked by a specific passage of a susceptibility gene through successive generations. The criteria for hereditary prostate cancer is a family with three generations affected, three first-degree relatives affected, or two relatives affected before the age of 55. Approximately 43% of men with a diagnosis of prostate cancer before the age of 55 have hereditary prostate cancer (26).

Excluding age and AH ancestry, the strongest risk factor for CaP is family history (27) as mentioned previously several germline SNPs have been associated with CaP risk and together explain about 30% of the genetic variance of CaP (27). Men identified with the top 1% risk profile of SNPs have a 4.7 fold increase in risk of developing CaP (18). The identification of prostate cancer SNPs led to the development of the Barcode 1 trial in 2017 predicting CaP risk from a saliva test. The Barcode 1 study (NCT03158922) is a screening study, which is still recruiting; designed to investigate the role of genetic profiling for targeting population based CaP screening. Men identified with a genetic risk equivalent to the top 10% of the population distribution will be invited for an MRI and transrectal ultrasound (TRUS) prostate biopsy with results correlated to a genetic score. The PROFILE feasibility study examined the role of upfront prostate biopsy in men with a genetic risk of CaP, reporting a cancer detection rate of 25% with 48% of

these being clinically significant (28). These preliminary results from this feasibility study lead to the current PROFILE study, which is still recruiting.

Several genes have been identified which confer a high risk of developing CaP. Carriers of a rare missense mutation (G84E) of HOXB13 gene have been shown to have a 33% risk of developing CaP compared to 12% risk of non carriers (29). This mutation appears to be more common in men with disease at a younger age and positive family history (30)

Germline mutations of BRCA 1/2 increase the risk of developing CaP. The relative risk of CaP by ≤65 yrs is estimated at 1.8 – 4.5 times greater for BRCA1 carriers (31) and 2.5 to 8.6 time greater for BRCA2 carriers (32). Several retrospective analyses have shown an association between BRCA status and higher risk of CaP recurrence, CaP specific-mortality and high risk disease with worse cancer specific survival (33–35). Targeted screening of BRCA1/2 carriers for earlier detection may therefore be beneficial. The IMPACT study (Identification of men with genetic predisposition to prostate cancer: targeted screening in BRCA1/2 mutation carriers and controls) is an international, multicentre study evaluating the role of PSA screening in men with these gene mutations. The interim results from the IMPACT study have shown that cancer incidence rates were higher in BRCA2 carriers as well as being diagnosed at a younger age (61 vs. 64 yrs) and more likely to have clinically significant disease than BRCA2 noncarriers (77% vs. 40%) (36).

Mutations of other DNA repair genes such as ATM, CHEK2, MSH1, MLH1, MSH2 and MSH6 have also been associated with a significantly increased risk of developing CaP (37). Lynch syndrome, formerly known as hereditary nonpolyposis colorectal cancer (HNPCC) is a disorder characterised by a familial predisposition to cancer development. The pattern of inheritance is autosomal dominant often due to a germline mutation in one of the DNA mismatch repair genes MLH1, MSH2, MSH6, PMS2 or deletion of EPCAM (38). The frequency of mutations of these genes in the general population is approximately 1:3100 to 1:370 (39). Patients with Lynch Syndrome have a 2.1-4.9 times higher risk of prostate cancer (38). The estimated cumulative risk of developing CaP by 70 in patients with these gene mutations is 30% compared with 9% in the general population (40). It also appears that the risk of development of less common cancer in patients with lynch syndrome is dependent on the sex of the patient and specific mutated gene the patient carries. Mutations in MSH2 appear to be more significantly associated with risk of developing CaP with a reported 10 fold risk of CaP by the age of 60 (41). Within the IMPACT study there is a lynch syndrome arm with men recruited with MSH2, MSH6 or MLH1 gene mutations, interim results for this arm are still awaited.

The BARCODE 2 study (NCT02955082) specifically aims to look at DNA repair gene mutations and how they can affect response to treatment in men with metastatic castrate resistant CaP.

1.2.5 Hormonal factors

Prostate cancer risk has been shown to be 38-83% in men with elevated levels of insulin-like growth factor-1 (IGF-1) (42).

1.2.6 Dietary factors

Several studies have looked at the associated risk of developing CaP relating to several dietary factors however the evidence is largely inconclusive. Epidemiologic studies conducted to date suggest that high intakes of saturated fats, meats cooked at high temperatures and calcium are associated with an increased risk of advanced CaP (43).

Lycopene, present in cooked tomatoes and tomato-based products are thought to reduce the risk of CaP progression and development of advanced CaP. However results from epidemiological studies are inconsistent (44).

1.3 Pathology

Adenocarcinoma of the acinar or ductal epithelium is the most common prostatic malignancy accounting for >95% of all prostate malignancies.

1.3.1 Anatomy of the prostate

McNeal first described the zonal anatomy of the prostate (45). Four anatomic structures were described which bare significant relevance to the development of adenocarcinomas in the prostate; the peripheral zone which constitutes over 70% of the prostate, the central zone constituting 25%, the

preprostatic region or transition zone and the anterior fibromuscular stroma. (Figure 3.)

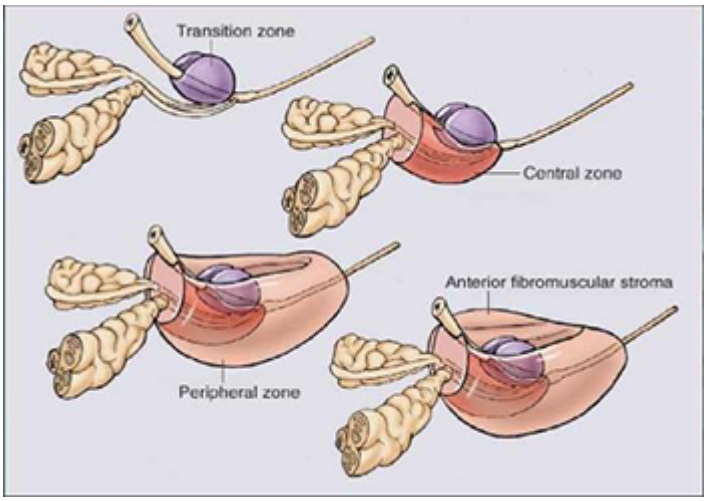


Figure 3: Zonal anatomy of the prostate (reused with permission from John Wiley and Sons, licence number 4531521330165) (45)

The majority of prostate tumours arise from the peripheral zone with 70% of tumours found here and a further 20% are found in the transitional zone (46).

1.3.2 Precursors of prostate cancer and molecular basis of prostate cancer

1.3.2.1 High-grade prostatic intraepithelial neoplasia (HGPIN) and epithelial cell lineage

Prostatic intraepithelial neoplasia (PIN) represents the pre-invasive end of cellular proliferation, consisting of typical benign prostatic acini and ducts lined by cytologically atypical cells with nuclear and nucleolar enlargement (47). Previously PIN was categorised into low-grade and high-grade

depending on the presence of prominent nucleoli but now only high-grade is reported since low-grade has no prognostic value.

The total incidence of isolated PIN averages 9% (range, 4%-16%) of prostate biopsies (48). The incidence and extent of PIN appears to increase with age and race with also geographical location appearing to influence incidence. African-American men aged between 50-60 years appear to have a greater prevalence of PIN than Caucasians (49,50).

The mean volume of PIN found in prostates with cancer increases with increasing pathological stage, Gleason grade, positive surgical margins and perineural invasion (51). These findings combined with the facts that both PIN and CaP are usually multicentric in nature, with both commonly found in the peripheral zone, have similar proliferative and apoptotic indices and similar genetic alterations, underscore the close biological relationship of PIN and cancer. This highlights the role of the former as a precursor lesion. PIN is defined by a series of well defined architectural and cytological changes which results in the progressive loss of the epithelial two-cell arrangement with disappearance of basal cells (52). The overall proliferative role of the affected epithelium throughout these changes may remain the same or be increased as the balance between cell proliferation and apoptosis changes. As apoptosis regulatory mechanisms falter there is a tendency for the number of malignant cells to increase.

The significance of HGPIN when detected on a prostate biopsy without associated prostate cancer is dependent on the number of biopsy cores with HGPIN present. If less than four cores are involved, the risk of malignancy being found on second prostate biopsy is approximately 26% however if more than four cores have HGPIN then the chance of finding cancer on a second biopsy rises to over 40% (53).

1.3.2.2 Atypical small acinar proliferation (ASAP)

Atypical small acinar proliferation (ASAP) is a histological finding of atypical glands which are suspicious for prostate cancer but do not show enough cytological changes to amount to cancer. Studies have shown that in patients with ASAP present in initial prostate biopsies, the subsequent risk of cancer being detected at second biopsy is approximately 40% (54).

Previous clinical practice had been to consider immediate re-biopsy in patients with four or more cores of HGPIN and ASAP however in the era of active surveillance with the adjunct of multiparametric MRI the incidence of clinically significant CaP at re-biopsy is of similar incidence to those patients with benign tissue and generally these patients are enrolled in a PSA surveillance programme (55,56).

1.3.2.3 Basal and luminal-like stem cells in prostate cancer

The prostate consists of two distinctive epithelial layers: a basal epithelium located on the basement membrane and a luminal epithelium, which is separated from the basal membrane by the basal layer (see figure 4.). The

exact nature and origin of prostate cancer remains controversial. The simplest explanation would be a direct transformation of normal luminal cells into malignant cells with loss of growth control (see figure 4). There is growing evidence however that there is a hierarchy of cells within a given cancer (57). In prostate cancer this hierarchy is likely to be dominated by an aberrant luminal cell. There is evidence however that tumour development is driven or possibly initiated from a stem like cell with different properties to the tumour mass (58).

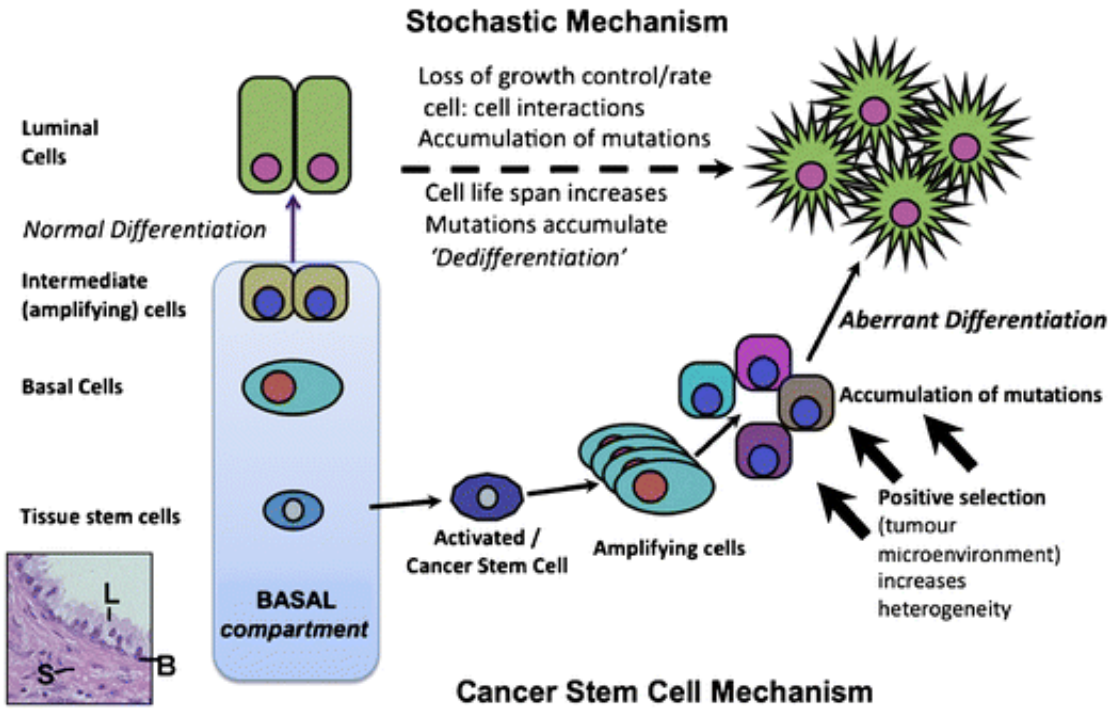


Figure 4: Normal prostate cell architecture and the origins of prostate cancer (reused with permission from Springer Nature, licence number 4531530049774) (59)

The predominant subtype of CaP is adenocarcinoma with features of luminal secretory cells and an absence of basal cells. A small number of less common

histological subtypes include small cell carcinoma and squamous cell carcinoma. Both of these subtypes are associated with poorer prognosis, aggressive disease and treatment resistance (60). Squamous cell carcinomas have features of basal cells and can occur synchronously with adenocarcinoma or alone. It is therefore plausible that based on their different phenotypes that different histological variants of prostate cancer arise from different cells of origin.

In vivo studies have shown stem like basal cells can generate luminal cells (61). Furthermore treatment naïve prostate basal cells have been shown to initiate acinar type prostate adenocarcinoma (62). Similarly basal cells from a BPH-1 human prostate cell line have been shown to initiate prostate cancer in response to combined oestrogen and testosterone treatment (63). Human prostate cancer cells with a basal phenotype have also been shown to produce luminal cancer cells in vitro (64). This suggest that prostate cancers may have basal stem like cells which they use to ensure continuous production of luminal-like tumour propagating cells CaP to aid continued development and growth.

1.3.2.4 Cell proliferation and regulation

1.3.2.4.1 Proliferation

Assessment of the number of cells proliferating within a tumour may indicate the course of tumour progression. Several studies that have assessed the mitotic index and Ki67 immunohistochemistry have suggested some prognostic significance of tumour cell proliferative indices in CaP. The

mitotic index and a range of clinical and histological features of 303 prostatic adenocarcinomas were assessed and found that the Gleason score, tumour invasiveness and metastasis at diagnosis as well as progression and progression free survival, were all related to the mitotic index (65). This suggests that there may be some prognostic value in using proliferation indices in the management of CaP.

Expression of Ki67 is required throughout the cell cycle and the expression of which is strictly associated with cell proliferation, leading to its use in routine pathology as a "proliferation marker" to assess the growth indices of tumour cells (66). Ki67 expression and correlation has been widely reported in all malignancies (67). The significance of Ki67 in prostate cancer has been reported in several studies. Ki67 expression has been shown to provide additional prognostic information in addition to a given Gleason score and prostate-specific antigen (PSA) in localised CaP, suggesting that it could be used as a novel biomarker to predict the need for treatment (68). Increased Ki67 expression levels were seen in patients with recurrence of their CaP following treatment with radiotherapy and hormone therapy and were significantly associated with biochemical failure, metastasis and disease specific mortality (69,70). Similarly Ki67 expression was associated with a shorter length of progression free survival in a cohort of patients with high-risk, localised prostate cancer (71). These suggest an important role of proliferation in CaP development and in treatment stratification strategies in treatment of CaP.

1.3.2.4.2 Regulation of apoptosis

The balance between proliferation and apoptosis regulates tumour growth and progression. We know that the rates of apoptosis are altered during CaP initiation, progression and process of metastasis (72). Androgens are the main regulator of the balance between proliferation and apoptosis in CaP. Development of CaP depends on androgenic stimulation for growth and continued survival whilst androgen deprivation causes cancer regression due to lowering of cell proliferation and increased apoptosis. Studies have shown that withdrawal of androgens from normal prostate cells results in epithelial cell death, caused by an increase in intracellular transcription of multiple genes normally suppressed by testosterone including, C-fos, c-myc4 and TGF-β resulting in cellular apoptosis (73,74). As prostate cancers comprise heterogeneous cells, although many will undergo apoptosis in response to anti-androgen therapy, some androgen independent cells will continue to proliferate leading to development of a subtype that is no longer androgen sensitive. Androgen-independent cells have intact cell death programmes but they fail to initiate these resulting in unregulated cell proliferation (75). Overexpression of anti-apoptotic proteins BCL-2 and BCL-XL, activation of pro-survival proteins such as AKT and NF-KB and loss of tumour suppressor genes such as p53 and PTEN and BIN1 all lead to an inhibition of apoptosis (76).

The BCL-2 family is a group of proteins that function as either pro or anti-apoptotic molecules. Several members of this family have been shown to be associated with CaP. Both BCL-2 and BCL-XL are both anti-apoptotic

proteins that block the release of cytochrome C and apoptosis inhibitory factor into the cytoplasm (77,78). Both BCL-2 and BCL-XL have been found to be over expressed in CaP (79).

1.3.2.5 Epithelial to mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a normal physiological process by which cells of epithelial origin convert to cells bearing mesenchymal characteristics. It is proposed that cancer cells use this process during their process of metastasis as supposed 'circulating tumour cells'. There is growing evidence that EMT states occur in CaP and may contribute to progression and development of metastasis. Adhesion molecules allow structural and functional interface between epithelial cells and the extracellular environment, promoting and maintaining cellular attachment. Studies have shown a loss of adhesion molecule E-cadherin during progression from normal prostatic epithelium to invasive prostate cancer (80).

TGF-β signalling is the most extensively studied pathway in regards to its role in EMT and cancer progression. TGF-β regulates cell growth, differentiation and matrix production through auto-regulatory functions related to other peptide growth factors. In prostate cancer various isoforms of TGF-β are down or up-regulated resulting in diminished sensitivity to the inhibitory or anti-proliferative effects of TGF-β resulting in pro-oncogenic activity. TGF-β has been shown to induce EMT states in various prostate

cancer cell lines and shown to predict disease recurrence in prostate cancer patient following radical prostatectomy (81,82).

1.3.2.6 Tumour suppressor genes

Several tumour suppressor genes have been identified as being responsible for suppressing neoplastic or metastatic phenotypes within prostatic epithelium. The specific functions for the majority of these genes is not well defined or understood but is thought to largely related to promoting cellular differentiation and arrest of cell proliferation by growth-inhibitory cytokines (83).

1.3.3 Prostate cancer and Gleason score

CaP is graded by the Gleason grading system (Figure 5.). The Gleason classification system was developed by Dr Donald Gleason in 1966 and remains the most widely reported classification system. The grading system involves grading the cancer glandular architecture using low power microscopy from grade 1 to 5 according to its gland-forming differentiation (Figure 5.). From well differentiated glands with a score of 1 to poorly differentiated anaplastic glands with a score of 5. Reporting of the specific score varies according to whether the tissue specimen is a prostate biopsy or radical prostatectomy specimen. The International Society of Urological Pathology (ISUP) Consensus group stipulated in 2005 that for TURP specimens and needle biopsies the most prevalent grade of cancer is combined with the most aggressive grade present to give an overall score. If the most prevalent grade is the same as the most aggressive grade then it is

reported twice. In the case of radical prostatectomy specimens, the most prevalent and second most prevalent grade is added together. If there is a smaller amount of a higher grade present then this is reported as a tertiary grade.

The 2014 ISUP Gleason grading conference stipulated that the various Gleason scores were classified into 5 overall grade groups according to their overall Gleason score (see Table 1.) (84).

The importance of Gleason score is that it correlates well with prognosis and remains the most important prognostic indicator after radical curative treatment (85,86).

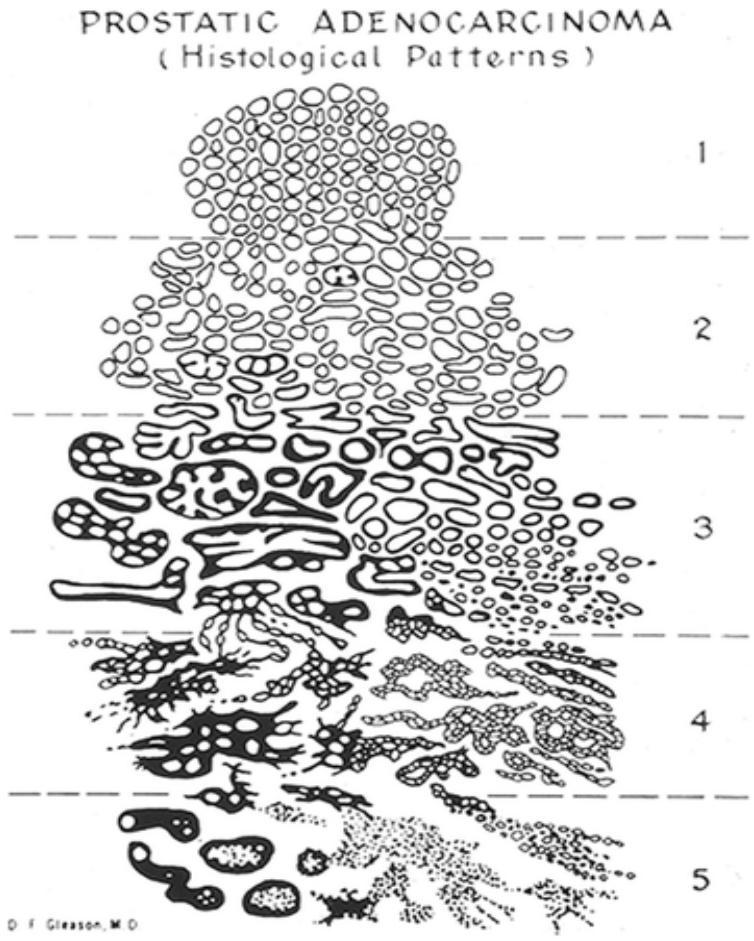


Figure 5: Gleason Grading system of Prostate Cancer. (Reused with permission from BMC publishers) (87) 1 – small, uniform glands 2 – More stroma between glands 3 – Distinctly infiltrative margins 4 – Irregular masses of neoplastic glands 5 – Only occasional gland formation.

Gleason Score	ISUP Grade
2-6	1
7 (3+4)	2
7 (4+3)	3
8 (4+4 or 3+5 or 5+3)	4
9-10	5

Table 1: International Society of Urological Pathology 2014 Grade Groups (88)

1.4 Diagnosis and classification

CaP presents in a variety of ways. It can be diagnosed amongst a plethora of lower urinary tracts symptoms, during an episode of acute or chronic urinary retention, visible or non-visible haematuria or asymptomatically as an elevated PSA test. Advanced prostate cancer can present with signs of malignancy with bone pain, spinal cord compression, obstructive uropathy, significantly elevated PSA or typical red flags of disseminated malignancy.

Diagnosis of CaP is largely made via clinical examination of the prostate by digital rectal examination (DRE) and measurement of the serum PSA level which leads to resultant histological assessment via a biopsy if appropriate.

Currently there is no recommendation for UK CaP screening due to the potential for over diagnosis and resultant overtreatment of patients.

Asymptomatic men can request a DRE and PSA testing after being counselled about the risks and benefits of PSA testing with their general practitioner (GP).

1.4.1 PSA

PSA testing is integral to the current diagnosis, staging and management of CaP patients and monitoring post treatment. PSA is a kallikrein serine protease encoded by the KLK3 gene and is almost exclusively secreted by the epithelial cells of the prostate. Its principal biological function is the liquefaction of semen. PSA is not cancer-specific with elevated levels can be due to benign prostatic hyperplasia (BPH), prostatitis, urinary tract infections, urinary retention and instrumentation of the urinary tract. Despite its low specificity for the diagnosis of CaP on prostate biopsies, PSA in combination with DRE and transrectal ultrasound (TRUS) is the most commonly used method for CaP detection and the most commonly used serum biomarker. A major limitation of PSA is the fact that although increasing levels of PSA are associated with a higher risk of CaP there is no optimal threshold value. Indeed studies have shown that even when patients have an extremely low PSA (PSA $\leq$ 1.0 ng/ml) they still have a 9% risk of CaP (89). Additionally, PSA value does not correlate with CaP aggressiveness (90). Currently although there is no 'normal' value for PSA, age-specific thresholds are used to identify PSA abnormalities. These thresholds specify expected PSA levels for specific age ranges. This aims to compensate for the fact that PSA levels rise with age, irrespective of the presence of CaP, and to increase the sensitivity in younger men and

specificity in older men. Previously the UK prostate Cancer Risk Management Programme had recommended age-related referral values (91), see table 2. Age-specific PSA cut-offs for detecting CaP are highly variable and may just reflect the various demographics and clinical characteristics of a particular population (92) and it was felt that there would be a considerable risk of missing a high proportion of clinically significant cancers in older men and potentially increase the rate of invasive investigations in younger men. Two large PSA-based screening trials have evaluated PSA testing in men aged 55-69 years with biopsy recommended in those with PSA ≥3.0 ng/ml (93,94). This has led to a new recommended referral value for men aged 50-69 of 3ng/ml in UK from NICE (95).

Age Related PSA Thresholds and associated risk of CaP		
Age (years)	**PSA (ng/ml)**	**Risk of CaP (%)**
50 – 59	3	6.6% to 23.9%
60-69	4	26.9%
≥70	5	41% (PSA 4-10)

Table 2: Age related PSA thresholds and associated risk of CaP (89)

The widespread use of PSA has resulted in the increased diagnosis of men with localised, early stage CaP (4,96). These tumours may not become clinically significant during their lifetime. Patients with these tumours are typically over diagnosed, resulting in unnecessary treatment and potential

significant treatment related side effects. This is one of the main reasons why CaP screening methods based on PSA measurement remains controversial. There is a great clinical need for accurate screening for CaP to decrease unnecessary prostate biopsies.

Several adjuncts to PSA have been used to improve diagnostic accuracy for early CaP detection and use as a prognostic tool in the follow-up of CaP patients. These include PSA density, PSA velocity, PSA doubling time, free:total PSA ratio and PSA isoforms (97,98). There is limited evidence regarding the feasibility of these various PSA adjuncts. They are largely confined to managing patients during periods or programmes of surveillance rather than during the initial diagnosis process.

1.4.2 Emerging biomarkers

As a result of the limitations of the PSA test along with technological advancements in the fields of molecular profiling and detection techniques, several new novel potential biomarkers have been discovered that have higher CaP specificity than PSA. Biomarkers are molecules whose detection or evaluation provides diagnostic as well as prognostic information about a disease process beyond the standard patient clinical factors available to the clinician. Biomarkers can be proteins, metabolites, RNA transcripts, DNA or epigenetic modifications of DNA. They can be detected through patient samples (bodily fluids such as urine/blood) or tissue samples (biopsy or surgical resection). The ideal biomarker should be safe and easy to measure preferably non-invasive, highly sensitive and specific and the ability to

improve clinical decision-making in conjunction with patient clinical characteristics on an individualised patient basis. Although it would be ideal for a single biomarker to have all these characteristics the reality is that panels of biomarkers are likely required to yield greater results (99).

1.4.2.1 Prostate cancer antigen 3 (PCA3)

Prostate cancer antigen 3 (PCA3) is a urinary biomarker that is a non-coding prostate –specific mRNA. It is overexpressed in prostate cancer tissue compared to benign tissue. The polymerase chain reaction (PCR) nucleic acid amplification test measures the ratio of the concentration of PCA3 to PSA mRNA in a post-DRE first catch urine sample. Currently the recommended cut-off value for biopsy is a score of ≥ 25 which is associated with a >25% chance of a positive biopsy result. PCA3 has been shown to be highly variable with a variable sensitivity, specificity, positive predictive value and negative predictive values ranging on the cut-off score chosen (ranging cut-offs 25-35) (100,101). These studies however essentially show that detection of CaP rises with rising PCA3 levels. The use of PCA3 in conjunction with PSA testing was shown to lower the number of unnecessary prostate biopsies in patients considered for initial or repeat biopsy (102). PCA3 has shown some potential as a prognostic marker, correlating with tumour aggressiveness (103). However the role of PCA3 remains controversial and limited with several studies assessing the role of PCA3 as a marker of CaP aggressiveness in relation to clinical/pathological stage, Gleason score, tumour volume and extra prostatic extension at

prostate biopsy showing no significant correlation (100,104,105). As a result PCA3 is not currently routinely used in clinical practice.

1.4.2.2 Transmembrane protease, serine 2-ETS fusion (TMPRSS2-ERG)

Transmembrane serine protease (TMPRSS2) is secreted by prostate epithelial cells in response to ligand exposure and this gene then becomes fused with sequences of the ETS family of transcriptional activators namely ERG. The resultant abnormal chromosomal rearrangement TMPRSS2-ERG results in overexpression of ERG and inhibits normal prostate differentiation (106). TMPRSS2-ERG is a urine biomarker that is highly CaP specific and has been found in approximately 50% of CaP cases (107,108). Expression of TMPRSS2-ERG was associated with a higher tumour stage but no association was seen with Gleason score, metastases, CaP specific death, biochemical recurrence or all cause mortality (108). The presence of urinary TMPRSS2-ERG in combination with serum PSA >10 ng/ml and detectable urinary PCA3 improved CaP detection rates (109). These suggest that TMPRSS2-ERG may be suitable as part of a biomarker panel used in combination with others to improve diagnosis. Indeed given that CaP is such a heterogeneous disease with each individual tumour displaying its own characteristics, a panel of biomarkers would be ideal to improve diagnosis of significant disease. A major challenge remains the ability to accurately predict the risk of harbouring particularly aggressive disease instead of insignificant cancer.

1.4.2.3 Oncotype DX® test

More recently genomic tools have been developed with the purpose of risk stratifying patients affected by disease to help clinicians decide on the most appropriate form of management or follow-up schedule for a particular disease profile. Oncotype DX® Prostate Cancer Assay is a multi gene RT-PCR expression array that was developed for use with fixed paraffin-embedded prostate needle biopsies. The assay measures 12 cancer related genes that represent four biological pathways; the androgen pathway the androgen pathway (*AZGP1, KLK2, SRD5A2*, and *FAM13C*), cellular organization (*FLNC, GSN, TPM2,* and *GSTM2*), proliferation (*TPX2*), and stromal response (*BGN, COL1A1,* and *SFRP4* and 5 reference genes which algorithmically calculates a Genomic Prostate Score (GPS) (110). A higher score correlates with a higher probability of adverse pathology at radical prostatectomy with men diagnosed with low or low-intermediate risk CaP on prostate biopsy. This assay has been validated clinically as a predictor of aggressive disease in this subgroup (111,112) and allows clinicians to discriminate patients with indolent prostate cancer from aggressive prostate cancer to help make the most appropriate treatment decisions, in this context for suitability of enrolment in active surveillance.

1.4.2.4 Decipher™ test

Decipher™ is another genomic test used to assess the probability of developing metastases and biochemical recurrence after radical prostatectomy. It is based on 22 expressed RNA biomarkers involved in multiple biological pathways (cell differentiation, proliferation, structure,

adhesion and motility, immune modulation, cell-cycle progression, androgen signalling) (113). Decipher™ has undergone multiple validation studies (114–117) demonstrating its ability to predict risk of metastasis at and biochemical recurrence 10 years and 3 years post radical prostatectomy respectively. Currently it has been approved in the United States for CaP patients treated with radical prostatectomy and adverse disease (pT3 and/or positive margins or PSA rise following surgery) to evaluate risk of clinical progression (development of metastases).

1.4.2.5 Prolaris

The Prolaris (Myriad Genetics, Salt Lake City, UT) score is a quantitative measure of the average expression of 31 cell cycle progression (CCP) genes and 15 reference genes in either a prostate biopsy or radical prostatectomy specimen. It is recommended for patients with low risk disease on a biopsy and a life expectancy of 10 years or more and is aimed at helping patients make the decision between active surveillance and active treatment although it may have some use in high-risk patients with adverse pathological features after surgery too (118). Studies have shown that the Polaris CCP score is able to predict the risk of biochemical recurrence after radical prostatectomy and 10-year survival in patients managed conservatively and the role of the CCP as an independent predictor of CaP death, biochemical recurrence and development of metastasis after radical prostatectomy (119,120,120).

1.4.2.6 ConfirmMDx

ConfirmMDx (MDxHealth) is a tissue based assay that assesses the genetic alterations surround the tumour lesions ("halo" effect) in the hope of reducing the need for repeat prostate biopsies (121). The test identifies the hypermethylation pattern of CpG island promoter regions of three genes (GSTP1, APC and RASSF) in men after a negative biopsy. ConfirmMDx has achieved a negative predictive value of 88-96% for high-grade cancer (122–124)

Genomic Test	Tissue type	Main Findings/Results
PCA3	Post-DRE urine	PCA3 score predicts biopsy outcome in combination with PSA, DRE and other clinical parameters (AUC = 0.71 – 0.75)
TMPRSS2-ERG	Post-DRE urine	Sensitivity 24.3% - 37% Specificity 93%. PPV 94%. When combined with PCA3 and PSA (AUC = 0.88)
Oncotype DX	Prostate biopsy	Genomic prostate score combined with clinical parameters (age, PSA, clinical stage and biopsy Gleason score is a predictor of high-grade or high stage disease and biochemical recurrence. Each 20-point increase in genomic prostate score was associated with a 2.3-fold increased risk of high-grade disease and a 1.9-fold increased risk of non–organ-confined disease. (AUC 0.63-67)
Decipher	Radical prostatectomy	Decipher score in addition to clinical variables predict 10-year distant metastasis after surgery (AUC = 0.81). Ability to predict occurrence of metastases (AUC 0.83-85)
Prolaris	Prostate biopsy	Cell cycle progression score is an independent predictor of CaP death, biochemical recurrence and metastasis after radical prostatectomy and radiation therapy.
Confirm MDX	Prostate biopsy	Methylation status of three genes (GSTP1, APC and RASSF) is able to identify men at higher need of repeat biopsy (sensitivity 68%, specificity 64 % and NPV of 88-96%, AUC = 0.742)

Table 3: Summary of genomic tests and summary of main findings/results (112,125–128)

1.4.3 Prostate biopsy techniques

Transrectal ultrasound (TRUS) guided prostate biopsies are the current main standard of care for diagnosis of CaP based on PSA level and/or suspicious DRE. The standard technique employed is the "double sextant" extended biopsy pattern described by Naughton et al (129), which involves taking 6 cores from the lateral peripheral zone of each prostatic lobe. These are taken via the rectum with the aid of a rectal ultrasound probe to visualise the prostate.

Newer techniques such as transperineal template saturation biopsies or targeted biopsies with the adjunct of multiparametric magnetic resonance imaging (MRI) is becoming more commonplace, with recent evidence from the PROMIS and PREDICT studies (130,131) highlighting the superiority of these techniques over standard TRUS sampling techniques.

Recent trends has been away from local anaesthetic TRUS guided prostate biopsies towards local anaesthetic transperineal targeted multiparamteric targeted prostate biopsy which has been shown to be safe, feasible and tolerable to be performed in the ambulatory setting (132). Freehand local anaesthetic transperineal template biopsies techniques have been associated with minimal complications and achieve higher cancer detection rates, as well as improving CaP diagnostic pathway waiting times (133,134).

1.4.3 Staging and risk stratification

1.4.3.1 TNM classification of prostate cancer

Once CaP has been diagnosed on a biopsy it is staged according to a combination of clinical and imaging findings. Staging is to assess the local extent of the disease in the prostate as well as to look for evidence of metastatic disease. The staging system used is The Tumour, Node, Metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC) (See Table 2.).

2010 TNM Staging System of prostate cancer	
Localised disease	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically unapparent tumour neither palpable nor visible by imaging
T1a	Tumour incidental histological finding in ≤ 5% of resected tissue
T1b	Tumour incidental histological finding in > 5% of resected tissue
T1c	Tumour identified by needle biopsy (e.g. due to elevated PSA level)
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
Local Extension	
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumour invades seminal vesicle(s)
T4	Bladder invasion, fixed to pelvic side wall, or invasion of adjacent structures
Metastatic disease	
N1	Positive regional lymph nodes
M1	Distant Metastasis

Table 4: 2010 TNM Staging System of Prostate Cancer (135)

1.4.3.2 D'Amico risk stratification

The D'Amico risk stratification system (136) is used to categorise localised CaP. Patients are divided into three risk groups (low, intermediate or high) based on PSA, Gleason score and clinical stage (See Table 3). This allows a framework to guide appropriate treatment options.

Definition			
Low-risk	**Intermediate-risk**	**High-risk**	
PSA <10 ng/ml and GS <7 (ISUP grade 1) and cT1-2a	PSA 10-20 ng/ml or GS 7 (ISUP grade 2/3) or cT2b	PSA > 20 ng/ml or GS > 7 (ISUP grade 4/5) or cT2c	any PSA any GS cT3-4 or cN+ any ISUP grade
Localised			Locally Advanced

Table 5: Modified D'Amico Risk Stratification from EAU guidelines (88)

1.4.3.3 Nomograms

A variety of staging nomograms (137–140) exist which integrate various specific clinical data to calculate the probability of various clinical outcomes including risk of extraprostatic extension, seminal vesicle invasion, lymph node metastasis and post treatment recurrence depending on which particular nomogram is used. The PREDICT prostate nomogram has been endorsed for use in CaP by NICE within the UK (92). The nomogram

provides individualised patient long-term prostate cancer-specific and overall survival estimates, estimating the potential treatment benefit on overall survival. This nomogram has been validated in a large external cohort and shown to be a robust and generalised model, which aids clinical decision-making reducing potential over-treatment of patients with CaP (141,141,142)

Despite the usage of these various staging tools it is still unclear what the optimal management for each individual patient diagnosed with CaP is. As the natural history of the disease is extremely heterogeneous, ranging from an incidental tumour, extremely unlikely to lead to a cancer specific mortality to extremely aggressive disease resulting in early metastasis and premature death. For patients diagnosed with high-risk disease the benefits of treatment are clear. However for patients diagnosed with low or intermediate risk disease (which accounts for the majority of patients diagnosed with CaP) (90), it is difficult to know whether radical treatment is necessary or not. Many of these low and intermediate risk tumours may remain indolent and unlikely to impact on patient life expectancy. In turn, undergoing unnecessary radical treatment may lead to significant life-altering side effects.

1.4.3.4 Multiparametric MRI

There have been significant advances made in the use of MRI imaging in prostate cancer diagnosis and staging. Multiparametric MRI is now routinely used for local staging in prostate cancer. The recent PROMIS study (130)

also highlighted the accuracy of multiparametric MRI in predicting the presence of high risk and intermediate risk (Gleason primary pattern 4) CaP. This has led to increasing use of MRI to direct the need for performing prostate biopsies in patients, and in some cases allows biopsies to be targeted to suspicious MRI lesions. Although this highlights the emerging validity of using MRI as a significant adjunct in CaP detection and allows avoidance of unnecessary biopsies, it has not yet replaced the need for biopsies to be performed for definitive diagnosis where necessary.

1.5 Treatment of localised prostate cancer

Localised CaP can be treated in a variety of ways. In many cases there is no single treatment option that can be recommended as being better than another. Often the decision to undergo a particular treatment is made after a careful counselling process, with all suitable options being considered.

1.5.1 Active surveillance

Active surveillance is often advocated for patients found to have low risk CaP in whom there is a low risk of progression and metastasis Active surveillance involves deferring radical treatment and adopting a close follow-up schedule with PSA monitoring and prostatic imaging with multiparametric MRI.

Whilst undergoing active surveillance, if there any concerns about disease progression patients will then be offered radical treatment. Although there is widespread acknowledgement that active surveillance is the best way of

managing patients with low risk CaP there is much controversy about the actual regime of monitoring for patients enrolled with widespread variation between centres. NICE has issued a recommended protocol for active surveillance with patients recommended to undergo a multiparametric MRI where they haven't already and PSA monitored every 3-4 months for the first year. At 12 months a DRE is recommended with a repeat multiparametric MRI, from year 2 and thereafter a PSA is recommended every 6 months with a DRE every 12 months. If there is concern about clinical or PSA changes at any time during active surveillance then re-assessment is recommended with either multiparametric MRI or re-biopsy (92). Patients often have a multiparametric MRI and transperineal template mapping biopsies of the prostate prior to enrolment in an active surveillance programme to ensure no under-staging of CaP grade and volume.

1.5.2 Radical prostatectomy

Radical prostatectomy involves surgical excision of the prostate gland. Historically this was performed via an open incision in the lower midline of the abdomen. More recently minimally invasive laparoscopic and robotic assisted surgical approaches have superseded this. Although minimally invasive robotic approaches have significantly reduced post-operative hospital stays, there are still significant risks associated with surgery including erectile dysfunction (50%), urinary incontinence (5-10%) and rectal injury (0.5%) amongst others.

Although radical prostatectomy is the only modality of treatment for localised CaP that has evidence to support its ability to reduce cancer-specific mortality (143), this data was obtained in the "pre PSA era". Recent studies have failed to show significant difference between patients undergoing radical prostatectomy versus watchful waiting (no active treatment) with regard to disease specific mortality (144). The PROTECT study compared treatments for localised prostate cancer which showed that at 10 years, prostate cancer specific mortality was low irrespective of treatment assigned (surgery, radiotherapy and active surveillance) with no significant difference among treatments. Surgery and radiotherapy were associated with reduction in disease progression and development of metastases than active monitoring. The results of PROTECT suggests that treatment particularly of low-risk prostate cancer has minimal effects on survival within the first 10 years after diagnosis irrespective of treatment (145,146). Nevertheless there is widespread acceptance that radical prostatectomy does prevent progression of localised CaP in younger men with intermediate or high-risk disease.

1.5.3 Radical radiotherapy

Radical radiotherapy can be delivered either by external beam therapy or seed brachytherapy. External beam radiotherapy is given in combination with androgen deprivation hormonal therapy (ADT), as evidence has shown significantly improved outcomes over radiotherapy alone(147). Despite advances in radiotherapy technology, there remain significant side effects largely related to unintended dose delivery to structures adjacent to the

prostate. These consist of bladder and bowel toxicity symptoms of cystitis, haematuria, incontinence, proctitis, diarrhoea, and erectile dysfunction as well as the risk of radiation-induced secondary malignancies.

Although patients with localised disease can be seen as 'suitable for all options' radiotherapy is often the treatment of choice in more elderly and less medically fit patients. Another important aspect when counselling patients particularly with regards to high-risk disease is the potential need for multi-modal therapy with adjuvant treatment. It is important to recognise, that if a patient underwent surgery initially and then developed a recurrence, it is easier to deliver salvage radiotherapy than perform salvage surgery.

1.6 Treatment of locally advanced prostate cancer and biochemical recurrence

Around 17-31% of men present with high-risk localised or locally advanced disease (148). Often these patients require treatment as if left untreated the associated 10- and 15-year CaP specific mortality rates are 28.8% and 35.5% respectively (149). Although treatment is associated with survival benefit there is no consensus on the optimal treatment for these patients. Treatment strategies often comprise of combination therapy with local and systemic therapies in a 'multimodal' approach.

Currently the European Association of Urology (EAU) CaP guidelines recommend radical prostatectomy with extended pelvic lymph node

dissection in a multimodal combination with possible post-operative radiotherapy and ADT, or external beam radiotherapy at a dose of 76-78 Gy or external beam radiotherapy with brachytherapy boost with long term androgen deprivation in men with a life expectancy greater than 10 years (150). There is no consensus on which treatment strategy is best with patients often managed according to surgeon experience and patient factors after careful counselling. There is reasonable evidence that both surgery and radiation treatment are good options provided they are delivered in combination with other treatments (151).

The definition of biochemical recurrence (BCR) differs according to the modality of the primary treatment. Post radical prostatectomy BCR is defined as PSA >0.2ng/ml, and post –RT >2ng/ml above nadir (152). Once a PSA relapse has been diagnosed it is important to differentiate whether it is likely to be a local or distant metastatic recurrence. This can often be deduced from initial clinical and pathological factors (T stage, Gleason score, PSA) and PSA kinetics post treatment (PSA doubling time and interval to BCR). Options for BCR after radical prostatectomy consist of salvage radiotherapy or ADT with delayed radiotherapy. For BCR after radiotherapy, options include ADT or local treatments such as salvage radical prostatectomy, cryotherapy, brachytherapy and HIFU however weak evidence means these are often only recommended within a trial setting. There are multiple phase 3 trials ongoing reviewing the management of BCR which will hopefully help guide future practice in BCR (RADICALS – NCT00541047 and RAVES – NCT00860652) (150).

1.7 Treatment of metastatic prostate cancer

The mainstay and first-line treatment for patients presenting with metastatic disease is ADT (153). EAU guidelines strongly recommend treating both asymptomatic and symptomatic patients with metastatic disease or discussing deferred ADT in well-informed patients (150) . The median overall survival for patients who present with metastatic disease is 42 months (153). In patients who are deemed fit with good performance status combined treatments (docetaxel, abiraterone, apalutamide) with ADT are recommended. The treatment options available for patients with metastatic disease are now numerous (154). The four-year follow-up of STAMPEDE , showed that ADT plus upfront docetaxel or abiraterone produced no significant differences between the two regimens in median OS, metastasis-free survival or CaP specific survival (155) . There is a continual debate with regards to which treatment to initially add to ADT and then how best to sequence further treatment thereafter. There are multiple on-going trials that will hopefully help determine the best treatment sequencing.

1.8 Racial differences in prostate cancer

1.8.1 Introduction

Men from western cultures, and especially black men, are known to have significantly higher CaP incidence and death rates. As previously mentioned, the incidence of CaP is 60% greater and the mortality rate 2-3 times higher in AH men compared with Caucasian men (9,156). Asian populations have the lowest CaP incidence and mortality but attain similar cancer rates of

those in western countries after migration to western countries (157). Autopsy studies from several countries have shown similar rates of clinically insignificant or low risk CaP despite significantly different CaP specific death rates amongst these racial populations (158). This suggests that although rates of clinically insignificant CaP is common to all racial groups, other unknown factors are promoting progression of the clinically insignificant low risk tumours into clinically significant tumours, resulting in more advanced and aggressive disease.

1.8.2 Identifiable risk factors for disparity

CaP in AH men presents at a younger age, with higher grade disease and more advanced stage, at time of diagnosis (159–161). This accounts for the disparity in increased CaP specific mortality rates seen in AH men. Several explanations have been proposed to account for this disparity. For example modifiable risk factors such as, demographic characteristics, socioeconomic status, associated co-morbidities and access to healthcare may limit cancer screening in AH populations, resulting in delayed presentation. Another explanation is accounted for by differences in tumour biology, related to dietary, hormonal or molecular factors, resulting in more aggressive disease.

1.8.3 Modifiable risk factors

In the United States (US) racial and ethnic minorities have a significantly higher rate of poverty, lower educational status and less access to health care or source of primary care. Further more, statistics from the National Centre for Health Statistics in the US, 49.5% of AH men were found to have a

poverty rate of over 20% compared to 7% of Caucasian population (162). It's plausible therefore that this bears some significance in disparity of disease presentation between these racial groups. However even when these factors are controlled for, it is apparent that tumour biological and genetic factors remain significant (163).

1.8.4 Molecular, Genetic and Biological Risk Factors

1.8.4.1 Introduction

Racial disparity can be potentially explained by inherent differences in genetic and tumour biology in AH men, attributable to hormonal, dietary or molecular factors.

Several studies have highlighted significant discrepancies in tumour-specific gene expression between AH and Caucasian populations (164). These genes could be influenced by environmental factors such as diet, obesity, hypertension and inflammation resulting in changes to tumour biological pathways. Several biological mechanisms and molecular pathways have been shown to be differentially expressed in CaP tumours from AH and Caucasian men, including those regulating apoptosis and proliferation, cell adhesion and EMT, inflammation and immune response pathways (165–167).

1.8.4.2 Diet and Obesity

AH men are reported to have a higher fat content in their diet and are more obese with higher rates of hypertension (168–170). Dietary fat intake has

been consistently associated with CaP risk and some studies have shown that it may even promote more aggressive disease (171,172). The fact that body mass influences serum androgen levels, which could affect CaP growth mechanisms suggests that a relationship between obesity and CaP could exist. Evidence however, trying to establish this link, has been largely inconsistent with no clear association demonstrated despite several studies (173,174). However a study reviewing recurrence rates of CaP, in AH and Caucasian men, undergoing radical prostatectomy showed that obesity was associated with higher grade cancer and higher rates of recurrence after surgery (169). Interestingly the biological mechanisms associated with obesity and hypertension result in an inflammatory response with release of tumour necrosis factor – α (TNF- α), resultant release of inflammatory cytokines, release of reactive oxides and activation of nuclear transcription factor – kB (NF- kB). NF-kB is a transcription factor involved in the regulation of a wide variety of biological responses including up regulation of androgen signalling pathways and is reported to increase CaP proliferation (175). Androgen receptor (AR) protein expression has been shown to be 22% higher in AH men than Caucasian men (176), suggesting CaP may occur at a younger age and more rapidly in AH due to differences in androgenic stimulation.

1.8.4.3 Molecular Pathways

1.8.4.3.1 Apoptosis and Proliferation

An epidemiology study in Detroit examining radical prostatectomy specimens showed increased CaP volume and greater Gleason grade in AH

men compared to Caucasian men. Furthermore metastatic disease occurred at 4 times more commonly in AH men (177). The author proposed that these findings supported the hypothesis that CaP grows more rapidly in AH men with an earlier transformation to aggressive CaP. As previously discussed, normal prostate growth is regulated by a careful balance between proliferation and apoptosis. The possibility that aberrant signalling pathways controlling this fine balance could lead to CaP is well documented (178,179). Androgen expression stimulates cell proliferation as well as inhibiting the rate of glandular epithelial cell death. It is therefore plausible that in AH men, increased androgen receptor expression could result in altered gene expression, resulting in a state of down regulation of apoptotic pathways and concomitant increases in proliferation of malignant glandular epithelial cells, resulting in more aggressive disease.

The bcl-2 family of apoptosis regulatory products has previously been discussed and can either be pro-apoptotic (bax, bak, bad) or anti-apoptotic (bcl-2, bcl-X_L, Mcl-1) (180,181). Anti-apoptotic activity of bcl-2 would lead to cell proliferation and accumulation of genetic abnormalities resulting in progression of cancer. Overexpression of bcl-2 has been shown to correlate with tumour progression to androgen independent disease states (182–184). Apoptosis and cell proliferation has been compared previously in age matched AH and Caucasian patients who had undergone a radical prostatectomy for localised disease. Prostate tumour cells were analysed for expression of bcl-2, bax and ki67. Interestingly bcl-2 was detected at significantly higher levels in tumours from Caucasian men than AH men, and

the rate of cell proliferation was similar (166). The author suggested that down regulation of bcl-2 expression may be potentially responsible for loss of apoptotic control and suggests that the difference in expression may be related to raised testosterone levels seen in AH men. Despite showing a down regulation of bcl-2, it is clear that there is a difference in kinetics of tumour growth between the two racial groups. This may explain the differences seen in clinical behaviour of CaP between the two ethnic groups.

1.8.4.3.2 Cell Adhesion, Migration and changes to EMT

EMT is increasingly thought to be involved in cancer progression and development through the process of enabling epithelial-derived cancer cells to migrate to distant sites during metastasis. It is this process that could account for earlier metastatic development and more aggressive disease seen in AH men.

As previously discussed EMT is a normal physiological process that involves reversible transdifferentiation of epithelial cells in response to local stimuli. Prostate cancers exhibit EMT-like states with changes in various genes associated with invasive or aggressive behaviour. The problem with CaP is that many of these processes are seen in benign tissue and are often difficult to differentiate from the changes seen in malignant cells. One potential explanation for EMT processes is that local tumour microenvironments that resemble EMT states, might promote high-grade patterns. Findings from several human prostate cancer cell lines studies have shown that cells with

more mesenchymal features exhibit a more aggressive and invasive phenotype (185).

The mechanisms involved in EMT-like states is extremely diverse and complex but essentially involves inducers (cell extrinsic inductive stimuli), controllers (cell-intrinsic means of interpreting stimuli) and effectors (proteins that mediate EMT) (186). A potential hypothesis is that local microtumour environments send signals or stimuli to induce EMT-like states such as growth factors TGF-β, fibroblast growth factor (FGF), insulin-like growth factor (HGF), platelet-derived growth factor (PDGF) as well as hypoxic states (inducers). These trigger downstream signalling of transcription factors via MAPK, SMAD, GSK3β and NFkB pathways which results in activity of transcription repressors in the ZEB, Twist and Snail pathways (controllers). The overall results of this process is decreased epithelial cell adhesion molecules such as E-cadherin and induction of other mesenchymal proteins (effectors) (186) (see Figure. 6).

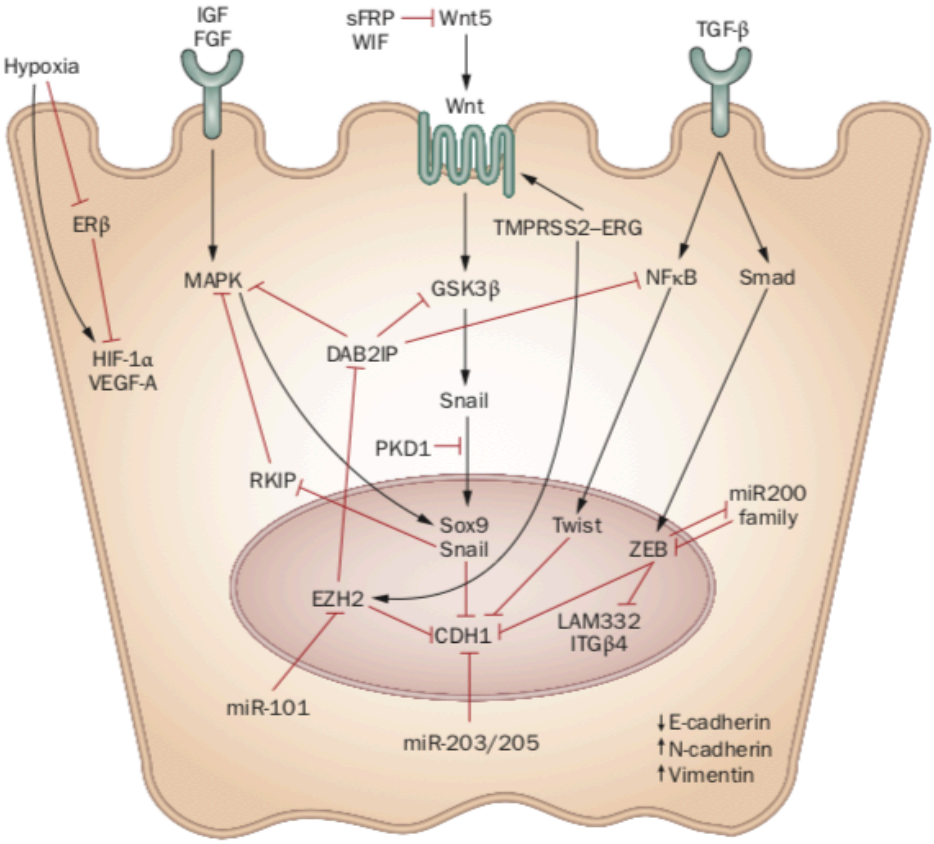


Figure 6: Mechanism of EMT-like states (reused with permission from Springer Nature, licence number 4531560043149) (186)

Several studies have shown that known gene EMT pathways and processes are differentially expressed in AH CaP specimens compared to Caucasian CaP tumours (164,165,167). This suggests that EMT processes in tumour-adjacent stroma may be responsible for the differences observed in tumour behaviour, particularly the aggressive and invasive characteristics seen in CaP tumours in AH men. Kinseth et al (167) showed that extracellular matrix (ECM), integrin family and signalling mediators of EMT pathways were all down regulated in the stroma of AH men. Many genes involved with cell adhesion and stress fibre formation via integrin signalling were down regulated in AH tumours. Decreased cell adhesion and disruption of

the normal ECM are classical features of EMT (187). Down-regulation of these signalling pathways suggests increased levels of EMT in AH tumours.

1.8.4.3.3 Inflammation

It is thought that about 20% of all human cancers are caused by chronic infection or chronic inflammatory states (188). Chronic inflammation is thought to be a possible mechanism in CaP development (189,190). The causes of prostatic inflammation are unclear but several mechanisms have been proposed including dietary factors, hormonal factors, urine reflux, trauma and direct infection.

The molecular mechanisms that are thought to underlie the pathogenesis of inflammation-associated carcinogenesis are complex and are heavily interlinked with host immune response mechanisms. Initially highly reactive compounds including hydrogen peroxide and nitric oxide are released from phagocytic inflammatory cells which then leads to oxidative damage to the DNA in the epithelial cells. These epithelial cells must then be replaced and cells that undergo DNA synthesis in the presence of these DNA altering states give rise to mutated epithelial cells. An inflammatory state also gives rise to increased cytokine release, which promotes epithelial cell proliferation. Inflammatory cells also migrate readily through the ECM resulting in increased EMT states facilitating epithelial cell invasion and possible metastasis.

Histologically, prostate biopsy lesions that contain either acute or chronic inflammatory infiltrates are associated with atrophic epithelium or focal epithelial atrophy (191). Compared to normal epithelium these areas of focal epithelial atrophy display increased epithelial proliferation rates described as proliferative inflammatory atrophy (PIA) (191). Transition has been seen from these areas of PIA to high grade PIN (192).

Interestingly studies have shown that inflammation is more prevalent in non-tumour prostate biopsy specimens from AH men than Caucasian men (193). Powell et al (165) showed that inflammatory cytokines IL6, IL8 and IL1B were significantly more expressed in CaP from AH men than CaP in Caucasian men. These inflammatory cytokines have been linked to CaP development (194,195). Increased levels of IL6 has been associated with higher Gleason scores and aggressive CaP (195). IL1B has been shown to increase production of IL8, which promotes prostate cell proliferation and migration (194). Kinseth et al (167) also demonstrated differential expression, of several cytokine signalling pathways including IL6, between AH and Caucasian tumour tissue samples.

1.8.4.3.4 Immune Response

The interaction of inflammation and immunoregulation is complex. Both the innate and humoral immune system are capable of providing antitumour activity. Tumours however are able to develop mechanisms to suppress and evade the immune system. In some cases the immune system itself has been shown to facilitate tumour progression and development (196).

Prostate cancer like many human cancers develops in an immunologically intact host, progressing from localized low-grade disease to eventual metastatic disease. This progression involves an extensive interaction with the host immune system. Although the mechanisms of evasion and modulation of the immune system in CaP are not fully understood, it is thought that immune effector cells which recognize tumour antigens become desensitised and incapable of recognising and killing tumour cells (197–200). This process is thought to occur through induction of regulatory or suppressor T cells. Regulatory T cells are a subset of $CD4^+$ T cells that act to suppress autoreactive T cells and the process of autoimmunity. High levels of T regulatory cells have been shown to confer a poorer prognosis in several solid tumours (201). CaP patients have been shown to have higher levels of regulatory T cells in their peripheral blood compared to normal donors (202). The presence of regulatory T cells has also been demonstrated using immunohistochemistry (IHC) surrounding CaP lesions (203). Interestingly one of the many roles of IL-6 is B cell activation, highlighting the close association between inflammation and immune response.

Due to the powerful capabilities of the anti-tumour immune system, researchers have looked at immunotherapeutic treatment options for CaP treatment. These efforts have focussed on vaccination and immune checkpoint blockade. The goal of immunotherapy treatments has been to activate a population of effector T cells able to mediate specific lysis of

tumour cells. Several antigen-specific approaches have been developed over the past several years (204). In these approaches a tumour-associated antigen is directly targeted either by loading the antigen into vaccine vector. Several vaccines have shown promise through clinical trials, the most notable including Sipuleucel-T (Provenge, Dedreon Corp.) and PROSTVAC-VF (Bavarian Nordic), however these have not shown any significant overall survival advantages (205,206).

Recent studies have focused on the concept of immune checkpoints. These are a collection of molecules expressed by previously activated immune cells that limit the on-going immune response. The aim is by blocking these checkpoint molecules; the immune system response against tumour cells can be sustained. The most substantially studied checkpoint molecule is cytotoxic T lymphocyte antigen-4 (CTLA-4), which is expressed by activated T cells. Of the antibodies specific for CTLA-4, Ipilimumab (MDX-010; Bristol-Myers Squibb/Medarex) and Tremelimumab (CP-675206; Pfizer) are the currently most well developed agents. Ipilimumab has been evaluated in patients in prostate cancer in phase II trials and has shown a reduction in PSA levels (207).

Another checkpoint molecule gathering interest in immunotherapy treatment is programmed cell death 1 (PD-1). PD-1 is expressed by activated T cells and when engaged by its ligand PDL-1 results in T cell inhibition and apoptosis. PD-1 blockade results in an antitumour response (208,209). There have been very promising results from clinical trials using

monoclonal antibodies for PD-1 blockade in several advanced malignancies (210,211). Although PD-1 has been less well studied in CaP, prostate infiltrating lymphocytes have been found to express PD-1 (212). This suggests that PD-1 blockade may be a promising strategy in prostate cancer immunotherapy.

The Checkmate 650 trail – NCT02985957 evaluated the combination treatment of Nivolumab and ipilimumab in patients with metastatic castrate resistant prostate cancer. This study was designed on findings that ipilimumab can increase tumour-infiltrating T-cells in the prostate with upregulation of the PD-1/PD-L1 inhibitory pathway. The overall response rate was 25% and 10%, median overall survival was 19 and 15.2 months in the chemotherapy-naïve and chemotherapy treated men respectively (213). This is better than historical survival data for treatment with immune-checkpoint monotherapies (9.6-11.2 months) (214–216). There were however considerable grade 3/4 adverse events (42-53%) and treatment related deaths (4.4%) associated with treatment, with approximately 38% of patients in each cohort discontinuing treatment due to side effects also (213).

Kinseth et al (167) found that 20% of the differentially expressed genes found between AH and Caucasian CaP samples in stroma tissue were related to immune processes. Furthermore significant numbers of differentially expressed tumour associated pathway genes were related to immune responses. Wallace et al (164) showed significant differences in tumour

immunobiology between AH and Caucasian men with the majority of differentially expressed genes relating to immune response and cytokine signalling pathways. This heavily suggests that immune processes may be linked to racial disparities in CaP.

1.9 Aims and objectives

The aim of this research project was to:

1. Identify a panel of prostate cancer associated protein biomarkers that could potentially be used to predict clinical outcomes – these being development of metastatic disease and prostate cancer specific death.
2. To review if these biomarkers displayed differential protein expression in AH and Caucasian men, to try to explain the disparity in aggressive disease between these two populations and to potentially review the molecular causes or pathways for these differences.

By being able to identify patients potentially at risk of developing more aggressive disease means treatment could be targeted more appropriately.

2.0 Materials and methods

2.1 Study design overview

This study involved the comparison of a panel of candidate biomarkers, selected according to techniques described below, against two different tissue microarrays – one Caucasian-predominant historic TMA and a newly constructed AH TMA.

A historical TMA was previously constructed, from predominantly Caucasian men of patients diagnosed with CaP between 1999 and 2001, by Dr Des Powe, Histopathology Department, Nottingham University Hospitals. A new TMA was constructed by, Mr Philip Goodall and Dr Des Powe, with tissue from AH patients diagnosed with CaP obtained from University of Birmingham human tissue biorepository. Both TMAs were then immunostained for protein expression of a selection of candidate biomarkers, selected from bioinformatics techniques and a thorough literature review described below. Staining was reviewed and scored independently by Philip Goodall and Des Powe.

The North West 7 Research Ethics Committee approved the use of the tissue samples from the historic predominantly Caucasian TMA – Greater Manchester Central REC number 10/H1008/72 previously. The East Midlands – Nottingham 1 Research Ethics Committee approved the construction of the new AH TMA and further use thereafter – East Midlands Nottingham 1 REC number 15/EM/0499.

Patient outcome data for the patients incorporated into the historic predominant Caucasian TMA was previously collated into a Microsoft excel spreadsheet by Mr Will Green as part of a previous thesis project. Multiple clinical outcome data parameters were recorded, as discussed below. Relevant clinical outcome data and protein biomarker expression was statistically analysed using SPSS to determine the ability of biomarker protein expression to predict prostate specific survival, tumour recurrence, metastasis development and treatment failure.

2.2 Patient cohort and data collection

The historic TMA was populated with archived wax embedded CaP tissue from 365 patients diagnosed with CaP between 1999 to 2001. These were consecutive non-selected patients that underwent 'best-practice' treatment at Nottingham City Hospital, Nottingham, UK. Initial CaP diagnosis was made from either prostate needle core biopsy, TURP specimens or radical prostatectomy specimens. Patients were asked if they would allow their tissue to be used for research purposes, with those in agreement incorporated into the TMA. Previously Green et al had used this TMA and clinical cohort to demonstrate that Ki67 was predictive of CaP specific survival and development of future metastases and DLX-2 – a novel biomarker, was predictive of development of metastases (217)

The majority of the patients incorporated into the TMA were diagnosed with CaP incidentally from tissue taken during a TURP and therefore treatment naive. This was largely in the context of treatment for urinary retention or

lower urinary tract symptoms and not diagnosis of CaP, as this procedure is not intended to detect or diagnose CaP. This means the majority of patients were diagnosed with coincidental CaP at TURP (T1a/b disease). This doesn't reflect current practice in the modern 'PSA screening' era which has led to a dramatic increase in the number of men
investigated for CaP with the use of standard TRUS biopsy and more recently transperineal template biopsy with the use of multiparametric MRI. This has led to the majority of patients being diagnosed with organ confined CaP.

The change in practice in diagnosis of CaP in the PSA screening era has led to many criticisms of studies carried out prior to this on the basis they are not relevant to current clinical practice. Despite this, although the number of patients diagnosed with CaP via TURP is smaller, it is still a relevant population. The management of these patients is often very similar to biopsy detected CaP, including the use of active surveillance if asymptomatic with a low PSA and Gleason grade. As well as this, the collected clinicopathological data has over 13 years of extended follow-up data, providing a powerful data tool in relevant analysis. The patient cohort characteristics incorporated into the TMA are shown in Table 4 below.

Clinical Variable		Number of Patients
Method of CaP Detection	TURP	279 (76%)
	Radical Prostatectomy	26 (7%)
	TRUS Biopsy	54 (15%)
	Not recorded	6 (2%)
PSA (ng/ml) at diagnosis	<4	34 (9%)
	>4	237 (65%)
	Not recorded	94 (26%)
Gleason Score	≤7	141 (39%)
	≥8	156 (43%)
	Not recorded	68 (19%)
D'Amico Risk group	Low	54 (15%)
	Intermediate	33 (9%)
	High	202 (55%)
	Unclear	76 (21%)
Androgen Deprivation Therapy	Yes	197 (54%)
	No	87 (24%)
	Unclear	81 (22%)
Castrate Resistance	Yes	127 (65% of those patients castrate)
	No	70 (35% of those castrate)
Metastatic Disease at diagnosis	Yes	44 (12%)
	No	257 (70%)
	Not recorded	64 (18%)
Development of subsequent metastatic disease (in those without metastases at diagnosis)	Yes	85 (23%)
	No	167 (46%)
	Not recorded	69 (19%)
Death due to prostate cancer	Yes	134 (37%)
	No	92 (25%)
	Unknown	87 (24%)
	Still Alive	52 (14%)

Table 6: Clinical Characteristics of prostate cancer patients incorporated into historic TMA comprising Nottingham derived patients

A prepopulated Microsoft Excel spreadsheet database was previously created and populated by Mr Will Green and included clinical factors for the patients incorporated in to the TMA. The following factors were recorded and incorporated into the database from reviewing patient medical notes and hospital IT results system software ('NOTIS'); racial origin, date of diagnosis, initial PSA, initial DRE, mode of tissue diagnosis, Gleason 1 score, Gleason 2 score, Overall Gleason score, Initial D'Amico Risk, bone mets at diagnosis, radical prostatectomy histology, active surveillance, radiotherapy, Date of radiotherapy, subsequent development of mets, date of mets, location of mets, months to mets from diagnosis, chemotherapy, androgen deprivation therapy, death, date of death, prostate cancer related death, other cause of death, months to all cause death, months to prostate cancer specific death, months to castrate resistance. Patient identifiable information was removed other than a derived 'patient analysis number'. Gleason scoring was modified in 2005 by the International Society of Urological Pathology (ISUP) consensus (218) therefore all cases were histologically reviewed and Gleason scored according to contemporary ISUP guidelines.

Given the lack of AH patients within the Nottingham patient cohort incorporated into the previous TMA, AH CaP specimens were sourced from University of Birmingham human tissue biorepository. 35 AH patients' CaP specimens were received and incorporated into a newly created TMA. The CaP tissue specimens obtained were a mixture of post radical prostatectomy and TURP specimens, the majority being post radical prostatectomy

specimens. The tissue was obtained from patients diagnosed and treated for CaP from 2001 to 2015. The patient characteristics of the AH CaP tissue incorporated into the TMA is shown below in Table 5.

Clinical Variable		Number of Patients (%)
Method of CaP Detection	TURP	3 (9%)
	Radical Prostatectomy	32 (91%)
PSA (ng/ml) at diagnosis	<4	1 (3%)
	>4	34 (97%)
Gleason Score	≤7	31 (89%)
	≥8	4 (11%)
D'Amico Risk group	Low	5 (14%)
	Intermediate	20 (57%)
	High	9 (26%)
	Unclear	1 (3%)
Antigen Deprivation Therapy	Yes	1 (3%)
	No	34 (97%)
Castrate Resistance	Yes	1 (100% of those patients castrate)
	No	34
Metastatic Disease at diagnosis	Yes	0 (0%)
	No	35 (100%)
Development of subsequent metastatic disease (in those without metastases at diagnosis)	Yes	1 (3%)
	No	34 (97%)
Death due to prostate cancer	Yes	0 (0%)
	No	35 100%)

Table 7: Patient characteristics of AH patients incorporated into new AH TMA.

AH patient factors were requested from Birmingham Biobank including; current age, date of specimen, Gleason score 1, Gleason score 2, overall

Gleason score, T stage at diagnosis, PSA at diagnosis, PSA at surgery, diagnosis method, Prostate cancer specimen type, biochemical recurrence, commencement of androgen therapy, development of castrate resistance, months to development of castrate resistance, treatment with radiotherapy, treatment with chemotherapy, death, mets at diagnosis, subsequent development of mets, date of mets, location of mets, months to mets from diagnosis. AH patients identifiable information was removed and given a unique 'generated patient analysis number' and incorporated into the previously created Microsoft excel spreadsheet relating to the historic TMA to allow combined analysis.

2.3 Tissue microarray construction

Mr Will Green and Dr Des Powe constructed the previous TMA, using archival wax-embedded TURP and radical prostatectomy samples sourced by the Nottingham Health Science BioBank.

A new TMA was constructed by Mr Philip Goodall and Dr Des Powe using archived AH wax embedded TURP and radical prostatectomy samples, sourced from University of Birmingham BioBank. Histology sections were reviewed by a Consultant Uropathologist (Dr Geoffrey Hulman – University Hospital Nottingham), Mr Philip Goodall and Dr Des Powe for evidence of CaP. At least two 0.6mm diameter donor cores were taken from the identified tumour sites per patient using an automated TMA Grand Master instrument (3DHistech LTD, Hungary) and placed in paraffin blocks. Each block is able to accommodate up to 100 cores. All patients were

represented with at least two cores with some patients represented by four cores. Cores were aligned in 2 blocks in a specific grid pattern from A1 to H10 for the first block and A1 and I4 for the second block, with each core serially numbered. Each core was given a unique 'NPN' number, starting from 4000 for core A1 in the first block continuing in sequence to 4115 for core I4 in block two. This was incorporated in to the previous Excel spreadsheet created by Mr Will Green that included the NPN number relating to the cores in each of the historic TMA's previously created, starting from 2000 to 2968. A significant numbering gap was left to allow for future incorporation of new specimens and for appropriate segregation between data sets to exist. Generation of the NPN allowed for subsequent analysis of biomarker scores and clinicopathological data relating to specific tissues samples from specific patients without compromising confidential personal data.

TMA sections from both TMA's were cut at a thickness of 4μm on a microtome for IHC.

2.4 Selection of candidate biomarkers

2.4.1 Artificial neural network analysis

The panel of candidate biomarkers were selected via two methods. The first was using a bioinformatic ANN approach to analyse a commercially available AH CaP gene expression array to identify genes that were associated with metastasis development. An ANN approach was utilised because of the ability of these techniques to assess and assimilate vast gene

data arrays with regards to a specific outcome or question (in this case gene association with metastasis development) and are able to predict futures outcomes based on previously learnt behaviour. The second was based on a thorough and comprehensive literature review of the molecular pathways thought to be responsible for the more aggressive disease seen in AH men.

Artificial neural analysis of a publically available prostate cancer DASL RNA gene expression profiling microarray (data source – series GSE41969) (165), consisting of both Caucasian and AH samples, was performed by Professor Graham Ball (Nottingham Trent University) identifying genes and therefore potential candidate biomarkers associated with increased risk of development of metastases. The gene microarray consisted of 517 genes in 639 tumour samples (270 African Heritage, 369 European American men) and 163 matched normal (control) samples (80 African Heritage, 83 European American Men). Categorised Gleason's grade between the 2 groups was significantly different, with African Heritage group having a higher prevalence of high Gleason's grade (categorised as Gleason's grade 7 (4+3), 8, 9 and 10) . The ANN modelling used a supervised learning, multi-layered approach with five hidden outputs and randomised initial weights. The learning rate and momentum were set to 0.1 and 0.5 respectively. The outputs were coded as 0 if the patient showed no evidence of metastasis within 5 years and 1 if metastasis was present. Prior to training each model the data was randomly divided into three subsets; 60% for training, 20% testing (to assess model performance during the training process) and 20% for independent validation (to independently validate the model on

previously unseen data) (219). Learning occurs after input of the training data and stops when the error of the generated outputs fails to increase for a specified number of training cycles. When this process is complete the ANN model is validated on the remaining 20% set aside for independent validation. This process of random sample cross- validation was reiterated 50 times for each input generating robust generalised models. The models are then trained over 50 randomly selected subsets with ANN predictions generated from average mean square error values calculated for each input generated. The highest-ranking values and those shown to be most capable of class prediction (development of metastasis) were then selected for further inclusion in subsequent models with each of the remaining inputs then sequentially added to the previous best input, generating an n – 1 model with two inputs. Training was then repeated with the process repeated and generation of an n – 2 model with three inputs and continued until no significant improvement was gained by addition of further inputs. This process was repeated generating a final model of gene expression most associated with prediction of metastasis development in both AH and Caucasian sub groups.

The ANN model was visualised with the use of cytoscape (220), allowing visualisation of the ANN model generated. Genes were identified from the cytoscape interaction network maps according to their level of influence, with identification of potential 'hub' genes. 'Gene interaction maps' were generated for genes associated with metastasis development in both the AH and Caucasian groups. The AH gene map generated from the African

Heritage gene microarray was used to identify potential 'hub' genes. The AH interaction map is shown below in Figure 8. Two AH specific genes were identified from this interaction map for their novelty and significant level of interaction – CYB561 (Cytochrome B561 – Gene accession number NM_001017916) and EPB41L4A (Erythrocyte Membrane Protein Band 4.1 Like 4A – Gene accession number NM_022140).

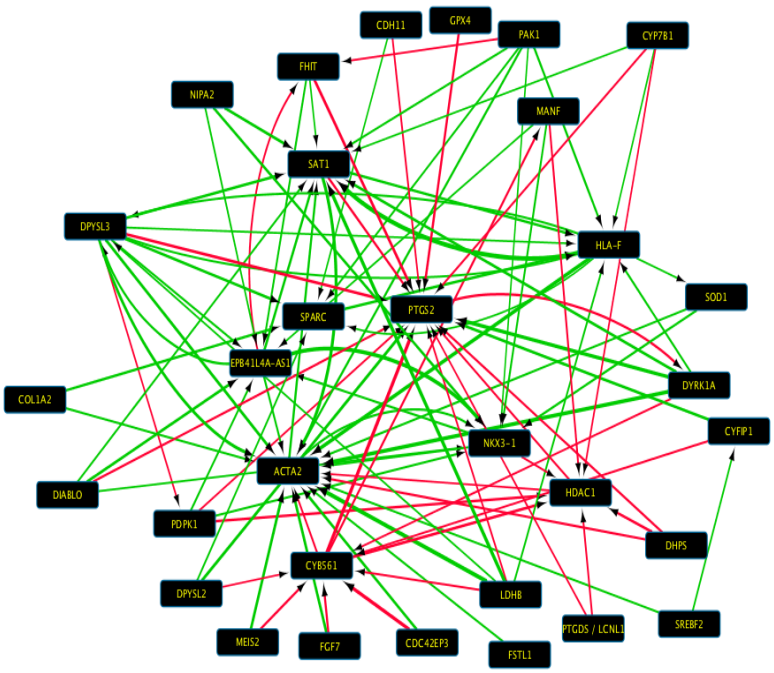


Figure 7: African Heritage Cytoscape Artificial Neural Network Gene Interaction map. The colour indicates whether a particular gene positively (green) or negatively influences (red) with the arrow showing the path of influence. The thickness of the line represents the degree of influence.

2.4.2 Validation of previous studies

DLX2 (Distal-less homeo box 2 – Gene accession number NM_004405.2) and proliferation marker Ki67 were included to validate results from Mr Will Green's previous work (217).

Immunoglobulin free light chains (FLCs) lambda and Kappa were included for their increased association in tumour associated inflammation pathways (Dr Des Powe) in poor prognosis basal type breast cancer (221).

2.4.3 Literature review

The remaining candidate biomarkers were selected from a curated extensive literature search using Ovid Medline search engine to identify biomarkers associated with different prostate cancer molecular pathways thought to potentially be able to predict disease specific survival as well as differential expression in Caucasian and AH populations. TGF-β (Transforming Growth Factor β 1 – Gene accession number NM_000660) was included given its multifunctional involvement in cell proliferation, differentiation and survival/apoptosis of cells. PD-L1 (Programmed death ligand 1 – gene accession number NM_014143) was included because of its relation to the immune system pathway. PTEN (Phosphatase and tensin homolog – gene accession number NM_00314) was selected for its role as a tumour suppressor gene. The following searches were performed using Ovid Medline:

1. 'prostate cancer', 'race' – this search returned 10 results, of which 7 papers were suitable for review.

2. ‘prostate cancer’, ‘African’ – this search returned 16 results of which 16 papers were suitable for review.
3. ‘prostate cancer’, ‘epithelial-mesenchymal transition’ – this search returned 5 papers of which 5 were suitable for review
4. ‘prostate cancer’, ‘genomics’ – this search returned 49 papers of which 24 were suitable for review.
5. ‘prostate cancer’, ‘inflammation’ – this search returned 5 papers of which 3 were suitable for review.

The selected candidate biomarkers are shown below in table 6.

Gene Name	Gene accession Number	Gene Protein
CYB561	NM_001017916	Cytochrome B561
EPB41L4A	NM_022140	Erythrocyte Membrane Protein Band 4.1 Like 4A
DLX2	NM_004405.2	Distal-less homeo box 2
Ki67	NM_002417	Proliferation marker
Kappa FLC	NC_000002.12	Immunoglobulin kappa constant
Lambda FLC	NC_000022.11	Immunoglobulin lambda constant 1
TGF-β	NM_000660	Transforming Growth Factor β 1
PD-L1	NM_014143	Programmed death ligand 1
PTEN	NM_00314	Phosphatase and tensin homolog – gene accession number

Table 8: Selected candidate biomarkers

2.5 Immunohistochemistry

Selected biomarker antibodies and dilutions used for IHC staining of both TMAs are shown in table 7.

Gene Name	Gene Protein	Antibody Manufacturer	Clone	Positive Control	Dilution
CYB561	Cytochrome B561	Abcam Ab185304	Rabbit Polyclonal	Human stomach	1:200
EPB41L4A	Erythrocyte Membrane Protein Band 4.1 Like 4A	SIGMA HPA036580	Rabbit Polyclonal	Human tissue – optimised on TMA	1:300
DLX2	Distal-less homeo box 2	Abcam Ab18188	Polyclonal	Mouse Brain	1:1500
Ki67	Proliferation marker	Leica NCL-L-MM1	Clone MM1	Colorectal Tissue	1:25
Kappa FLC	Immunoglobulin kappa constant	Unknown	Mouse Monoclonal	Optimised on TMA	1:100
Lambda FLC	Immunoglobulin lambda constant 1	Unknown	Mouse Monoclonal	Optimised on TMA	1:100
TGF-β	Transforming Growth Factor β 1	Abcam Ab92486	Rabbit polyclonal	Mouse muscle lysate	1:100
PD-L1	Programmed death ligand 1	Merk/Millipore Abc324	Rabbit polyclonal	Human Tonsil	1:400
PTEN	Phosphatase and tensin homolog – gene accession number	Unknown	Unknown	Unknown	Unknown

Table 9: Antibodies used for immunohistochemical staining of TMAs.

Optimisation of antibody dilutions were initially performed using recommendation positive and negative control tissues at dilutions where suggested by the antibody suppliers. The kappa and lambda FLC antibody was resourced from Dr Des Powe and optimised on the historic TMA. Dr Wakkas Fadhil, Nottingham University QMC Hospital IHC department, stained the TMAs for PTEN independently with a previously optimised antibody. Positive and negative control samples were analysed and

reviewed by Mr Philip Goodall and Dr Des Powe for staining verification. In samples where it was not possible to differentiate between the controls, dilutions were titrated as appropriate to establish optimal antibody dilutions. The optimal Ventana auto-stainer protocol was established for each antibody by comparing ultraview and optiview procedures with or without additional amplification steps (see Appendices A-C).

After optimising each biomarker antibody, 4µm sections from each individual TMA block were mounted on Dako REALTM capillary cap microscope slides, 75µm (grey), S2024 (Dako UK Ltd). TMA sections representing the entire Caucasian and AH cohorts underwent IHC using The Benchmark Ultra Ventana™ multi-system auto staining system according to the optimal protocol established from optimisation for each biomarker antibody. The Ventana staining protocol used for each biomarker antibody is listed in table 8 below with detailed steps of each protocol described in Appendices A-C. Once slides had been stained they were mounted using a Leica Biosystem automated slide mounting system.

Gene Name	Gene Protein	Ventana Protocol/ Protocol Number	Ventana Procedure
CYB561	Cytochrome B561	UV SCC1 + 32 4000	U ultraView DAB
EPB41L4A	Erythrocyte Membrane Protein Band 4.1 Like 4A	OV SCC1 + 32 4991	U OptiView DAB IHC v5
DLX2	Distal-less homeo box 2	UV SCC1 + 32 4000	U ultraView DAB
Ki67	Proliferation marker	Ki67 Roche RTU 4954	U ultraView DAB
Kappa FLC	Immunoglobulin kappa constant	UV SCC1 + 32 4000	U ultraView DAB
Lambda FLC	Immunoglobulin lambda constant 1	UV SCC1 + 32 4000	U ultraView DAB
TGF-β	Transforming Growth Factor β 1	UV SCC1 + 32 4000	U ultraView DAB
PD-L1	Programmed death ligand 1	OV SCC1 + 32 4991	U OptiView DAB IHC v5

Table 10: Ventana staining protocol for each biomarker antibody

Immunostained TMA sections were assessed to determine the most appropriate IHC scoring technique for quantifying protein expression dependent on what type of cell staining was present. Some antibody's generated different types of staining (cytoplasmic and nuclear). Where this was evident both types of cell staining was scored. This wasn't seen for every antibody though. Mr Philip Goodall and Dr Des Powe independently assessed slides for protein expression without knowledge of patient clincopathological data for each tissue core. H-Scoring was used for CYB561 membranous (>30 positive – AH TMA), EPB41L4A cytoplasmic (>12 positive Caucasian TMA, >175 AH TMA), Ki67 nuclear (>110 positive, AH TMA), DLX2 (>10 positive, AH TMA) and TGF-β cytoplasmic (>10 positive Caucasian TMA, >90 positive AH TMA). The H-score was achieved by summing the product of percentage cells showing each level of staining

intensity where 0=absence of staining, 1=weak staining, 2=moderate staining and 3=strong staining intensity (222). Percentage nuclear scoring was used for PD-L1 (>60% positive Caucasian, >90% positive AH) and TGF-β (>60% positive Caucasian, >90% positive AH). Presence absence scoring was used for Kappa and Lambda FLC (staining of mast cells) (AH and Caucasian TMA), PD-L1 (cytoplasmic staining) and PTEN (cytoplasmic staining). PD-L1 staining was particularly difficult to optimise. Unfortunately we were limited with the specific PD-L1 antibody available to us due to availability and cost restrictions. We were unable to identify any cell surface staining with the antibody used hence the use of presence/absence cytoplasmic staining and percentage nuclear staining was used. PD-L1 staining thresholds are very specific in order to be validated and we knew that our results with regards to PD-L1 would be limited and potentially affect our confidence in any significant results generated. Staining thresholds used for dichotomous categorisation were chosen using the software program X-tile or by those documented in previous studies (217,223). IHC was only performed in the AH TMA for biomarkers DLX2 and Ki67 as this has previously been performed on the Caucasian TMA previously with results previously published and were included for further biomarker validation. The number of patients that could be scored for each individual marker for each cohort is shown below in table 9 and 10 for each TMA respectively. No biomarker had a score reflecting the whole cohort as tissue core samples were lost in the processing of the cutting the microtome sections from the particular TMA.

We used REMARK (Reporting recommendations for tumour MARKer prognostic studies) guidelines for reporting on prognostic biomarkers in the whole patient series. REMARK guidelines are an internationally recognised set of parameters developed to ensure a robust standard of study design, pre-planned hypotheses, patient and specimens, assay methods and statistical analysis (224).

Biomarker	Number of cancer patients scored	Number of patients positive	Percentage Positive
CYB561A	147	1	0.7%
EPB41L4A	144	41	28.5%
Kappa FLC	139	1	0.7%
Lambda FLC	139	48	34.5%
PD-L1 Nuclear	131	69	52.7%
PD-L1 Cytoplasmic	131	97	74%
TGF-β Nuclear	144	78	18.3%
TGF-β Cytoplasmic	144	124	86.1%
PTEN	121	91	75.2%

Table 11: The number of patients within Caucasian Cohort dichotomously categorised for each biomarker

Biomarker	Number of cancer patients scored	Number of patients positive	Percentage Positive
CYB561A	33	20	60.6%
EPB41L4A	33	15	45.5%
DLX2	36	36	100%
Ki67	36	0	0%
Kappa FLC	33	2	6.1%
Lambda FLC	33	29	87.9%
PD-L1 Nuclear	33	12	36.4%
PD-L1 Cytoplasmic	33	33	100%
TGF-β Nuclear	33	8	24.2%
TGF-β Cytoplasmic	33	31	93.9%
PTEN	32	32	100%

Table 12: The number of patients within the AH cohort dichotomously categorised for each biomarker.

2.6 Statistical Analysis

Statistical analysis was performed using SPSS (Version 24; IBM, US). Kaplan-Meier plots with log-rank tests were used to analyse biomarker protein expression association with disease specific survival (DSS), time to metastasis development and time to development of castrate resistant disease. Pearson chi-square tests were performed to assess individual biomarker protein expression association with specific clinicopathological data variables including PSA, Gleason score, Gleason groups, D'Amico risk group, development of castrate resistance, requirement for further treatment with androgen deprivation treatment (ADT), race and association with proliferation marker Ki67 and biomarker DLX-2 and for further analysis and consolidation of findings shown in previous studies. African heritage samples included in the predominantly Caucasian TMA were

included in the AH cohort for statistical analysis. Variable expression of biomarker protein expression and association with race was demonstrated with combined analysis of both samples from the Caucasian and AH TMAs using the AH specific scoring thresholds. The significance level used for analysis was $P<0.05$.

3.0 Results of biomarkers from bioinformatic selection

3.1 The role of bioinformatics in prostate cancer

Recent advances in scientific research particularly in the field of gene expression profiling and development of genetic biomarkers have resulted in vast amounts of data to process and analyse. This has given rise to the field of bioinformatics, combining biological and technological science. To enable evaluation of large data sets machine learning and predictive approaches such as artificial neural network (ANN) algorithms have been utilised, particularly in the field of gene biomarker research. ANNs are a form of artificial intelligence based on human neural networks. The basic concept of neural networks is a connection of artificial neurons. Connection weights modulate the effects of input signals with nonlinear characteristics exhibited by neurons represented by a transfer function. The impulses from neurons is the weighted sum of the input signals, transformed by the transfer function. The learning ability of artificial neurons is achieved by adjusting the weights according to chosen learning algorithms (225).

A key advantage of ANNs compared with conventional methods like logistic regression is their ability to resolve complex non-linear relations among variables, without the need for any prior assumptions about these relations. ANNs are able to address a particular question by analysing data pattern recognition, which may correspond to a specific clinical outcome or disease process.

The most common form of ANNs are multilayer networks. These consist of three types of artificial neurons: input, hidden and output. In feed-forward networks, the signal flows from input to output neurons (See Figure. 7).

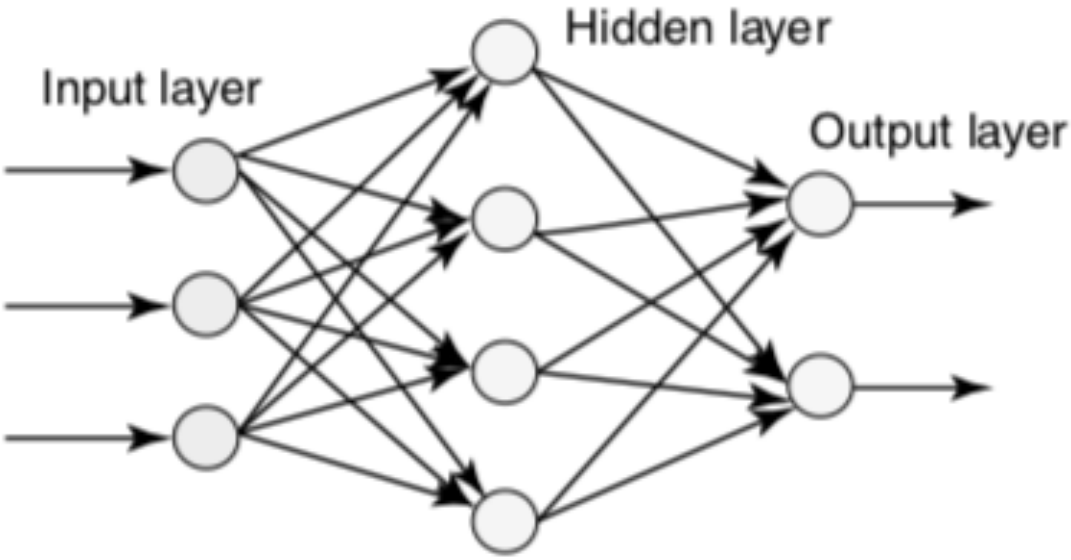


Figure 8: Multi-layered Artificial Neural Network (Reused with permission from John Wiley and Sons, licence number 4531561145751) (225)

Links or artificial synapses connect the layers carrying a value or weight, which transmits data between the neurons. The signals from the input layer of neurons will be processed by the hidden layer of neurons according to a defined set of parameters, with an activation function applied to the sum resulting in an output result generated by the output neurons. A neural network has to be configured so that the set of inputs produces the desired set of outputs. One method to configure this is to set the weights according to previous knowledge. Another method is to train the ANN with teaching patterns or learning algorithms and letting it learn and adjust the weights according to a specified learning rate, resulting in the desired outcome.

ANNs can be used to review large scale gene subsets to assess the prognostic potential of each gene individually according to a specific prognostic indicator and then adding in further genes sequentially to review the connections between them (226). This allows potential targeted novel gene biomarkers to be generated from large gene data pools.

Association between candidate biomarkers protein expression and clinicopathological variables are shown below. Significant Kaplan-Meier modelling results for prostate cancer specific survival, time to metastasis and time to development of castrate resistance are shown below for respective biomarkers. Unfortunately, Kaplan-Meier modelling was unable to be performed in the AH cohort due to a lack of data. No data was obtained for the AH Cohort for association with DLX2 as all samples were positive for expression for the specified staining threshold. Examples of biomarker staining patterns are shown in each individual biomarker section respectively.

3.2 CYB561A

Associations between biomarker CYB561A protein expression and clinicopathological variables are shown below in table 11. Association of CYB561A protein expression with race is shown in table 12. No data was obtained from the Caucasian cohort as only one patient sample was positive for expression.

There was no association seen with CYB561A protein expression in the AH patient cohort and serum PSA, Gleason groups, D'Amico risk groups, requirement for ADT and Ki67. Decreased membranous expression of CYB561A was shown to be associated with development of castrate resistance (x^2=5.077, p=0.024). Increased membranous expression of CYB561A was seen in AH patients (x^2=91.823, p=<0.001).

Clinical Variable		CYB561A					
		Caucasian Cohort			AH Cohort		
		CYB561A Negative	CYB561A Positive		CYB561A Negative	CYB561A Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	-	-	-	0 (0%)	1 (100%)	0.706 (p=0.401)
	>4	-	-		13 (41.9%)	18 (58.1%)	
Gleason Groups (Gleason Score)	1 (≤6)	-	-	-	3 (30%)	7 (70%)	2.071 (p=0.723)
	2 (3+4=7)	-	-		7 (41.2%)	10 (58.8%)	
	3 (4+3=7)	-	-		1 (50%)	1 (50%)	
	4 (8)	-	-		0 (0%)	1 (100%)	
	5 (9-10)	-	-		2 (66.7%)	1 (33.3%)	
D'Amico Risk Groups	Low	-	-	-	1 (25%)	3 (75%)	0.505 (p=0.777)
	Intermedia-te	-	-		8 (42.1%)	11 (57.9%)	
	High	-	-		3 (33.3%)	6 (66.7%)	
Development of Castrate Resistance	No	-	-	-	10 (33.3%)	20 (66.7%)	**5.077 (p=0.024)**
	Yes	-	-		3 (100%)	0 (0%)	
Androgen Deprivation Therapy (ADT)	No	-	-	-	12 (37.5%)	20 (62.5%)	1.587 (p=0.208)
	Yes	-	-		1 (100%)	0 (0%)	
Association with Ki67	Ki67 Negative	-	-	-	12 (37.5%)	20 (62.5%)	1.587 (p=0.208)
	Ki67 Positive	-	-		1 (100%)	0 (0%)	
Association with DLX2	DLX2 Negative	-	-	-	-	-	-
	DLX2 Positive	-	-		-	-	

Table 13: Association of CYB561A protein expression with clinicopathological variables. Significant p-values highlighted in bold.

		Combined Caucasian and AH Cohort Analysis CYB561A		
		CYB561A Negative	CYB561A Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	29 (96.7%)	1 (3.3%)	**91.823 (p=<0.001)**
	Afro-Caribbean	13 (39.4%)	20 (60.6%)	
	Black - Other	-	-	
	Indian	2 (100%)	0 (0%)	
	Not Specified	111 (100%)	0 (0%)	

Table 14: Association of CYB561A protein expression with race. Significant p-values highlighted in bold.

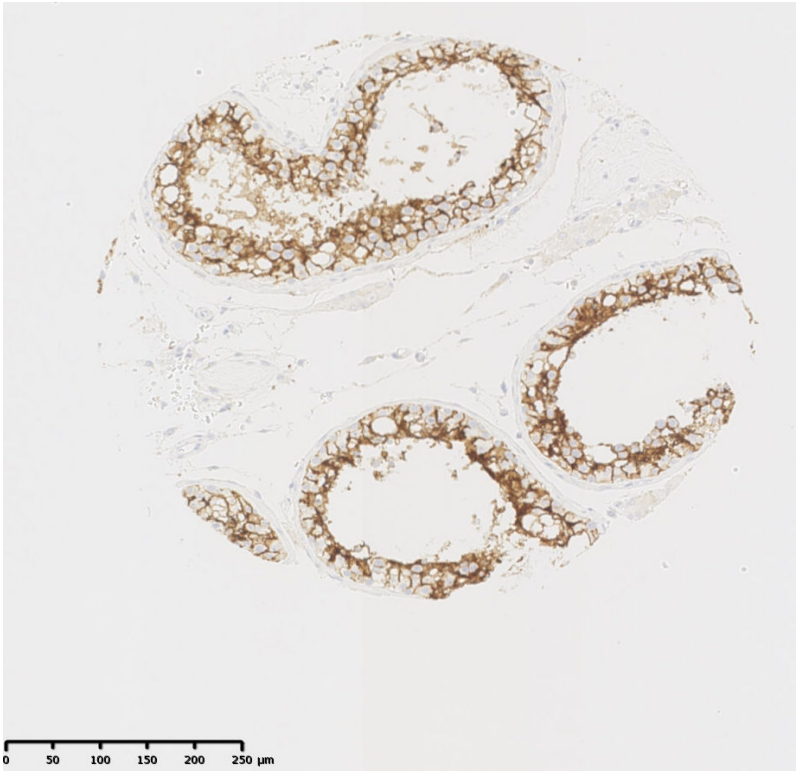


Figure 9a.

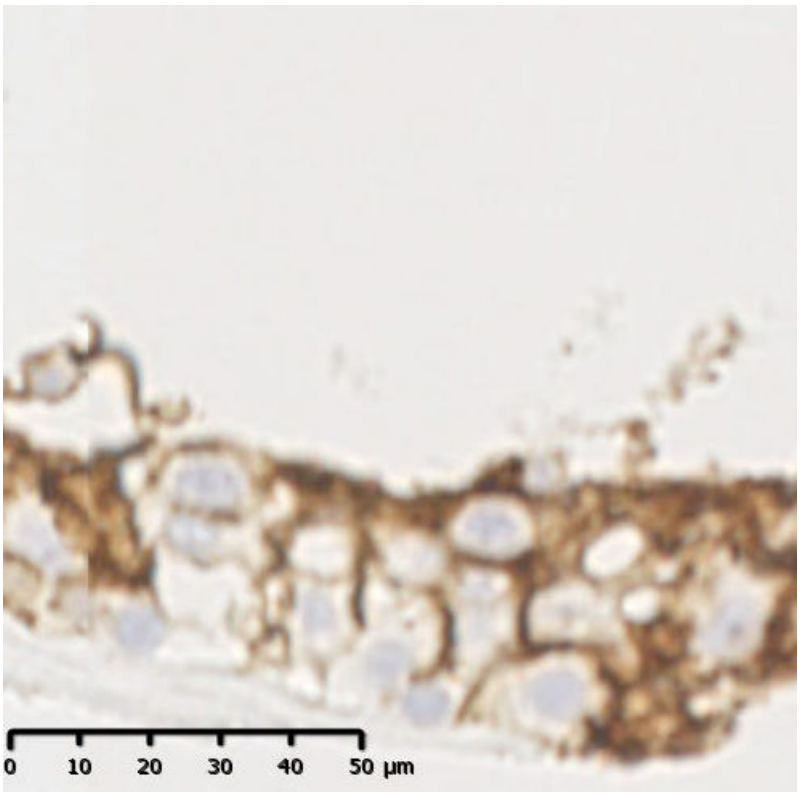


Figure 9b.

Figure 9: Example of IHC staining of CYB561A in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+5=10, PSA 100, TURP specimen)

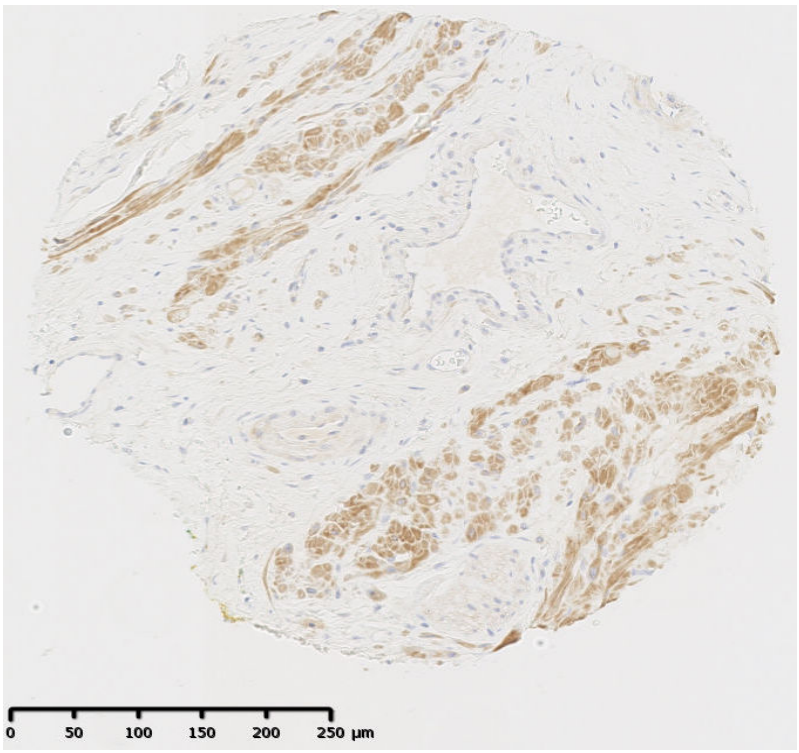


Figure 10a.

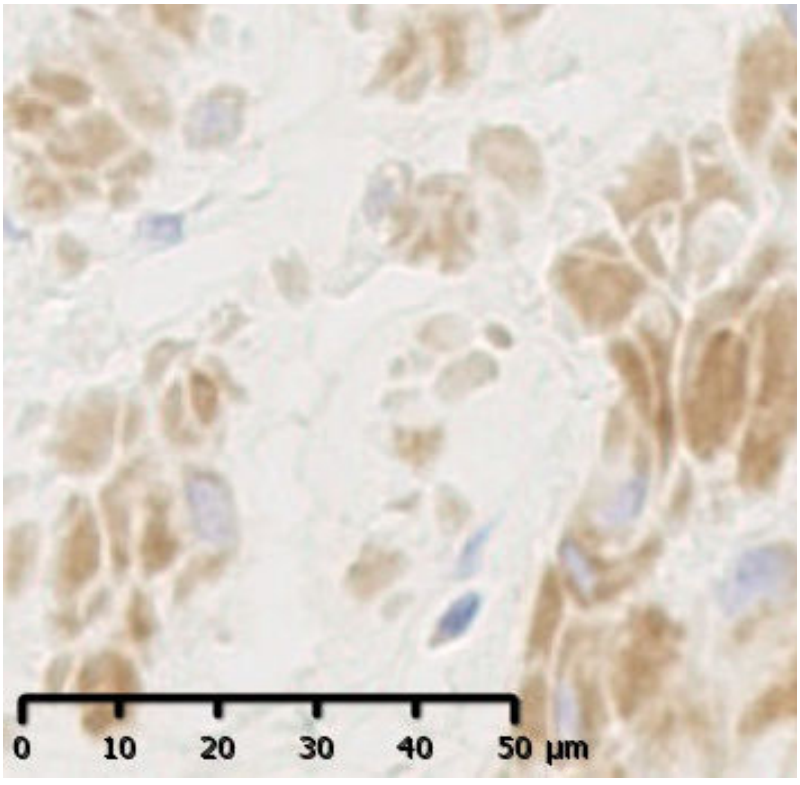


Figure 10b.

Figure 10: Example of IHC staining of CYB561A in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4=10, PSA 100, TURP specimen)

3.3 EPB41L4A

Associations between biomarker EPB41L4A protein expression and clinicopathological variables are shown below in table 13. Association of EPB41L4A protein expression with race is shown in table 14. Kaplan-Meier modelling for biomarker protein expression and prostate cancer specific survival, time to metastasis and castrate resistance are shown in table 15 and figure 11.

Increased EPB41L4A protein expression was positively associated with time to development of castrate resistance (x^2=7.320, p=<0.007) in the Caucasian cohort. There was no association between EPB41L4A expression within the Caucasian cohort and prostate cancer specific survival or time to metastasis development. No association was seen with EPB41L4A expression in the Caucasian or AH patient cohort with regards to serum PSA, Gleason groups, D'Amico risk groups, development of castrate resistance, requirement for ADT and Ki67. Decreased cytoplasmic expression of EPB41L4A in the Caucasian population was shown to be associated with increased expression of DLX2 expression (x^2=4.351, p=0.037). Increased cytoplasmic expression of EPB41L4A was seen in AH patients (x^2=48.255, p=<0.001).

Clinical Variable		EPB41L4A					
		Caucasian Cohort			AH Cohort		
		EPB41L4A Negative	EPB41L4A Positive		EPB41L4A Negative	EPB41L4A Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	3 (75%)	1 (25%)	0.024 (p=0.876)	0 (0%)	1 (100%)	1.170 (p=0.279)
	>4	90 (71.4%)	36 (28.6%)		17 (54.8%)	14 (45.2%)	
Gleason Groups (Gleason Score)	1 (≤6)	1 (100%)	0 (0%)	3.249 (p=0.517)	6 (60%)	4 (40%)	2.071 (p=0.723)
	2 (3+4=7)	10 (83.3%)	2 (16.7%)		10 (58.8%)	7 (41.2%)	
	3 (4+3=7)	6 (54.5%)	5 (45.5%)		1 (50%)	1 (50%)	
	4 (8)	21 (75%)	7 (25%)		0 (0%)	1 (100%)	
	5 (9-10)	41 (67.2%)	20 (32.8%)		1 (33.3%)	2 (66.7%)	
D'Amico Risk Groups	Low	5 (62.5%)	3 (37.5%)	0.311 (p=0.856)	2 (50%)	2 (50%)	2.199 (p=0.333)
	Intermedi -ate	6 (66.7%)	3 (33.3%)		12 (63.2%)	7 (36.8%)	
	High	83 (70.9%)	34 (29.1%)		3 (33.3%)	6 (66.7%)	
Development of Castrate Resistance	No	32 (69.6%)	14 (30.4%)	0.033 (p=0.856)	17 (56.7%)	13 (43.3%)	0.599 (p=0.439)
	Yes	59 (71.1%)	24 (28.9%)		1 (33.3%)	2 (66.7%)	
Androgen Deprivation Therapy (ADT)	No	14 (63.6%)	8 (36.4%	1.047 (p=0.306)	18 (56.3%)	14 (43.8%)	1.237 (p=0.266)
	Yes	70 (74.5%)	24 (25.5%)		0 (0%)	1 (100%)	
Association with Ki67	Ki67 Negative	57 (69.5%)	25 (30.5%)	0.021 (p=0.884)	18 (56.3%)	14 (43.8%)	1.237 (p=0.266)
	Ki67 Positive	4 (66.7%)	2 (33.3%)		0 (100%)	1 (100%)	
Association with DLX2	DLX2 Negative	13 (59.1%)	9 (40.9%)	**4.351 (p=0.037)**	-	-	-
	DLX2 Positive	59 (80.8%)	14 (19.2%)		-	-	

Table 15: Association of EPB41L4A protein expression with clinicopathological variables. Significant p-values highlighted in bold.

		Combined Caucasian and AH Cohort Analysis EPB41L4A		
		EPB41L4A Negative	EPB41L4A Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	28 (100%)	0 (0%)	**48.255 (p=<0.001)**
	Afro-Caribbean	18 (54.4%)	15 (45.5%)	
	Black - Other	-	-	
	Indian	1 (50%)	1 (50%)	
	Not Specified	109 (95.6%)	5 (4.4%)	

Table 16: Association of EPB41L4A protein expression with race. Significant p-values highlighted in bold.

EPB41L4A – Caucasian cohort					
Prostate cancer specific survival		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
0.045	0.831	2.428	0.119	7.320	0.007

Table 17: Kaplan-Meier modelling results demonstrating the association between EPB41L4A protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months). Significant associations are shown in bold.

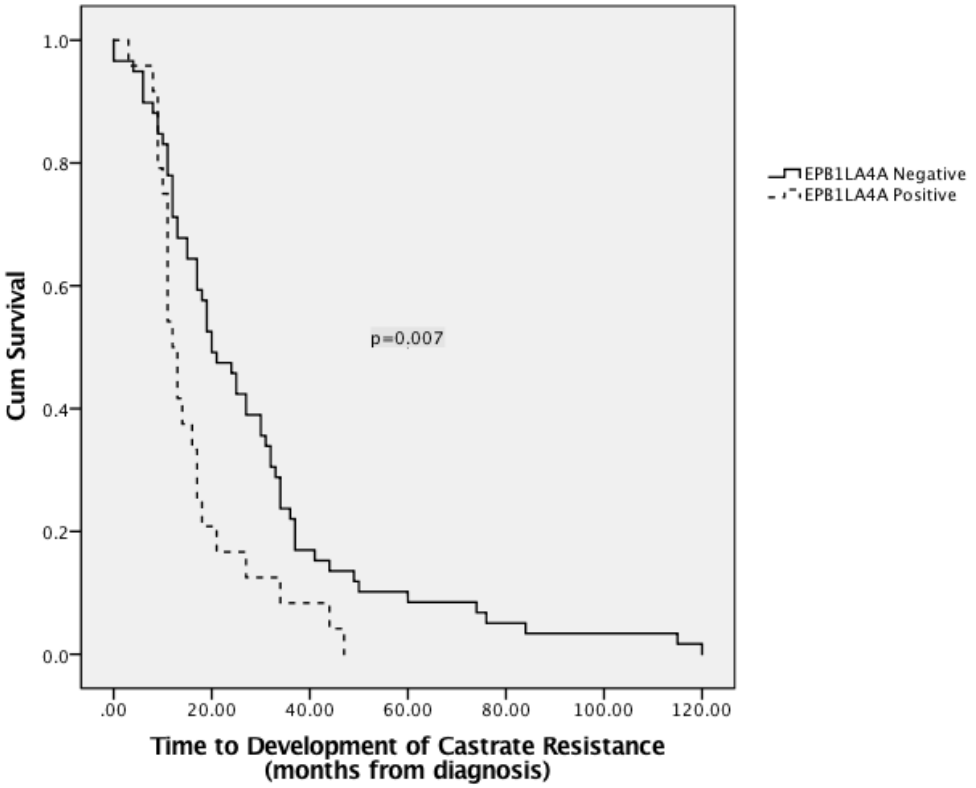


Figure 11: Kaplan Meier Chart demonstrating the association between EPB41L4A protein expression and Time to Development of Castrate Resistance

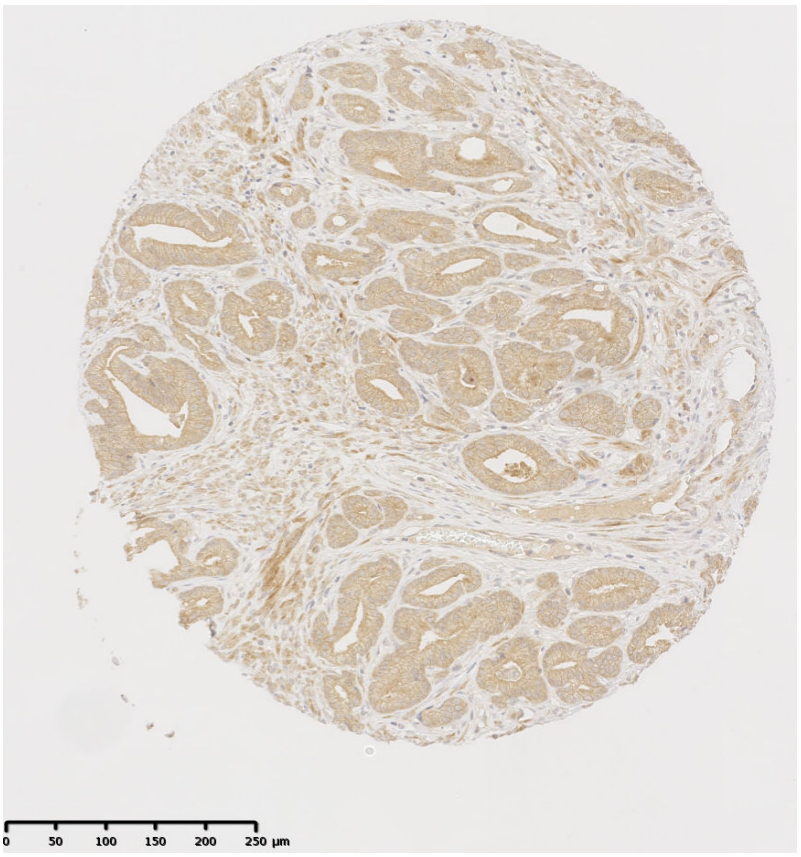


Figure 12a.

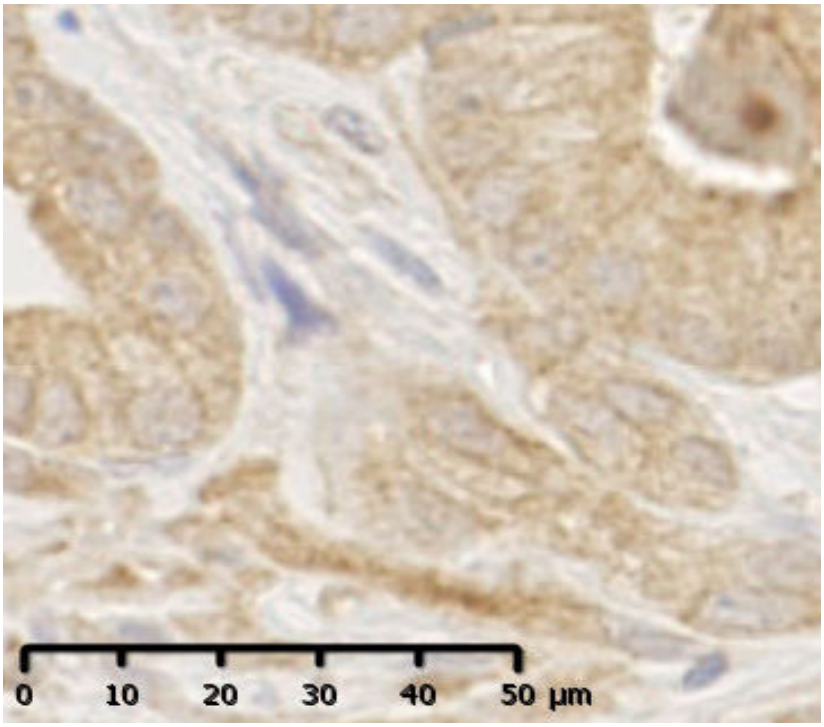


Figure 12b.

Figure 12: Example of IHC staining of EPB41L4A in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+3=10, PSA 152, TURP specimen)

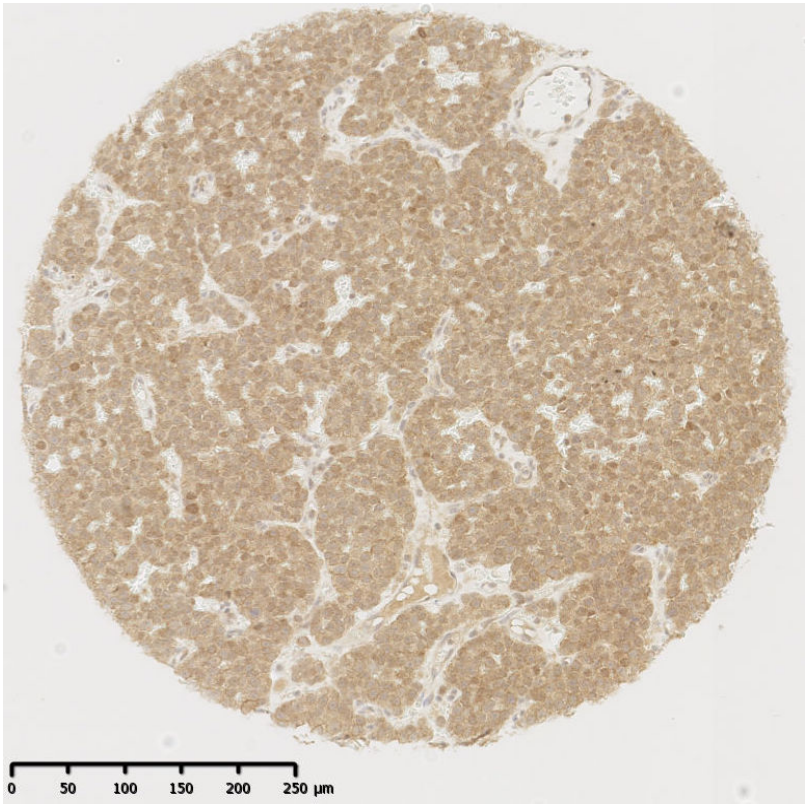
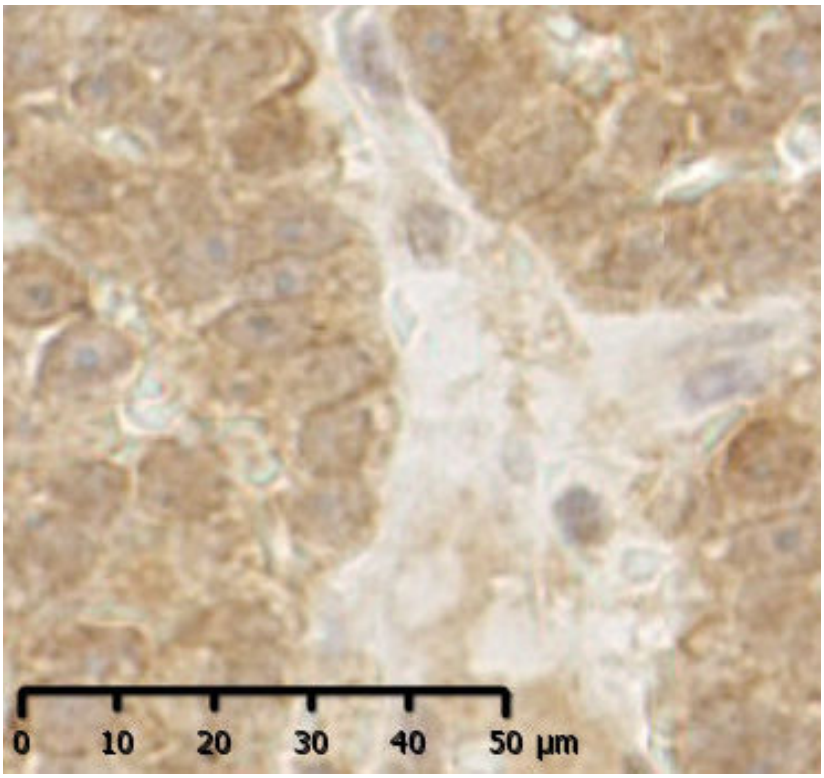


Figure 13a.



Fugure 13b.

Figure 13: Example of IHC staining of EPB41L4A in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4 =9, PSA 100, TURP specimen)

4.0 Results of biomarkers from previous studies and literature review

Association between candidate biomarkers protein expression and clinicopathological variables are shown below. Significant Kaplan-Meier modelling results for prostate cancer specific survival, time to metastasis and time to development of castrate resistance are shown below for respective biomarkers. Unfortunately, Kaplan-Meier modelling was unable to be performed in the AH cohort due to a lack of data. No data was obtained for the AH Cohort for association with DLX2 as all samples were positive for expression for the specified staining threshold. Examples of biomarker staining patterns are shown in each individual biomarker section respectively.

4.1 DLX2

No data was obtained for the AH Cohort for association with DLX2 as all samples were positive for expression for the specified staining threshold. Association of DLX2 with race is shown in table 16. Increased cytoplasmic protein expression of DLX2 was seen in AH patients (x^2=12.605, p=0.006).

		Combined Caucasian and AH Cohort Analysis DLX2		
		DLX2 Negative	DLX2 Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	13 (29.5%)	31 (70.5%)	**12.605 (p=0.006)**
	Afro-Caribbean	0 (0%)	35 (100%)	
	Black - Other	-	-	
	Indian	0 (0%)	3 (100%)	
	Not Specified	23 (19.6%)	79 (77.5%)	

Table 18: Association of DLX2 protein expression with race. Significant p-values highlighted in bold.

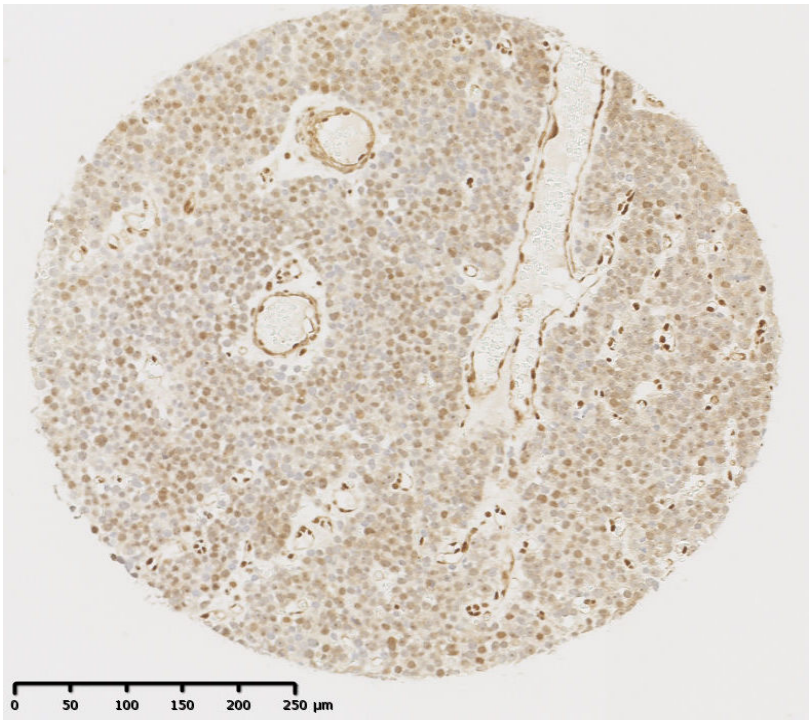


Figure 14a.

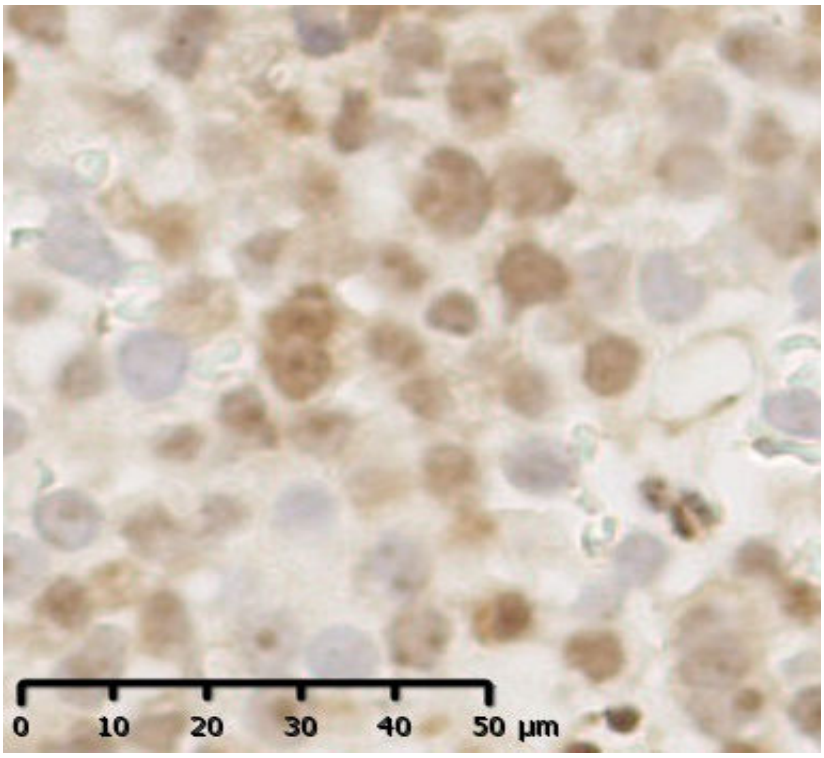


Figure 14b.

Figure 14: Example of IHC staining of DLX2 in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4 =9, PSA 100, TURP specimen)

4.2 Ki67

No data was obtained for the AH Cohort for association with Ki67 as only one sample was positive for expression for the specified staining threshold. There was no association between Ki67 protein expression and race, results are shown in table 17.

		Combined Caucasian and AH Cohort Analysis Ki67		
		Ki67 Negative	Ki67 Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	39 (92.9%)	3 (7.1%)	6.823 (p=<0.146)
	Afro-Caribbean	34 (97.1%)	1 (2.9%)	
	Black - Other	1 (100%)	0 (0%)	
	Indian	1 (50%)	1 (50%)	
	Not Specified	88 (92.6%)	7 (7.4%)	

Table 19: Association of Ki67 protein expression with race.

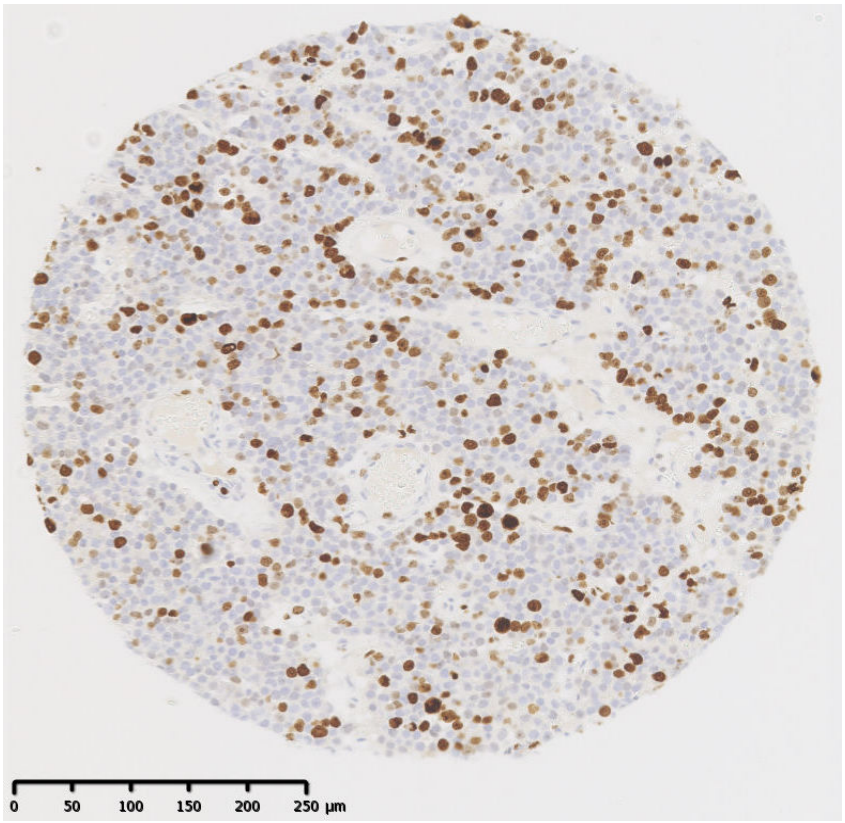


Figure 15a.

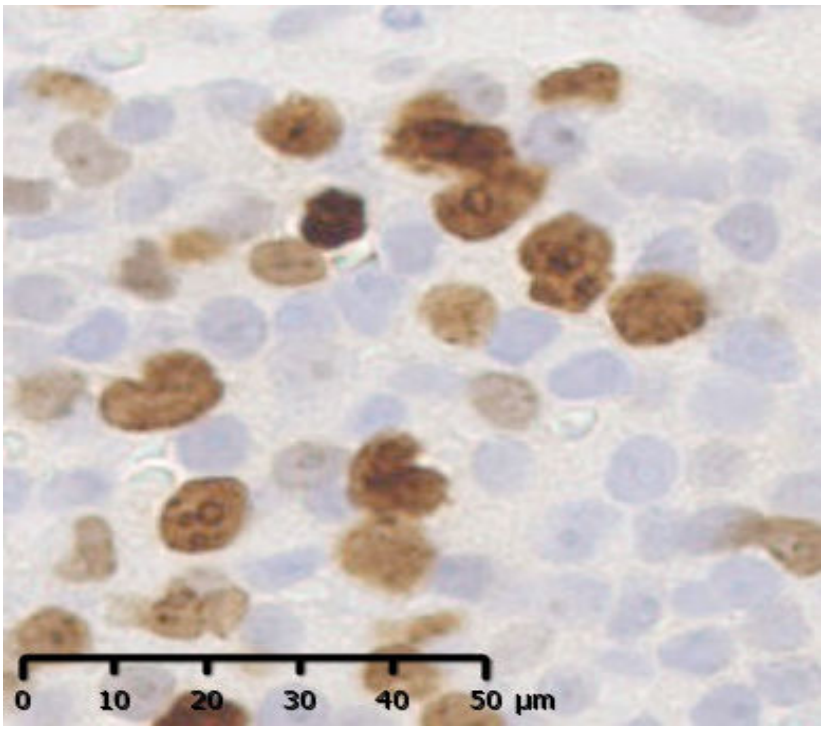


Figure 15b.

Figure 15: Example of IHC staining of Ki67 in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4 =9, PSA 100, TURP specimen)

4.3 Immunoglobulin Free Lights Chains – Lambda and Kappa

4.3.1 Lambda FLC

Associations between biomarker Lambda FLC protein expression and clinicopathological variables are shown below in table 18. Association of Lambda FLC with race is shown in table 19. Kaplan-Meier modelling for biomarker protein expression and prostate cancer specific survival, time to metastasis and castrate resistance are shown in table 20 and figures 16 and 17.

There was no association seen with Lambda FLC protein expression in the Caucasian or AH patient cohort with regards to serum PSA, Gleason groups, D'Amico risk groups and development of castrate resistance. There was no association in the Caucasian cohort with need for ADT, or expression of Ki67 or DLX2. Presence of lambda FLC expression was shown to be negativity associated with the need for ADT (x^2=7.477, p=0.006) and Ki67 expression (x^2=7.477, p=0.006) in the AH cohort. Increased expression of Lambda FLC was seen in AH patients (x^2=48.255, p=<0.001) and showed a positive association with development of castrate resistance (x^2=7.320, p=<0.007). Within the Caucasian cohort presence of lambda FLC expression showed a negative association with prostate cancer specific survival (x^2=10.496, p=0.001) and a positive association with development of metastatic disease (x^2=5.223, p=0.022).

Clinical Variable		Lambda FLC					
		Caucasian Cohort			AH Cohort		
		Lambda FLC Negative	Lambda FLC Positive		Lambda FLC Negative	Lambda FLC Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	5 (100%)	0 (0%)	1.540 (p=0.215)	0 (0%)	1 (100%)	0.147 (p=0.701)
	>4	93 (76.2%)	36 (23.8%)		4 (12.9)	27 (87.5%)	
Gleason Groups (Gleason Score)	1 (≤6)	1 (100%)	0 (0%)	3.249 (p=0.517)	1 (10%)	9 (90%)	9.457 (p=0.051)
	2 (3+4=7)	11 (84.6%)	2 (15.4%)		1 (5.9%)	16 (94.1%)	
	3 (4+3=7)	6 (66.7%)	3 (33.3%)		0 (0%)	2 (100%)	
	4 (8)	21 (77.8%)	6 (22.2%)		0 (0%)	1 (100%)	
	5 (9-10)	45 (76.3%)	14 (23.7%)		2 (66.7.3%)	1 (33.3%)	
D'Amico Risk Groups	Low	7 (87.5%)	1 (12.5%)	0.940 (p=0.625)	1 (25%)	3 (75%)	2.259 (p=0.323)
	Intermedi -ate	7 (87.5%)	1 (12.5%)		1 (5.3%)	18 (94.7%)	
	High	86 (76.8%)	26 (23.2%)		2 (22.2%)	7 (77.8%)	
Development of Castrate Resistance	No	39 (88.6%)	5 (11.4%)	2.33 (p=0.127)	3 (10%)	27 (90%)	1.394 (p=0.238)
	Yes	62 (77.5%)	18 (22.5%)		1 (33.3%)	2 (66.7%)	
Androgen Deprivation Therapy (ADT)	No	18 (90%)	2 (10%)	1.224 (p=0.269)	3 (9.4%)	29 (90.6%)	**7.477 (p=0..006)**
	Yes	73 (79.3%)	19 (20.7%)		1 (100%)	0 (100%)	
Association with Ki67	Ki67 Negative	63 (80.8%)	15 (19.2%)	0.103 (p=0.748)	3 (9.4%)	29 (90.6%)	**7.477 (p=0.006)**
	Ki67 Positive	6 (85.7%)	1 (14.3%)		1 (100%)	0 (100%)	
Association with DLX2	DLX2 Negative	17 (77.3%)	5 (22.7%)	0.009 (p=0.922)	-	-	-
	DLX2 Positive	54 (78.3%)	15 (21.7%)		-	-	

Table 20: Association of lambda FLC protein expression with clinicopathological variables. Significant p-values highlighted in bold.

		Combined Caucasian and AH Cohort Analysis Lambda FLC		
		Lambda FLC Negative	Lambda FLC Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	17 (63%)	10 (37%)	30.992 (p=<0.001)
	Afro-Caribbean	4 (12.1%)	29 (87.9%)	
	Black - Other	-	-	
	Indian	1 (50%)	1 (50%)	
	Not Specified	73 (66.4%)	37 (33.6%)	

Table 21: Association of Lambda FLC protein expression with race. Significant p-values highlighted in bold.

Lambda FLC – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
10.496	**0.001**	**5.223**	**0.022**	0.106	0.744

Table 22: Kaplan-Meier modelling results demonstrating the association between Lambda FLC protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months). Significant associations are shown in bold.

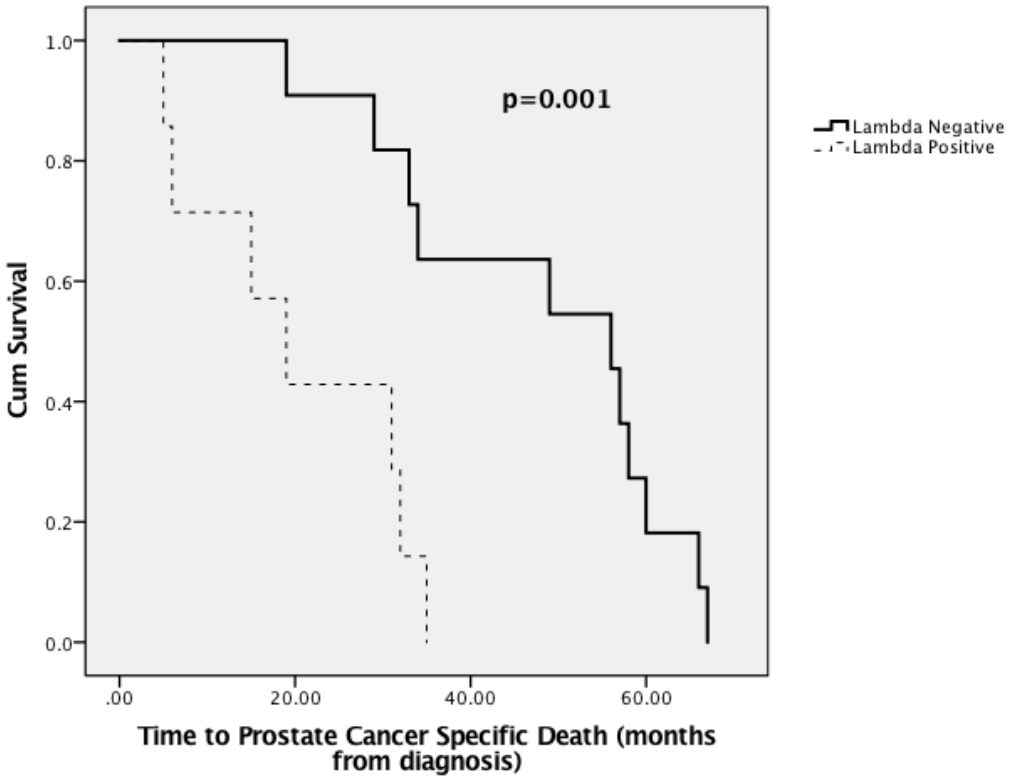


Figure 16: Kaplan-Meier chart demonstrating the association between Lambda FLC protein expression and prostate cancer specific death

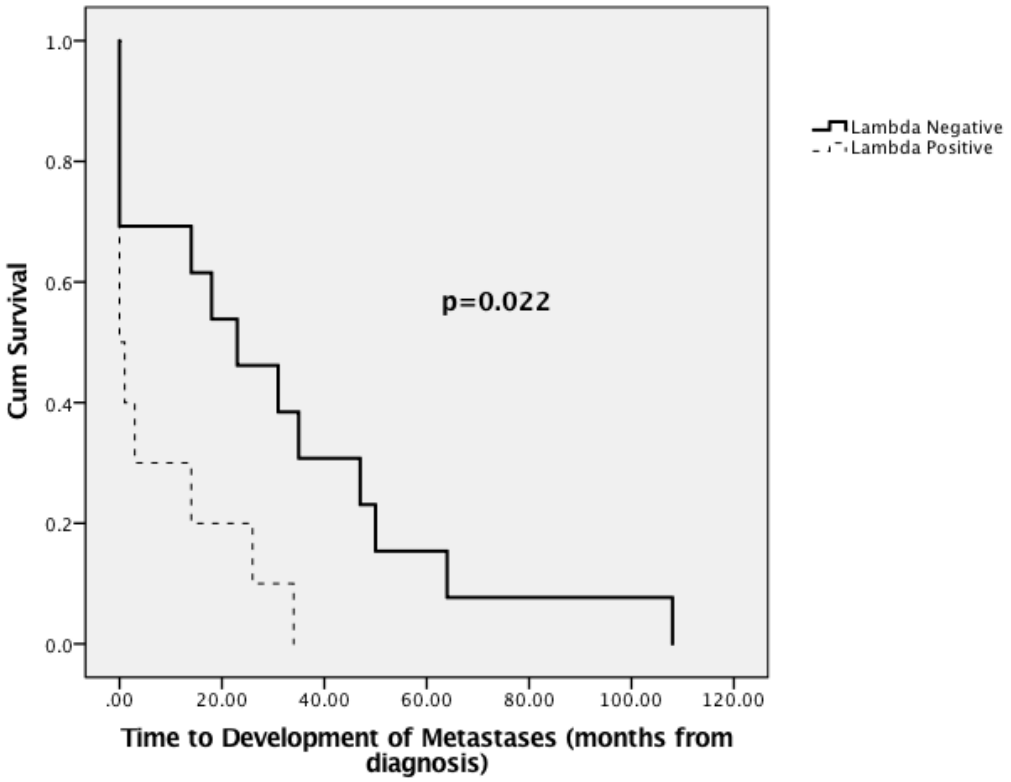


Figure 17: Kaplan Meier Chart demonstrating the association between Lambda FLC protein expression and Time to development of metastases (months from diagnosis)

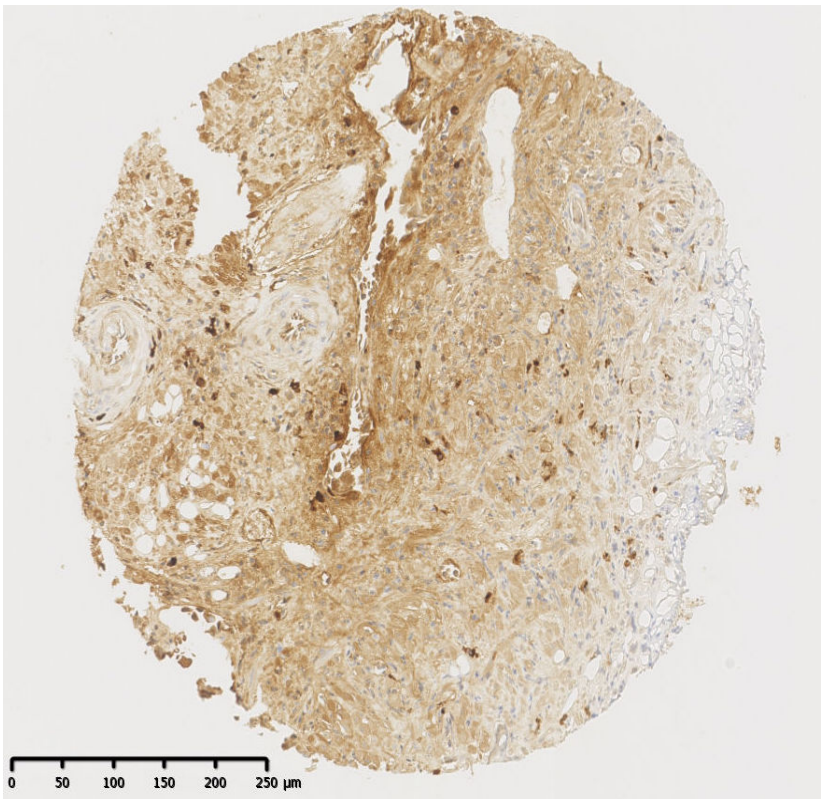


Figure 18a.

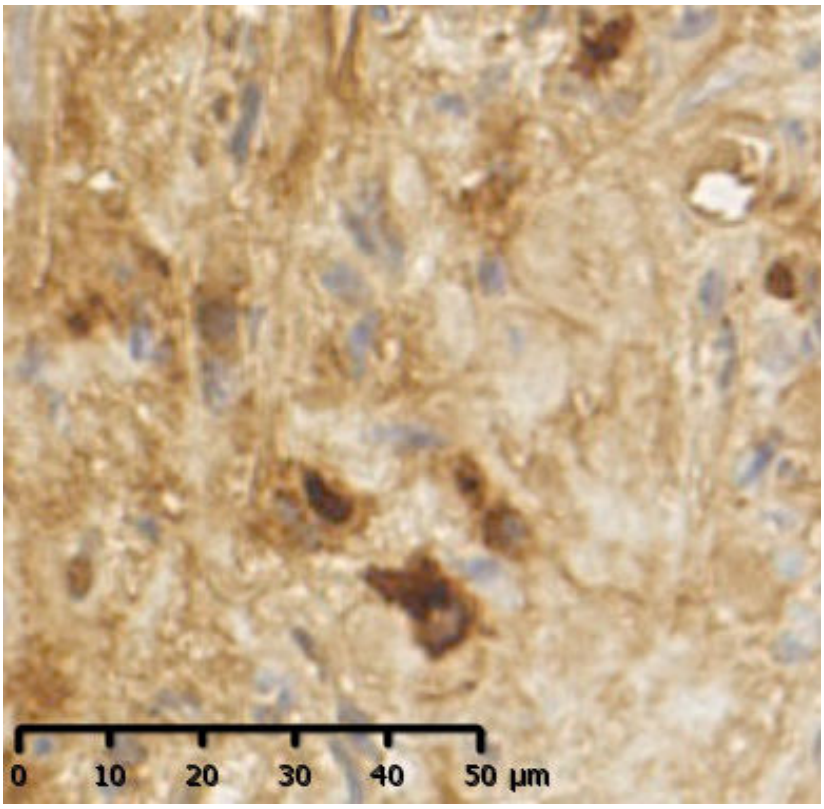


Figure 18b.

Figure 18: Example of IHC staining of Lambda FLC in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 1, TURP specimen)

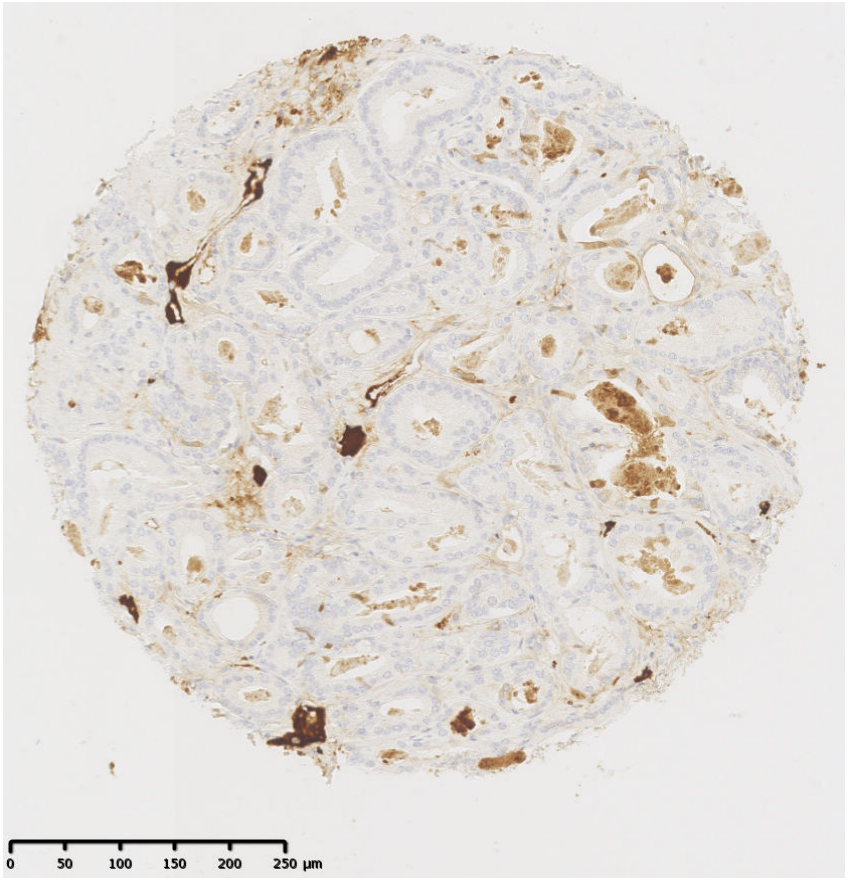


Figure 19a.

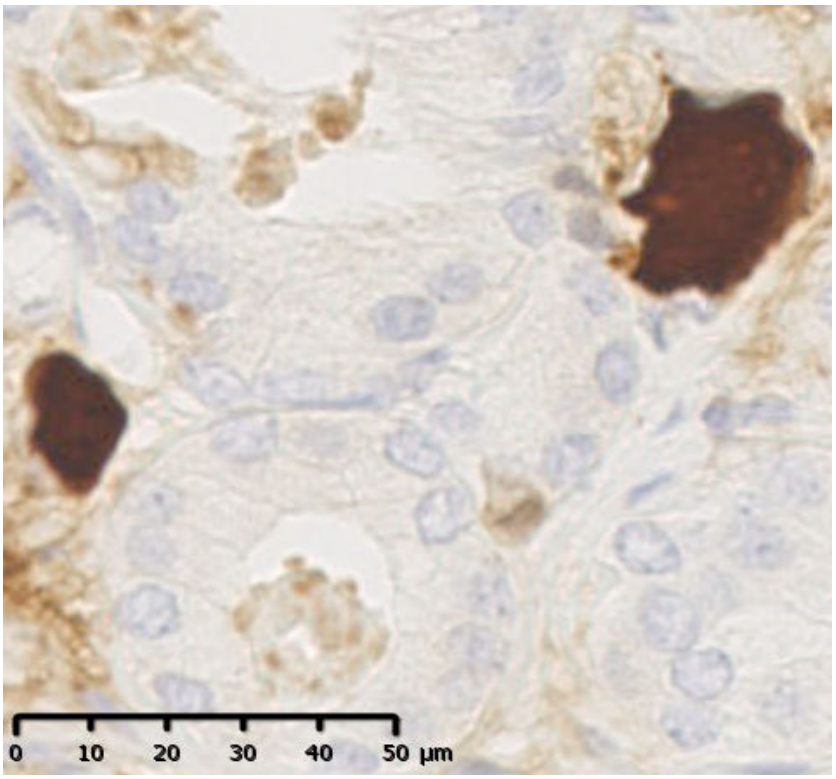


Figure 19b.

Figure 19: Example of IHC staining of Lambda FLC in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 11, radical prostatectomy specimen)

4.3.2 Kappa FLC

Associations between biomarker Kappa FLC protein expression and clinicopathological variables are shown below in table 21. Association of Kappa FLC with race is shown in table 22. Kaplan-Meier modelling for biomarker protein expression and prostate cancer specific survival, time to metastasis and castrate resistance couldn't be performed, as there wasn't enough patients samples positive for Kappa FLC protein expression.

Within the Caucasian or cohort there was no association seen with regards to Kappa FLC protein expression and serum PSA, Gleason groups, need for ADT, Ki67 expression and DLX2 expression. There was no data for development of castrate resistance as all the patient samples were negative. Decreased expression of Kappa FLC was associated with a higher D'Amico risk group (x^2=14.993, p=0.001). Within the AH cohort there was no association seen with any clinicopathological variables. No association was seen between Kappa FLC protein expression and race.

Clinical Variable		Kappa FLC					
		Caucasian Cohort			AH Cohort		
		Kappa FLC Negative	Kappa FLC Positive		Kappa FLC Negative	Kappa FLC Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	5 (100%)	0 (0%)	0.041 (p=0.839)	1 (100%)	0 (100%)	0.069 (p=0.793)
	>4	121 (99.2%)	1 (0.8%)		29 (93.5%)	2 (6.5%)	
Gleason Groups (Gleason Score)	1 (≤6)	1 (100%)	0 (0%)	7.376 (p=0.117)	9 (90%)	1 (10%)	8.410 (p=0.078)
	2 (3+4=7)	12 (92.3%)	1 (7.7%)		17 (100%)	0 (0%)	
	3 (4+3=7)	10 (100%)	0 (0%)		1 (50%)	1 (50%)	
	4 (8)	25 (100%)	0 (0%)		1 (100%)	0 (0%)	
	5 (9-10)	59 (100%)	0 (0%)		3 (100%)	0 (0%)	
D'Amico Risk Groups	Low	7 (87.5%)	1 (12.5%)	**14.993 (p=0.001)**	4 (100%)	0 (0%)	1.460 (p=0.482)
	Intermedi -ate	9 (100%)	0 (0%)		17 (89.5%)	2 (10.5%)	
	High	110 (0%)	0 (0%)		9 (100%)	0 (0%)	
Development of Castrate Resistance	No	-	-	-	28 (93.3%)	2 (6.7%)	0.213 (p=0.645)
	Yes	-	-		3 (100%)	0 (0%)	
Androgen Deprivation Therapy (ADT)	No	20 (100%)	0 (0%)	0.224 (p=0.636)	30 (93.8%)	2 (6.3%)	0.067 (p=0.796)
	Yes	89 (98.9%)	1 (1.1%)		1 (100%)	0 (100%)	
Association with Ki67	Ki67 Negative	78 (98.7%)	1 (1.3%)	0.090 (p=0.765)	30 (93.8%)	2 (6.3%)	0.067 (p=0.796)
	Ki67 Positive	7 (100%)	0 (0%)		1 (100%)	0 (0%)	
Association with DLX2	DLX2 Negative	21 (100%)	0 (22.7%)	0.303 (p=0.582)	-	-	-
	DLX2 Positive	69 (98.6%)	1 (1.4%)		-	-	

Table 23: Association of Kappa FLC protein expression with clinicopathological variables. Significant p-values highlighted in bold.

		Combined Caucasian and AH Cohort Analysis Kappa FLC		
		Kappa FLC Negative	Kappa FLC Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	26 (100%)	0 (0%)	4.506 (p=0.212)
	Afro-Caribbean	31 (93.9%)	2 (6.1%)	
	Black - Other	-	-	
	Indian	2 (100%)	0 (0%)	
	Not Specified	109 (99.1%)	1 (0.9%)	

Table 24: Association of Kappa FLC protein expression with race.

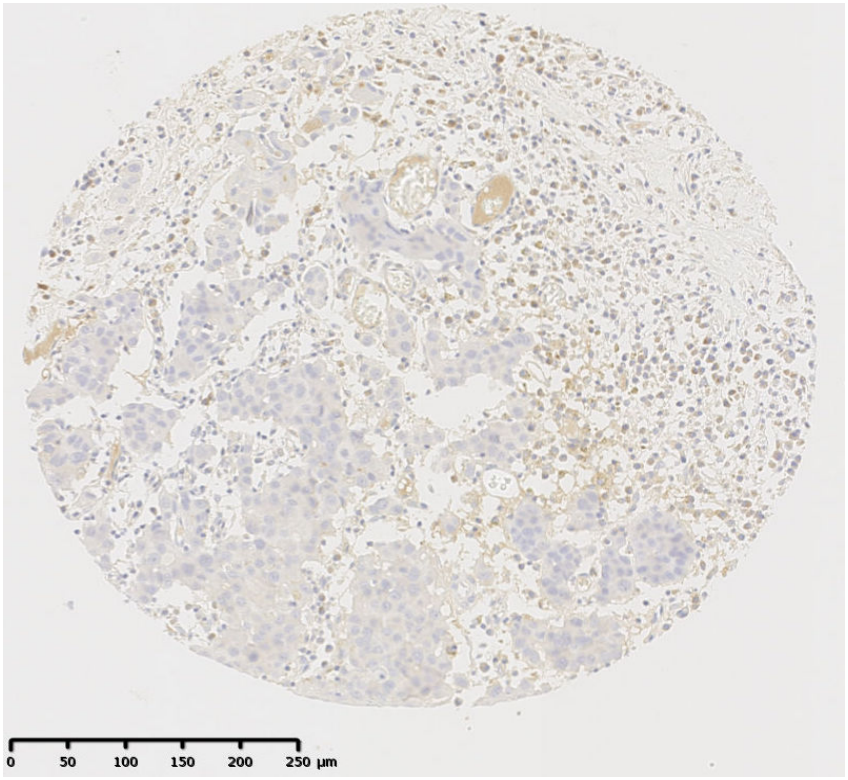


Figure 20a.

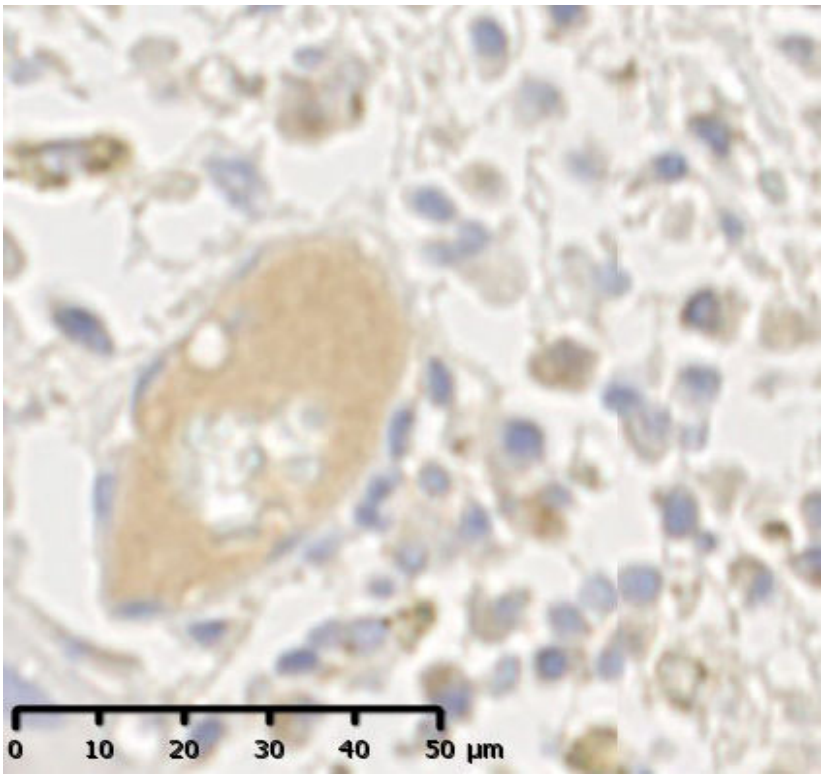


Figure 20b.

Figure 20: Example of IHC staining of Kappa FLC in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 8, TURP specimen)

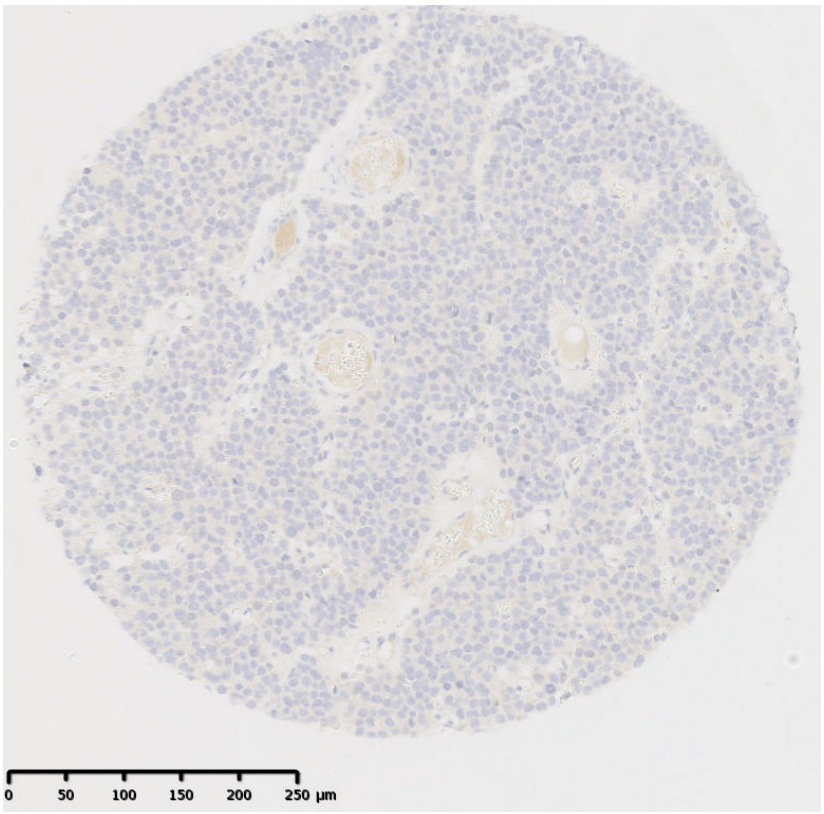


Figure 21a.

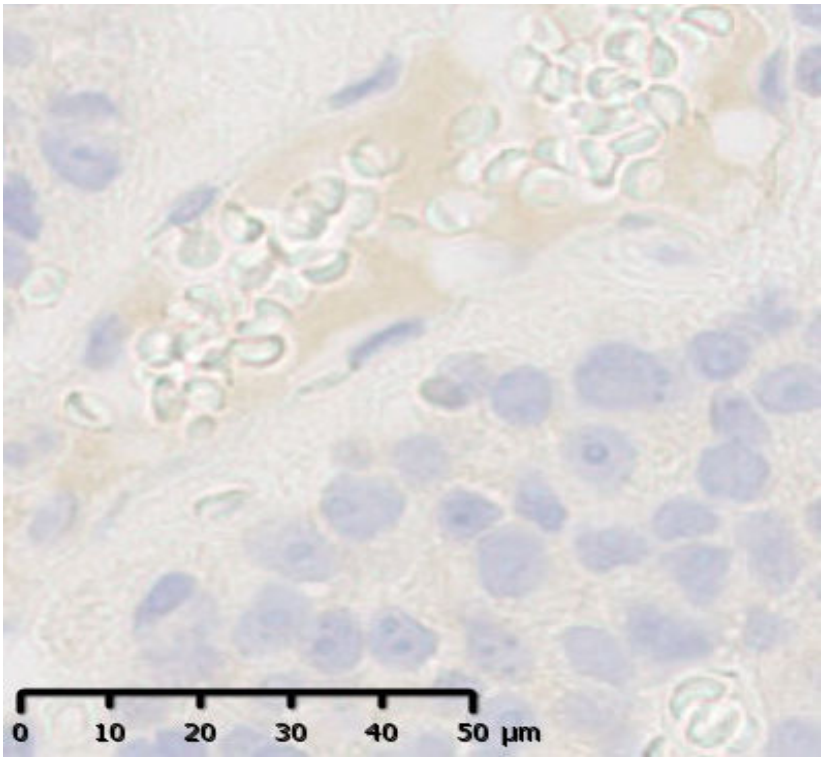


Figure 21b.

Figure 21: Example of IHC staining of Kappa FLC in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4 =9, PSA 100, TURP specimen)

4.4 PD-L1 nuclear and cytoplasmic protein expression

Associations between biomarker PD-L1 nuclear and cytoplasmic and clinicopathological variables and Kaplan-Meier modelling for prostate cancer specific survival, time to metastasis and castrate resistance are shown below in tables 23-28 and figure 22. No data was obtained for the AH Cohort for association with PD-L1 cytoplasmic as all samples were positive for expression for the specified staining threshold.

No association seen with PD-L1 nuclear or PD-L1 cytoplasmic protein expression in both the Caucasian and African cohorts and serum PSA, Gleason groups, D'Amico risk group, development of castrate resistance, need for ADT, Ki67 expression and DLX2 expression (Caucasian cohort). No association was seen with regards to prostate cancer specific survival or time to development of metastases (Caucasian cohort). Increased PD-L1 nuclear and cytoplasmic protein expression was seen in AH patients (x^2=16.343, p=<0.001 and x^2=11.639, p=0.009). Nuclear expression of PD-L1 showed a positive association with development of castrate resistance (x^2=4.062, p=<0.044) (Caucasian cohort).

Clinical Variable		PD-L1 Nuclear					
		Caucasian Cohort			AH Cohort		
		PD-L1 Nuclear Negative	PD-L1 Nuclear Positive		PD-L1 Nuclear Negative	PD-L1 Nuclear Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	2 (66.7%)	1 (33.3%)	0.146 (p=0.702)	1 (100%)	0 (100%)	0.541 (p=0.462)
	>4	65 (55.6%)	52 (44.4%)		20 (64.5%)	11 (35.5%)	
Gleason Groups (Gleason Score)	1 (≤6)	1 (100%)	0 (0%)	4.290 (p=0.368)	6 (60%)	4 (40%)	0.810 (p=0.937)
	2 (3+4=7)	6 (46.2%)	7 (53.8%)		11 (64.7%)	6 (35.3%)	
	3 (4+3=7)	4 (44.4%)	5 (55.6%)		1 (50%)	1 (50%)	
	4 (8)	18 (72%)	7 (28%)		1 (100%)	0 (0%)	
	5 (9-10)	30 (55.6%)	24 (44.4%)		2 (66.7%)	1 (33.3%)	
D'Amico Risk Groups	Low	4 (57.1%)	3 (42.9%)	1.567 (p=0.457)	2 (50%)	2 (50%)	0.337 (p=0.845)
	Intermedi -ate	6 (75%)	2 (25%)		12 (63.2%)	7 (36.8%)	
	High	56 (52.3%)	51 (47.7%)		6 (66.7%)	3 (33.3%)	
Development of Castrate Resistance	No	27 (62.8%)	16 (37.2%)	1.624 (p=0.203)	20 (66.7%)	1 (33.3%)	1.310 (p=0.252)
	Yes	38 (50.7%)	37 (49.3%)		1 (33.3%)	2 (66.7%)	
Androgen Deprivation Therapy (ADT)	No	11 (57.9%)	8 (42.1%)	0.066 (p=0.797)	21 (65.6%)	11 (34.4%)	1.805 (p=0.179)
	Yes	47 (54.7%)	39 (45.3%)		0 (0%)	1 (100%)	
Association with Ki67	Ki67 Negative	44 (58.7%)	31 (41.3%)	0.006 (p=0.938)	20 (66.7%)	10 (33.3%)	1.879 (p=0.170)
	Ki67 Positive	4 (57.1%)	3 (42.9%)		0 (100%)	1 (100%)	
Association with DLX2	DLX2 Negative	14 (66.7%)	7 (33.3%)	1.115 (p=0.291)	-	-	-
	DLX2 Positive	37 (53.6%)	32 (46.4%)		-	-	

Table 25: Association of PD-L1 nuclear protein expression with clinicopathological variables.

		Combined Caucasian and AH Cohort Analysis PD-L1 Nuclear		
		PD-L1 Nuclear Negative	PD-L1 Nuclear Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	23 (88.5%)	3 (11.5%)	**16.343 (p=0.001)**
	Afro-Caribbean	21 (63.6%)	12 (36.4%)	
	Black - Other	-	-	
	Indian	0 (0%)	1 (100%)	
	Not Specified	92 (88.5%)	12 (11.5%)	

Table 26: Association of PD-L1 nuclear protein expression with race. Significant associations are shown in bold.

PD-L1 Nuclear – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
0.024	0.877	0.7	0.791	**4.062**	**0.044**

Table 27: Kaplan-Meier modelling results demonstrating the association between PD-L1 nuclear protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months). Significant associations are shown in bold.

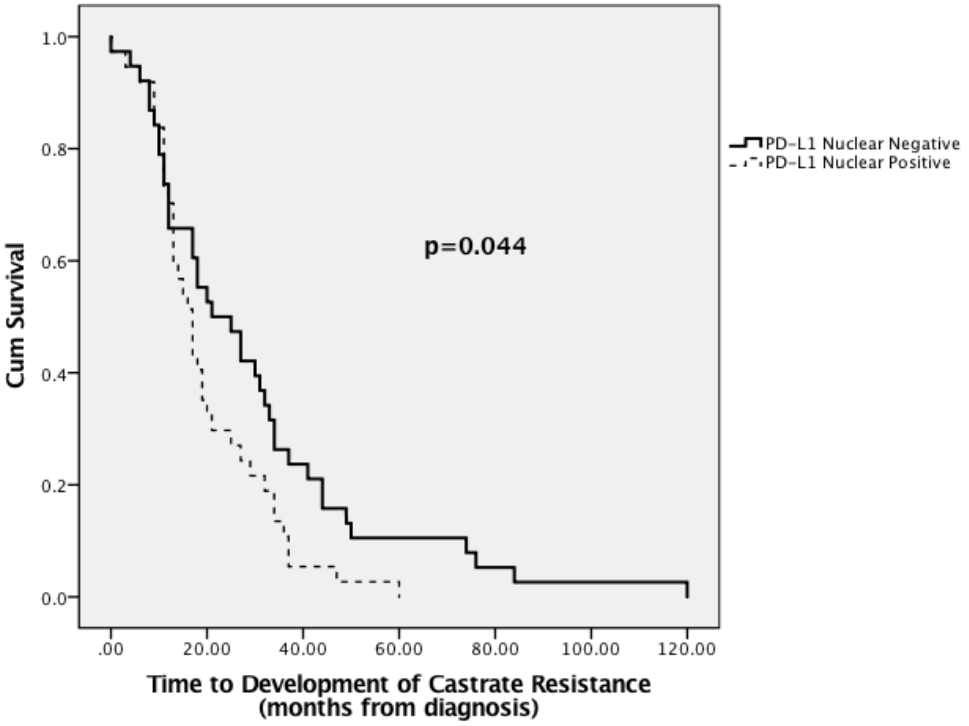


Figure 22: Kaplan Meier Chart demonstrating the association between PD-L1 nuclear protein expression and time to development of castrate resistance

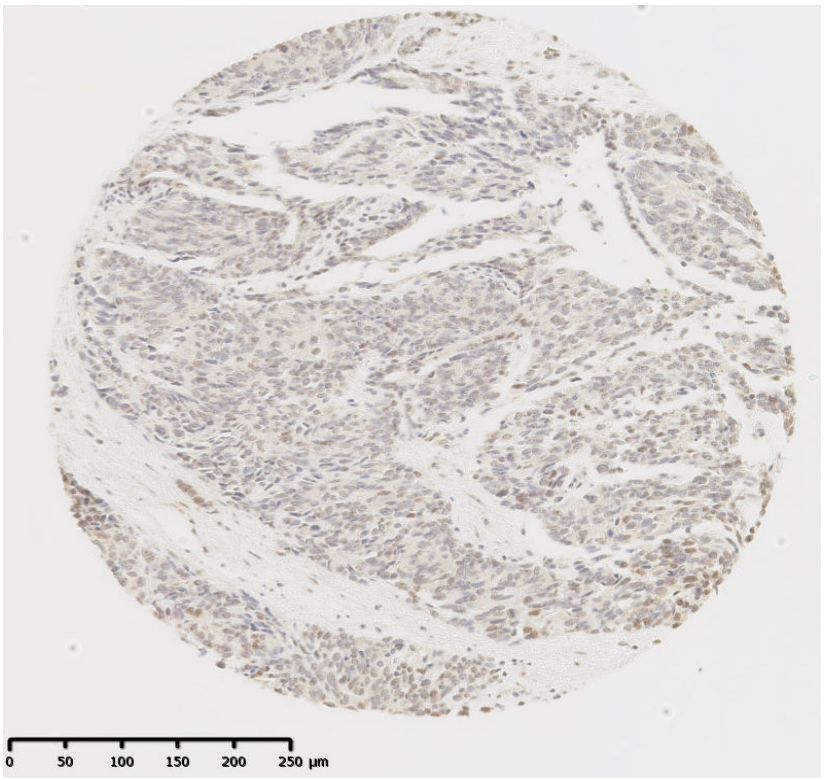


Figure 23a.

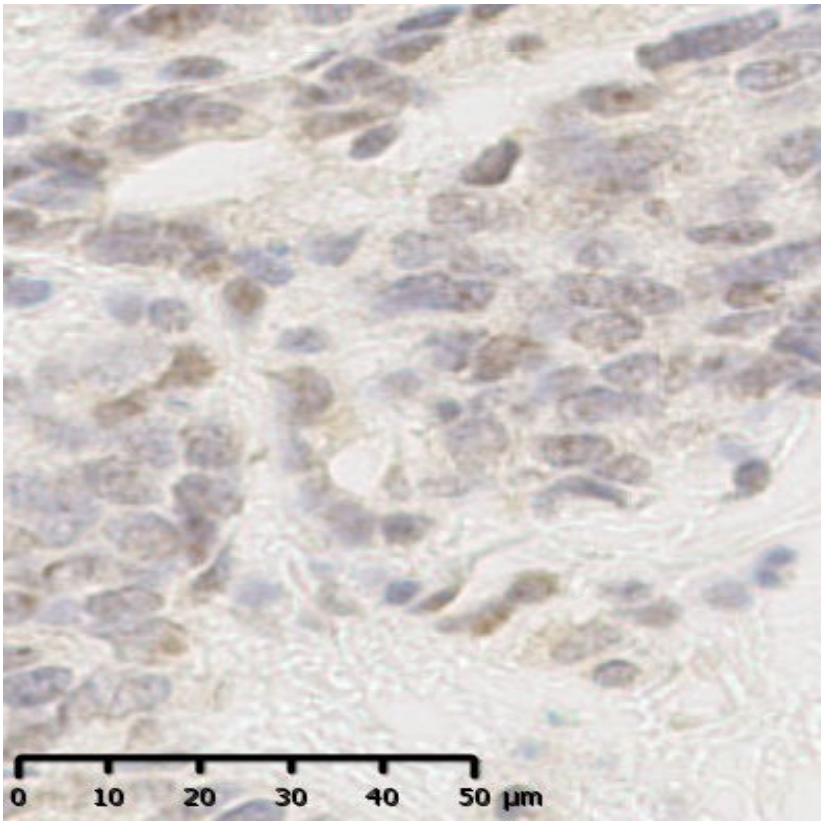


Figure 23b.

Figure 23: Example of IHC staining of PD-L1 in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+3 =7, PSA 1, TURP specimen)

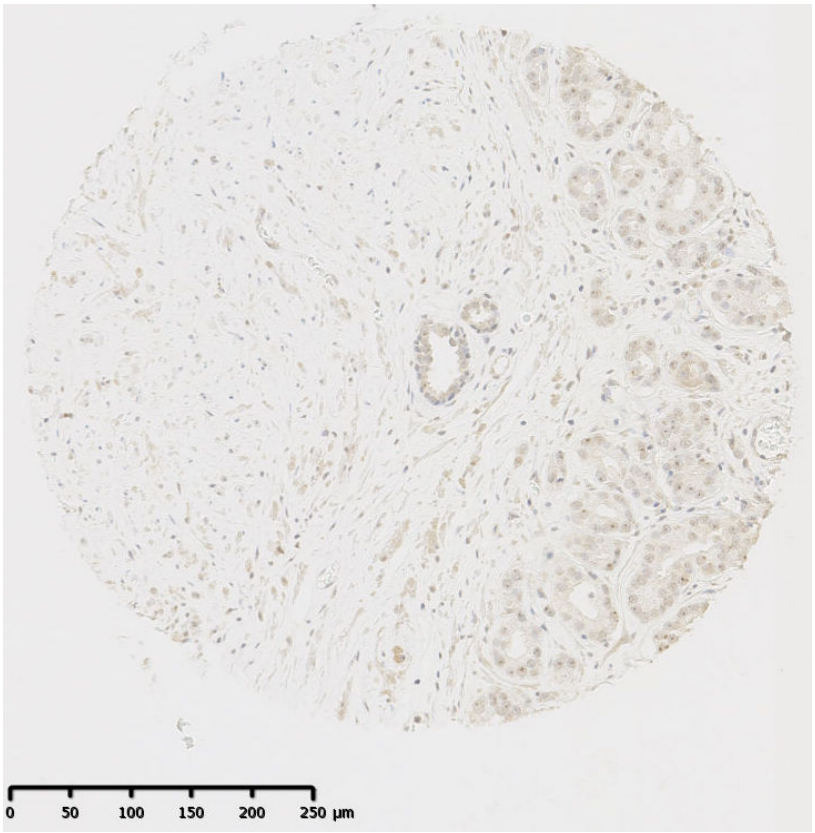


Figure 24a.

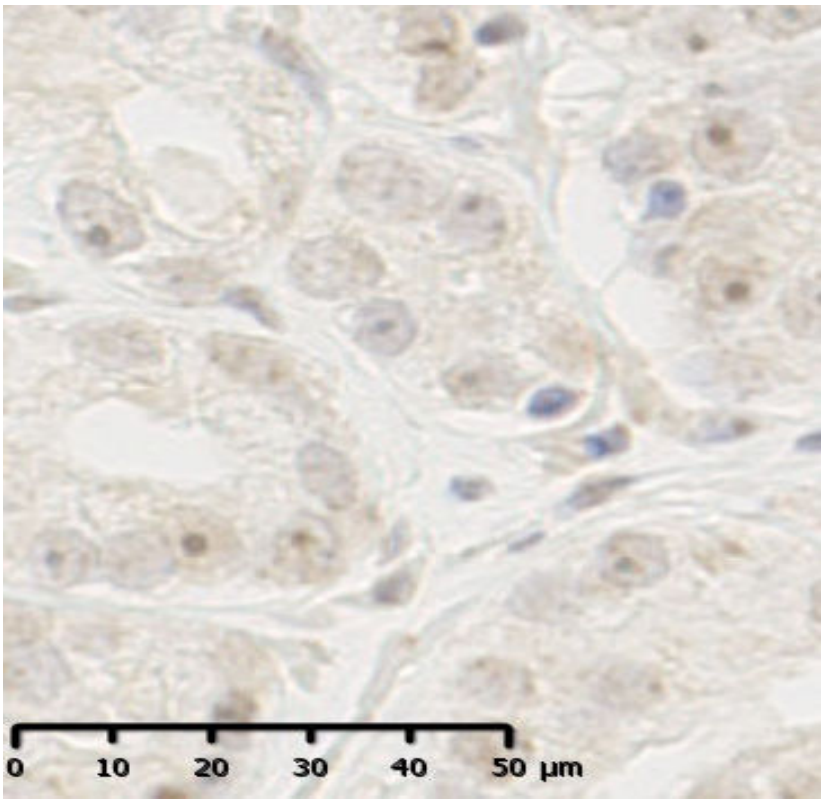


Figure 24b.

Figure 24: Example of IHC staining of PD-L1 in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+3 =7, PSA 8, radical prostatectomy specimen)

Clinical Variable		PD-L1 Cytoplasmic					
		Caucasian Cohort			AH Cohort		
		PD-L1 Cytoplasmic Negative	PD-L1 Cytoplasmic Positive		PD-L1 Cytoplasmic Negative	PD-L1 Cytoplasmic Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	1 (33.3%)	2 (66.7%)	0.114 (p=0.736)	-	-	-
	>4	29 (24.8%)	88 (75.2%)		-	-	
Gleason Groups (Gleason Score)	1 (≤6)	0 (0%)	1 (100%)	4.688 (p=0.321)	-	-	-
	2 (3+4=7)	3 (23.1%)	10 (76.9%)		-	-	
	3 (4+3=7)	3 (33.3%)	6 (66.7%)		-	-	
	4 (8)	11 (44%)	14 (56%)		-	-	
	5 (9-10)	12 (22.2%)	42 (77.8%)		-	-	
D'Amico Risk Groups	Low	2 (28.6%)	5 (71.4%)	3.115 (p=0.211)	-	-	-
	Intermedi -ate	4 (50%)	4 (50%)		-	-	
	High	24 (22.4%)	83 (77.6%)		-	-	
Development of Castrate Resistance	No	12 (27.9%)	31 (72.1%)	0.653 (p=0.419)	-	-	-
	Yes	16 (21.3%)	59 (78.7%)		-	-	
Androgen Deprivation Therapy (ADT)	No	6 (31.6%)	13 (68.4%)	0.286 (p=0.593)	-	-	-
	Yes	22 (25.6%)	64 (74.4%)		-	-	
Association with Ki67	Ki67 Negative	21 (28%)	54 (72%)	0.001 (p=0.974)	-	-	-
	Ki67 Positive	2 (28.6%)	5 (71.4%)		-	-	
Association with DLX2	DLX2 Negative	7 (33.3%)	14 (66.7%)	0.871 (p=0.351)	-	-	-
	DLX2 Positive	16 (23.2%)	53 (76.8%)		-	-	

Table 28: Association of PD-L1 cytoplasmic protein expression with clinicopathological variables.

		Combined Caucasian and AH Cohort Analysis PD-L1 Cytoplasmic		
		PD-L1 Cytoplasmic Negative	PD-L1 Cytoplasmic Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	8 (30.8%)	18 (69.2%)	**11.639 (p=0.009)**
	Afro-Caribbean	0 (0%)	33 (100%)	
	Black - Other	-	-	
	Indian	0 (0%)	1 (100%)	
	Not Specified	26 (25%)	78 (75%)	

Table 29: Association of PD-L1 cytoplasmic protein expression with race. Significant associations are shown in bold.

PD-L1 Cytoplasmic – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
0.196	0.658	0.039	0.844	0.058	0.809

Table 30: Kaplan-Meier modelling results demonstrating the association between PD-L1 cytoplasmic protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months).

4.5 TGF-β nuclear and cytoplasmic

Associations between biomarker TGF-β nuclear and cytoplasmic protein expression and clinicopathological variables and Kaplan-Meier modelling for prostate cancer specific survival, time to metastasis and castrate resistance are shown below in tables 29-34 and figure 25. No data was obtained from the AH cohort as all patients were positive for expression for the specified

staining threshold. No association was seen with nuclear expression of TGF-β and prostate cancer specific death, time to metastasis, time to development of castrate resistance, serum PSA, Gleason groups, D'Amico risk groups, development of castrate resistance, requirement for ADT, Ki67 or DLX2 expression in either Caucasian or AH cohort respectively. Increased nuclear expression of TGF-β was seen in AH patients (x^2=27.295, p=<0.001).

No association was seen with cytoplasmic expression of TGF-β and time to metastasis, time to development of castrate resistance, serum PSA, Gleason groups (AH cohort only), D'Amico risk groups, development of castrate resistance, requirement for ADT, Ki67 or DLX2 expression (Caucasian cohort only) in either Caucasian or AH cohort respectively. Within the Caucasian cohort, increased cytoplasmic expression of TGF-β showed positive association with Gleason score groups (x^2=11.969, p=0.018). Decreased expression showed a negative association with prostate cancer specific survival (x^2=8.846, p=0.003). Increased cytoplasmic expression of TGF-β was seen in AH patients (x^2=33.896, p=<0.001).

Clinical Variable		TGF-β Nuclear					
		Caucasian Cohort			AH Cohort		
		TGF-β Nuclear Negative	TGF-β Nuclear Positive		TGF-β Nuclear Negative	TGF-β Nuclear Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	4 (80%)	1 (20%)	2.336 (p=0.126)	1 (100%)	0 (0%)	0.289 (p=0.591)
	>4	57 (45.2%)	69 (54.8%)		24 (77.4%)	7 (22.6%)	
Gleason Groups (Gleason Score)	1 (≤6)	1 (100%)	0 (0%)	3.727 (p=0.444)	9 (90%)	1 (10%)	8.295 (p=0.081)
	2 (3+4=7)	8 (61.5%)	5 (38.5%)		14 (82.4%)	3 (17.6%)	
	3 (4+3=7)	6 (46.2%)	7 (53.8%)		1 (50%)	1 (50%)	
	4 (8)	16 (57.1%)	12 (42.9%)		0 (0%)	1 (100%)	
	5 (9-10)	24 (42.1%)	33 (57.9%)		1 (33.3%)	2 (66.7%)	
D'Amico Risk Groups	Low	5 (62.5%)	3 (37.5%)	2.881 (p=0.237)	3 (75%%)	1 (25%)	1.126 (p=0.569)
	Intermedi -ate	2 (22.2%)	7 (77.8%)		16 (84.2%)	3 (15.8%)	
	High	53 (45.3%)	64 (54.7%)		3 (66.7%)	3 (33.3%)	
Development of Castrate Resistance	No	23 (48.9%)	24 (51.1%)	0.305 (p=0.581)	23 (76.7%)	7 (23.3%)	0.149 (p=0.700)
	Yes	36 (43.9%)	46 (56.1%)		2 (66.7%)	1 (33.3%)	
Androgen Deprivation Therapy (ADT)	No	13 (59.1%)	9 (40.9%)	0.286 (p=0.593)	24 (75%)	8 (25%)	0.330 (p=0.566)
	Yes	46 (48.9%)	48 (51.1%)		1 (100%)	0 (0%)	
Association with Ki67	Ki67 Negative	38 (46.9%)	43 (53.1%)	0.736 (p=0.391)	22 (73.3%)	8 (26.7%)	0.359 (p=0.549)
	Ki67 Positive	4 (57.1%)	3 (42.9%)		1 (100%)	0 (0%)	
Association with DLX2	DLX2 Negative	10 (50%)	10 (50%)	0.074 (p=0.786)	-	-	-
	DLX2 Positive	34 (46.6%)	39 (53.4%)		-	-	

Table 31: Association of TGF-β nuclear protein expression with clinicopathological variables.

		Combined Caucasian and AH Cohort Analysis TGF-β Nuclear		
		TGF-β Nuclear Negative	TGF-β Nuclear Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	0 (51.7%)	14 (48.3%)	**27.295 (p=<0.001)**
	Afro-Caribbean	0 (0%)	33 (100%)	
	Black - Other	-	-	
	Indian	2 (100%)	0 (0%)	
	Not Specified	48 (43.2%)	63 (56.8%)	

Table 32: Association of TGF-β nuclear protein expression with race. Significant associations are shown in bold.

TGF-β Nuclear – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
0.28	0.597	0.009	0.924	0.003	0.959

Table 33: Kaplan-Meier modelling results demonstrating the association between TGF-β nuclear protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months).

Clinical Variable		TGF-β Cytoplasmic					
		Caucasian Cohort			AH Cohort		
		TGF-β Cytoplasmic Negative	TGF-β Cytoplasmic Positive		TGF-β Cytoplasmic Negative	TGF-β Cytoplasmic Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	1 (20%)	4 (80%)	20.172 (p=0.678)	0 (0%)	1 (100%)	0.069 (p=0.793)
	>4	17 (13.5%)	109 (86.5%)		2 (6.5%)	29 (93.5%)	
Gleason Groups (Gleason Score)	1 (≤6)	0 (0%)	1 (100%)	**11.969 (p=0.018)**	1 (10%)	9 (90%)	5.482 (p=0.241)
	2 (3+4=7)	4 (30.8%)	9 (69.2%)		0 (0%)	17 (100%)	
	3 (4+3=7)	1 (7.7%)	12 (92.3%)		0 (0%)	2 (100%)	
	4 (8)	8 (28.6%)	20 (71.4%)		0 (0%)	1 (100%)	
	5 (9-10)	3 (5.3%)	54 (94.7%)		1 (33.3%)	2 (66.7%)	
D'Amico Risk Groups	Low	3 (37.5%)	5 (62.5%)	5.419 (p=0.067)	0 (0%)	4 (100%)	0.706 (p=0.702)
	Intermedi -ate	0 (0%)	9 (100%)		1 (5.3%)	18 (94.7%)	
	High	15 (12.8%)	102 (87.2%)		0 (0%)	9 (100%)	
Development of Castrate Resistance	No	9 (19.1%)	38 (80.9%)	1.150 (p=0.283)	2 (6.7%)	28 (93.3%)	0.213 (p=0.645)
	Yes	10 (12.2%)	72 (87.8%)		0 (0%)	3 (100%)	
Androgen Deprivation Therapy (ADT)	No	4 (18.2%)	18 (81.8%)	0.064 (p=0.800)	2 (6.3%)	30 (93.8%)	0.067 (p=0.796)
	Yes	15 (16%)	79 (84%)		0 (0%)	1 (100%)	
Association with Ki67	Ki67 Negative	11 (13.6%)	70 (86.4%)	0.003 (p=0.958)	2 (6.7%)	28 (93.3%)	0.071 (p=0.790)
	Ki67 Positive	1 (14.3%)	6 (85.7%)		0 (0%)	1 (100%)	
Association with DLX2	DLX2 Negative	3 (15%)	17 (85%)	0.183 (p=0.668)	-	-	-
	DLX2 Positive	14 (19.2%)	59 (80.8%)		-	-	

Table 34: Association of TGF-β cytoplasmic protein expression with clinicopathological variables. Significant associations are shown in bold.

		Combined Caucasian and AH Cohort Analysis TGF-β Cytoplasmic		
		TGF-β Cytoplasmic Negative	TGF-β Cytoplasmic Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	19 (65.5%)	10 (34.5%)	**33.896 (p=<0.001)**
	Afro-Caribbean	2 (6.1%)	31 (93.9%)	
	Black - Other	-	-	
	Indian	2 (100%)	0 (0%)	
	Not Specified	65 (59.1%)	45 (40.9%)	

Table 35: Association of TGF-β cytoplasmic protein expression with race. Significant associations are shown in bold.

TGF-β Cytoplasmic – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
8.846	**0.003**	3.365	0.067	0.196	0.658

Table 36: Kaplan-Meier modelling results demonstrating the association between TGF-β cytoplasmic protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months).

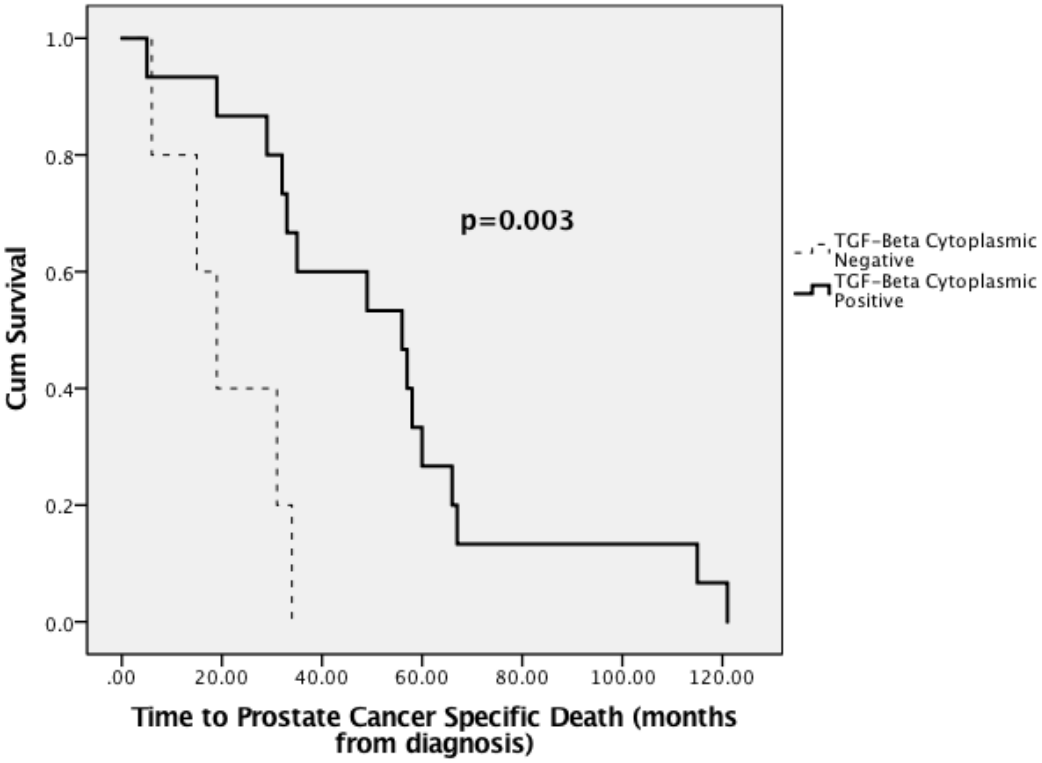


Figure 25: Kaplan-Meier chart demonstrating the association between TGF-β cytoplasmic protein expression and prostate cancer specific death

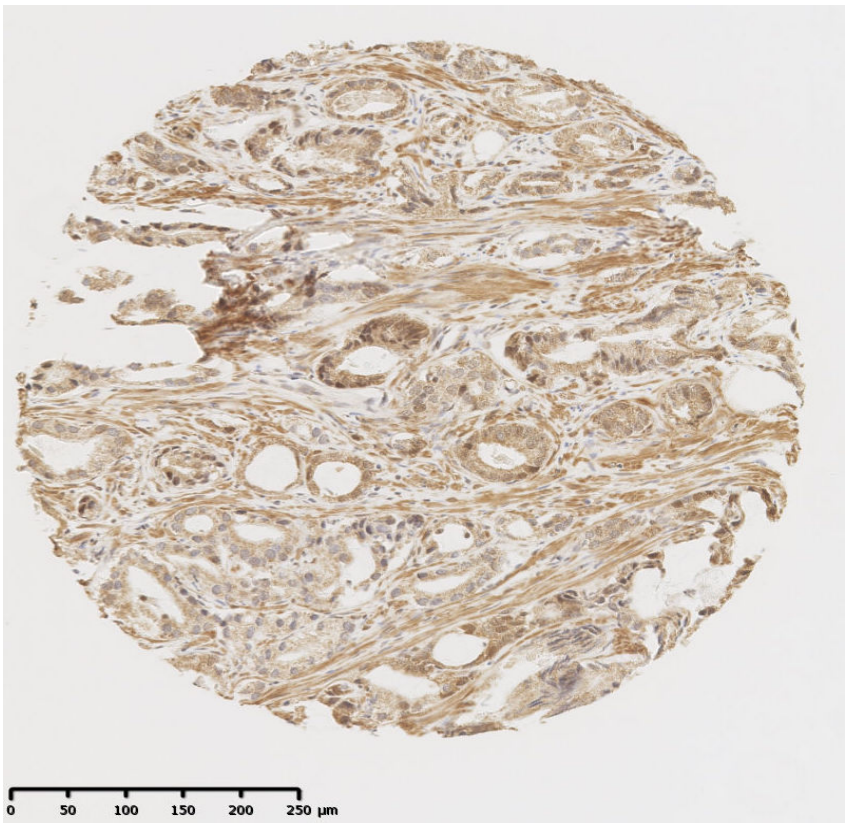


Figure 26a.

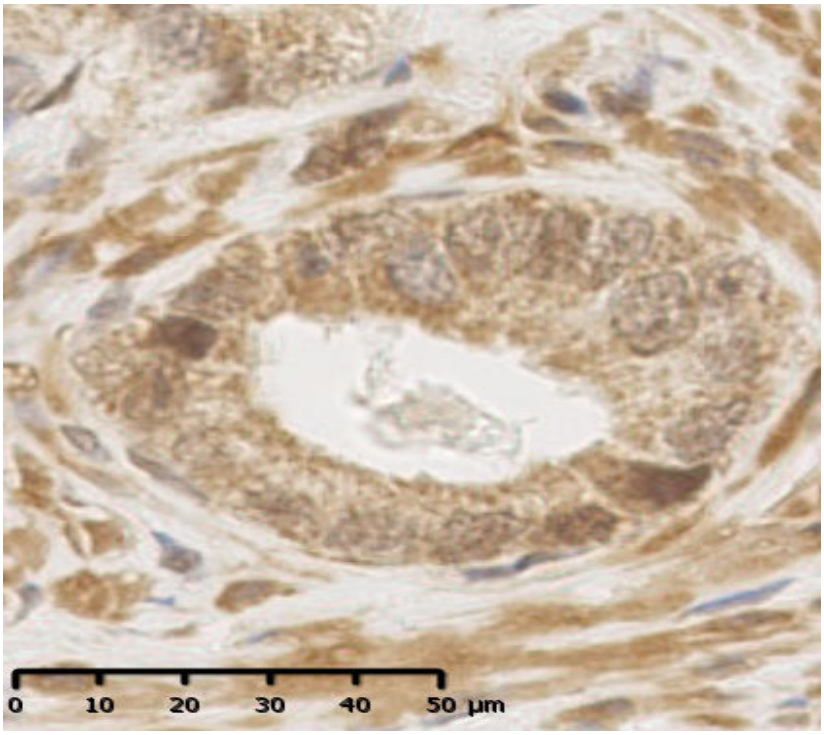


Figure 26b.

Figure 26: Example of IHC staining of TGF-β in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 39, TURP specimen

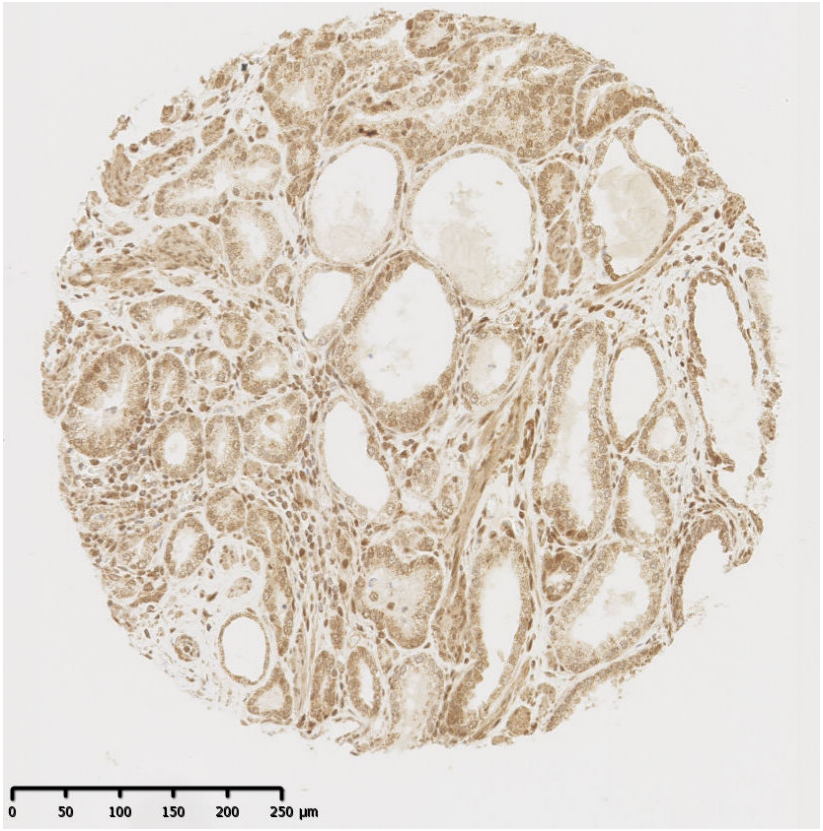


Figure 27a.

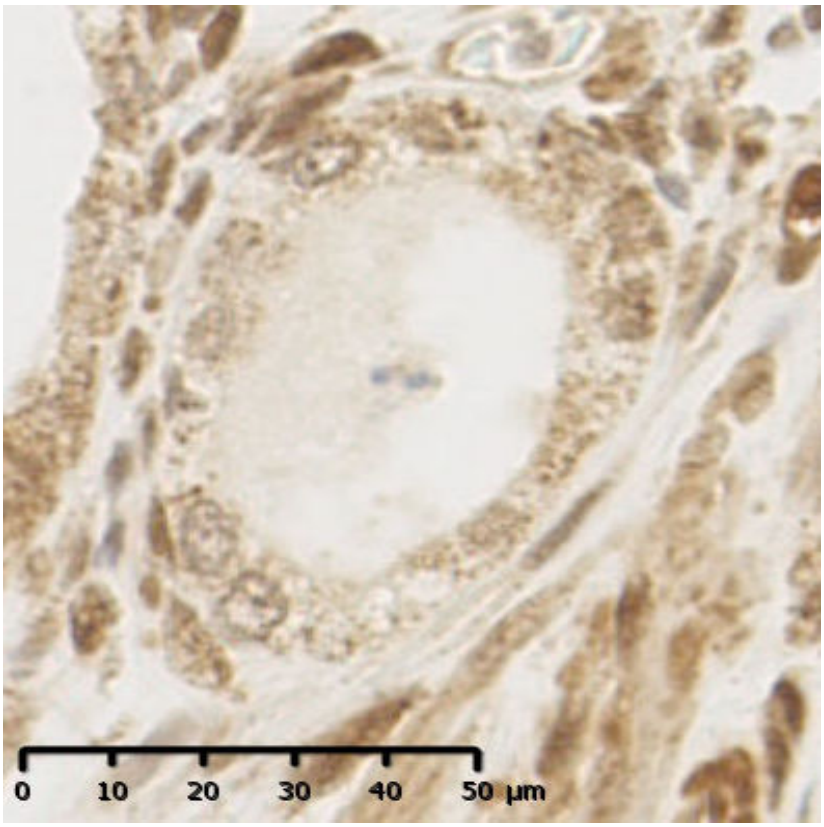


Figure 27b.

Figure 27: Example of IHC staining of TGF-β in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 11, radical prostatectomy specimen)

4.6 PTEN

Associations between biomarker PTEN protein expression and clinicopathological variables and Kaplan-Meier modelling for prostate cancer specific survival, time to metastasis and castrate resistance are shown below in tables 35-37. No data was obtained from the AH cohort as all patients were positive for expression for the specified staining threshold.

There was no association with presence of PTEN and prostate cancer specific death, time to metastasis, time to development of castrate resistance, serum PSA, Gleason groups, D'Amico risk groups, development of castrate resistance, requirement for ADT, Ki67 or DLX2 expression. Increased expression of PTEN was seen in AH patients (x^2=13.751, p=<0.003).

Clinical Variable		PTEN					
		Caucasian Cohort			AH Cohort		
		PTEN Negative	PTEN Positive		PTEN Negative	PTEN Positive	
		Number (%)	Number (%)	Chi Square (p-Value)	Number (%)	Number (%)	Chi Square (p-Value)
PSA (ng/ml) at diagnosis	<4	2 (50%)	2 (50%)	1.795 (p=0.180)	-	-	-
	>4	23 (21.5%)	84 (78.5%)		-	-	
Gleason Groups (Gleason Score)	1 (≤6)	0 (0%)	1 (100%)	2.849 (p=0.583)	-	-	-
	2 (3+4=7)	3 (27.3%)	8 (72.7%)		-	-	
	3 (4+3=7)	2 (33.3%)	4 (66.7%)		-	-	
	4 (8)	8 (34.8%)	15 (65.2%)		-	-	
	5 (9-10)	10 (18.9%)	43 (81.1%)		-	-	
D'Amico Risk Groups	Low	1 (16.7%)	5 (83.3%)	0.444 (p=0.801)	-	-	-
	Intermedi -ate	2 (33.3%)	4 (66.7%)		-	-	
	High	25 (25%)	75 (75%)		-	-	
Development of Castrate Resistance	No	10 (25.6%)	29 (74.4%)	0.000 (p=0.993)	-	-	-
	Yes	18 (25.7%)	52 (74.3%)		-	-	
Androgen Deprivation Therapy (ADT)	No	5 (27.8%)	13 (72.2%)	0.177 (p=0.674)	-	-	-
	Yes	18 (23.1%)	60 (76.9%)		-	-	
Association with Ki67	Ki67 Negative	18 (25.4%)	53 (74.6%)	0.225 (p=0.636)	-	-	-
	Ki67 Positive	1 (16.7%)	5 (83.3%)		-	-	
Association with DLX2	DLX2 Negative	7 (35%)	13 (65%)	0.976 (p=0.323)	-	-	-
	DLX2 Positive	15 (23.8%)	48 (76.2%)		-	-	

Table 37: Association of PTEN protein expression with clinicopathological variables.

		Combined Caucasian and AH Cohort Analysis PTEN		
		PTEN Negative	PTEN Positive	
		Number (%)	Number (%)	Chi-Square (p-value)
Association with Race	White British	4 (20%)	16 (80%)	13.838 (p=0.003)
	Afro-Caribbean	0 (0%)	32 (100%)	
	Black - Other	-	-	
	Indian	1 (100%)	0 (0%)	
	Not Specified	25 (25.3%)	74 (74.7%)	

Table 38: Association of PTEN protein expression with race. Significant associations are shown in bold.

PTEN – Caucasian cohort					
Prostate cancer specific mortality		Time to metastasis development		Time to development of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
2.729	0.099	0.204	0.652	0.01	0.922

Table 39: Kaplan-Meier modelling results demonstrating the association between PTEN protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months).

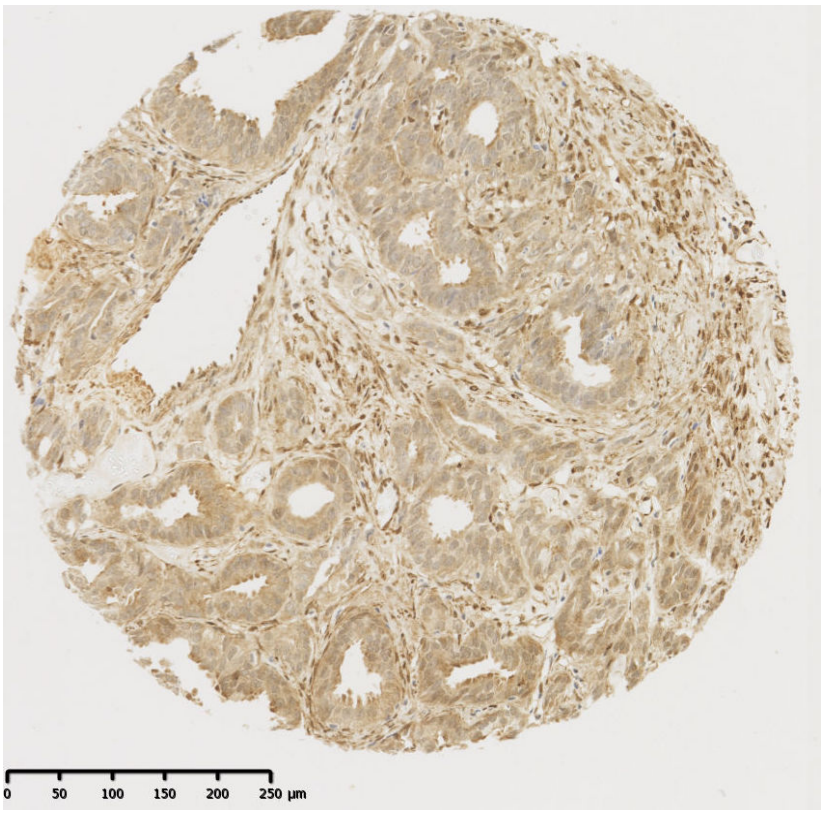


Figure 28a.

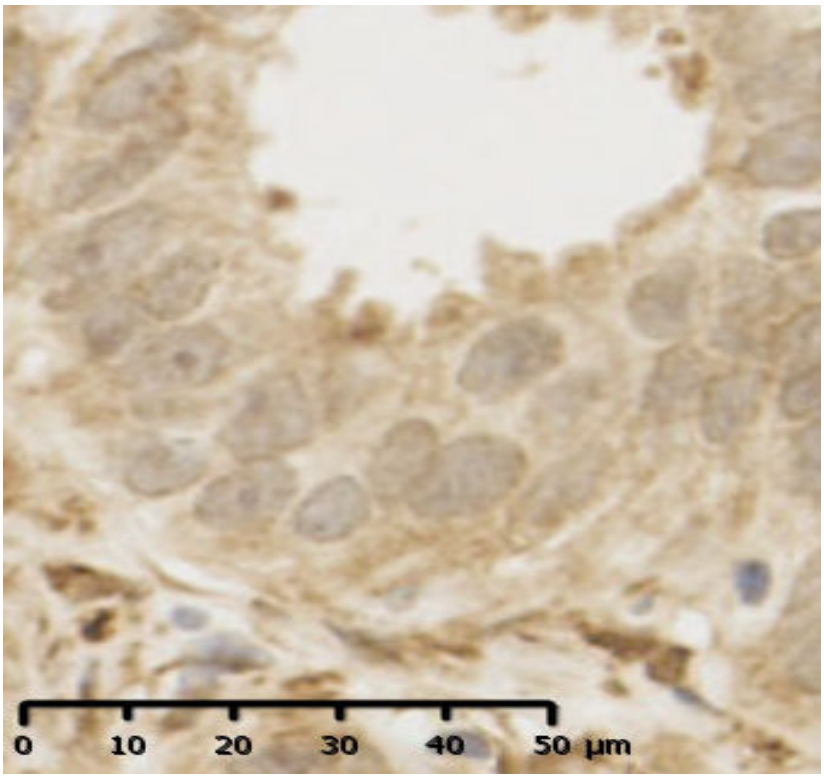


Figure 28b.

Figure 28: Example of IHC staining of PTEN in Caucasian tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 4+5 =9, PSA 61, TURP specimen)

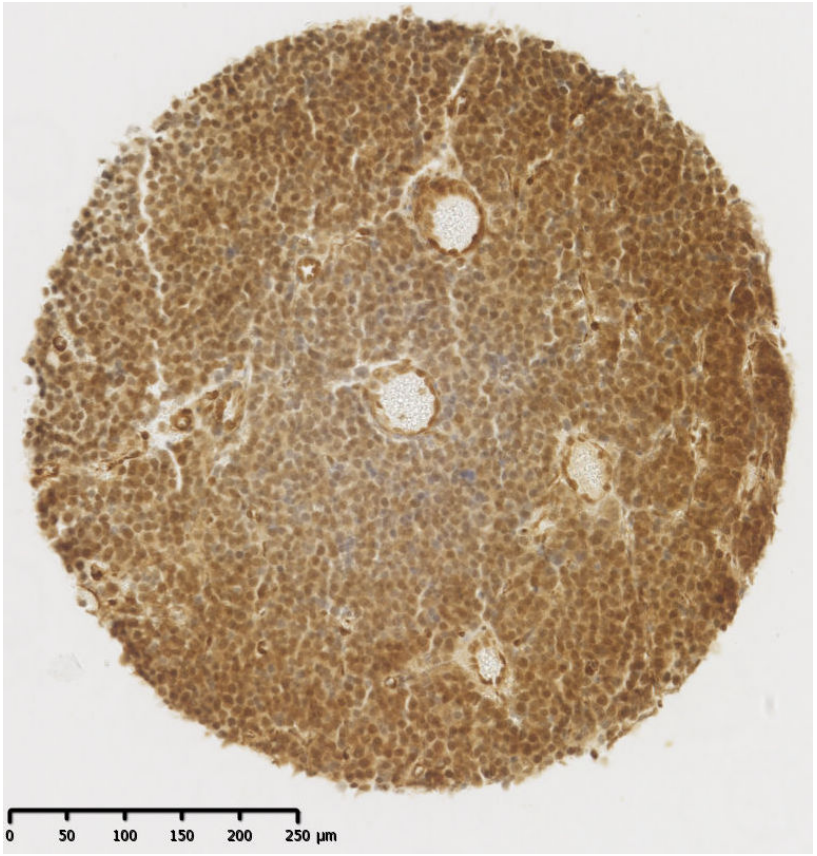


Figure 29a.

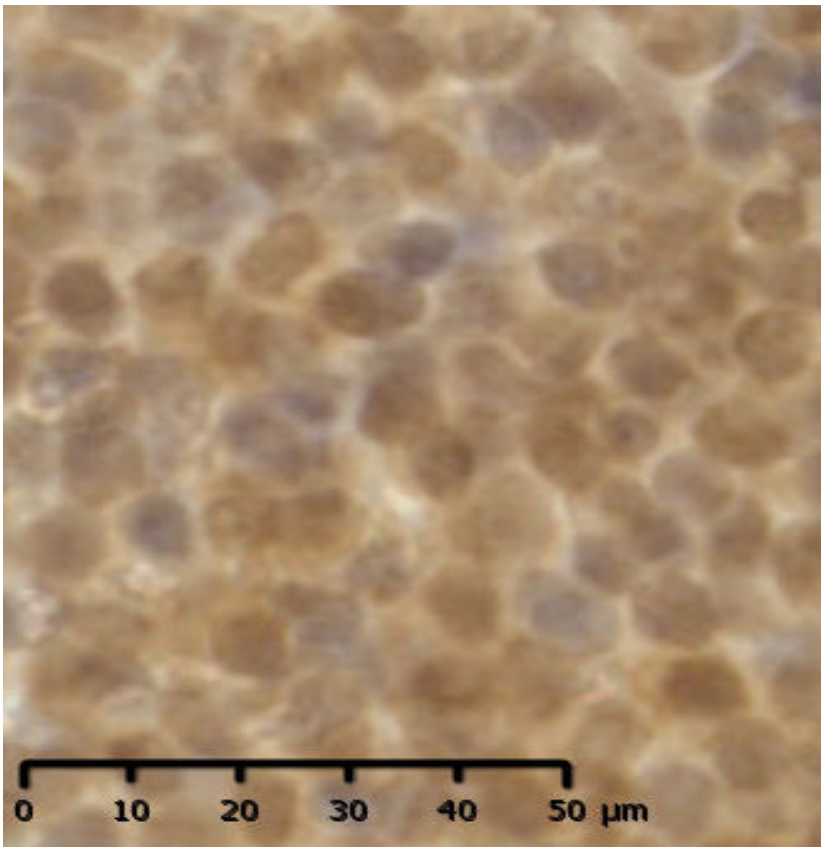


Figure 29b.

Figure 29: Example of IHC staining of PTEN in AH tissue at x10 (a) and x40 (b) magnification (Patient characteristics: Gleason 5+4 =9, PSA 100, TURP specimen)

4.7 Biomarker combination analysis

In combination, presence of Lambda FLC expression and decreased TGF-β cytoplasmic protein expression were highly predictive of prostate cancer specific survival (x^2=12.537, p=<0.001) and development of metastatic disease (x^2=6.864, p=0.009). See table 38 and figures 30-31.

Combined TGF-β and Lambda FLC– Caucasian cohort		
Prostate cancer	**Time to metastasis**	**Time to development**

specific mortality		development		of Castrate resistance	
x^2	p-value	x^2	p-value	x^2	p-value
12.537	<0.001	6.864	0.009	0.739	0.39

Table 40: Kaplan-Meier modelling results demonstrating the association between combined Lambda FLC and TGF-β cytoplasmic protein expression and prostate cancer specific death, time to metastasis (months) and time to development of castrate resistance (months).

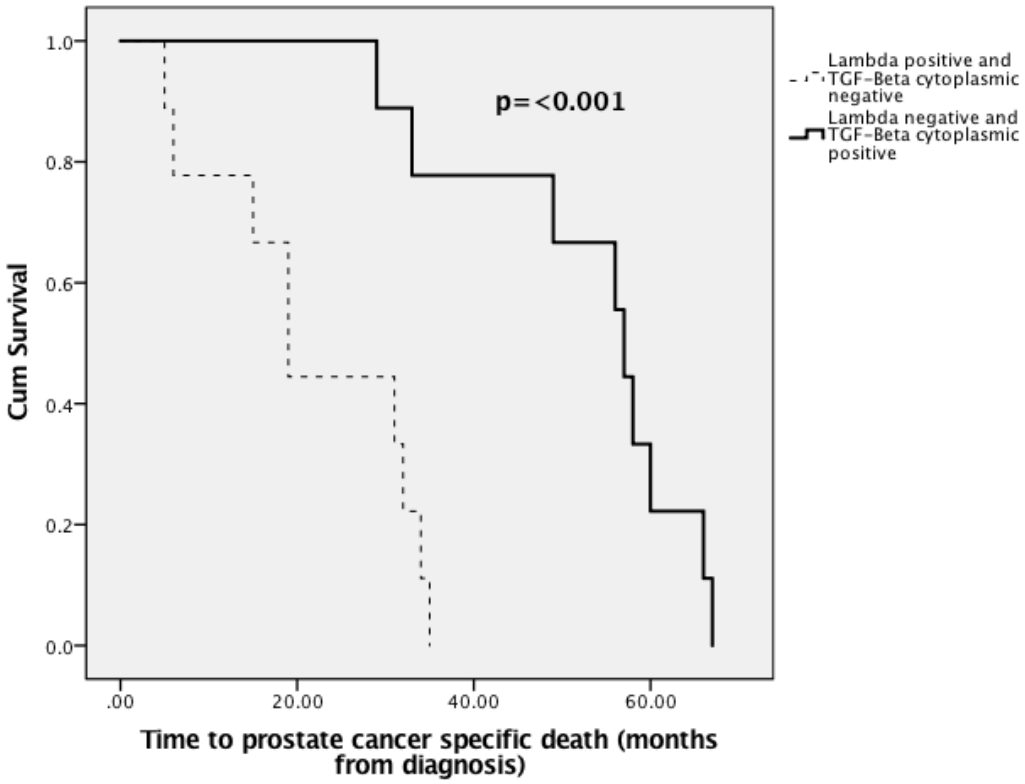


Figure 30: Kaplan Meier Chart demonstrating the association between Lambda FLC and TGF-β cytoplasmic protein expression and prostate cancer specific death (months from diagnosis).

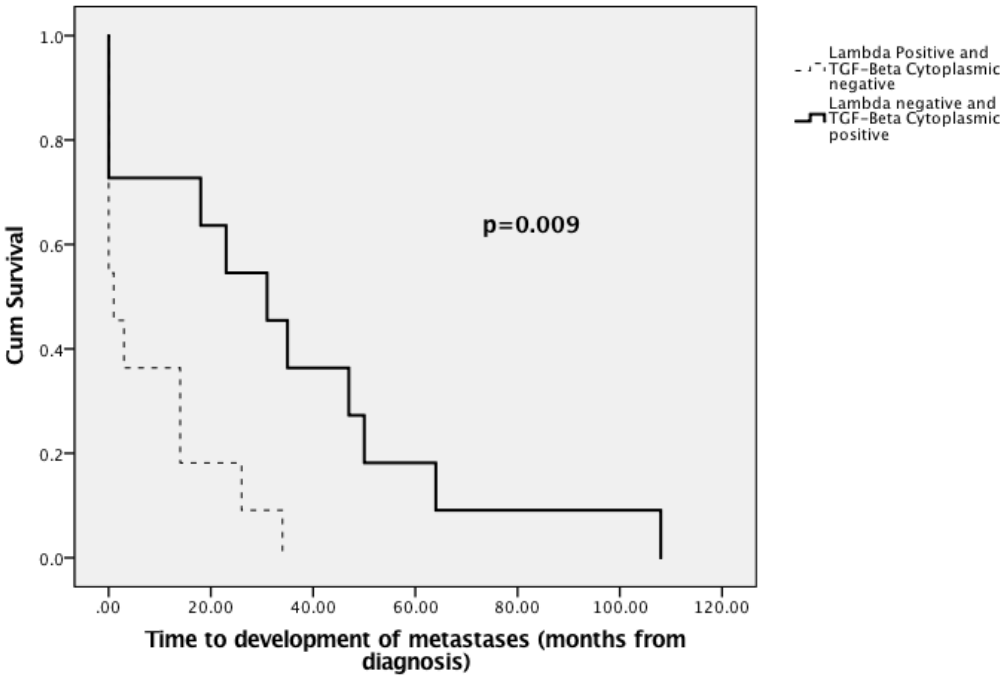


Figure 31: Kaplan Meier Chart demonstrating the association between Lambda FLC and TGF-β cytoplasmic protein expression and Time to development of metastases (months from diagnosis).

5.0 Discussion

5.1 Overview

This study identifies potential future biomarkers of CaP that could be used to add prognostic benefit to current CaP staging methods. In particular, we sought to identify a prostate cancer biomarker that would indicate AH patients at significant risk of developing aggressive or advanced disease.

We employed artificial neural network analysis and thorough research of biological pathways to identify genes associated with increased risk of metastasis development in AH patients to identify a panel of new CaP biomarkers that could identify patients at risk in this specific population.

We created a new TMA of CaP from AH patients to allow analysis of relevant protein expression against multiple recorded clinical outcomes and to compare this to a historic predominant Caucasian TMA.

We have demonstrated several biomarkers with the potential to predict disease progression and clinical outcome in an unselected group of prostate cancer patients. We have shown biomarkers Lambda FLC, TGF-β, EPB1LA4A ad PDL-1 have a statistically significant relationship with key prognostic outcomes; prostate cancer mortality (lambda FLC, TGF-β), development of prostate cancer metastasis (lambda FLC) and time to development of castrate resistance (EPB1LA4A, PDL-1). We have previously summarised the potential biological pathways these biomarkers may be involved with in the development of CaP. The markers lambda FLC and EPB1LA4A are

entirely novel in CaP and this work is the first study to identify them as potential prognostic markers in CaP. Unfortunately we have been unable to demonstrate these results in our AH sample population due to limitations with the size of the AH cohort, the limited maturity of the clinicopathological follow-up data and the disparity in the numbers of high risk CaP patients between the two cohorts.

Although we did demonstrate differential expression in the majority of our biomarkers, with increased protein expression of Kappa FLC, Lambda FLC, EPB1LA4A, CYB561A, DLX-2, PTEN, PD-L1 and TGF-β in AH CaP samples compared to Caucasian samples, these results need to be carefully considered in view of the limited AH data set and disparity of high risk disease between the two cohorts. Our recommendation would be to review and validate these identified biomarkers in a wider AH population with more mature (longer post-diagnosis follow-up) clinicopathological data with CaP risk matched controls.

5.2 AH background and wider context

AH men have an approximately 3-fold greater risk of developing prostate cancer and are more likely to present with CaP at a younger age (10,227), with a 30% higher death rate compared to Caucasian men (228). There are multiple potential reasons for this disparity as discussed earlier.

However there are other non-biological factors that may be associated with this increased risk. In the UK it was recognised that there was a health

inequality in prostate cancer in AH men. In the UK there is limited high-quality data on CaP and AH men, which may be contributing to this inequality. In addition to this lack of national and local ethnic data there has been little research on CaP in different ethnic groups within the UK. Without data derived from high quality research into CaP in ethnically diverse communities, local commissioning services lack the evidence needed to fund new services. In particular this may result in a failure to meet local black and minority ethnic communities (BME) need for increased access to prostate cancer diagnosis and treatment.

Despite the increased risk of CaP, awareness of this risk is lowest amongst AH men. In a UK study, 64% of Caucasian men had heard of CaP compared to only 37% of AH men (229). In a study performed by The Prostate Cancer Charity, 58% of AH men correctly identified CaP as the most common form of cancer in men compared with 69% of the general population of the UK and only 15% of AH men knew they were at an increased risk of developing CaP (230).

There is a lack of awareness of racial disparities in CaP amongst primary care providers as well, with some general practitioners (GPs) being unaware of the increased risk of CaP in younger AH men. There have been anecdotal reports of AH men being denied a PSA test on the basis they were too young to develop CaP (231). The combination of decreased awareness of CaP amongst AH men and lack of perceived risk in this population by health care professionals is almost certainly a contributing factor to the ongoing racial

disparity in outcomes. BME communities have often cited poorer experiences of healthcare interactions compared to Caucasian people. First generation AH immigrants are much less likely to engage with healthcare professionals largely due to feelings of the healthcare system being unresponsive to their particular needs combined with a perceived lack of cultural sensitivity (232).

Studies have highlighted the most common barriers to prostate cancer detection in AH men being poor knowledge, lack of financial/insurance cover (American men), mistrust of healthcare professionals and poor relationships with healthcare systems, fear of cancer diagnosis, fear of death and fear of testing procedures. Other barriers and fears identified were, "threat to manhood", reluctance to talk about sex-related health issues, complacency, a belief that a DRE threatens male sexuality and a belief that CaP is related to sexual behaviour (230).

Disadvantaged groups (including BME groups) have higher incidence and mortality rates and poorer outcomes from cancer. Several studies have shown that the incidence of cancer and treatment success is associated with socioeconomic status (233). In response to the unmet cancer need of BME communities in the UK Rose Thompson founded "BME Cancer Communities" providing cancer information to and supporting BME and low-income communities. A study conducted by BME Cancer Communities in Nottingham (where it is based) in 2009 showed that 36% of BME people in Nottingham didn't know what the signs or symptoms of cancer were and

47% did not know how to reduce their own cancer risk. Whilst at the same time 55% of people had a family member directly affected by cancer (234). In addition to increased incidences of some cancer in BME groups inequality in cancer outcomes can also be accounted for by numerous other factors; lifestyle choices such as smoking, lack of awareness, language barriers, lack of engagement and uptake in cancer screening, poor previous experiences, unemployment and poverty (231).

In 2010, the coalition government made a commitment to saving 5,000 lives from cancer every year by 2015 in the cancer strategy "Improving Outcomes: A Strategy for Cancer" (221). The strategy also made health inequality a priority for NHS cancer services, realising that addressing these inequality gaps would improve overall survival rates. This led to the report by Rose Thompson "Hear Me Now" (231) in 2013 setting out clear recommendations on improving outcomes of CaP in AH men. These included a need for high quality ethnicity equality data collection at a national level to measure prostate cancer inequalities, and increased local level support of BME populations to promote awareness of CaP in black men in an engaging and culturally sensitive approach. Although hoped this report would lead to wide scale national changes the most notable impact was seen at a local level. Within Nottinghamshire particularly there was a notable increased engagement in local BME groups such as The Friends and Bredrins (FAB) charity and the BME community. There was specific investment in urology BME outreach clinics for outreach urology nurses to see BME men and counsel them with regards to assessment and screening for CaP.

Mortality rates from CaP vary across the various regions of England. Mortality rates in the East Midlands are higher than the England Average with Nottingham having the second highest death rate from CaP as well as the highest black population in the East Midlands (231). Nottingham was identified as a "lead city" in tackling health inequality in BME groups. The FAB charity has been set up in Nottingham: it is a volunteer run charity aimed at raising awareness about CaP and to provide support to AH men affected by CaP with educational talks, group peer-to-peer support and advice.

It was this recognition of this health inequality amongst BME groups particularly observed in Nottingham that acted as a significant impetus for this research and thesis project.

Several recent high profile clinical research studies have sought to look at whether understanding specific genetic features can improve prostate cancer diagnosis in men with high risk of the disease including high risk groups such as AH men specifically. The PROFILE study (28,235) is currently recruiting men with a family history of prostate cancer and healthy African/Caribbean men aged 40-69. Men recruited will have blood and tissue samples to collect genetic information and then they will be monitored for development of CaP over 5 years using PSA testing with men choosing whether to undergo an MRI scan or prostate biopsy as well. It is anticipated the study will assess whether men with specific genetic features

are more likely to develop cancer and may benefit from targeted CaP screening. The largest known multiancestry meta-analysis of prostate cancer genome-wide association studies has recently shown that men of African ancestry have a mean genetic risk score that was 2.18 times higher than men of European ancestry (236). The genetic risk score identified could be used in future to identify men at risk and need targeted screening particularly in AH men given their higher incidence rates. It is hoped this research and future studies can play an important role in reducing health inequalities among AH men with prostate cancer by allowing them and others at high genetic risk to be identified and treated earlier.

5.3 Biological background

There is a considerable need to develop alternative prostate cancer biomarkers with better diagnostic and prognostic capabilities able to distinguish patients more likely to develop higher risk aggressive disease.

We know AH patients with CaP have a higher risk profile compared to their Caucasian counterparts. This study has identified potential biological pathways associated with CaP progression and development of metastasis within AH populations, which we discussed earlier. We have identified several tumour biomarkers associated with these biological prostate tumour pathways. Although our results relating to CaP progression, survival and development of metastases were in an unselected cohort of patients and not specific to the AH cohort, the results still have some clinical relevance to

this cohort. We discuss their relevant biological pathways and our findings below.

5.3.1 Inflammation, cell adhesion, migration and alteration to EMT

TGF-β is a multifunctional cytokine, comprising three isoforms that has been linked to the regulation of tumour initiation, progression and metastasis. It is able to exert this influence through its ability to regulate both tumour and host cell populations. It is involved in mediation of cell cycle regulation via epithelial cell proliferation and apoptosis, as well as regulation of inflammatory and immune pathways. Ultimately this allows TGF-β to control tumour progression through modification of tumour cell behaviour and interactions with adjacent cell populations in the tumour microenvironment.

TGF-β signalling is mediated through SMAD and non-SMAD pathways regulating transcription, translation, protein synthesis, microRNA synthesis and post-translational modifications (237–240).

TGF-β has been studied in multiple epithelial cancers. It appears that it can function as both a tumour suppressor and a tumour promoter (241). In benign epithelia and early tumour growth stages it acts as an inducer of growth arrest and is generally considered anti-proliferative and pro-apoptotic. However in advanced tumour stages where TGF-β pathways are altered it promotes tumour growth and progression with pro-survival and anti-apoptotic effects (242). This is known as the TGF-β paradox (243).

In contrast with its role in regulating epithelial cell proliferation TGF-β is seen as a pro-growth signaller in the mesenchyme. In response to injury, the influx of inflammatory cells leads to an increase in TGF-β at the site of injury. TGF-β then results in fibroblast proliferation and resultant remodelling of the extracellular matrix (244). TGF-β induces EMT in cancer cells (245). EMT is characterised by loss of E-cadherin and expression of proteins such as vimentin, fibronectin and N-cadherin leading to progression and metastasis of cancer cells which is driven by TGF-β (246).

Another key step in cancer progression and invasiveness is evasion of the host immune system. It is well known that cytotoxic lymphocytes, such as natural killer and T-cells can effectively destroy cancer cells. TGF-β plays a key role in repression of the host immune system within the microtumour environment, suppressing the anti-tumour activity of natural killer cells, T-cells, neutrophils, dendritic cells monocytes and macrophages whilst stimulating their recruitment (247,248). As a result TGF-β associated inflammation can promote tumourigenesis due to secretion of growth factors, cytokines, chemokines, proteases modifying the extra-cellular matrix stimulating cancer cell growth, motility and invasion.

We have previously discussed the study by Kinseth et al (167) which demonstrated the majority of differentially expressed genes in matched prostate cancer specimens in AH and Caucasian men related to genes associated with tumour adjacent stroma rather than tumour tissue. This

suggested that altered immune, inflammatory and EMT processes particularly in the tumour adjacent stroma may be responsible for the aggressive nature of CaP in AH men. Interestingly they found that SMAD-3, TGF-β 2 and TGF-β receptor 3 was down regulated in AH men samples compared to Caucasian samples. Powell et al (165) demonstrated that TGF-β 1 as well as multiple related TGF-β SMAD-independent pathway mediators were up regulated in AH prostate cancer specimens compared to Caucasian specimens. We demonstrated that loss of TGF-β cytoplasmic protein expression was associated with worse prostate cancer specific survival (x^2=8.846, p=0.003). Mean survival was 21 months vs. 53.5 months for patients positive and negative for TGF-β cytoplasmic protein expression respectively. This was demonstrated in the Caucasian population only. We also demonstrated variable expression between populations with increased cytoplasmic expression of TGF-β in AH samples (x^2=33.896, p=<0.001), validating previous findings by Powell et al (165) .

There are multiple reasons for our variable expression of TGF-β within CaP tissue samples. One is the fact the biomarker we used for IHC was related to one TGF-β isoform only, TGF-β 1, however all three isoforms of TGF-β have been shown to be virtually identical and exhibit indistinguishable actions in vitro (249). TGF-β expression is also highly variable due to heterogeneous role with both suppressive and promoting effects. TGF-β has been shown to have a key role in androgen control of prostatic growth regulating stromal proliferation and differentiation in BPH. Descazeaud et al (250) demonstrated Increased TGF-β 2 receptors in relation to prostatic volume.

Zeng et al (251) demonstrated that TGF-β receptor 2 expression was down regulated in HGPIN and prostate cancer compared with BPH. This relates to the complexities of TGF-β signalling and likely paradox in tumour biology as previously discussed, where in benign cells or very early tumour cells, TGF-β expression might be increased. Whereas in advanced tumours, TGF-β expression may be lost. Interestingly gain or loss of TGF-β signalling can promote increased aggressive and invasive behaviour. Gene expression profiles representing gain or loss of TGF-β signalling in mammary carcinoma cells have been shown to correlate with poor prognosis in human breast cancer (252–254). Increased TGF-β 1 expression by tumour cells has been shown to correlate with tumour progression in non-small cell lung carcinoma, colorectal cancer and gastric cancer (255–257). This highlights the extremely diverse and complex nature of TGF-β mediated signalling within the tumour microenvironment, with down- or up-regulation leading to aberrant expression and increasingly aggressive and invasive disease, and dual tumour suppressive and promoting effects.

Due to these complexities, utilising therapies that target TGF-β pathways is potentially challenging and may even lead to poorer outcomes. Despite this monoclonal neutralizing antibodies targeting TGF-β receptors have been evaluated in the treatment of metastatic breast cancer and malignant pleural mesothelioma (258). Another therapy involving inhibition of the kinase activity of the TGF-β receptor has been developed (259). Although these clinical trials have shown promise, they have been aimed at treating late-stage metastatic disease and the challenge remains in targeting only the

tumour promoting aspects of TGF-β, along with identifying the patients that will respond well.

EPB41L4A is a member of the band 4.1 protein superfamily. The 4.1 proteins, encoded by the EPB41 (erythrocyte protein band 4.1) genes are components of the cortical cytoskeleton underlying the cell membrane (260). They contribute to the organisation of cell polarity, adhesion and motility affecting transport through the cell membrane and response to cellular growth factors. The 4.1 proteins are part of a larger family of proteins that activate the reorganisation of the cytoskeleton, its attachment to the cell membrane and changes to the ECM during cancer progression leading to cell migration and invasion. EPB41L4B expression has been shown to be increased in high grade melanoma cells (261). Furthermore expression of EPB41L4B was shown to be up regulated in CaP cell lines, with increased expression also leading to decreased adhesion of cancer cells to collagen, suggesting a role in metastasis development (262). The work presented in this thesis is the first to report on the prognostic potential of EPB41L4A as a novel biomarker demonstrating increased expression of this protein is associated with earlier development of castrate resistant disease (x^2=7.320, p=<0.007). Mean time to development of metastatic disease for patients positive for EPB41L4A protein expression was 16.5 months compared to 28.1 months in negative patients. An increased proportion of AH patients were positive for EPB41L4A protein expression (x^2=48.255, p=<0.001).

DLX-2 was included in our analysis to validate previous findings demonstrated by Green et al (217), who identified DLX-2 as a novel marker of increased risk of metastasis development in prostate cancer. DLX gene family is involved in embryonic development, tissue homeostasis, cell cycle and apoptosis (263). DLX2 has been shown to be involved with shifting TGF-β function from tumour suppression to tumour promoting by repressing TGF-β receptor 2 and cell cycle inhibitor p21CIp1 and increasing mitogenic transcription factors c-myc and epidermal growth factor (264), leading to increased EMT. We demonstrated increased expression of DLX-2 in AH patients (x^2=0.237, p=0.001).

5.3.2 Immune response

As a result of inflammation, the tumour microenvironment is flooded with innate immune cells such as macrophages, neutrophils, mast cells, dendritic cells, natural killer cells and myeloid-derived suppressor cells as well as adaptive immune cells T and B lymphocytes (196). The development of cancer cells in response to chronic inflammation is outlined in figure 32. Antigens presented by early tumour cells are carried to lymphoid organs by dendritic cells (DCs) activating T and B cells, resulting in both tumour promoting and anti-tumour effects. Activation of B cells results in chronic activation of innate immune cells in the tumours. This promotes tumour development through the release of pro-survival molecules that modulate gene expression and alter the ECM and adjacent tumour stroma resulting in altered cell progression, cell survival and altered angiogenic vasculature. Activated T cells elicit an anti-tumour effect through T cell mediated tumour

cell lysis. In established tumours the balance between tumour promotion and anti-tumour immunity is tilted towards pro-tumour promotion due to environmental and microenvironmental changes. T cells are classified according to their effector functions $CD8^+$ cytotoxic cells and $CD4^+$ helper T Cells. T cells can similarly act as both tumour suppressors or promoters determined by their effector function (265). The tumour promoting effects of T cells and tumour associated immune cells are mediated by cytokines. Through the activation of downstream effectors such as NF-KB, AP-1, STAT and SMAD transcription factors, cytokines promote anti-tumour activity (IL-12, TRAIL, IFNγ) or enhance tumour progression (IL-6, IL-17, IL-23) and affect cancer cell regulation and survival (TRAIL, FasL, TNF- α, EGFR ligands, TGF-β, IL-6) (266). Powell et al (165) demonstrated that several of these cytokines are overexpressed in CaP found AH men compared to Caucasian men. Similarly Kinseth et al (167) demonstrated that these cytokine pathways were significantly altered when comparing gene expression arrays between ethnic groups.

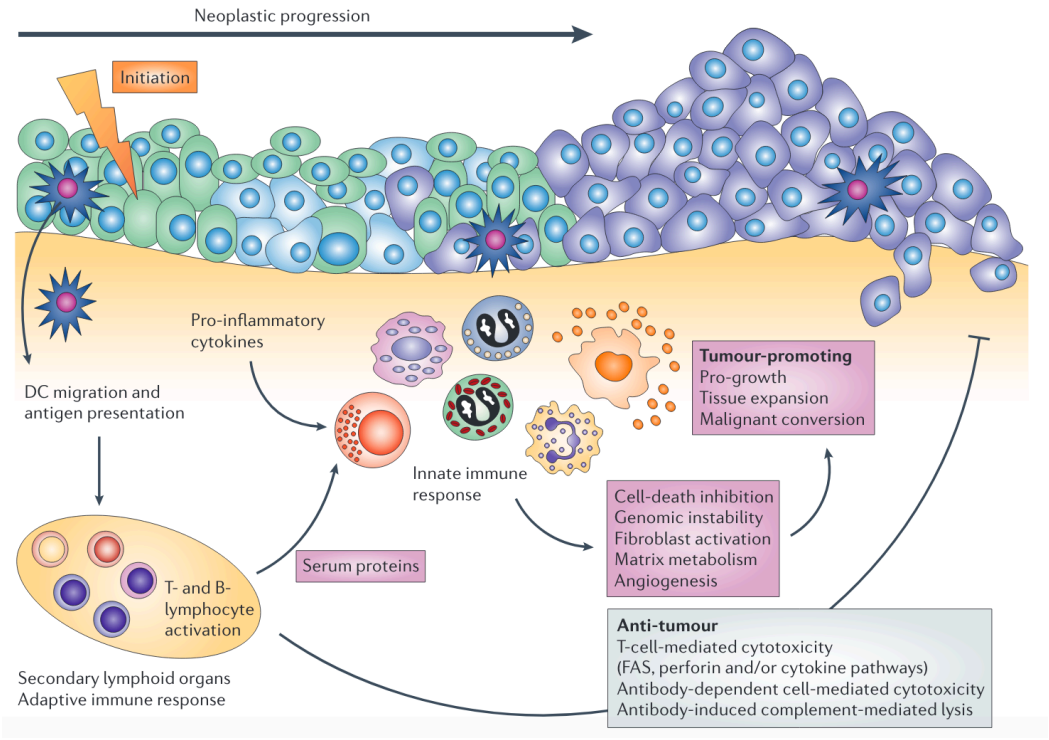


Figure 32: The role of the innate and adaptive immune-cell function during inflammation-associated cancer development (reused with permission from Nature Publishing Group, Licence number 4514801238451) (196)

Co-stimulatory and co-inhibitory molecules control antigen specific T Cell responses respectively. Co-inhibitory molecules prevent inappropriate immunity responses and limit the scale and duration of any given immune response. The main co-inhibitory molecules, termed 'checkpoint molecules' are CDLA-4 and PD-1 (267). PD-1 is expressed on activated $CD8^+$ cytotoxic cells and $CD4^+$ helper T Cells as well as natural killer cells, B cells and dendritic cells. The counter receptor of PD-1 is PD-L1 (also called B7-H1), expression of which has been seen on cancer cells (268–270). Normal PD-1 signalling in T cells serves to minimise damage to tissues from excessive inflammation and development of autoimmunity. Ligation of PD-L1 results in the formation of PD-L1/T-cell receptor (TCR) that effectively turns off T

cell activation. This in turn leads to down-regulation of the RAS and P13k/AKT pathways with suppression of cell cycle progression and T cell activation. PD-1 ligation by PD-L1 on other antigen-presenting cells (APC) such as macrophages or tumour cells inhibits the production of several cytokines and survival factors such as BCL-XL (271) see figure 33.

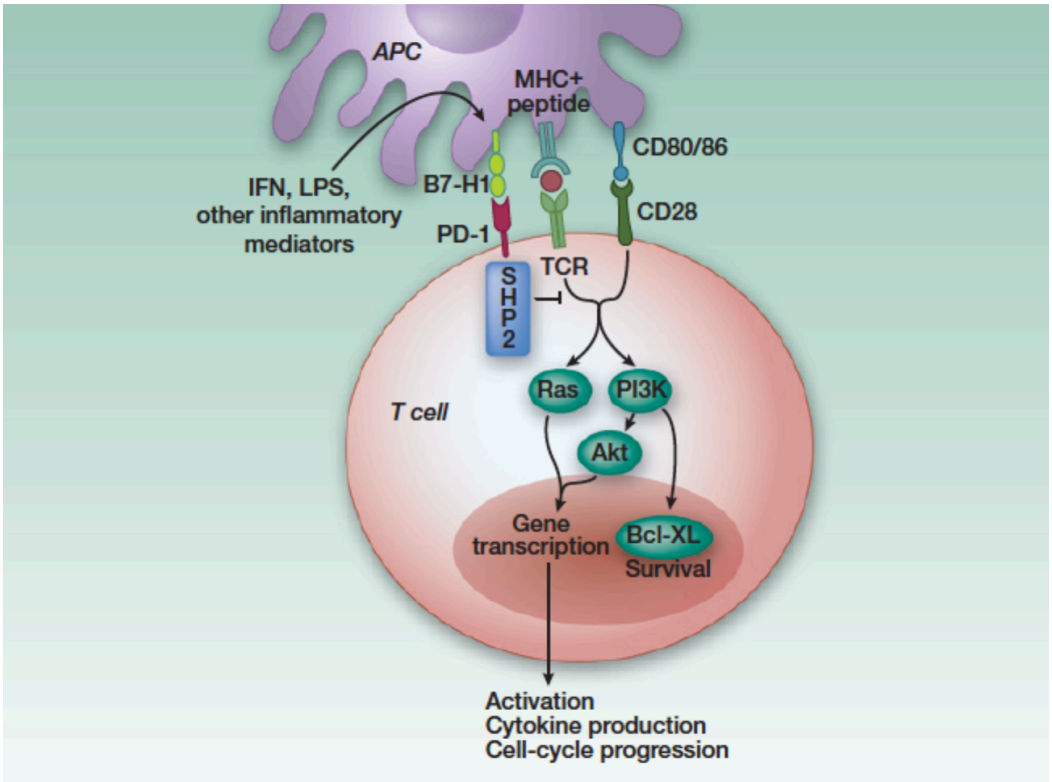


Figure 33: PD-L1 signalling (reused with permission from American Association for Cancer Research, license number 4515300836034) (272)

In the tumour microenvironment PD-L1 expression is induced in tumour cells and within the tumour microenvironment in response to inflammatory cytokines produced by T cells infiltrating into tumour tissue. It has been

reported that expression levels of PD-L1 correlate with advanced stage of cancer and poor prognosis (273). PD-L1 expression has been shown to be increased within an aggressive subset of breast cancer, basal type (274). In urothelial cancer tumour associated expression of PD-L1 was significantly associated with high rates of postoperative recurrence and poor survival rates (275). PD-L1 expression is also greatly increased in treatment resistant castrate resistant metastatic prostate cancer (276) and increased expression of PD-L1 in prostate cancer tissue has been associated with shorter biochemical-recurrence free survival (277). We demonstrated similarly that increased nuclear expression of PD-L1 was associated with development of castrate resistance (x^2=4.062, p=<0.044) and both nuclear and cytoplasmic protein expression of PD-L1 was increased in AH prostate cancer tissue. Mean time to development of castrate resistant disease was 19.9 months vs. 29.4 months for patients positive and negative for PD-L1 nuclear protein expression. We have to be cautious with interpreting these results however as we did not use an approved pharma (Roche/Dako) IHC kit. Utilising these kits for IHC requires meeting exacting and stringent parameters with regards to IHC thresholds; we therefore cannot guarantee the accuracy of these results.

Several clinical trials are underway evaluating treatment with monoclonal antibodies designed to disrupt PD-1/PD-L1 interaction in patients with advanced melanoma, non-small cell lung cancer, renal cell carcinoma and colorectal cancer with promising results with up to 36% of patient having an objective response to treatment (216,278).

Mast cells are key regulators of inflammation and modulate the immune response through angiogenesis, tissue degradation and regulation of T cell tolerance (279,280). Multiple tumours show evidence of mast cells within the ECM and tumour adjacent stroma. Mast cells appear to have a vital role with maintaining tumour growth through tumour related angiogenesis (281,282). Angiogenesis is vital for the expansion of tumours and development of metastasis. Mast cells produce a variety of potent cytokine mediators of angiogenesis such as VEGF, FGF-2, TGF-β, TNF-α, IL-8, NGF, tryptase, chymase, heparin and histamine (283). The alterations of these mediators within the ECM leads to remodeling and subsequent release of angiogenic factors leading to tumour associated angiogenesis and at advanced stages, invasion. Furthermore mast cells are able to promote cell proliferation and inhibition and modulation of the immune system. It appears that during tumour growth, tumour cells release various chemotactic factors recruiting mast cells to the tumour microenvironment. Within the tumour microenvironment mast cells become activated releasing multiple mediators resulting in a ECM remodeling with resultant angiogenesis, tumour cell proliferation, recruitment and activation of other tumour activated macrophages and subsequent immunosuppression/modulation.

FLCs can activate mast cells in a antigen specific fashion making up an integral part of the immune system response to antigen exposure (284).Within a large breast carcinoma population with long term clinical

follow-up, patients with triple negative basal type aggressive cancer phenotype showed increased expression of lambda FLC. This showed significant association with decreased survival, decreased disease free survival and increased time to metastasis formation (221).

We found that increased lambda FLC expression was associated with a decreased prostate cancer specific survival (x^2=10.496, p=0.001) and an increased time to development of metastatic disease (x^2=5.223, p=0.022). Mean survival time was 20.4 months vs. 48 months and mean time to development of metastatic disease was 7.8 months vs. 30 months for patients positive and negative for lambda FLC protein expression respectively. Increased expression of lambda FLC was also seen in AH patients (x^2=48.255, p=<0.001) where expression also showed a positive association with development of castrate resistance (x^2=7.320, p=<0.007). These findings validate those by Kormelink et al (221) and are completely novel with regards to prostate cancer.

Although we opted to assess FLCs because of their association with tumour related inflammation pathways, FLCs are involved with recruitment of tumour infiltrating leukocytes in particular macrophages. Another possible option for assessing the role of inflammatory pathways may have to assess for tumour infiltrating macrophages the role of which has previously been noted in advanced aggressive CaP, castrate resistant states and associated with a worse prognosis (285–288).

5.4 Limitations

In order to assess the validity of this work, it is important to examine the limitations of the project.

Firstly the majority of tissue incorporated into the historical predominant Caucasian TMA was taken from patients undergoing a TURP procedure rather than a diagnostic biopsy. Patients who are likely to be undergoing a TURP procedure are generally older than those patients who would be undergoing an elevated PSA related prostate biopsy. Therefore these patients are by the fact they are older, more likely to have higher volume and higher risk prostate cancer. This does not reflect the typical trend of patients that are currently undergoing PSA testing and subsequent prostatic biopsy, who are considerably younger. The studied cohort is still relevant as a group of patients with higher risk disease in the context of our study. It would be necessary to review our candidate biomarkers in a sample of patient more representative of current CaP diagnosis and management.

Although our newly constructed AH TMA predominantly comprises radical prostatectomy specimens, there were a significantly smaller proportion of higher risk disease patients and a smaller cohort in comparison to the predominant Caucasian-predominant TMA. The patients incorporated into the two TMA's had significantly different presentations at diagnosis and largely treated differently from radical prostatectomy in AH TMA and watchful wait in the Caucasian TMA. Therefore the difference in results and staining expression seen could be related to clinical or disease

characteristics alone. A more appropriate design method would have been to match Caucasian and AH patients on these clinical or disease characteristics.

A major limitation with the newer AH cohort was the limitation of long-term clinicopathological data with only approximately 2 years of clinical follow-up data. This limited relevant statistical analysis with regards of survival and metastasis development.

We used 2 TMA's for assessment of our biomarkers. Although the use of TMAs is a universally accepted method for assessment of large numbers of tissue samples with multiple biomarkers it does have some limitations. Staining of tissue samples can be variable with biomarker protein expression subjective and variable depending on the way the tissue samples are fixed.

When selecting the potential biomarkers from the bioinformatics analysis, the genes identified were largely selected for their potential novelty and significant level of interaction. However, there were genes that had significant interactions that were overlooked because of not seemingly being novel. An example of this is alpha-smooth muscle actin (ACTA2). Although this gene is not 'novel 'it could be potentially novel in its use and possible relation to CaP aggressiveness. Stromal phenotypes of CaP with an increased number of fibroblasts has been associated with aggressive disease and castrate resistance (289).

5.5 Conclusions

There is a clear disparity of incidence, mortality and aggressiveness of CaP between AH and Caucasian men respectively. As such there is a need to understand the mechanisms and possible reasons for this in order to be able to objectively identify patients that are at increased risk of developing high risk, aggressive disease at an earlier stage.

We have identified several biomarkers relating to the inflammatory and immune pathways associated with CaP, with the potential to predict disease progression and clinical outcome. Furthermore we have shown that these are differentially expressed in AH men. This suggests that inflammation and immune pathways as well as those regulating EMT within the tumour microenvironment and stroma could influence the development and progression of more aggressive CaP in AH men.

We have identified Lambda FLC as a novel biomarker in CaP and this is the first to report its potential prognostic utility in CaP. We have shown that this biomarker is able to predict disease specific survival and metastatic disease in CaP, showing great promise as a prognostic biomarker in a retrospective cohort.

We have also shown that expression of lambda FLC is also significantly increased in CaP from AH men suggesting a role in development and progression of aggressive forms of CaP. This marker is relatively cheap and

easy to assay with minimal tissue required, making it an ideal potential biomarker. The techniques used to quantify its expression are routinely used in most hospitals meaning that its use and analysis are technically and logistically possible.

It is important that these findings are now applied to a wider AH cohort particularly with an extended period of clinical follow-up data to assess the prognostic implications of lambda FLC expression in AH men.

Interestingly despite the advances in CaP diagnosis and treatment in recent years, AH men remain poorly represented in CaP clinical trials (290). This is largely due to underreporting of race and studies failing to account for it in subsequent analysis. The implications of this are significant, as CaP clinic trials and studies not proportionally representing the population being studied leads to selection bias and as a result conclusions and findings may not necessarily be applicable to AH men. There are however multiple CaP clinical trials on going actively involving AH patients with one currently reviewing metastatic CaP treatment in Caucasian and AH men with Abiraterone and the PROFILE study currently actively recruiting (235,291). This study highlights the continued need to encourage better participation and enrolment of AH in high quality CaP clinical trials and ensure that racial data is collected and reviewed in subsequent analysis.

Inflammation is a very complex process with the interplay of several hundreds of different genes and pathways. Therefore there are many genes

that contribute to the process of development of CaP. Several studies including ours have shown how certain genes within a pathway contribute to an increased risk of CaP and development of aggressive disease. Larger studies within different ethnic populations are required to thoroughly characterise the associations of these genes and pathways. Traditional methods looking at single genes are valuable. However these approaches are fast being surpassed by methods that allow analysis of the genes within a complete pathway using a chip-based or bead-based array. These approaches provide a comprehensive analysis of the genes involved with the process of inflammation. Analysis using genome wide sequencing methods of immune and inflammatory pathways within a large cohort of Caucasian and AH men would allow a greater understanding of these pathways and genes with the development of aggressive disease.

The role of immunotherapy and anti-inflammatory strategies in treatment of several other cancers has shown significant survival benefits and has produced significant results with regards to tumour control in several malignancies. Inhibition of CTLA-4 by ipilimumab represented the first drug used in immune checkpoint inhibition being licensed for use in advanced metastatic melanoma (292). More recently anti PD-1 or anti PD-L1 antibody immune checkpoint inhibition in treatment of tumours expressing PD-L1 has led to significant advances in the treatment of metastatic malignancy melanoma, non-small cell lung cancer, renal cell cancer, head and neck cancer, urothelial cancer and Hodgkin's lymphoma with trials shown significant improvement of survival rates in many advanced metastatic

cancers (293–299). Several other malignancies (ovarian cancer, hepatocellular carcinoma, mesothelioma, gastric cancer and B-cell non-Hodgkin lymphoma) are currently under clinical investigation to determine the benefits of checkpoint inhibition (300–302).

Mutations in DNA repair genes have been identified as significant cause of genomic instability and hallmark of carcinogenesis. Identification of mismatch repair genes (MMR) and potential therapeutic targeting has been a significant area of development over recent years. We know that hereditary germline mutations in DNA repair genes are associated with a higher risk of CaP, with mutations in BRCA2 increasing the risk of developing CaP and their role in development and progression of breast, ovarian and pancreatic cancers also well documented (31,32). Inherited mutations in several other DNA repair genes appears to associated with increased CaP risk also (27). Over recent years exploiting the vulnerabilities of tumour cells with DNA repair genes defects has been reviewed in different tumour types with most success seen in ovarian and breast cancers. Poly (ADP-ribose) polmerases (PARPs) are a family of enzymes involved in transcriptional regulation and in detecting and localising DNA repair proteins to DNA strand breaks triggering a damage response and repair process. PARP inhibitor olaparib has shown efficacy in the treatment of ovarian cancer (303–306). Phase 2 trials in patients with metastatic castration resistant CaP and identified defects in DNA-repair genes treated with olaparib have shown encouraging response rates (307).

The role of immunotherapy treatment in prostate cancer in early trials has shown some promise. This work has been confined to patients with late stage disease with a significant tumour burden. The challenge remains in assessing the benefit to patients with early stage disease. This would probably require careful integration of multi-modal therapy with immunotherapy and anti inflammatory treatments added to existing treatment.

Inflammation and the modulation of the immune system and inflammatory response is key in tumour development and progression. Greater levels of inflammation are seen within CaP from AH men suggesting an important role of inflammation in development and progression of CaP and a possible explanation for the increased high risk CaP seen in this population. We have identified Lambda FLC, a marker of the inflammatory response, as a potential novel biomarker in CaP. We have demonstrated its ability to predict disease specific survival and metastatic disease in patients with CaP. Anti-inflammatory and immunotherapy approaches targeting the tumour microenvironment in CaP, may prevent tumour promoting inflammatory cell recruitment or migration of tumour cells inhibiting progression and resultant metastatic spread.

6.0 Bibliography

1. Cancer Registration Statistics, England - Office for National Statistics [Internet]. [cited 2017 Nov 14]. Available from: https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancerregistrationstatisticsengland/2014

2. GLOBOCAN Cancer Fact Sheets: prostate cancer [Internet]. [cited 2017 Nov 14]. Available from: http://globocan.iarc.fr/old/FactSheets/cancers/prostate-new.asp

3. Prostate cancer statistics [Internet]. Cancer Research UK. 2015 [cited 2017 Nov 14]. Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer

4. Cooperberg MR, Lubeck DP, Meng MV, Mehta SS, Carroll PR. The Changing Face of Low-risk Prostate Cancer: Trends in Clinical Presentation and Primary Management. J Clin Oncol Off J Am Soc Clin Oncol. 2004 Jun 1;22(11):2141–9.

5. Williams N, Hughes LJ, Turner EL, Donovan JL, Hamdy FC, Neal DE, et al. Prostate-specific antigen testing rates remain low in UK general practice: a cross-sectional study in six English cities. BJU Int. 2011 Nov;108(9):1402–8.

6. Cancer Registration Statistics, England Statistical bulletins - Office for National Statistics [Internet]. [cited 2017 Nov 14]. Available from: https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancerregistrationstatisticsengland/previousReleases

7. Prostate cancer incidence statistics [Internet]. Cancer Research UK. 2015 [cited 2017 Nov 14]. Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence

8. Lloyd T, Hounsome L, Mehay A, Mee S, Verne J, Cooper A. Lifetime risk of being diagnosed with, or dying from, prostate cancer by major ethnic group in England 2008-2010. BMC Med. 2015 Jul 30;13:171.

9. Howlader N, Ries LAG, Mariotto AB, Reichman ME, Ruhl J, Cronin KA. Improved Estimates of Cancer-Specific Survival Rates From Population-Based Data. JNCI J Natl Cancer Inst. 2010 Oct 20;102(20):1584–98.

10. Ben-Shlomo Y, Evans S, Ibrahim F, Patel B, Anson K, Chinegwundoh F, et al. The Risk of Prostate Cancer amongst Black Men in the United Kingdom: The PROCESS Cohort Study. Eur Urol. 2008 Jan;53(1):99–105.

11. Evans S, Metcalfe C, Patel B, Ibrahim F, Anson K, Chinegwundoh F, et al. Clinical presentation and initial management of black men and white men with prostate cancer in the United Kingdom: the PROCESS cohort study. Br J Cancer. 2010 Jan 19;102(2):249–54.

12. Kheirandish P, Chinegwundoh F. Ethnic differences in prostate cancer. Br J Cancer. 2011 Aug 9;105(4):481–5.

13. Eeles RA, Kote-Jarai Z, Giles GG, Olama AAA, Guy M, Jugurnauth SK, et al. Multiple newly identified loci associated with prostate cancer susceptibility. Nat Genet. 2008 Mar;40(3):316–21.

14. Kote-Jarai Z, Easton DF, Stanford JL, Ostrander EA, Schleutker J, Ingles SA, et al. Multiple novel prostate cancer predisposition loci confirmed by an international study: the PRACTICAL Consortium. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2008 Aug;17(8):2052–61.

15. Macinnis RJ, Antoniou AC, Eeles RA, Severi G, Al Olama AA, McGuffog L, et al. A risk prediction algorithm based on family history and common genetic variants: application to prostate cancer with potential clinical impact. Genet Epidemiol. 2011 Sep;35(6):549–56.

16. Zheng SL, Sun J, Wiklund F, Smith S, Stattin P, Li G, et al. Cumulative association of five genetic variants with prostate cancer. N Engl J Med. 2008 Feb 28;358(9):910–9.

17. Schumacher FR, Al Olama AA, Berndt SI, Benlloch S, Ahmed M, Saunders EJ, et al. Association analyses of more than 140,000 men identify 63 new prostate cancer susceptibility loci. Nat Genet. 2018;50(7):928–36.

18. Eeles RA, Olama AAA, Benlloch S, Saunders EJ, Leongamornlert DA, Tymrakiewicz M, et al. Identification of 23 new prostate cancer susceptibility loci using the iCOGS custom genotyping array. Nat Genet. 2013 Apr;45(4):385–91, 391e1-2.

19. Okobia MN, Zmuda JM, Ferrell RE, Patrick AL, Bunker CH. Chromosome 8q24 variants are associated with prostate cancer risk in a high risk population of African ancestry. The Prostate. 2011;71(10):1054–63.

20. Haiman CA, Chen GK, Blot WJ, Strom SS, Berndt SI, Kittles RA, et al. Genome-wide association study of prostate cancer in men of African ancestry identifies a susceptibility locus at 17q21. Nat Genet. 2011 Jun;43(6):570–3.

21. Fact Sheets by Cancer [Internet]. [cited 2017 Nov 14]. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

22. Chu LW, Ritchey J, Devesa SS, Quraishi SM, Zhang H, Hsing AW. Prostate Cancer Incidence Rates in Africa. Prostate Cancer [Internet]. 2011

[cited 2019 Apr 11];2011. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3200287/

23. Hemminki K, Czene K. Age specific and attributable risks of familial prostate carcinoma from the family-cancer database. Cancer. 2002 Sep 15;95(6):1346–53.

24. Steinberg GD, Carter BS, Beaty TH, Childs B, Walsh PC. Family history and the risk of prostate cancer. The Prostate. 1990;17(4):337–47.

25. Bruner DW, Moore D, Parlanti A, Dorgan J, Engstrom P. Relative risk of prostate cancer for men with affected relatives: systematic review and meta-analysis. Int J Cancer. 2003 Dec 10;107(5):797–803.

26. Potter SR, Partin AW. Hereditary and Familial Prostate Cancer: Biologic Aggressiveness and Recurrence. Rev Urol. 2000;2(1):35–6.

27. Eeles R, Goh C, Castro E, Bancroft E, Guy M, Al Olama AA, et al. The genetic epidemiology of prostate cancer and its clinical implications. Nat Rev Urol. 2014 Jan;11(1):18–31.

28. Castro E, Mikropoulos C, Bancroft EK, Dadaev T, Goh C, Taylor N, et al. The PROFILE Feasibility Study: Targeted Screening of Men With a Family History of Prostate Cancer. The Oncologist. 2016;21(6):716–22.

29. Karlsson R, Aly M, Clements M, Zheng L, Adolfsson J, Xu J, et al. A population-based assessment of germline HOXB13 G84E mutation and prostate cancer risk. Eur Urol. 2014 Jan;65(1):169–76.

30. Ewing CM, Ray AM, Lange EM, Zuhlke KA, Robbins CM, Tembe WD, et al. Germline mutations in HOXB13 and prostate-cancer risk. N Engl J Med. 2012 Jan 12;366(2):141–9.

31. Leongamornlert D, Mahmud N, Tymrakiewicz M, Saunders E, Dadaev T, Castro E, et al. Germline BRCA1 mutations increase prostate cancer risk. Br J Cancer. 2012 May 8;106(10):1697–701.

32. Kote-Jarai Z, Leongamornlert D, Saunders E, Tymrakiewicz M, Castro E, Mahmud N, et al. BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. Br J Cancer. 2011 Oct 11;105(8):1230–4.

33. Castro E, Goh C, Olmos D, Saunders E, Leongamornlert D, Tymrakiewicz M, et al. Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2013 May 10;31(14):1748–57.

34. Gallagher DJ, Gaudet MM, Pal P, Kirchhoff T, Balistreri L, Vora K, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate

cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2010 Apr 1;16(7):2115–21.

35. Taylor RA, Fraser M, Livingstone J, Espiritu SMG, Thorne H, Huang V, et al. Germline BRCA2 mutations drive prostate cancers with distinct evolutionary trajectories. Nat Commun [Internet]. 2017 Jan 9 [cited 2020 Nov 30];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5227331/

36. Page EC, Bancroft EK, Brook MN, Assel M, Hassan Al Battat M, Thomas S, et al. Interim Results from the IMPACT Study: Evidence for Prostate-specific Antigen Screening in BRCA2 Mutation Carriers. Eur Urol. 2019 Dec 1;76(6):831–42.

37. Leongamornlert D, Saunders E, Dadaev T, Tymrakiewicz M, Goh C, Jugurnauth-Little S, et al. Frequent germline deleterious mutations in DNA repair genes in familial prostate cancer cases are associated with advanced disease. Br J Cancer. 2014 Mar 18;110(6):1663–72.

38. Ryan S, Jenkins MA, Win AK. Risk of prostate cancer in Lynch syndrome: a systematic review and meta-analysis. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2014 Mar;23(3):437–49.

39. de la Chapelle A. The Incidence of Lynch Syndrome. Fam Cancer. 2005 Sep 1;4(3):233–7.

40. Grindedal EM, Moller P, Eeles R, Stormorken AT, Bowitz-Lothe IM, Landro SM, et al. Germ-Line Mutations in Mismatch Repair Genes Associated with Prostate Cancer. Cancer Epidemiol Biomarkers Prev. 2009 Sep 1;18(9):2460–7.

41. Barrow PJ, Ingham S, O'Hara C, Green K, McIntyre I, Lalloo F, et al. The spectrum of urological malignancy in Lynch syndrome. Fam Cancer. 2013 Mar;12(1):57–63.

42. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet Lond Engl. 2004 Apr 24;363(9418):1346–53.

43. Gathirua-Mwangi WG, Zhang J. Dietary Factors and Risk of Advanced Prostate Cancer. Eur J Cancer Prev Off J Eur Cancer Prev Organ ECP. 2014 Mar;23(2):96–109.

44. Key TJ, Appleby PN, Allen NE, Travis RC, Roddam AW, Jenab M, et al. Plasma carotenoids, retinol, and tocopherols and the risk of prostate cancer in the European Prospective Investigation into Cancer and Nutrition study. Am J Clin Nutr. 2007 Sep;86(3):672–81.

45. McNeal JE. The zonal anatomy of the prostate. The Prostate. 1981 Jan 1;2(1):35–49.

46. McNeal JE, Redwine EA, Freiha FS, Stamey TA. Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread. Am J Surg Pathol. 1988 Dec;12(12):897–906.

47. Bostwick DG, Liu L, Brawer MK, Qian J. High-Grade Prostatic Intraepithelial Neoplasia. Rev Urol. 2004;6(4):171–9.

48. Steiner MS. High grade prostatic intraepithelial neoplasia is a disease. Curr Urol Rep. 2001 May 1;2(3):195–8.

49. Sakr WA, Grignon DJ, Crissman JD, Heilbrun LK, Cassin BJ, Pontes JJ, et al. High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. Vivo Athens Greece. 1994;8(3):439–43.

50. Sakr WA. Prostatic Intraepithelial Neoplasia: A Marker for High-Risk Groups and a Potential Target for Chemoprevention. Eur Urol. 1999;35(5–6):474–8.

51. Qian J, Wollan P, Bostwick DG. The extent and multicentricity of high-grade prostatic intraepithelial neoplasia in clinically localized prostatic adenocarcinoma. Hum Pathol. 1997 Feb 1;28(2):143–8.

52. Bostwick DG. Progression of prostatic intraepithelial neoplasia to early invasive adenocarcinoma. Eur Urol. 1996;30(2):145–52.

53. Netto GJ, Epstein JI. Widespread High-grade Prostatic Intraepithelial Neoplasia on Prostatic Needle Biopsy: A Significant Likelihood of Subsequently Diagnosed Adenocarcinoma. Am J Surg Pathol. 2006 Sep;30(9):1184.

54. Epstein JI, Herawi M. Prostate Needle Biopsies Containing Prostatic Intraepithelial Neoplasia or Atypical Foci Suspicious for Carcinoma: Implications for Patient Care. J Urol. 2006 Mar 1;175(3):820–34.

55. Wiener S, Haddock P, Cusano J, Staff I, McLaughlin T, Wagner J. Incidence of Clinically Significant Prostate Cancer After a Diagnosis of Atypical Small Acinar Proliferation, High-grade Prostatic Intraepithelial Neoplasia, or Benign Tissue. Urology. 2017 Dec 1;110:161–5.

56. Srirangam V, Rai BP, Abroaf A, Agarwal S, Tadtayev S, Foley C, et al. Atypical Small Acinar Proliferation and High Grade Prostatic Intraepithelial Neoplasia: Should We Be Concerned? An Observational Cohort Study with a Minimum Follow-Up of 3 Years. Curr Urol. 2017 Nov;10(4):199–205.

57. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997 Jul;3(7):730–7.

58. Maitland NJ, Collins AT. Prostate cancer stem cells: a new target for therapy. J Clin Oncol Off J Am Soc Clin Oncol. 2008 Jun 10;26(17):2862–70.

59. Maitland NJ, Frame FM, Polson ES, Lewis JL, Collins AT. Prostate cancer stem cells: do they have a basal or luminal phenotype? Horm Cancer. 2011 Feb;2(1):47–61.

60. Humphrey PA. Histological variants of prostatic carcinoma and their significance. Histopathology. 2012 Jan;60(1):59–74.

61. Collins AT, Habib FK, Maitland NJ, Neal DE. Identification and isolation of human prostate epithelial stem cells based on α2β1-integrin expression. J Cell Sci. 2001 Nov 1;114(21):3865–72.

62. Goldstein AS, Huang J, Guo C, Garraway IP, Witte ON. Identification of a cell of origin for human prostate cancer. Science. 2010 Jul 30;329(5991):568–71.

63. Taylor RA, Toivanen R, Frydenberg M, Pedersen J, Harewood L, Australian Prostate Cancer Bioresource, et al. Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status. Stem Cells Dayt Ohio. 2012;30(6):1087–96.

64. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 2005 Dec 1;65(23):10946–51.

65. Vesalainen SL, Lipponen PK, Talja MT, Alhava EM, Syrjänen KJ. Proliferating cell nuclear antigen and p53 expression as prognostic factors in T1-2M0 prostatic adenocarcinoma. Int J Cancer. 1994 Jul 15;58(2):303–8.

66. Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, et al. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol. 1993 Nov;123(3):513–22.

67. Ross W, Hall PA. Ki67: from antibody to molecule to understanding? Mol Pathol. 1995 Jun 1;48(3):M113–7.

68. Berney DM, Gopalan A, Kudahetti S, Fisher G, Ambroisine L, Foster CS, et al. Ki-67 and outcome in clinically localised prostate cancer: analysis of conservatively treated prostate cancer patients from the Trans-Atlantic Prostate Group study. Br J Cancer. 2009 Mar;100(6):888–93.

69. Khor L-Y, Bae K, Paulus R, Al-Saleem T, Hammond ME, Grignon DJ, et al. MDM2 and Ki-67 Predict for Distant Metastasis and Mortality in Men Treated With Radiotherapy and Androgen Deprivation for Prostate Cancer: RTOG 92-02. J Clin Oncol. 2009 Jul 1;27(19):3177–84.

70. Verhoven B, Yan Y, Ritter M, Khor L-Y, Hammond E, Jones C, et al. Ki-67 is an Independent Predictor of Metastasis and Cause-Specific Mortality for Prostate Cancer Patients Treated on Radiation Therapy Oncology Group (RTOG) 94-08. Int J Radiat Oncol Biol Phys. 2013 Jun 1;86(2):317–23.

71. Antonarakis ES, Keizman D, Zhang Z, Gurel B, Lotan TL, Hicks JL, et al. An immunohistochemical signature comprising PTEN, MYC, and Ki67 predicts progression in prostate cancer patients receiving adjuvant docetaxel after prostatectomy. Cancer. 2012 Dec 15;118(24):6063–71.

72. Tang DG, Porter AT. Target to apoptosis: a hopeful weapon for prostate cancer. The Prostate. 1997 Sep 1;32(4):284–93.

73. Isaacs JT, Lundmo PI, Berges R, Martikainen P, Kyprianou N, English HF. Androgen regulation of programmed death of normal and malignant prostatic cells. J Androl. 1992;13(6):457–64.

74. Kyprianou N, Isaacs JT. Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol Baltim Md. 1989 Oct;3(10):1515–22.

75. Denmeade SR, Tombal B, Isaacs JT. Apoptotic pathways in prostate cancer. Adv Cell Aging Gerontol. 2001;6:23–54.

76. Chakraborty M, Qiu SG, Vasudevan KM, Rangnekar VM. Par-4 drives trafficking and activation of Fas and Fasl to induce prostate cancer cell apoptosis and tumor regression. Cancer Res. 2001 Oct;61(19):7255–63.

77. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis. Science. 1997 Feb 21;275(5303):1132–6.

78. Kim CN, Wang X, Huang Y, Ibrado AM, Liu L, Fang G, et al. Overexpression of Bcl-X(L) inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cancer Res. 1997 Aug;57(15):3115–20.

79. Bruckheimer EM, Gjertsen BT, McDonnell TJ. Implications of cell death regulation in the pathogenesis and treatment of prostate cancer. Semin Oncol. 1999 Aug;26(4):382–98.

80. Umbas R, Schalken JA, Aalders TW, Carter BS, Karthaus HF, Schaafsma HE, et al. Expression of the cellular adhesion molecule E-cadherin is

reduced or absent in high-grade prostate cancer. Cancer Res. 1992 Sep 15;52(18):5104–9.

81. Zhang Q, Helfand BT, Jang TL, Zhu LJ, Chen L, Yang XJ, et al. Nuclear Factor-κB-Mediated Transforming Growth Factor-β-Induced Expression of Vimentin Is an Independent Predictor of Biochemical Recurrence after Radical Prostatectomy. Clin Cancer Res. 2009 May 15;15(10):3557–67.

82. Ao M, Williams K, Bhowmick NA, Hayward SW. Transforming Growth Factor-β Promotes Invasion in Tumorigenic but not in Nontumorigenic Human Prostatic Epithelial Cells. Cancer Res. 2006 Aug 15;66(16):8007–16.

83. Stanbridge EJ. Human tumor suppressor genes. Annu Rev Genet. 1990;24:615–57.

84. Epstein JI, Egevad L, Amin MB, Delahunt B, Srigley JR, Humphrey PA, et al. The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. Am J Surg Pathol. 2016 Feb;40(2):244–52.

85. Catalona WJ, Smith DS. 5-year tumor recurrence rates after anatomical radical retropubic prostatectomy for prostate cancer. J Urol. 1994 Nov;152(5 Pt 2):1837–42.

86. Prediction of Progression Following Radical Prostatectomy: A Multivariate Analysis of 721 Men with Long-term Follow-up. Am J Surg Pathol. 1996 Mar 1;20(3):286–92.

87. Gordetsky J, Epstein J. Grading of prostatic adenocarcinoma: current state and prognostic implications. Diagn Pathol. 2016 Mar 9;11(1):25.

88. Professionals S-O. Prostate Cancer [Internet]. Uroweb. [cited 2018 Mar 6]. Available from: https://uroweb.org/guideline/prostate-cancer/

89. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, et al. Prevalence of Prostate Cancer among Men with a Prostate-Specific Antigen Level ≤4.0 ng per Milliliter. N Engl J Med. 2004 May 27;350(22):2239–46.

90. Cooperberg MR, Broering JM, Carroll PR. Time Trends and Local Variation in Primary Treatment of Localized Prostate Cancer. J Clin Oncol. 2010 Mar 1;28(7):1117–23.

91. Prostate cancer risk management programme: supporting documents [Internet]. GOV.UK. [cited 2020 Dec 1]. Available from: https://www.gov.uk/government/collections/prostate-cancer-risk-management-programme-supporting-documents

92. Prostate cancer: diagnosis and treatment | Guidance and guidelines | NICE [Internet]. [cited 2015 Feb 15]. Available from: https://www.nice.org.uk/guidance/cg175

93. Schröder FH, Hugosson J, Roobol MJ, Tammela TLJ, Zappa M, Nelen V, et al. Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. The Lancet. 2014 Dec 6;384(9959):2027–35.

94. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia D, Church TR, et al. Prostate cancer screening in the randomized Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial: mortality results after 13 years of follow-up. J Natl Cancer Inst. 2012 Jan 18;104(2):125–32.

95. PSA testing | Diagnosis | Prostate cancer | CKS | NICE [Internet]. [cited 2020 Dec 1]. Available from: https://cks.nice.org.uk/topics/prostate-cancer/diagnosis/psa-testing/

96. Rosser CJ, Parker A. RE: THE PROSTATE SPECIFIC ANTIGEN ERA IN THE UNITED STATES IS OVER FOR PROSTATE CANCER: WHAT HAPPENED IN THE LAST 20 YEARS? J Urol. 2005 Sep 1;174(3):1154–5.

97. Catalona WJ, Southwick PC, Slawin KM, Partin AW, Brawer MK, Flanigan RC, et al. Comparison of percent free PSA, PSA density, and age-specific PSA cutoffs for prostate cancer detection and staging. Urology. 2000 Aug 1;56(2):255–60.

98. Carter HB, Pearson JD. PROSTATE-SPECIFIC ANTIGEN VELOCITY AND REPEATED MEASURES OF PROSTATE-SPECIFIC ANTIGEN. Urol Clin. 1997 May 1;24(2):333–8.

99. Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. Beyond PSA: The next generation of prostate cancer biomarkers. Sci Transl Med. 2012 Mar 28;4(127):127rv3.

100. Marks LS, Fradet Y, Deras IL, Blase A, Mathis J, Aubin SMJ, et al. PCA3 Molecular Urine Assay for Prostate Cancer in Men Undergoing Repeat Biopsy. Urology. 2007 Mar 1;69(3):532–5.

101. Tinzl M, Marberger M, Horvath S, Chypre C. DD3PCA3 RNA Analysis in Urine – A New Perspective for Detecting Prostate Cancer. Eur Urol. 2004 Aug 1;46(2):182–7.

102. Crawford ED, Rove KO, Trabulsi EJ, Qian J, Drewnowska KP, Kaminetsky JC, et al. Diagnostic Performance of PCA3 to Detect Prostate Cancer in Men with Increased Prostate Specific Antigen: A Prospective Study of 1,962 Cases. J Urol. 2012 Nov 1;188(5):1726–31.

103. Merola R, Tomao L, Antenucci A, Sperduti I, Sentinelli S, Masi S, et al. PCA3 in prostate cancer and tumor aggressiveness detection on 407

high-risk patients: a National Cancer Institute experience. J Exp Clin Cancer Res. 2015 Feb 6;34:15.

104. Deras IL, Aubin SMJ, Blase A, Day JR, Koo S, Partin AW, et al. PCA3: A Molecular Urine Assay for Predicting Prostate Biopsy Outcome. J Urol. 2008 Apr 1;179(4):1587–92.

105. Hessels D, Gunnewiek JMTK, Oort I van, Karthaus HFM, Leenders GJL van, Balken B van, et al. DD3PCA3-based Molecular Urine Analysis for the Diagnosis of Prostate Cancer. Eur Urol. 2003 Jul 1;44(1):8–16.

106. Nguyen P-N, Violette P, Chan S, Tanguay S, Kassouf W, Aprikian A, et al. A panel of TMPRSS2:ERG fusion transcript markers for urine-based prostate cancer detection with high specificity and sensitivity. Eur Urol. 2011 Mar;59(3):407–14.

107. Perner S, Mosquera J, Demichelis F, Hofer MD, Paris PL, Simko J, et al. *tmprss2-erg* Fusion Prostate Cancer: An Early Molecular Event Associated With Invasion. Am J Surg Pathol. 2007 Jun 1;31(6):882–8.

108. Pettersson A, Graff RE, Bauer SR, Pitt MJ, Lis RT, Stack EC, et al. The TMPRSS2:ERG Rearrangement, ERG Expression, and Prostate Cancer Outcomes: A Cohort Study and Meta-analysis. Cancer Epidemiol Prev Biomark. 2012 Sep 1;21(9):1497–509.

109. Salami SS, Schmidt F, Laxman B, Regan MM, Rickman DS, Scherr D, et al. Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer. Urol Oncol Semin Orig Investig. 2013 Jul 1;31(5):566–71.

110. Knezevic D, Goddard AD, Natraj N, Cherbavaz DB, Clark-Langone KM, Snable J, et al. Analytical validation of the Oncotype DX prostate cancer assay – a clinical RT-PCR assay optimized for prostate needle biopsies. BMC Genomics. 2013 Oct 8;14:690.

111. Cullen J, Rosner IL, Brand TC, Zhang N, Tsiatis AC, Moncur J, et al. A Biopsy-based 17-gene Genomic Prostate Score Predicts Recurrence After Radical Prostatectomy and Adverse Surgical Pathology in a Racially Diverse Population of Men with Clinically Low- and Intermediate-risk Prostate Cancer. Eur Urol. 2015 Jul 1;68(1):123–31.

112. Klein EA, Cooperberg MR, Magi-Galluzzi C, Simko JP, Falzarano SM, Maddala T, et al. A 17-gene Assay to Predict Prostate Cancer Aggressiveness in the Context of Gleason Grade Heterogeneity, Tumor Multifocality, and Biopsy Undersampling. Eur Urol. 2014 Sep 1;66(3):550–60.

113. Discovery and Validation of a Prostate Cancer Genomic Classifier that Predicts Early Metastasis Following Radical Prostatectomy [Internet]. [cited 2019 Apr 15]. Available from:

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0066855

114. Validation of a Genomic Classifier that Predicts Metastasis Following Radical Prostatectomy in an At Risk Patient Population [Internet]. [cited 2019 Apr 15]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4097302/

115. Genomic Prostate Cancer Classifier Predicts Biochemical Failure and Metastases in Patients After Postoperative Radiation Therapy - ScienceDirect [Internet]. [cited 2019 Apr 15]. Available from: https://www.sciencedirect.com/science/article/pii/S0360301614005525

116. Ross A, Feng F, Ghadessi M, Erho N, Crisan A, Buerki C, et al. A genomic classifier predicting metastatic disease progression in men with biochemical recurrence after prostatectomy. Prostate Cancer Prostatic Dis. 2014 Mar;17(1):64–9.

117. Klein EA, Yousefi K, Haddad Z, Choeurng V, Buerki C, Stephenson AJ, et al. A Genomic Classifier Improves Prediction of Metastatic Disease Within 5 Years After Surgery in Node-negative High-risk Prostate Cancer Patients Managed by Radical Prostatectomy Without Adjuvant Therapy. Eur Urol. 2015 Apr 1;67(4):778–86.

118. Shore ND, Kella N, Moran B, Boczko J, Bianco FJ, Crawford ED, et al. Impact of the Cell Cycle Progression Test on Physician and Patient Treatment Selection for Localized Prostate Cancer. J Urol. 2016 Mar 1;195(3):612–8.

119. Bishoff JT, Freedland SJ, Gerber L, Tennstedt P, Reid J, Welbourn W, et al. Prognostic Utility of the Cell Cycle Progression Score Generated from Biopsy in Men Treated with Prostatectomy. J Urol. 2014 Aug 1;192(2):409–14.

120. Cuzick J, Swanson GP, Fisher G, Brothman AR, Berney DM, Reid JE, et al. Prognostic value of an RNA expression signature derived from cell cycle proliferation genes in patients with prostate cancer: a retrospective study. Lancet Oncol. 2011 Mar 1;12(3):245–55.

121. Wojno KJ, Costa FJ, Cornell RJ, Small JD, Pasin E, Van Criekinge W, et al. Reduced Rate of Repeated Prostate Biopsies Observed in ConfirmMDx Clinical Utility Field Study. Am Health Drug Benefits. 2014 May;7(3):129–34.

122. Neste LV, Partin AW, Stewart GD, Epstein JI, Harrison DJ, Criekinge WV. Risk score predicts high-grade prostate cancer in DNA-methylation positive, histopathologically negative biopsies. The Prostate. 2016;76(12):1078–87.

123. Partin AW, Van Neste L, Klein EA, Marks LS, Gee JR, Troyer DA, et al. Clinical Validation of an Epigenetic Assay to Predict Negative Histopathological Results in Repeat Prostate Biopsies. J Urol. 2014 Oct 1;192(4):1081–7.

124. Stewart GD, Van Neste L, Delvenne P, Delrée P, Delga A, McNeill SA, et al. Clinical Utility of an Epigenetic Assay to Detect Occult Prostate Cancer in Histopathologically Negative Biopsies: Results of the MATLOC Study. J Urol. 2013 Mar 1;189(3):1110–6.

125. Gittelman MC, Hertzman B, Bailen J, Williams T, Koziol I, Henderson RJ, et al. PCA3 Molecular Urine Test as a Predictor of Repeat Prostate Biopsy Outcome in Men with Previous Negative Biopsies: A Prospective Multicenter Clinical Study. J Urol. 2013 Jul 1;190(1):64–9.

126. Leyten GHJM, Hessels D, Jannink SA, Smit FP, de Jong H, Cornel EB, et al. Prospective Multicentre Evaluation of PCA3 and TMPRSS2-ERG Gene Fusions as Diagnostic and Prognostic Urinary Biomarkers for Prostate Cancer. Eur Urol. 2014 Mar 1;65(3):534–42.

127. Alford AV, Brito JM, Yadav KK, Yadav SS, Tewari AK, Renzulli J. The Use of Biomarkers in Prostate Cancer Screening and Treatment. Rev Urol. 2017;19(4):221–34.

128. Cucchiara V, Cooperberg MR, Dall'Era M, Lin DW, Montorsi F, Schalken JA, et al. Genomic Markers in Prostate Cancer Decision Making. Eur Urol. 2018;73(4):572–82.

129. Naughton CK, Miller DC, Mager DE, Ornstein DK, Catalona WJ. A PROSPECTIVE RANDOMIZED TRIAL COMPARING 6 VERSUS 12 PROSTATE BIOPSY CORES: IMPACT ON CANCER DETECTION. J Urol. 2000 Aug 1;164(2):388–92.

130. Ahmed HU, El-Shater Bosaily A, Brown LC, Gabe R, Kaplan R, Parmar MK, et al. Diagnostic accuracy of multi-parametric MRI and TRUS biopsy in prostate cancer (PROMIS): a paired validating confirmatory study. Lancet Lond Engl. 2017 Feb 25;389(10071):815–22.

131. Kasivisvanathan V, Rannikko AS, Borghi M, Panebianco V, Mynderse LA, Vaarala MH, et al. MRI-Targeted or Standard Biopsy for Prostate-Cancer Diagnosis. N Engl J Med. 2018 Mar 18;0(0):null.

132. Bass EJ, Donaldson IA, Freeman A, Jameson C, Punwani S, Moore C, et al. Magnetic resonance imaging targeted transperineal prostate biopsy: a local anaesthetic approach. Prostate Cancer Prostatic Dis. 2017;20(3):311–7.

133. Kum F, Elhage O, Maliyil J, Wong K, Walker NF, Kulkarni M, et al. Initial outcomes of local anaesthetic freehand transperineal prostate biopsies in the outpatient setting. BJU Int. 2020;125(2):244–52.

134. Stefanova Veselina, Buckley Roger, Flax Stanley, Spevack Les, Hajek David, Tunis Adam, et al. Transperineal Prostate Biopsies Using Local Anesthesia: Experience with 1,287 Patients. Prostate Cancer Detection Rate, Complications and Patient Tolerability. J Urol. 2019 Jun 1;201(6):1121–6.

135. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 2010 Jun;17(6):1471–4.

136. D'Amico AV, Moul J, Carroll PR, Sun L, Lubeck D, Chen M-H. Cancer-Specific Mortality After Surgery or Radiation for Patients With Clinically Localized Prostate Cancer Managed During the Prostate-Specific Antigen Era. J Clin Oncol. 2003 Jun 1;21(11):2163–72.

137. Eifler JB, Feng Z, Lin BM, Partin MT, Humphreys EB, Han M, et al. An updated prostate cancer staging nomogram (Partin tables) based on cases from 2006 to 2011. BJU Int. 2013 Jan 1;111(1):22–9.

138. Briganti A, Larcher A, Abdollah F, Capitanio U, Gallina A, Suardi N, et al. Updated Nomogram Predicting Lymph Node Invasion in Patients with Prostate Cancer Undergoing Extended Pelvic Lymph Node Dissection: The Essential Importance of Percentage of Positive Cores. Eur Urol. 2012 Mar 1;61(3):480–7.

139. Vugt HA van, Roobol MJ, Kranse R, Määttänen L, Finne P, Hugosson J, et al. Prediction of prostate cancer in unscreened men: External validation of a risk calculator. Eur J Cancer. 2011 Apr 1;47(6):903–9.

140. Pre-Radical Prostatectomy [Internet]. Memorial Sloan Kettering Cancer Center. [cited 2018 Mar 13]. Available from: https://www.mskcc.org/nomograms/prostate/pre_op

141. Thurtle DR, Jenkins V, Pharoah PD, Gnanapragasam VJ. Understanding of prognosis in non-metastatic prostate cancer: a randomised comparative study of clinician estimates measured against the PREDICT prostate prognostic model. Br J Cancer. 2019 Oct;121(8):715–8.

142. Thurtle D, Bratt O, Stattin P, Pharoah P, Gnanapragasam V. Comparative performance and external validation of the multivariable PREDICT Prostate tool for non-metastatic prostate cancer: a study in 69,206 men from Prostate Cancer data Base Sweden (PCBaSe). BMC Med. 2020 Jun 16;18(1):139.

143. Bill-Axelson A, Holmberg L, Ruutu M, Häggman M, Andersson S-O, Bratell S, et al. Radical Prostatectomy versus Watchful Waiting in Early Prostate Cancer. N Engl J Med. 2005 May 12;352(19):1977–84.

144. Wilt TJ, Brawer MK, Jones KM, Barry MJ, Aronson WJ, Fox S, et al. Radical Prostatectomy versus Observation for Localized Prostate Cancer. N Engl J Med. 2012 Jul 19;367(3):203–13.

145. Bryant RJ, Oxley J, Young GJ, Lane JA, Metcalfe C, Davis M, et al. The ProtecT trial: analysis of the patient cohort, baseline risk stratification and disease progression. Bju Int. 2020 Apr;125(4):506–14.

146. Hamdy FC, Donovan JL, Lane JA, Mason M, Metcalfe C, Holding P, et al. 10-Year Outcomes after Monitoring, Surgery, or Radiotherapy for Localized Prostate Cancer. N Engl J Med. 2016 Oct 13;375(15):1415–24.

147. D'Amico AV, Manola J, Loffredo M, Renshaw AA, DellaCroce A, Kantoff PW. 6-Month Androgen Suppression Plus Radiation Therapy vs Radiation Therapy Alone for Patients With Clinically Localized Prostate Cancer: A Randomized Controlled Trial. JAMA. 2004 Aug 18;292(7):821–7.

148. Cooperberg MR, Cowan J, Broering JM, Carroll PR. High-Risk Prostate Cancer in the United States, 1990-2007. World J Urol. 2008 Jun;26(3):211–8.

149. Rider JR, Sandin F, Andrén O, Wiklund P, Hugosson J, Stattin P. Long-term Outcomes Among Noncuratively Treated Men According to Prostate Cancer Risk Category in a Nationwide, Population-based Study. Eur Urol. 2013 Jan 1;63(1):88–96.

150. Professionals S-O. EAU Guidelines: Prostate Cancer [Internet]. Uroweb. [cited 2021 Jan 12]. Available from: https://uroweb.org/guideline/prostate-cancer/

151. Moris L, Cumberbatch MG, Van den Broeck T, Gandaglia G, Fossati N, Kelly B, et al. Benefits and Risks of Primary Treatments for High-risk Localized and Locally Advanced Prostate Cancer: An International Multidisciplinary Systematic Review. Eur Urol. 2020 May 1;77(5):614–27.

152. Cornford P, Bellmunt J, Bolla M, Briers E, De Santis M, Gross T, et al. EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part II: Treatment of Relapsing, Metastatic, and Castration-Resistant Prostate Cancer. Eur Urol. 2017 Apr;71(4):630–42.

153. James ND, Spears MR, Clarke NW, Dearnaley DP, De Bono JS, Gale J, et al. Survival with Newly Diagnosed Metastatic Prostate Cancer in the 'Docetaxel Era': Data from 917 Patients in the Control Arm of the STAMPEDE Trial (MRC PR08, CRUK/06/019). Eur Urol. 2015 Jun;67(6):1028–38.

154. Yu EY, Gillessen S, Mottet N. What Do the Guidelines Say for Metastatic Prostate Cancer Starting Androgen Deprivation Therapy? National

Comprehensive Cancer Network, European Society for Medical Oncology, and European Association of Urology recommendations. Eur Urol Focus. 2019 Mar;5(2):162–4.

155. Sydes MR, Spears MR, Mason MD, Clarke NW, Dearnaley DP, de Bono JS, et al. Adding abiraterone or docetaxel to long-term hormone therapy for prostate cancer: directly randomised data from the STAMPEDE multi-arm, multi-stage platform protocol. Ann Oncol Off J Eur Soc Med Oncol. 2018 May 1;29(5):1235–48.

156. Powell IJ. Epidemiology and Pathophysiology of Prostate Cancer in African-American Men. J Urol. 2007 Feb 1;177(2):444–9.

157. Wynder EL, Fujita Y, Harris RE, Hirayama T, Hiyama T. Comparative epidemiology of cancer between the United States and Japan. A second look. Cancer. 1991 Feb 1;67(3):746–63.

158. Yatani R, Chigusa I, Akazaki K, Stemmermann GN, Welsh RA, Correa P. Geographic pathology of latent prostatic carcinoma. Int J Cancer. 1982 Jun 15;29(6):611–6.

159. Powell IJ. Prostate cancer in the African American: is this a different disease? Semin Urol Oncol. 1998 Nov;16(4):221–6.

160. Freeman VL, Leszczak J, Cooper RS. Race and the histologic grade of prostate cancer. The Prostate. 1997 Feb;30(2):79–84.

161. Sakr WA, Grignon DJ, Haas GP. Pathology of premalignant lesions and carcinoma of the prostate in African-American men. Semin Urol Oncol. 1998 Nov;16(4):214–20.

162. Ward E, Jemal A, Cokkinides V, Singh GK, Cardinez C, Ghafoor A, et al. Cancer disparities by race/ethnicity and socioeconomic status. CA Cancer J Clin. 2004 Apr;54(2):78–93.

163. Marlow NM, Halpern MT, Pavluck AL, Ward EM, Chen AY. Disparities Associated with Advanced Prostate Cancer Stage at Diagnosis. J Health Care Poor Underserved. 2010 Feb 18;21(1):112–31.

164. Wallace TA, Prueitt RL, Yi M, Howe TM, Gillespie JW, Yfantis HG, et al. Tumor Immunobiological Differences in Prostate Cancer between African-American and European-American Men. Cancer Res. 2008 Feb 1;68(3):927–36.

165. Powell IJ, Dyson G, Land S, Ruterbusch J, Bock CH, Lenk S, et al. Genes Associated with Prostate Cancer Are Differentially Expressed in African American and European American Men. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2013 May;22(5):891–7.

166. Guo Y, Sigman DB, Borkowski A, Kyprianou N. Racial differences in prostate cancer growth: apoptosis and cell proliferation in Caucasian and African-American patients. The Prostate. 2000 Feb 1;42(2):130–6.

167. Kinseth MA, Jia Z, Rahmatpanah F, Sawyers A, Sutton M, Wang-Rodriguez J, et al. Expression differences between African American and Caucasian prostate cancer tissue reveals that stroma is the site of aggressive changes. Int J Cancer. 2014 Jan 1;134(1):81–91.

168. Gokce N, Holbrook M, Duffy SJ, Demissie S, Cupples LA, Biegelsen E, et al. Effects of race and hypertension on flow-mediated and nitroglycerin-mediated dilation of the brachial artery. Hypertens Dallas Tex 1979. 2001 Dec 1;38(6):1349–54.

169. Amling CL, Riffenburgh RH, Sun L, Moul JW, Lance RS, Kusuda L, et al. Pathologic variables and recurrence rates as related to obesity and race in men with prostate cancer undergoing radical prostatectomy. J Clin Oncol Off J Am Soc Clin Oncol. 2004 Feb 1;22(3):439–45.

170. Whittemore AS, Lele C, Friedman GD, Stamey T, Vogelman JH, Orentreich N. Prostate-specific antigen as predictor of prostate cancer in black men and white men. J Natl Cancer Inst. 1995 Mar 1;87(5):354–60.

171. West DW, Slattery ML, Robison LM, French TK, Mahoney AW. Adult dietary intake and prostate cancer risk in Utah: a case-control study with special emphasis on aggressive tumors. Cancer Causes Control. 1991 Mar 1;2(2):85–94.

172. Giovannucci E, Rimm EB, Colditz GA, Stampfer MJ, Ascherio A, Chute CC, et al. A Prospective Study of Dietary Fat and Risk of Prostate Cancer. JNCI J Natl Cancer Inst. 1993 Oct 6;85(19):1571–9.

173. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Willett WC. Height, body weight, and risk of prostate cancer. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 1997 Aug;6(8):557–63.

174. Severson RK, Grove JS, Nomura AM, Stemmermann GN. Body mass and prostatic cancer: a prospective study. BMJ. 1988 Sep 17;297(6650):713–5.

175. Sonnenberg GE, Krakower GR, Kissebah AH. A novel pathway to the manifestations of metabolic syndrome. Obes Res. 2004 Feb;12(2):180–6.

176. Gaston KE, Kim D, Singh S, Ford OH, Mohler JL. Racial differences in androgen receptor protein expression in men with clinically localized prostate cancer. J Urol. 2003 Sep;170(3):990–3.

177. Powell IJ, Bock CH, Ruterbusch JJ, Sakr W. Evidence Supports a Faster Growth Rate and/or Earlier Transformation to Clinically Significant Prostate Cancer in Black Than in White American Men and Influences Racial Progression and Mortality Disparity. J Urol [Internet]. 2010 May [cited 2018 Apr 8];183(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3840791/

178. Tu H, Jacobs SC, Borkowski A, Kyprianou N. Incidence of apoptosis and cell proliferation in prostate cancer: relationship with TGF-beta1 and bcl-2 expression. Int J Cancer. 1996 Oct 21;69(5):357–63.

179. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. The Prostate. 1996 Apr;28(4):251–65.

180. Bcl-2 and the regulation of programmed cell death. J Cell Biol. 1994 Jan 1;124(1):1–6.

181. Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med. 1997 Jun;3(6):614–20.

182. Furuya Y, Krajewski S, Epstein JI, Reed JC, Isaacs JT. Expression of bcl-2 and the progression of human and rodent prostatic cancers. Clin Cancer Res Off J Am Assoc Cancer Res. 1996 Feb;2(2):389–98.

183. Colombel M, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, et al. Detection of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers. Am J Pathol. 1993 Aug;143(2):390–400.

184. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res. 1992 Dec 15;52(24):6940–4.

185. Drake JM, Barnes JM, Madsen JM, Domann FE, Stipp CS, Henry MD. ZEB1 Coordinately Regulates Laminin-332 and β4 Integrin Expression Altering the Invasive Phenotype of Prostate Cancer Cells. J Biol Chem. 2010 Oct 29;285(44):33940–8.

186. Nauseef JT, Henry MD. Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? Nat Rev Urol Lond. 2011 Aug;8(8):428–39.

187. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646–74.

188. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002 Dec 19;420(6917):860–7.

189. Hayes RB, Pottern LM, Strickler H, Rabkin C, Pope V, Swanson GM, et al. Sexual behaviour, STDs and risks for prostate cancer. Br J Cancer. 2000 Feb;82(3):718–25.

190. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, et al. Inflammation in prostate carcinogenesis. Nat Rev Cancer. 2007 Apr;7(4):256–69.

191. De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am J Pathol. 1999 Dec;155(6):1985–92.

192. Putzi MJ, De Marzo AM. Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia. Urology. 2000 Nov 1;56(5):828–32.

193. Eastham JA, May RA, Whatley T, Crow A, Venable DD, Sartor O. Clinical Characteristics and Biopsy Specimen Features in African-American and White Men Without Prostate Cancer. JNCI J Natl Cancer Inst. 1998 May 20;90(10):756–60.

194. Tsai C-Y, Lee T-S, Kou YR, Wu Y-L. Glucosamine inhibits IL-1beta-mediated IL-8 production in prostate cancer cells by MAPK attenuation. J Cell Biochem. 2009 Oct 1;108(2):489–98.

195. Finley DS, Calvert VS, Inokuchi J, Lau A, Narula N, Petricoin EF, et al. Periprostatic adipose tissue as a modulator of prostate cancer aggressiveness. J Urol. 2009 Oct;182(4):1621–7.

196. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006 Jan;6(1):24–37.

197. Degl'Innocenti E, Grioni M, Boni A, Camporeale A, Bertilaccio MTS, Freschi M, et al. Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization. Eur J Immunol. 2005 Jan;35(1):66–75.

198. Drake CG, Doody ADH, Mihalyo MA, Huang C-T, Kelleher E, Ravi S, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell. 2005 Mar;7(3):239–49.

199. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med. 2005 Apr 18;201(8):1257–68.

200. Anderson MJ, Shafer-Weaver K, Greenberg NM, Hurwitz AA. Tolerization of tumor-specific T cells despite efficient initial priming in a primary murine model of prostate cancer. J Immunol Baltim Md 1950. 2007 Feb 1;178(3):1268–76.

201. Dranoff G. The therapeutic implications of intratumoral regulatory T cells. Clin Cancer Res Off J Am Assoc Cancer Res. 2005 Dec 1;11(23):8226–9.

202. Miller AM, Lundberg K, Ozenci V, Banham AH, Hellström M, Egevad L, et al. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol Baltim Md 1950. 2006 Nov 15;177(10):7398–405.

203. Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, DeMarzo AM, et al. Phenotypic Analysis of Prostate-Infiltrating Lymphocytes Reveals TH17 and Treg Skewing. Clin Cancer Res Off J Am Assoc Cancer Res. 2008 Jun 1;14(11):3254–61.

204. Drake CG. Prostate cancer as a model for tumour immunotherapy. Nat Rev Immunol. 2010 Aug;10(8):580–93.

205. Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2006 Jul 1;24(19):3089–94.

206. Gulley JL, Arlen PM, Madan RA, Tsang K-Y, Pazdur MP, Skarupa L, et al. Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. Cancer Immunol Immunother CII. 2010 May;59(5):663–74.

207. Slovin SF, Beer TM, Higano CS, Tejwani S, Hamid O, Picus J, et al. Initial phase II experience of ipilimumab (IPI) alone and in combination with radiotherapy (XRT) in patients with metastatic castration-resistant prostate cancer (mCRPC). J Clin Oncol. 2009 May 20;27(15_suppl):5138–5138.

208. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A. 2002 Sep 17;99(19):12293–7.

209. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. Int Immunol. 2005 Feb;17(2):133–44.

210. Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, et al. Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. Clin Cancer Res Off J Am Assoc Cancer Res. 2008 May 15;14(10):3044–51.

211. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol Off J Am Soc Clin Oncol. 2010 Jul 1;28(19):3167–75.

212. Ebelt K, Babaryka G, Frankenberger B, Stief CG, Eisenmenger W, Kirchner T, et al. Prostate cancer lesions are surrounded by FOXP3+, PD-1+ and B7-H1+ lymphocyte clusters. Eur J Cancer Oxf Engl 1990. 2009 Jun;45(9):1664–72.

213. Sharma P, Pachynski RK, Narayan V, Fléchon A, Gravis G, Galsky MD, et al. Nivolumab Plus Ipilimumab for Metastatic Castration-Resistant Prostate Cancer: Preliminary Analysis of Patients in the CheckMate 650 Trial. Cancer Cell. 2020 Oct 12;38(4):489-499.e3.

214. Antonarakis ES, Piulats JM, Gross-Goupil M, Goh J, Ojamaa K, Hoimes CJ, et al. Pembrolizumab for Treatment-Refractory Metastatic Castration-Resistant Prostate Cancer: Multicohort, Open-Label Phase II KEYNOTE-199 Study. J Clin Oncol. 2020 Feb 10;38(5):395–405.

215. Kwon ED, Drake CG, Scher HI, Fizazi K, Bossi A, van den Eertwegh AJM, et al. Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial. Lancet Oncol. 2014 Jun 1;15(7):700–12.

216. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. N Engl J Med. 2012 Jun 28;366(26):2443–54.

217. Green WJ, Ball G, Hulman G, Johnson C, Schalwyk GV, Ratan HL, et al. KI67 and DLX2 predict increased risk of metastasis formation in prostate cancer–a targeted molecular approach. Br J Cancer. 2016 Jul;115(2):236–42.

218. Epstein JI, Allsbrook WCJ, Amin MB, Egevad LL, Committee and the IG. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol. 2005 Sep;29(9):1228.

219. Lancashire LJ, Powe DG, Reis-Filho JS, Rakha E, Lemetre C, Weigelt B, et al. A validated gene expression profile for detecting clinical outcome in breast cancer using artificial neural networks. Breast Cancer Res Treat. 2009 Apr;120(1):83–93.

220. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 2003 Jan 11;13(11):2498–504.

221. Kormelink TG, Powe DG, Kuijpers SA, Abudukelimu A, Fens MHAM, Pieters EHE, et al. Immunoglobulin free light chains are biomarkers of poor prognosis in basal-like breast cancer and are potential targets in tumor-associated inflammation. Oncotarget. 2014 Mar 26;5(10):3159–67.

222. McCarty KS, Miller LS, Cox EB, Konrath J, McCarty KS. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. Arch Pathol Lab Med. 1985 Aug;109(8):716–21.

223. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res Off J Am Assoc Cancer Res. 2004 Nov 1;10(21):7252–9.

224. for the Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics, McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer. 2005 Aug;93(4):387–91.

225. Abraham A. Artificial Neural Networks. In: Handbook of Measuring System Design [Internet]. American Cancer Society; 2005 [cited 2018 Apr 28]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/0471497398.mm421

226. Lancashire LJ, Rees RC, Ball GR. Identification of gene transcript signatures predictive for estrogen receptor and lymph node status using a stepwise forward selection artificial neural network modelling approach. Artif Intell Med. 2008 Jun;43(2):99–111.

227. on behalf of the PROCESS study group, Metcalfe C, Evans S, Ibrahim F, Patel B, Anson K, et al. Pathways to diagnosis for Black men and White men found to have prostate cancer: the PROCESS cohort study. Br J Cancer. 2008 Oct;99(7):1040–5.

228. NCIN Homepage [Internet]. [cited 2019 Jan 10]. Available from: http://www.ncin.org.uk/home

229. Rajbabu K, Chandrasekera S, Zhu G, Dezylva S, Grunfeld EA, Muir GH. Racial origin is associated with poor awareness of prostate cancer in UK men, but can be increased by simple information. Prostate Cancer Prostatic Dis. 2007 Sep;10(3):256–60.

230. Roberts A. Barriers preventing early detection of prostate cancer in Black African/Caribbean men living in Westminster. :52.

231. Hear-me-now.pdf [Internet]. [cited 2019 Jan 10]. Available from: http://www.nbpa.co.uk/wp-content/uploads/2014/04/Hear-me-now.pdf

232. 'It don't make sense to worry too much': the experience of prostate cancer in African-Caribbean men in the UK - NANTON - 2011 - European Journal of Cancer Care - Wiley Online Library [Internet]. [cited 2019 Jan 10]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2354.2009.01155.x

233. Origins of socio-economic inequalities in cancer survival: a review | Annals of Oncology | Oxford Academic [Internet]. [cited 2019 Jan 10]. Available from: https://academic.oup.com/annonc/article/17/1/5/160750

234. BME_ProstatecancerREPORT_FINAL.pdf [Internet]. [cited 2019 Jan 10]. Available from: https://www.baun.co.uk/files/1513/5790/2147/BME_ProstatecancerREPORT_FINAL.PDF

235. The PROFILE Study: Germline Genetic Profiling: Correlation With Targeted Prostate Cancer Screening and Treatment - Full Text View - ClinicalTrials.gov [Internet]. [cited 2020 Nov 30]. Available from: https://clinicaltrials.gov/ct2/show/NCT02543905

236. Conti DV, Darst BF, Moss LC, Saunders EJ, Sheng X, Chou A, et al. Trans-ancestry genome-wide association meta-analysis of prostate cancer identifies new susceptibility loci and informs genetic risk prediction. Nat Genet. 2021 Jan;53(1):65–75.

237. Hata A, Davis BN. Control of microRNA Biogenesis by TGFβ Signaling Pathway ----a novel role of Smads in the nucleus. Cytokine Growth Factor Rev. 2009;20(5–6):517–21.

238. Mu Y, Gudey SK, Landström M. Non-Smad signaling pathways. Cell Tissue Res. 2012 Jan 1;347(1):11–20.

239. Shi Y, Massagué J. Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. Cell. 2003 Jun 13;113(6):685–700.

240. Hussey GS, Chaudhury A, Dawson AE, Lindner DJ, Knudsen CR, Wilce MCJ, et al. Identification of an mRNP Complex Regulating Tumorigenesis at the Translational Elongation Step. Mol Cell. 2011 Feb 18;41(4):419–31.

241. Inman GJ. Switching TGFβ from a tumor suppressor to a tumor promoter. Curr Opin Genet Dev. 2011 Feb 1;21(1):93–9.

242. Pasche B. Role of transforming growth factor beta in cancer. J Cell Physiol. 2001 Feb;186(2):153–68.

243. Wendt MK, Tian M, Schiemann WP. Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression. Cell Tissue Res. 2012 Jan;347(1):85–101.

244. Xiao L. TGF-beta 1 induced fibroblast proliferation is mediated by the FGF-2/ERK pathway. Front Biosci. 2012;17(7):2667.

245. Zavadil J, Cermak L, Soto-Nieves N, Böttinger EP. Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J. 2004;23(5):1155–65.

246. Vimentin affects the mobility and invasiveness of prostate cancer cells - Zhao - 2008 - Cell Biochemistry and Function - Wiley Online Library [Internet]. [cited 2019 Jan 17]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/cbf.1478

247. Wrzesinski SH, Wan YY, Flavell RA. Transforming Growth Factor-β and the Immune Response: Implications for Anticancer Therapy. Clin Cancer Res. 2007 Sep 15;13(18):5262–70.

248. Li MO, Wan YY, Sanjabi S, Robertson A-KL, Flavell RA. TRANSFORMING GROWTH FACTOR-β REGULATION OF IMMUNE RESPONSES. Annu Rev Immunol. 2006;24(1):99–146.

249. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med. 2000 May 4;342(18):1350–8.

250. Descazeaud A, Weinbreck N, Robert G, Vacherot F, Abbou CC, Labrousse F, et al. Transforming growth factor β-receptor II protein expression in benign prostatic hyperplasia is associated with prostate volume and inflammation. BJU Int. 2011;108(2b):E23–8.

251. Zeng L, Rowland RG, Lele SM, Kyprianou N. Apoptosis incidence and protein expression of p53, TGF-β receptor II, p27Kip1, and Smad4 in benign, premalignant, and malignant human prostate11Accepted for publication 0, 2003. Hum Pathol. 2004 Mar 1;35(3):290–7.

252. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, et al. Abrogation of TGFβ Signaling in Mammary Carcinomas Recruits Gr-1+CD11b+ Myeloid Cells that Promote Metastasis. Cancer Cell. 2008 Jan 8;13(1):23–35.

253. Forrester E, Chytil A, Bierie B, Aakre M, Gorska AE, Sharif-Afshar A-R, et al. Effect of Conditional Knockout of the Type II TGF- b Receptor Gene in Mammary Epithelia on Mammary Gland Development and Polyomavirus Middle T Antigen Induced Tumor Formation and Metastasis. Cancer Res. 2005;8.

254. Bierie B, Stover DG, Abel TW, Chytil A, Gorska AE, Aakre M, et al. Transforming Growth Factor- Regulates Mammary Carcinoma Cell Survival and Interaction with the Adjacent Microenvironment. Cancer Res. 2008 Mar 15;68(6):1809–19.

255. Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K. Transforming growth factor-β1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. Cancer. 2001;91(5):964–71.

256. Saito H, Tsujitani S, Oka S, Kondo A, Ikeguchi M, Maeta M, et al. The expression of transforming growth factor-β1 is significantly correlated with the expression of vascular endothelial growth factor and poor prognosis of patients with advanced gastric carcinoma. Cancer. 1999;86(8):1455–62.

257. Tsushima H, Kawata S, Tamura S, Ito N, Shirai Y, Kiso S, et al. High Levels of Transforming Growth Factor b1 in Patients With Colorectal Cancer: Association With Disease Progression. 1996;110(2):8.

258. Trachtman H, Fervenza FC, Gipson DS, Heering P, Jayne DRW, Peters H, et al. A phase 1, single-dose study of fresolimumab, an anti-TGF-β antibody, in treatment-resistant primary focal segmental glomerulosclerosis. Kidney Int. 2011 Jun;79(11):1236–43.

259. Bueno L, de Alwis DP, Pitou C, Yingling J, Lahn M, Glatt S, et al. Semi-mechanistic modelling of the tumour growth inhibitory effects of LY2157299, a new type I receptor TGF-beta kinase antagonist, in mice. Eur J Cancer Oxf Engl 1990. 2008 Jan;44(1):142–50.

260. Sun C-X. Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. J Cell Sci. 2002 Nov 1;115(21):3991–4000.

261. Shimizu K, Nagamachi Y, Tani M, Kimura K, Shiroishi T, Wakana S, et al. Molecular Cloning of a Novel NF2/ERM/4.1 Superfamily Gene, Ehm2, That Is Expressed in High-Metastatic K1735 Murine Melanoma Cells. Genomics. 2000 Apr 15;65(2):113–20.

262. Wang J, Cai Y, Penland R, Chauhan S, Miesfeld RL, Ittmann M. Increased expression of the metastasis-associated gene Ehm2 in prostate cancer. The Prostate. 2006;66(15):1641–52.

263. Tang P, Huang H, Chang J, Zhao G-F, Lu M-L, Wang Y. Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma. World J Gastroenterol. 2013 May 7;19(17):2697–703.

264. Yilmaz M, Maass D, Tiwari N, Waldmeier L, Schmidt P, Lehembre F, et al. Transcription factor Dlx2 protects from TGFβ-induced cell-cycle arrest and apoptosis. EMBO J. 2011 Sep 6;30(21):4489–99.

265. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, et al. CD4+ T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. Cancer Cell. 2009 Aug 4;16(2):91–102.

266. Lin W-W, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007 May 1;117(5):1175–83.

267. Chen L. Co-inhibitory molecules of the B7–CD28 family in the control of T-cell immunity. Nat Rev Immunol. 2004 May 1;4:336.

268. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol. 2008 Jun 1;8:467.

269. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed death ligand 2 in cancer-induced immune suppression. Clin Dev Immunol. 2012;2012:656340–656340.

270. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med. 1999 Dec 1;5:1365.

271. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and Its Ligands in Tolerance and Immunity. Annu Rev Immunol. 2008;26(1):677–704.

272. Sznol M, Chen L. Antagonist Antibodies to PD-1 and B7-H1 (PD-L1) in the Treatment of Advanced Human Cancer. Clin Cancer Res. 2013 Mar 1;19(5):1021–34.

273. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 Is Associated with Poor Prognosis in Renal Cell Carcinoma Patients with Long-term Follow-up. Cancer Res. 2006 Apr 1;66(7):3381–5.

274. Soliman H, Khalil F, Antonia S. PD-L1 expression is increased in a subset of basal type breast cancer cells. PloS One. 2014;9(2):e88557.

275. Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K, Ueda S. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother. 2007 May 14;56(8):1173–82.

276. Bishop JL, Sio A, Angeles A, Roberts ME, Azad AA, Chi KN, et al. PD-L1 is highly expressed in Enzalutamide resistant prostate cancer. Oncotarget. 2015;6(1):234.

277. Thoma C. Prostate cancer: PD-L1 expression is common and indicates poor prognosis. Nat Rev Urol. 2015 Dec 1;13(1):5–5.

278. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. N Engl J Med. 2012 Jun 28;366(26):2455–65.

279. Wasiuk A, de Vries VC, Hartmann K, Roers A, Noelle RJ. Mast cells as regulators of adaptive immunity to tumours. Clin Exp Immunol. 2009 Feb;155(2):140–6.

280. Lu L-F, Lind EF, Gondek DC, Bennett KA, Gleeson MW, Pino-Lagos K, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature. 2006 Aug;442(7106):997–1002.

281. Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nat Med. 2007 Oct;13(10):1211–8.

282. Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev. 1999 Jun 1;13(11):1382–97.

283. Ribatti D, Crivellato E, Roccaro AM, Ria R, Vacca A. Mast cell contribution to angiogenesis related to tumour progression. Clin Exp Allergy. 2004;34(11):1660–4.

284. Redegeld FA, van der Heijden MW, Kool M, Heijdra BM, Garssen J, Kraneveld AD, et al. Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. Nat Med. 2002 Jun 17;8:694.

285. Lanciotti M, Masieri L, Raspollini MR, Minervini A, Mari A, Comito G, et al. The Role of M1 and M2 Macrophages in Prostate Cancer in relation to Extracapsular Tumor Extension and Biochemical Recurrence after Radical Prostatectomy [Internet]. Vol. 2014, BioMed Research International. Hindawi; 2014 [cited 2021 Feb 24]. p. e486798. Available from: https://www.hindawi.com/journals/bmri/2014/486798/

286. Erlandsson A, Carlsson J, Lundholm M, Fält A, Andersson S-O, Andrén O, et al. M2 macrophages and regulatory T cells in lethal prostate cancer. The Prostate. 2019;79(4):363–9.

287. Fang L-Y, Izumi K, Lai K-P, Liang L, Li L, Miyamoto H, et al. Infiltrating macrophages promote prostate tumorigenesis via modulating androgen receptor-mediated CCL4-STAT3 signaling. Cancer Res [Internet]. 2013 Sep 15 [cited 2021 Feb 24];73(18). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3833080/

288. Ammirante M, Luo J-L, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature. 2010 Mar 11;464(7286):302–5.

289. Blom S. Fibroblast as a critical stromal cell type determining prognosis in prostate cancer. :9.

290. Oren O, Beach DF. Representation of African-American patients in clinical studies of prostate cancer. J Clin Oncol. 2016 May 20;34(15_suppl):e18057–e18057.

291. George DJ, Heath EI, Sartor AO, Sonpavde G, Berry WR, Healy P, et al. Abi Race: A prospective, multicenter study of black (B) and white (W) patients (pts) with metastatic castrate resistant prostate cancer (mCRPC) treated with abiraterone acetate and prednisone (AAP). J Clin Oncol. 2018 Jun 7;36(18_suppl):LBA5009–LBA5009.

292. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010 Aug 19;363(8):711–23.

293. Beckermann KE, Johnson DB, Sosman JA. PD-1/PD-L1 blockade in renal cell cancer. Expert Rev Clin Immunol. 2017 Jan;13(1):77–84.

294. Beckermann KE, Jolly PC, Kim JY, Bordeaux J, Puzanov I, Rathmell WK, et al. Clinical and immunologic correlates of response to PD-1 blockade in a patient with metastatic renal medullary carcinoma. J Immunother Cancer. 2017;5:1.

295. Garon EB. Current Perspectives in Immunotherapy for Non-Small Cell Lung Cancer. Semin Oncol. 2015 Oct;42 Suppl 2:S11-18.

296. Garon EB. Selecting patients for immune checkpoint inhibition in lung cancer. Clin Adv Hematol Oncol HO. 2015 Aug;13(8):490–2.

297. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med. 2015 Jun 25;372(26):2521–32.

298. Sonpavde G. PD-1 and PD-L1 Inhibitors as Salvage Therapy for Urothelial Carcinoma. N Engl J Med. 2017 Mar 16;376(11):1073–4.

299. Allen PB, Gordon LI. PD-1 blockade in Hodgkin's lymphoma: learning new tricks from an old teacher. Expert Rev Hematol. 2016 Oct;9(10):939–49.

300. Lordick F, Shitara K, Janjigian YY. New agents on the horizon in gastric cancer. Ann Oncol Off J Eur Soc Med Oncol. 2017 Aug 1;28(8):1767–75.

301. Khanna S, Thomas A, Abate-Daga D, Zhang J, Morrow B, Steinberg SM, et al. Malignant Mesothelioma Effusions Are Infiltrated by CD3+ T Cells Highly Expressing PD-L1 and the PD-L1+ Tumor Cells within These Effusions Are Susceptible to ADCC by the Anti-PD-L1 Antibody Avelumab. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2016 Nov;11(11):1993–2005.

302. Gaillard SL, Secord AA, Monk B. The role of immune checkpoint inhibition in the treatment of ovarian cancer. Gynecol Oncol Res Pract. 2016 Nov 24;3(1):11.

303. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with

BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet Lond Engl. 2010 Jul 24;376(9737):235–44.

304. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: A preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol. 2014 Jul;15(8):852–61.

305. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N Engl J Med. 2012 Apr 12;366(15):1382–92.

306. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med. 2009 Jul 9;361(2):123–34.

307. Mateo J, Carreira S, Sandhu S, Miranda S, Mossop H, Perez-Lopez R, et al. DNA-Repair Defects and Olaparib in Metastatic Prostate Cancer. N Engl J Med. 2015 Oct 29;373(18):1697–708.

7.0 Appendix

APPENDIX A

THE *VENTANA™* MULTI-SYSTEM AUTO-STAINER PROTOCOL

All slides placed on a heat plate at 60°C for 20 minutes to melt the paraffin embedded wax.
Slides then placed in *Ventana™* system to undergo the following:

PROTOCOL: SCC1 32
PROTOCOL NO.: 4000
PROCEDURE: U ultraView DAB

1. **Deparaffinisation**
   Slide warmed to 72°C from medium temperatures.

2. **Cell conditioning**
   ULTRA Conditioner #1 [EDTA Buffer].

3. **Warm slide to 95°C on a hot plate**

4. **Incubate for 64 minutes in EDTA buffer**

5. **Primary antibody titration**
   Hand apply primary antibody.

6. **Incubate for 32 minutes**

7. **Counterstain**
   Apply one drop of Haematoxylin II counterstain.
   Apply coverslip.

8. **Incubate for 12 minutes**

APPENDIX B

THE *VENTANA™* MULTI-SYSTEM AUTO-STAINER PROTOCOL

All slides placed on a heat plate at 60°C for 20 minutes to melt the paraffin embedded wax.
Slides then placed in Ventana™ system to undergo the following:

PROTOCOL: OV STCC1 32 PXb
PROTOCOL NO.: 4991
PROCEDURE: U OptiView DAB IHC v5

1. **Deparaffinisation**
   Warm up slide to 72°C from medium temperatures.

2. **Cell conditioning**
   ULTRA Conditioner #2 [Citrate Buffer].

3. **Warm slide to 95°C on a hot plate**

4. **Incubate for 64 minutes in citrate buffer**

5. **Pre-primary peroxidase inhibition**

6. **Primary antibody titration**
   Hand apply primary antibody.

7. **Incubate for 32 minutes**

8. **Counterstain**
   Apply one drop of Haematoxylin II counterstain.
   Apply coverslip.

9. **Incubate for 12 minutes**

APPENDIX C

THE *VENTANA™* MULTI-SYSTEM AUTO-STAINER PROTOCOL

All slides placed on a heat plate at 60°C for 20 minutes to melt the paraffin embedded wax.
Slides then placed in *Ventana™* system to undergo the following:

PROTOCOL: Ki67 Roche RTU
PROTOCOL NO.: 4954
PROCEDURE: U ultraView DAB

1. **Deparaffinisation**
   Slide warmed to 72°C from medium temperatures.

2. **Cell conditioning**
   ULTRA Conditioner #1 [EDTA Buffer].

3. **Warm slide to 95°C on a hot plate**

4. **Incubate for 36 minutes in citrate buffer**

5. **Ki67 antibody added**
   One drop of Ki67 antibody
   Apply Coverslip

6. **Incubate for 24 minutes**

7. **ultraWash**
   Apply one drop of UV HRP UNIV MULT. Apply coverslip, and incubate for 8 minutes
   Apply one drop of UV DAB and one drop of UV DAB H2O2. Apply coverslip, incubate for 8 minutes
   Apply one drop of UV COPPER, apply coverslip, and incubate for 4 minutes

8. **Counterstain**
   Apply one drop of Haematoxylin II counterstain.
   Apply coverslip.

9. **Incubate for 12 minutes**

APPENDIX D

SPSS data output for EPB41L4A and Kaplan-Meier Modelling for association between biomarker expression and Time to development of castrate resistance.

Case Processing Summary				
EPB41L4A	Total N	N of Events	Censored	
			N	Percent
.00	59	59	0	0.0%
1.00	24	24	0	0.0%
Overall	83	83	0	0.0%

Survival Table

EPB41L4A		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
.00	1	.000	yes	.	.	1	58
	2	.000	yes	.966	.024	2	57
	3	4.000	yes	.949	.029	3	56
	4	6.000	yes	.	.	4	55
	5	6.000	yes	.	.	5	54
	6	6.000	yes	.898	.039	6	53
	7	8.000	yes	.881	.042	7	52
	8	9.000	yes	.	.	8	51
	9	9.000	yes	.847	.047	9	50
	10	10.000	yes	.831	.049	10	49
	11	11.000	yes	.	.	11	48
	12	11.000	yes	.	.	12	47
	13	11.000	yes	.780	.054	13	46
	14	12.000	yes	.	.	14	45
	15	12.000	yes	.	.	15	44
	16	12.000	yes	.	.	16	43
	17	12.000	yes	.712	.059	17	42
	18	13.000	yes	.	.	18	41
	19	13.000	yes	.678	.061	19	40
	20	15.000	yes	.	.	20	39
	21	15.000	yes	.644	.062	21	38

	22	17.000	yes	.	.	22	37
	23	17.000	yes	.	.	23	36
	24	17.000	yes	.593	.064	24	35
	25	18.000	yes	.576	.064	25	34
	26	19.000	yes	.	.	26	33
	27	19.000	yes	.	.	27	32
	28	19.000	yes	.525	.065	28	31
	29	20.000	yes	.	.	29	30
	30	20.000	yes	.492	.065	30	29
	31	21.000	yes	.475	.065	31	28
	32	24.000	yes	.458	.065	32	27
	33	25.000	yes	.	.	33	26
	34	25.000	yes	.424	.064	34	25
	35	27.000	yes	.	.	35	24
	36	27.000	yes	.390	.063	36	23
	37	30.000	yes	.	.	37	22
	38	30.000	yes	.356	.062	38	21
	39	31.000	yes	.339	.062	39	20
	40	32.000	yes	.	.	40	19
	41	32.000	yes	.305	.060	41	18
	42	33.000	yes	.288	.059	42	17
	43	34.000	yes	.	.	43	16
	44	34.000	yes	.	.	44	15
	45	34.000	yes	.237	.055	45	14
	46	36.000	yes	.220	.054	46	13
	47	37.000	yes	.	.	47	12
	48	37.000	yes	.	.	48	11
	49	37.000	yes	.169	.049	49	10
	50	41.000	yes	.153	.047	50	9
	51	44.000	yes	.136	.045	51	8
	52	49.000	yes	.119	.042	52	7
	53	50.000	yes	.102	.039	53	6
	54	60.000	yes	.085	.036	54	5
	55	74.000	yes	.068	.033	55	4
	56	76.000	yes	.051	.029	56	3
	57	84.000	yes	.034	.024	57	2
	58	115.000	yes	.017	.017	58	1
	59	120.000	yes	.000	.000	59	0
1.00	1	3.000	yes	.958	.041	1	23
	2	8.000	yes	.917	.056	2	22
	3	9.000	yes	.	.	3	21

	4	9.000	yes	.	.	4	20
	5	9.000	yes	.792	.083	5	19
	6	10.000	yes	.750	.088	6	18
	7	11.000	yes	.	.	7	17
	8	11.000	yes	.	.	8	16
	9	11.000	yes	.	.	9	15
	10	11.000	yes	.	.	10	14
	11	11.000	yes	.542	.102	11	13
	12	12.000	yes	.500	.102	12	12
	13	13.000	yes	.	.	13	11
	14	13.000	yes	.417	.101	14	10
	15	14.000	yes	.375	.099	15	9
	16	16.000	yes	.333	.096	16	8
	17	17.000	yes	.	.	17	7
	18	17.000	yes	.250	.088	18	6
	19	18.000	yes	.208	.083	19	5
	20	21.000	yes	.167	.076	20	4
	21	27.000	yes	.125	.068	21	3
	22	34.000	yes	.083	.056	22	2
	23	44.000	yes	.042	.041	23	1
	24	47.000	yes	.000	.000	24	0

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
EPB41L4A	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
.00	28.136	3.202	21.859	34.412	20.000	2.987	14.146	25.854
1.00	16.500	2.250	12.090	20.910	12.000	.919	10.200	13.800
Overall	24.771	2.430	20.008	29.534	17.000	1.366	14.322	19.678

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	7.320	1	.007

Test of equality of survival distributions for the different levels of EPB41L4A.

APPENDIX E

SPSS output data for Lambda FLC and Kaplan-Meier Modelling, for association between biomarker expression and prostate cancer specific survival and time to development of metastases.

Lambda FLC and Prostate Cancer Specific Survival

Case Processing Summary				
LAMBDA FLC	Total N	N of Events	Censored	
			N	Percent
negative	11	11	0	0.0%
positive	7	7	0	0.0%
Overall	18	18	0	0.0%

Survival Table							
LAMBDA FLC		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
negative	1	19.000	yes	.909	.087	1	10
	2	29.000	yes	.818	.116	2	9
	3	33.000	yes	.727	.134	3	8
	4	34.000	yes	.636	.145	4	7
	5	49.000	yes	.545	.150	5	6
	6	56.000	yes	.455	.150	6	5
	7	57.000	yes	.364	.145	7	4
	8	58.000	yes	.273	.134	8	3
	9	60.000	yes	.182	.116	9	2
	10	66.000	yes	.091	.087	10	1
	11	67.000	yes	.000	.000	11	0
positive	1	5.000	yes	.857	.132	1	6
	2	6.000	yes	.714	.171	2	5
	3	15.000	yes	.571	.187	3	4
	4	19.000	yes	.429	.187	4	3
	5	31.000	yes	.286	.171	5	2
	6	32.000	yes	.143	.132	6	1
	7	35.000	yes	.000	.000	7	0

Means and Medians for Survival Time								
	Mean				Median			
			95% Confidence Interval				95% Confidence Interval	
LAMBDA FLC	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
negative	48.000	4.953	38.293	57.707	56.000	12.661	31.184	80.816
positive	20.429	4.720	11.177	29.680	19.000	5.237	8.735	29.265
Overall	37.278	4.744	27.980	46.575	33.000	2.121	28.842	37.158
a. Estimation is limited to the largest survival time if it is censored.								

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	10.496	1	.001
Test of equality of survival distributions for the different levels of LAMBDAlowPerPat.			

Lambda FLC and Time to Development of Metastases

Case Processing Summary				
			Censored	
LAMBDA FLC	Total N	N of Events	N	Percent
negative	13	13	0	0.0%
positive	10	10	0	0.0%
Overall	23	23	0	0.0%

Survival Table							
				Cumulative Proportion Surviving at the Time			
LAMBDA FLC		Time	Status	Estimate	Std. Error	N of Cumulative Events	N of Remaining Cases
negative	1	.000	yes	.	.	1	12
	2	.000	yes	.	.	2	11
	3	.000	yes	.	.	3	10
	4	.000	yes	.692	.128	4	9
	5	14.000	yes	.615	.135	5	8
	6	18.000	yes	.538	.138	6	7
	7	23.000	yes	.462	.138	7	6
	8	31.000	yes	.385	.135	8	5
	9	35.000	yes	.308	.128	9	4
	10	47.000	yes	.231	.117	10	3

	11	50.000	yes	.154	.100	11	2
	12	64.000	yes	.077	.074	12	1
	13	108.000	yes	.000	.000	13	0
positive	1	.000	yes	.	.	1	9
	2	.000	yes	.	.	2	8
	3	.000	yes	.	.	3	7
	4	.000	yes	.	.	4	6
	5	.000	yes	.500	.158	5	5
	6	1.000	yes	.400	.155	6	4
	7	3.000	yes	.300	.145	7	3
	8	14.000	yes	.200	.126	8	2
	9	26.000	yes	.100	.095	9	1
	10	34.000	yes	.000	.000	10	0

Means and Medians for Survival Time

LAMBDA FLC	Mean		95% Confidence Interval		Median		95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
negative	30.000	8.772	12.807	47.193	23.000	10.185	3.036	42.964
positive	7.800	3.986	.000	15.612	.000	.	.	.
Overall	20.348	5.662	9.251	31.445	14.000	10.302	.000	34.192

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	5.223	1	.022

Test of equality of survival distributions for the different levels of LAMBDA FLC.

APPENDIX F

SPSS output data for PD-L1 nuclear and Kaplan-Meier modelling, for association between biomarker expression and time to development of castrate resistance.

Case Processing Summary				
PDL1nuc	Total N	N of Events	Censored	
			N	Percent
.00	38	38	0	0.0%
1.00	37	37	0	0.0%
Overall	75	75	0	0.0%

Survival Table							
PDL1nuc		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
.00	1	.000	yes	.974	.026	1	37
	2	4.000	yes	.947	.036	2	36
	3	6.000	yes	.921	.044	3	35
	4	8.000	yes	.	.	4	34
	5	8.000	yes	.868	.055	5	33
	6	9.000	yes	.842	.059	6	32
	7	10.000	yes	.	.	7	31
	8	10.000	yes	.789	.066	8	30
	9	11.000	yes	.	.	9	29
	10	11.000	yes	.737	.071	10	28
	11	12.000	yes	.	.	11	27
	12	12.000	yes	.	.	12	26
	13	12.000	yes	.658	.077	13	25
	14	17.000	yes	.	.	14	24
	15	17.000	yes	.605	.079	15	23
	16	18.000	yes	.	.	16	22
	17	18.000	yes	.553	.081	17	21
	18	20.000	yes	.526	.081	18	20
	19	21.000	yes	.500	.081	19	19
	20	25.000	yes	.474	.081	20	18
	21	27.000	yes	.	.	21	17
	22	27.000	yes	.421	.080	22	16
	23	30.000	yes	.395	.079	23	15
	24	31.000	yes	.368	.078	24	14
	25	32.000	yes	.342	.077	25	13

	26	33.000	yes	.316	.075	26	12
	27	34.000	yes	.	.	27	11
	28	34.000	yes	.263	.071	28	10
	29	37.000	yes	.237	.069	29	9
	30	41.000	yes	.211	.066	30	8
	31	44.000	yes	.	.	31	7
	32	44.000	yes	.158	.059	32	6
	33	49.000	yes	.132	.055	33	5
	34	50.000	yes	.105	.050	34	4
	35	74.000	yes	.079	.044	35	3
	36	76.000	yes	.053	.036	36	2
	37	84.000	yes	.026	.026	37	1
	38	120.000	yes	.000	.000	38	0
1.00	1	.000	yes	.973	.027	1	36
	2	3.000	yes	.946	.037	2	35
	3	6.000	yes	.919	.045	3	34
	4	9.000	yes	.	.	4	33
	5	9.000	yes	.	.	5	32
	6	9.000	yes	.838	.061	6	31
	7	11.000	yes	.	.	7	30
	8	11.000	yes	.	.	8	29
	9	11.000	yes	.	.	9	28
	10	11.000	yes	.730	.073	10	27
	11	12.000	yes	.703	.075	11	26
	12	13.000	yes	.	.	12	25
	13	13.000	yes	.	.	13	24
	14	13.000	yes	.	.	14	23
	15	13.000	yes	.595	.081	15	22
	16	14.000	yes	.568	.081	16	21
	17	15.000	yes	.541	.082	17	20
	18	16.000	yes	.514	.082	18	19
	19	17.000	yes	.	.	19	18
	20	17.000	yes	.	.	20	17
	21	17.000	yes	.432	.081	21	16
	22	18.000	yes	.405	.081	22	15
	23	19.000	yes	.	.	23	14
	24	19.000	yes	.351	.078	24	13
	25	20.000	yes	.324	.077	25	12
	26	21.000	yes	.297	.075	26	11
	27	25.000	yes	.270	.073	27	10
	28	27.000	yes	.243	.071	28	9
	29	29.000	yes	.216	.068	29	8
	30	32.000	yes	.189	.064	30	7
	31	34.000	yes	.	.	31	6
	32	34.000	yes	.135	.056	32	5
	33	36.000	yes	.108	.051	33	4

	34	37.000	yes	.	.	34	3
	35	37.000	yes	.054	.037	35	2
	36	47.000	yes	.027	.027	36	1
	37	60.000	yes	.000	.000	37	0

Means and Medians for Survival Time								
	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
PDL1nuc	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
.00	29.368	4.073	21.385	37.352	21.000	5.548	10.126	31.874
1.00	19.865	2.086	15.776	23.954	17.000	1.808	13.456	20.544
Overall	24.680	2.356	20.062	29.298	18.000	1.442	15.173	20.827
a. Estimation is limited to the largest survival time if it is censored.								

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.062	1	.044
Test of equality of survival distributions for the different levels of PDL1nucLowPatCutoff60.			

APPENDIX G

SPSS output data for TGF-Beta cytoplasmic and Kaplan-Meier modelling, for association between biomarker expression and time to prostate cancer specific death.

Case Processing Summary

TGFbetaCyto	Total N	N of Events	Censored	
			N	Percent
.00	5	5	0	0.0%
1.00	15	15	0	0.0%
Overall	20	20	0	0.0%

Survival Table

TGFbetaCyto		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
.00	1	6.000	yes	.800	.179	1	4
	2	15.000	yes	.600	.219	2	3
	3	19.000	yes	.400	.219	3	2
	4	31.000	yes	.200	.179	4	1
	5	34.000	yes	.000	.000	5	0
1.00	1	5.000	yes	.933	.064	1	14
	2	19.000	yes	.867	.088	2	13
	3	29.000	yes	.800	.103	3	12
	4	32.000	yes	.733	.114	4	11
	5	33.000	yes	.667	.122	5	10
	6	35.000	yes	.600	.126	6	9
	7	49.000	yes	.533	.129	7	8
	8	56.000	yes	.467	.129	8	7
	9	57.000	yes	.400	.126	9	6
	10	58.000	yes	.333	.122	10	5
	11	60.000	yes	.267	.114	11	4
	12	66.000	yes	.200	.103	12	3
	13	67.000	yes	.133	.088	13	2
	14	115.000	yes	.067	.064	14	1
	15	121.000	yes	.000	.000	15	0

Means and Medians for Survival Time

TGFbetaCyt	Mean[a]	Median

o			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
.00	21.000	5.167	10.872	31.128	19.000	4.382	10.412	27.588
1.00	53.467	8.207	37.380	69.553	56.000	14.169	28.228	83.772
Overall	45.350	7.002	31.625	59.075	34.000	2.236	29.617	38.383
a. Estimation is limited to the largest survival time if it is censored.								

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	8.846	1	.003
Test of equality of survival distributions for the different levels of TGFbetaCyto			

APPENDIX H

SPSS output data for Lambda FLC and TGF-Beta cytoplasmic expression and Kaplan-Meier modelling, for association between combined biomarker expression and time to prostate cancer specific death and time to development of metastases

Lambda FLC and Prostate Cancer Specific Survival

Case Processing Summary				
LambdaNegTGFbetapos	Total N	N of Events	Censored	
			N	Percent
.00	9	9	0	0.0%
1.00	9	9	0	0.0%
Overall	18	18	0	0.0%

Survival Table							
LambdaNegTGFbetapos		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
.00	1	5.000	yes	.889	.105	1	8
	2	6.000	yes	.778	.139	2	7
	3	15.000	yes	.667	.157	3	6
	4	19.000	yes	.	.	4	5
	5	19.000	yes	.444	.166	5	4
	6	31.000	yes	.333	.157	6	3
	7	32.000	yes	.222	.139	7	2
	8	34.000	yes	.111	.105	8	1
	9	35.000	yes	.000	.000	9	0
1.00	1	29.000	yes	.889	.105	1	8
	2	33.000	yes	.778	.139	2	7

	3	49.000	yes	.667	.157	3	6
	4	56.000	yes	.556	.166	4	5
	5	57.000	yes	.444	.166	5	4
	6	58.000	yes	.333	.157	6	3
	7	60.000	yes	.222	.139	7	2
	8	66.000	yes	.111	.105	8	1
	9	67.000	yes	.000	.000	9	0

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
LambdaNegTGFbetacutoff10pos	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
.00	21.778	3.919	14.097	29.458	19.000	2.981	13.156	24.844
1.00	52.778	4.496	43.965	61.590	57.000	1.491	54.078	59.922
Overall	37.278	4.744	27.980	46.575	33.000	2.121	28.842	37.158

a. Estimation is limited to the largest survival time if it is censored.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	12.537	1	.000

Test of equality of survival distributions for the different levels of LambdaNegTGFbetacutoff10pos.

Lambda FLC and Time to Development of Metastases

Case Processing Summary

LambdaNegTGFbetapos	Total N	N of Events	Censored

			N	Percent
.00	11	11	0	0.0%
1.00	11	11	0	0.0%
Overall	22	22	0	0.0%

Survival Table

LambdaNegTGFbetapos		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
.00	1	.000	yes	.	.	1	10
	2	.000	yes	.	.	2	9
	3	.000	yes	.	.	3	8
	4	.000	yes	.	.	4	7
	5	.000	yes	.545	.150	5	6
	6	1.000	yes	.455	.150	6	5
	7	3.000	yes	.364	.145	7	4
	8	14.000	yes	.	.	8	3
	9	14.000	yes	.182	.116	9	2
	10	26.000	yes	.091	.087	10	1
	11	34.000	yes	.000	.000	11	0
1.00	1	.000	yes	.	.	1	10
	2	.000	yes	.	.	2	9
	3	.000	yes	.727	.134	3	8
	4	18.000	yes	.636	.145	4	7
	5	23.000	yes	.545	.150	5	6
	6	31.000	yes	.455	.150	6	5
	7	35.000	yes	.364	.145	7	4
	8	47.000	yes	.273	.134	8	3
	9	50.000	yes	.182	.116	9	2
	10	64.000	yes	.091	.087	10	1
	11	108.000	yes	.000	.000	11	0

Means and Medians for Survival Time

LambdaNegTGFbetapos	Mean[a]			Median		
	Estimate	Std. Error	95% Confidence Interval	Estimate	Std. Error	95% Confidence Interval

			Lower Bound	Upper Bound			Lower Bound	Upper Bound
.00	8.364	3.649	1.212	15.515	1.000	.	.	.
1.00	34.182	9.842	14.891	53.473	31.000	9.358	12.658	49.342
Overall	21.273	5.845	9.816	32.730	14.000	9.926	.000	33.455
a. Estimation is limited to the largest survival time if it is censored.								

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	6.864	1	.009
Test of equality of survival distributions for the different levels of LambdaNegTGFbetapos.			