Exosomal RNA as a source of urine biomarkers for prostate cancer

A thesis submitted to the University of East Anglia
For the degree of Doctor in Medicine

By

Marcelino Yazbek Hanna

School of Medicine, Health Policy and Practice
University of East Anglia
Norwich NR4 7TJ

2017

© This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that it's copyright rests with the author and that no quotation from the thesis, nor any information derived therefrom, may be published without the author's prior, written consent.

Abstract

Introduction

In this study we exploited the recent development of methods that have enabled the analysis of RNA present in urine exosomes of prostate cancer patients. We report RNA expression patterns that contain diagnostic and prognostic information for prostate cancer, and association with response to hormone treatment.

Methods

First catch urine following digital rectal examination were collected from 662 men. 3 groups of patients were used: Low, Intermediate, and High-risk according to NICE stratification criteria, and two control groups: benign and advanced disease. 50-gene transcript expression analysis using NanoString technology was performed on 192 samples. Exosomal RNA Next-Generation Sequencing was performed on 18 samples for novel biomarker discovery.

Results

Expression analysis showed that PCa-specific transcripts such as TMPRSS2/ERG fusion transcripts were identifiable in exosomes from PCa urine samples. LPD analysis highlighted expression levels of 15 transcripts with diagnostic potential (significantly up-regulated in cancer samples in comparison to benign control) and 17 transcripts with prognostic potential (differentialy expressed in high risk and advanced disease in comparison to lower grade disease).

I also report two gene transcripts (SERPINB5/Maspin, HPRT) that were significantly differentially expressed in patients who failed to respond to hormone deprivation therapy for high risk/metastatic disease. Three genes (STEAP4, ARexons4_8 and NAALADL2) were significantly differentially expressed in patients who relapsed within 12 months of hormone treatment initiation.

Next-Generation Sequencing of twenty samples identified 45 genes to be significantly differentially expressed between non-cancer and cancer samples (28 were up regulated and 17 down regulated). 33 out of the 45 genes showed a significant linear trend in association with cancer risk.

Conclusions

Urine Exosomal RNA contains PCa specific transcripts. Gene expression analysis and Next Generation Sequencing identified genes that are significantly differentially expressed between cancer and non-cancer cases as well as prognostic genes and genes that can predict response to hormone treatment

Acknowledgments

Firstly, I would like to thank Professor Colin Cooper for taking me on in his lab and providing me with the highest level of support and supervision to explore such an interesting field. Also a big thank you to Professor Dylan Edwards who initially engaged me to work in his lab and offered me full support and supervision prior to moving to Professor Cooper's lab. I owe a very special and hearty gratitude to Dr Jeremy Clark for his priceless support and advice throughout my journey, as well as to Dr Rachel Hurst, and Lin Cooley for their help. I also owe a very special thank you to Mr Mills and Mr Rochester who gave me infinite support and helped make this project possible, first by giving me a post at the NNUH that enabled a large amount of sample collection from various clinics and secondly by providing continuous advice and support throughout the project. I also owe a big thank you to Professor Sethia whose original introduction to Professor Edwards made this thesis possible. His continued support and encouragement over the last few years have been invaluable. The last three years have been a most worthwhile experience thanks to all of you.

Fundamental to this thesis was the data analysis, which was done in collaboration with Dr Brewer and Helen Curley. I owe them both a very big thank you for their patients and advice and for the amazing work they have done. I also want to thank all the staff at the outpatient clinic at the NNUH starting with sister Rooney, and staff nurse Andria Clayton, and the reception staff for their priceless support, help and patients throughout this project which would certainly not be possible to complete without their help.

I also want to thank the Biochemistry laboratory staff at the NNUH for allowing us to use their facility at the beginning of this project, as well as Kathryn Andrews at the R&D department.
The research was kindly and generously supported by the Movember prostate cancer charity to which I also owe a big thank you.

Also, a big thank you for all the patients who provided samples for the purpose of this project.
Last but not least, I thank members of my family; particularly my wife Ksenija for her support and encouragement throughout, and in particular during the writing of this thesis and also my parents for their keen interest and support.

National and Internaltional Presentations

1. **Exosomal RNA as a source of urine biomarkers for prostate cancer.**

   Marcelino Yazbek Hanna*, Jeremy Clark, Rachel Hurst, Mark Rochester, Robert Mills, Krishna Sethia, Dylan Edwards, Colin Cooper.*

   - **Moderated Poster Presentation**. BAUS Liverpool 2016.

2. **Urine Biomarkers for Prostate Cancer.**

   Marcelino Yazbek Hanna*, Jeremy Clark, Rachel Hurst, Mark Rochester, Robert Mills, Krishna Sethia, Dylan Edwards, Colin Cooper.*

   - **Moderated Poster Presentation.** 29th Annual EAU Congress Stockholm 2014.
   - **Podium Presentation.** Academic BAUS Cambridge 2014.

3. **Exosomes Derived Urine Biomarkers for Prostate Cancer.**

   Marcelino Yazbek Hanna*, Jeremy Clark, Rachel Hurst, Mark Rochester, Robert Mills, Krishna Sethia, Dylan Edwards, Colin Cooper.*

   - **Un-Moderated Poster Presentation.** Academic EAU Meeting Dresden Germany 2013.

4. **Prostrate Cancer Biomarker in a Bubble.**

   Marcelino Yazbek Hanna*, Jeremy Clark, Rachel Hurst, Mark Rochester, Robert Mills, Krishna Sethia, Dylan Edwards, Colin Cooper.*

   - **Moderated Poster Presentation**. BAUS Manchester 2013.

5. **Exosomal RNA, Urine Biomarkers for prostate cancer. Our experience.**

   Marcelino Yazbek Hanna*, Jeremy Clark, Rachel Hurst, Mark Rochester, Robert Mills, Krishna Sethia, Dylan Edwards, Colin Cooper.*

   - **Podium Presentation.** Academic BAUS London 2013.

Table of Contents

Chapter

List of Figures

List of Tables

Abbreviations

AATF: Apoptosis antagonizing transcription factor
ABCB9: ATP-binding cassette, sub-family B (MDR/TAP), member 9
ACTR5: Actin-Related Protein 5 Homolog (Yeast)
ADT: Androgen-deprivation therapy
AGR2: Anterior gradient 2 homolog
ALAS1: Aminolevulinate, delta-synthase 1
AMACR: Alpha-Methylacyl-CoA Racemase
ANPEP: Alanyl (Membrane) Aminopeptidase
AR: Androgen receptor
ARHGEF25: Rho guanine nucleotide exchange factor (GEF) 25
AS: Active Surveillance
AUA: American urological association
AURKA: Aurora Kinase A
B2M: Beta-2-microglobulin
BIK: BCL2-interacting killer (apoptosis-inducing)
BPH: Benign prostate hyperplasia
BRAF: V-Raf Murine Sarcoma Viral Oncogene Homolog B
C16orf91: Chromosome 16 Open Reading Frame 91
C1orf216: Chromosome 1 Open Reading Frame 216
CAMKK2: Calcium/Calmodulin-Dependent Protein Kinase Kinase 2 Beta
CB<1: Clinically benign and PSA<1
CBN: Clinically benign and PSA normal to age
CCDC88B: Coiled-coil domain containing 88B
CDC20: Cell Division Cycle 20 Gene
CDKN3: Cyclin-Dependent Kinase Inhibitor 3
CKAP2L: Cytoskeleton associated protein 2-like
CKAP2L: Cytoskeleton associated protein 2-like
CLIC2: Chloride intracellular channel 2
CLU: Clusterin identified as a therapeutic target
CP: Ceruloplasmin (ferroxidase)
CT: Computer tomography
DLX1: Distal-less homeobox 1
DNA: Deoxyribonucleic acid
DNAJC27: DnaJ (Hsp40) homolog, subfamily C, member 27
DRE: Digital rectal examination
EAU: European association of urology
ELISA: Enzyme-linked immunosorbent assay
EMC9: ER membrane protein complex subunit 9
ERC2: ELKS/RAB6-interacting/CAST family member 2
ERG: V-Ets Avian Erythroblastosis Virus E26
ERMP1: Endoplasmic reticulum metallopeptidase 1
ERSPC: European Randomized study of Screening for Prostate Cancer
FBC: Full blood count
FDA: Food and Drug Administration
FISH: Fluorescence in situ hybridisation
FoxM1: Forkhead box protein M1gene
FSH: Follicle-stimulating hormone

G: Gleason
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GH-PIN: High-grade prostatic intraepithelial neoplasia
GOLM1: Golgi membrane protein 1
HIST1H2BF: Histone cluster 1, H2bf
HMBOX1: Homeobox containing 1
HOXC4: Homeobox C4
HOXC6: Homeobox C6
HPC1: Hereditary Prostate Cancer 1
HPN: Hepsin
HPRT: Hypoxanthine-guanine phosphoribosyltransferase 1
HPSE2: Heparanase 2
HPX: Hereditary Prostate Cancer X
IGF: Insulin like growth factor
IMPDH2: Inosine Monophosphate Dehydrogenase 2
KLK2: Kallikrein 2
KLK4: Kallikrein 4
KNTC1: Kinetochore associated 1
LFT: Liver function test
LH: Luteinising hormone
LHRH: Luteinising hormone releasing hormone
LUTS: Lower Urinary Tract Symptoms
MCTP1: Multiple C2 domains, transmembrane 1
MDK: Midkine. Neurite Growth-Promoting Factor 2
MFSD2A: Major facilitator superfamily domain containing 2A
MIR146A: MicroRNA 146a
MKi67: Marker Of Proliferation Ki-67
MMP: Matrix Metalloproteinase
MMP25: Matrix metallopeptidase 25
MMP26: Endometase/matrilysin-2/matrix metalloproteinase 26
MRI: Magnetic resonance imaging
MYOF: Myoferlin
NAALAD2: N-acetyl-L-aspartyl-L-glutamate peptidase-like 2
NAALADL1: N-acetylated alpha-linked acidic dipeptidase 1
NEBL: Nebulette
NHS: National Health Service
NICE: National institute for health and care excellence
NLRP3: NLR family, pyrin domain containing 3
NNUH: Norfolk and Norwich University Hospital
NT5C3B: 5'-nucleotidase, cytosolic IIIB
NUDT6: Nudix (nucleoside diphosphate linked moiety X)-type motif 6
OGT: O-linked β-N-acetylglucosamine transferase
P: Prostatitis
PALM3: Paralemmin 3
PBS: Phosphate buffer saline
PCA: Principal component analysis
PCa: prostate cancer
PCA3: Prostate cancer gene 3
PCPT: Prostate cancer prevention trial
PECI: Peroxisomal 3,2-trans-enoyl-CoA isomerase

PIN: Prostatic intraepithelial neoplasia
PLCB2: Phospholipase C, beta 2
PLCO: Prostate, Lung, Colorectal and Ovarian
PPAP2A: Phosphatidic acid phosphatase type 2A
PPIRES: Public and Patient Involvement in Research
PPP1R12B: Protein phosphatase 1, regulatory subunit 12B
PRR5L: Proline rich 5 like
PSA: Prostate specific antigen
PSGR: Prostate-specific G-protein coupled receptor
PSTPIP1: Proline-serine-threonine phosphatase interacting protein 1
PTPRC: Protein Tyrosine Phosphatase, Receptor Type, C
R&D: Research and development
RIN: RNA Integrity Number
RNA: Ribonucleic acid
RNASE10: Ribonuclease, RNase A family, 10 (non-active)
RP: Radical prostatectomy
RP9: Retinitis pigmentosa 9
RPL18A: Ribosomal protein L18a
RPL36: Ribosomal protein L36
RPS10: Ribosomal protein S10
RPS11: Ribosomal protein S11
RT-PCR: Reverse transcription polymerase chain reaction
RT: Radical treatment
SLC12A1: Solute carrier family 12
SMIM1: Small integral membrane protein 1
SPINK1: Serine protease inhibitor Kazal-type 1
ST6GALNAC1: ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
STEAP2: Six transmembrane epithelial antigen of the prostate family member 2
STEAP4: Six transmembrane epithelial antigen of the prostate family member 4
SULF2: Sulfatase 2
SULT1A1: Sulfotransferase Family, Cytosolic, 1A, Phenol-Preferring member 1
TBP: TATA box binding protein
TDRD1: Tudor domain containing 1
TERT: Telomerase Reverse Transcriptase
TGF-ß: Transforming growth factor-beta
TIMP4: Tissue inhibitor of metalloproteinases 4
TMEM47: Transmembrane protein 47
TMEM86A: Transmembrane protein 86A
TMPRSS2: Transmembrane protease, serine 2
TRUS: Trans rectal Ultrasound
TTC39B: Tetratricopeptide repeat domain 39B
TURP: trans urethral resection of the prostate
U&E: Urea and Electrolytes
UEA: University of East Anglia
UPK2: Uroplakin 2
WW: Watchful waiting
ZBTB34: Zinc finger and BTB domain containing 34

1. Introduction

1.1 Prostate cancer – an Introduction

The increased testing with serum prostate specific antigen (PSA) in healthy men as well as aging populations and public awareness led to increase in prostate cancer (PCa) diagnosis making it the most common cancer diagnosed in men (Cancer research UK, 2014 http://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence/common-cancers-compared), and the sixth leading cause of death in males worldwide (1,2). Its incidence is strongly age, country and race related with the highest incidence being in older men and the highest detection rate in Guadeloupe and lowest in south-central Asia. However these statistical figures may not represent an accurate picture due to poor cancer registration in some developing nations (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/prostate/incidence/uk-prostate-cancer-incidence-statistics) (3). PCa has a highly unpredictable clinical behaviour which is due to its innate multifocality and heterogeneity of progression rate. Unlike most other cancers, a large proportion of patients have clinically insignificant and indolent disease that will pose no real risk to their life. However due to the limitation of the available diagnostic and prognostic measures to identify aggressive PCa these patients often undergo unnecessary investigation and radical treatments. This has led to the questioning of prostate cancer screening by many, as several trials have shown no significantly decrease in prostate cancer-specific mortality in screened populations (3-5). Detection of prostate cancer by PSA testing and needle biopsy alone is also unreliable as 30 to 40% of anterior tumour can be missed (6,7), as well as a significant proportion of peripheral zone tumours particularly in large prostate glands where the 10-core standard biopsy may not adequately sample the entire prostate (8).

There is therefore an unmet need for diagnostic biomarkers that are more specific for detecting prostate cancer *per se*, and which can also discern indolent from clinically significant disease. Such biomarkers would retain the beneficial effect of early detection, while minimising the problems of over-diagnosis and over-treatment.

1.1.1 The Epidemiology of prostate cancer

In 2010 prostate cancer was reported to be the most common cancer in most Western populations including the UK where it accounted for approximately 25% of all new diagnosed cancer cases in men in England and Wales (Cancer Research UK) (3,9,10).
The detection rate of prostate cancer has risen in part due to increased detection via the PSA testing since 1986 (11) and diagnosis via surgical treatment for benign prostatic hyperplasia (BPH) (11) (12). In the UK alone PCa incidence increased by approximately 10,000 cases between 2001 and 2010 (3) however the mortality rate remained unchanged (Table 1.1 and Figure 1.1 provide a comparison of incidence rate and mortality rate in the UK. Both figures were adopted from Cancer research UK (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence#heading-Three)).

Table 1.1: Prostate cancer associated incidence and mortality rate in the UK.

Mortality	England	Wales	Scotland	Northern Ireland	UK
Incidence Numbers 2001	26,027	1,746	1,860	509	**30,142**
Age standardised Rates	89.9	92.6	64.9	63.1	79.2
Incidence Numbers 2010	34,892	2,462	2,679	942	**40,975**
Age standardised Rates	106.4	114.0	82.1	96.5	104.5
Mortality Numbers in 2003	8,582	579	786	217	**10,164**
Age standardised Rates	27.3	28.6	26.7	25.5	27.2
Mortality Numbers in 2010	9,082	849	547	243	**10,721**
Age standardised Rates	23.8	23.7	22.8	23.5	23.8

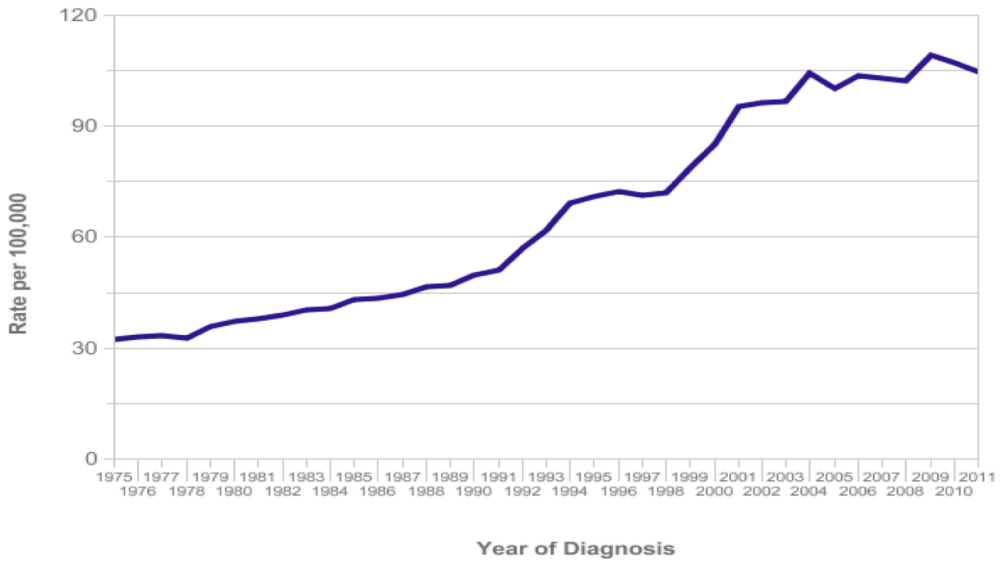


Figure 1.1: The incidence of PCa in the UK over the last two decades (illustration adopted from Cancer research UK. Prostate cancer incidence statistics). Rates are per 100,000 men.

1.1.2 Aetiology and risk factors

Various risk factors are associated with prostate cancer including:

(1) Age: the most prominent risk factor in prostate cancer is aging. In the UK approximately 36% of cases are diagnosed in men aged 75 years and over, and only 1% were diagnosed in the under-50s based on histological diagnosis registered by the office for national statistics in the UK (Cancer Research UK). The incidence rate is 166 per 100,000 for men aged 55-59, 560 per 100,000 for men aged 65-69 and 800 per 100,000 for men aged 75-79 (13) (3). Much of prostate cancer's detected incidence in various parts of the world comes from autopsy studies which reported an increase incidence of PCa in older men with a detection rate as high as 100% in men aged 100 (14). The prostate cancer prevention trial also reported an increased incidence of PCa in older men - this study was based on 5519 men and reported age related incidences of 0.7% in men aged 55-60, 20.7% in men 60-64, 31.5% in men age 65-69 and 47.1% in men over 70 years of age, these findings were based on PSA screening and histological diagnosis (TRUS biopsy)(15). To date the natural history of prostate cancer is not fully understood however it was thought that with increasing age, the production of steroid hormones changes, resulting in a favourable environmental conditions for the development and progression of cancer (16,17).

(2) Hereditary: A meta-analysis investigating the risk of developing prostate cancer in the presence of a family history showed that relative risk increases with increasing numbers of affected family members (18), and malignant PCa is relatively more common at younger ages (19,20).

Several genes have been reported to be involved in familial risk of prostate cancer including *HPC1, HPC2, HPC20, CAPB, HPCX, BRAC1, BRAC2 and HOXB13* (21,22), some of which will be briefly discussed here. The *HPC1* gene (Hereditary Prostate Cancer 1 on chromosome 1 at q24-q25) (23) and the *HPCX* gene (Hereditary Prostate Cancer X–linked, located on the X chromosome at q27-28). Men who inherit the PCa-linked *HPC1* allele tend to develop prostate cancer before the age of 65 years (24) The *HPCX* gene has been reported to account for 16% of all familial prostate cancer cases and is particularly noticeable in cases of affected brothers due to the fact that it is X-linked and so not inherited from the father - the Y chromosome being inherited from the father and the X from the mother) (25).

The G84E mutation in *HOXB13* in prostate cancer was initialy reported by in 2012. This mutation was particularly associated with men below the age of 55 years old that had a positive family history for prostate cancer (26), these finding was confirmed by another study examining the frequency of G84E in *HOXB13* in in 2,443 hereditary prostate cancer families recruited from an International Consortium for Prostate Cancer Genetics (ICPCG). Others also reported i) a significant increase in prostate cancer in men carrying the mutation, ii) an associated clinical characteristic of high-risk disease compared to non carriers (51%/30%), and iii) significant over-transmittion of the mutation from parents to offspring.

Several studies also reported an increased risk of prostate cancer in *BRAC1+2* mutation carriers in comparison to non-carriers (five to two relative risk). The highest risk was for carriers of a *BRCA2* mutation, particularly as these patients developed poorly differentiated aggressive disease (27-30). Another study reported that most of *BRAC2* mutation carriers develop early onset disease (31).

Genome-wide association studies have brought attention to inherited SNPs (Single Nucleotide Polymorphisms). One study reported 8 out of 300 SNP's on 8q24 (rs12543663, rs10086908, rs1016343, rs13252298, rs6983561, rs620861, rs6983267 rs10090154) to be significantly associated with PCa susceptibility, though with no evidence for a link with disease aggressiveness. A risk score based on those 8 SNPs identified a 2-fold increased risk of PCa between the top and the bottom 1% of the population, with 8% of the top 1% being explained by familial risk with first degree relatives (32). A genome wide association study

identified 7 further PCa susceptibility loci in 7 regions of 5 different chromosomes (2p21, 2q31, 4q22, 4q24, 8p21, 11p15, 22q12), the SNP on chromosome 4 having the strongest association with family history (rs7679673). By using a polygenic risk score based on these SNPs and previously identified ones, they reported that subjects in the top 1% of the risk distribution had a three-fold increased risk for prostate cancer and the top 10% have a relative increased risk of 2.3 fold in comparison with the general population. They also reported that 21.5% of familial PCa could be related to SNPs (33). Another study on 2609 men showed that prostate cancer specific genetic variants can be used to improve prediction of prostate cancer (34).

Epigenetic factors such as DNA-methylation can accompany genomic instability in PCa development. DNA-methylation can act as an ancillary to DNA mutations, leading to up- and down-regulation of genes and interruption of their normal functions, which may, for example predispose to increases in genetic alterations through the inactivation of tumour suppressor genes such as DNA repair genes, leading to the development of cancer (35).

(3) Race: African-American men have been reported to have a higher incidence of prostate cancer with more aggressive disease. In the USA, people of African-American descent tend to be more affected by the disease and have poorer outcomes in terms of mortality when compared to their white counterparts (36-38). Men of Asian or Oriental origin have the lowest reported incidence (22), whereas Caucasians have an incidence between that of African-American and Asian populations. A higher incidence of the disease in second generation migrant Japanese and Chinese men in the USA as compared to those in their native countries support an argument for the contribution of environmental factors, diet and life style in the casualty of the disease rather than of differences in genetic structure or function (39).

(4) Diet: Several studies have reported a correlation between diet and the incidence of prostate cancer, in particular consumption of high levels of animal protein and calcium as reported by the EPIC (European Prospective Investigation into Cancer and Nutrition) in 2008. An 8.7 year follow up on 142,251 men showed that consumption of 35 g a day of dairy protein increased the risk of prostate cancer by 32%. Several studies have linked dairy protein with an enhanced activity of growth hormones particularly Insulin like Growth factor-I (IGF-I) which has been targeted in some studies for prostate cancer therapy (40)

(41-43) (44). Dairy calcium may increase PCa by supressing the synthesis of 1,25-dehydroxyvitamin-D (45).

Others have correlated prostate cancer with animal fat. This theory was strongly supported by a prospective study on a cohort of 51,529 U.S. men, aged 40 through to 75, that found directly correlation between total fat consumption and risk of advanced prostate cancer (46). Potential mechanisms of action include fat- induced changes in the hormonal milieu, induction of oxidative stress, and/or insulin-like growth factor-1 (IGF-1) (47).

(5) Other factors: There are a numbers of other factors that have been reported to influence prostate cancer including, obesity, vasectomy, physical inactivity, sexual activity, sexual transmitted disease, infection with human papillomavirus, smoking, and alcohol consumption (48).

1.2 Clinical presentation and diagnosis

Presentation: The majority of prostate cancer patients are asymptomatic. Diagnosis in such cases is based on abnormalities detected by screening with serum levels of prostate-specific antigen (PSA) or findings on digital rectal examination (DRE). In addition, prostate cancer can be an incidental pathologic finding when tissue is removed during transurethral resection to manage obstructive symptoms from benign prostatic hyperplasia. Patients may also present with symptoms of metastatic disease.

Symptoms of primary disease are usually secondary to prostate volume rather than cancer symptoms *per se*. These syptoms usually include lower urinary tract symptoms (LUTS) urine retention and or haematuria. However patients with benign prostatic hyperplasia alone will exhibit similar symptoms.

Symptoms of advanced disease result from any combination of lymphatic, haematogenous, or contiguous local spread. Skeletal manifestations are especially common, symptoms depends on the site of metastasis that usualy manifest as a localised bone pain. Other symptoms comprise lower limb neurological defect due to spinal cord compression which is the most common sign in axial metastasis (more then 70% of people who die of prostate carcinoma have metastatic disease in their bones (49)). Beside bones, liver and lungs can

also be affected. Metastasis can also take the lymphatic route leading to enlarged lymph nodes that can cause symptoms due to pressure on other organs such as the ureters and swelling in the lower limb due to lymph congestion seconadary to blockage in the lymph circulation. Symptoms of malignancy including lethargy, weight loss and anaemia, are also common and usually are secondary to marrow infiltration or destruction by metastasis.

Diagnosis can be attained by combination of clinical history, examination, and investigations: clinically a raised PSA or abnormal DRE raise the suspicion of PCa, histologically prostate tissue can be obtained through TRUS-guided biopsy of the prostate or prostate tissue through TURP (in which case cancer in not expected (TURP is done as a treatment for symptomatic BPE (benign enlargement of the prostate)), and radiologically by the mean of CT or MRI staging. Clinically a raised PSA and or abnormal DRE are an indication for trans rectal biopsy of the prostate. A DRE provides a rudimentary assessment of the local extent of the tumour and clinical staging. The histological assessment provides histological grading on the disease aggressiveness. According to the American Joint Committee on Cancer (AJCC) clinical staging is as follows: T1 - tumour present, but not detectable by DRE, T2: the tumour can be felt (palpated) on DRE, but has not spread outside the prostate, T3: the tumour has spread through the prostatic capsule (not detectable by DRE), T4: the tumour has invaded other nearby structures.
Radiological staging by means of magnetic resonance imaging (multi-parametric MRI), Imaging technology is used in some centres in first line investigation of patients with raised PSA, followed up with a subsequent target and random biopsy in case of radiologically identifiable disease. The advantage of this is being able to identify clinically impalpable disease, anterior lobe tumours (small foci or anterior lobe tumours), and preventing biopsy-related artefacts in patients that require a post biopsy MRI for staging porposes (to assess whether the tumour is localised to within the prostate capsule, or has invaded locally, or metastasised to lymph nodes). MRI and CT (Computer Tomography) scans are typically used post-biopsy in most centres for staging. In clinically advanced disease (PSA>100 and/or locally advanced tumour on DRE) a bone nucleotide scan can be used to detect bone metastasis.

1.3 Histopathology of prostate cancer

1.3.1 Macroscopic pathology

The prostate gland consists of three main zones, which differ histologically and biologically. The Peripheral zone constitutes the bulk of the prostate, forming about 70% of the glandular part of the organ, and is the sub-capsular portion of the posterior aspect of the prostate gland that surrounds the distal urethra where its ducts open. The Central zone surrounds the ejaculatory ducts and forms about 25% of the glandular prostate; its ducts open mainly into the middle prostatic urethra. The Transition zone constitutes about 5% of the prostate and consists of two small lobes that surround the urethra proximal to the ejaculatory ducts. Its ducts open close to the sphincteric part of the urethra (50) (Figure 1.2).

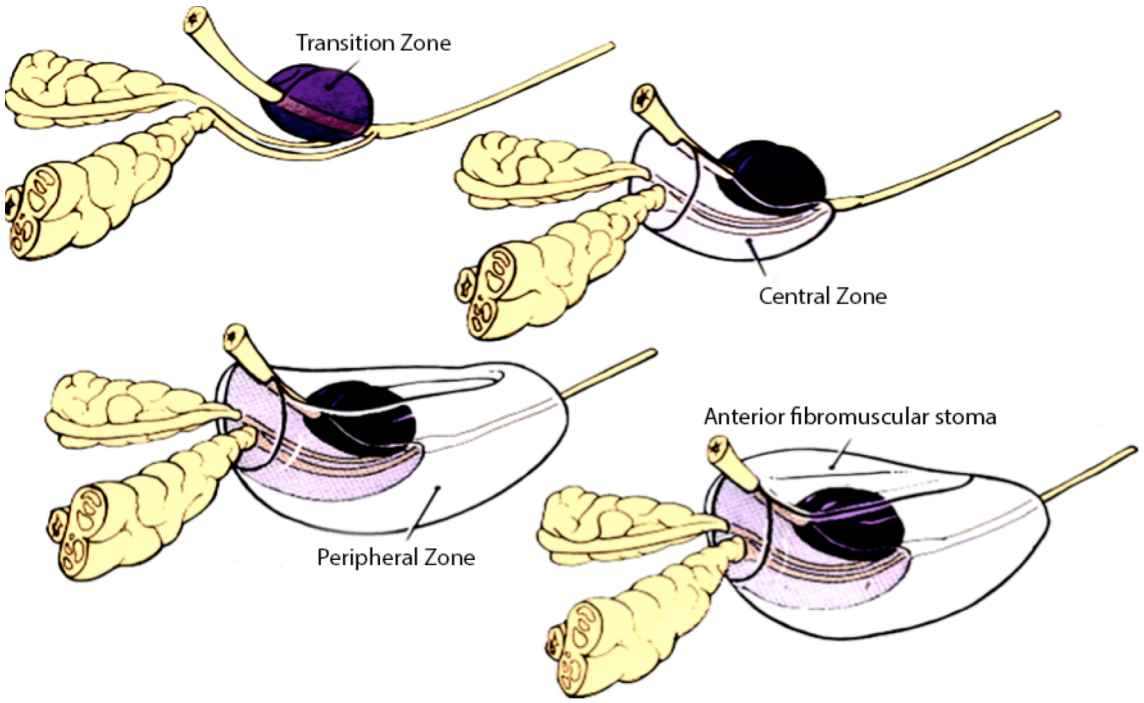


Figure 1.2: The various components of the prostate around the urethra. Illustration adopted from Elsevierimages.com (http://www.elsevierimages.com/image/25271.htm).

The majority of prostate malignancies arise in the Peripheral zone, which accounts for approximately 75% of all prostate cancers. The remaining 25% are found in the Transition zone (20%) and Central zone (5%).

Tumours in different prostatic zones have different pathological behaviours. Peripheral zone tumours are usually large in volume and are well known for their heterogeneity (Gleason scores varying from 3 to 5) and multifocality. Transition zone tumours arise in or near foci of benign prostatic hyperplasia and are smaller and better differentiated (Gleason 1-2) (51). Central zone carcinomas are the rarest, but highly aggressive with a distinct route of spread from the gland via the ejaculatory ducts and seminal vesicles routes that contrasts with spread of tumours from the other zones (52).

Most prostate malignancies (95%) are adenocarcinoma. The remaining morphological variants are uncommon; they include ductal carcinoma variants, mucinous carcinoma, adenosquamous carcinoma and sarcomatoid carcinoma and metastases from other sites (53).

1.3.2 Microscopic pathology

The stem cells of prostate acini have been hypothesised to be the origin of prostate cancer (54). Between these cells and the final secretory cells, different intermediate or transit cells can be observed [stem cells, early progenitors intermediate stem cells, late progenitors intermediate stem cells, secretory cells (Figure 1.3, 1.4), and every one of them has been proposed to be able to evolve into malignant cells, perhaps explaining the biological variability of prostatic cancer (55).

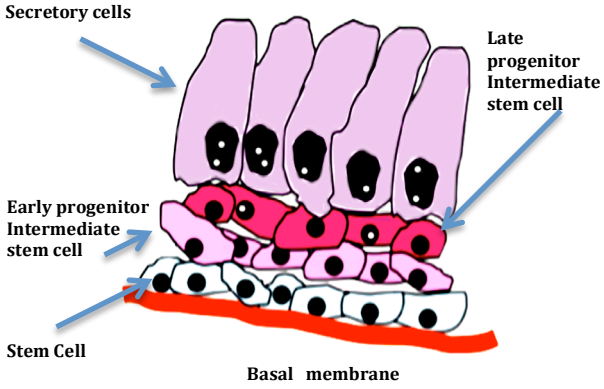


Figure 1.3: Stem cell model of the normal prostate acini (Adopted from Maitland & Collins (55)).

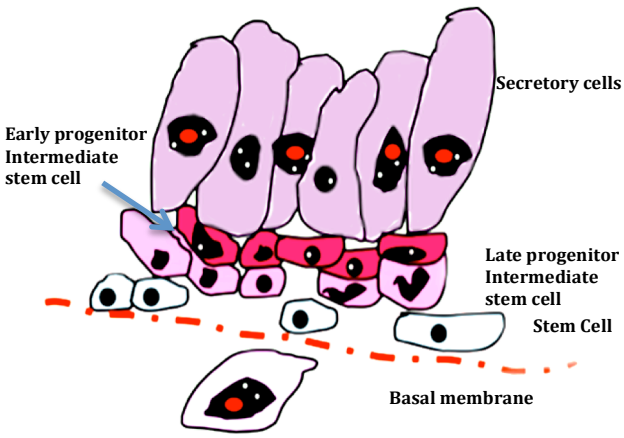


Figure 1.4: Stem cell model showing the malignant transformation of prostate acini, with stem cell loss, malignant transformation of intermediate stem cells and secretory cells. Every one of these cells can be the final differentiation of the prostate cancer, and for this reason the prostate cancer has different phenotypes (Adapted from Maitland & Collins (55)).

Histologically, Gleason's grading system is by far the most common prostate cancer grading method accepted and widely used. It is based on tissue architecture and the degree of tumour differentiation, cytological features do not play a role in the assessment (56). This grading system relies on identifying the 2 or 3 most dominant architectural patterns that get allocated as grades from 1 to 5, from the most differentiated (Gleason 1), to the least differentiated (Gleason 5). The two scores added together provide a 'Gleason sum', which range from 2 to 10 however in cases where there is a 3 dominant grade it does not get added to the over all Gleason sum but may have clinical weight when it comes to offer treatment options. Gleason grading is an independent predictor of outcome and correlates with crude survival, tumour-free survival, and cause-specific survival (57). In addition to the Gleason grading system other microscopic features such as micro-vascular invasion and perineural infiltration can help predict the aggressiveness of the disease (58).

1.3.3 PIN

HG-PIN (High Grade Prostatic Intraepithelial Neoplasia) is a histopathological change that is considered to be the most likely precursor of invasive carcinoma of the prostate (59,60). There are numerous publications that support this hypothesis, including the many shared similarities between PIN and carcinoma of the prostate, such as the pattern of spread through

prostatic ducts, the multifocal nature, the predominance in the Peripheral zone, and the association with raised PSA (61-64). Other morphological similarities that PIN shares with prostate cancer but to a lesser extent include: 1) neovascularisation; studies showed that the number of microvessels in HG-PIN is greater than that in benign prostatic epithelium, but less than that in adenocarcinoma (65), 2) basal cell layer disruption is present in 56% of cases of HG-PIN and the amount of disruption increases with increasing grade of PIN (61), 3) evidence of increased expression of a proteolytic enzyme (type IV collagenase) in PIN and cancer when compared with benign epithelium, this is thought to induce fragmentation of the stroma during invasion (61,66). 4) The frequency, severity, and extent of PIN increases in prostates with cancer compared to benign glands, 82% and 43% respectively as shown by several studies (61,62,67,68). 5) One of the strongest lines of evidence that support PIN as a precursor of carcinoma of the prostate is that they share similar genetic alterations such as the *TMPRSS2/ERG* fusion gene (69-72). Benign prostatic acini and ducts consist the histological architecture of PIN, where they are lined by atypical dysplastic cells depending on its grade (Figure 1.5). In HG-PIN there is partial destruction of the basal cell layers shown by 34BE12 cytokeratin immunostaining, in contrast in prostate adenocarcinoma there is a complete destruction.

The cytological changes in PIN include prominent nuclei and nuclear enlargement in most cells, as well as crowding and increased density of cytoplasm (73).

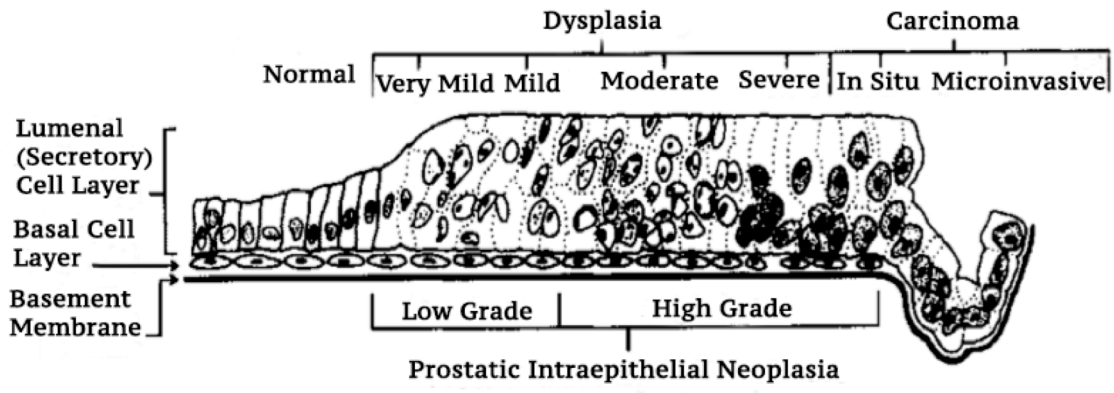


Figure 1.5: Changes in the prostatic epithelium through increasing grades from PIN to early invasive carcinoma, according to the disease-continuum concept. Low grade PIN consists of very mild dysplasia. High grade PIN, corresponds to moderate to severe dysplasia and carcinoma *in situ*. The disruption of the basal cell layer accompanies the architectural and cytological features of high grade PIN and appears to be a requirement for stromal invasion. The basement membrane is retained with high grade PIN and early invasive carcinoma. (Illustration taken from a review by D. Bostwick (73)).

The incidence and extent of PIN appears to increase with patient age and predates the onset of prostate cancer by more than 10 years. It was reported that the onset of PIN in men in their 20s and 30s were 9% and 22% frequency respectively, they also showed that most foci of PIN in young males were low grade, and higher grade were found more commonly with advancing age (67,74). There is also evidence that PIN is influenced by race and geographical location and has increased sensitivity to androgens, characteristics that are strongly shared with prostate cancer (73,75). Several studies reported a higher frequency of PIN in Afro-American men compared to Caucasian men in the same age group (76-78).
Despite the resemblances between HG-PIN and prostatic carcinoma, it is not clear what proportion of HG-PIN progresses to invasive carcinoma, and which remains stable.

1.4 Clinico-pathological staging

The TNM prostate cancer staging is used to assess the extent of the disease including; extent of local tumour 'T', lymph node status 'N', and distant metastasis 'M', which impacts on the appropriate treatment modality for the patient. The TNM staging system is widely accepted for this purpose (79).
Based on the TNM staging system, tumours can be classified into 3 main categories (Figure 1.6):

(1) Organ confined disease (T1 to T2 N0M0)
(2) Locally advanced disease (T3 to T4)
(3) Advanced disease with metastasis (N1, M1)

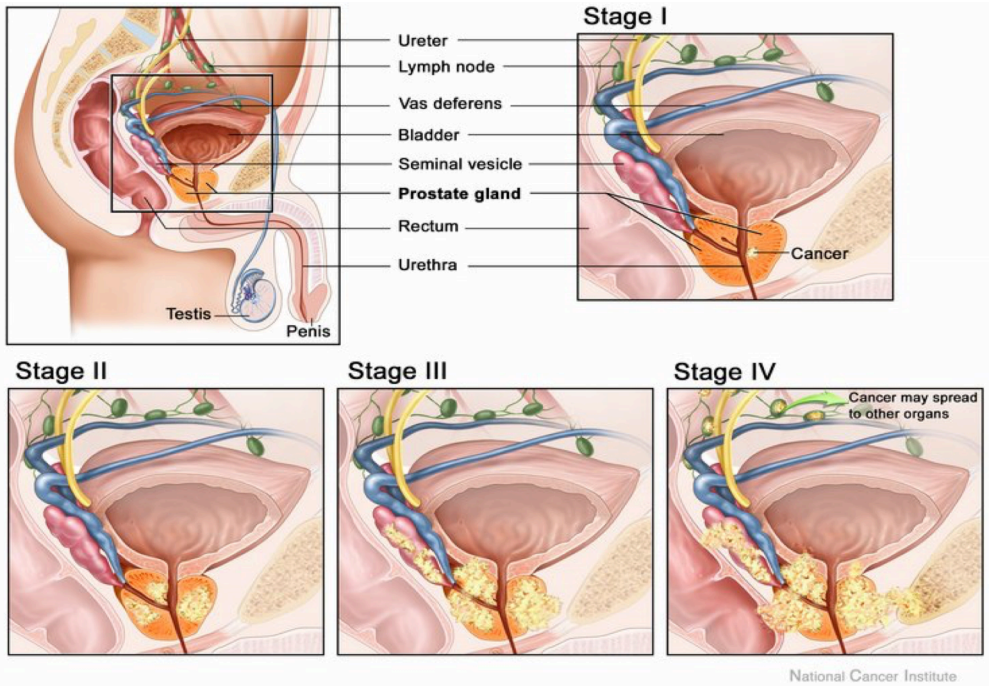


Figure 1.6: The TNM staging of prostate cancer. Illustration taken from The National Cancer Institute (http://www.yourcancertoday.com/Cancers/Prostate-Cancer/78).

1.5 Treatment

Decisions on prostate cancer treatment are based on adequate disease staging histologically and radiologically, taking into consideration the patient suitability for the treatment (age and comorbidities).

1.5.1 Localised and locally advanced prostate cancer

This stage is defined by primary tumour that is confined to the prostate gland and has not breached the prostate capsule and invaded to adjacent organs. This could be determined from the clinical, histological, and radiological staging. The treatments options for this group of patients are dependent on the cancer progression risk as reported by the National Institute for Health and Care Excellence (NICE) (table 1.2).

Table 1.2: NICE Risk stratification for men with localised prostate cancer
This table is illustrated from NICE clinical guidelines
(http://www.nice.org.uk/nicemedia/live/14348/66226/66226.pdf)

Level of risk	PSA		Gleason Score		Clinical Stage
Low risk	<10 ng/ml	And	≤6	And	T1-T2a
Intermediate risk	10-20 ng/ml	Or	7	Or	T2b
High risk	>20 ng/ml	Or	8-10	Or	≥ T2c
High-risk localised prostate cancer is also included in the definition of locally advanced prostate cancer.					

1.5.1.1 low risk group

For men in the low risk group all treatment options are offered including:

1- **Active surveillance** can be offered if subsequent radical treatment is suitable in case of disease progression (the patient is reassessment with serial PSA, DRE, Biopsy and MRI as required until the end of the active surveillance period when the tumour is reclassified).

The European Association of Urology (EAU) recommends that patients should be informed of two randomised trials (80) (81) that compared radical prostatectomy (RP) vs. Watchful waiting (WW) in localised PCa before offering these treatment options. In the SPCG-4 study (80) (confined to men < 65 years of age) the survival benefit was similar before and after 9 years of follow-up. The number of patients that were needed to be treated to avert one death was 15 overall and seven for men < 65 years of age. However, physical symptoms, anxiety, and a depressed mood were lower, and a sense of well-being and self-assessed quality of life were better in the RP group than in the WW group. In the PIVOT-trial (81), a preplanned analysis of a sub-group of men with low-risk tumours showed that RP did not significantly reduce all-cause mortality. This option is more suitable for T1a and b disease. Reports stress that T1c tumours are mostly significant and should not be left untreated because up to 30% of T1c tumours are locally advanced at final histopathological analysis (82,83). The 2011 EAU Guidelines suggest that a PSA doubling time in <3 yr or a biopsy progression indicates the need for active intervention (84).

2- **Radical treatment** with curative intent, in the form of radical surgery (radical prostatectomy) or radiotherapy (external beam and brachytherapy) (85). Stage T2a patients with a 10-year life expectancy should be offered radical prostatectomy (RP) because 35-55% of them will have disease progression within 5 years if not treated (86,87). The outcome in terms of mortality when comparing radical radiotherapy (RT) and prostatectomy in patients with localised disease, has been shown to be similar (88).

1.5.1.2 Intermediate Risk

A policy of watchful waiting (WW) has been proposed for some patients with intermediate-risk localised tumours (89), however, when the tumour is palpable or visible on imaging, and is clinically confined to the prostate, disease progression can be expected in most long-term survivors - the median time to progression of untreated T2 disease has been reported as 6-10 years. Stage T2b disease will progress in >70% of patients within 5 years (90) for this reason radical prostatectomy is the recommended standard treatment for patient with a life expectancy of >10 years (91). These recommendations are based on two large randomised control trials that showed a significant reduction in disease-specific mortality in favour of RP when comparing RP to WW (81,92).

1.5.1.3 High Risk

This group of patients is subclassified to 1) high-risk (T3aN0M0) and 2) very-high-risk (T3b-T4N0 or N1) according to the EAU guidelines 2013.

1) For patients in the high-risk group, the treatment options remain controversial. The management decisions are made after case-by-case discussion by a multidisciplinary team, including urologists; radiation oncologists, medical oncologists and radiologists, and treatment should be offered to patients with regard to their own individual circumstances.

WW is only offered to patients with < 10 years life expectancy.

Surgical treatment is an option for patients with clinical stage T3a however it has been traditionally discouraged, mainly because patients have an increased risk of positive surgical margins and lymph node metastases and/or distant relapse (93,94).

The recommended treatment option for this group of patients is a combination of androgen deprivation treatment and radiotherapy. Several randomised studies of radiotherapy combined with ADT versus radiotherapy alone have shown a clear advantage for

combination treatment, (95).

2) Patients in the very-high-risk group generally have a significant risk of disease progression and cancer-related death if left untreated. The optimal treatment approach therefore often necessitates multiple modalities in the form of RP or Radical Radiotherapy in combination with hormonal treatment, both of these treatment modalities showed comparable results in a recent US study (96).

1.5.2 Metastatic disease

The main treatment modality for patients with distant metastasis is hormone deprivation by means of surgical or medical castration. This treatment option is also suitable for individuals who have a locally advanced (non-organ confined) tumour, patients not fit for radical treatment or those with short life expectancy and patients who opt to avoid medical treatment. Men with locally advanced PCa in whom local therapy is not mandatory, WW is a treatment alternative to hormone depreviation with equivalent oncologic efficacy (84).

The principle of hormone treatment prove the fact that prostate cells depend on androgens such as testosterone and dihydrotestosterone for growth and survival via stimulation of the cytoplasmic androgen receptor (AR). Androgen withdrawal by hormonal manipulation or surgical castration increase apoptosis and abates the tumour (97,98). The former treatment modality relies on the use of luteinizing hormone-releasing hormone (LHRH) analogues to induce a state of androgen depletion by causing a depletion of pituitary gonadotropin releasing hormone (99), while surgical castration is achieved by bilateral orchiectomy (the androgen hormone is mainly produced by the testicles under the influence of LH and FSH). Other modes of anti-androgen treatment include the use of steroid or non-steroid based androgen receptor inhibitors, as well as oestrogen therapy (this will influence the androgen release from the adrenal gland).

Hormone depletion is initially effective but eventually fails with development of castrate resistance tumour, a stage that is inevitable in the natural course of prostate cancer. The median time to castrate resistant stage is 18 months, with lifetime expectancy of 12 months from the point of developing resistance (100) although these figures are improving on the new chemotherapy treatments (101). Various molecular mechanisms are involved to bring about this stage of androgen-withdrawal insensitivity. Some of these mechanisms are briefly explained here:

1) AR gene amplification resulting in high levels of androgen receptor, which allow cancer cells to respond to low levels of androgens, or by allowing enhance ligand binding that enable these cells to use very low level of androgen for growth (102,103).

2) Activation of AR by ligand dependent binding. AR mutations can broaden its specificity and allow its activation by non androgenic steroid molecules as well as antiandrogens (104) (105). Patients who show signs of disease progression despite antiandrogen treatment usually have the antiandrogen medication withdrawn, which can lead to disease regression (106-108). The majority of these mutations are thought to be in the ligand binding domains (98,109,110). AR can also be activated by ligand-independent mechanisms such as by growth factors including insulin-like growth factor, keratinocyte GF, epidermal GF, cytokines, IL6 and deregulation of the signal transduction pathways for example by overexpression of the tyrosine kinase receptor Her-2/neu (111,112). Other factors that may contribute to cancer growth in states of androgen sensitivity or insensitivity are released by bone stromal cells, osteoblasts, osteoclasts and ECM as a result of interaction with metastatic cancer cells (113).

3) Co-activators and co-repressors of AR regulation: the balance between co-activators and co-repressors that are normally used for intermediary signalling between AR and downstream transcription modulation can influence AR activation particularly in androgen independant disease (mechanisms unknown) (114).

4) Bypass Pathway: by which AR activation is completely bypassed, allowing cancer cells to develop the ability to survive independently of androgen stimulation, by decreasing apoptosis through the Ras/Raf/MAP kinase cascade (115-117) and inactivation of tumour-suppressor genes (109). Neuroendocrine cells can survive in a low androgen environment, and their proliferation can stimulate disease progression by the action of secreted neuropeptides, such as serotonin and bombesin, which can increase the proliferation of neighboring cancer cells in a low-androgen environment (118).

1.6 Controversy in the screening and diagnosis of prostate cancer

1.6.1 Screening controversy

Due to the innate heterogeneity of prostate cancer, the decision about whether to pursue early PCa detection is complex, as treatment may not necessarily be to the benefit of all patients. On the other hand, prostate cancer remains the second most common cause of male cancer deaths, catching it early could in theory save lives. Differentiating between patients whose cancer will remain clinically insignificant and those whose disease will progress and kill them is a challenge that we need to overcome in order to reduce over-treatment and offer appropriate treatment for aggressive disease. Screening for prostate cancer to detect aggressive disease is a priority as the ethos of screening is to detect the disease at an early curable stage that can prolong and improve a patient's quality of life. To date, screening for prostate cancer relies mainly on PSA testing and digital rectal examination (DRE), each of which lack sensitivity and specificity. There is therefore a real clinical challenge and an unmet need for improved diagnostic screening and follow up of patients with prostate cancer (119). Current screening investigations and their drawbacks are discussed below.

1.6.1.1 Digital rectal examination (DRE)

The DRE is examiner-dependent, and serial examinations over time are best. A nodule detectable by DRE is usually suspicious for malignancy and warrants evaluation; in addition, findings such as prostate asymmetry, difference in texture, and sponginess are important clues and should be considered in conjunction with the PSA level.

Cysts, stones and benign nodules can resemble the above findings and cannot be accurately differentiated from cancer based on DRE findings alone. Therefore, a high index of suspicion is maintained.

In case of cancer detection, the DRE findings form the basis of clinical staging of the primary tumour (ie, tumour clinical [T] stage). It can also sometimes form the basis of clinical management particularly in advanced stage (local metastasis) where the patient can be spared a prostate biopsy and get treated with hormone manipulation. However in cases where a tumours is present and there is no corresponding increase in PSA, the DRE finding are not relied upon and patients will still need to undergo a biopsy in order to confirm a diagnosis of PCa. In my current practice at the NNUH, most patients diagnosed with prostate cancer have abnormal PSA readings and or abnormal DRE (small impalpable

disease), a small number of patients get diagnosed following histological examination from tissues obtained through transurethral resection of the prostate.

1.6.1.2 Prostate Specific Antigen (PSA)

PSA is a 33-kDa-glycoprotein enzyme with serine protease activity encoded by the *KLK3* gene; it is a member of the tissue kallikrein family of serine proteases that also includes KLK2 and KLK4. Mature PSA is formed as a result of two proteolytic cleavages of two inactive precursor peptides, pre-proenzyme PSA (pre-proPSA) and pro-PSA. It is primarily produced by epithelial cells lining of the acini and ducts of the prostate gland from which it is secreted into the prostatic ducts in high concentrations (120). Its main function is to facilitate sperm motility by liquefying the seminal fluid through the breakdown of semenogelin and fibronectin (122). It was also found to affect the function of the IGF by the breakdown of its binding protein-3 (IGFBP3) (121). In cancer it was found to facilitate metastasis by activating the latent transforming growth factor (TGF-ß) (122,123).
Serum PSA is broken down by the liver with a 2.2- to 3.2-day serum half-life, its serum concentration is normally low, however it can be affected by various conditions other than for prostate cancer such as: benign prostatic hyperplasia, infection or inflammation of the prostate, urine infection, urine retention, instrumentation for example urethral catheterisation or cystoscopy, digital rectal examination, and sexual intercourse (120,124), limiting its specificity and clinical utility particularly as screening test. Due to those limitations prostate cancer screening remain controvertial. Some completely oppose it as it is thought to lead to unnecessary invasive diagnostic tests (TRUS biopsy) that may identify clinically insignificant cancer and unnecessary treatment and treatment-related complications, as well as financial burden to the health care system (125,126); in the other hand some consider it necessary as it is thought to reduce metastatic burden and mortality that reduce the costs to the health care system (127).

Over-diagnosis can be defined in many ways, and includes: the diagnosis of cancer that would not be diagnosed clinically, the diagnosis of a cancer that will not kill a given patient left untreated and in an epidemiologic sense, it is the difference in ‘incidence’ in a screened population and a matched unscreened population. There is clear evidence that screening is closely associated with over-diagnosis and that it increases the incidence/mortality ratio from 2 to approximately 5 in the United States where screening is prevalent (128). To

evaluate the efficacy of PCa screening, two large randomised trials have been published: the Prostate, Lung, Colorectal, and Ovary (PLCO) trial in the United States and the European Randomised Study of Screening for Prostate Cancer (ERSPC) in Europe (129,130). After a follow-up period of 7 years in the PLCO trial, the incidence of PCa per 10,000 man-years was 116 in the group that received regular PSA and DRE, and 95 in the control group (an incidence ratio of 1.22) (129). The incidence of death per 10,000 man-years was 2.0 in the screened group and 1.7 in the control group (rate ratio: 1.13). So it was concluded that PCa-related mortality in screen-detected individuals was very low and not significantly different between the two study groups. On the other hand in the ERSPC trial with a median follow-up of 9 years, the cumulative incidence of PCa was 8.2% in the screened group and 4.8% in the control group (130). The absolute risk difference was 0.71 deaths per 1000 men. This means that 1410 men would need to be screened and 48 additional cases of PCa would need to be treated to prevent 1 death from PCa. The ERSPC investigators concluded that PSA based screening reduced the rate of death from PCa by 20% but was associated with a high risk of overtreatment.

Like other serine proteases, serum PSA exists mostly in a complexed and inactive form; however, a small proportion remains in a free but active form. Free VS Bound PSA: Some studies suggested that determining the Free to Total PSA ratio in serum can improve the PSA specificity by allowing for a clearer distinction between patients with PCa and patients with benign prostate hyperplasia (BPH). For example men with a PSA between 4-10ng/ml and a PSA ratio of 25% have a 10% risk of PCa which increases to 60% if the PSA ratio is >25% (131). However other studies suggest that these calculation can only be useful in men with a prostate volume of less than 40 gram in size (132), and there is no clear guidance or set ratio for clinical use. For this reason free to total PSA ratio is only used in some units to determine whether to rebiopsy patients with persistently raised PSA.

Despite all the efforts put into these trials the question remains as to whether early detection of organ confined prostate cancer with earlier treatment would improve life expectancy.

1.6.2 Diagnosis controversy

Prostate cancer diagnosis relies on: transrectal ultrasound scan (TRUS) guided prostate biopsy, histological and radiological staging, all of which lack sensitivity and specificity that more often than not will expose patients to more invasive procedures and morbidity. Each of these tests is discussed individually below.

1.6.2.1 Trans-rectal ultrasound scan (TRUS) guided prostate biopsy

It is well established that standard sextant prostate biopsies underestimate prostate cancer incidence. Some studies have reported 15 to 23% false negative results, even in patients who have undergone repeat biopsies, this being particularly true for younger patients with lower PSA readings (133,134). For this reason some patients are required to undertake more invasive diagnostic tools such as extended TRUS biopsies or template biopsies of the prostate.

A study undertaken with 2,887 patients, reported that an extended 12 site biopsy scheme may be more appropriate in patients with a normal rectal examination which are <60 years old or have a PSA of <7 ng/ml, since they may harbour smaller tumours that are more susceptible to sampling error (135).

1.6.2.2 Histology

To overcome the complexity of tumour heterogeneity and multifocal pattern of prostate cancer, the Gleason score was introduced. However histological grading relies on predicting cancer grade in the small amount of tissue removed by thin-core needle biopsies (The average 20-mm, 18-G core samples are a 0.04% of the average gland volume (40 ml). This makes biopsies prone to sampling errors that mostly manifest as missing higher or lower grade components resulting in under-grading in 42% of cases and over-grading in 15% of cases. As a result this leads to discrepancies between biopsy and post-prostatectomy histologies that can greatly affect the survival rate and clinical outcomes (57,136). It is also appreciated the tumours may be lumped into the same group whereas they may behave differently biologically. An example being that of a Gleason 7 (4+3) tumour, which is likely to be more aggressive then a Gleason 7 (3+4) tumour (53,57,137,138). (See paragraph 1.3.2 for more explanation about Gleason grading).

1.6.2.3 Magnetic resonance imaging MRI

At present MRI and other imaging modalities are used in the staging of prostate cancer. MRI is particularly useful to assess the extent of local metastasis, but has previously had no established role in cancer detection (139). There are however newly emerging technologies such as proton three-dimensional magnetic resonance spectroscopic imaging and diffusion-weighted MRI that if used in combination increase the specificity for prostate cancer detection (140). These are playing an increasing role in screening for prostate cancer but have a high cost to the health service.

To date prostate cancer screening, diagnosis management and follow up still remain a challenge. For this reason researchers have been looking for the ideal biomarker(s) for several decades, as summarised below.

1.7 Prostate cancer biomarkers

1.7.1 Biomarker definition

A dictionary definition of a biomarker is a measurable indicator of some biological state or condition. Biomarkers are often measured and evaluated to examine normal biological processes or pathogenic processes.

A biomarker is a molecule whose detection provides information about a disease beyond the standard clinical parameters that are gathered by the clinician (http://www.cancer.gov/dictionary). Based on biological properties, biomarkers may also be specific cells, molecules, or genes, gene products, enzymes, or hormones.

To qualify a biomarker for clinical use, several criteria are required including:

1- Safety:

   Sampling access: the marker must be present in peripheral body tissue and/or fluid (e.g., blood, urine, saliva).

2- Sensitivity:

   Must be associated as sensitively as possible with damage of a particular tissue, in a quantifiable manner.

3- Specificity:

It must have a high specificity for the disease.

4- Implementable in the clinic:

It must be easy to detect or quantify in assays that are both affordable and robust.

1.7.2 Biomarker limitations

Although interest in biomarkers is increasing, controversies regarding what constitutes a robust biomarker and how to rigorously investigate biomarkers remain.

Sawyers *et al.,* (141) stated seven common roles for biomarkers to address specific clinical questions when managing patients suspected to have a malignancy this broadly included:

1. Disease disposition: patient's risk of developing cancer.
2. Screening: earlier detection of patients with cancer.
3. Diagnostic: high sensitivity and specificity.
4. Grading and prognosis: disease aggressiveness and mortality risk. Most likely clinical outcome if therapy is not administered.
5. Predictive: Which therapy is most appropriate?
6. Monitoring: Is therapy effective? Does the patient's disease recur?
7. Pharmacogenomics: Do genetics predict response to therapy or the risk for an adverse reaction to the prescribed therapeutic dose?

A single prostate cancer biomarker required to cover all the above-mentioned criteria would be a major challenge. Several studies have suggested that a multiplex panel of biomarkers can outperform PSA or any other single marker (142), hence in the last decade the search has changed from looking for a single markers to a panel of biomarkers that in combination can more accurately assess the clinical needs of the patient.

1.7.3 Prostate cancer molecular biomarkers

Since the adoption of PSA testing in 1980, there have been a number of advances in DNA analysis and RNA transcriptome profiling, via methods such as whole-genome sequencing and microarrays. These new technologies have enabled detailed analyses of cancer biology (143,144) and have led to the discovery of several biomarkers from for example: tissue samples (obtained either by biopsy, surgical resection or tissue cultures) circulating tumour cells, and bodily fluids (blood and urine). These biomarkers include proteins, metabolites, RNA transcripts, DNA mutations and epigenetic modifications of DNA. A few biomarkers

have shown potential clinical utility, but none have actually fulfilled clinical needs. Some of the most popular biomarkers are discussed below.

- **PCA3 (prostate cancer gene 3 or *DD3*)**. The *PCA3* gene consists of 4 exons and is located on chromosome 9 at q21–22 (145,146). It encodes a prostate-specific noncoding mRNA that is known to be overexpressed in over 90% of prostate cancer tissues in comparison to benign prostate (145,146). In 2009 an RNA-based urine test based on transcription-mediated amplification became available as a diagnostic test (147). The FDA approved the *PCA3* test in 2012, to be used only in the clinical setting where a patient has a negative prostate biopsy in the presence of consistently rising PSA. It has superior specificity to PSA but inferior sensitivity (58 and 69% respectively (148)). Unlike PSA, *PCA3* levels are independent of prostate size (149).

- **AMACR (Alpha-Methylacyl-CoA Racemase).** Several studies have reported *AMACR* mRNA and protein to be overexpressed in prostate cancer cells when compared with benign prostate epithelial cells (150,151). This gene encodes a protein that is localised to mitochondria and peroxisomes. It is an isomerase enzyme that plays a key role in peroxisomal β-oxidation of dietary branched-chain fatty acids and C27-bile acid intermediates (152) by catalysing the conversion of (R)-α-methyl-branched-chain fatty acyl-CoA esters to their (S)-stereoisomers. This pathway may have two aspects of relevance for prostate carcinogenesis: (i) the main sources of branched chain fatty acids in humans (milk, beef, and dairy products) have been implicated as dietary risk factors for prostate cancer (153); and (ii) peroxisomal β -oxidation generates hydrogen peroxide (154), a potential source of procarcinogenic oxidative damage (155,156). Analysis of mRNA levels of *AMACR* revealed an average 9-fold up-regulation in clinical prostate cancer specimens compared with normal. Western blot and immunohistochemical analysis have confirmed up-regulation at the protein level and localises the enzyme predominantly to the peroxisomal compartment of prostate cancer cells. On needle biopsy specimens, AMACR has demonstrated high sensitivity (97%) and specificity (100%) as a diagnostic biomarker for prostate cancer (157). Low *AMACR* gene expression has also been correlated with metastasis and biochemical recurrence of prostate cancer (158). However, *AMACR* is not specific to prostate cancer (159) and has been reported as not being suitable for non-invasive detection in urine (142). It has been found to be most useful as a tissue biomarker when prostate biopsy cores yield ambiguous pathological results.

- **GSTP1 (Glutathione S-Transferase Pi 1).** This gene encodes the eukaryotic peptide chain release factor GTP-binding subunit ERF3A, an enzyme that plays an important role in detoxification of xenobiotics by catalysing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. One of the most common alterations found in prostate cancer is the Hypermethylation of the CpG Island at the promoter of *GSTP1* which leads to the loss of *GSTP1* expression (160). These changes are found in approximately 90% of prostate cancer tissue but not normal prostate tissue. It is also found to correlate with disease recurrence independent of Gleason score and pathological stage (161-164). These findings have also been reported in urine DNA, with an ability to identify the presence of prostate cancer with sensitivities ranging from 19 to 76% and specificity from 56 to 100% (164,165). However, using *GSTP1* CpG island hypermethylation as a single marker for molecular screening and diagnosis of prostate cancer is limited due to its low sensitivity, and presence in other cancers (166).

- **TMPRSS2/ERG fusion gene (transmembrane protease serine 2 / v-ets avian erythroblastosis virus E26 oncogene homolog).** *ERG* is member of the ETS (Erythroblast Transformation Specific) family that also contain *ETV1, ETV4* and *ETV5* (167) which can be involved in chromosomal translocations in malignancies in several organs. In prostate cancer the most common translocation is the *TMPRSS2/ERG* gene fusion formed as a result of a chromosomal rearrangement that fuses the androgen sensitive promoter of *TMPRSS2* with 3' coding exons of *ERG,* bringing *ERG* transcription (168) under the control of androgen regulation. The encoded protein is a transcription factor that can regulate multiple genes and cellular pathways leading to PCa (169). A *TMPRSS2/ERG* is found in ~50% of prostate cancer and is specific for this disease (170). This gene fusion, which leads to ERG overexpression, can be an early event in prostate cancer due to its expression in PIN lesions (171) (172). However these findings has been questioned by some authors as they showed that ERG overexpression is less common in (PIN) (173). *ERG* rearrangements are hypothesised to promote carcinogenesis by activating cell differentiation programs and modulating the prostate cancer cell phenotype by a wide range of processes, including: cell growth, proliferation, differentiation and migration. It has been reported to function via the disruption of AR signaling (174-176), activation of the Wnt pathway, induction of epithelial-to-mesenchymal transition (177), by activating TGF-b/BMP signaling

(178), and cooperation with PI3K to drive carcinogenesis (179,180).

Formation of a *TMPRSS2/ERG* alone is not linked to a poorer prognosis *per se* (167), however, there is an associated worse patient survival when i) two or more copies of a *TMPRSS2/ERG* are present, ii) when combined with the loss of the *PTEN* tumour suppressor gene (181-183), iii) when linked with AR overexpression, possibly by promoting the development of a more poorly differentiated invasive cancer cells.

ERG overexpression in normal prostate tissue can induce the expression of genes in the plasminogen pathway which can lead to invasion. *ERG* down-regulation in the metastatic prostate cancer cell line (VCap) inhibited the invasive nature of these cells (184). This inhibition effect is thought to be secondary to a decrease in the expression of the proto-oncogene *c-MYC* which is caused by the down-regulation of *ERG* (169). An alternative hypothesis is the down-regulation of genes involved in cell death pathways and overexpression of genes involved in the WNT pathway and histone deacetylase 1 (HDAC1) (185). *TMPRSS2/ERG* rearrangements appear to differ in incidence between different prostatic zones. Some studies have reported that cancers arising in the para-urethral region (Transition zone) have a lower prevalence of *TMPRSS2/ERG* rearrangements compare to the Peripheral zone (186).

However, as *TMPRSS2/ERG* is absent in about 50% of prostate cancers, its use as a biomarker will only be as part of a multiplexed assay with other biomarkers (187,188). For example in a study of more than 1300 men, combined measurement of *PCA3* and *TMPRSS2/ERG* in urine was shown to outperformed serum PSA alone for prostate cancer diagnosis (191). Beside its diagnostic advantages in prostate cancer *TMPRSS2/ERG* also has prognostic value as shown by studies who demonstrated that *TMPRSS2/ERG* expressing tumours have increased risk of recurrence after radical treatment (189,190).

Those cancers lacking *ERG* alterations may harbour overexpression of *SPINK1* (191) or mutations in the *SPOP* gene (192). Prostate cancer may also be assigned to different prognostic categories based on copy number of *ERG* alteration (193) or by examining a combination of *ERG* and *PTEN* gene status sometimes in combination with other genes (194,195). A combination of *AURKA* and *MYCN* gene amplifications predicted the occurrence of lethal neuroendocrine prostate cancer (196). Analysis of microarray expression profiles and combinations of expression profiles and patterns of gain and loss have also been used to identify diagnostic categories and prognostic biomarkers (197).

- **Matrix Metalloproteinase MMPs or matrixins.** These proteins are a family of zinc

binding, calcium dependent endopeptidases. They belong to the 'Metzincin' superfamily of endopeptidases, which consist of three further multi-gene families, the serralysins, the astacins and the adamalysins (*ADAMs*) (198). MMPs are reported to participate in numerous disease processes including prostate cancer. Matrix metalloproteinases have been implicated in invasion and metastasis of human malignancies by breaching the extracellular matrix and thereby facilitating metastasis.

- **SPINK1 (Serine protease inhibitor Kazal-type 1).** This gene encodes a trypsin inhibitor protein (199) that is secreted from the pancreatic acinar cells. It functions as a serine protease inhibitor, involved in inflammation response and prevention of trypsin-catalysed premature activation of zymogens within the pancreas and the pancreatic duct (200,201). SPINK1 overexpression is reported in association with prostate cancer, particularly with high grade disease giving it a prognostic potential (202,203). SPINK1 over-expression has been associated with approximately 10% of *ETS* rearrangement-negative cancers, and more aggressive disease (191,204,205). Its overexpression was also demonstrated in the aggressive 22RV1 prostate cancer cell line, where its knockdown weakens their invasiveness (191). *SPINK1* also has the potential to serve as a PCa biomarker in that urine sediment multiplexed qPCR assay showed that *SPINK1* outperformed serum PSA or *PCA3* alone as diagnostic markers. SPINK1 expression is also an independent predictor of biochemical recurrence after resection (191,206). *SPINK1* overexpression has been associated with Chromophobe renal cell carcinoma (207).

1.7.4 Circulating tumour cells

Circulating tumour cells (CTCs) originate from primary tumours or metastatic deposits and find their way to the blood by invading blood vessels. In PCa, circulating tumour cells that exhibit features of prostate cancer such as expressing PSA, AMACR or genomic abnormalities such as *AR*-amplification, *PTEN* loss, and *TMPRSS2*/*ETV* fusions, have been isolated from patients with metastatic disease giving them a potential diagnostic use. Some studies have shown that CTCs from whole blood can be also used as prognostic markers in patients with metastatic disease (183,208). They also have the potential to provide information of the molecular structure of an individual patient's tumour, to profile for elements that predict for sensitivity or resistance to therapy (209). So far enumeration of CTCs – as measured by the Cell Search assay – has been approved by the FDA only for use

as an aid to monitor men with metastatic disease in combination with other clinical assessments, however measurement of CTCs to determining patient response and drug efficacy is still under research.

Due to the invasiveness and difficulty acquiring tissue for screening and diagnosis of prostate cancer, researcher looked for less invasive more accessible sources of biomarkers such as urine as discussed below:

1.8 Urine biomarkers

Urine offers a non-invasive source of prostate bio-molecules that have the potential to be used as biomarkers. Due to the anatomic connection between the prostate and the urinary tract (urethra) via the prostatic ducts and the main ejaculatory duct (figure 1.2), prostatic exfoliates including cells, proteins and microvesicles have a direct access to urine flow particularly when stimulated by prostatic massage. This offers the ability to measure gene expression from all foci of cancer within a prostate, and thereby assess the heterogeneity of prostate cancer (142,187,210). Its differentially expressed transcripts in PCa tissue were also found to be differentially expressed in urine samples (211).

DNA, RNA, and protein-based markers harvested from cells, microvesicles or whole urine can thus be considered for the detection of prostate cancer. The challenge however is to find a set of markers that has good performance characteristics and at the same time is easy to detect in urine.

To date, several urine markers has been reported (Table 1.3), some of which have been proposed as potential diagnostic markers for early PCa detection e.g. *GSTP-1* (glutathione-S-transferase P1), *PCA3* (prostate cancer antigen 3, DD3), *TB-15* (thymosin b15) and *TMPRSS2*/*ERG* (212) of which only *PCA3* has progressed to clinical use.

Table 1.3 Urine biomarkers

		Type of marker			
		DNA	**RNA**	**Protein**	**Metabolite**
8-OhdGa	8-Hydroxydeoxyguanosine	+			+
AMACR	a-Methylacyl coenzyme A racemase		+	+	
ANXA3	Annexin A3			+	
BHUAEa	Basic human urinary arginine amidase			+	
BIRC5	Baculoviral IAP repeat-containing 5 (alias survivin)		+	+	
F3	Coagulation factor III (thromboplastin, tissue factor)			+	
FGF1	Fibroblast growth factor 1 (acidic)			+	
FN1	Bladder tumour fibronectin			+	
GOLM1	Golgi membrane protein 1 (alias GOLPH2)		+		
GSTP1	Glutathione S-transferase P 1	+			
LOH[a]	Loss of heterozygosity	+			
MCM5	Minichromosome maintenance complex component 5			+	
MMP9	Matrix metalloproteinases 9			+	
PCA1[a]	Prostate cancer antigen 1			+	
PCA3	Prostate cancer antigen 3		+		
PIP[a]	Prostatic inhibin-like peptide			+	
PSA	Urinary prostate specific antigen			+	
S100A9	S100 calcium binding protein A9 (alias calgranulin B)			+	
SAR[a]	Sarcosine				+
SPINK1	Serine peptidase inhibitor, Kazal type 1		+		
SRD5A2	Steroid 5-alpha-reductase type 2			+	
TERT	Telomerase reverse transcriptase		+		
TF	Urinary transferring			+	
TFF3	Trefoil factor 3		+		
TMSB15A	Thymosin beta 15a			+	
VEGF	Vascular endothelial growth factor			+	

Prostate cancer cells can be detected in urine; however, these can break up shortly after urine sample collection. We therefore aimed in this study to exploit other biological products that could withstand surviving in urine for longer periods and could be used as a source of biomarkers. Exosomes and other microvesicles appeared to have this property.

1.8.1 Exosomes

It is well documented that eukaryotic cells release extracellular vesicles including apoptotic bodies, exosomes, and other microvesicles (213,214). Extracellular vesicles differ in their cellular origins and sizes, for example, apoptotic bodies are released from the cell membrane as the final consequence of cell fragmentation during apoptosis, and they have irregular

shapes with a range of 1–5 mm in size (214,215).

Exosomes are specialised vesicles, 30 to 100nm in size with a cup-shape morphology that are actively secreted by a variety of normal and tumour cells and are present in many biological fluids, including serum and urine. They carry membrane and cytosolic components including protein and RNA into the extracellular space (216-218). These microvesicles form as a result of inward budding of the cellular endosomal membrane resulting in the accumulation of intraluminal vesicles within large multivesicular bodies. Through this process trans-membrane proteins are incorporated into the invaginating membrane while the cytosolic components are engulfed within the intraluminal vesicles that form the exosomes, which will then be released, into the extracellular space (219,220) (Figure 1.7). This process is usually regulated by multiple enzymes including: calpain, flippase, floppase, scramblase and gelsolin (221).

So far urine exosomes have been examined in several studies for renal and prostatic pathology and have been reported to be stable in urine. mRNA isolated from urine exosomes had a better preserved profile than cell-isolated mRNA from the same samples (222,223), which makes them much better for potential biomarker use.

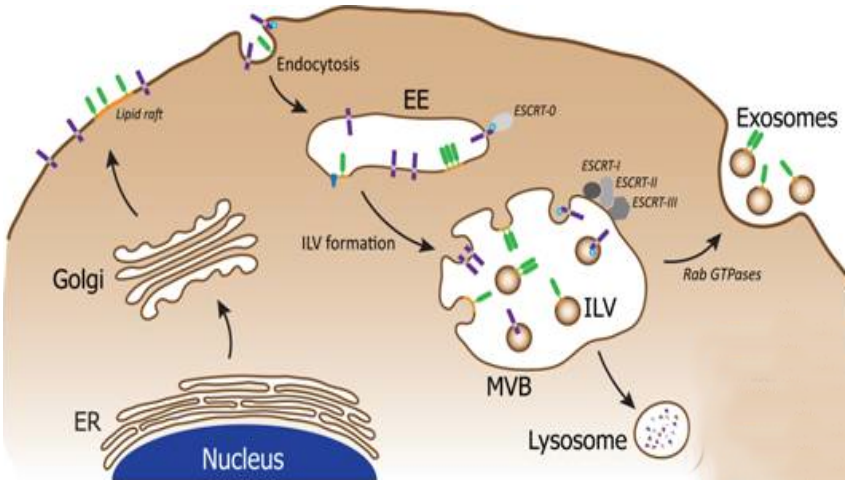


Figure 1.7: A schematic representation showing the intracellular formation and release of microvesicles into extracellular space.
Invagination of the cellular plasma membrane forms the endosome, in turn budding from the limiting membrane into the lumen of endosomes generates intraluminal vesicles (ILVs) in the so called multi-vesicular body (MVB).
Owing to the biophysical properties, MVBs can be exocytic, (ie can fuse with the plasma membrane with subsequent release of their contents as exosomes). This schema was adopted from (http://www.biochemistry.unimelb.edu.au/research/res_hill-areas.html).

1.8.1.1 Exosome Function

Exosomes function as a means of transport for biological material between cells within an organism. As a consequence of their origin, exosomes exhibit the mother-cell's membrane and cytoplasmic components such as proteins, lipids and genomic materials. Some of the proteins they exhibit regulate their docking and membrane fusion, for example the Rab proteins, which are the largest family of small GTPases (224). Annexins and flotillin aid in membrane trafficking and fusion events (225). Exosomes also contain proteins that have been termed exosomal-marker-proteins, for excample Alix, TSG101, HSP70 and the tetraspanins CD63, CD81 and CD9. Exosome protein composition is very dependant on the cell type of origin. So far a total of 13,333 exosomal proteins have been reported in the ExoCarta database, mainly from dendritic, normal and malignant cells (Figure 1.8).

Besides proteins, 2,375 mRNAs and 764 microRNAs have been reported (Exocarta.org) which can be delivering to recipient cells. Exosomes are rich in lipids such as cholesterol, sphingolipids, ceramide and glycerophospolipids (226-228) which play an important role in exosome biogenesis, especially ILV formation (229).

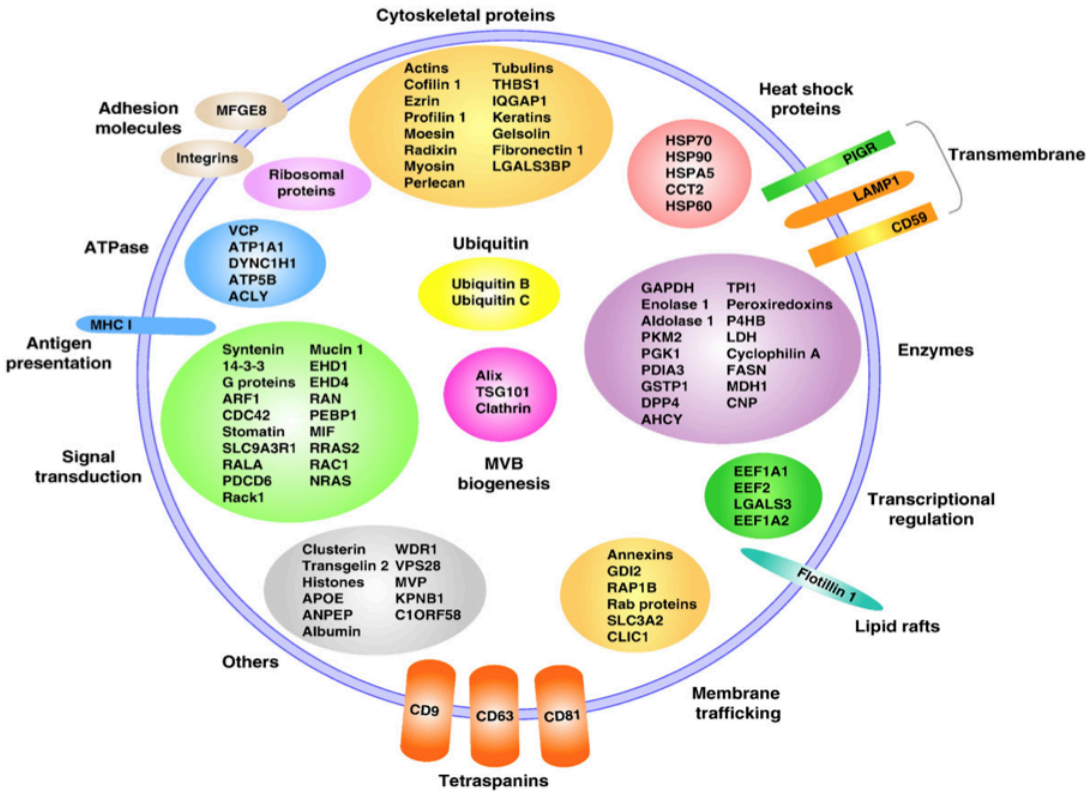


Figure 1.8: A graphical representation of the protein composition of exosomes categorised by function performed. This graph was adopted from a review by Mathivanan et al.,(214).

1.8.1.2 Mechanisms of exosome mediated intracellular communication

A number of mechanisms have been described that are used by exosomes for interaction with target cells.

1) Fusion with a target cell: this leads to the transfer of lipids, proteins and RNAs to the target cell. The proteins and lipids can change the target cell's membrane characteristics eg the transfer of CD41 antigen from platelet-derived SMVs to tumour and endothelial cell membranes (230,231), and transfer of arachidonic acid from platelet derived microvesicles to leucocyte and endothelial cells (232). The transferred RNAs can be translated into functional proteins within the recipient cell (233).

2) Release of their cargo by endocytosis, internalise through distinct endocytic pathways without fusing with the plasma membrane.

3) Binding to a target cell and initiating a signalling response: exosomes are characterised by membrane proteins such as LAMP-2 that are not abundant in the cell of origin (234). Membrane proteins can bind and interact with a target cell in two ways: i) in a juxtacrine fashion and ii) by protease cleaved exosomal membrane proteins that can act as ligands for cell surface receptors in the target cell (214,235,236).

4) Exosomes can release their cargo resulting in the release of signalling molecules and proteins into the extracellular space. For example tumours cells can secrete vesicles that contain metalloproteinases which when released can be responsible for extracellular matrix digestion and increased mobility of tumour cells (237).

1.8.1.3 Exosomes in malignancy

The role of exosomes in cancer remains to be fully elucidated; they appear to function as both pro- and anti-tumour effectors. Either way cancer cell-derived exosomes appear to have distinct biologic roles and molecular profiles. They can have unique gene expression signatures (RNAs, miRNAs) and proteomics profiles compared to exosomes from normal cells (238,239). Large numbers of differentially expressed mRNAs in exosomes from melanocytes compared with melanoma-derived exosomes has also been reported (238). This indicates that exosomal mRNAs may contribute to important biological functions in normal cells, as well as promoting malignancy in tumour cells. This study also found that cancer cell-derived exosomes have a closer relationship to the originating cancer cell than normal cell derived exosomes do to a normal cell, which highlights the potential of using

exosomes as a source of diagnostic biomarkers (238). mRNA expression in melanoma exosomes has been linked to the advancement of the disease (233) supporting the idea that exosomes can promote tumour growth. A similar finding was reported in glioblastoma (240), highlighting their potential as prognostic markers.

Experiments in mice have shown that cancer-derived exosomes can induce an anti-tumour immune response. It has been demonstrated that exosomes isolated from malignant effusions are an effective source of tumour antigens which are used by the host to present to CD8+ cytotoxic T cells, dramatically increasing the antitumour immune response (241).

On the other hand exosomes have also exhibited an involvement in cancer development and metastasis as described below:

1- Contribution to cancer invasion by promoting the proteolytic cascade required for the localised degradation of the extracellular matrix via lytic enzymes. In a melanoma study, exosomes exhibited uPA and MMPs proteins that are associated with inducing cell adhesion, migration and metastatic growth (242). Novel exosomal derived proteins such as sytenin-1 have been shown to enhance melanoma cell migration invasion and metastasis (243,244). Exosomal activity in cancer is not restricted to intracellular activity only. A study on human fibrosarcoma and melanoma cell line exosomes which contained the full-length (60kDa) and the proteolytically processed (43kDa) form of MT1-MMP, reported an efficient extracellular matrix (ECM) degrading enzyme which plays an important role in tissue homeostasis and cell invasion. Furthermore they demonstrated that the exosomal MT1-MMP was functionally active and able to activate pro-MMP-2 and degrade type 1 collagen and gelatin and promote metastasis (237).

2- Induction of angiogenesis, a lifeline for the tumour mass:

Exosomes are rich in pro-angiogenic growth factors, including VEGF, FGF-2, and also proteases (245-249) that play a key role in activating the VEGF/VEGFR pathway in endothelial cells and promote angiogenesis (250).

Annexin A1, a protein that functions as a key regulator of pathological angiogenesis has been found in malignant cell-exosomes (251,252), as has annexin A2, which has multiple roles in regulating cellular function, including angiogenesis, proliferation, apoptosis, cell migration, adhesion and invasion (253).

3- Induction of transformation

A melanoma study concluded that normal melanocytes can acquire invasiveness through up-take of melanoma-derived exosomes (238). In an *in vivo* study on mice, others showed that exosomes facilitated the ability of aggressive melanoma to metastasise to bone marrow by the transfer of Met oncoprotein from tumour-derived exosomes to bone marrow progenitor cells (254). They also showed that reducing Met expression in exosomes diminished this effect. Tumour derived exosomes also induced vascular permeability at the pre-metastatic sites to facilitate metastasis. Exosome production was increased in the melanoma cells, and the Rab family (RAB1A, RAB5B, RAB7 and RAB27A) that regulate membrane trafficking and exosome formation, were highly expressed. Rab27A RNA interference decreased exosome production and resulted in reduction in tumour growth and metastasis (254). The introduction of melanoma exosomes into sentinel lymph nodes (by injecting melanoma exosomes into mice foot pads) was reported to produce a molecular signal that affected melanoma cell recruitment, extracellular matrix deposition, and vascular proliferation in those lymph nodes (255). Melanoma metastasis was correlated to Stabilin 1 and VEGF-B expression that promote and maintain the survival of neovasculature that is necessary for melanoma growth leading to poor prognosis (256). While up-regulation of ephrin receptor beta4 promoted migration and proliferation of melanoma cells (257) (258). These findings demonstrated that melanoma exosomes are capable of directly tuning a remote lymph node toward a microenvironment that facilitates growth and metastasis in lymph nodes even in the local absence of tumour cells. Thus melanomas and perhaps other tumours can take advantage of an efficient exosomal messenger mechanism to prepare a site for eventual metastasis (259). A similar study demonstrated that proteins and exosomes secreted by tumour cells have the potential to modulate their microenvironment and facilitate angiogenesis and metastasis (260). In an *in vivo* study on lung cancer cell lines, it was demonstrated that the most aggressive type of cancer cells (A549) exhibited the strongest response to platelet-derived exosomes that are known to play a role in cancer metastasis. They also demonstrated in this study that when mice were injected with tumour cells (Large Lung cell Carcinoma, LLC) covered with platelet derived microvesicles they had significantly more metastatic foci in their lungs and bone marrow when compared to genetically identical animals injected with LCC cells not covered with platelet derived microvesicles (230).

4- Modulating the immune response and preventing cytotoxic effects on tumour cells. Kim *et al.,* (261) showed in a study that microvesicles carrying Fas ligand, resulted in T-cell apoptosis and consequently prevented the cytotoxic effects on tumour cells. Another study showed that MV-associated CD46, helped cancer cells to escape from complement-induced lysis (262), and that fusion of MVs with monocytes inhibited their differentiation and promoted immunosuppressive cytokine release (263).

5- Drug resistance: A study on prostate cancer cell lines showed that PCa vesicles are involved in drug resistance and that cancer cells (DU145), that are normally sensitive to camptothecin treatment, become resistant to camptothecin-induced apoptosis after being co-cultured with vesicles isolated from the camptothecin-resistant cell line RC1. Conversely, RC1 cells, cocultured with vesicles isolated from DU145, underwent apoptosis when treated with camptothecin, suggesting the role of vesicles in mediating drug resistance and susceptibility (264).

1.8.1.4 Exosomes and prostate cancer

Several studies have examined the role of exosomes in prostate cancer. Some showed in their work that prostate cancer derived vesicles can stimulate fibroblast activation and lead to cancer development by increasing cell motility and preventing cell apoptosis. Similarly vesicles from activated fibroblasts are, in turn, able to induce migration and invasion in the PC3 cell line (265). Others reported that vesicles from hormone refractory PCa cells are able to induce osteoblast differentiation via the Ets1 which they contained, suggesting a role for vesicles in cell-to-cell communication during the osteoblastic metastasis process (266,267). Cell-to-cell communication was also emphasised in another study that showed that vesicles released from the human prostate carcinoma cell line DU145 are able to induce transformation in a non-malignant human prostate epithelial cell line (264).

Besides the *in vivo* evidence on the active role of exosomes in cancer and cancer metastasis, it was also reported that exosomes are present in high levels in the urine of cancer patients (268), and that unlike cells, exosomes have remarkable stability in urine (269). Furthermore presence of exosomes was verified in prostatic secretions in 2006 (270), identifying them as a potential source of prostate cancer biomarkers.

A study using nested PCR-based approach, showed that tumour exosomes are harvestable from urine samples from PCa patients and that they carry biomarkers specific to PCa

including *KLK3*, *PCA3* and *TMPRSS2/ERG* mRNAs. *PCA3* transcripts were detectable in all patients including subjects with low grade disease, however *TMPRSS2/ERG* transcripts were only detectable in high Gleason grades. They also demonstrated in this study that i) mild prostate massage increased the exosomal secretion into the urethra and subsequently into the collected urine fraction ii) that tumour exosomes are distinct from exosomes shed by normal cells, and iii) they are more abundant in cancer patients (210). This study took the first step in developing new methods and identifying novel markers for the diagnosis and prognosis of PCa.

1.9 Gene transcripts tested in this study

This project was performed as a part of a larger international project (Movember GAP Global Action for Prostate cancer) that has been led by Professor Cooper and Dr Jeremy Clark. The 50 genes including the housekeeping genes selected for the first set of gene expression analysis were chosen by 7 different groups that are participating in the Movember project (See table 1.4).

- **Matrix metalloproteinase and serine proteases**

TIMP4, Maspin (SERPINB5), MMP26 and Hepsin are markers that belong to families of matrix metalloproteinase and serine proteases that participate in many aspects of tumour growth and metastasis.

- **MMP26 (Endometase/matrilysin-2/matrix metalloproteinase 26)** is one of the smallest members of the MMP family of zinc-catalysed proteolytic enzymes. Its activity is regulated by specific Tissue Inhibitors of MetalloProteinases including *TIMP1*, *TIMP2* and *TIMP4* with the latter having the greatest inhibitory potency (271,272). It is known to promote the invasion of human prostate cancer cells via cleavage of the basement membrane and multiple components of the ECM such as fibronectin, type IV collagen, fibrinogen, gelatins, vitronectin, as well as non-ECM proteins such as insulin-like growth factor-binding protein- 1 and α-1 protease inhibitor (271,273-275). It also activates the zymogen form of *MMP-9*, an enzyme that plays a critical role in ECM remodeling (276). *MMP-26* mRNA has been shown to be widely expressed in epithelial cell prostate carcinomas (271,275) with

significantly higher levels in PCa cells when compared to prostatitis, benign prostate hyperplasia and normal prostate tissue (276). Several studies suggested that *MMP26* plays a role in early cancer stages prior to development of invasive disease. This theory has been supported by several studies on epithelial tissues that express *MMP26* including breast cancer. The expression of *MMP26* in human breast tissue was shown to be significantly higher during pre-invasive ductal carcinoma *in situ*, when compared to infiltrating ductal carcinoma, atypical intraductal hyperplasia, and normal breast epithelia adjacent to them (272). In a similar study on prostate cancer tissue, using Western blot analysis and immunohistochemistry, it was shown that *MMP-26* is significantly overexpressed in HGPIN when compared to adjacent cancer areas in the same tissues and that it has the lowest expression in non-neoplastic tissues (277), however a comparison between Gleason grades was not made in this study. As HGPIN is considered the pre-invasive precursor form of prostate cancer, they concluded that *MMP26* plays an important role during disease progression. Another study using quantitative real time PCR on human prostate tissue, showed significant overexpression and Gleason correlation of *MMP26* compared to benign tissue, however HGPIN was not included in this study (278).

- **TIMP4 (tissue inhibitor of metalloproteinases 4)** is one of a family of four molecules that are produced and secreted into the extracellular milieu. It is a potent endogenous inhibitor of *MMP-26* (272) and plays an key part in the degradation of the extracellular matrix that is integral in tumourigenesis (198) (279). Their overall structure can be divided into a highly conserved N-terminal domain responsible for its MMP inhibition, and a variable C- terminal domain which may impart distinct properties to the four TIMPs (280). Similar to *MMP26*, *TIMP-4* is thought to play an important role in disease progression. *TIMP-4* was reported to be overexpressed in breast DCIS compare to IDC and normal tissue (272), similar findings was reported in prostate cancer (281). Due to the direct inhibition of the MMPs in a 1:1 fashion, the relative levels of the TIMP and the activated MMP determines the proteolytic potential of tumours in some contexts. Hence much of the cumulative data relating to TIMPs and MMPs in prostate cancer indicate that TIMP expression decreases in cancer, while the ratio of MMPs to TIMPs increases. In other contexts, an increase in TIMPs is associated with tumour progression (279). In Pancreatic adenocarcinoma studies showed that unlike *MMP26*, *TIMP-4* intensity tends to diminish with higher cancer grades with its expression lowest in poorly differentiated tumours, a similar finding was reported in a study on prostate adenocarcinoma (278,282).

- **HPN (Hepsin)** is a trans-membrane serine protease, expressed in human tissues such as liver, kidney and prostate (283,284). It physiological function is not fully understood, however in vitro studies it has been shown to activate clotting factors VII, XII, and IX, pro-urokinase, and pro-hepatocyte growth factor (pro-HGF) (285-287). In prostate cancer studies, *Hepsin* was shown to be consistently unregulated with approximately10-fold increase in cancer tissue when compared to benign control, its up-regulation was also shown to correlate with the disease progression as shown by several studies. At mRNA level several studies reported correlation between *Hepsin* overexpression and prostate cancer grades with the highest expression in higher grade disease (288,289) (278). These finding was confirmed by using a monoclonal antibody against *Hepsin* in various prostate tissues, where weak expression of *Hepsin* in normal prostate tissue, BPH and low-grade disease (G2/3) were reported and high *Hepsin* expression in advanced prostate cancer (G4/5) and bone metastasis (290,291). Low *Hepsin* expression was also reported in hormone-refractory prostate cancer when compared to clinically localised disease (292). The role of *Hepsin* in prostate cancer is not fully understood however studies have shown that it has the ability to promote cancer progression in several mechanisms. Being a proteolytic enzyme it can degrade extracellular matrix protein allowing cancer metastasis, *Hepsin*-overexpressing in transgenic mice was shown to have a role in the disorganisation of the basement membrane and promotion of cancer progression and metastasis (293). Beside its direct proteolytic ability, *Hepsin* can also activate proteases of the plasminogen/plasmin pathway by converting pro-uPA to active uPA which in turn activates matrix-degrading metalloproteinases (294). *In vitro* studies have also shown that *Hepsin* can activate growth factors such as pro-HGF (Pro Hepatocyte Growth Factor) (290,295) which is a potent stimulator for the receptor tyrosine kinase c-Met, which in turn plays an important role in tumour progression (296) (297). In an *in vitro* study, it was shown that in cases of human prostate cancer metastasis to bone, the proteolysis of the bone matrix protein (DQ-collagen I and DQ-collagen IV) was reduced by inhibiting the matrix metallo- serine and cysteine proteases (298). This study also reported that the secretion of cathepsin was increased in DU145 cells (Metastatic hormone sensitive Human prostate cancer cell line) when they were grown *in vitro* on human bone fragments. By using a SCID-human model of prostate bone metastasis, they also reported increased secretion of cathepsin B protein and activity in DU145, PC3, and LNCaP bone tumours.

• **Maspin (mammary serine protease inhibitor)** is a serine protease inhibitor and a member of the serpin superfamily. It has been characterised as a class II tumour suppressor by its ability to promote apoptosis and inhibit cell invasion. However the detailed molecular mechanism of its function as a tumour suppressor is still poorly understood. So far, several *Maspin* targets have been identified including inhibition of serine protease urokinase-type plasminogen activator (uPA) that plays an important role in human prostate cancer metastasis to bone (as described below) (299) (300). As well as targeting the single-chain tissue-type plasminogen activator (sc-tPA) (301), interferon regulatory factor 6 (IRF6) (302), β1-integrin (303) (304) collagen I (305) and glutathione S-transferase (GST) (306) all of which play an important role in cancer growth and metastasis. uPA is a serine protease that is present in several physiological locations, including blood stream and the extracellular matrix. It is also expressed in both osteoblast and osteoclast cells during bone remodeling whether in physiological or pathological state (10–15). One of its functions is to convert plasminogen to plasmin (21) - another serine protease capable of cleaving fibrin in thrombolysis (22, 23), degrading extracellular matrix (ECM) components (24, 25), and activating other zymogen proteases such as pro-MMP-9 (26–28) that can promote tumour growth by osteolysis and angiogenesis leading to cancer growth and bone metastasis. In an *in vivo* study in rats it was demonstrated that uPA promoted prostate cancer metastasis to bone (307), thus by controlling uPA activity, *Maspin* has the ability to affect MMP-dependent proteolysis and bone metastasis. In a mouse model it was shown that *Maspin* has the ability to inhibit mammary tumour cell growth invasion and motility (308) (29–33). This explains *Maspin* up-regulation in premalignant prostate cancer epithelial cells and constant down-regulation at the critical transition from noninvasive, low-grade to highly invasive, high-grade prostate cancer as reported by some studies (309), similar findings was documented in breast cancer (310). A study on lung adenocarcinoma, reported that *Maspin* expression was associated with a better-differentiated phenotype and better prognosis (311), on the other hand several studies reported its down-regulation on progression to tumour invasion and metastasis (306,312).

• **GOLM1 (Golgi membrane protein 1)** is a resident cis-Golgi membrane protein of unknown function. *GOLM1* dysregulation has been reported in 20 common types of cancer including kidney, bladder, prostate (313) and hepatocellular cancer where its upregulation were initially detected (314). The epithelial origin of *GOLM1* in prostate cancer was also documented (315) as well as its up-regulation in PCa at the mRNA level (316) (317).

GOLPH2 (aka *GOLM1*) expression was reported to be significantly higher in prostate cancer tissue compared to benign. Its upregulation was detected in (84%) of *AMACR*-negative prostate cancer cases (318). Multiplexing *GOLM1* mRNA with other biomarkers including *SPINK1, PCA3* and *TMPRSS2/ERG* was shown to be a significant predictor of PCa (206), furthermore a secretory form of GOLM1 protein was identified in culture supernatants of a prostate cancer cell line. This secretion was inhibited by brefeldin A which is a protein transport inhibitor (319) (320). A full length version was detectable in the urine of PCa patients (315), the origin of which was thought to be either due to secretion or alternatively released from within exosomes (321).

- **HOXC4 and HOXC6 (Homeobox C4 and Homeobox C6)** *HOX* or Homeotic genes are developmental genes that play a critical role in embryogenesis by coding functional regulatory proteins (322). In the human genome there are a total of 39 *HOX* genes located on 4 different chromosomes (323) each of which contains a homeodomain (324). *HOX* genes have been known to play important roles in the development of cancers, including poor cell-differentiation, a main feature of malignant cells. Embryogenesis studies demonstrated that the lack of *HOX* expression can lead to undifferentiated cells (325), on the other hand *HOXC8* over-expression has been shown to be associated with failure of cell differentiation in prostate cancer, suggesting that it is involved in the acquisition of the invasive and metastatic character of this malignancy (326). Similarly another study reported over-expression of *HOXC8, HOXC4* and *HOXC6* in malignant cell lines of PCa and lymph node metastases using RT-PCR (327). They also demonstrated that over-expression of *HOXC8* in LNCaP PCa cells supressed transactivation via the androgen receptor suggesting that *HOX* gene dysregulation plays a role in androgen independence by requiring adaptation to low androgen signalling (327). In whole genome profiling comparing 28 PCa samples and 12 normal prostates, a study demonstrated upregulation of *HOXC6* along with 55 other genes in the tumour samples (328). They also showed that silencing *HOXC6* expression (using small-interfering RNA (*HOXC6* siRNA)) in both androgen-dependant LNCaP cells and C4-2 androgen-independent cell lines lead to decreased cell proliferation rates by inducing apoptosis. It was also demonstrated that over-expression of *HOXC6* prevented LNCaP cells from *HOXC6* siRNA-induced apoptosis possibly by promoting cell survival by modulating AR-stimulated gene expression, repressing expression of filamin A (FLNA) and preventing apoptosis by targeting tumour suppressor p53 regulating targets such as (IGFBP-3 and PA26) (329) (330). Besides *HOXC6* and *HOXC4* other *HOX* genes play a significant

role in cancer by promoting tumour vascularisation, metastasis and cell proliferation (331).

- **KLK2 (Human Kallikrein 2)** is a member of the kallikrein gene family and is located on chromosome 19 at q13-4 (332). It is a serine protease with trypsin-like activity, and is mainly expressed in the prostate gland (333). Its expression is regulated by androgens and androgen receptor (AR) signaling (334) and is often co-expressed with *KLK3* (PSA) within the same tissue. One of its functions is to cleave PSA into its enzymatically active mature form (335). It also plays important roles in prostate cancer initiation and metastasis (336). Via its protease activity, and by activating members of the matrix metalloprotease family and uPA (uPA function is discussed earlier in this chapter) it promotes extracellular matrix degradation and metastasis (337). *KLK2* has also been found to enhance AR transactivation (ARA70) that may result in alteration of PCa formation and promotion of prostate cancer cell growth (338). In castrate resistant prostate cancer specimens, *KLK2* over-expression was found to correlate with high cell proliferation rate and a lower cell apoptotic index, while knock down has the opposite effect (338). Serum levels of *KLK2* have been shown to differentiate organ-confined from non-organ-confined prostate cancer, different disease grades, and also, benign from malignant disease when the PSA levels are low (339-341).

- **KLK4 (Human Kallikrein4)** is a member of the human KLK family that is androgen regulated (342,343). Unlike other kallikreins that encode an extracellular functional protein, *KLK4* is primarily localised to the cell nucleus and cytoplasm (343-345). It is highly expressed in prostate epithelial basal cells. Several studies have shown that the expression of *KLK4* is significantly higher in prostate cancer tissue when compared to benign and that it has a proliferative effect on cancer cells possibly through cell cycle regulation (346). Ectopic expression of *KLK4* was also reported to dramatically increases cell proliferation and motility in PCa cell lines, and that its over-expression has significant effects on cell cycle–related gene expression (346). It was also demonstrated that knockdown of endogenous *KLK4* in LNCaP cells by small interfering RNA has significant effects on inhibiting cellular proliferation. Similarly a study on the PC-3 prostate cancer cell line reported that *KLK4* transfection induced cellular migration and invasion through repression of E-cadherin and increased expression of vimentin in these cells (345).

- **DLX1 (Distal-less homeobox 1)** encodes a member of a homeobox transcription

factor gene family (347). It encodes a nuclear protein (348) that regulates transcriptional signals from multiple TGF-{beta} superfamily members. *DLX1* was initially found to be expressed in the proximal and distal component of the first pharyngeal where it controls craniofacial patterning, and the differentiation and survival of inhibitory neurons in the forebrain (347). The androgen regulated prostate-expressed *DLX1* was reported to be up regulated in prostate cancer by several authors, however its role in cancer is not fully understood. In a study on human prostate specimens obtained by radical prostatectomies *DLX1* was reported to be the most significantly over-expressed of 26 genes including the *PCA3* gene in transition zone disease when compared to benign tissue (349). In 'Ingenuity' pathway analysis they showed that *DLX1* significantly represented 2 biological functions: cellular movement in benign tissue and epithelial carcinoma respectively. Similarly other studies documented that *DLX1* and *DLX2* expression resulting in altered regulation of genes in prostate cancer cells and epithelial-neuronal cell conversion (348) (350).

- **TDRD1 (Tudor domain containing 1)** belongs to a family of Tudor domain containing proteins. It was initially identified as a testicular cancer-related gene (351), Physiologically, its main role is in spermatogenesis where it represses transposable elements and prevents their mobilisation, a process that appears to be essential for germline integrity (352) as its knockout in mice is associated with defective spermatogenesis (353). *TDRD1* is not transcribed in normal prostate epithelium (351), however it is known to be over-expressed in prostate cancer (354,355). Several studies indicated that the over-expression of *ERG* alone is not sufficient for the development of prostate cancer (173,356,357) however its coexpression with *TDRD1* can promote PCa, a study using RNA expression array analysis and qRT-PCR, found that *TDRD1* was strongly and strictly coexpressed with *ERG* in primary prostate cancer (358). Another study using a transcriptome-wide analysis of 28 primary prostate cancers found that *TDRD1* is highly coexpressed with *ERG* (359,360), In this cohort, 14 tumours over-expressed *ERG* with *TDRD1* ranking highest of the genes that were coexpressed with it. Two other studies using expression arrays, also found that *TDRD1* ranked highest among differentially expressed genes with *ERG*-positive prostate cancer (178,361) they also showed that (*TDRD1*) is the most differentially expressed gene between *ERG* rearrangement-positive and -negative prostate cancer with it being significantly over-expressed in *ERG*-positive compared with *ERG*-negative and normal prostate tissue samples (178) (359). Others reported that *TDRD1* over-expression in *ERG*-positive prostate cancer is secondary to DNA methylation of the *TDRD1* promoter by *ERG*, leading to *TDRD1* over-

expression (353).

- **CAMKK2 (Calcium/Calmodulin-Dependent Protein Kinase Kinase 2, Beta)** This protein phosphorylates the downstream kinases CaMK1 and CaMK4, components of the calcium/calmodulin-dependent (CaM) kinase cascade. It also phosphorylates AMP-activated protein kinase (AMPK). In prostate cancer the expression of this gene is reported to be up-regulated by several authors (316,362) (363), some of whom reported an increase in its expression in the transition from PIN to PCa (364). In a mouse model (transgenic adenocarcinoma of mouse prostate), it was reported that increased CaMKK2 expression was associated with cancer progression, with it being higher in castration resistant xenografts and markedly higher in the AR-expressing PCa cell line LNCaP (365). In LNCaP *CaMKK2* mRNA and protein appear to be induced by androgen hormone (dihydrotestosterone), its withdrawal suppressed CaMKK2 expression (365). These findings are in concordance with earlier studies which showed an increase in *CaMKK2* mRNA expression with exposure to synthetic androgen R1881 (363,366). Other study also showed that the knockdown of CaMKK2 expression in LNCaP cells arrested the cell cycle at its G1 phase reducing cell proliferation (365). They also reported that AR induced CaMKK2 expression in turn feeds back to positively regulate the transcriptional activity of the AR forming a regulatory feedback loop that is important in prostate cancer progression. In contrast, the expression of CAMKK2 has been reported to diminished in high grade PCa and that its down-regulation is associated with poor prognosis. They also concluded from this study that androgen deprivation therapy may cause down-regulation of CAMKK2 that in turn could lead to AR hypersensitivity to androgen and disease progression (367).

- **IMPDH2 (Inosine Monophosphate Dehydrogenase 2)** This gene encodes a rate-limiting enzyme that plays a key role in the *de novo* synthesis of purine nucleotides and is thus involved in maintaining cellular guanine deoxy- and ribonucleotide pools needed for DNA and RNA synthesis. This is particularly true in lymphocytes which have a dependent biosynthesis pathway, making *IMPDH* a target for immunosuppressive therapy. Two isoforms of *IMPDH* exist in humans, type I and type II , both encoding proteins of 514 amino acids (368). *IMPDH II* up-regulation stimulates *IMPDH* activity, which in turn is up-regulated in cancers and is associated with rapidly proliferating tumour cells (369) (370). In contrast, IMPDH inhibition leads to a decrease in DNA and RNA synthesis by depleting

guanine nucleotides in cells which in turn leads to cell cycle block and cell death (371). In a study on gene expression microarrays for prostate cancer biomarkers it was shown that IMPDH II is significantly over-expressed in prostate cancer in comparison to benign tissue. They also showed that serum levels of IMPDH II were also significantly raised in patients with prostate cancer and were associated with clinicopathological features (372). In contrast, inhibition of IMPDH induces cell growth arrest and cell death in the androgen-independent prostate cancer cell line PC-3 (373).

- **Androgen receptor (AR) splicing (AR exon 3 to 9).** AR-regulated genes play a role in the state of hormone deprivation. The androgen receptor (AR) is a nuclear hormone receptor that regulates target gene expression. The androgen receptor is known to play essential roles in prostate cancer from cell viability, to proliferation and invasion in both castrate resistant and hormone sensitive prostate cancer cells (374,375). This signaling pathway is the key molecular determinant in castrate resistance PCa (376) and makes it a potential target for treatment with second generation AR antagonists as shown by several authors (377,378). In androgen sensitive prostate cancer, it is known that AR regulates genes such as *KLK3* and *TMPRSS2*/*ETS* fusions that in turn regulate cell cycle progression through G1/S cell-cycle regulation (379,380). In androgen-independent castrate resistant cells the regulatory effect of AR is believed to continue through selective regulation of expression of FoxA1, CDC20 and CDKN3 that up-regulate the M-phase cell cycle. Hormone treatments are aimed at androgen receptor silencing, however progression to castration resistant PCa gradually takes place via mechanisms believed to reactivate the androgen receptor axis (381), mitosis stimulation via growth factor pathways, stress induced pro-survival gene and cytoprotective chaperone (382). AR has a complex protein signature due to expression of splice variants that are still not fully understood or targeted by any of the existing therapies (383). So far, 13 variants of AR have been documented, with AR3 being the most extensively characterised. AR variants have been shown to be dramatically elevated in castrate resistant prostate cancer as well as in specimens of hormone naïve patients who progressed after curative surgery, suggesting that AR3 is associated with prostate cancer progression (384) (385) (386) as demonstrated by study on 9 *AR* splicing variants in CRPC that found that most of these *AR* splicing variants were dramatically elevated (386).

- **STEAP2/STAMP1 (six transmembrane epithelial antigen of the prostate family member 2)** is a *STEAP* family member that encodes a multi-pass membrane protein. It has

been shown to be localised to the Golgi complex, the plasma membrane, and vesicular tubular structures in the cytosol by immunofluorescence microscopy. It may have a role in endocytic and secretory trafficking pathways (387) acting as ferroreductase and cupric reductase, stimulating cellular uptake of both iron and copper (388). It is expressed in several tissues including placenta, heart and prostate. *STEAP2* over-expression was also reported in prostate cancer cells compared to normal epithelial cells that had been micro-dissected from the same prostate gland. It was also reported that *STEAP2* is a highly androgen-regulated gene in androgen receptor-positive cells, however this did not prove true in receptor-negative cells (387) (389). In addition, ectopic expression of *STAMP2* in a prostate cancer cell line appeared to increase cell proliferation and cancer progression. Furthermore, it was demonstrated that *STEAP2* contains a domain associated with apoptosis and cancer (anti-apoptotic), suggesting its involvement in cell cycle regulation (390). In an *in vitro* and growth of human tumour xenografts *in vivo* study it was reported that monoclonal antibodies to *STEAP1* inhibit intercellular communication (391).

- **STEAP4/STAMP2 (six transmembrane epithelial antigen of the prostate 4)** is a member of the *STEAP* family that functions as a metalloreductase exhibiting a strong iron reductase activity (392), its loss has been reported to lead to metabolic syndrome (393). In mice it functions to control inflammatory response, adipocyte development and metabolism. Similar to *STEAP2*, *STEAP4* expression is reported in various normal tissues including placenta, lung, heart, prostate and adipose (389) (394), and LNCaP prostate cancer cells (389). One study (395), reported that *STEAP4* inhibits anchorage independent cell growth through regulation of phospho-Y397 on focal adhesion kinase (FAK). Furthermore in androgen-independent prostate cancer cells (DU145) they reported that CpG sequences in the *STEAP4* promoter region were frequently methylated, and that demethylation treatment induced the expression of *STEAP4* in this cell line; this was in contrast with the androgen-dependent prostate cancer cell line LNCaP in which no methylation was reported.

- **MDK: Midkine (Neurite Growth-Promoting Factor 2)** This gene encodes a retinoic acid-induced, heparin-binding growth factor that is highly expressed during embryogenesis, is involved in neurogenesis and epithelial to mesenchymal transition. The protein promotes cell growth, survival, migration, and angiogenesis, particularly in neoplasia where its expression has been correlated with poor clinical outcome (396,397) (398) (399). *MDK* has been reported to be over-expressed in prostate cancer by several

authors, its expression shown to be associated with cell survival and proliferation (399,400). The mechanism of *MDK* action is not fully understood, however it is thought to be the product of neuroendocrine-like tumour cells that are believed to arise through a neuroendocrine-differentiation (NED) process from malignant luminal epithelial cells or possibly from PCa stem cell differentiation (401). These cells play an important role in AR signaling reactivation through neuropeptide secretion in the absence of androgens in castration resistant prostate cancer (402,403). *MDK* was also reported to be up-regulated in CRPC and that its up-regulation is associated with neuroendocrine differentiation (404). In a study on PC-3 and LNCaP cell lines, midkine expression was reported to be increasingly raised in end-stage prostate cancer and was thought to be induced by several factors including cytokines particularly interleukin-1beta and TNFα that strongly induced midkine expression via the nuclear factor-kappa B pathway (399). Midkines were also induced by growth factors including epidermal growth factor, androgen, insulin-like growth factor-I, and hepatocyte growth factor. The carcinogenic effect of midkines appeared to be through the activation of signal-regulated kinase 1/2 and the mitogen-activated protein kinase pathways' p38 and by partially inhibiting TNFα-induced apoptosis. *Midkine* mRNA was detectable in a urine test study that demonstrated its over-expression and therefore usefulness as a diagnostic and prognostic urine marker for urothelial transitional cell carcinoma (405).

- **TERT (Telomerase Reverse Transcriptase)** The gene encode a protein enzyme with reverse transcriptase activity, and an RNA, which serves as a template for the telomere repeat. It is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. Its activity is high in the foetus, however it is repressed post-natally and is very low in normal adult somatic cells. Deregulation of telomerase expression is known to be associated with prostate cancer, where the reactivation of the telomerase and telomerase over-expression is thought to prevent post-mitotic cell apoptosis (406). Its expression was positively correlated with aggressive disease (146). Telomerase expression in urine sediments of patients undergoing radical prostatectomy as a treatment for PCa was reported positive in approximately 74% of patients and was thought to be predictive of biochemical recurrence in these patients. There may also be a possible link between increased ERG expression and *TERT* reactivation, as *TERT* positive cases also had elevated levels of ERG which was associated with higher recurrence rate (205).

- **FOXM1: (Forkhead box protein M1)** plays a key role in cell cycle mitotic division at the S and G2/M phases (407), and regulates the expression of a large array of G2/M-specific genes, such as *Plk1*, *Nek2*, *Cyclin B2* and *CENPF*. It also plays an important role in maintenance of chromosomal segregation and genomic stability (408). It's main role in cancer remains unknown; however, it is thought to promote oncogenesis through an abnormal impact on its roles in cell cycle and chromosomal/genomic maintenance. In a transgenic mouse studies, that used two mouse lines in which they had put the Rosa26 promoter to drive expression of the human FoxM1b cDNA transgene: i) a TRAMP mouse PCa that recapitulates multiple stages of human prostate cancer (409) and ii) LADY TG mice that develop multifocal low grade PIN that progresses to high-grade PIN and early invasive prostate carcinomas with progressive neuroendocrine differentiation (410) prostate cancer. Another study showed that the increased expression of FoxM1b accelerated development, proliferation, and growth of prostatic tumours in both mice lines. Furthermore by using prostate cancer cell lines (PC-3, LNCaP, or DU-145) on soft agar they demonstrated that when *FoxM1* levels was depleted by small interfering RNA transfection a significant reduction in proliferation and growth was observed. They concluded from their study that FoxM1 regulates development and proliferation of prostate tumours, and that FoxM1 could be a novel target for prostate cancer treatment (411). Cell cycle progression genes were originally identified as having RNA expression that fluctuated as cells progressed through the different stages of the cell cycle (412). For this reason it has been proposed that these genes may be useful for prediction of outcome in prostate cancer. In breast cancer, genes that have their expression regulated as a function of cell cycle progression (CCP) have proven to have prognostic value and have changed clinical care. (413-416). These findings led some authors to study the expression of 31 CCP genes including *FoxM1*, *CDC20* and *CDKN3* in prostate cancer RNA extracts, using qRT-PCR. A comparison of disease progression was made between 2 groups of patients (post radical prostatectomy, and localised T1a/b disease on TURP) in which they reported that the expression of CCP genes was higher in actively growing cells. Their cell cycle score was predictive of outcome in both cohorts and provided substantially more prognostic information than did clinical variables alone. They also reported that heterogeneity in the hazard ratio for the CCP score was not noted in any case for any clinical variables (417).

- **CDC20 (Cell Division Cycle 20 Gene)** This gene encodes a protein that plays an essential role in the regulation of cell division by activating the anaphase promoting

complex that initiates chromatid separation and entrance into anaphase; it also plays a role in the S and M phases. In prostate cancer, CDC20 is regulated by the androgen receptor, it prevents cell apoptosis and increases cell proliferation with its highest expression being in metastatic disease (418). CDC20 is inhibited by RASSF1a, tumour suppressor that normally prevents mitotic progression in PCa However methylation of RASSF1a in approximately 75% of PCa leads to its inactivation and disease progression (419).

- **CDKN3 (Cyclin-Dependent Kinase Inhibitor 3)** This gene encodes a protein that is known to prevent the activation of CDK2 kinase. Its mutation and/or over-expression had been reported in several cancers including PCa where it is AR regulated. CDKN3 is one of a large subset of AR target genes associated with control of cell division in castrate resistant prostate cancer cells as reported by several authors (420). It was also identified as one of a subset of cell cycle progression genes that can provide a prognostic score for the risk of disease recurrence after radical prostatectomy and the risk of death in conservatively managed prostate cancer diagnosed by TURP (417).

- **MKi67 (Marker Of Proliferation Ki-67)** This gene encodes a nuclear coding protein that is necessary for cellular proliferation. In immunohistochemical analysis on formalin fixed tissue taken from prostate needle biopsies of 111 patients it was shown that Ki67 labelling index (LI) strongly correlated to Gleason grade and an increase in proliferative activity from low-grade to high-grade disease (421). They also suggested a correlation between Ki67 expression and tumour volume and related death. In another prospective study on 279 needle biopsies of the prostate it was shown that increased Ki67LI strongly correlated with cancer invasion to the seminal vesicles and that it is an independent prognostic factor in biopsies with low grade and low volume prostate cancer. They also showed in this study that a combination of Gleason score, number of positive cores, percentage of tumour in each biopsy and Ki67LI can predict risk of recurrence following radical prostatectomy (422). Other studies also highlighted the prognostic use of Ki67 in preoperative core needle biopsies of PCa (423,424).

- **AURKA (Aurora Kinase A)** This gene encodes a serine/threonine kinase that is involved in cell cycle progression by regulating a number of the processes that are crucial to mitosis, including centrosome maturation, chromosome separation, and regulation of the microtubule network that forms mitotic spindles. *AURKA* maps to human chromosome 20 at

q13, a locus that is frequently altered (amplified or over-expressed) in human cancers including breast and colon, where it is associated with tumour development and progression (425). In prostate cancer, *AURKA* amplification has been identified in 65% of PCas (hormone naïve and treated) from patients with poorly differentiated neuroendocrine carcinoma (NEPC, also known as small cell carcinoma of the prostate) and in 86% of metastases. Neuroendocrine prostate cancer (NEPC), also referred to as anaplastic prostate cancer, is a lethal tumour that is known to have aggressive clinical features. It is AR-negative and has AR independent progression. They are known to secrete neuroendocrine proteins such as chromogranin A and express high levels of transcription factors that are characteristic of neural processor cells (426,427). They have a predilection to metastasise to visceral organs, lytic bone disease, a poor response to androgen ablation, and only briefly respond to chemotherapy (428). A meta-analysis of gene expression, reported *AURKA* as one of the top 10 genes that are likely to drive prostate cancer development (429).

- **CLU (Clusterin identified as a therapeutic target)** *CLU* is a nine-exon gene that is located on chromosome 8 at p21-p12 (430). This gene encodes a secreted protein that functions as an extracellular stress-induced cyto-protective chaperone that protects cells against apoptosis and cytolysis. It also plays a role in cellular signalling and transcriptional regulatory networks that stabilise the cell phenotype at times of stress. This includes therapeutic stressors such as treatment induced apoptosis caused by androgen or oestrogen withdrawal, radiation, cytotoxic chemotherapy, and biologic agents leading to treatment resistance (431,432). *CLU* over-expression in castrate resistance prostate cancer makes it an attractive target for cancer therapy: hence attempts to knockdown *CLU* using an antisense oligonucleotide and siRNA undertaken in a randomised study, which showed increased patient survival when they were treated with Docetaxel-Custirsen compared to Docetaxel alone (382). *CLU* was also identified in several urine studies as a prognostic marker for prostate cancer at both cellular (433) and exosomal levels (434).

- **BRAF (V-Raf Murine Sarcoma Viral Oncogene Homolog B)** This gene encodes a serine/threonine kinase protein that regulates the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signalling pathways, which in turn effect cell division, differentiation, and secretion. *BRAF* mutations have been associated with various cancers, including PCa (435). A study on a large cohort of patients with PCa showed that RAF pathway rearrangements tend to occur in advanced disease and that the expression of

SLC45A3-BRAF or *ESRP1-RAF1* induce a neoplastic phenotype that is sensitive to RAF activated protein kinase inhibitors (436). A study done on a genetically engineered mice model reported that BRAF oncoprotein induced activation of the ERK1/2 and MAPK signalling pathways and disruption of PTEN that in turn lead to PI3K-Akt-mTOR signalling pathway activation causing up-regulation of c-Myc that lead to castration resistant metastatic prostate cancer. They also showed in this study that targeting these pathways with therapeutic treatment such as Rapamycin and PD0325901 weakened c-Myc levels and reduced metastasis (437).

- **OGT (O-linked β-N-acetylglucosamine transferase)** This gene encodes an enzyme that adds the O-GlcNAc moiety to the free hydroxyl of select serine and threonine residues (438). In cancer, OGT over-expression is the result of altered metabolism that renders ATP production independent of oxygenation (439). Cancer cells generate ATP by glycolysis hence their increased utilisation rates for glucose and glutamine to compensate for the lack of oxygen (Warburg effect) (440). This phenomenon is also thought to alter other signaling pathways including the mTOR (mammalian target of rapamycin), AMP-activated protein kinase, and the hexosamine biosynthetic pathway (HBP) (441). OGT is also thought to regulate a number of cancer associated proteins such as p53, c-Myc and Snail (442-444). In breast cancer, metabolic alteration has been shown to increase OGT expression that has a profound effect on cancer phenotype, growth and invasion that may be secondary to FoxM1 up-regulation. Reducing OGT levels blocked breast cancer growth *in vivo* and *in vitro* (16). Similar finding was reported in prostate cancer where OGT was also found to be over-expressed in cancer tissue compared with normal epithelium, and linked to a poor clinical outcome. Another study showed that OGT inhibition in a prostate cancer cell line (PC3-ML) lead to reduction of the aggressive phenotype, matrix metalloproteinase (MMP)-2 and MMP-9 expression, as well as inhibition of cell growth and bone metastasis. Findings were also associated with decreased FoxM1 levels (445).

- **Chronic inflammation and prostate cancer** Several authors have reported a link between chronic inflammation and prostate cancer (446) thought to be linked to several oncogenic mechanisms including DNA damage (due to increased reactive oxygen and nitrogen species) and down-regulation of anti-tumour activity (447-449). One cause of chronic inflammation is thought to be epidemiological; link has ben reported between PCa and diets rich in fats and meat, and low in fruits and vegetables to PCa incidence, which has

been hypothesised to be as result of increased eicosanoid and prostaglandin production in response to high fatty acid intake (446). We discuss below several genes that may be involved.

- **PECI (Peroxisomal 3,2-trans-enoyl-CoA isomerase)** PECI is an auxiliary enzyme that catalyses an isomerisation step required for the beta-oxidation of unsaturated fatty acid. PECI is downstream from AMACR in the peroxisomal-branched chain fatty acid β-oxidation, and is also up-regulated in prostate cancer (450,451).

- **SULT1A1 (Sulfotransferase Family, Cytosolic, 1A, Phenol-Preferring member 1)** This gene encodes a cytosolic enzyme that catalyses the sulphate conjugation of hormones, such as catecholamine, phenolic drugs, and neurotransmitters. SULT1A1 can also bioactivate dietary and environmental pro-carcinogens and promutagens such as the N-hydroxy metabolite of the food-borne heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo (4, 5-b) pyridine that may lead to chronic inflammation and carcinogenesis (452,453). It can also activate the carcinogenic N-hydroxyarylamines to DNA-binding products and can modulate cancer risk. In humans, it was shown that there is a strong association between increased *SULT1A1* activity and prostate cancer risk irrespective of race, and a link to high consumption of over-cooked meat (454).

OR52A2/PSGR (Prostate-specific G-protein coupled receptor) *PSGR* is a human prostate tissue-specific gene and a member of the G-protein coupled odorant receptor family that maps to chromosome 11 at p15. It has a high prostrate tissue-specific expression where it may play an important role in early prostate cancer development and progression. It is significantly over-expressed in PCa in comparison to benign tissue (455). In a quantitative real-time PCR experiment on 220 RNA specimens, PSGR was reported to have 89% specificity in detecting prostate cancer, its over-expression had prognostic value as it was associated with higher grade pT3 disease, and higher levels of serum PSA (456). In a transgenic mouse model, it was demonstrated that over-expression of *PSGR* led to chronic inflammatory response which in turn led to PIN (457). Furthermore this study showed that PSGR signaling led to the activation of the nuclear factor-κB (NF-κB) or RELA through the phosphatidylinositol-3-kinase/Akt (PI3K/AKT) pathway in the initial phase of prostate disease (457). Using qRTPCR on post-prostate massage urine sediment, PSGR was shown to be detectable in urine and that its over-expression is comparable to PCA3 in predicting

prostate cancer (458). Another study also demonstrated its usefulness in a multiplex gene panel in increasing the sensitivity and specificity of their urine test in detecting PCa (459).

- **PPAP2A (Phosphatidic acid phosphatase type 2A)** This gene encodes an enzyme that is a member of the phosphatidic acid phosphatase family (PAP). It is an important membrane glycoprotein that plays a role in the hydrolysis and uptake of lipids from the extracellular space, and regulates cell signaling by modifying the concentrations of lipid phosphates to activate intracellular signaling cascades (460). In the prostate, PPAP2A expression is stimulated by androgens via activation of sterol regulatory element-binding proteins (SREBPs), resulting in an increase in lipogenesis that serves the synthesis of key membrane components (phospholipids, cholesterol), which in turn leads to carcinogenesis. This gene was shown to be up-regulated in LNCaP cells where it led to increased cell proliferation, survival, and altered lipid metabolism (461) (462). Studies have also shown that its down-regulation can lead to apoptosis in cancer cell lines and reduced tumour growth which makes it a potential therapeutic target for PCa (461).

- **ANPEP (Alanyl (Membrane) Aminopeptidase) (Prognostic))** encodes a membrane-bound zinc-dependent protease called aminopeptidase N (APN) (463) that regulates post secretory neuropeptides and their access to cellular receptor. It is also involved in intracellular signaling. In cancer, it is though to play an important role in neoangiogenesis and vascular endothelial growth factor (VEGF) expression as well as facilitating invasion and metastasis of various malignancies, including PCa (464-466). In contrast to other cancers (pancreatic and colon) where APN over-expression is associated with poor prognosis, in localised PCa, APN over-expression appears to be associated with good prognosis (467). Several reports shown that APN is down-regulated in PCa in comparison to benign tissue including a study using immunohistochemistry analysis on 278 samples in which they showed that APN is significantly down regulated in PCa comparing to benign tissue as a result of epigenetic silencing of ANPEP as a result of aberrant promoter hypermethylation. Hence ANPEP expression in PCa can be a potential prognostic factor (467).

- **PSMA (Prostate-specific membrane antigen)** This gene is located on the short arm of chromosome 11 and encodes a type II transmembrane glycoprotein that is known for its

enzymatic activities. It acts as a glutamate carboxypeptidase that belongs to the M28 peptidase family (468). Its expression was reported in a number of normal and cancerous tissues (469,470). The encoded protein is also known as the prostate-specific membrane antigen, a trans-membrane protein expressed in all types of prostatic tissue, however in PCa its over-expression is highly restricted to the epithelial cells and was reported to be diagnostic and prognostic as its up-regulation was shown to be grade dependent (471). Furthermore its its activity increases as cells become more androgen independent (472,473). The mechanism of PSMA involvement in PCa is poorly understood however some studies showed that through the stimulation of phospho-p38 (P-p38) PSMA increases cell proliferation, migration and survival (474). Its usefulness as a prostate cancer diagnostic marker are questionable at the serum level as variable results has been reported (475), however its diagnostic usefulness appear to improve when used in a gene panel for urine analysis (459).

- **NAALADL2 (N-acetyl-L-aspartyl-L-glutamate peptidase-like 2)** is a member of the glutamate carboxypeptidase II family, it is known to be over-expressed in prostate and colon cancer. Its expression in prostate cancer is prognostic, being associated with disease stage and grade. Its over-expression has also been shown to predict poor survival following radical surgical treatment for PCa. Unlike NAALAD1, NAALADL2 is localised to the basal cell surface and promotes cancer progression by endorsing adhesion to extracellular matrix proteins. It also has effects on cell migration and invasion, and promotes cancer development and progression through regulating the levels of Ser133 phosphorylated C-AMP-binding protein (CREB) (476).

- **AGR2 (Anterior gradient 2 homolog (Xenopus laevis))** is also known as secreted cement gland protein XAG-2 homolog). This gene is located on chromosome 7 at p21, a region known for genetic alterations leading to cancers in different organs including the prostate. It encodes a member of the endoplasmatic reticulum (ER) disulphide isomerase (PDI) family (477,478) that is known to facilitate the bio-activation of protein through the ER for secretion or membrane association. When these processes fail proteins accumulate in the ER, initiating ER stress, cell-cycle arrest, and apoptosis (478). In PCa AGR2 is highly up-regulated in comparison to benign tissue. Its up-regulation is associated with high disease grades, particularly metastatic disease hence its expression level is associated with poor survival (479,480) giving it a prognostic value as a biomarker. This androgen regulated gene

(481) has also been shown to play an important role in promoting bone metastasis (482) as its over-expression can lead to cancer cell proliferation (483) and its knockdown to cell cycle arrest at the G0/G1 phase. Studies also showed that, silencing AGR2 in PCa cells lead to significant reduction in cellular attachment to fibronectin collagen I, collagen IV, laminin I and fibrinogen as well as lost of integrin expression (α4, α5, αV, β3 and β4 integrins); which in turn led to failure in cellular adhesion and reduction in tumour cell migration (483). There was also significant reduction in Caspase-3 expression which is a key regulator of both extrinsic and intrinsic death signalling pathways, causing a higher resistance to apoptosis inflicted by tumour necrosis factor inducing ligand (TRAIL) suggesting that AGR-2 stimulates prostate cancer metastasis by regulation of cellular adhesion and apoptosis (482). Urine analysis for *AGR2* showed that its transcripts are detectable and that urine *AGR2*/*PSA* transcript ratios have better diagnostic accuracy than serum total PSA alone (481).

1.9.1 Genes used for kidney, bladder and blood control

- **UPK2 (Uroplakin 2)** is a gene that encodes the bladder specific uroplakin 2 protein. Unlike other uroplakins, UPK2 contains one transmembrane domain (484) (485) and maps to chromosome 11, at 11q23. It forms part of specialised plasma membrane that covers the urothelium, called AUM. It is believed to strengthen the urothelium by preventing cell rupture during bladder distention. It was shown to be over-expressed in the urine of patients with bladder cancer (485,486).

- **SLC12A1 (solute carrier family 12)** This kidney-specific protein encodes a membrane transport protein that acts as a co-transporter of solutes including sodium, potassium, and chloride ions across the cell membrane. It is mainly expressed in the thick ascending limb of the loop of Henle and the macula densa of the nephrons where it plays a key role in sodium reabsorption into the cells, hence mutations can play an important role in hypertension (487,488). *SLC12A1* mRNA was detected in urinary exosomes in a study in a quest for renal urine biomarkers (489).

- **PTPRC (Protein Tyrosine Phosphatase, Receptor Type, C).** The protein encoded by this gene regulates T-cell and B-cell activation through antigen receptor signalling. It is mainly expressed in monocytes, neutrophils, B and T- lymphocytes. It has been used as a blood control in several prostate cancer studies analysing gene expression in circulating tumour cells (490,491).

1.9.2 Housekeeping Genes

- **HPRT (Hypoxanthine-guanine phosphoribosyltransferase 1)** is an enzyme encoded by the *HPRT1* gene. This gene has similar expression levels in bladder, blood, prostate, and prostate cancer. No prognostic assocation with its expression levels was reported, for this reason, this gene is an established housekeeping gene for PCa that is frequently used in multi-gene expression profiling of prostate cancer (492) (493). A study comparing 13 endogenous control genes for normalisation of gene expression measurements in tumour tissues also report this gene to be usable for this purpose (494).

- **GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)** This gene encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family. It has been established as a housekeeping gene for PCa and was previously used in multi-gene expression profiling of primary prostate cancer (492). Another study comparing 13 endogenous control genes for normalisation of gene expression measurements in tumour tissues also report this gene to be usable for this purpose (494). It has also been used for normalisation of gene expression data as well as internal controls in miR-644a by several authors (495)**.**

- **TBP (TATA box binding protein) and ALAS1 (Aminolevulinate, delta-, synthase 1)** As well as GAPDH and HPRT these genes has been established as housekeeping genes for PCa and are frequently used in multi-gene expression profiling of primary prostate cancer (492) (493).

- **B2M (Beta-2-microglobulin)** This gene encodes a serum protein that has been reported to have an even expression between bladder, blood, and prostate. This gene was also shown to be one of the most stable genes for the normalisation of expression studies in invasive breast tumour studies (496) as well as prostate cancer (493).

Table 1.4: Summary of the genes tested in this study

Gene	Gene name	Function in PCA	Expression	Diagnostic Expressed in low grade disease HGPIN	Prognostic increase impression in higher grade disease	AR Regulated	Location	Name of the Author that chose the gene
KLK2	Kallikrein 2	- Cancer initiation. - Extracellular matrix degradation - Metastasis. - AR transactivation and cell growth	Up-regulated	yes	yes	yes	Epithelial	Cooper
PSA/KLK 3 (Exons 1,2,3)	Prostate specific antigen/Kallikrein 3	- Modulates the function of IGF - Facilitate tumour invasion	Up-regulated	Yes	Yes	Yes	Epithelial	Cooper, Doll
KLK4	Kallikrein 4	- Cell cycle regulation and proliferative effect. - Cell migration and invasion	Up-regulated	Yes	?	Yes	Epithelial	Cooper
SPINK1	Serine protease inhibitor Kazal-type 1	- Serine protease inhibitor - Disease progression	Up-regulated	?	Yes	no	?	Cooper
PCA3	Prostate cancer gene 3 or DD3	- Unknown	Up-regulated	Yes	Yes	?	?	Cooper, Schalken, Nelson, Doll
AMACR	Alpha-Methylacyl-CoA Racemase	- Initiation - Progression	Up-regulated	Yes	no	?	Epithelial	Cooper
TMPRSS2/ ERG		- Activate androgen regulated transcription factor - Cell growth, proliferation, differentiation and migration	Up-regulated	Yes	Yes	?	Epithelial	Cooper, Schalken
MMP26	Endometase/matrilysin-2/matrix metalloproteinase 26	- Disease progression and metastasis - Degrade ECM	Up-regulated	Yes	Yes	no	Epithelial	Cooper
TIMP4	Tissue inhibitor of metalloproteinases	- Disease progression and	Down-regulated	no	yes	no	Epithelial	Cooper

	4	metastasis - Degrade ECM						
HPN	**Hepsin**	- Promote metastasis - Degrade ECM and - Proteolitic activity	Up-regulated	no	Yes	no	Epithelial	Cooper
Maspin	**Maspin**	- Tumour suppressor - Promote apoptosis - Inhibit cell invasion	Down-regulated	yes	yes	no	Epithelial	Cooper
GOLM1	Golgi membrane protein 1	- Unknown	Up-regulated	yes	no	no	Epithelial	Cooper
HOXC4 and HOXC6	Homeobox C4 and Homeobox C6	- Poor cell differentiation - Cell proliferation - Vascularization and metastasis	Up-regulated	yes	?	no	Epithelial	Schalken
DLX1	Distal-less homeobox 1	Unknown	Up-regulated	yes	?		Transitional	Schalken
TDRD1	Tudor domain containing 1	Direct target of ERG	Up-regulated	yes	?	?	?	Schalken
CAMKK2	Calcium/Calmodulin-Dependent Protein Kinase Kinase 2, Beta	- Cell cycle regulation and proliferative effect. - Induce AR transcriptional activity leading to progression	Up-regulated	Yes	Yes	Yes	Transitional	Mills
IMPDH2	Inosine Monophosphate Dehydrogenase 2	- Cell cycle regulation and proliferative effect.	Up-regulated	Yes	?	?	?	Mills
ARexon3, ARexon4-8 ARexon9	Androgen Receptor splicing	- Cell viability - Proliferation - Progression	Up-regulated	Yes	Yes	Yes	All	Cooper
STEAP2/ STAMP1 **STEAP 4**	Six transmembrane epithelial antigen of the prostate family member 2	- Cell growth - Metastasis	Up-regulated	Yes	Yes	Androgen dependent	?	Mills
MDK	Midkine. Neurite Growth-Promoting Factor 2	- Cell growth and survival - Cell migration - Angiogenesis	Up-regulated	Yes	Yes	yes	?	Cooper
TERT	Telomerase	- Prevent	Up-	Yes	Yes	no	?	Cooper

	Reverse Transcriptase	apoptosis	regulated					
FoxM1	Forkhead box protein M1gene	- Up-regulate cell cycle that lead to cell development and proliferation.	Up-regulated	Yes	Yes and recurrence predictor	Yes	?	Mills
CDC20	Cell Division Cycle 20 Gene	- Up-regulate cell cycle - Prevents cell apoptosis - Proliferation	Up-regulated	Yes	Yes	Yes	?	Mills
CDKN3	Cyclin-Dependent Kinase Inhibitor 3	- Up-regulate cell cycle	Up-regulated	Yes	Yes and recurrence predictor	Yes	?	Mills
MKi67	Marker Of Proliferation Ki-67	- Development and proliferation.	Up-regulated	Yes	Yes and recurrence predictor	Yes	?	Cooper
AURKA	Aurora Kinase A	- Up-regulate cell cycle that lead to cell development and proliferation.	Up-regulated	no	Yes And metastasis predictor	no	Neuro endocrine	Cooper
CLU	Clusterin identified as a therapeutic target	- Stress gene protects cells against apoptosis and cytolysis	Up-regulated	no	Yes And potential of therapeutic target	no	?	Cooper
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B	- Cell division, differentiation, and secretion	Up-regulated	no	Yes And potential of therapeutic target	no	?	Cooper
OGT	O-linked β-N-acetylglucosamine transferase	- Altered metabolism - Affect cancer phenotype, growth and invasion - FoxM1 up-regulation	Up-regulated	no	Yes And potential of therapeutic target	no	?	Mills
PECI	Peroxisomal 3,2-trans-enoyl-CoA isomerase	- Altered metabolism - Down stream from AMCR	Up-regulated	Yes	no	no	?	Mills

		- Bio-activate dietary and environmental pro-carcinogens - Chronic inflammation						
SULT1A1	Sulfotransferase Family, Cytosolic, 1A, Phenol-Preferring member 1	- Altered metabolism - Bio-activate dietary and environmental pro-carcinogens	Up-regulated	Yes	No	No	?	Mills
OR52A2/PSGR	Prostate-specific G-protein coupled receptor	- Bio-activate dietary and environmental pro-carcinogens - Activation of the nuclear factor-ϰB - Chronic inflammation	Up-regulated	Yes	Yes	No	?	Cooper, Doll
PPAP2A	Phosphatidic acid phosphatase type 2A	- Intracellular signaling - Cell proliferation, survival, lipid metabolism, and differentiation	Up-regulated	Yes	Yes	Yes	?	Mills, Guido, Jenster
ANPEP	Alanyl (Membrane) Aminopeptidase	- Intracellular signaling - Neoangiogenesis - Metastasis	Down-regulated	yes	yes	?	?	Mills, Guido, Jenster
NAALADL1	N-acetylated alpha-linked acidic dipeptidase 1	- Stimulation of the phospho-p38 leading to increases cells proliferation, migration and survival	Up-regulated	Yes	Yes	?	Epithelial	Nills, Doll
NAALAD2	N-acetyl-L-aspartyl-L-glutamate peptidase-like 2	- Endorsing adhesion to extracellular matrix proteins - Cell migration and invasion	Up-regulated	Yes	Yes And recurrence predictor	?	?	Mills
AGR2	Anterior gradient 2 homolog	- Cell proliferation and adhesion - promoting bone metastasis	Up-regulated	yes	Yes And Potential of therapeutic target	?	?	Mills
UPK2	Uroplakin 2	Bladder control	-	-	-	-	-	Cooper

SLC12A1	Solute carrier family 12	Kidney Control	-	-	-	-	-	Cooper, Mills
PTPRC	Protein Tyrosine Phosphatase, Receptor Type, C	Blood control	-	-	-	-	-	Cooper
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	House keeping Gene	-	-	-	-	-	Cooper
TBP	TATA box binding protein	House keeping Gene	-	-	-	-	-	Cooper
ALAS1	Aminolevulinate, delta-, synthase 1	House keeping Gene	-	-	-	-	-	Cooper
B2M	Beta-2-microglobulin	House keeping Gene	-	-	-	-	-	Cooper
HPRT	Hypoxanthine-guanine phosphoribosyltransferase 1	House keeping Gene	-	-	-	-	-	Cooper

1.10 Hypothesis

In current clinical practice, PSA is still widely used as a first line test in the diagnosis of prostate cancer despite its known drawbacks. The reason for that is the lack of alternatives, which may be explained by the heterogeneity and multifocality of prostate cancer that create difficulty for a single biomarker to be representative for the whole picture. I believe that a multiplex biomarker test could have a better chance at overcoming these issues and be successfully incorporated into the existing diagnostic pathway for prostate cancer. Several studies have shown the usefulness of urine tests for PCa diagnosis, and other studies the important role of exosomes in prostate cancer. However, urine exosomal biomarkers are still very much underexplored. I believe that by studying gene expression in these microvesicles we can potentially identify markers that can differentiate between cancer and benign tissue (diagnostic) as well as predicting the biopsy outcomes and potential metastasis (prognostic).

1.11 Study Aims

Urine-based diagnostic tests are non-invasive and are potentially of considerable clinical utility for prostate cancer patients. Prostate cells and macromolecules have direct anatomical access to urine through the prostatic urethra and ducts that connect the prostate to the urinary tract. Biological products that represent the prostate as a whole can therefore be detected in first catch urine samples. Prostatic biomarkers in urine are boosted after stimulation of the prostate via digital rectal examination (DRE), as has been demonstrated by several authors including the study that identified and designed the clinically implemented PCA3 test.

Aim 1: The initial idea for this project was to use urine sediment as a source of biomarkers. However urine does not provide an ideal living environment for cells, and so arose the first real challenge to the study, to which exosomes appeared to be the answer.
Aim 2: To identify a good source of biomarkers that could serve the clinical purpose of a) screening and diagnosis for PCa, and b) identification of patients with aggressive disease that would benefit from radical treatment.
Aim 3: To work out a way to collect and process urine samples from patients and maximise the amount of prostate biological material with the least amount of clinical involvement and patient distress.
Aim 4: To develop methods for extracting the best possible quality and quantity of exosomes and their RNA from urine while preserving the biological materials such as cells and proteins for future analysis.
Aim 5: To carry out multiplex analysis of biomarkers using 200 urine samples from patients from clinics at the NNUH.

For biomarkers to be accepted into clinical use they must be verified in prospective multicenter clinical trials. Our long-term aim is therefore to link to other centers both in this country and worldwide to set up multicenter studies.

All detailed discussion on the above information is presented in Chapter 5

2. Methods

2.1 Study setup and patient recruitment

2.2.1 Public and Patients' Involvement in the Research Committee

All the forms used in this project for recruitment including the patient invitation letter, information sheet and consent form were reviewed and revised by the public and patients involvement in research committee (PPIRES) (See Appendix 5 for forms).

2.2.2 Ethical approval

The research recruitment process was planned prior to ethical approval application. All ethical issues including patient's involvement in the study, consent, sample anonymity, inclusion and exclusion criteria was addressed as the following:

- Invitation letter and information sheet: forms (see Appendix 1 and Appendix 2) were provided to all men attending the PSA clinic. This is a dedicated clinic to investigate patients that have been referred to the NNUH due to a raised serum PSA test and/or an abnormal digital rectal examination. In addition, I collected samples from a small number of men attending the haematuria clinic, which was a dedicated clinic to investigate patients referred with haematuria (blood in the urine). The majority of the haematuria patients are normal on further clinical investigation.

These documents invite patients to participate in the research study and give them information about the degree of their involvement, ie providing a urine sample directly after a direct examination. It also explained the difference between the routine digital rectal examination which they would have outside the context of the study and the one they would have should they opt to participate in the project. It was also explained that their involvement or denial to participate in the research would not affect their clinical course in anyway, and that they are free to withdraw their consent in the study at any time without providing a reason and without prejudicing future medical care. These forms also clarified that the research outcome would not benefit them personally. Contact details of the chief investigator were included in these forms in case patients required further information or subsequently decided to opt out of the study.

- Consent form: Patient consent to join the study was requested during my consultation with them on the day of the clinic and prior to the examination of the prostate. I

fully discussed the study with the patients, and encouraged them to ask any questions related to the research.

All individuals asked to consider taking part in research were given full information about the research in written forms that were presented in non-technical language to ease understanding. Forms given to patients before the clinic including the invitation letter and information sheet that explained the project intention in detail and the degree of patient involvement. Patients were informed that their participation was voluntary and that refusal would not affect in any way their clinical course. In addition, if they decide to participate then they could subsequently withdraw their consent for the study at any time without providing a reason and without prejudicing future medical care. I also explained that there was no direct benefit to them from this project as this was a pilot study that required further research in order to reach clinical practice and that information resulting from this research will be published in due time. Male patients who were unable or not willing to consent or undergo a rectal examination for any reason were excluded. (see appendix 3).

- Digital rectal examination (DRE): All male patients seen in the PSA and haematuria clinic would normally (outside the context of this study) have a digital rectal examination as part of their routine clinical investigations. This usually involves one finger swipe across the surface of the prostate to detect any abnormality or asymmetry that would raise suspicion for prostate cancer and would warrant further investigation with a biopsy. The DRE would also provide a clinical stage of the disease in case of cancer diagnosis. However for the purpose of the research the DRE technique was changed from one, to three swipes across the surface of the prostate, aiming to depress the prostate surface by about 1 cm. This technique was adopted from the procedure used for the PCA3 urine test, and has enable us to maintain the diagnostic purpose of this examination while increasing prostatic secretions into the urethra and subsequently the urine. Adopting this system for doing the DRE also helped us standardise the procedures and reduce any variability in our data.

- Urine collection: All patients attending the PSA and Haematuria clinics would normally have to provide a urine sample for dipstick analysis outside the context of the study; however this normally happened when the patient first turned up at the clinic in order to facilitate the clinics flow. This routine was changed for the purpose of the research in order to obtain a sample following DRE. However a very small number of men were able to provide 2 samples ie before and after DRE (4 patients).

- Urine sample anonymisation: All urine samples were labelled with a unique code that is only identifiable by the principle investigator. The file with this important linking information was maintained in an encrypted form and backed up on a secure NHS server with password protection.

- Inclusion criteria: All male patients who were not thought to have prostate cancer were recruited into the control arm, while men who have an established diagnosis of prostate cancer and were not on treatment went into the cancer arm.

- Exclusion criteria: Male patients that were post prostate cancer treatment such as radical surgery or radiotherapy patients, or who were unable to consent or undergo rectal examination for any reason were excluded.

2.2 Clinical Procedure

Patient recruitment took place on a weekly basis in two specialised clinics (PSA and Haematuria) at the Norfolk and Norwich University Hospitals NHS Foundation Trust (NNUH).

Prostate cancer assessment clinic: Patients with suspected PCa were recruited from the PSA clinic on an average of 9 patients a week these are patients refered by their GP due to raised PSA or clinically abnormal DRE. All patients had a history taken, examination and investigation including DRE and TRUS biopsy (when indicated) as a part of their clinical workout. Patients diagnosed with PCa based on histological finding were included in the cancer arm. Patients with benign histology were followed up for a period of time, some of which were included in the benign arm only if there PSA normalised and their biopsy is negative for PCa. Diagnosed cancer patients in the high and advanced risk group had further radiological investigations in the form of MRI, CT or bone scan in case of clinical suspicion of metastasis.

Benign patients: were recruited from the haematuria clinic on an average of 2 to 3 samples per week. Men attending the haematuria clinic also underwent DRE and serum PSA testing as part of the haematuria investigation workout. All the patients recruited from this clinic are

patients with a normal PSA, have a clinically benign prostate and have a normal haematuria investigation including urine dipstick testing for infection, kidney function test and full blood count as well as renal ultrasound scan and bladder telescopic investigation. Three patients were found to have bladder cancer. Their urine samples were used with caution taking these finding into consideration).

All patients with raised PSA had a prophylactic dose of antibiotics (750mg of ciprofloxacin) and trans-rectal ultrasound TRUS guided biopsy 30 min after the initial consultation.

2.3 Data collection

2.3.1 History

I designed and took the patient data required for the study. (See Section 3.6 and Appendix 6 for details)

2.3.2 Examination

Patient examination data, including the digital rectal examination finding, were carefully recorded and interpreted by myself to state the clinical stage (as per the prostate cancer TNM classification) as follows.

1- No palpable abnormality
2- (T1 disease are not palpable clinically hence could not comment on this stage)
3- T2a: the tumour is palpable in half or less than half of one of the prostate gland's two lobes.
4- T2b: the tumour is palpable in more than half of one lobe, but not both
5- T2c: the tumour is palpable in both lobes but within the prostatic capsule
6- T3: the tumour has spread through the prostatic capsule
7- T4: the tumour has invaded other nearby structures (feels solid and fixed)

Findings that suggested distant metastasis to the bones and lymph nodes such as neuropathy, lower limb oedema and retention of urine were also documented and addressed in the treatment plan.

2.3.3 Digital Rectal Examination (DRE)

A DRE was performed on all patients as part of their standard clinical investigation see 3.7.2 for more information

All patients were consulted and examined by myself in order to standardise the DRE technique and minimise operator related variability. At the start of the project, all DREs were done as per the PCA3 test protocol using 3 swipes on each lobe in order to maximise the quantity and quality of the prostate microvesicle RNA obtained (497,498).

The aim of the DRE is to persuade the prostatic biosecretions through the prostatic tubules and duct into the prostatic urethra, which will be then carried out by the flowing urine and enhance the detection of biomarkers in urine samples as demonstrated by several studies including the PCA3 test (498-500). Another evidence was provided by Hendriks *et al,* (501) who demonstrated that prostate biomarkers are significantly higher in urine after DRE in comparison to sample without DRE, this has also been proven true for urinary exosomes as shown by another study (502).

2.3.4 Trans-rectal ultrasound TRUS

We recorded the following data for all patients who underwent this procedure:

1- Histological findings included: numbers of cores taken from each lobe, number of positive core in each lobe and percentage of cancers in them, and whether there is perineural and or vascular invasion. Gleason major and minor and the overall score.
2- Ultrasound detected abnormalities such as, calcified lesion, hypoechoic lesion, extra capsular spread, and benign gland.
3- Prostate volume measured in ml by multiplying the prostate height, depth, and width.

All the TRUS biopsies were performed in the same day of the clinical consultation (if no contra indication such as UTI) as a one-stop service for prostate cancer (Clinic in the morning, TRUS biopsy in the afternoon)

2.3.5 Radiological staging

The entire radiological investigations results were recorded including MRI, CT and bone scan results. These Scans were done within two weeks from presentation (as a two week wait rule basis for PCa).

2.3.6 PSA

Follow-up PSA measurements were taken for all patients at 3, 6, 12 and 24 months, except for those patients that had benign disease (normal PSA and clinically benign prostate). Treatment plan and treatment outcome data was collected retrospectively using the hospital intranet system.

2.4 Sample collection

Urine samples were collected from 662 men consented to participate in the study in the period between January 2012 and 2014. Only two patients refused to consent for sample collection during this period.

20-25 ml urine samples were collected from all patients immediately after clinical examination (DRE) in polypropylene 'Universal' tubes (Sterilin) containing 5 ml of preservative cell culture media/EDTA (RPMI+++ and 10xRPMI). All patients are required to have a urine dipstick test prior to prostate biopsy in order to i) exclude urinary tract infection (nitrites, bloods and protein as well as pH) which is a contraindication for the procedure and ii) identify variables that might affect the research data.

2.4.1 Sample Labelling

All samples were labelled with a unique code from which only the principle investigator could identify the patients. I created and maintained the simple code that referred to the experiment and the sample number (Ex M1-1 = Marcelino experiment 1 sample 1). This code correlated to patients notes according to the date of the experiment and the sequence the patients were seen in the clinic on the day.

2.5 Sample fraction analysis performed by Movember groups

Urine sediments were separated into several fractions and analysed by several groups as part of the Movember GAP (Global action plan) Urine Biomarker Consortium (except for the 1st 44 samples that were only used for exosomal RNA analysis), which was led by our laboratory and coordinated by Jeremy Clark at UEA. The Consortium consisted of 12 teams in 7 countries working on 5 urine fractions analysed in laboratories worldwide by: Mass Spec, ELISA, DNA-methylation. Expression analysis of RNA from cells and microvesicles was by NanoString, qRT-PCR and RT-PCR. When the study is finished (Dec 2016) data will be combined with clinical information to determine the optimal combination of biomarkers and fractions for prostate cancer diagnosis and prognosis (Figure 2.1).

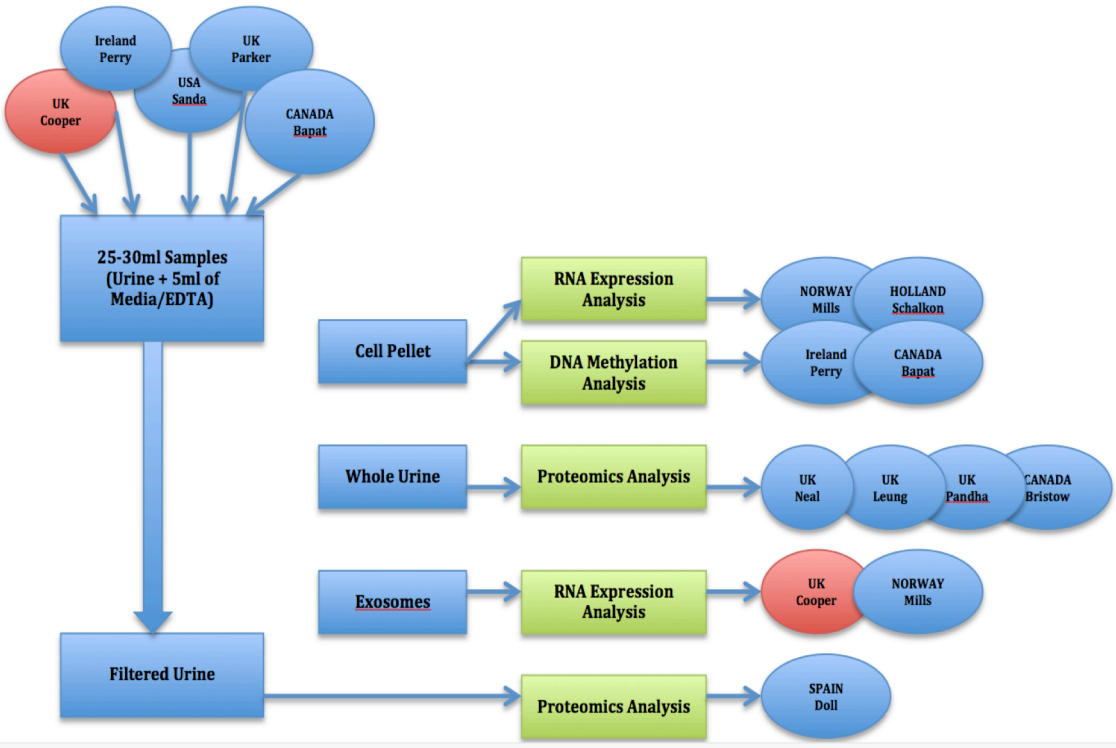


Figure 2.1 : Source of urine samples and, fractions and analysis laboratories.

2.6 Sample processing

2.6.1 Sample processing at the NNUH

At the beginning of the project samples where collected and placed in wet ice until the end of the clinic (ie a maximum of three hours) before transport to the laboratory area at the University of East Anglia (UEA) for processing. This method was applied for the first 100 samples until we acquired a centrifuge in a room near the NNUH clinic. Subsequent to this, the first steps of the urine processing took place in the NNUH as follows: i) 1 ml of whole urine was used to prepare a slide for FISH (Fluorescence *in situ* Hybridisation) by Rachel Hurst - for a parallel line of research running alongside this project in our laboratories. ii) 3 ml of whole urine was aliquoted for proteomic analysis (sample stores on dry ice until transport to the laboratory area for storage at -80°C for future analysis). iii) The urine sample was then centrifuged at 2000g for 5 min to sediment and extract the cell pellet, which was then stored on ice or in a refrigerator at -6°C (Cell RNA extraction was carried out 3 hours later in the laboratory). iv) The supernatant was decanted into a 50ml polypropylene tube (Fisher cat. 35-2070) and placed on ice. All the samples were then transported to the laboratory area at UEA for further work. The change in the processes of sample treatment took place in an attempt to improve microvesicular RNA yields (discussed in detail in chapter 3 (3.7.3.5). The sample handling and transportation was in accordance with health and safety regulations for transport of human tissue, in summary all the samples were double bagged with absorbent material to prevent accidents in case of leakage and then placed in a secure box.

2.6.2 Sample processing at the Laboratory

The urine was distributed and processed for multiple biological fractions as shown in Figure 2.3.

The urine cell pellet and supernatant was processed at the UEA laboratory as follows:

1- Cell pellet: Initially, RNA-only was extracted from the cell pellet using the Qiagen RNeasy kit as manufacturer's instructions. Later, both DNA and RNA were extracted from the cell pellet using the Qiagen AllPrepKit as per the manufacturer protocol. Nucleotide extractions were initially performed by myself, and subsequently by Dr Rachel Hurst.

2- Exosome microvesicle harvesting and RNA extraction: The urine supernatant was centrifuged at 3,400g for 10 min at 10°C to sediment any residual cell debris, which was then discarded. The supernatant was then poured into a 30 ml syringe and filtered through both 0.80 and 0.45um Minisart filters (16592k, Sartorius) into a labelled Ultra-15 100 Da MWCO filter device for microvesicle harvesting (Figure 2.2). This device contained a micropore filter with a cut off of 100 Da. The microvesicles were thereby sieved out of the urine by centrifugation at 3,400g for 15min or until the volume was reduced to <200μl. 15ml of PBS (Phosphate-Buffered Saline which was prepared earlier in the laboratory by dissolving 1 PBS tablet in 100ml of sterile water) was added and the sample was re-spun until the volume was 200μl. The PBS wash step was then repeated twice more. The remaining liquid in the upper part of the filter was then transferred into a 1·5ml Ambion non-stick tube by pipetting, ready for RNA extraction.

Figure 2.2: Filters used in the exosomal harvesting process. Left hand side: filtering the urine through a 0.80 um minisart filter (16592k, Sartorius) into a (right hand side) Ultra-15 100,000 Da MW cut-off filter device.

2.6.3 Exosomal RNA Extraction

Exosomal RNA extraction was performed using the Qiagen MicroNeasy kit as follows.

- 700µl of Qiagen 'RLT' buffer supplied in the Qiagen MicroRNeasy kit was added to the ultra-15 filter device to rinse out any remaining microvesicles. RLT was then transferred to the pre-prepared and labelled Ambion non-stick tube.
- 3·5µl of DTT (Dithiothreitol; Clelands reagent) was added. Ethanol (100%) was added to the samples to create a 35% solution which was mixed by vortexing. The solutes were then loaded to the pre-labelled Qiagen micro column and centrifuged at maximum speed (13,000g) for 10 seconds in a microcentrifuge. The column was then washed with 350µl of 'RW1 Buffer' (supplied in the Qiagen kit) and spun at maximum speed for 10 seconds (this step was repeated twice), in the meantime a DNase solution was prepared while the columns rested on ice (10µl of DNase I solution was mixed with 70µl of 'Buffer RDD' for each sample and mixed by inversion). 80µl of the DNase mix was then added directly to the membrane of each 'Mini Elute Column' and left at room temperature for 15 minutes. The columns was then washed with 350µl of 'Buffer RW1' and 2 rounds of 500µl of 'Buffer RPE', spinning at max speed for 15 seconds between each step. This was then followed by 2 steps of ethanol wash using 80% ethanol and centrifugation at maximum speed for 2 minutes.
- Each 'Mini Elute Column' was then placed in a new collection tube to avoid alcohol contamination and spun at maximum speed for 5 minutes with the tube lid open to allow the filter to dry, followed by a 10 min air dry. 19 µl of pre heated (45°C) Qiagen nuclease free water (provided in the kit) was then added to the column which was then placed in a fresh non stick Ambion collection tube (into which 1ul of (1ug/ul) glycogen had previously been added) and left to rest for 2 minutes at room temperature. The tube was then centrifugated for 1 minute at maximum speed to elute the RNA which was stored at -80°C

The RNA was quantified using a Thermo Scientific spectrophotometer which measured the relative absorbencies of light of two wavelengths (260:280 absorbance ratio). The quality with respect to RNA length was assessed using an Agilent 2100 Bioanalyzer system with an Agilent RNA 6000 Pico kit as per the manufacturer's instructions. The process included gel preparation (which rendered the chip as an integrated electrical circuit once loaded) and the Gel-Dye mix (that intercalates directly with the RNA during the chip run). 9 RNA samples

could assessed on one chip: samples were heated to 70°C and placed on ice to cool down. The samples were then loaded into the Pico chip along with a ladder solution (the RNA 6000 ladder standard is run on every chip as a reference for data analysis). The chip was then placed in the Bioanalyzer machine. An electrode was inserted into each well in turn, and the RNA was driven by voltage electrophoretically through the gel, which separated the molecules by size. The RNA strands with intercalated dye molecules were detected as they run off the end of the gel by laser-induced fluorescence and the data was translated into gel-like images and electropherograms as shown in Figure 2.3. The data was analysed using the internal reference ladder. The RNA 6000 ladder contains six RNA fragments ranging in size from 0·2 to 6 kb (0·2 kb, 0·5 kb, 1·0 kb, 2·0 kb, 4·0 kb, and 6·0 kb). The software automatically compares the RNA samples to the ladder fragments to determine their concentration and identify the 26s and 18s ribosomal RNA peaks. The RNA Integrity Number (RIN score) was generated for each sample on a scale of 1-10 (1=lowest; 10=highest) as an indication of RNA quality. Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample (Aligent.com) The 18s/28s ratio and an estimation of concentration is also produced.

The interpretation of the Bioanalyzer graphs was done according to the Agilent 2100 b Bioanalyzer data interpretation. The average microvesicle/exosomal RNA yields was 340ng. (https://www.urmc.rochester.edu/fgc/documents/Bioanalyzer_Interpretations_forcustomers.pdf)

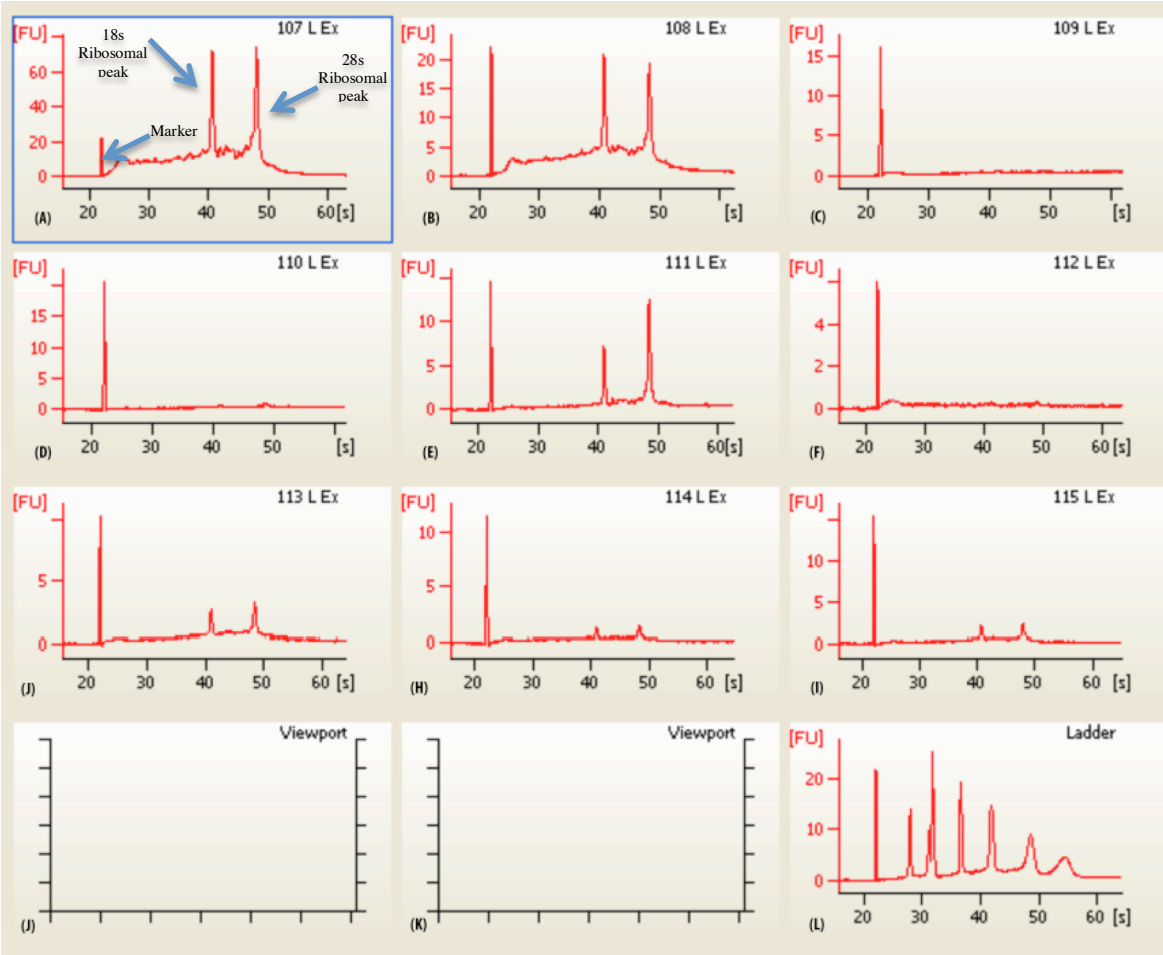


Figure 2.3: Examples of the Bioanalyzer chip analysis of urine RNA samples. Images are labeled A to L, A being top left, C top right down to L bottom right. A, B and E are examples of good RNA quality. The best example is E as the 28S peak should ideally be 2x the height of the 18S peak. Where the 18S peak is taller than the 28S peak, and the base line is sloping toward the left (ie sloping down from 28 to 18S) it represents heavily degraded RNA. L is a plot of the ladder only, and J and K are empty wells.

2.6.4 Amplification

Due to low exosomal RNA yields form some samples, it was decided to amplify all the samples prior to multiple gene expression analysis. The NuGEN Ovation PicoSL WTA System V2 was used to amplify the whole transcriptome, converting the RNA into double stranded cDNA. The Ovation system is capable of amplifying an input amount of RNA in the range of 500pg to 50ng. However; based on previous expression pattern validation experiments undertaken by Professor Cooper's group to optimise the quantity of RNA used

for amplification, a range of 10 to 20ng per sample was chosen for this study. Exceptions were made for samples collected from advanced patients (patients with PSA>100 and clinical stage T3/4 ie local metastasis) from which low RNA yields were obtained in the majority of cases (see Results for further explanation of low yields from this group). For these patients we used as low as 2ng or 0·5ng/µl for amplification.

The amplification procedure was carried out using the Ovation Kit as per the manufacturer protocol in three steps starting with 1) first strand cDNA synthesis 2) second strand cDNA synthesis then 3) purification and SPIA amplification. (see http://www.nugen.com/nugen/index.cfm/products/cs/microarrays-and-qpcr/ovation-pico-wta-system-v2/ for details). 20 samples were amplified at one time, which made this procedure time efficient. A PCR purification step using the Qiagen MiniElute Reaction Cleanup Kit then followed. The cleaning step was again done as per the manufacturer protocol (http://www.qiagen.com/gb/products/catalog/sample-technologies/dna-sample-technologies/dna-cleanup/minelute-pcr-purification-kit/). Samples were stored at -20°C. The cDNA was quantified using a Nanodrop spectrophotometer. A total of 352 samples were amplified. The RNA input amounts used were based on Bioanalyzer quantification. 325 samples had sufficient RNA for amplification, (average RNA yields were 9·7ng/µl SD= 12). Post amplification cDNA yields from these samples were (132·6ng/µl SD= 92·4). 23 samples were amplified using low RNA yields with an average of 1·2ng/µl. 14 of these samples had a post amplification cDNA average of 134·9ng/µl and 17 with average of 61.48ng/µl. Samples with cDNA yield below 100ng/µl were not used for gene expression analysis).

2.7 Expression analyses

2.7.1 NanoString Analysis

2.7.1.1 General principles of NanoString analysis

NanoString's nCounter technology is a based on double hybridisation of two adjacent ~50bp probes to their target RNA/cDNA (Figure 2.4). The first probe hybridisation is used to pull the target mRNA down on to a hard surface. The excess unbound RNA is then washed away. The second probe is then hybridised to the RNA. This probe has a multi-colour

barcode attached to it. The nucleotides are then stretched out under an electrical current, and the image is recorded. The barcodes number and type are counted, and this is the data output. Up to 800 different barcodes are possible, and therefore up to 800 different target RNAs can be detected in a single assay.

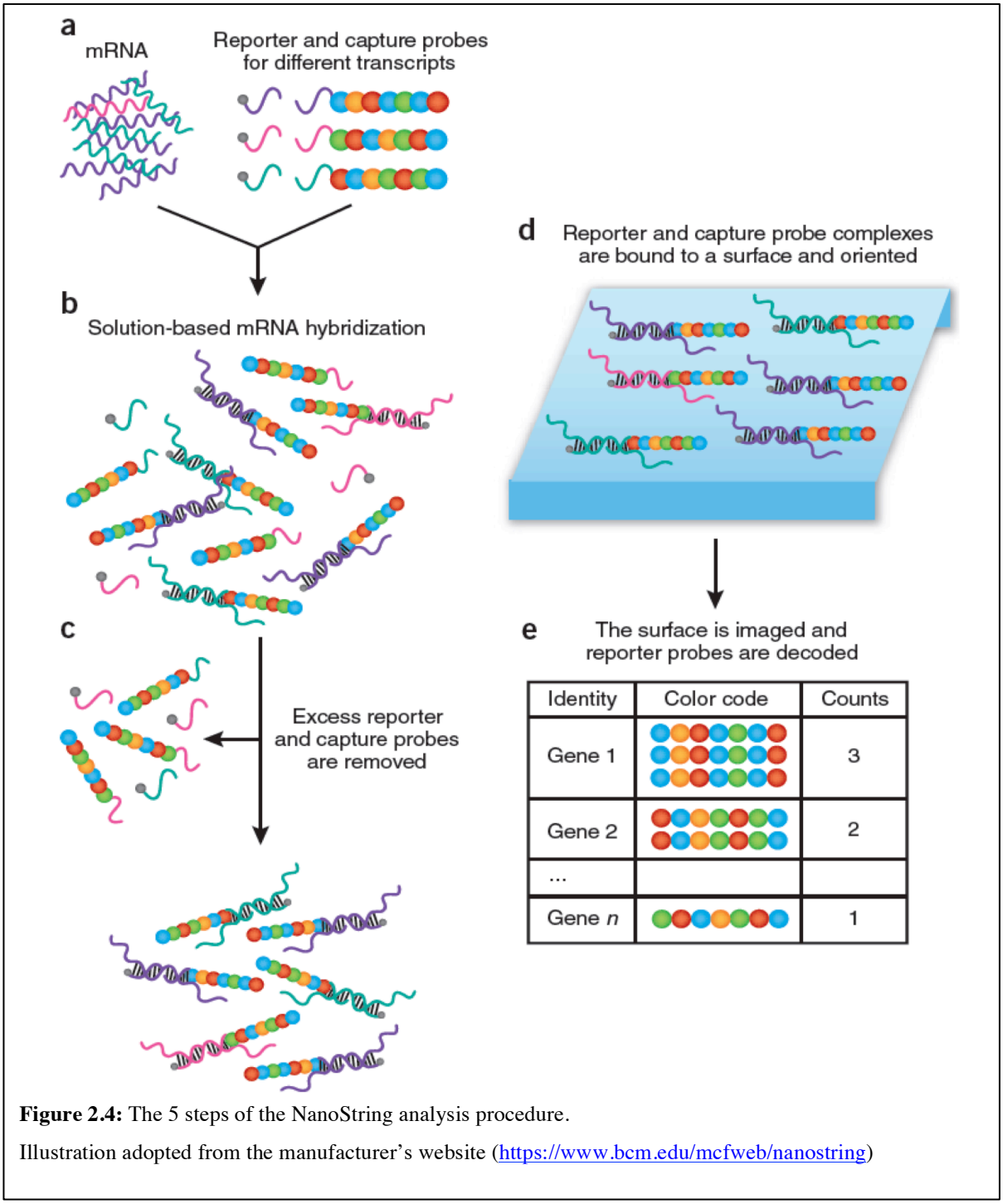


Figure 2.4: The 5 steps of the NanoString analysis procedure.

Illustration adopted from the manufacturer's website (https://www.bcm.edu/mcfweb/nanostring)

2.7.1.2 NanoString validation

The NanoString system is designed to work with RNA. As we were using cDNA, the system was not guaranteed to work. Nugen assured us that both strands of cDNA should be present in the cDNA amplification products in equal amounts, and so should work in the NanoString system. It was therefore decided that we would run a pilot test with NanoString analysis.
Pilot NanoString gene analysis using the nCounter human cancer 236-gene reference assay on 12 samples (6 from the High risk group: G8-10 PSA>20, 3 from the Benign control: PSA<1 and clinically benign prostate, and 3 from the Low risk group: Gleason 6 PSA 0<10) were initially undertaken to asses the feasibility of NanoString on our amplified cDNA samples. A total of 300ng in 10µl (30ng/µl) per sample was prepared in 0·5ml non-stick tubes and sent to the NanoString laboratory in Seattle, USA for gene expression analysis.

2.7.1.3 192 samples with 50-gene NanoString analysis

192 cancer and benign samples were analysed using the NanoString technology including an LNCaP/VCaP cell line mix RNA sample (LNCaP are an androgen-sensitive human prostate adenocarcinoma cell line derived from the left supraclavicular lymph node metastasis. VCaP are an androgen independent prostatic human prostate adenocarcinoma cell line derived from a bone metastasis).
For better correlation of the gene analysis with the clinical data, patients were grouped into a cancer arm, which was again sub-grouped into smaller groups based on the NICE stratification criteria for local metastasis. For information on the PCa groups and the control groups see Section 3.4.2
A total of 300ng in 10µl (30ng/µl) per sample was prepared in 0·5ml non-stick tubes and sent to the NanoString laboratory in Seattle, USA for gene expression analysis. A total of 50 genes were used including 5 housekeeping genes (*HPRT*, *B2M*, *TBP*, *GAPDH* and *ALAS1*) a bladder control gene (*UPK2*), kidney control (*SLC12A*) and blood control (*PTPRC*). Due to the lack of publications on exosomal derived prostate cancer transcripts, the choice of our genes were based on literature review and a on previous studies conducted in our laboratory (more details can be found in chapter 3 (3.1.3))

The analysed data was return in the form of a spreadsheet including the following:

1- Sample ID and scanner ID.

2- Field of View (FOV) count and counted: The digital analyser images the lanes in separate units, called fields of view, this critical step can be enabled due to optical issues, such as an inability to focus due to bubbles or insufficient oiling of the cartridge, for this reason the digital analyser reports the numbers of FOVs successfully imaged as FOV Counted, and the attempts of imaging in order to get a successful count as (FOV Count). Significant discrepancy between the number of FOV (FOV Count) and (FOV Counted) may be indicative of an issue with imaging performance. In our data set there were no discrepancies between the FOV count and FOV counted (mean 280 SD=0 and 279.59 SD= 1.75 respectively)

3- Binding Density: in order to get accurate molecular counts, the digital analyser counts only the codes that are unambiguously distinguishable, for this reason codes that overlap with each other do not get counted. The overlapping codes does not usually impact the data unless there are too many of them in the image, for this reason, the data analyser calculates the binding density for each lane in order to determine image saturation (The binding density is a measure of the number of optical features per square micron) which is useful for determining whether or not data collection has been compromised by image saturation. According to the manufacturer, the range for binding density is between 0.05 and 2.25 (A binding density greater than 2.25 is indicative of a large number of overlapping reporters on the slide surface suggesting that there are significant numbers of codes ignored). The mean Binding density of our samples were 0.472 SD=0.12.

4- Gene expression count per sample: in our data-set the range was between 1 (minimum) and 159,370 (maximum) counted transcripts.

2.7.3 Statistical methods

Due to the complexity of data analysis in correlating gene expressions and cancer grade, stage and variable clinical finding, the data analysis were carried out by the UEA bio-informations Dr Dan Brewer and Helen Curley using several analysis techniques. Helen

Curley performed these analyses as part of her PhD and has given me permission to show them in this thesis:
For the comparisons of correlations between gene expression levels and the presence of cancer, they first used standard statistical methods including:

2.7.3.1 Principal Component Analysis (PCA)

PCA is a statistical procedure used in multivariate analysis. The main purpose of using this test was to reduce the dimension of the dataset with minimal loss of information. It works by converting a set of observations with correlated variables into a set of values with linearly, uncorrelated variables called principal components (PC) that could be visualised. A set of orthogonal standardised linear combinations can then be set to explain all of the variations in the dataset. We used this analysis with an aim to identify variation differences in our dataset. This test has identified 2 clusters of samples and an outlier that did not correlate to the data, this was found later to be a contaminated sample and was excluded from further analysis. We then used a divisive Hierarchical Cluster Analysis (HCA) to confirm the PCa finding and identified the samples in each cluster so that gene expression analysis can be carried out.

2.7.3.2 Hierarchical Cluster Analysis (HCA)

HCA is a method of cluster analysis, in which a hierarchy of clusters can be built producing a dendrogram, or other type of tree diagrams, as final output (503,504). Each association level of the dendrogram represents a partitioning of the data set into a specific number of clusters (503). Based on the dendrogram the number of clusters can be defined. Cluster splits is then performed recursively as one moves down the hierarchy. HCA is used to identify biologically relevant structure in large data sets.
Having identified the samples in each cluster we then used the Wilcoxon test (a non-parametric statistical hypothesis test) to identify the up-regulated genes in these samples (the total number of genes identified was 25) and the Chi-Squared test to assess the likelihood of samples in with category similar to the identified cluster to belong to the same cluster and maintain the same gene expression. These analyses identified two clusters of samples of which one was due to poor gene expression secondary to poor RNA quantity, for this reason those samples were excluded from further analysis (more details are available in chapter 4).

2.7.3.3 Latent Process Decomposition analyses (LPD)

This is a computational technique used to cluster samples in an unsupervised probabilistic approach, it uses a combinatorial mixture to represent samples over a limited set of latent processes, which are expected to correspond to biological processes. Unlike clustering by dendrogram which can not objectively assess the most probable number of structures, which underlie the data, LPD can assess the optimal number of sample clusters and represent samples and gene expression levels using a common set of latent variables. In contrast to clustering by dendrogram, observations are not assigned to a single cluster and thus, gene expression levels are modelled via combinations of the latent processes identified by the algorithm. Simply put, each sample can have a presence in more than one group, this enables us to see how strongly samples fit within the different groups. We used LPD analysis to determine whether distinct subgroups of patients could be identified.

2.7.3.4 Kaplan Meier (KM)

Is the most common method used for estimating survival functions. Designed to deal with data that has incomplete observations using censoring. It works by using a start point and an end point for each subject. In this study, we used the KM analysis to study survival for patients on hormones treatment for advanced prostate cancer we use the start point as when the hormones treatment began and the end point will be when resistance to the treatment and subsequent progression was monitored for each patient. Data is often incomplete due to patients dropping out of the study or insufficient follow up of patients, here censoring is used to ensure there is no bias.

2.8 Second generation sequencing

In order to get in-depth understanding of the role of PCa exosomes and get a comprehensive view of the exosomal transcriptome that will enable us to identify a range of gene expression changes and the detection of novel transcripts in both coding and non-coding RNA species; we sequenced 18 samples as follows: 7 in the High risk group (Gleason 8-10, PSA<100), 7 Intermediate risk group (G7, PSA<20) and 4 Benign controls (PSA value normal to patients age and clinically benign prostate) using Next Generation Sequencing, known as NGS or

RNA-seq. This sequencing method is free from many of the limitations of previous technologies, such as the dependence on prior knowledge of the organism, as required for microarrays and PCR and have the ability to access allele-specific expression and novel promoters and isoforms (505,506).

RNAseq was performed by Oxford Gene Technologies (Oxford) as follows: 100ng of total exosomal RNA from 18 samples were prepared with the Illumina TruSeq RNA Sample Prep Kit v2, and sequenced using the Illumina HiSeq 2000 platform and TruSeqv3 chemistry. An average of 48,334,002 paired end reads were sequenced per sample. A total of 67.61 Gigabases (966680055 reads) of sequence data were read and aligned at high quality.

The Illumina HiSeq2000 sequencing system workflow is based on three steps: libraries preparation from nucleic acid sample, amplification to produce clonal clusters and sequencing using massively parallel synthesis.

2.8.1 Processing of RNA-seq data

RNA-seq processing converts samples of purified RNA to cDNA and sequences them on a high-throughput platform (507). This process generates millions of short (25 to 300 bp) reads taken from both ends of each cDNA fragment. In order to optimise the results, low quality bases (q<30) were clipped from the read ends, we also removed the TruSeq indexed adapter and TruSeq universal adapter (explanation can be found below) using Cutadapt v1.3. Reads containing more than 5% Ns, or less than 32bp or those with low complexity were also removed as well as ribosomal RNA.

TruSeq indexed and universal adapters, are short nucleotide sequences, which allow DNA fragments to bind to a flow cell for next generation sequencing, PCR enrichment of adapter ligated DNA fragments only and indexing of samples so multiple DNA libraries can be mixed together into 1 sequencing lane.

TruSeq Universal Adapter:

5’AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCT 3’

TruSeq Indexed Adapter

5’GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-NNNNNN-

ATCTCGTATGCCGTCTTCTGCTTG 3’

"N" is any nucleotide, and the 6 of them together are a unique sequence which can readily be identified as unique to 1 library. (http://tucf-genomics.tufts.edu/ documents/protocols /TUCF_Understanding_Illumina_TruSeq_Adapters.pdf).

2.8.2 Analysis of RNA Seq data

The DESeq2 package (Differential analysis of count data) was used to calculate the differential expression between samples and compare benign vs cancer (the cancer group was a combination of Intermediate and High-risk samples), and linear trend where each group of samples is assigned a numerical value (benign=0, intermediate=1 and high=2).

DESeq2 package analyses raw counts of sequencing reads in the form of a matrix of integer values, as obtained, from RNA-Seq sequencing experiment to allow assessing the measurement precision correctly. The value in the i-th row and the j-th column of the matrix tells how many reads have been mapped to gene i in sample j. Results tables are generated using the function results, which extracts a results table with log2 fold changes, p values and adjusted p values. It can also be useful to examine the counts of reads for a single gene across the groups (508).

PRINSEQ were used to filter, the sequence data. It generates summary statistics of the sequences in graphical and tabular format. PRINSEQ provides summary statistics for the data including read length, GC content, sequence complexity and quality score distributions, number of read duplicates, occurrence of Ns and poly-A/T tails, assembly quality measures and tag sequences.

2.9 Schematic representation of samples used for analysis in this study

3. Study Design, Set up, optimisation and Implementation in the Hospital and Laboratory

3.1 Introduction

Prostate cancer (PCa) is a complex disease that exhibits genetic, as well as morphological heterogeneity and multifocality that increases with stage and grade. This makes it extremely difficult to manage clinically as it can behave completely differently in different patients, some of which have an indolent disease that would require no treatment while the others may have an aggressive disease that would shorten their life significantly. Due to the poor understanding of the natural history of this disease the possibility of achieving specific and sensitive early detection is hard particularly when using the available clinical tools which include: DRE, PSA testing, TRUS biopsy and MRI imaging, each of which lack specificity and/or sensitivity. In order to overcome these challenges, researchers have explored avenues for discovery of new prostate cancer biomarkers for decades. However despite some progress in developing new biomarkers, including urine biomarkers, none are in routine clinical use. Obstacles to clinical implementation of these biomarkers are: 1) the difficulty in sampling all areas of the prostate for disease 2) detection of small foci of progressing tumour, and 3) the lack of direct correlation of these biomarkers to the clinical outcome and disease behaviour.

For these reasons the study described here was designed to analyse urine from prostate cancer patients. PCa biomarkers can be harvested from the urine and could potentially contain biomarkers from every part of the prostate. In addition, the urine can be interrogated for many different types of biomarker information (RNA, DNA, Protein and metabolite), with integration of these multiple data streams as the end point of the study, correlating biomarker data with the clinical characteristics of the research subjects with the aim of discovering a set of biomarkers that would overcome the problems of heterogeneity and multifocality of the disease. This study demanded a robust clinical set up that would link continuously with the laboratory analyses, a system to recruit a large number of patients whose clinical parameters that would cover all the variabilities generally found in prostate cancer patients as well as developing and optimising new methods for biomarkers discovery. The focus of this project is to identify new biomarkers that can, at the time of early diagnosis, be used to i) detect PCa *per se*, and ii) distinguish between aggressive cancer that requires immediate treatment, and clinically irrelevant disease. The study could improve our understanding of the conversion of indolent prostate cells to a rapidly growing prostate cancer, and identify the changes that underlie a cancer becoming androgen independent and

so untreatable by conventional androgen withdrawal therapy.

3.2 Study setup

This study is part of an international consortium run from UEA/NNUH and funded by Movember. The study was set up to get rapid results by using the specialised analytical methods of the collaborator's laboratories. See figure 2.1 (chapter 2) for overview of the study.

The protocol for urine collection and fractionation was designed at UEA. Urine samples were fractionated as described in the Methods (section 2) and sent out to the specialist laboratories for analysis. UEA specialised in the harvest and analysis of exosomal RNA. At the end of the project data will be collated for bioinformatic meta-analysis by Dr Dan Brewer at UEA.

My role in the study was to: 1) gain ethical approval for the study, 2) set up the patient recruitment and sample collection from PCa patients and controls, 3) develop the urine fractionation protocols for exosomal RNA harvest, 4) Set up and maintain a file of the patient numbers and relevant clinical data, 5) collect follow-up data on patients, 5) prepare samples ready for Nanostring expression analysis, 6) provide insight and direction into what clinical questions needed to be answered.

3.2.1 Patient recruitment

In order to maximise patient recruitment, it was essential to interest the patient in the research. The documents describing the study (Protocol), Letter of Invitation, Patient Information Sheet, and Consent form had to be clearly written so that the patient could understand the study and what it entailed for them. Clear presentation of the study information to the patient would help the patient understand the potential impact of the study, clearly describe their role, what we were doing and why, and enable them to make an informed decision on whether to take part in the study. The documents were written with the patient's point of view in mind, and written in a layman's level of understanding, while covering all points of clinical and scientific interest. The documents were then assessed by public members who took part in a 'Public and patient involvement in research' group to weigh the public views about the study and to suggest changes to the created documents for

better understanding. Changes were then carried out according to the public feed back. It was found that the general idea was well supported. All the forms were then reviewed and approved by the PPIRES committee and by the Research Ethics Committee (forms are included in appendix 5). Agreement to allow the study to be carried out at the NNUH was sought from the R&D department. Full ethical approval for the study was obtained on the 12/03/2012. NHS approval to start the collection at the NNUH was gained on 15/03/2012.

3.2.2 Effect of the study on the patients normal clinical course

Recruitment into the study was voluntary, and the patient's clinical course was not altered because of the study. No research data from the study is returned to the patient.

All research participants were patients attending our clinics for evaluation of urological symptoms and would normally (outside any research setting) undergo an assessment, which includes history, abdominal, genital and rectal examination to exclude any prostatic pathology. The only change made for the purpose of this study is with respect to the digital rectal examination. The rectal examination is not usually standardised and is often clinician dependent; it usually involves a single swipe across the whole surface of the prostate in order to identify any underlying pathology (firm tissue or asymmetry). This was altered to two or three strokes per lobe –see section 2.3.3 for details

3.2.3 Patient Inclusion and exclusion criteria

In order to cover all the aspects of PCa screening, from diagnosis, prognosis and follow up, we tailored our recruitment process to included patients throughout their clinical journey, from the initial appointment for investigation, to treatment and follow up. The nature of the PSA assessment clinic (designed to see patients on the two week wait rule when refered due to raised PSA or clinically abnormal prostate with the suspicsion of prostate cancer) means not all urine samples would prove to be from cancer patients, for this reason the sample was categorized after the assessment, some of which would be benign that could be used in the benign arm (For further explanation about the different categories see section 3.4).

All patients attending the Prostate Specific Antigen (PSA) assessment clinic were eligible for recruitment. I also recruited patients for the control arm of the study from other

outpatient urology clinics for 'non-PCa' controls. These were men that were considered to be suitable candidates for the research and would normally – as part of their standard clinical treatment – have a digital rectal examination. This including patients who presented with haematuria (blood in the urine) but were found on further investigation to have no strong contra-indications for inclusion in the study; and patients with lower urinary tract symptoms caused by benign prostate enlargement in the presence of normal PSA.

3.3 Sample collection and labelling

Urine was collected post-DRE as described in Section 2.4. The DRE was performed for 2 reasons: 1) diagnostic i.e. a clinical requirement, outside the context of this research, and 2) to increase the amounts of prostate secretions in the urine. It was important that the samples could be centrifuged within 30 min of collection (see 3.8.1.6). In order to do this, I organised the setting up of a centrifuge and other essential equipment in a small area in the sluice room in the clinical department (see 3.7). The samples were centrifuged to sediment the cell pellet, this was then snap frozen on dry ice for subsequent RNA and DNA extraction in the laboratory at UEA. The supernatant was decanted into another sterile tube, placed on ice and transported to the laboratory at the end of the clinic (3 hours later) for further processing. The sample handling and transportation was in accordance with health and safety regulations for the transport of human tissue - in summary, samples were double bagged with absorbent material in case of leakage. These were then placed in a secure box. In case of leakage bags were handled at all times using gloves and eye protection.

Samples were collected straight after DRE for 2 reasons, 1) to minimise delays in the clinic so patients would not have to wait to provide a urine sample and 2) to prevent retrograde flow of the prostatic secretion into the bladder and dilution into a large urine volume that will lead to loss of the genomic material. For the purpose of comparing prostatic biomarker levels in pre- and post-DRE samples, a few samples were collected both before, and after DRE.

3.3.1 Sample labelling and anonymisation

All urine samples destined for the laboratory were labelled with a unique code from which only the principal investigator could identify the patients – see Section 2.4.1. I maintained a file with the important information linking the patient ID to the unique code identifier in an encrypted form and backed up on a secure NHS server with password protection. It was necessary to have this information so that clinical follow up data could be gathered on the patients.

3.4 Patient cohort

3.4.1 Patient motive for PSA check

Men were referred by their general practitioner to the NNUH for further investigation after a positive PSA test. There were a number of reasons for the PSA test: 1) screening as part of the health care prostate awareness program "Wellmen". Lower urinary tract symptoms such as physical obstruction from benign or malignant prostate cancer manifest as 1) storage and/or voiding LUTS including slow urine flow, straining to pass urine, feeling of incomplete emptying of the bladder, hesitancy to initiate a flow, frequency and urgency to pass urine as well as nocturia, 2) family history of prostate cancer, 3) urinary tract infection or, 4) haematuria (blood in the urine) (Figure 3.1). 63% of these patients were diagnosed with prostate cancer based on histological findings, which is consistent with the national figures. The vast majority of the study population consisted of patients of white background, with only 2 patients from an Indian background (no race variability that would affect our results). There was no association between cancer diagnosis and reason for PSA check.

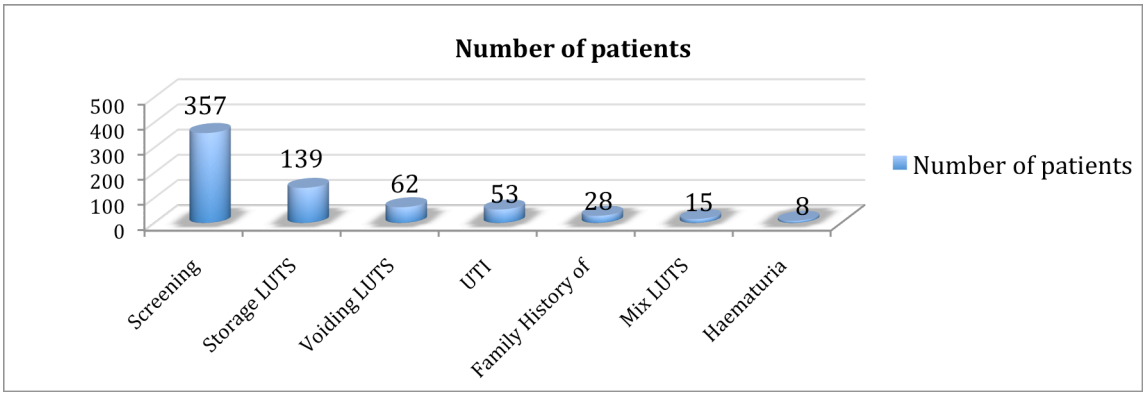


Figure 3.1: Patients in the study: reason for PSA testing.

3.4.2 Patient distribution into clinical groups

The cancer and benign arms of patients were designed as follows:

3.4.2.1 Cancer arm

The cancer arm was divided into 4 main groups based on the NICE risk stratification criteria for localised prostate cancer:

1- **Low risk group**: Patients with PSA <10ng/ml **and** Gleason score of 6 **and** clinical stage of T1a to T2a.

[T1 is when tumour is not detectable clinically or with imaging i.e. detected only histologically by biopsy or TURP. T2a: is when the tumour is clinically detectable (palpable on DRE) in half or less than half of one of the prostate gland's two lobes]

2- **Intermediate risk group**: Patients with PSA of 10-20ng/ml **or** Gleason score 7 **or** clinical stage of T2b to T2c (T2b: is where the tumour is clinically detectable in more than half of one lobe, but not both, T2c: is when the tumour is in both lobes but still contained within the prostatic capsule)

3- **High Risk Group**: Patients with PSA> 20 **or** Gleason score of 8 -10 **or** clinical stage of ≥ T2c (T2c is when the tumour envolve two lobes of the prostate, T3 is when the tumour has spread through the prostatic capsule and T4 is when the tumour has invaded other nearby structures)

4- **Advanced Group (positive control)**: PSA ≥ 100ng/ml with any Gleason score and clinical stage T3-T4. This group of patients is part of the high-risk group according to the NICE stratification criteria, however for the purpose of this study we set this group apart as a positive control. The reason for that is it is well known that patients with PSA of 100 or above have 100 % risk of cancer metastasis with 100% predictive value as reported in the literature (509).

In order to gain in-depth information and precise correlation of our biomarkers for diagnostic and prognostic accuracy, I subdivided some of the NICE groups into smaller groups according to disease aggressiveness (Figure 3.2). The reason for these subdivisions is because some of these patients fell in between two categories in the NICE stratification criteria, for example a patient with Gleason score of 7 and PSA of 20 would be classified in the high risk group according to NICE due to the highest variable (PSA>20) however this

patient's disease may not behave similar to a patient with PSA of 20 and Gleason 8-10 which would be truly in the high risk category. Also, it is well known that PSA readings are not always representative of the disease -see Section 2.3.6.

Similarly patients in the intermediate risk group with Gleason 7 (4+3) disease would be expected to behave more aggressively than a patient with a Gleason 7 (3+4) disease as the first patient will have more of the less differentiated cells (4 > 3). Hence we grouped these patients into subcategories taking into consideration all these variables (Figure 3.2) in order to give our analysis a better diagnostic and prognostic meaning.

The intermediate risk group is sub-divided into Intermediate-High Gleason (Ih) (G4+3 and PSA<20), and Intermediate-Lower Gleason score (I)(G3+4 and PSA<20) and Intermediate Low (IL) (G6 PSA>10).

We divided the high-risk group into High (H) (G7 PSA>20) and High-High (Hh) (G8-10 PSA<100).

No subdivision was made to the low risk group (L) or patients who were found to have prostatitis (P)

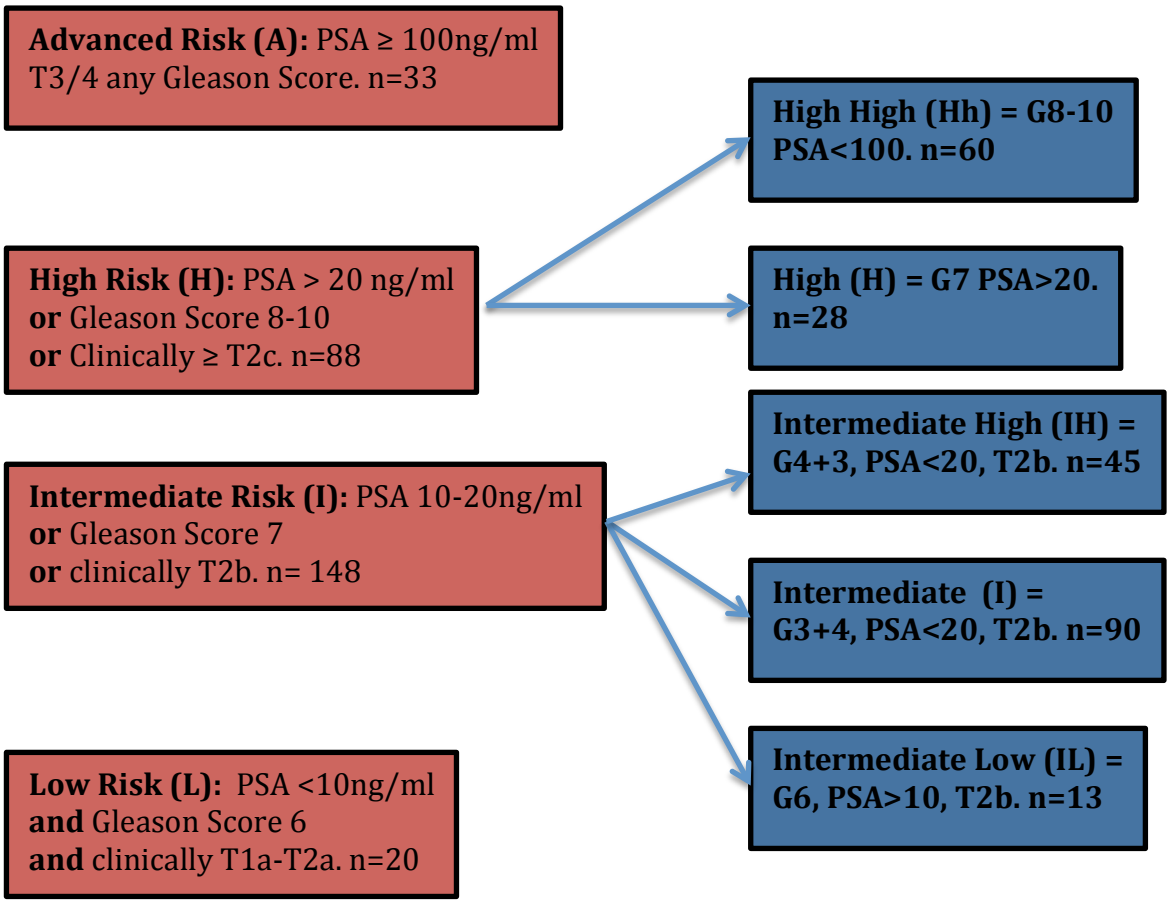


Figure 3.2: Patient clinical subgroups. 289 cancer patients devided into 4 groups and 5 subgroups

All Cancer patients were followed up and offered treatment that is recommended for their disease stage and grade and followed up on a 3 monthly basis with a repeat PSA test.

3.4.2.2 Benign Arm

The benign group was sub-divided into:

1- **HG-PIN and Atypia**: Patients with raised PSA and histological finding of Atypia or high-grade prostatic inter-epithelial neoplasia (HG-PIN).

2- **Prostatitis**: patients with raised PSA and histological finding of inflammatory changes in the absence of neoplasia

3- **Raised PSA negative biopsy**: patients with raised PSA and histological finding of normal tissue.

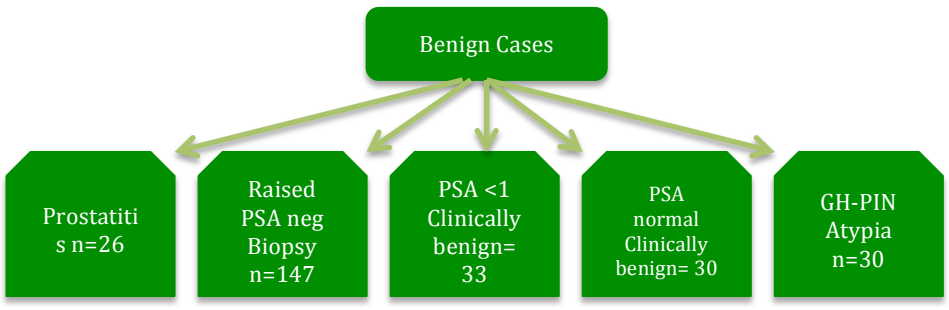
4- **Benign/non-cancer control group**: Patients with a clinically benign prostate (ie no palpable nodules or size asymmetry between the lobes) and PSA normal to Age or below 1ng/ml (according to NICE guidelines, PSA of 2.7ng/ml for men aged 40-49, PSA 3.9ng/ml for men aged 50-59, PSA of 5ng/ml for men aged 60-69 and PSA of 7.2ng/ml for men aged 70-75). These patients were recruited outside the context of the PSA-referral clinic, they presented with various urological complaints that required a DRE, but did not have prostate cancer or a raised PSA.

This group of clinically benign patients was again subdivided into two smaller groups:

a) Patients with PSA<1 (CB1)

b) PSA>1 but normal to age (CBN)

We used both these sub-categories in the benign control accepting that there is a small risk that some of these patients would still have prostate cancer, which is most probably, insignificant due to the fact that they are asymptomatic and have clinically undetectable disease in the presence of normal PSA. Should these patients have PCa it would be expected to be low volume, low-grade disease that would require no clinical attention. The reasons for using these sub-groups as 'non-cancer' controls is explained in the Discussion.



All the patients recruited for the benign control group were seen due to haematuria (blood in the urine) with normal haematuria investigations.

All patients with raised PSA and negative biopsy were followed up at 3 months with a repeat PSA. Patients whose PSA had normalised were discharged, and those with rising PSA were offered a repeat biopsy.

3.4.3.3 Patient's clinical distribution and risk factors

In order to identify the breadth of the clinical problem we grouped patients according to their risk groups (Figure 3.3) and found that the majority of patients in the cancer arm fell in the intermediate risk category, while in the benign arm, patients with raised PSA and negative biopsy were predominant. These findings clearly highlight the clinical problem associated with PSA screening, as it is clearly demonstrated here that a large proportion of these PSA referral patients (Raised PSA negative biopsy), had unnecessary anxiety and invasive procedures such as TRUS biopsy, as well as creating unnecessary workload and financial burden to the health care system. In addition, these patients would require further PSA monitoring and possibly more biopsies in order to exclude prostate cancer. Hence the urgent need for novel biomarkers that will help identify these patients.

All PSA-referral patients underwent trans-rectal prostate biopsy as per the clinical requirement except patients in the advanced risk group (27 patients) as their diagnosis was made on clinical findings and no biopsies were necessary. Patients in the benign control group with PSA<1 or PSA normal for age were also not biopsied, as there was no clinical requirement for biopsy in order to exclude cancer.

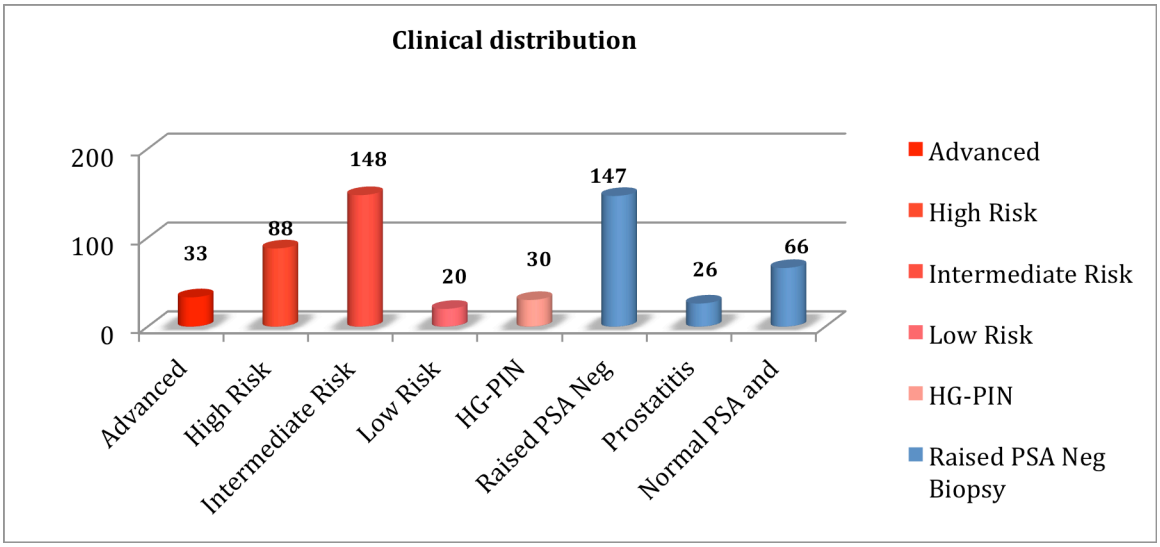


Figure 3.3: Patient distribution according to clinical groups.

3.5 Patient Cohort and rational

In order to get clinically analysable data we initially matched our sample size to that of the published urine marker *PCA3* (*DD3*) study of 200 patients (497). However, after a revision of the numbers of the various groups of patients we are studying in this project with hypothetical statistical analysis it became apparent that the initial number we opted for was too small and had to be increased to approximately 1200 patients as explained below.

The increased numbers of samples is necessary because of the following reasons:

A) prostate cancer has an extremely diverse range of progression. Therefore we needed to collect a large number of samples to enable us to have sufficient statistical power from each of the patient groups, which include: i) negative controls, including patients with PSA <4ng/ml and PSA <1ng/ml. ii) BPH (Benign prostatic hyperplasia) iii) patients with low risk of progression and metastasis (PSA >4 and <10ng/ml or Gleason <6) iv) patients with intermediate risk of progression and metastasis (PSA >10 and <20ng/ml or Gleason 7) v) patients with high risk of progression and metastasis, (PSA >20 or Gleason 8-10).

B) The majority of the patients that have prostate cancer will be in the intermediate risk of progression, some in the High-risk/advanced category and some in the low risk.

Approximately 10% of these will progress each year, but we do not know which ones. This means that we must collect additional samples so that we have sufficient numbers of those that will progress.

C) Approximately one third of the patients referred to the NNUH PSA clinic were from the High PSA negative biopsy group.

D) Prostate cancer can progress down multiple pathways with varying risk of metastasis. So, to fully assess patients' risk of progression in the different risk categories (see above) we require large sample numbers.

To look for multiple comparisons of correlations between gene expression levels and the presence of cancer, we used standard statistical methods for assessing correlations and, Hierarchical Clustering and Latent Process Decomposition analyses to determine whether distinct subgroups of patients can be identified. In these analyses we specifically looked to see whether identifiable patient groups exist that could have avoided prostate biopsy. Dr Dan Brewer hypothesised the following: to calculate the power of individual markers the statistical question was reduced to a test of equal proportions with a binary outcome (cancer present/absent) and a binary variable (biomarker positive or negative; below or above

median of expression). We calculated the minimum difference in proportion of patients that have cancer in the biomarker positive and negative groups that could be detected by the study when using a significance level of 0.05 and a power level of 0.8 using the bpower function in the R statistical programming language. For a 200 patient cohort this percentage varied from 30% to 20% for a marker positive in between 10% and 40% of cases. This calculation only considers a comparison of two variables. However in the case of 3 different primary cancer risk categories comparison with two non-cancer groups of patients and metastatic disease, we needed to increase the numbers so that any two way comparison consists of 200 patients in order to get a similar power. Thus for 6 groups we needed to collect 600 patients. The fall out rate due to poor sample quality and because risk groups can only be determined after the sample has been collected is about 50%. So overall, Dr Brewer calculated that we needed to collect 1200 specimens to appropriately power this study.
The initial expression analysis was performed on 192 samples as a pilot study, which will then be followed by a larger number to validate the data.

3.6 Clinical Data collection

Biomarker data is of no use when used independently of other information, thus, it is highly important to collate appropriate clinical data. For this reason collection of the relevant clinical information on the day of the consultation is vital to avoid revising large number of patient notes at a later date or even arranging further consultations to gather missing data. Taking into consideration the risk factors of prostate cancer, and medical treatments that can affect the disease such as 5-alpha-reductase inhibitors, I created a proforma to collect and organise the clinical data in a manner that would facilitate statistical analysis. The collected data included, patient's demographic (age and ethnicity) previous and current PSA check and the reason for PSA testing, symptoms including lower urinary tract symptoms (LUTS), haematuria or urinary tract infection, recent blood results including Urea and Creatinine (U&E) and Liver function test (LFT) (Patients U&E's and liver and kidney function tests could be Deranged due to metastatic disease), full blood count (FBC) (anaemia due to bone metastasis) as well as past medical history and current medication, family history of prostate cancer and social history including smoking, alcohol consumption and occupation. I also documented patient 'detailed histological findings' including Gleason score information

(when PCa was found on biopsy), as well as the number of cores taken during the biopsy and the percentage of the disease involved, whether or not there is perineural invasion, the ultrasound scan finding and prostate volumes. Investigations including MRI scan, bone scans and post-radical prostatectomy histology results were also reported as well as PSA follow-up for all the patients.

An international prostate symptoms score (IPSS) sheet to assess the patients urinary flow symptoms was provided in advance to all patients attending the prostate cancer one-stop clinic. (See appendix 4 for the IPPS score sheet and appendix 6 for the clinical information collected on all patients).

3.7 Protocol optimisation and results

3.7.1 Functional set up of the Clinic

Two clinics a week were set up for patient recruitment. The success of this project relies on clinical and scientific work harmony, for this reason a good clinical set up where clinical and lab work can go alongside each other was key. In order to achieve that, I utilised the PSA clinic on a weekly basis where I could consult a maximum of 10 patients referred by their GP due to raised PSA for further investigations. I also set up a room in an adjacent clinical area, which was equipped with the essential tools to carry out the initial steps in processing the urine samples including centrifugation and slide preparations for FISH (Fluorescence *in situ* hybridisation) (this was for a parallel line of research running alongside this project in our labs). I also accessed the haematuria clinic (where patients were investigated for the presence of blood in urine) to collect samples for the benign/non-cancer control arm, as these patients would be young men and likely to have a healthy prostate gland (see 3.4.2.2). I have also organised regular meetings that gathered scientists and clinicians together to discuss the project and ensure that the research aims are based on clinical needs.

A total of 662 men were recruited at the end of this study period with an average age of 68 (Age range 35-98).

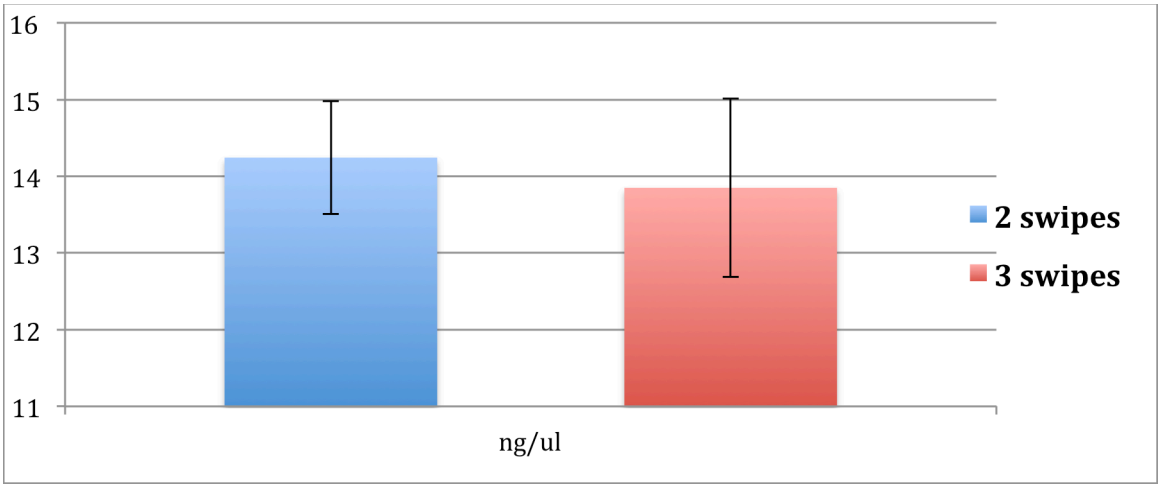
We had good compliance from our patients with only 4 patients refusing to consent for the study.

(See appendix 1 to 4 for letter of invitation, consent form and PPRiES sample sheet)

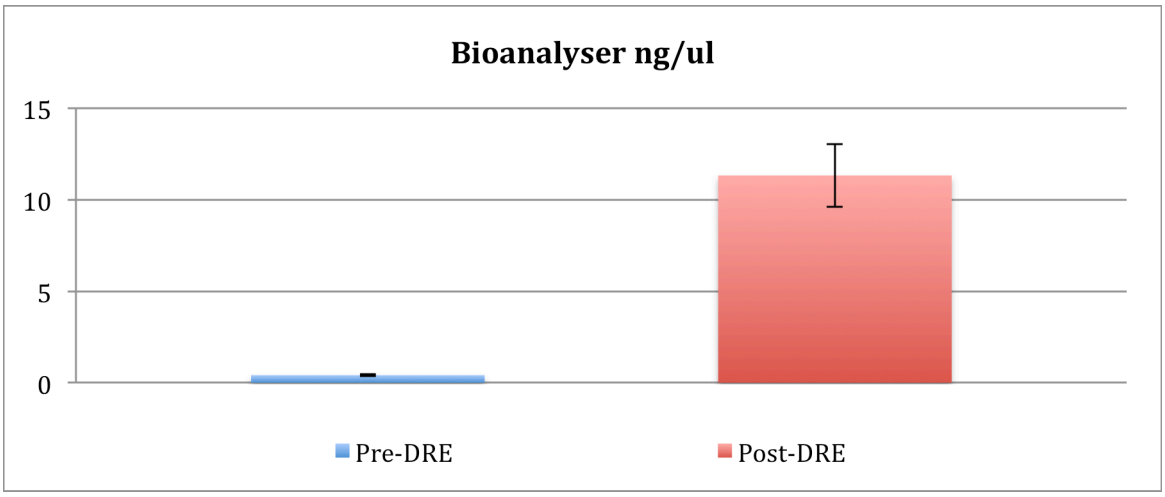
3.7.2 DRE optimisation

During the course of this study I noted that the DRE technique we used (PCA3 technique of 3 swipes) could cause slight discomfort to patients. For this reason I tested different DRE techniques comparing the PCA3 3 swipes per lobe depressing the prostate tissue 1cm each time to 2 swipes each lobe instead to find out whether a lighter prostate massage would affect the RNA quantity and the patients experience. The experiment was carried out on two consecutive clinics on seven patients in each clinic. The RNA as measured by a Nanodrop spectrophotometer showed no significant difference between the two sets of samples (see figure 3.4). However it is worth noting that RNA yield varies significantly between samples. Following this experiment I continued using the 2-swipe technique for the rest of the project with no change in our RNA yields. Clinically it is quite apparent that there is a variability in prostate glands in term of size, anatomy (some are high riding and difficult to reach in order to perform a consistent DRE) and texture (cancer tissue feels very hard and patients with Advanced cancer had prostates that were impossible to depress during DRE). All of which can cause variability in the amount of prostatic fluids that can be persuaded out of the gland by DRE.

We also looked at the difference in the RNA yields between samples before and after DRE, Patient was asked to provide a sample on arrival to the clinic and another sample later on after DRE (after the consultation). This has proven the prostatic origin of the exosomes (Figure 3.4).



A



B

Figure 3.4: A) Shows the mean difference in the RNA quantity in ng/ul between the two groups of patients who had 2 and 3 swipe prostate massage prior to the urine sample collection. This is an average of 10 samples in each group. 3-swipes average 13.7 ng/ul SD +/- 7.58 vs 2 swipes 14.1 ng/ul SD +/- 8.92
B) Shows the mean difference in the RNA yields in ng/ul between samples Pre and Post DRE taken from the same patients.

3.7.3 Urine processing optimisation

Due to the nature of urine as a waste product it does not provide the ideal milieu for cells to survive long enough to allow RNA extraction several hours after the sample collection, for this reason several issues had to be addressed in order to optimised the protocol (See Sections 2.6.2 and 2.6.2).

In order to improve the exosomal RNA, and speed up the protocol I improved on each of the processing steps, and some additional features of the protocol as described below.

These improvements reduced the exosomal RNA harvesting time significantly, which enabled us to complete the RNA extraction in the same day. Performing the extraction in a single day has itself led to improvements in RNA yields and quality.

3.7.3.1 Cell Pellet extraction

Urine was initially collected from patients immediately after the digital rectal examination and stored on ice for 3 hours (ie until the end of the clinic) before processing in the UEA lab. The cellular RNA yields at this point were poor (average of 4·6ng/ul), and in many cases were not sufficient for multiplex gene analysis. Despite trialling several cooling techniques with wet ice, crushed ice, and water/ice mix, no changes in the RNA yields were found. Microscopic examination (using the cytospin and H&E staining) of the individual sample pellets after centrifugation showed no evidence of cells, both in wet and dry ice, However there were abundant numbers of cells when the sample was spun immediately (Figure 3.5 A) suggesting that the cells were either affected by freezing the sample which caused the cells to burst and lose RNA or that urine was just not suitable for cell survival for this period of time. I tested both theories; firstly by testing and correcting the urine medium to find out whether I could prolong the cell survival. Urine pH was initially tested on 5 samples (pH range 6 to 8), and corrected by adding 10xPBS to a neutral pH of 7, however this had no effect on the cell survival as measured by no change in the RNA yields, nor microscopic detection of cells. The next test was to treat the urine with cell culture media (RPMI+++ and 10xRPMI) aiming to prolong cell longevity in urine; here there were a noticeable improvement in the cell RNA yields (24.0 ng/ul SD 28.78). 25 samples used in this series) from previously 4.6 ng/ul SD 1.60, 8 samples used in this series) with the same

RNA extraction technique (Qiagen RNA kit) used in both experiments.

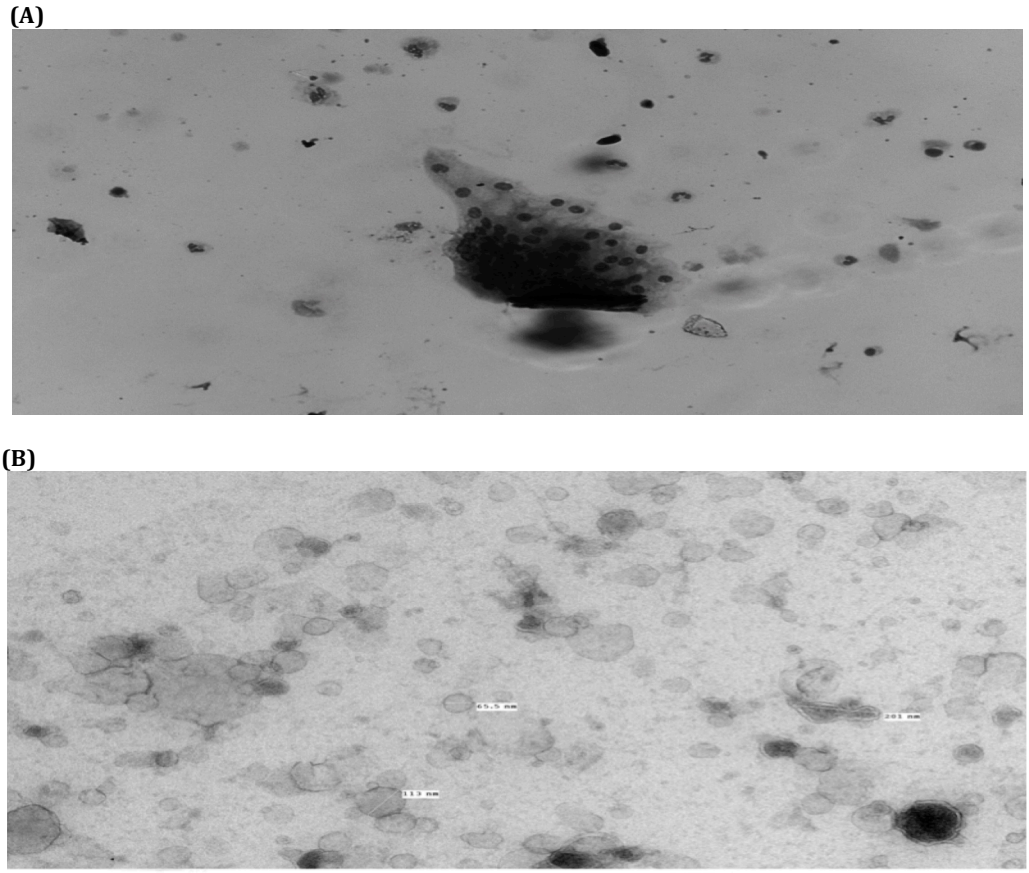


Figure 3.5: Microscopic examination of urine samples showing cells and microvesicles.
(A) Epithelial cells in urine following immediate processing by centrifugation after sample collection, (B) Microvesicles post microfiltration harvest (measuring 50-120 nm). The presence of microvesicles in the micro-filtered urine samples was confirmed by electron microscope analysis by Rachel Hurst at UEA (figure 3.5 B).

As the prime aim of this study was to investigate exosome/microvesicle derived urine biomarkers rather then cell derived biomarkers, my concentration shifted toward optimising exosomal RNA extraction using an already established protocol for exosomal extraction (developed by Professor Cooper's group).

3.7.3.2 Urine pH and Exosomal RNA yield

These samples contained cell-culture media (see 3.8.1.2), which controlled the pH to some extent. However, a range of pHs were found in samples- see figure 3.6 No significant difference in RNA yields from urine samples at different pHs were found

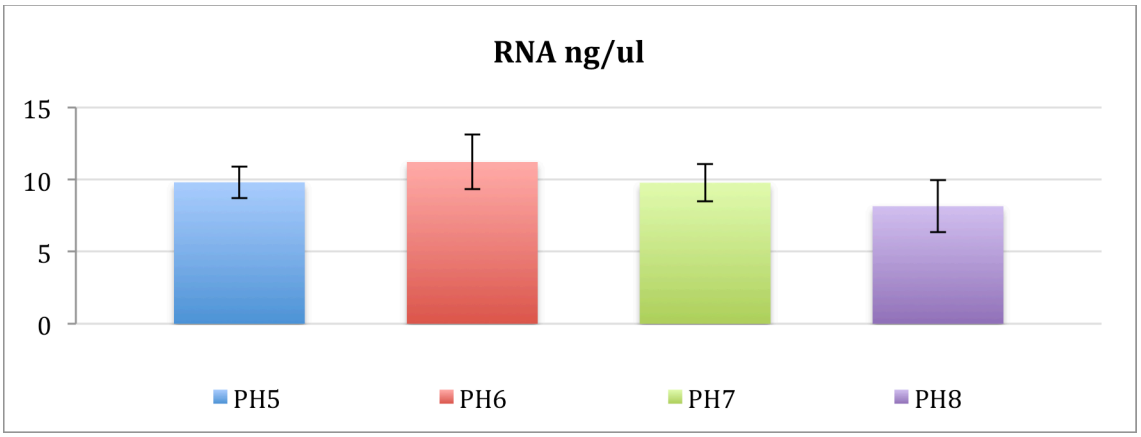


Figure 3.6: Effect of urine pH on exosomal RNA yields.

3.7.3.3 Urine Volume and Exosomal RNA yield

The existing protocol stated that 30 ml of urine was to be collected per patient. However some patients were not able to provide the whole 30 ml sample as well as provide another sample for urine dipstick testing (which is a clinical requirement to exclude urine infection prior to the TRUS biopsy). The question was, was 30ml of urine absolutely necessary. In order to answer this I compared urine samples from 30 patients, 10 of which had provided a 10ml sample, 10 had provided 20ml and 10 patients with sample volumes of over 20 ml as shown in figure 3.7 below. This experiment showed that a 20 ml urine sample appears to be the optimal amount of urine to gather the prostatic secretion. The smaller sample volume appears to gather just over half of the prostatic secretions. This effect may be due to the physical urethral volume. The urethral capacity is normally about 10ml, and I hypothesised that as we are aiming the get the prostatic secretion from the urethra we would expect the first 10ml of passing urine to collect the majority of the prostatic secretions, with the next 10 ml of flow-through washing the residual secretions. Collection of a large sample volume appears to give a poorer yield – reasons unknown.

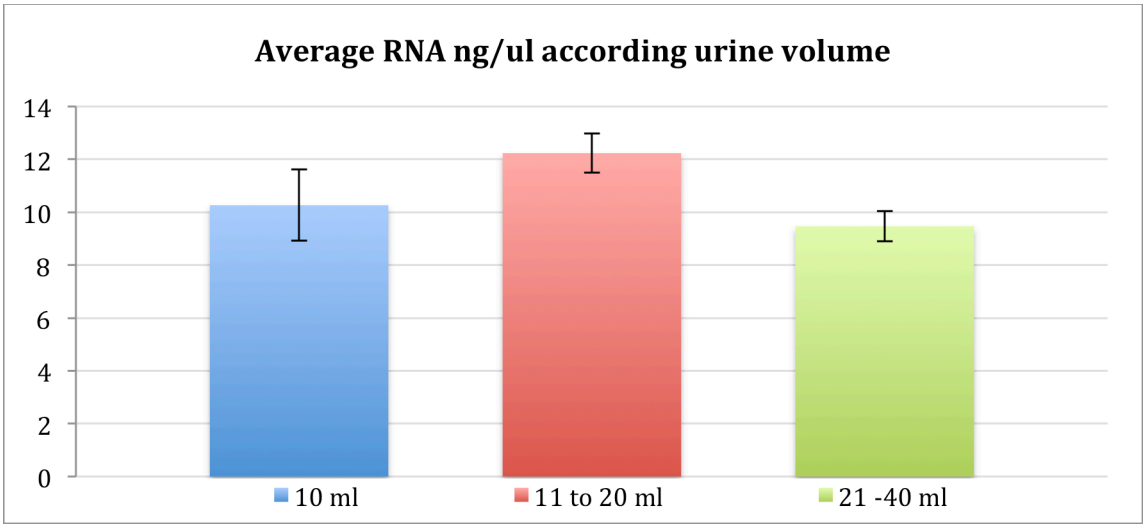


Figure 3.7: The difference in average RNA yields in samples collected using different urine volumes.

3.7.3.4 Urine Dip stick results and Exosomal RNA yield

Results taken from a urine dipstick appeared to have no obvious link with exosomal RNA yields. The average RNA yields harvested from urine samples positive for leucocytes only were 8.8 ng/ul (of 31 patients), those positive for blood only was 13.5 ng/ul (40 patients), protein 7.7 ng/ul (10 patients). The rest of the patients had a combination of leucocytes and or protein and blood. Interestingly we found that patients who had positive nitrites suggesting bacterial infection had very low yields of exosomal RNA and very high yields of cellular RNA (4.2 ng/ul and 455 ng/ul respectively) in comparison to the rest of the samples. One explanation for this could be that the exosomes could be removed by the bacterial or immunological cells, or are adhering to the bacterial or immunological cells that are abundant in these samples, and are thus removed by centrifugation prior to filtration harvest. It is also possible that a some of the cellular RNA was bacterial in origin.

3.7.3.5 Processing speed

Acquiring a centrifuge at the clinical site enabled me to harvest the cell pellet by centrifugation immediate (within 30min) of collection rather than after 3 hours on ice. Immediate centrifugation dramatically improved the exosomal RNA yields while decreasing the cell RNA yields. Exosomal RNA yields increased to 32.2ng/ul +/- SD 52.7. (n=25).
This was the only protocol change, the exosomal RNA extraction itself being unchanged and performed 3 hours later as in the original protocol. It was hypothesised that the exosomes

might be sticking to the cells, and the longer the cells are kept in the urine samples in the company of the exosomes; the more exosomes bind to the cells and come over in the cellular fraction. See figure 3.8.

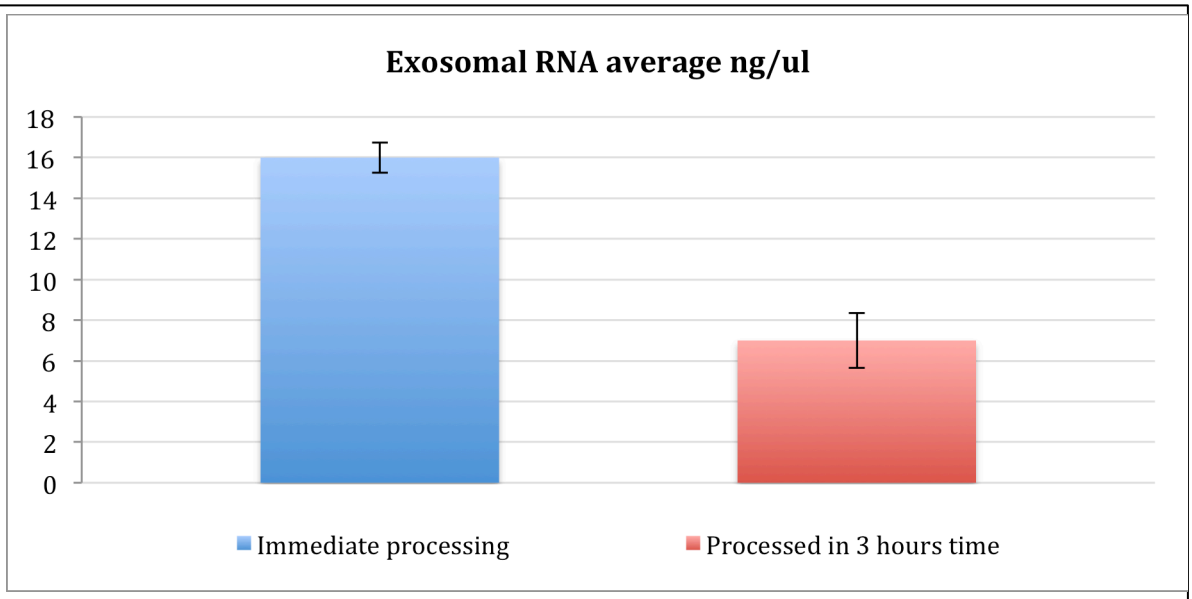


Figure 3.8: Statistically significant differences in RNA yields when the samples were processed immediately in comparison to 3 hours ice incubation (P=0.01)

3.7.3.6 Qiagen RNeasy kit improvements

Small improvements were made to the Qiagen RNeasy protocol:

a) Increasing the amount of buffer (RLT) used to lyse the exosomes from 350ul to 700ul, improved the RNA yields from an average of 7.3ng/ul to13ng/ul.

b) Wash step: All the centrifugation steps were reduced to 10 minutes each with no effect on the RNA yields.

3.7.4 Amplification

As yields of RNA from many of the samples were too low to directly perform expression analysis on, it became necessary to use an amplification procedure, which converted the RNA into cDNA.

The amplification process was carried out using the NuGEN Ovation® Pico and PicoSL WTA Systems V2. According to the manufacturer's instructions the kit can amplify as little as 500 picogram of RNA. However a range of input amounts had been previously tested in

our labs (data not published) and 10 to 20ng of RNA was found to be a good compromise to gain consistent results. Though as little as 1ng could be used if necessary and still maintain reasonable consistency of expression.

I had measured all of the exosomal RNA samples on a Bio-analyzer (Agilent 2100 Bioanalyzer system) using a Pico RNA Assay Chip to estimate quality and quantity of the RNA prior to amplification. Due to the low yields of RNA in the advanced and high-risk group we opted to compromise on these samples by amplifying as low as 2.5ng of RNA per sample. For the rest of the samples a total of 10-20ng per sample was used. There were no significant difference in the post amplification cDNA yields between the high and low input amount groups (See table 3.1) and there were no obvious effects on the gene analysis.

Table 3.1: Average Bioanalyzer measured RNA yields and the RIN scores pre-amplification and average cDNA yields post amplification. RIN is the RNA integrity score which is an indication for RNA quality (score 1-10) more explanation available in the Chapter 3 (text 2.5).

Samples Amplified	RNA Bio analyzer ng/ul	SD	RIN score	SD	cDNA Nano drop ng/ul	SD
Total amplified n=334	7.65	8.78	4.84	1.33	180.97	58.94
RNA yields >2ng/ul n=232	10.60	9.08	4.71	1.14	193.89	50.65
RNA yields <2ng/ul n=102	0.95	0.52	5.15	1.68	152.99	64.86

3.8 Exosomal RNA yields and Clinical category

Exosomal RNA yields varied between samples, ranging from 2 to 57 ng/ul with a mean of 10ng/ul (SD 10.4 ng/ul). The mean 260:280 optical density ratio was 2.26 (SD 4.04).

The RNA yields varied significantly with cancer grade and stage, with for example a mean of 13 ng/ul in the intermediate risk group to 3.5ul/ul in the advanced risk group, see figure 3.9.

Exosomes are well documented to be more abundant in cancer tissue in comparison to benign (238,268) which is consistent with my findings that exosomal RNA yields are low in benign samples, higher in low risk samples, and peak in the samples from the intermediate risk group. Yield then decreases in high, and is lower again in the advanced-risk samples.

The cell RNA quantity and quality followed a similar trend.

This variability could be related to the change in the anatomical structure of the prostate gland in these cancer patients. In high-grade disease (Gleason 8-10) there is great disruption of the inner prostatic tubules and ducts that normally provide egress of the prostatic secretions into the urethra. The passage of secretions into the urine is therefore limited. Another reason for the low yields in the high-grade and advanced groups could be the prostatic massage by DRE itself. The prostate in high Gleason tumours becomes very hard due to packed cancer cells invading the surrounding tissue. This makes it impossible to depress the surface of the prostate during DRE to persuade egress of prostatic secretions. However in the intermediate risk group, (patients of Gleason 7 disease) there is no great disruption of the prostatic anatomy, hence the high RNA yields.

As can be seen, the amounts of exosomal RNA from patient's samples post prostatectomy are very low, this implies that the majority of the exosomal RNA is prostatic in origin and not from the bladder, kidney, urethra etc.

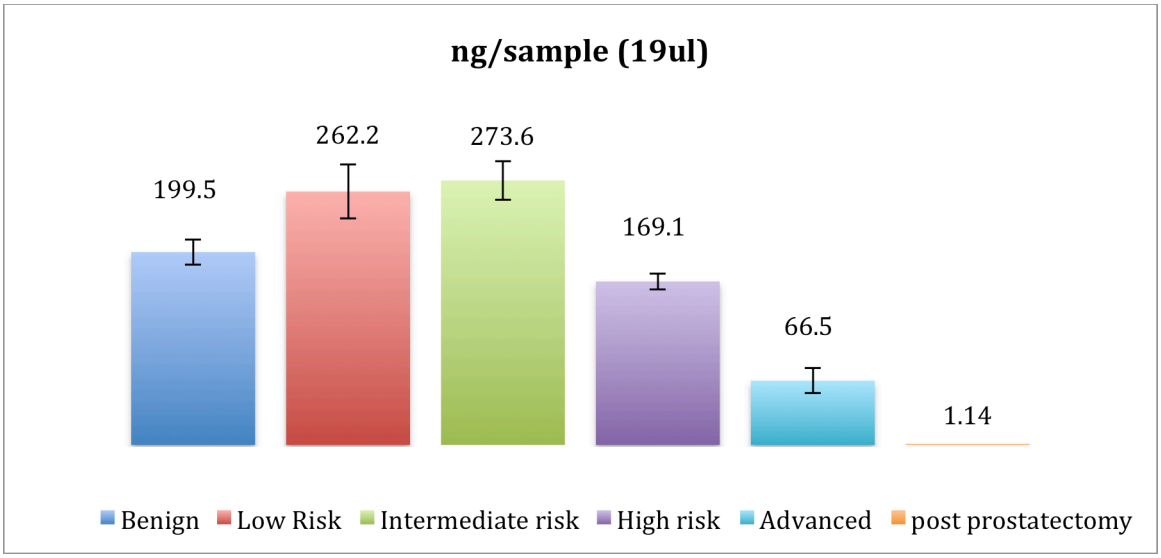


Figure 3.9: Exosomal RNA yields in relation to patient's disease, and prostatectomy

Detailed discussion on the above information is presented in Chapter 5

4: Results of the Study

4.1 Clinical Parameters and Patient History

A number of features were examined.

4.1.1 Alcohol consumption

66% of patients in this cohort drank alcohol on a regular basis with an average of 19.8 units per week (range from 2-100 units). 53% of patients that reported alcohol consumption were diagnosed with prostate cancer (figure 4.1). The $\boldsymbol{X}$-square test, which was performed to assess the significance of alcohol consumption on the different risk groups, showed a significant difference with (p=0.0001 and $\boldsymbol{X}$ =27.74)

The information provided by patients about the amount and the period of alcohol consumption in units was not expected to be entirely accurate (see Discussion) For this reason the amount of alcohol and the period of alcohol consumption was not taken into consideration in the analysis.

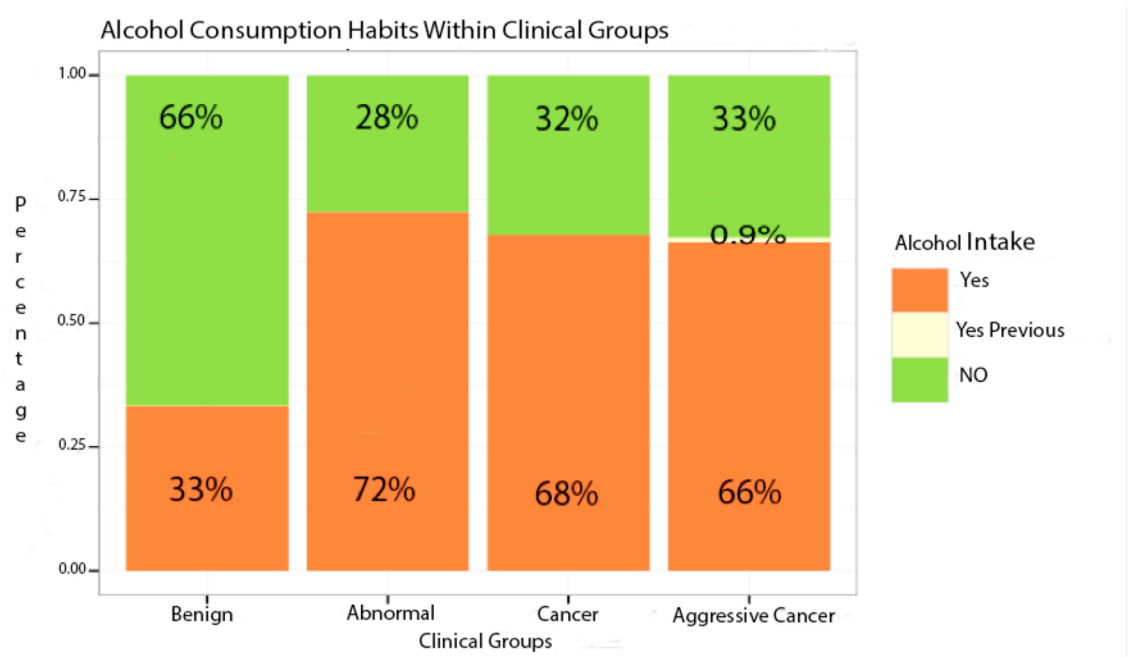


Figure 4.1: Distribution of patients in relation to alcohol consumption and prostate cancer.
507 patients
Benign = CB1 and CBN. (Yes n= 14, No n= 28)
Abnormal = raised PSA negative biopsies, HG-P, Prostatitis. (Yes n= 147, No n= 56)
Cancer = Intermediate (IH and I) and Low risk (Yes n=103, No n=49)
Aggressive Cancer = Advanced and High risk (Yes n=73, Yes Previous n= 1, No n=36)

4.1.2 Smoking

Cigarette smoking had been reported to be associated with an increased risk of prostate cancer (510).

175 patients (33%) reported that they smoked or had smoked within the past 15 years. 106 of them (60%) were diagnosed with prostate cancer (figure 4.2). In the cancer group there was no correlation between the numbers of cigarettes smoked daily and cancer grade, it is worth noting however that we did not accurately document the length of period these patients smoked or their pack years, which may have an effect on these findings. However statistical analysis using the X-square test showed that there was a significant association (p=0.03 and X=14.17) between smoking habits and clinical group. Comparing non-cancer to cancer also yielded a significant result (p=0.02, X= 12.13).

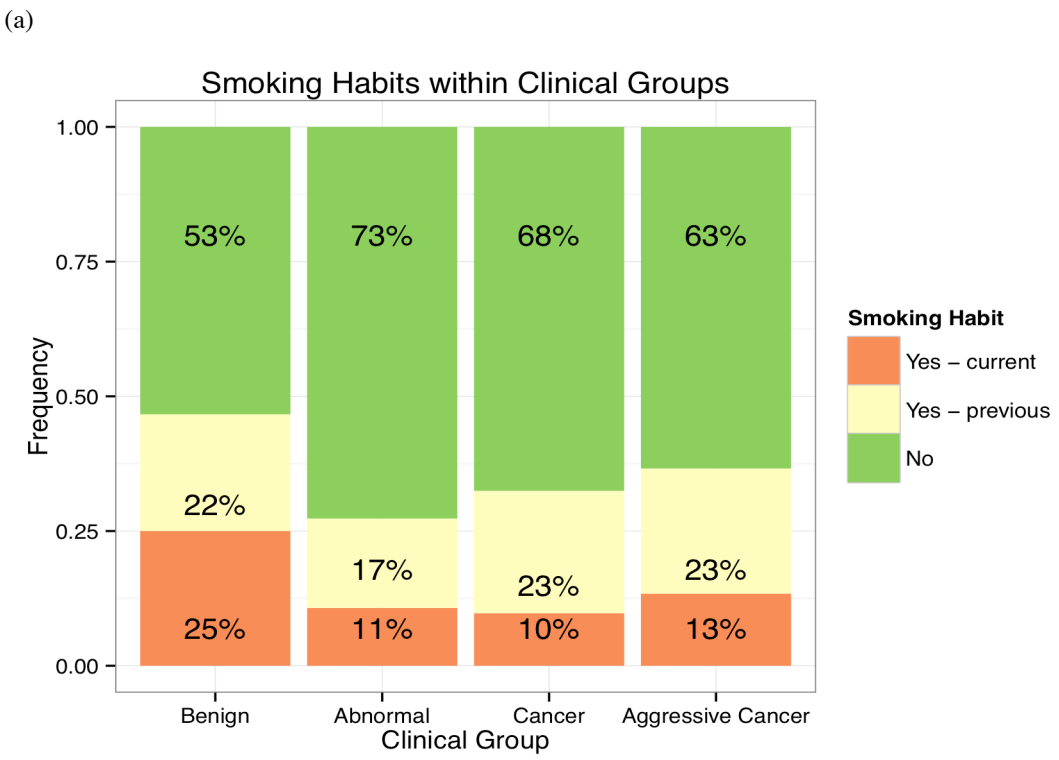


Figure 4.2: Distribution of patients in relation to smoking and prostate cancer.
531 patients
Benign = CB1 and CBN. (Yes n= 15, Yes Previous n= 13, No n= 32)
Abnormal = raised PSA negative biopsies, HG-P, Prostatitis. (Yes n= 22, Yes Previous n= 34, No n= 149)
Cancer = Intermediate (IH and I) and Low risk. (Yes n= 15, Yes Previous n= 35, No n= 104)
Aggressive Cancer = Advanced and High risk. (Yes n= 15, Yes Previous n= 26, No n= 71)

4.1.3 Medication

Beside the social risk factors above, I also examined patient's previous medication history with 5-alpha-reductase inhibitors. The reason for this is because 5-alpha-reductase inhibitors have been reported as being preventative for PCa in the Prostate Cancer Prevention Trial (PCPT), which randomised 18,882 patients with PSA <3ng/ml to either a placebo arm or a chemoprevention arm (patients receiving 5 alpha-reductase inhibitors). A 25% reduction in relative risk of developing prostate cancer was reported in the PCPT trial in patients receiving 5 alpha-reductase inhibitors, however these patients had higher incidence of developing high-grade disease.

In our study 85 patients (15%) used these medications for a period of time prior to their presentation to our clinic (2 months to 15 years). 43 patients (50%) were diagnosed with prostate cancer, 11 (13%) of them were on 5-alpha reductase inhibitors of which 8 (9%) was diagnosed with high grade disease and 3 intermediate risk disease. This distribution of patients between high grade and intermediate grade disease was not mirrored in patients on alpha-blockers only, see table 4.1 and 4.2. Patient numbers were too small to make any firm conclusions.

Table 4.1: Distribution of patients in relation to alpha-blockers and 5 alpha reductase inhibitors intake.

	Total number of patients on medication	**Alpha blockers**	**5 alpha-reductase inhibitors**	**Combination of both**	**Neither Drug**
Cancer	43	32	8	3	247
Non-cancer	31	31	3	5	183
HG-PIN	11	10	0	1	22

Table 4.2: Distribution of cancer patients disease grade in relation to alpha-blockers and 5-alpha reductase inhibitor intake.

Cancer Patients	**High Risk**	**Intermediate**	**Low**
5 alpha-reductase inhibitors	6	2	0
Alpha blockers	16	15	1
Combination of both	2	1	0
Neither drug	99	129	19

4.1.4 Family history of prostate cancer

12% of patients attending the PSA referral clinic reported evidence of a family history of prostate cancer. 54% of men reporting family history were diagnosed with having the disease themselves. In contrast 44% of patients who did not have a reported family history of PCa were diagnosed with the disease. Statistical analysis using the *X*-square test shows no significant association between family history and prostate cancer (p=0.53 and $\boldsymbol{X}$ =2.21) (Figure 4.3 A, and table 4.3). Most of the patients who reported a family history of PCa and who were diagnosed with the disease had an affected young close relative (32% had an affected brother or father by the age of 60) (Figure 4.4). An affected brother was predominantly reported (50% affected brother, 38% affected father). Most of these patients were diagnosed with intermediate risk disease in a similar age range to their affected relatives (Figure 4.5). However the group of patients who had an affected father (13 patients 38%) were diagnosed themselves with predominantly higher risk disease (69% high risk) in comparison to the ones with affected brother (12.5% high risk) (figure 4.6). The statistical analysis reported no significance when patients had a close relative affected with the disease *X*-square test (p=0.66 and $\boldsymbol{X}$ =1.6) (Figure 4.3 B and table 4.4).

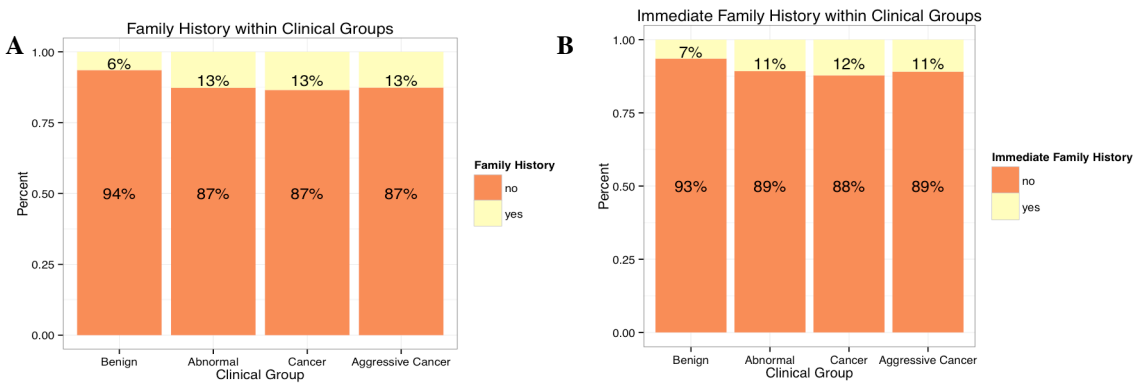


Figure 4.3: (A) Patient distribution according to their family history of prostate cancer. (B) Distribution of patients in correlation to immediate family history of prostate cancer.

Table 4.3: Distribution of patients according to their diagnosis and their family history of PCa n=542 samples

	Yes	No
Benign	4	58
Abnormal	26	179
Cancer	21	135
Aggressive Cancer	15	104

***X*-square test shows there is no significance (p=0.53 and $\boldsymbol{X}$ =2.21)**

Table 4.4 Family History First Order Family – 542 samples

	Yes	No
Benign	4	58
Abnormal	22	179
Cancer	19	135
Aggressive Cancer	13	104

***X*-square test shows there is no significance (p=0.66 and X =1.6)**

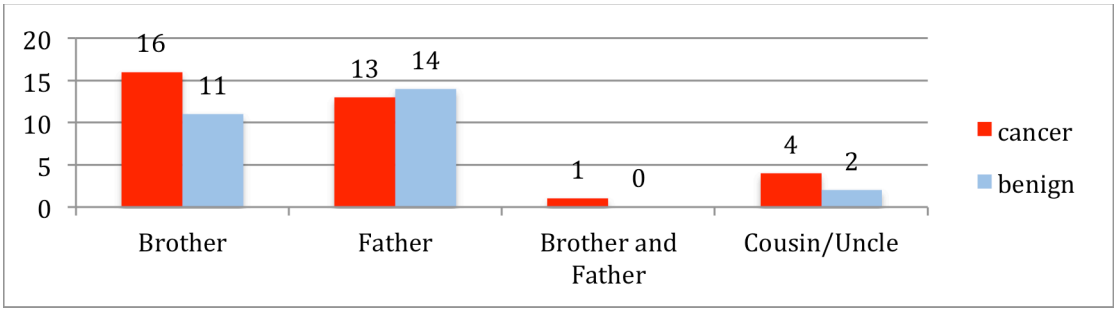


Figure 4.4 Prostate cancer patients with positive family history, distribution in relation to their reported positive relatives. The red group is patients who had positive family history and were themselves positive for PCa. The blue are patients who had a positive family history but were negative themselves.

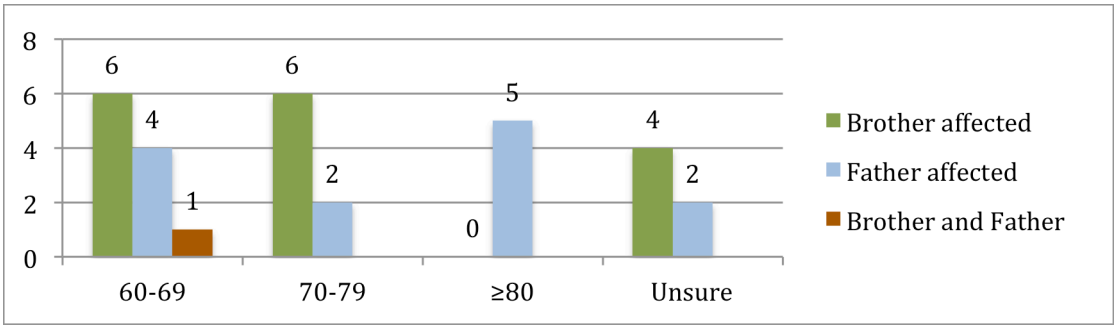


Figure 4.5: Distribution of cancer patients (number of patients) according to the age of their affected relatives at the time they were diagnosed with the disease.

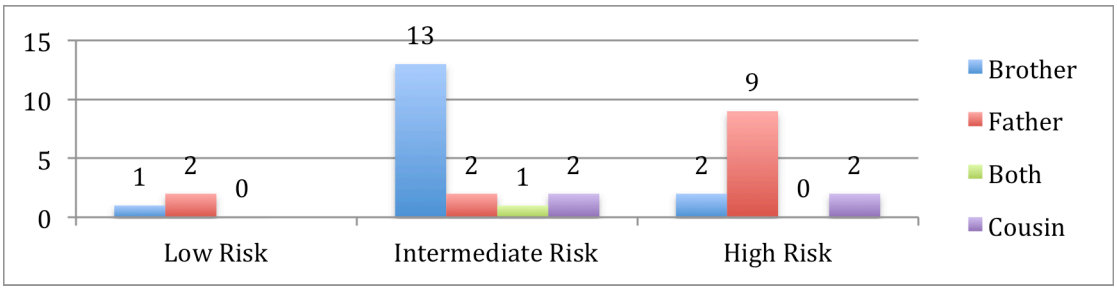


Figure 4.6 Distribution of cancer patients according to their disease grade (Low, Intermediate and high) in relation to the affected relatives.

4.2 NanoString Expression Analysis

Further information on NanoString technology can be found in chapter 2.

4.2.1 NanoString: Pilot NCounter human Cancer Gene Set analysis

NanoString probes are single stranded, designed to hybridise and detect mRNAs, and are not able to hybridise to the complementary strand of cDNA. As sample amounts were limited, it had been necessary to amplify them as cDNA. We were therefore unsure how effectively NanoString technology would work. The Nugen Company that manufactures the Ovation amplification kit assured us that it amplified both strands equally, and the cDNA and its complement would be in the amplification products in equal proportions. However, NanoString Inc informed me that this sort of sample had never been tested with NanoString before, and so, I carried out a pilot analysis on 12 samples (6 in the high risk group: G8-10 PSA>20, 3 benign control: PSA<1 and clinically benign prostate, and 3 in the low risk group: Gleason 6 PSA 0<10) prior to analysing large number of samples. This pilot was using an off-the-shelf 'cancer gene' assay, which would tell us: i) whether the overall process was working, ii) whether prostate specific genes would be detectable and iii) whether we can differentiate cancer from benign disease. However this pilot study was not aimed to identify cancer markers nor significant variable expression as we used very small number of samples from the extreme ends ie High risk and Benign with no normalisation.

The nCounter human cancer 236-gene reference assay detected expression of 189 out of 236 genes in our samples In addition, 20 genes showed differential expression between cancer and non-cancer samples including *AR*, *PTEN*, and *RAF1* (see figure 4.7 Data analysis by Dan Brewer). This initial study confirms the utility of these analyses in that i) the NanoString analysis method seemed to work well on these samples, and, the NanoString company reported that they thought that the analyses had worked very well; ii) there are many transcribed mRNAs harvestable from microvesicles with the methods used; iii) there are differences in expression between samples with PCa and samples from patients with no evidence of PCa.

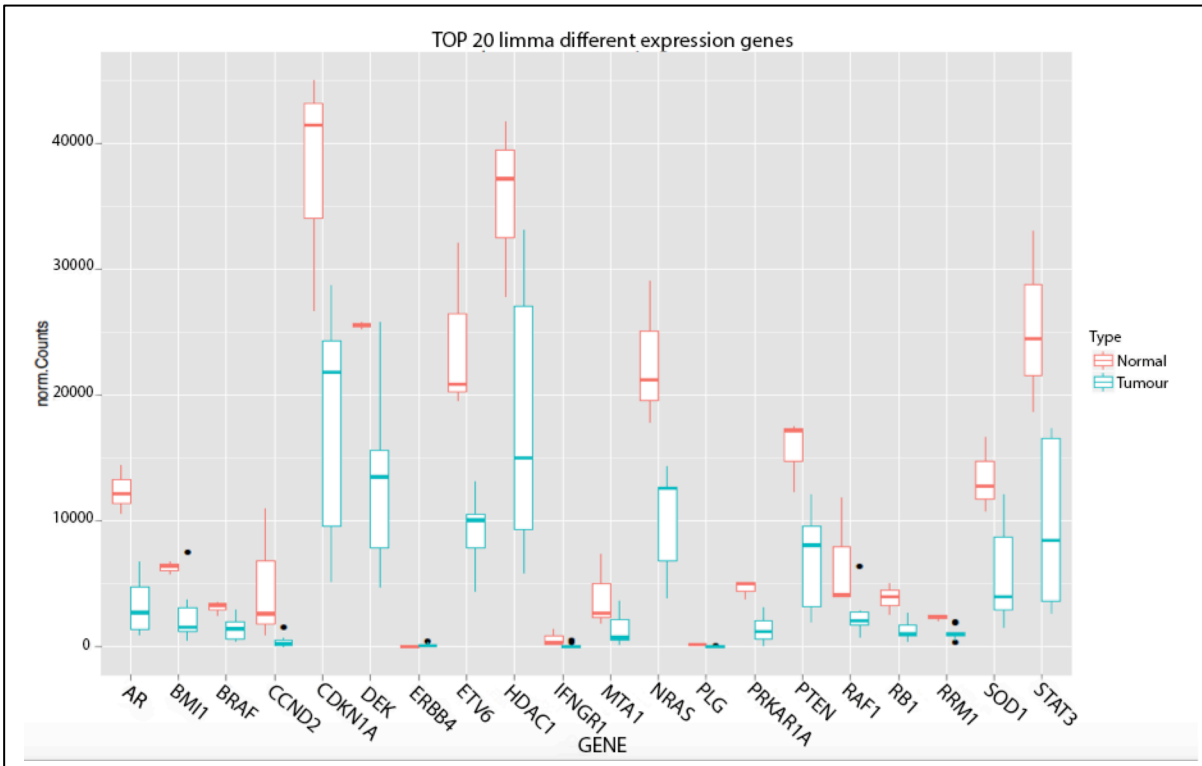


Figure 4.7: Differential gene expression between benign and cancer patients.
The data analysis was done using LIMMA (LInear Models for Microarray and RNA-sequence data Analysis. Software used for the assessment of differential expression used in count data) shows the differential gene expression between cancer and benign samples in 20 out of 236 genes of the n-counter reference assay. 'Normal' are samples from patients with no evidence of PCa.

4.3 NanoString: Custom 50-probe analysis

A total of 192 samples and a positive PCa control consisting of an equal mixture of RNA from metastatic PCa cell lines LnCaP and VCaP were used for the NanoString analysis of 50 gene transcripts.

The sample distribution was as follows:

- Benign Arm: 42 samples i) 18 CB1 (PSA<1 and clinically benign prostate gland) ii) 24 CBN (PSA normal to age and clinically benign prostate).
- Cancer Arm: 150 samples of which 132 divided into; 10 Low, 71 Intermediate and 51 High-risk groups as per NICE stratification criteria (see table 1.2 chapter 1 for more

details). The remaining 18 were in the advanced/metastasised risk group (Patients with clinically T3/T4 prostate and PSA >100) as a positive control arm (Figure 8).

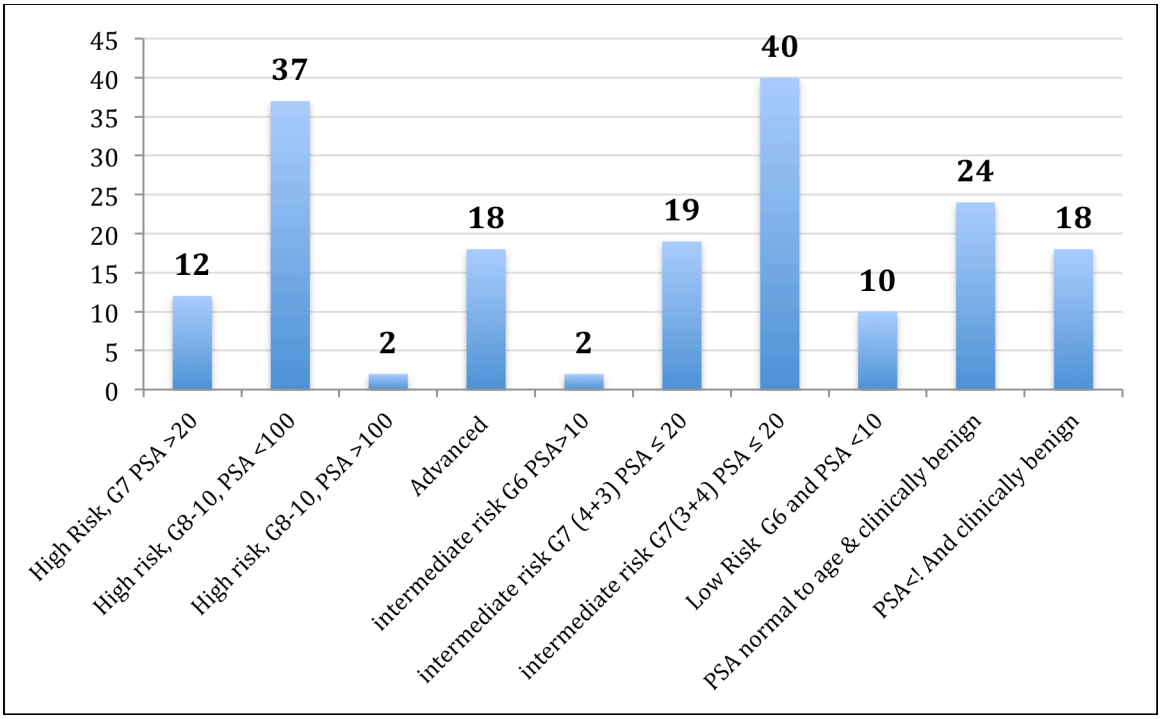


Figure 8: Showing the distribution of samples used for Nanostring analysis according to risk groups

50 genes were analysed in this cohort based on previous literature review, and genes suggested by members of the Movember funded GAP1 Urine Biomarker consortium. (see chapter 1, and table 1.4 for further information on the genes and their functions.)

4.3.1 Gene Probe Selection

The choice of the gene transcripts investigation in the study was based on 1) previous studies conducted in our lab (data not published), and 2) genes reported as being differentially expressed in PCa tissues, 3) control genes. (See table 1.4, section 1.10 for more detailed information on the gene transcripts investigated, and the rational for choosing each gene.)

Gene transcripts identified in the laboratory of my supervisor Dylan Edwards include a subset of degradome components that were altered in expression in prostate cancer. These include some up-regulated transcripts: members of the serine protease family, *Hepsin*, and MMP family member *MMP26*. Down-regulated transcripts genes include *Maspin* and *TIMP4*. Another 41 genes were chosen based on a literature review for transcripts that were

published as having diagnostic and prognostic potential.

The final group of transcripts were controls, and included 5 housekeeping genes (*HPRT*, *B2M*, *TBP*, *GAPDH* and *ALAS1*) and transcripts that are specifically expressed in the following tissues: bladder (*UPK2*), kidney (*SLC12A*) and blood (*PTPRC*). Genes were also included that were prostate specific (*KLK3*, *PCA3*), and prostate cancer specific (*TMPRSS2*/*ERG* fusion gene). The tissue-specific genes were chosen to provide fundamental information on the origin of the microvesicles in the urine from which we were extracting the RNA.

This initial set of 50-gene transcript analysis was designed as a pilot study to confirm the validity of the approach, and to identify the potential of these markers, which, if they proved to be useful would be extended to a wider cohort in a continuation study.

4.3.2 Data Analysis: Identifying genes of interest and sample clustering according to disease groups.

PCA and LPD analyses that I present and have discussed below were performed with the assistance of Dr Dan Brewer and Helen Curley.

4.3.2.1 Principal Component Analysis (PCA)

Principal component analysis (PCA) is a statistical procedure to analyse large multivariate datasets by reducing their dimentionality. It transforms a matrix of data so that the majority of the structure (i.e. variance) in a dataset can be viewed in a 2D plot. This transformation is defined in such a way that a sample's expression profile is represented by variables (called principal components) that account for as much of the variability of the data. Each principle component had the constraint that it is linearly uncorrelated to all other principal components. For example principal component 1 (PC1) will have the largest possible variance and PC2 will have the second largest possible variance and be uncorrelated to PC1.

The PCa analyses plotting PC1 against PC2 are shown in Figure (Figure 4.10). In addition to the main cluster two separate clusters (Clusters A and B) appeared to be present. One sample was identified as an outlier from the initial analysis ("H19.5" from the advanced group

category) (Figure 4.9). After further investigation, it became apparent that this sample was contaminated at the mRNA extraction step, and so was removed from further analysis.

The two potential outlaying clusters (Cluster A and B) looked interesting as they stood apart from the rest of the samples. These were therefore tested further by K-means to confirm that they were distinct groups. K-means clustering aims to partition the data into K-groups such that each observation belongs to the group with the nearest mean. K-means clustering (k=5) confirmed that Cluster A and Cluster B were distinct (Figure 4.10)

Figure 4.9: PCa data analysis reveals a remote outlier (H19.5).

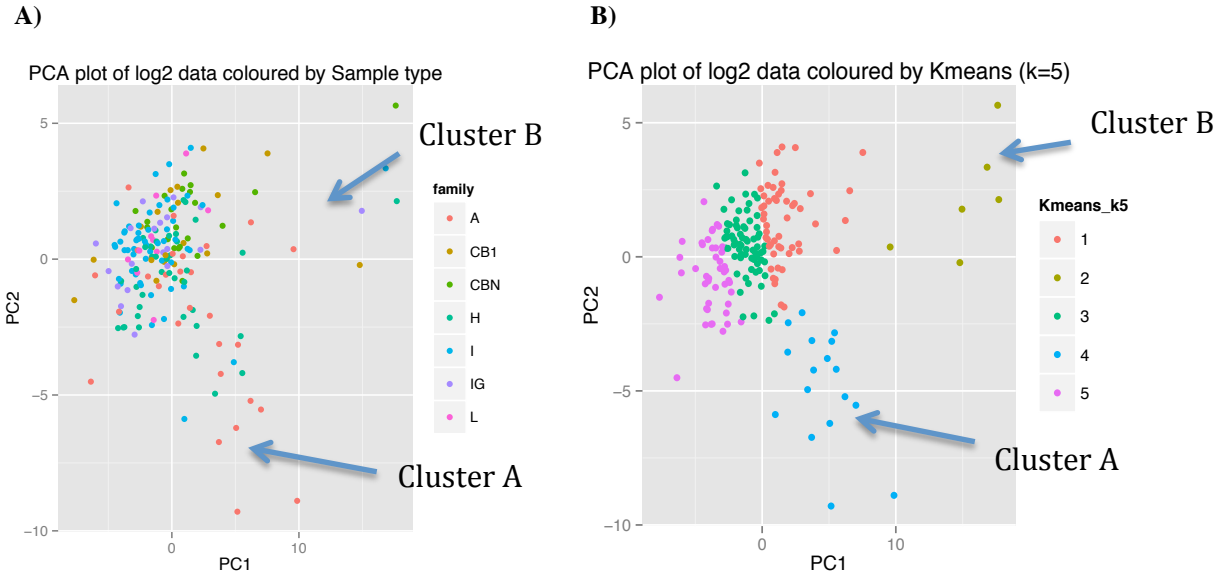


Figure 4.10 A) PCa identified three clusters. **A)** main cluster and two separate clusters A and B. PC1 has 28% of the variance and PC2 8%. Sample clinical groups are: A=Adv, CB1 and CBN are clinically benign samples, L= Low, I= Intermediate, H= high-risk of metastasis, **B)** K-means clustering identifying the 5 clusters shown in different colours. Cluster A corresponds to as K-means group 4 and Cluster B corresponds to K-means group 2.

The 5 groups revealed by K-means clustering are shown in different colours in Figure 4.10B. Notably Cluster A corresponded to K-means group 4 while Cluster B corresponsed to K-means group 2. Samples from Cluster A and Cluster B also grouped together when hierarchical clustering was performed (Figure 4.11)

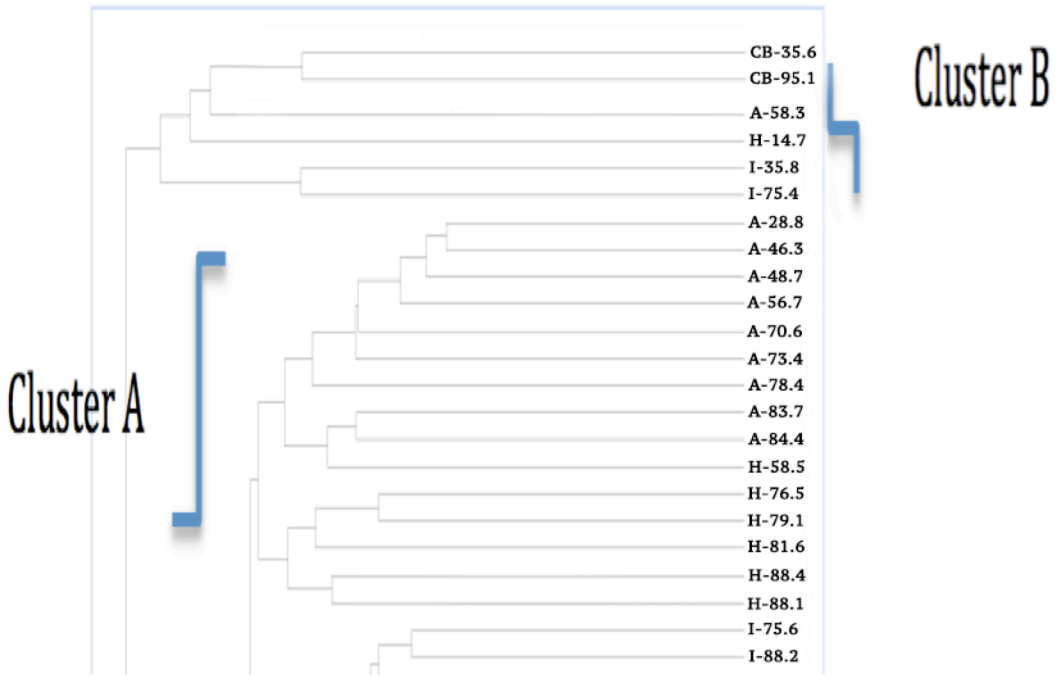


Figure 4.11. A dendrogram showing samples belonging to the PCa identified clusters A (17 samples) and B (6 samples).
A=Advance risk, I= intermediate risk, H= high, CB= benign risk groups.

4.3.2.2 The composition of samples belonging to Cluster A

Cluster A contained 17 samples, the majority of which were from the Advanced and High-risk clinical groups (9 Advanced, 6 High risk and 2 in the Intermediate risk groups), no benign samples were in this cluster. This suggests that there is a biological reason underlying the cluster formation. There is a significant over-representation of Advanced or High-risk cases in Cluster A compared to the remaining samples (Chi-squared test; $p = 6.67x10^{-06}$).

4.3.2.3 Genes differentially expressed in Cluster A.

25 genes were found to be significantly differentially expressed (Mann–Whitney *U* test;$p < 0.05$) in Cluster A samples in comparison to the rest of the cohort (table 4.5). 21 genes were up-regulated and 4 down-regulated.

DLX1, *AR*_truncation_exon, *MMP26*, and *Timp4* are the most significantly up-regulated genes. *DLX1* and *AR* are known to be significantly expressed in prostate cancer and have been associated with cancer progression and metastasis in several studies (See Chapter 1.9). *MMP26* is also known to be up-regulated in PCa and plays an important role in disease progression (511). *Timp4* promotes cancer progression by degrading the extracellular matrix that is integral in tumourigenesis. However *Timp4* is known to directly inhibit *MMP26* and its intensity and expression has been reported to diminish in higher cancer grades (See paragraph 1.9). In contrast to this, our analysis reports that the expression of *Timp4* is increased (fold change +1.29) – though it has to be noted that although the majority of our samples are from patients in a Advanced risk group (metastatic) and High risk group rather then Intermediate risk disease disease. These differences may also reflect the use of exosomes in our studies rather than cellular mRNA in published reports.

SPINK1, *SLC12A1*, *UPK2*, are the most down-regulated genes in Cluster A. *SPINK1* is known to be overexpressed in PCa at the cellular level particularly in association with high grade disease and ETS-negative cancers (See paragraph 1.7.3), in contrast to the data presented here. This could again be due to an exosome v cell comparison, or due to the fact that the high risk cancer in our group were selected from the cancer subgroup that did not over-express *SPINK1*.

SLC12A1 and *UPK2* are kidney-specific and bladder-specific transcripts previously reported to be present in urinary exosomes (See paragraph 1.9.1). Their expression has not been documented in prostate cancer. Their relative downregulation in Cluster A is likely to be due, at least in some part, to over-representation of prostate derived exosomal RNA in the PCa samples having the effect of diluting out the kidney and bladder markers.

Table 4.5. The 25 significant ($p<0.05$) genes identified by the Wilcoxon test when comparing Cluster A (PCA and K-means) against all other samples. '+' and '-' mean relatively up and down-regulated respectively.

Gene	Wilcoxon P-value	Fold Change
SPINK1	1.46E-10	-0.50
*KLK3*_PSA_exons2-3	4.24E-10	+0.25
UPK2	5.06E-10	-0.80
SLC12A1	2.50E-09	-0.74
KLK4	6.06E-09	+0.13
KLK2	1.19E-08	+0.17
STEAP2	1.22E-08	+0.18
OR52A2_PSGR	2.64E-08	+0.55
*KLK3*_PSA_exons1-2	4.93E-08	+0.24
FOLH1_PSMA_NAALAD1	6.05E-08	+0.21
PPAP2A	3.53E-07	+0.13
*AR*exons4-8	7.92E-07	+0.19
*AR*_truncation_exon	7.30E-06	+1.26
MMP26	7.38E-06	+1.13
STEAP4	8.48E-06	+0.13
GOLM1	1.43E-05	+0.49
CLU	6.39E-05	+1.02
B2M	0.0001	+0.21
Timp4	0.0001	+1.29
PCA3	0.0002	+0.46
SERPINB5/Maspin	0.0003	+0.29
CDC20	0.0003	+0.54
DLX1	0.0007	+1.50
HPRT	0.0019	+0.10
HPN	0.0040	-0.12

4.3.2.4 Cluster B: gene identification

Cluster B contained 6 samples with one advanced case one high, two intermediate and two benign) There appeared to be no obvious clinical distinction of patients present in this cluster. However I noted that all samples in this group except one had low RNA yields RNA yields (mean for 5 samples with low RNA= 0.7492ng/ul, SD= 0.41 prior to amplification). For this reason these samples were removed from further analysis.

4.3.2.5 Latent Process Decomposition (LPD) analysis

Latent Process Decomposition (LPD) is a unsupervised clustering technique developed by Colin Campbell at Bristol University using a statistical Baysian approach to group samples (512). Our group has previously used this technique to confirm that breast cancers contain basal and ERBB2 overexpressing subgroups and to show that metatstatic cancers can be divided into two clinically distinct groups (513). The first step is to find the optimal number of groups that the data should be divided in to. In the second step, the gene expression profile for each sample is decomposed and a probability that the sample belongs to each group is obtained. If the probability of membership is greater than 0.5 the sample is assigned to that group. The LPD analysis was completed on the nanostring dataset using all the samples excluding the LNCAP, H19_5, and the cluster B samples. The optimal number of processes was four (result not shown). Assignments to the four groups is shown in Table 4.6 and in Figure 4.13 (Further information on these analyses can be found in Chapter 2)

18 out of 185 samples were not assigned to one of the four LPD groups as shown in table 4.6 (LPD-NA), the rest of the samples were clustered into 4 clinically different LPD processes with an uneven distribution of clinical categories (X-squared = 94.0012 P-value = 2.665e-07).

LPD group 1 (LPD1) predominantly consists of patients in the high-risk group; LPD2 predominantly contains patients in the benign risk group, LPD3 has patients in the intermediate and high-risk group and LPD 4 are mainly cancer patients in the intermediate risk group (Figures 4.12, 4.13).

Table 4.6: LPD representing all samples except the LNCAP, H_19_5 and the 5 cluster B samples.
The total number of samples is shown, as well as the number of samples from the clinical sub groups. *X*-squared = 94.0012 P-value = 2.665e-07. Pearson's chi squared test for LPD groups 1-4 across all clinical categories. A = Advance, H = High risk, IG = Intermediate risk Gleason 4+3, I = Intermediate risk Gleason 3+4 and Gleason 6 PSA>10, L = Low risk, CB1 = clinically benign PSA < 1, CBN = clinically benign PSA normal to age.

	A	H	IG	I	L	CB1	CBN	Total
LPD1	9	1	0	1	0	0	0	11
LPD2	3	5	2	2	2	6	12	32
LPD3	3	7	5	9	1	2	0	27
LPD4	10	16	11	37	7	10	8	99
LPD-NA	4	9	0	4	0	0	1	18
Total	29	38	18	53	10	18	21	187

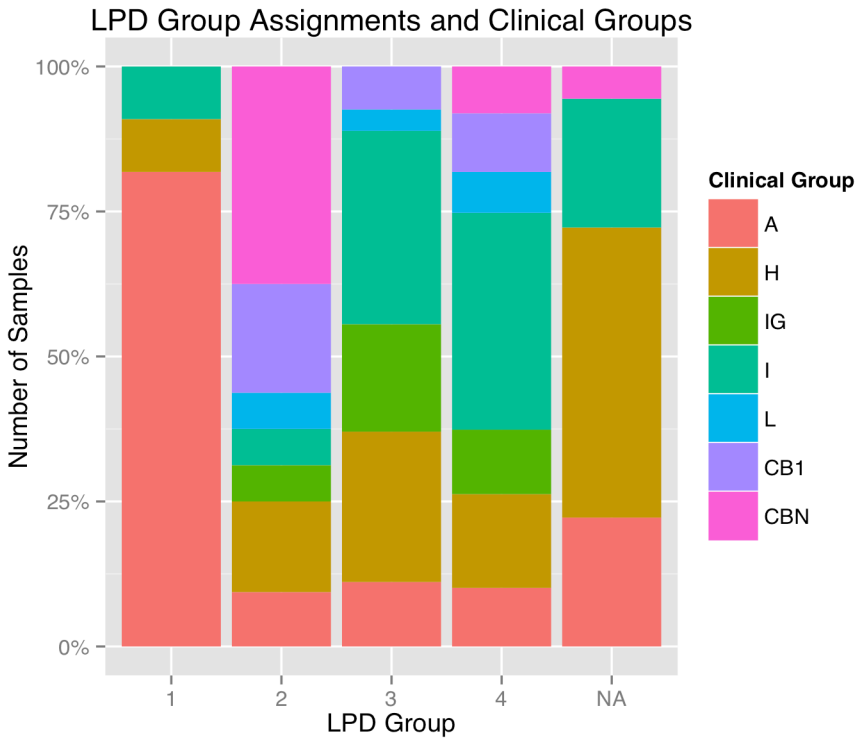


Fig 4.12: LPD Graph colour representation: A= Advanced, H= high risk, IG (IH)= Intermediate high risk, I= Intermediate, L= low risk, CB1= benign PSA<1, CBN= benign PSA normal to age.

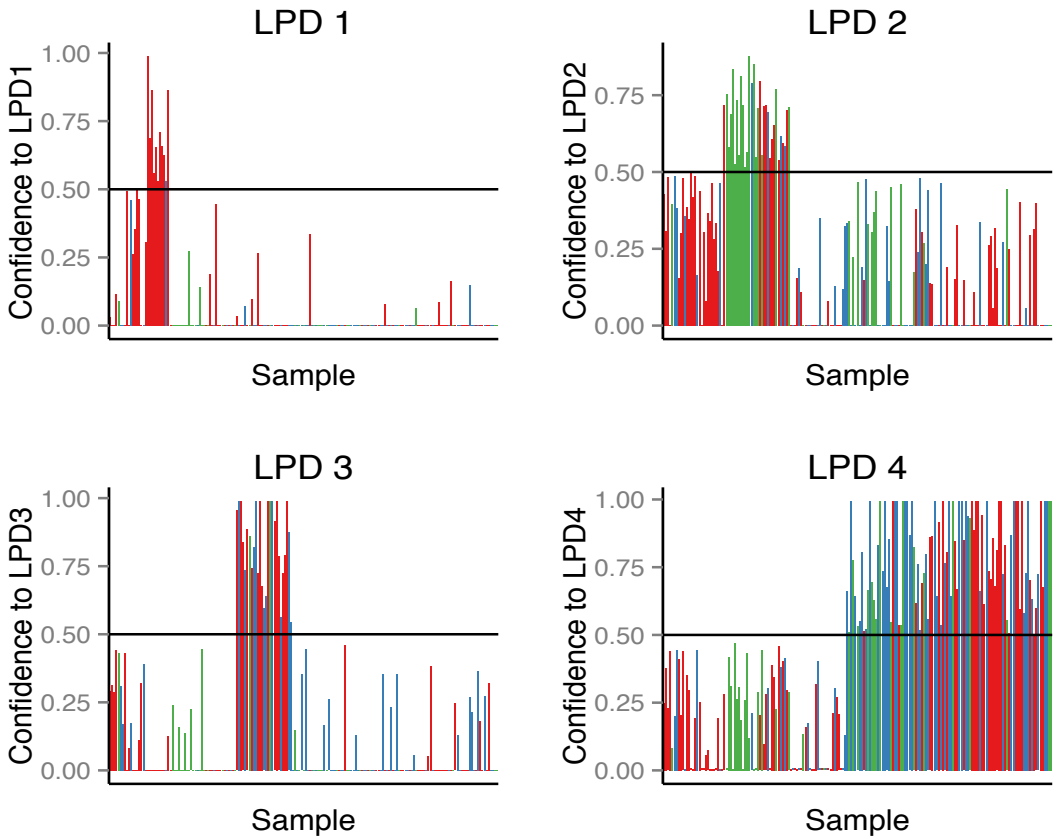


Figure 4.13: Samples belonging to each LPD group. Each of the 187 samples is represented in the same order in each of the four plots.

The expression levels of the samples in one LPD group were compared to all the other samples (tables 4.7-4.10). This was done for each LPD group in turn; for example, table 4.7 is where samples in LPD1 was compared to all the other samples. This method was used to identify probes with differential expression using the Wilcoxon test. FC means log2 fold change. A negative log2 fold change means that the expression of that RNA was lower in the samples belonging to LPD group 3 compared to its expression in all other samples.

Table 4.7: LPD Group 1 (Predominantly patients from the Advanced and High risk group)
Top significant genes

Gene name	LPD1 P-value	LPD1 FC
*KLK3*_PSA_exons2_3_	6.87E-08	-0.308
KLK4	8.32E-08	-0.166
STEAP2	3.58E-07	-0.183
UPK2	5.61E-07	0.829
KLK2	7.12E-07	-0.377
SPINK1	7.77E-07	0.560
FOLH1_PSMA_NAALAD1	2.20E-06	-0.171
SLC12A1	2.26E-06	0.726
*KLK3*_PSA_exons1_2_	2.53E-06	-0.310
PPAP2A	8.03E-06	-0.143

Table 4.8: LPD Group 2 (predominantly patients in the Benign group)
Top significant genes

Gene name	LPD2 P-value	LPD2 FC
Timp4	2.12E-08	-1.207
DLX1	1.34E-07	-1.561
HPN	3.54E-07	-0.159
TDRD	1.22E-06	-0.908
*ERG*_3prime	6.24E-06	-1.536
*KLK3*_PSA_exons1_2_	1.53E-05	-0.121
CDC20	1.71E-05	-0.515
HOXC4	2.04E-05	-1.054
STEAP2	2.72E-05	-0.050

Table 4.9: LPD group 3 (predominantly cancer patients in the High-Intermediate risk and Intermediate risk groups)
Top significant genes:

Gene name	LPD3 P-value	LPD3 FC
HOXC6	3.01E-13	0.253
*ERG*_5prime	7.05E-13	0.405
TDRD	1.50E-09	0.760
PCA3	1.10E-08	0.223
IMPDH2	1.89E-08	0.097
AMACR	4.17E-08	0.188
FOLH1_PSMA_NAALAD1	8.13E-08	0.102
Timp4	4.02E-07	1.341
HOXC4	5.65E-07	0.648

Table 4.10: LPD group 4 (predominantly cancer patients in the Intermediate risk)
Top significant genes:

Gene name	LPD4 P-value	LPD4 FC
*KLK3*_PSA_exons2-3	3.26E-12	0.081
*KLK3*_PSA_exons1-2	1.82E-10	0.115
KLK4	3.84E-07	0.052
CLU	5.47E-07	0.666
B2M	6.57E-07	0.096
STEAP2	1.11E-06	0.047
CDC20	1.78E-06	0.516
SERPINB5/Maspin	3.90E-06	0.175
*AR*_truncation_exon	8.75E-06	0.817
*AR*exons4_8	5.17E-05	0.073

These analyses yielded some very interesting results. In particular, samples in LPD1 exhibited high levels of expression of the bladder marker *UPK2* and of *SPINK1* – the latter being not only overexpressed in a proportion of prostate cancer but also in the normal kidney. One possible interpretation of this result is that in advanced disease, the prostate is usually very firm, and thereby difficult to massage during DRE. This could lead to a relative

increase in the representation of markers from the kidney and bladder. It may be possible to overcome the differences in the efficiency of DRE by normalisation of the data to a prostate specific marker such as *KLK2* or *KLK3*. Further analysis would be needed to test the efficacy of this approach.

LPD group 3 (predominantly intermediate risk) is characterised by the overexpression of the *HOXC6, TDRD1, DLX1, NAALADL2, IMPDH2 FOLH1* and *AMACR* genes. *HOXC6*, TDRD1, and DLX1 have recently been reported as a three gene urine panel overexpressed in patients with aggressive disease (514). *NAALADL2* and *IMPDH2* overexpression is linked to aggressive prostate cancer (476,515) and *FOLH1* and *AMACR* are established prostate markers. LPD2, which contains many of the cases of benign cancer, had relative underexpression of genes known to be associated with prostate cancer including *3'-ERG*, *DLX1, HPN and CDC20*.

4.3.2.7 Wilcoxon Test on all samples

With Dan Brewer and Helen Curley we applied a one-way "Wilcoxon Rank Sum" test on all the samples to identify genes that had significantly different expression across categorical groups. The Wilcoxon test is a non-parametric rank so works with data that is not normally distributed. The test allows the arrangement of genes based on the significance of the changes between 2 selected classification groups.

The groups compared in this test are as the following:

1. Prognostic genes: Aggressive vs. Non-Aggressive (A, H vs. I, L, CBN and CB1).
2. Diagnostic genes: Cancer vs. Non-Cancer/clinically benign (A, H, I, L vs. CBN and CB1).
3. Control groups: Advanced vs. Clinically benign.

The result of the Wilcoxon test was as follows:

1- Prognostic genes: 17 genes that can differentiate between high risk disease (where it is significantly differentially expressed) and lower grade and benign disease as shown in table 4.11.

2- Diagnostic genes: 15 genes were identified that are significantly over expressed in cancer sample in comparison to benign and two under expressed genes as shown in table 4.12

3- Control groups: 21 genes were identified that are significantly differentially expressed in advanced (positive control group) in comparison to the benign control group. These genes can also be utilised as prognostic genes, as shown in table 4.13

Table 4.11. Genes where p<0.05 when the Wilcox test is used (on log2 transformed data) to compare the aggressive/non-aggressive samples.

Aggressive vs. Non-Aggressive		
Gene Name	**P-value**	**FC**
SPINK1	1.86E-07	0.2081515
SLC12A1	2.25E-06	0.415704
UPK2	0.000126187	0.2558604
KLK4	0.000246849	-0.03544626
KLK2	0.000257111	-0.0346426
HPN	0.000316275	0.08247698
STEAP2	0.00039577	-0.03398385
SULT1A1	0.000536153	0.1499247
PPAP2A	0.003973885	-0.01849959
*AR*exons4_8	0.007376703	-0.04742768
BRAF (melanoma)	0.007910603	-0.04782195
STEAP4	0.01281419	-0.03681462
*KLK3*_PSA_exons2-3	0.01722231	-0.01606706
*KLK3*_PSA_exons1-2	0.02712317	-0.03678167
AGR2	0.03086049	-0.09692415
HOXC4	0.03369962	0.1502574
PTPRC	0.04916936	0.1339369

Table 4.12. Genes where p<0.05 when the Wilcox test is used (on log2 transformed data) to compare the cancer/non-cancer samples.

Cancer Vs. Non-Cancer		
Gene Name	**P-value**	**FC**
DLX1	4.07E-07	1.371327
HPN	2.05E-06	0.1314081
Timp4	1.05E-05	1.013754
*ERG*_5prime	1.39E-05	0.2522371
*ERG*_3prime	1.52E-05	1.258378
PCA3	2.80E-05	0.227959
HOXC6	6.70E-05	0.2403505
HOXC4	0.000101547	0.6434544
SULT1A1	0.001282921	0.1177117
TDRD	0.002203598	0.6536242
HPRT	0.009890869	-0.03327144
GAPDH	0.02326512	0.01399529
PPAP2A	0.02424608	-0.02004526
CLU	0.02707516	0.2870932
CDKN3	0.03217008	0.2278353
*KLK3*_PSA_exons1-2	0.03895625	0.07032925
PTPRC	0.04451618	0.1367218

Table 4.13. Genes where p<0.05 when the Wilcox test is used (on log2 transformed data) to compare the extreme (CBN/Advanced) samples.

Advanced vs. Clinically Benign		
Gene Name	**P-value**	**FC**
SPINK1	9.35E-07	0.4654994
SLC12A1	3.98E-05	0.671614
KLK4	0.000192694	-0.128272
UPK2	0.000350423	0.5319335
STEAP2	0.000441177	-0.1456561
PPAP2A	0.000441177	-0.1163773
KLK2	0.000855373	-0.1147816
HOXC4	0.000951311	1.128014
HOXC6	0.002358381	0.3051628
HPRT	0.003133012	-0.1692933
GOLM1	0.004125507	-0.2682384
SULT1A1	0.004513196	0.2458821
STEAP4	0.005386093	-0.09001821
*AR*exons4_8	0.005875767	-0.1588067
HPN	0.01053577	0.0941864
FOLH1_PSMA_NAALAD1	0.01053577	-0.1165729
*ERG*_5prime	0.01252864	0.356371
TMPRSS2/ERG	0.0144308	-0.4794675
*KLK3*_PSA_exons2_3_	0.02098788	-0.1021957
NAALADL2	0.0260251	-0.1135113
OR52A2_PSGR	0.02791597	-0.3714729

Again in this data set, *UPK2* and *SLC12A1* (bladder and kidney markers respectively) appear to have prognostic value. Although they are not known to be expressed by prostate cancer, their expression in the advanced and high risk samples (whether it is due to reduced exosomal RNA from the prostate at this stage as explained above or due to other unknown reasons) is significant to differentiate between high risk cancer and low risk or benign as shown in table 4.11 and 4.13. Interestingly *KLK2* and *KLK3* underexpression also appears to differentiate between the same groups as for *UPK2* and *SLC12A1*. Even though these men's serum PSA-protein levels are high, their urine exosomal *KLK3* levels appear to be the opposite, again possibly due to relatively lower levels of prostate exosomal RNA in these High-risk samples. Looking at the LPD analysis (table 4.7, 4.8, 4.10) we can see that *KLK3* is under-expressed in the advanced group in comparison to the intermediate risk group and benign samples (as expected) which again supports the theory that the more advanced the

tumour the less representation of the epithelial exosomes in urine as unable to reach the urine. This may apply to most epithelial genes as well, such as AMACR.

4.4 Identification of prognostic genes capable of predicting response to hormone manipulation and progression in patients with metastatic disease

Patients in the advanced and high-risk group that were treated with Androgen Deprivation Therapy (ADT) by LHRH agonist/antagonist, (Leuprorelin, Goserelin/Degarelix) were followed up at three monthly intervals with repeat PSA testing.

Some of the patients with aggressive disease fail to respond to ADT, their PSA shows a marginal drop within the first 6-months but never drop below 60ng/ml (failed initial response). Others have an initial response (PSA drops to normal) and relapse within the first 6 months (early relapse), or relapse within 7 to 24 months (late relapse). Some men respond significantly longer than this (delayed relapse). Therefore, it is of clinical importance to be able to identify patients in those different clinical groups and offer them different treatment strategies such as maximum androgen blockage (adding anti-androgens such as ceproterone acetate or Bicalutamide) or chemotherapy if they become castrate resistant. For these reasons I proposed that we examine gene expression patterns in this groups of patients.

EAU and NICE guidance did not specify time intervals for relapse. The criteria that I chose in this study and the choice of clinical groups are as follows: i) failure to respond, ii) early relapse iii) late relapse and iv) delayed relapse. According to EAU guidelines, patient survival expectancy after seven months of ADT treatment depends on the minimum treatment-related PSA levels exhibited by the patient: i) patients with PSA below 0.2 ng/mL, have an expected median survival of 75 months, ii) patients with PSA < 4 ng/mL, 44 months and iii) patients with PSA> 4 ng/mL, 13 months (516). However, the guidelines state that these predictions need further confirmation. NICE guidance has more sophisticated criteria taking into consideration patients presenting with a PSA under or over 60, a Gleason score below or over 8, patient performance status less than or more than one, and presence of distant metastasis (axial and visceral). Again the survival results are similar to the EAU guidance. None of the guidelines specified relapse time to specific ADT treatment.

4.4.1 Prediction of Failure to Respond to Hormone Manipulation Therapy

40 patients 21 with local metastasis and 19 with distant metastasis underwent androgen depreviation therapy with LHRH agonist, leuprorelin/goserelin (NB: these patients get started on antiandrogen initially such as Bicalutamide for 4 weeks only to prevent disease flare up when the LHRH agonist commenced). All patients were monitored at 3-monthly intervals with repeat PSA testing. In this cohort of 40 patients we identified 8 patients (20%) who failed to respond to the initial treatment; 7 whose PSA remained above 60ng/ml (most had presenting PSA >100 ng/ml) and one man whose PSA dropped to 20 at 3 months and rose again to over 60. 7 of these patients had extensive widespread bone metastasis (as per their bone scan results) and none of the patients had visceral metastasis (Figure 4.14).

To identify genes that can predict response to hormone treatment, with Helen Curley we compared gene expression levels in 32 patients who had good initial response and the 8 patients who failed to respond (failed initial response). The Wilcoxon test identified two genes that significantly differentiated between the two groups, *SERPINB5*/*Maspin* and *HPRT*. Further analysis by scatterplot (Figure 4.15) also revealed a difference in the expression of these genes between the two groups: low expression values of *SERPINB5*/*Maspin* and *HPRT* in patients with poor response to hormone treatment and relative over-expression in patients with good response (Figure 4.15). 5 out of the 32 patients had early relapse (PSA reduced to normal levels but rose again within 6 months). These 5 patients as well as the 8 who failed the initial treatment were given maximum androgen blockage by adding anti-androgens. Expression analyses were not performed on them separately due to the small numbers.

A scatterplot shows that there is no correlation between the expression values for these two genes across all samples receiving HT (p-value = 0.11). The two genes had lower expression values for negative responses to HT and higher expression values for positive responses.

Figure 4.14: showing patients response to hormone treatment over 12-month period

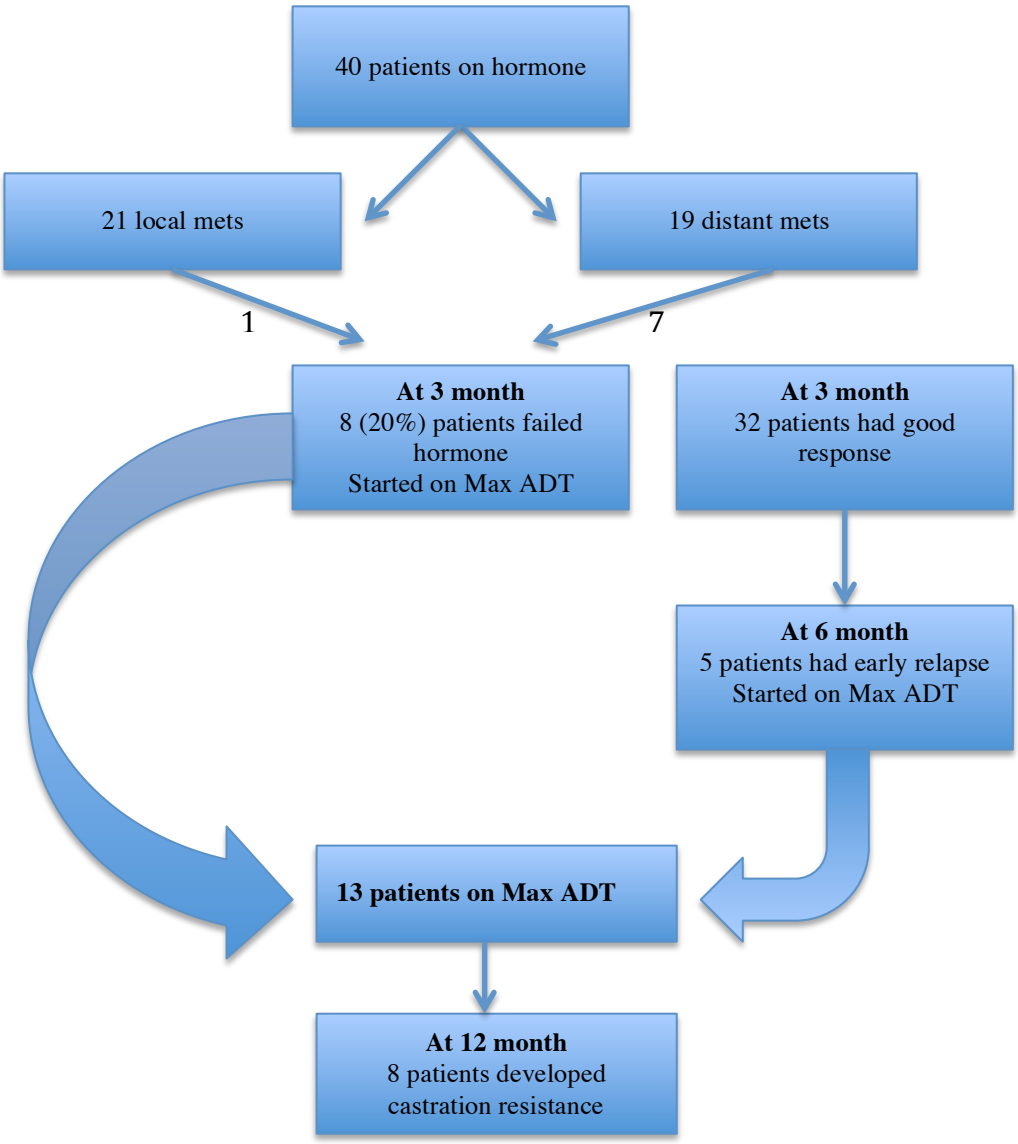


Figure 4.15: (A) A scatterplot of the 2 genes (*HPRT* expression vs. *SERPINB5/Maspin*) expression for all patients undergoing ADT. Samples of patients that responded to ADT are indicated in green, and red for patients who did not respond to ADT. **(B)** A boxplot showing the expression values of *HPRT* and *SERPINB5/Maspin* in the two patient groups (Green for patient responding to ADT and Red for patients who did not respond}.

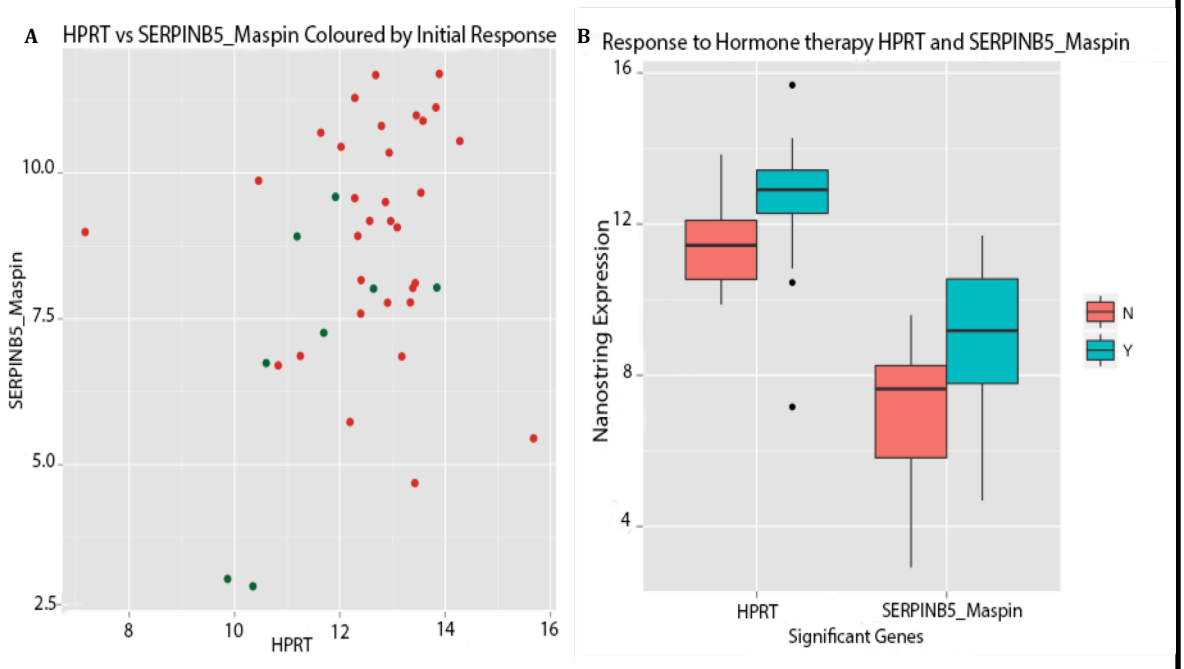


Table 4.14: Comparison of the two genes identified by the Wilcox tests as significantly expressed between patients who responded and the once who failed to respond to initial hormone manipulation, their p-value, adjusted p-value and fold change.

Gene	P-value	Adjusted P-value (Hochberg)	Fold Change
SERPINB5_Maspin	0.02	0.92	-0.26
HPRT	0.03	0.92	-0.17

4.4.2 Development of Early Castration Resistance

It is known that patients with advanced disease will inevitably develop castration resistance however, this is much earlier in some than in others, and depends on the extent of the disease and response to treatment. For this reason we looked at patients who developed castration resistance - these are patients who failed initial ADT treatment (LHRH agonist/antagonist alone) or had an early relapse, and who were therefore given maximum androgen blockage by combining a LHRH agonist/antagonist with an anti-androgen such as

Cyproterone acetate or Bicalutamide. In this cohort, we had 13 patients (7 distant metastasis and 6 local metastasis, see paragraph 4.4.1 for more information on these patients).

Eight of the 13 patients on maximum androgen blockage developed castration resistant (rising PSA despite maximum androgen blockage with combination of antiandrogen and LHRH agonist) in the first 12 months indicating early development of castration resistance and disease progression (Figure 4.14).

The Wilcoxon test identified three gene probes: *STEAP4*, *AR*exons4_8 and *NAALADL2* (Figure 4.16) (Table 4.15) as being significant before adjustments to the P-values were made (Table 4.15). In these Early Castrate Resistance analyses, neither *HPRT* (p=0.05), or *SERPINB5*/*Maspin* (p=0.14) were significant.

All genes were relatively under-expressed in the samples that relapsed in 12 months (figure 4.15).

Figure 4.16: Boxplot showing the differences in expression for the three significant genes in patients who became castrate resistant in the first 12 months (red, P=Progressed) and those who didn't (Green, N=Not progressed). Expression for all three genes is down regulated in samples that progressed.

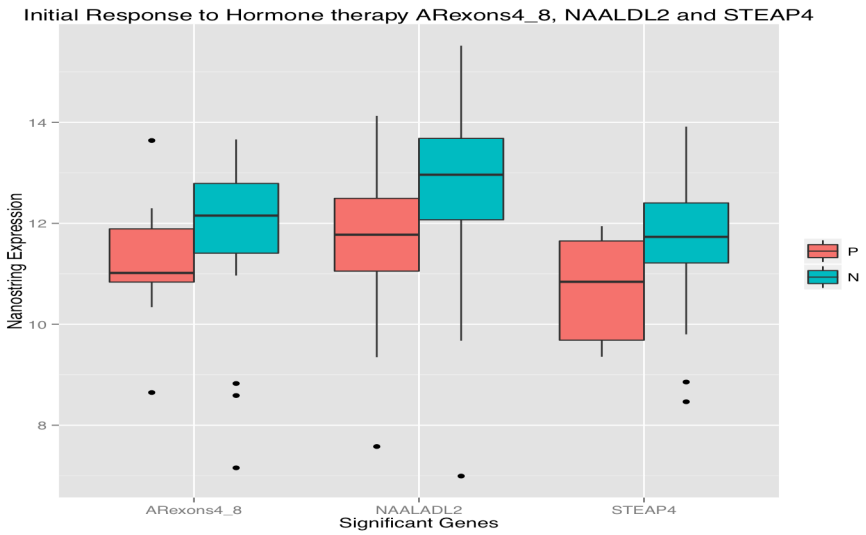


Table 4.15: Comparison of the genes identified by the Wilcox test between samples of patients who relapsed in the first 12 month after been given maximum androgen blockage and those who didn't. We also included in the two genes that were previously significant when comparing positive and negative initial responses. *Fold change is lower in Negative Response Samples.

Gene	P-value	Adjusted P-value (Hochberg)	Log_2 Fold Change
STEAP4	0.03	0.96	0.11
*AR*exons4_8	0.04	0.96	0.14
NAALADL2	0.04	0.96	0.14

The Kaplan Meier (KM) estimator (Figure 4.16) test was used to examine links between the expression of *SERPINB5*/*Maspin* and/or *HPRT*, and the development of resistance (rising PSA) over 24 months.

Patients were then divided into low and high expression groups for each of the 5 genes (*AR*exons 4-8, *NAALADL2*, *STEAP4*, *HPRT* and *SERPINB5*/*Maspin*) using cut-offs measured by K-means clustering. The Kaplen Meier (KM) curves were then constructed for the low and high expression groups to establish if there was a link with progression. The test

showed that lower expression of *AR*exons 4-8 and higher expression of *NAALADL2*, *HPRT* and *SERPINB5*/*Maspin* is linked with an improved chance of staying progression-free in the 24-month period. *STEAP4* expression however was not linked to progression due to the interweaved probabilities for high and low values. However, it is worth noting that these analyses were limited by a high number of censored subjects due to limited follow up time.

Figure 4.17: Kaplan Meier Curves for the 5 genes, independently using the red line for high expression and the green for low expression. A vertical drop in the red and green lines signifies disease progression in individual patients. (A) AR exons 4-8 high expression gives a higher probability of progression over the 24-month period and its low expression lower probability of progression. (B). Similarly *NAALADL2* its high expression gives a higher probability of progression for the first 16 months only and its low expression a lower probability of progression for the first 16 months. (C) *STEAP4*, high and low expression interwines, thus neither of which can give probability of progression. (D) *HPRT* high expression gives a lower probability of progression over 24 months and its low expression gives a higher probability of progression. (E) *SERPINB5*/*Maspin*, high expression gives a lower probability of progression compared to low expression.

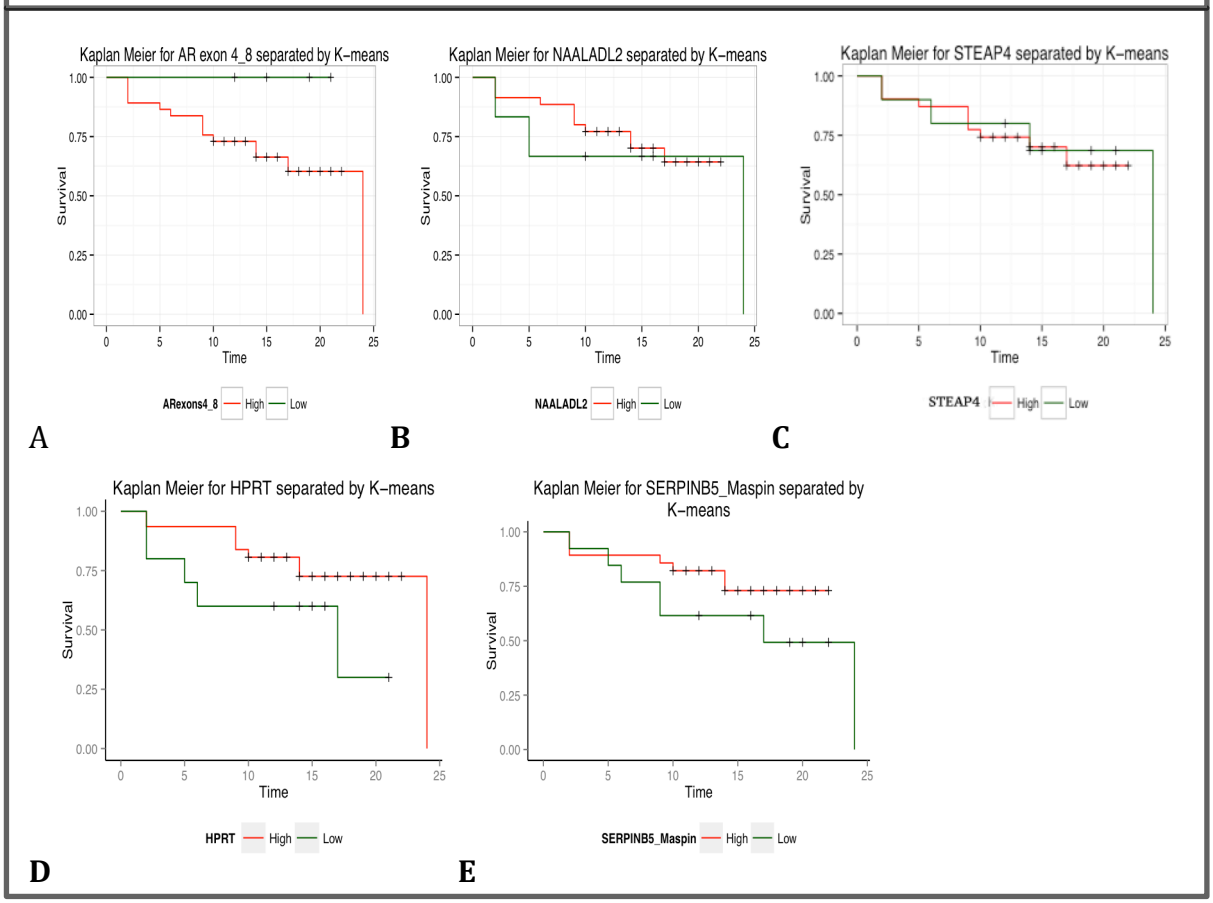


Table 4.16: Log rank and Cox test showing the statistical difference between the two groups (patients who progressed and who didn't progress) in the above KM curves (figure 4.15).

	Log Rank - Kmeans		**Cox - Kmeans**		**Cox - Continuous**	
	P-value	Chi-Sq	P-value	Hazard Ratio (exp)	P-value	Hazard Ratio (exp)
AR exons 4-8	0.69	0.2	0.68	0.67	0.46	0.87
NAALADL2	0.74	0.1	0.75	1.28	0.95	0.99
STEAP4	0.62	0.2	0.63	1.35	0.65	0.91
HPRT	0.1	2.8	0.13	0.4	0.21	0.82
SERPINB5_Maspin	0.05	3.8	0.05	3.05	0.02	0.73

4.5 Exosomal RNA Next-Generation Sequencing

Exosome/microvesicle gene expression is understudied, particularly in microvesicles derived from prostate. The gene probes that have been used so far in the study were selected by being differentially expressed in prostate cancer compared to normal prostate or by comparing aggressive to non-aggressive disease. As we are actually dealing with RNA extracted from exosomes rather than prostate tissue it was decided that we needed to know what range of RNA species were actually present in the urinary exosomes. Therefore in order to get an in-depth understanding of the role of these microvesicles and identify gene transcripts that would potentially be used for diagnostic and prognostic purposes, we used Next Generation Sequencing (NGS) to assess 18 exosomal RNA samples. I selected samples from the following clinical groups: 7 in the high risk group (Gleason 8-10, PSA<100), 7 intermediate risk group (G7, PSA<20) and 4 benign control (PSA value normal to patients age and clinically benign prostate). Further information on sequencing and analysis methods can be found in chapter 2. My clinical knowledge was central to sample selection, I prepared all the RNA samples ready for NGS analysis, and helped in data interpretation.

4.5.1 NGS data analysis

Dr Dan Brewer's analysis of the NGS data found 45 genes to be significantly differentially expressed between benign (4 cases) and cancer samples (14 cases) ($p < 0.05$ after multiple testing correction), with a $\log_2$ fold change ranging from 0.3 to 2.5 (Figure 4.18) of those genes 28 are up regulated and 17 down regulated in cancer. 33 genes showed a significant linear trend in association with cancer risk (27 genes showed increasing expression and 6 showed decreasing expression with increasing risk (Figure 4.18, 4.19)).

Figure 4.18. Boxplots showing statistically significant gene transcripts (n=45) that are differentially expressed between benign and cancer samples. 'DESeq' is the number of reads assigned to a specific gene normalised by the estimated size factor of the sample.

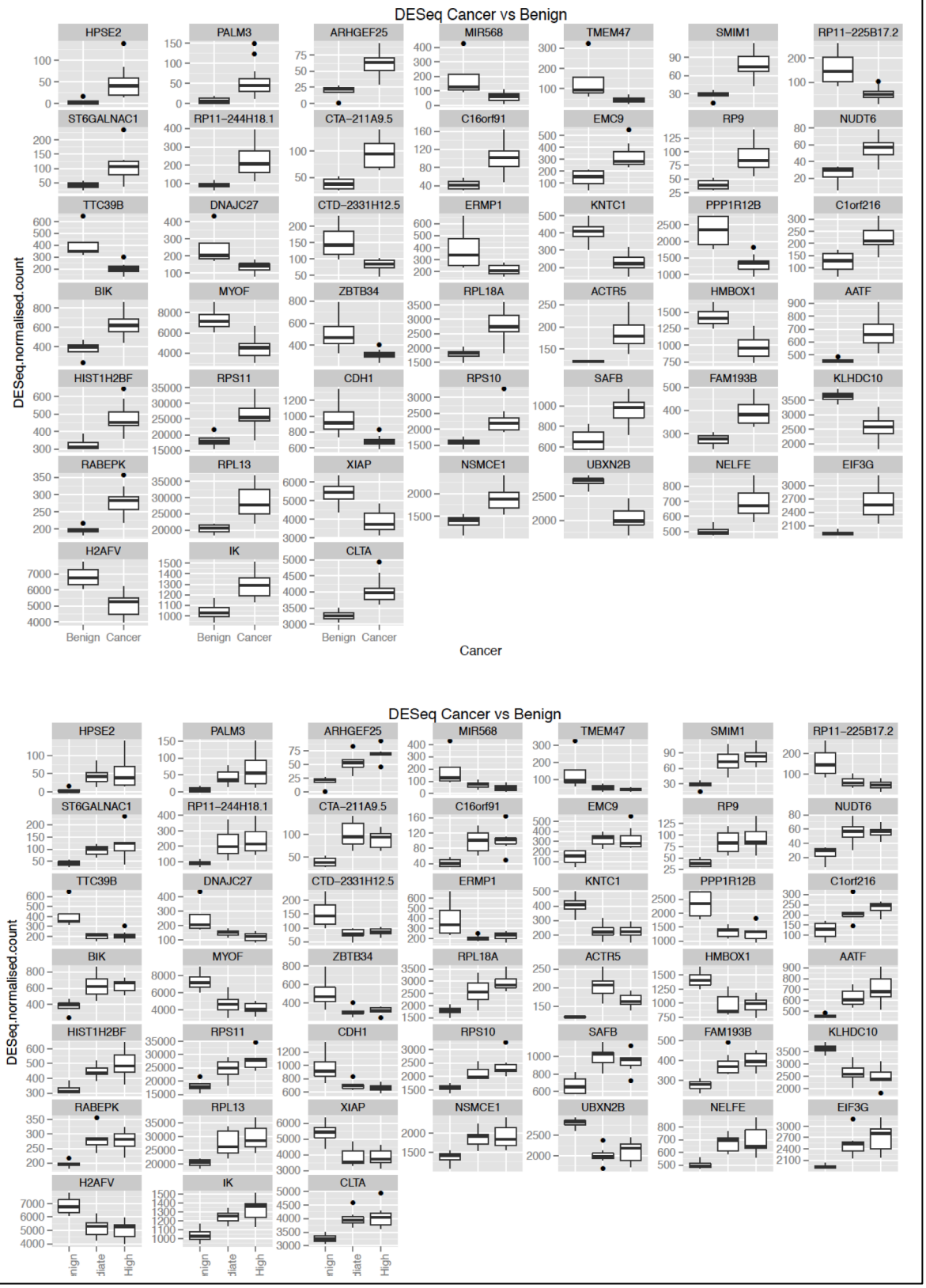
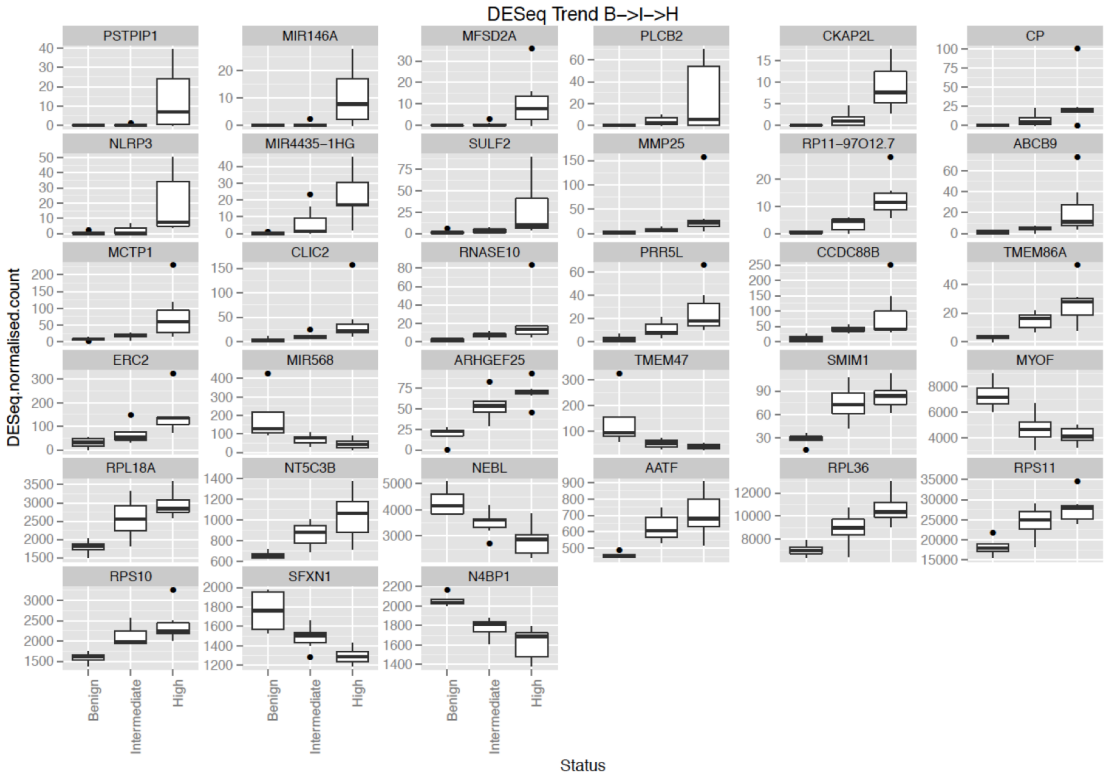
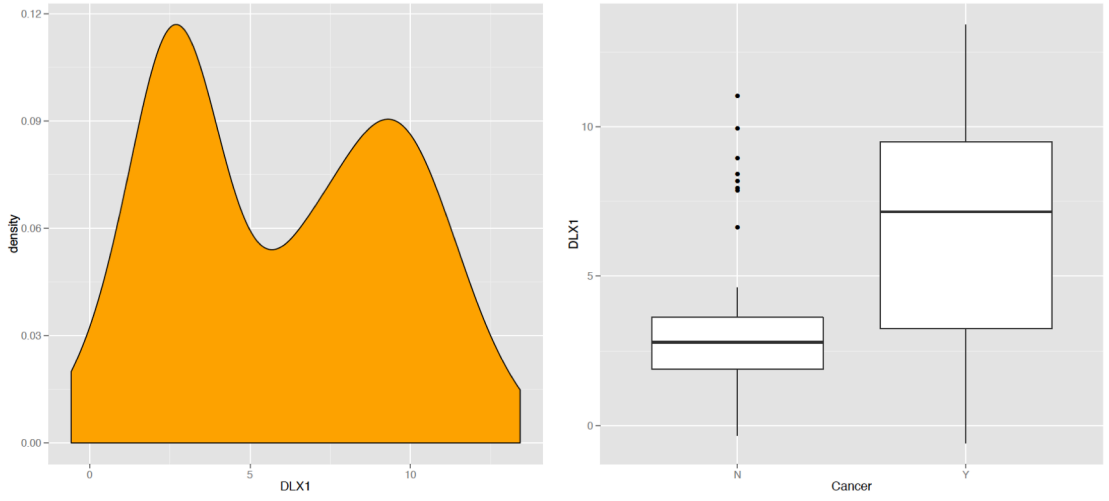


Figure 4.19. A) Boxplots showing statistically significant gene transcripts that show an increase or decrease trend with risk status. **B)** Boxplot showing in cancer vs benign samples.

A



B



PCA3 and *DLX1* were identified by the analyses as being significantly overexpressed between PCa and benign. This was reassuring, as both these genes are known to be overexpressed in PCa vs benign in whole-urine and exosomal RNA as reported in the literature and our LPD analysis respectively. This confirms that the system is working. *DLX1* was also selected, and this has been reported to be useful in urine analyses particularly as a diagnostic gene in urine sediments in patients with aggressive prostate cancer (514). Furthermore *DLX1* was recently identified to be a prognostic gene as a good predictor of high grade prostate cancer (using whole urine) as well as *HOXC6* although in our gene analysis (NaNostring) both these genes were identified as strong diagnostic genes (differentiate between benign and malignant) but not as prognostic genes (517). (Table 4.12). Similarly *DLX1* appear to be significantly over expressed in cancer patients in the third generation sequencing but did not correlate to high grade disease.

Matrix metalloproteinase 25 (*MMP25*) is another gene that was identified by the analysis to link to Gleason score, these finding are in agreement with a another study that demonstrated that the expression of *MMP25* correlates positively with Gleason score (511). The exact function of those transcripts in exosomes found in PCa patients remain to be identified.

The rest of the genes identified as differentialy expressed by analysis of RNAseq data did not match with the gene probes that had been chosen for the NanoString analysis and which were selected on published analyses of cell tissue samples. This difference emphasises the potential importance of this sequencing study in producing novel candidates for PCa diagnosis and prognosis.

Other genes identified by the analysis are the apoptotic genes *BIK* and *AATF*.
BIK (BCL2-Interacting Killer) is known for its pro-apoptotic activity (518), It was noted to be up-regulated in a number of cancer types inluding lung, prostate, colon, blood (leukemia); its induction is used in some cancer treatment (519). In Breast cancer it plays a critical role in inducing apoptosis by promoting estrogen starvation (520), its knock-down signicantly inhibits apoptosis (521). In prostate cancer its apoptotic function was also found to to be effective in both hormone sensitive and castrate resistant cells, the authors of the study concuded that it may have therapeutic function in PCa (522), beside cancer suppression it plays a role in controlling spermatogenesis (523).

AATF (Apoptosis Antagonising Transcription Factor): is a nuclear phosphoprotein known for its envolvement in cell cycle control and gene transcription, it exhibits a dual role, 1) the

regulation of cell proliferation and 2) growth arrest by apoptosis. It interfere with apoptosis via interaction with Dlk/ZIP kinase (a serine/threonine kinase known to induce apoptosis) leading to inhibition of apoptosis (524). It has also been found to correlate positively to Oct4 inhibition of apoptosis in stem cells, regulating cell growth in embryos (525). Its apoptotic-inhibitory function has also been documented in prostate cancer in patients on androgen deprivation therapy. The study concluded that the gene could be used in the prediction of outcomes in PCa patients on ADT (526). The encoded protein also exhibits DNA-binding transcription factor activity by the interaction of its leucine zipper contents with the Gal4 DNA-binding domain (524). It also plays a role in cell to cell adhesion in neuronal cells, and can function through interaction with different transcription factors, promoting cell cycle progression by binding to ribosomes and activating factor E2F and enhancing steroid receptor-mediated transactivation. By functioning in co-operation with *TSG101* (tumor susceptibility gene product, a co-regulator of nuclear hormone receptors) it was also shown to have coactivator activity on androgen receptor-mediated transcription (527) (528).

Some of the top genes identified by the sequencing analysis are shown in table 4.17.

Table: 4.17: Shows interesting genes identified by the sequencing analysis, their function and role in cancer

Gene name		Brief Function (Data taken from Gene Cards)	Involvement in Cancer in general	Involvement in Prostate Cancer	Expression in exosomal RNA
Up regulated genes includes					
ACTR5	Actin-Related Protein 5 Homolog (Yeast)	DNA Double-Strand Break Repair and Transcription-Coupled Nucleotide Excision Repair	Cell cycle progression coactivation of nuclear receptors. Overexpressed in several types of cancer (529)	Overexpressed in LNCaP cell line after exposure to androgen and anti-androgen (530)	Up-regulated. Higher expression in intermediate and high risk group
ARHGEF25	Rho guanine nucleotide exchange factor (GEF) 25	Positive regulation of Rho GTPase activity			Up-regulated
C16orf91	Chromosome 16 Open Reading Frame 91	Protein Coding gene			Up-regulated
C1orf216	Chromosome 1 Open Reading Frame 216	Protein Coding gene			Up-regulated
CTA-211A9.5	Transfer RNA Suppressor transcript	Non coding RNA			Up-regulated
EMC9	ER membrane protein complex subunit 9	Ubiquitination and de-ubiquitination activity within the cell			Up-regulated
HIST1H2BF	Histone cluster 1, H2bf	Innate immune response in mucosa, chromatin organization, nucleosome assembly, antibacterial humoral response, defense response to Gram-positive bacterium.	Up-regulated in non small cell lung cancer cell line (methylation modification)(531)and breast cancer (532)		Up-regulated
HPSE2	Heparanase 2 (inactive)	Encodes a heparanase enzyme that act on the extracellular matrix and cell surface. It is also envolved in the remodeling of the extracellular matrix, angiogenesis and tumor progression (533)	Has been reported in breast cancer (533), pancreatic and prostate cancer, as well as malignant melanoma where it is used as a molecular marker of cell invasion (534). In pancreatic cancer it regulates VEGF-C expression a cytokines that promote metastasis and angiogenesis (535)	Shown to be marginaly up regulated in plasma DNA in prostate cancer (536). It may have a role in PCa metastasis (537)	Up-regulated
MFSD2A	Major facilitator superfamily domain containing 2A	Transmembrane protein, which may be responsible for uptake and transport of fatty acid, phospholipids.	Lung cancer tumour suppressor gene. It has been shown to alter mRNA levels of genes involved in cell cycle and interact with the extracellular matrix attachment. (538)		Up-regulated in high risk group
MIR146A	MicroRNA 146a	Non-coding RNAs that are involved in post-transcriptional regulation of gene expression		Tum suppressor, and suppressor of metastasis, by regulating cell growth (539)	Up-regulated in high risk group
NUDT6	Nudix (nucleoside diphosphate linked moiety X)-type motif 6	Is thought to be the FGF2 (Fibroblast growth factor 2) antisense gene. FGF2 expression is Multifunctional: heparin-binding growth factor neuroectoderm development, angiogenesis, and wound healing.	Elevated levels of FGF2 are associated with proliferation of smooth muscle in atherosclerosis and with proliferation of tumors. The fibroblast growth factor-2 antisense gene inhibits nuclear accumulation of FGF-2 and delays cell cycle progression in C6 glioma cells. (540)	FGFs play a role in the growth of normal prostate tissue. Over expression of FGF2 was observed in prostate cancer epithelial cells with poor differentiation (541)	Up-regulated
PALM3	Paralemmin 3	ATP-binding protein. It negatively regulate			Up-regulated

		cytokine-mediated signaling pathway			
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	Protein coding gene involved in endocytosis, inflammatory response, cell adhesion, signal transduction nucleotide-binding domain and innate immune response		Chromosome translocation that result in the loss of PSTPIP1 gene leading to its downregulation in LNCaP cells has been documented (542)	Up-regulated in high risk group
RP11-244H18.1	LincRNA	P712P prostate specific transcript. non-coding mRNA located on chromosome 22.		Androgen driven in LNCaP cells (543).	Up-regulated
RP9	Retinitis pigmentosa 9	RNA splicing and Cognition, mainly. Its exact role is not fuly understood. Mainly expressed by B cells.			Up-regulated
SMIM1	Small integral membrane protein 1	A type II transmembrane phosphoprotein, responsible for the Vel-negative blood type. (544)			Up-regulated in high and intermediate risk group
ST6GALNAC1	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1	Affect cell to cell interaction, and cell interactions with the matrix.	It is mainly expressed in adenocarcinomas leading to the synthesis of sialyl Tn (sTn) antigen particularely in gastric CA, however, its role remain unknown (545)		Up-regulated
Some of the up regulated genes that trend up with cancer grades include					
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	Member of the MDR/TAP subfamily that act as a membrane transporter involved in multidrug resistance as well as antigen presentation. It is expressed in all tissues with its highest expression is in CD71	Inhibition of ABCB9 by microRNA 31 lead to decrease in DDP-induced apoptosis in lung cancer (546)	ABCB9 was identified as one of 16 genes predictive of prostate cancer recurrence after prostatectomy in DNA microarray-based gene expression profiles (547)	Trend-Up in High risk Group
CKAP2L	Cytoskeleton associated protein 2-like	Involved in mitotic progression. Very highly expressed in CD71			Trend-Up in High risk Group
CLIC2	Chloride intracellular channel 2	A member of the p64 family that regulate cellular processes including stabilisation of cell membrane potential, transepithelial transport, and regulation of cell volume. (548) Very highly expressed in CD31	A microarray gene expression profiling demonstrated its involvement in lung cancer metastasis (549)		Trend-Up
NLRP3	NLR family, pyrin domain containing 3	Involved in inflammatory response and apoptotic process by activation of cysteine-type endopeptidase activity, defense response, signal transduction and detection of biotic	Its activation can enhance proliferation and migration cancer cells by activation of the IL1 beta that Curtail anti cancer activity (552,553)		Trend-Up in High risk Group

		stimulus. Negative regulation of NF-kappaB transcription factor activity and interleukin-1 beta production. (550,551) Very highly expressed in CD33 myeloid, CD14 monocytes			
PLCB2	Phospholipase C, beta 2	Some of this gene function is the activation of phospholipase C activity synaptic transmission and phospholipid metabolic process as well as intracellular signal transduction. Expressed by Blood cells, myeloid, monocytes, NKs, Cd4, CD8 T-cells	Lung cancer tumor suppressor gene that regulates cell cycle progression and matrix attachment. (538) also identified to be expressed in leukemia(554)		Trend-Up in High risk Group
PRR5L	Proline rich 5 like	Regulation of protein phosphorylation and negative regulation of signal transduction it also regulate fibroblast migration. It also positively regulats phosphatidylinositol 3-kinase signalling, control cell proliferation by promoting cell apoptosis via interaction with mTORC2 (555). Its is highly expressed in CD56 NK cells.	PRR5L degradation lead to PKC-delta phosphorylation and cell migration mediated by mTORC2 (556)		Trend-Up
Some of the down regulated genes includes					
ERMP1	Endoplasmic reticulum metallopeptidase 1	Is a transmembrane metallopeptidase. In rat ovaries it is required for folliculogenesis, where its underexpression resulted in loss of follicules and structural disorganisations of the ovaries (557) highly expressed in B-lymphoblasts, NK cells	Over-expressed in breat cancer (identified as breast cancer oncogene) (558) where its silencing has been shown to significantly reduceinvasiveness and proliferation (559)		Down regulated
HMBOX1	Homeobox containing 1	Telomere involved in maintenance and inhibition of the NK cells acticity (560) It also has transcriptional repressor activity (561) Expressed in prostate, pancreas, thymus, testis and other tissues (562)	Overexpression of HMBOX1 significantly inhibited NK cell activities, including natural cytotoxicity against tumor cells (563).		Down regulated
KNTC1	kinetochore associated 1	Mitotic cell cycle protein complex assembly regulation of exit from mitosis chromosome			Down regulated

		kinetochore B-lymphoblasts (564) (565) Cell division (566)			

Further detailed discussions on the above information is presented in Chapter 5.

5. Discussion

5.1 Prostate cancer screening and diagnosis

Prostate cancer poses a number of clinical challenges in terms of its diagnosis, prognosis and treatment. The challenge posed by PCa is the clinical inability to accurately predict the disease's course of progression in individual patients. There are many indolent prostate cancers that could be safely left completely untreated, if only they could be reliably recognised as such. There are also aggressive types of PCa that for optimal survival would require early identification and possibly a more aggressive treatment regime. The mechanisms of development of castration resistant- and of androgen independent- disease are also poorly understood and such patients cannot be identified early enough to consider other treatment modalities. Under current clinical practice these patients are very challenging, and in many cases are managed by palliation. As accurate prediction of individual prostate cancer behaviour at the time of diagnosis is not currently possible, immediate radical treatment is considered in many cases (130), at the cost of numerous complications including impotence and incontinence.

We therefore set out in this study to identify a set of urine biomarkers that would potentially answer the following questions: (i) are there novel urine based RNA molecules that can assist in cancer diagnosis? (ii) can the detection of specific RNA transcripts be used to distinguish aggressive from non-aggressive cancer? (iii) can these biomarkers predict response to ADT treatment? and (iv) can these biomarkers predict resistance to ADT treatment?

5.2 Risk Factors for PCa

There are a number of risk factors documented in the literature associated with an increased risk of developing prostate cancer. I aimed to investigate some of these risk factors in this study however our study patient population from the Norwich and Norfolk University Hospital was not diverse or large enough for a thorough investigation.

5.2.1 Race

It is well documented in the literature that the incidence of PCa differs between men with different racial origin (36,37). However, the NNUH cohort was not diverse enough to draw any conclusions (only 0.8% of the population included in this cohort where of non-caucasian origin). Having a more diverse population could have strengthened the outcome in terms of developing a test that could be effective for men of all races, particularly those with a higher associated risk. It is unlikely in Norfolk that we would ever generate a large enough cohort to address this issue systematically.

5.2.2 Alcohol

There have been a number of conflicting results from studies with respect to alcohol consumption risk and PCa. A prospective study in the USA of 238 men showed no associated risk (510,567). In contrast, a study on 753 men showed a reduced risk of PCa in wine drinkers, thought by the authors to be due to hormonal milieu alterations as a result of chemical substances such as flavonoids in red wine (568). This study also investigated the consumption of other types of alcoholic drinks, none of which appeared to be significantly associated with an increase in PCa risk. This was in contrast to a Canadian study that showed an increased risk in men consuming beer (569). In my study, I documented alcohol consumption as units per week regardless of the type of alcohol consumed or the number of years these men had consumed alcohol. Our findings showed a statistically significant risk in alcohol consumption and PCa - however these results need to be carefully interpreted particularly in the absence of in-depth data and documentation.

5.2.3 Smoking

Several studies have reported an increased risk of PCa in smokers (9,510,567). Some studies reported an increased risk of High grade disease, metastasis, biochemical recurrence and development of castration resistance (570). It has also been reported that cigarette smoking whilst undergoing a course of external beam radio-therapy treatment is associated with an increased risk of prostate cancer-specific mortality and treatment-related toxicity (571).

The findings of my study were that 60% of patients who admitted to smoking were diagnosed with prostate cancer. However the prevalence of PCa was similar or even higher in non-smokers, and there were no correlation between the cigarette numbers smoked daily and cancer grade. However, due to anti-smoking publicity campaigns, and the government ban on smoking in public places, it has become an embarrassment for patients to admit to a clinician that they smoke. I had aimed to document smoking accurately in this project in order to assess any relationship with smoking, including any effect of smoking on our biomarker expression. However for the reasons mentioned above and due to an elderly patient-population presenting with prostate cancer, recording accurate history of smoking proved to be extremely difficult, and I am uncertain if the data are a true representation of the smoking population or not. One way around this problem could be to perform a blood test for nicotine, or other smoking-related chemicals, but this would only assess current habits and not previous history or how long a patient had been smoking. I also did not have ethical approval for taking a blood sample for such a test, and so it was outside the scope of this study.

5.2.4 Family history

It has been reported that the risk of getting PCa increases with the number of close relatives that have already been affected by the disease. The overall risk of familial PCa is documented to be 9% (18). Our findings are consistent with the reported figures with 12% of our patients reporting a family history of PCa. Most of these patients had an affected young close relative, and most of these patients were diagnosed with Intermediate risk disease in a similar age range to their affected relatives (information about the relative's disease grade and stage were not obtained in this study as we did not have ethical aproval to collect this information).

5.3 Urine as a source of PCa Diagnostic and Prognostic markers

Urine is a carrier of prostatic secretions and other biomaterials, and a source of prostate cancer biomarkers (572). The literature contains many examples of urine-based biomarkers that have been reported to have diagnostic (mainly) and in a few cases prognostic usefulness. DNA, RNA, protein and metabolite levels have all been reported as promising markers. However, despite significant progress only one urine biomarker (PCA3) to date has

succeeded in reaching clinical use. Despite it being easy to use, the PCA3 test is still in limited clinical use mainly due to the lack of large clinical trials to validate its proposed prognostic power (573) and guide the course of patient treatment towards surveillance or a radical approach. Its utility as a marker of treatment response has also been questioned since the PCA3 test showed variable results on patients with localised prostate cancer on 5-alpha reductase inhibitor dutasteride, on a pilot study with nine patients (573).

Other RNA urine biomarkers reported include *GOLPH2*/*GOLM1*, *SPINK1*, *AMACR*, *TFF3*, *TMPRSS2*/*ERG* fusion, *HOXC6* and *DLX1*, some of which have been shown to outperform serum PSA in the diagnosis and prognosis of PCa (157,206,517,574). Some of these markers such as the *TMPRSS2*/*ERG* fusion gene transcripts, when used in combination with *PCA3* have proved to enhance the utility of serum PSA for predicting prostate cancer risk as shown in a large multicenter study on 1312 men (575). An assay combining serum PSA with urinary *PCA3* and *TMPRSS2*/*ERG* (574) has shown similar results with 90% specificity and 80% sensitivity in diagnosing PCa. Although it has high specificity it is lacking in sensitivity and prognostic ability. Recently prostate cancer derived urine exosomes have shown to be a promising source of biomarkers; however to date there are only 3 studies published, all of which are on small cohorts of patients, the largest having 11 subjects. Two have reported the presence of genetic information specific for PCa including PCA3 and *TMPRSS2*/*ERG* (576,577), and the third has reported an ability to predict treatment response on patients undergoing radiotherapy treatment (268). To date, single biomarkers from any origin have failed to combine high specificity and sensitivity in detecting prostate cancer, nevermind predict prognosis and response to treatment. It was therefore hypothesised that multiple biomarkers could be used, each supplying a small amount of clinical information, which, when used together could provide superior detection of PCa *per se* and more accurate prognostic information. This hypothesis has been investigated by many, including a study who showed that a multiplexed model, including *GOLPH2*, *SPINK1*, *PCA3* and *TMPRSS2*/*ERG*, outperformed serum PSA and *PCA3* alone in detecting prostate cancer (206). Another study showed that the combination of *EZH2* and *TRPM8* added diagnostic power to *PCA3* (492). Both of the studies were performed using urine sedimentary fractions rather then exosomal mRNA.

Despite all these efforts none of those urine markers, with the exception of *PCA3* have progressed to clinical use. The reasons for this are many-fold and include: i) the chemical nature of urine itself which makes it difficult to optimise protocols for preservation of the biomaterials needed for testing - this is particularly true in the case of cell-based biomarkers;

ii) the heterogeneity of prostate cancer with multiple routes to progression that cannot be represented by one biomarker and iii) the lack of repeat and conformational studies. The current study was planned taking into consideration all the above, namely good clinical information on which we based our data analysis, a urine-stable biomaterial as the source of biomarkers (exosomes) and a large cohort of patients (192 in the current study and 1000 in the overall project) as well as a large number of biomarkers (50 in this pilot study) chosen as a panel for diagnostic and prognostic purposes. Our next-generation sequencing of urine exosomal RNA samples has opened the door to the development of new RNA biomarkers tailored to exosomes themselves rather than chosen because they have differential expression in tumour and normal tissue.

Our initial results showed that prostatectomy patients exhibited very low yields of exosomal RNA suggesting that most of the exosomal RNA prepared in our study was derived from the prostate. This result has given us the confidence in the source of our gene transcripts for analysis. However, as shown by samples in-group LPD1, high levels of *Uroplakin 2* and *SPINK1* RNA are detectable, suggesting an increase contribution from bladder and kidney respectively in patients at the clinically extreme categories of benign and advanced disease. Exosomal shedding from normal prostate cells has been reported to be less than from cancer cells (578), hence the relative increase in renal and bladder derived exosomes in advanced cancer patients is hypothesised to be due to an inability of prostate exosomes to reach the urethra due to the distorted luminal anatomy of advanced disease. To assess bladder contamination *Uroplakin 2* was specifically included (485,486). Midkine is another gene we included as both a test, and a control as it has been reported to be over expressed in the urine of patients with renal and bladder cancer as well as eight other cancers (http://www.cellmid.com.au/content_common/pg-cancer-treatment-and-detection.seo). We suspect that this issue would be more accentuated when analysing the whole urine.

The 662 samples generated in my study will generate a considerable amount of information on the relative uses of exosomal and sedimentary fractions as sources of prostate biomarkers in future analytic studies. My samples have provided a core set of samples for the Movember GAP1 initiative who's overall aims are to develop better markers for detecting prostate cancer and for distinguishing aggressive from non-aggressive disease. The samples have been analysed in the following ways (see figure 2.1):

1. 3,347 aliquots of whole urine have been analysed by 5 teams (Bristow- Toronto, Pandha-Guildford, Whitaker- Cambridge) for 11 different proteins by ELISA, and also for Mass Spectrometry analysis (Leung- Glasgow).

2. 414 aliquots of Cell cDNA has been analysed by 4 teams (Pandha - Guildford, Olivan - Barcelona, Mills - Oslo) for 7 RT-PCR targets, plus 167-probe analysis by NanoString (Cooper - Norwich).
3. 1,436 aliquots of exosomal cDNA have been analysed by 3 teams for 7 RT-PCR targets (Pandha - Guildford, Olivan - Barcelona, Cooper - Norwich), plus 167-probe NanoString analysis of 499 samples by Cooper, and 60 by Sanda -Atlanta.
4. 980 aliquots of cell DNA have been analysed for DNA-methylation patterns in 10 genes by Bapat - Toronto), and Perry - Dublin.
When all the data from the above analyses has finally been collated, then it will be meta-analysed to see what the best combination of markers is for PCa detection and prognostic markers.

5.4 Exosome, cell sediment and whole-urine biomarkers

To better understand the relative contributions of the cell and exosomal components we aimed to study each fraction separately rather than whole urine as such. This was a fundamental principal of the Movember GAP1 Urine Biomarker study. Also, knowing the source of the RNA is crucial in my opinion in order to appropriately interpret the information with maximum confidence.
Exosomal RNA may be more clinically informative than cellular RNA from urine. Exosomes are part of an inter-cellular communication system that can promote cancer proliferation and metastasis through their signaling pathways in recipient cells (230,238). They may therefore contain molecules directly associated with these processes. It is reported that exosomes from a prostate cancer cell line can contain αvβ6 integrin which, can be taken up by recipient cells and become expressed on their cell surface – the integrin appeared to be functional and enhance cell adhesion and migration (579). Some studies have also shown that exosomes can also be responsible for transferring drug resistance to other cells in prostate cancer (580). For these reasons studying exosomes could have a number of advantages as they have the potential to provide a vast array of information beyond diagnosis, such as prognosis, response to treatment, and possibly cancer resistance to certain medications including hormone ablation therapies. A biomarker comparison between urine sediment and exosome showed that exosomes were a more stable substrate in comparison to urinary sediment (particularly post-DRE), where gene expression analysis was compromised

by amorphous precipitation in 10% of the specimens (502). Similar findings were reported by another study that showed that expression of *KLK3*, *PCA3* and *ERG* were higher in exosomes (501).

The isolation of RNA from pure exosomal or pure cell pellet fractions may not be as clear-cut as it first appeared to be. It was noticed that the longer the whole urine was kept before sedimentation of the cell pellet, the higher the cell RNA yield was and correspondingly the lower the exosomal RNA yield. It was hypothesised that this was due to exosomal vesicles adhering to the cells over time, and their RNA becoming harvested with the cell RNA. This implies that RNA content from the sedimentary fraction could vary with time so for a clearer division, there needs to be rapid urine processing.

One could argue on the significance of studying exosomal RNA alone when instead we could study RNA from whole-urine as the PCA3 test does. This would negate any changes in RNA between the sediment and supernatant fractions, and could be a future avenue of exploration.

In this study, it was decided to focus on exosomal RNA, as whole-urine RNA poses some challenges, particularly the efficient extraction of RNA from large urine volumes. However it is noted that very little RNA is needed for amplification with a Nugen Ovation kit, and, only 1ng of RNA is needed for Illumina sequencing, so, poor yields may not be an obstacle for future analyses of whole-urine RNA if single or small number of genes need to be examined.

A study comparing urine exosomes and whole urine showed advantages in term of analytical limits with whole urine in comparison to exosomes and cell pellet. For example, the amounts of *KLK3* and *PCA3* were highest in whole urine, then exosomes and lowest in cell sediment. But for diagnostic use the cell pellet appeared to be of more use than whole urine and exosomes. For example the *PCA3*/*KLK3* ratio was significantly higher in the PCa samples in the cell pellet, while in the whole urine and exosomes there was no significant different between cancer and benign. *ERG* mRNA levels were also significantly higher in the PCa cell pellet (diagnostic) but not in the whole urine or exosome samples. However the number of samples used were small (29 patients in total, including 15 PCa, 4 of which were excluded from the analysis), the PCa predominantly being Low and Intermediate risk (3 out of 15 had T3-T4 disease). This study also did not include bladder and kidney control genes. mRNA amplification was not used in this study which may explain the higher sample dropout for the exosome fraction (501) (we know from our experience that exosomal mRNA

level drops in High grade disease as explained previously and that needs to be taken into account). The authors also concluded that the exosomal fraction has an advantage in term of stability which I think is one the most important factors in developing a clinically robust test.

Urine can vary significantly between individuals and even in the same individual between different times of the day (see 5.5 for more information). The effects of these variables on urine biomarkers is expected to vary according to fraction. The cell pellet fraction appears to be the most unstable, as urine does not provide a healthy medium; cell survival is limited and will vary with exposure time. The cell pellet has also been shown (data by Rachel Hurst, UEA, not presented here) to largely consist of white blood cells, with only a very small proportion staining for prostate and prostate cancer cell markers. In addition, when prostate cells become detatched and lose cell:cell and cell:ECM contact they will initiate anoikis cell death. Thus the expression patterns of the cell pellet can vary enormously from sample to sample. In contrast RNA from the exosomal fraction is a stable snapshot of molecules as they were produced by the mother cells. Exosomal mRNA is stable in the urine at room temperature or frozen for over 48 hours (data not shown in this thesis) which is consistent with other study's findings (501).

5.5 A Urine test for the detection of prostate cancer: pros and cons

Urine has gained research attention as a non-invasive source of biomarkers with the potential to represent multiple foci of PCa. As mentioned above, I have tested exosomal RNA yields in patients pre- and post- prostatectomy, RNA yields dropped from >100ng to ~1ng indicating the prostatic origin of the majority of the RNA.

However there are several points that need to be taken into consideration if a urine test is to be available for future clinical use. The first point is practicality: urine consistency is variable from day to day or even from hour to hour in individual men. It is dependent on numerous factors both physiological and pathological. Physiological factors include hydration and nutrition status, and pathological conditions include pH, glucose, bilirubin, ketone, protein, specific gravity blood, urobilinogen, nitrites, and leucocytes – all reasonably

assessable by dipstick. Indeed a potential limitation of this study is that I did not systematically examine multiple samples taken from the same patient over time. Such a study would however have been complicated due to the requirement for a prostate massage prior to each sample collection, which would have been quite difficult to achieve in a clinical setting, and outside our ethical approval limitations. Changes in urine composition may affect the prostate biomarkers directly, or may interfere in the test results eg the presence of a large amount of bacterial cells. Attempting to standardise some of these variables, such as pH was not found to be of great benefit in this project. The primary way I found that improved the quality and quantity of the prostate RNA biomarkers was by speedy processing of the urine, which indeed will pose a great challenge for a urine test to be practically used in the primary and secondary care. These difficulties are reflected in the PCA3 test that has a limited time from collection to processing, as well as an expensive kit to aid preservation of the RNA in urine. For the PCA3 test the patient has to provide 20 to 30 ml of first catch urine following DRE - giving more then the required 30 ml may invalidate the test. The urine must then be kept at 2-8°C or on ice, and then transferred to a urine specimen transport tube containing preservation media within four hours of collection otherwise the sample is rejected. Shipping arrangements must ensure that the sample arrives at an analysis lab within 5 days of collection or it will be rejected (see http://www.hologic.com/sites/default/files/) (498) These aspects need to be taken into consideration as they have financial implications particularly if the test is to be used on large patient numbers for screening.

The second point is sensitivity. I identified *TMPRSS2/ERG* gene expression in ~20% of clinically benign samples from men that had a normal serum PSA reading suggesting that the technique is sensitive, and that these patients probably have low volume clinically undetectable disease that was sampled by exosomes, or a larger tumour that does not have an associated raised sPSA. Positive *TMPRSS2/ERG* in urine has been reported to have 94% positive predictive value for detection of prostate cancer (187).

A third point is how does tumour position within the prostate affect sampling? Due to the anatomical luminal network of connections within the prostate that connect to the urinary tract it is thought that biomaterial from most of the prostate could reach the urine after a DRE (581). The DRE technique has been reported to be important in boosting PCa biomarkers in the urine (581), however DRE efficiency may be dependent on several factors, such as site of tumour within the prostate. It is known that 75% of prostate cancer

arises in the peripheral zone, the remaining 20% arising in the transition zone and 5% in the central zone. In the clinic, transition and central zone tumours can be missed, as they are hard to detect in the biopsy procedure. Theoretically tumours in the central and transition zone are more difficult to massage in comparison to peripheral zone disease. For this reason the PCA3 test described a particular DRE requirement to depress the surface of the prostate by one centimeter aiming to produce pressure in most of prostatic tissue (superficial and deep). However it is not known to which extent these tumours are being represented in our samples.

Point four: High grade and advanced disease: Something that has been clearly apparent in my clinical experience is that the prostate becomes hardened in advanced disease (T4 disease). The prostate becomes packed with cancer cells and fixed to the pelvic floor. This renders it physically very firm and un-depressable during DRE, thereby reducing the effectiveness of the DRE. In addition, biomarker access to the urethra will be affected by luminal access, and this maybe curtailed in poorly differentiated/High Gleason tumours which can have blind-dended lumen as demonstrated by several studies (502,582).

Point five: clinical factors affecting DRE efficiency: i) patients can have a large prostate with a cancer focus that can be difficult to reach e.g. at the base of the gland, ii) a 'high riding' prostate, which is an anatomically high prostate that is difficult to reach by digital rectal examination; iii) obese patients whose prostate can be difficult to reach, iv) the effectiveness of the clinician performing the DRE, a factor not relevant to this study as I performed all the DRE and sample collections myself.

In order to obtain accurate information on the above I suggest for future study that urine expression analysis should be examined in patients undergoing radical prostatectomy to identify two groups of patients: i) patients with peripheral disease only and ii) patients with anterior disease only. A direct comparison could then be made with exosomal biomarker readout, to assess the efficiency of tumour biomarker sampling in cancers from different locations. This would also allow insight into assessment of multifocal disease, and to the efficiency of detection of tumours with different Gleason patterns.

To examine the function of a DRE, I examined samples from the same patients with and without a DRE. I showed a significant difference in the RNA yields in urine samples taken from men pre- and post- DRE which is consistent with other studies (498-501) (Discussed further in 3.7.2). However when I examined the difference in RNA yields in two cohorts of patients: one with a DRE according to the PCA3 protocol (vigorous 3-swipe massage) (498)

and one with a less aggressive DRE using only two swipes, there were no difference in the yields or cancer gene expression analysis between the two groups. This suggests that as long as a DRE is performed, prostatic secretions including exosomal RNA will be present in the urine and perhaps able to represent the prostate entirely.
To further examine the use of exosomal material in patients with no DRE compared to DRE, I prepared 10 paired pre- and post-DRE samples that in future studies are scheduled to be analysed by NanoString for a comparison of PCa biomarkers. At the time of writing these analyses were not available.

As discussed above, the expression data showed that cancer specific genes were not highly expressed in samples from men with advanced disease (see tables 4.7, 4.9, 4.10). However, I have also shown in this study that exosomal RNA from patients with High-risk disease is still detectable in urine, even though it is in smaller amounts, and the expression signature was usable in predicting response to hormone treatment. Wilcoxon test analysis comparing advanced to non-advanced disease showed that expression of *KLK's* as well as *PCA3* and *HOXC6* were not significantly over expressed. However when we compared cancer to benign samples these transcripts were significantly overexpressed in cancer samples as expected (tables 4.11, 4.12).

5.6 PCa Clinical Groups and subgroups

The lack of understanding of the disease's natural history on an individual basis is reflected in the complexity of prostate cancer management, which poses a real clinical challenge. The intricacies of the NICE risk stratification can change depending on the understanding we gain about the disease through clinical experience and research; for example the NICE Guidelines for PCa risk stratification in 2012 changed to upgrade T2c from the Intermediate to the High-risk group. A systematic literature review in 2012 (583) showed that there are some further clinical subcategories that should be taken into consideration, such as i) the creation of a very-low risk category; ii) splitting the Intermediate-risk into low-Intermediate - and high-Intermediate risk groups; and iii) further clarification of the boundary between Intermediate and High-risk disease. Rodrigues also suggested that more prognostic parameters should be taken into consideration when it comes to assessing risk of metastasis and prognosis, such as percentage of positive-core biopsies and evidence of perineural

metastasis. In this study we adopted the NICE stratification criteria for PCa to facilitate our data analysis and to assess the differential gene expression between different risk groups. However in addition I felt that further sub-classification of the risk groups see paragraph 3.4.2) was necessary. However due to low numbers of patients in some of the subcategories we have mainly used subclassification in the Intermediate risk and High risk groups in order to gain in-depth information and precise correlation of our biomarkers for diagnostic and prognostic accuracy. Hence for the LPD analyses (paragraph 4.3.2.5) I subdivided the Intermediate risk group into Intermediate (G3+4 PSA 10-20 and T2c) and high-Intermediate (G4+3 PSA 10-20 and T2c) as my clinical experience has led me to expect these 2 subgroups of patients to behave differently and have different prognoses (defined as I and IH respectively). The high-Intermediate (G4+3) have more of the less-differentiated cancer cells and behave more aggressively than the low-Intermediate (G3+4) who in turn have more of the relatively well-differentiated cells. However, our data analysis showed no significant difference between the two sub-groups in term of gene expression which could be due to the low number of samples in the IH group (only 18 samples) vs I group (53 samples) as represented in table 4.6.

I subdivided the High-risk group into i) High risk (PSA>20 and <100 or G8-10 or T2c and above) and iii) Advanced (T3-4, G8-10 PSA>100 and patients with PSA >100 and clinically T3-4 with no available histology). The reasons for this are: i) it is well known that patients with PSA>100 have 100 % risk of cancer metastasis as reported in the literature (509), and so should be differentiated from the high risk group with lower PSAs; ii) some of these patients get diagnosed based on clinical findings ie PSA>100 and clinically T3-4 without histological evidence (for further explanation see paragraph 3.4.2). While these subgroups would be expected to behave clinically differently, all these patients would be offered clinically the same treatment (hormone deprivation therapy). For this reason NICE does not separate them.

Due to a lack of funding for expression analysis at the start of the project (these funds are available now and further analysis will taking place), we did not include the most clinically challenging group of patients. These patients present with a high PSA, but are negative for PCa on biopsy analysis. This group makes up 50 to 70% of patients in the US, (135,584) and approximately 30% of NNUH patients at the PSA clinic. There is a real clinical challenge in their management, particularly in the presence of continued rising PSA levels, as we know that 10-20% of these patients may still have cancer that was missed by the initial biopsy.

Much of this undiagnosed PCa will probably have only a limited clinical impact (585) while some can be aggressive disease (15), hence urologists have a low threshold for repeating a biopsy in cases of rising PSA. Rising or persistently raised PSA creates great anxiety for the patients and their clinicians, even though PSA cut-off is subjective. 10% of patients with a PSA below one can still have cancer (ERSPC Study), and most of these patients require routine follow up with serial PSA testing and repeat biopsies which carry significant morbidity. In the presence of negative serial biopsies we sometimes accept that the PSA level is high for these patients – PSA can be raised for an unknown reason, can reflect the prostate size, or is due to inflammatory changes (586) - as long as it remains stable without substantial variation (587). All these dilemmas created by PSA testing also have a significant financial side-effect on the health service, and stress on large numbers of men. For these reasons we will include this group of patients in the next set of analysis (samples from this group of patients have been collected and stored ready for analysis). Indeed this project, which I formulated during my clinical fellowship, is being used as the basis for a PhD project for a current clinical fellow. Specifically urine that I collected from men with raised PSA but negative for cancer on biopsy is being used to assess whether molecular approaches can be used to detect men who subsequently develop cancer.

Another interesting group of patients to study would be to follow-up patients found with High-Grade PIN and/or atypia in their initial biopsy, to identify whether our gene expression can predict progression in this group of patients. Again these patients will normally be candidates for repeat biopsies under current clinical practice.
The percentage of positive cores was not taken into consideration in these analyses (data was recorded and is available) as I thought it was likely to complicate the analyses. However, further analysis will take place in the future when the full data analyses has been completed, and, biopsy cores as well as other variables will be considered e.g. disease percentage, and Gleason grade found in the radical prostatectomy patients (Data also recorded).

Adding in further clinical data, combined with sub-division of patient subgroups should not only help us to study our gene expression signatures more accurately to provide a superior prognostic value, but may also be useful in prediction to treatment response.

5.7 Benign control group

Choosing a benign control group was very challenging due to the fact that there is no guarantee that any patient with a normal PSA will not have prostate cancer. The PCPT trial showed that there is 6.6% prevalence of PCa in patients with PSA<0.5ng/ml, 10.1% in PSA 0.6-1ng/ml, 17% in PSA 1.1-2 ng/ml, 23.9% in PSA 2.1-3 and 26.9% in PSA 3.1-4 ng/ml (588,589). Very young patients in their early adulthood may have been a good alternative, but their recruitment is not ethical, and also a comparison of their gene expression with men in their 60s may not be appropriate, as it would not reflect the benign changes that the prostate develops in older men. Similarly as stated above, patients with a raised PSA and negative biopsy cannot be guaranteed to be cancer free, as at least 23% of these would still have PCa (134,590). However, it should be noted that both the above groups of clinically benign patients are unlikely to have significant disease that would require treatment. For these reasons I opted to use two sub-groups of men with clinically benign prostate as non-cancer controls: i) those men with a PSA normal for age, and ii) men with a PSA below 1ng/ml.

I followed up some of these patients by checking the results of their PSA for a period of two years to identify subjects that had rising PSA indicating possible PCa, but none were found. However, it is worth noting that a proportion of these patients, particularly the younger ones did not have further PSA testing suggesting they are asymptomatic for prostate cancer and therefore had no clinical indication for further testing. Looking at the literature I found that different strategies for identifying a benign group have been adopted by different authors. For example a PCA3 study, used patients below the age of 45 with no known prostate cancer risk factors (498). It is however not clear whether these men had a PSA check at all. Another study, used patients with a benign histology on TRUS biopsy (206) while others used female and young men (age<27) (591) and some used men with a PSA below one. This shows that there isn't a well-defined benign control group that could be used across the board. For this reason different authors choose what is thought to be most appropriate.

5.8 NanoString Expression analysis

The large number of samples generated by this study, and the 50 gene probes that had been chosen for analysis meant that a cost effective but reliable means of analysis needed to be found. Four available options were considered:

- TaqMan quantitative RT-PCR gene expression analysis: this is a well-validated method of gene expression analysis but has negative aspects which include i) expense: cost for 50 gene probes was ~£110 per sample, a total of £21,300 for 193 samples, ii) time efficiency: sample preparation for the procedure is time consuming and prone to operator error. An alternative to setting up each assay by hand would be to use custom made TaqMan microfluidic cards, however, this would be even more expensive, and the card designs come with restrictions on the number of genes that can be interrogated, namely, combinations of 12, 24, 32 genes only. It would not be possible to purchase cards with 50 gene probes on them. This meant that number of genes would need to be compromised.
- Next Generation sequencing: is a good method for gene expression analysis due to the fact that it will analyse the whole transcriptome in order to identify the genes in question. However it was again thought not to be cost effective - around £1,340 per sample. Bioinformatics analysis is also time consuming because of the amount of data generated - approximately 30 million reads per sample.
- Microarray analysis: a good tool for evaluating differential gene expression, but again costs are around £450 per array, and the data from ~30,000 gene probes was considered to be more than we needed for this pilot study.
- NanoString: we have opted to use NanoString for several reasons including time efficiency i)- a large numbers of samples and genes can be analysed by NanoString inc. in a very short period of time, ii) it is a cost effective assay for large numbers of samples and multi-gene analysis in comparison to TaqMan, microarrays and next generation sequencing (NanoString £125 per sample inc labour, TaqMan Cards £110 plus labour, Microarray £450 per array, Next Generation sequencing £1,340) iii) gene expression data (including PCa analysis) has been reported to be of good quality (592,593), iv) NanoString is now in use for the FDA approved Prosigna test for breast cancer.

As NanoString is designed for RNA analysis, and we had Nugen Ovation WTA2 amplified cDNA, which had never before been analysed by NanoString, I performed a pilot test of 12 samples using the off-the-shelf nCounter human cancer 236-gene reference assay. These cDNA samples worked very well in the assay, and expression of 189 out of 236 genes were detectable. In this pilot, 20 genes showed differential expression between cancer and non-cancer samples including, *AR, BM11, BRAF, CCND2, CDKN1A, DEK, ERBB4, ETV5, HDAC1, IFNGR1, MTA1, NRAS, PLG, PRKAR1A, PTEN, RAF1, RRM1, SCD1, STAT3*. (figure 4.7) Some of these genes are known to be expressed in prostate cancer such as *AR*,

BRAF, and RAF. It is worth noting that this data was normalised to internal controls probes such as GAPDH (cell house keeper not exosomes). As discussed below, exosomal RNA in urine can come from a number of cell sources. Thus the expression of cancer markers is likely to be improved by adjusting them to be relative to a prostate tissue specific probe such as *KLK2*, or *KLK3* – the latter as used by the PCA3 test (498). However, *KLK* probes were not present on the nCounter human cancer assay, and therefore full expression analysis was not possible. The nCounter analysis did enable me to determine that amplified cDNA from urine exosomal RNA samples was adequate for analysis by NanoString, and that a wide range of transcripts were detectable in our samples.

These pilot analyses highlighted the need for a normalisation gene: Urinary exosomes are expected to originate from a number of cellular sources besides PCa itself, these include: prostate stromal tissue, immune cells, bladder, urothelium and kidney. Thus normalisation of the expression data relative to prostate or PCa is critical to fully understand the data. *KLK2* and *KLK3* (594,595) have been used as their expression is relatively even between normal prostate and PCa. Use of an appropriate normalising transcript will be particularly useful for examination of high-grade disease where exosomal representation may be compromised as discussed above. Groskopf *et al*., (498) has shown that normalisation of *PCA3* to *KLK3* ((*PCA3*/*KLK3*)x1000) has improved *PCA3* test diagnostic capability. The ratio being significantly higher in cancer samples in comparison to benign. It has also improved the specificity of the test, and similar finding have been reported by other authors (497). The data in the study presented here has not been normalised to *KLK2* or *3*, but this will take place in the larger project.

NanoString expression analysis (50 gene-transcript probes. See Chapter One Table 1.4 for gene information summary) of exosomal cDNAs identified genes that were significantly up regulated in cancer patients, mainly in the High risk and Advanced risk group (Cluster A). These included Kallikreins (2, 3 and 4), *MMP26*, *STEAP2, STEAP4, AR*exons4_8, *AR*_truncation_exon, *SERPINB5*/*Maspin, PPAP2A, CLU, OR52A2_PSGR* and *CDC2* which are expected to be upregulated in prostate cancer and in particular with high grade disease in consistence with a study at the cellular level (Table 4.5 Chapter 4). *B2M* was initially chosen as a house-keeping gene as some studies reported it to have an even expression between bladder, blood, and prostate (493,496) and was used as a housekeeping gene on a study on urine sediment (596) although other studies reported it to be up regulated in prostate cancer in particular with high grade disease with distant metastasis (493,597) which is in

consistence with our results particularly as most of the samples in this set are from the Advanced risk group. In contrast *SPINK1* and *Hepsin* (HPN) which are known to be up-regulated in prostate cancer tissue (see paragraph 1.9) were found to be down regulated in our data although the PCA analysis did not directly compare cancer vs benign disease (Cluster A vs all data). In this study *HPRT* was selected as a housekeeping gene as several studies had shown similar expression of this gene in bladder, blood, and the prostate, tissue and it has been frequently used as in multi-gene expression profiling of prostate cancers (See paragraph 1.9.2). However, in these analyses *HPRT* was upregulated in exosomal RNA in cluster A (High risk and Advanced risk group). Our results are in consistant with several studies that have shown that mutations in the X-linked *HPRT* gene are associated with metastatic prostate cancer
(107,598,599).

UPK2 and *SLC12A1* were used as bladder and kidney controls respectively although they were also differentially expressed in this analysis (Down-regulated).

Latent Process Decomposition (LPD) analysis was then utilised to look at the data in a different way. LPD clusters data in an unsupervised, probabilistic approach. LPD analysis was applied to all the samples except those identified in cluster B due to their low mRNA yields (6 samples). We identified 4 statistically different groups: LPD1 that predominantly consisted of samples in the High-risk and Advanced risk groups and was similar to cluster A identified in the PCA analysis; LPD2 contained patients in the Benign risk group, LPD3 had patients in the Intermediate and High-risk group; and LPD 4 which mainly contained cancer patients predominantly in the Intermediate risk group as shown in table 4.6.

The expression of the genes identified in LPD1 (High risk/Advanced patients) contrasted to those highlighted by the PCA analysis. Interestingly all the prostate and prostate cancer associated genes including *KLK2*, *KLK3*, *KLK4*, *STEAP2*, *PSMA*, *PPAP2A* were all relatively underexpressed compared to the other LPD groups, while the bladder and kidney control genes *UPK2*, *SLC12A1* and *SPINK1* were all overexpressed. A possible interpretation of this result is the difficulty in efficiently massaging the prostate in men with advanced tumours and the distorted intraprostatic anatomy that is likely to prevent PCa exosomes from reaching the urethra. This can lead to a relative increase in the representation of markers from the kidney and bladder as discussed in paragraph 4.3.2.5. Although

SPINK1 is known to be overexpressed in a proportion of prostate cancer it is also known to be expressed in the normal kidney, which may explain its overexpression.

AMACR, *PCA3*, *HOXC6*, *TORD*, *ERG*, *PSMA* and *IMPDH2* were identified as being overexpressed in LPD3, a group that predominantly contained Intermediate risk patients. The expression of these genes is known to be associated with prostate cancer as discussed previously. Interestingly bladder and renal genes do not appear to be overexpressed which is in agreement with the discussion above regarding DRE efficiency and intraprostatic anatomical distortion, which is expected to be at a lesser extent in this group of patients.

The non-parametric rank Wilcoxon test results were in agreement with the LPD findings in terms of gene expression. A comparison between cancer and benign risk groups identified 15 genes that looked promising diagnostically as they appeared to be upregulated in cancer including; *ERG, PCA3, DLX1, HOXC6, HOXC4, HPN, SUL1A1, TDRD, GAPDH, CLU,* and *CDKN3* (Table 4.12) and two downreguated genes including *HPRT* and *PPAP2A*. These were reassuring results particularly as *PCA3, ERG, TDRD, HOXC6, HOXC4* and *DLX1* are well known gene transcripts associated with prostate cancer, particularly in urine sediments (514,517). In contrast, *HPRT* was again found to be underexpressed in agreement with our PCA analysis discussed above (which may be due to samples selected by the different analysis ie PCA selected mainly high risk sample while Wilcoxon test compared cancer to benign). 17 prognostic genes were identified as significantly differentially expressed in High risk and Advanced disease in comparison to the Lower grade disease and Benign (table 4.11). Again these results are in agreement with the LPD analysis were we find that the prostate cancer genes are mainly under expressed including *AR, KLK's* and *STEAP2*, and the bladder and renal control genes are overexpressed. 21 genes were significantly differentially expressed in Advanced in comparison to the Benign control group (see section 4.3.2.6). Again, here the prognostic genes are in consistence with our previous results (LPD1 analysis) and included *SPINK1*, *KLK2*, *3* and *4*, *SLC12A1*, *STEAP2* and *4*.

The data from these analyses has created a solid ground for further analysis, which will be performed on the planned bigger project. In these future analyses, transcript expression patterns will be integrated with clinical parameters in order to maximise performance in terms of diagnostic and prognostic accuracy. The Prostate Cancer Prevention Trial Risk Calculator (PCPTRC) uses a multimodal risk assessment score to predict risk on an individual basis; parameters include PSA, DRE, age, family history, and previous biopsy information (600,601). A recent study showed a higher detection rate of clinically significant PCa was achievable when combining the clinical risk assessment score (ie PSA

Density, DRE, age, family history) with a two-gene risk score for whole urine expression levels of *DLX1* and *HOXC6*. Inclusion of the expression data significantly outperformed the PCPTRC on its own and improved the diagnosis and management of PCa patients (517). Van Neste found that the PSA Density (PSAD) PSA ng/ml divided by prostate volume in gram) was an important factor. It is known that prostate cancer cells do not secrete more PSA relatively to normal cells (594,595), and it is disruption of the basement membrane in a cancerous prostate that enables more PSA to enter the circulation system leading to an increase in the PSA level. Adjusting sPSA relative to the prostate volume (471,602) in several studies has shown a significant improvement in the prediction of TRUS biopsy results in comparison to sPSA alone, thus improving the diagnostic accuracy (603-605).
PSA volume data has not yet been obtained for our cohort, but will be used in the larger study.

Patients with metastatic disease are primarily treated with hormone deprivation therapy. However, the cancer invariably becomes resistant to treatment leading to disease progression and eventualy death. Treatment of patients with metastatic prostate cancer is clinically very challenging for a number of reasons, which include: 1) the variability in patient response to hormone treatment ie time prior to relapse and becoming castrate resistant, 2) the detrimental effects of hormone manipulation therapy on patients, 3) the myriad new treatment options available for castrate resistant patients.

The response to hormone manipulation/ablation therapy is highly variable (discussed in paragraph 4.4). Some men fail to respond to treatment while others relapse early i.e. within 6 months, the majority relapse within 18 months (late relapse) and the rest respond well to the treatment often taking several years before relapsing (delayed relapse). Early identification of patients who will have a poor response will provide a clinical opportunity to offer them a different treatment approach that may perhaps improve their prognosis. However there is no means currently to identify such patients except for when they exhibit biochemical progression with rising serum PSA, or become clinically symptomatic, in which case they get offered a different treatment strategy. This regime however goes hand in hand with a number of detrimental effects (606) such as bone loss (607) increased obesity, decreased insulin sensitivity increasing the incidence of diabetes, adversely altered lipid profiles leading to cardiovascular disease (608,609) and an increased rate of heart attacks (610). For these reasons offering hormone manipulation requires a lot of clinical consideration

particularly as most of the patients requiring such treatment are elderly patients and such treatment could overall be detrimental rather than beneficial (611).

Due to ever-emerging new treatments or second line therapies for patients with advanced metastatic cancer in the past decade, the treatment of men with castrate resistant prostate cancer is dramatically changing. Prior to 2004, the only treatment option for these patients was medical or surgical castration then palliation. Since then several chemotherapy treatments have emerged starting with docetaxel (612,613) which has shown to improve survival for these patients. This was followed by five additional agents (FDA-approved) including new hormonal agents targeting the androgen receptor (AR) such as the AR antagonist Enzalutamide, agents to inhibit androgen biosynthesis such as Abiraterone, two agents designed specifically to affect the androgen axis (614,615) sipuleucel-T, which stimulates the immune system (616) cabazitaxel chemotherapeutic agent (617) and radium-223, a radionuclide therapy (618). Other treatments include targeted therapies such as the PI3K inhibitor BKM120 and an Akt inhibitor AZD5363 (619-621) all of which are still under clinical trials. Therefore it is crucially important to be able to identify patients that would benefit from these expensive treatments and those that will not. While these agents have been tested in multiple disease states of castration resistant patients to determine if or when patients might benefit, the answer at present to this question is still not available. Identification of prognostic indicators capable of predicting response to hormone manipulation and to the above list of alternative treatments is very important and would have great clinical impact in managing these patients. In addition, the only current clinically available means to diagnose metastasis is by imaging. Markers that are being put forward include circulating tumour cells and urine bone degradation markers, both of which are still under research (622), A test for metastasis *per se* could radically alter patient treatment. The data within this thesis suggests that exosomal RNA may have the potential to overcome these issues, particularly as studies have shown a role fro exosomes in aiding metastasis (623)

To my knowledge exosomal gene expression analysis on patients treated with hormone manipulation to identify response to treatment has not been done before. In the current study, all patients treated with hormone ablation had their urine sample analysed and processed for gene expression analysis on the same day that they later started treatment. Any

patient presenting with clinically advanced PCa ie PSA>60 and a clinically malignant prostate T3/T4 on biopsy (if considered not suitable for other treatment strategy) was started on hormone deprivation therapy on the day of presentation. A 3-monthly follow up was arranged for all patients, with repeat PSA testing as an indication of progression. 40 patients (17 Advanced and 23 High-risk) that had local (21 patients) and distant metastasis (19 patients) were treated with hormone manipulation in this cohort. LHRH agonists or antagonists such as Goserelin or Leuprorelin acetate. 8 patients (20%) failed to respond to initial treatment with their PSA remaining above 60ng/μl, 7 of which had extensive widespread bone metastasis (as per their bone scan results) none of the patients had visceral metastasis. 5 patients had early relapse (PSA reduced to normal levels but rose again within 6 months). These 5 patients as well as the 8 who failed the initial treatment were subsequently given maximum androgen blockage by adding anti-androgens, followed by PSA follow up on a 3 monthly basis to detect whether they progress and become castrate resistant.

Gene expression analysis identified two genes (*SERPINB5*/*Maspin* and *HPRT*) that differentiated between two groups of patients: i) those who failed initial response (low expression values) and ii) those with a good initial response. Maspin is known as a class II tumour suppressor, which specifically inhibits uPA (urokinase-type plasminogen activator) that promotes tumour growth by osteolysis and angiogenesis leading to cancer growth and bone metastasis. uPA also affects expression of interferon regulatory factor 6 (*IRF6*) (302), β1-integrin (303,304) collagen I (305) and glutathione-S-transferase (*GST*) (306); all of which play an important role in cancer growth and metastasis. *Maspin* is known to be up-regulated in premalignant prostate cancer epithelial cells (309) and constantly down-regulated at the critical transition from noninvasive, low-grade to highly invasive, high-grade prostate cancer (309). To my knowledge *Maspin* exosomal expression has not been reported in the literature however our results suggest it is underexpressed in patients with poor response to hormonal treatment in comparison to patients with a good response. LPD analysis identified *Maspin* as overexpressed in Intermediate disease (table 4.10). In the LPD analysis *Maspin* was not identified as underexpressed in the advanced samples, however the LPD group confined mixture of patients with different response to treatment.

HPRT (Hypoxanthine-guanine phosphoribosyltransferase 1). This is an established housekeeping gene for PCa tissue that is frequently used in multi-gene expression profiling of prostate cancer (492,493). However its use as a housekeeping gene has been questioned as some studies have implicated it as tumour suppressor gene after finding mutations in this

gene that lead to cancer progression and metastasis (107,598,624). Although its role in exosomes is not yet known, our results may suggest that it has a role in castrate resistant cancer at the exosomal level. Further analysis in a larger number of samples will be required in order to validate our finding. On the LPD analysis *HPRT* was found to be underexpressed in the advanced disease group (table 4.13) and was also found to differentiate between cancer and benign disease, although *KLK3* normalisation was not applied to these data to obtain a clear picture of gene expression representation.

Three gene probes were identified that were differentially expressed in early development of castration resistance patients (paragraph 4.4.2): *STEAP4*, *ARexons4_8* and *NAALAD2*, (not predicted by *HPRT* and *Maspin* which were associated with failure to initial response to treatment). The down-regulation of these genes predicted development of castration resistance and relative over-expression was associated with a prolonged response to hormone therapy (See paragraph 4.4.2 for further explanations). The Kaplan Meier (KM) estimator test to evaluate prognosis showed an 85% chance of survival at 24 months in patients with gene overexpression in comparison to 45% in patients with downregulation.

STEAP4 is known as a tumour suppressor gene, its encoded protein inhibits independent cell growth through regulation of phospho-Y397 on focal adhesion kinase (FAK). In androgen-independent prostate cancer cells, some studies reported that that CpG sequences of the *STEAP4* promoter region were frequently methylated, and that demethylation treatment induced the expression of *STEAP4* in this cell line. This was in contrast with the androgen-dependent prostate cancer cell line LNCaP in which no methylation was reported (395). Our results are in general agreement in that *STEAP4* underexpression was found in patients with poor response to hormone manipulation treatment and early relapse (castrate resistant) in comparison to castrate sensitive samples.

The androgen receptor is known to be associated with castration resistant prostate cancer (hormone insensitivity). AR plays an essential role in prostate cancer from cell viability to proliferation and invasion (374-376,384,385)**.** Interestingly however its expression in NanoString analysis of exosomal cDNA was found to be down-regulated at the exosomal level in patients with poor response to hormone treatment, and relatively over-expressed in those with good response. This is contradictory to the reports documented on cellular RNA. Its expression in exosomes is not documented in the literature and further investigations are required.

Two *AR* probes were included in our analyses however while both probes gave a range of

signal strengths (ie appeared to be working), only one of them was associated with castration resistant PCa. This highlights the importance of targeting known specific transcript splice variants in expression analyses. It is well documented that castrate resistant PCa remains driven by AR signaling which remains activated through various mechanisms; one of which is *AR* transcript splicing resulting in shortened AR isoforms which mainly affect the dual-function COOH-terminal ligand-binding domain/AF-2 (AR is a protein with an NH2-terminal (NTD) transcriptional activation domain, a central DNA-binding domain (DBD), and a dual-function COOH-terminal ligand-binding domain) (385,625,626).

These shortened AR isoforms are constitutively active and can support various features of the CRPCa phenotype that play an important role in disease progression. However some of these AR variants have also been reported in benign cells as well as in androgen naïve PCa (385,625,626). Some studies have reported several *AR* splice variants (384,385) (627) that increase in expression during progression to castrate resistant PCa (384). Each of these AR variants has been associated with distinctive functional properties, which are thought to synergise to form a castration-resistant phenotype independent of the full-length AR. A recent study reported a set of genes that was regulated uniquely by AR variants, but not by full-length AR in the absence of androgen, some of which were directly modulated by the AR-variants (628). This study also reports a difference in the AR variant signature between benign, malignant and metastatic castrate resistant prostate cancer. Some studies have reported a correlation between AR variant and response to chemotherapy. Ome study (629) reported that men in which AR-V7 was detected in circulating tumour cells had a better response to taxanes in comparison to enzalutamide and abiraterone therapy whereas AR-V7 negative men all those treatments had comparable efficacy.

NAALADL2 is known to be over-expressed in hormone sensitive prostate cancer. Its over-expression has also been shown to predict poor survival following radical surgical treatment for PCa. It is known to promote cancer progression by endorsing adhesion to extracellular matrix proteins, migration and invasion by regulating the levels of Ser133 phosphorylated C-AMP-binding protein (CREB) (476)**.** However, its expression in castrate resistant cancer cells is not well documented nor is its correlation to androgen receptor expression. Our findings suggest that its expression is under-regulated in castrate resistant PCa perhaps due to AR signalling changes or other unknown factors.

5.9 Sequencing of Exosomal RNA

There is a lack of publications on exosomal RNA expression, particularly prostate-derived exosomes. This meant that our choice of gene probes for this study had to be based on differentially expressed genes in prostate cancer tissue as compared to normal prostate tissue. However my study was based on exosome derived RNA rather than prostate tissue, and so it was decided that the range of RNA species present in urinary exosomes had to be explored to attain new diagnostic and prognostic biomarkers, and also provide a better understanding of the role of these microvesicles in PCa.

Due to the improvement in exosomal RNA yields it was possible to carry out sequencing analysis on 100ng RNA without resorting to sequencing amplified cDNA samples. It was thought that sequencing amplified cDNA samples would possibly cause some doubt on the final data, however one could argue that sequencing amplified cDNA could be better for probe selection for NanoString gene analysis, which is, after all, performed on amplified cDNA. As there were no other published prostate exosomal sequences available for comparison, we opted to analyse the native RNA, using Next Generation Sequencing (NGS) to assess 18 exosomal RNA samples, comparing High- Intermediate- and Benign samples.

The analysis identified 45 genes that were significantly differentially expressed between benign and cancer samples (figure 4.18). There is little in term of match between the gene probes chosen for the NanoString analysis and the NGS except for the overexpression of *PCA3* and *DLX1*. This is not surprising, 1st as the gene choice for the Nanostring analysis was based on prostate tissue expression patterns rather then exosomal mRNA and 2nd cDNA was used in NanoString compared to mRNA in the NGS. Reassuringly *PCA3* and *DLX1* are known to be overexpressed in cancer, in particular, *PCA3* that has been reported to be over expressed in exosomal RNA from prostate cancer patients by several authors (576,577) which to some extent validate our data.

DLX1 up-regulation in urine is known to be associated with prostate cancer, and aggressive disease in particularly as reported by several authors (517). One study has identified *DLX1* as a promising biomarker for the diagnosis of prostate cancer, on an expression profiling on urine sediments (514).

Of the 45 genes identified by the NGS, 28 are up regulated and 17 down regulated in cancer showing a significant linear trend in association with cancer risk (Table 4.17). Of the up

regulated are the apoptotic genes *BIK* and *AATF* which are known to be significantly over expressed in many solid tumours such as lung, breast, colon and prostate (519,520). BIK apoptotic function was reported in both hormone sensitive and castrate resistant cells, and it was thought that its induction could have therapeutic potential (522). ATTF is known for its role in cell cycle regulation and growth arrest by apoptosis via interaction with Dlk/ZIP kinase (524). Its expression in prostate cancer was reported to be associated with higher mortality in patients on ADT (526) (see 4.5.1 for further explanations). Although both these proteins appear to work in opposition to each other they were both up-regulated in cancer exosomal samples. However, unlike *BIK*, *AATF* had a trend with cancer stage (table 4.17). Other upregulated genes included *ACTR5* that plays a role in cell cycle progression and nuclear co-activation, its overexpression has been documented in LNCaP Cells (529,530). Genes that play a role in tumour proliferation and progression such as *HPSE2* (remodelling of the extracellular matrix and cell surface promoting disease progression and metastasis (533,537)), *NUDT6* (541) and *ST6GALNAC1* (545).

MMP25 was identified to link positively to Gleason score (table 4.17). It is one of a large family of MMP's that promote cancer progression and metastasis by its proteolytic function leading to degradation of the basement membrane and extracellular matrix, as well as induction of the epithelial-mesenchymal transition, neovascularisation, and regulation of growth factor and chemokine activity (630). Althought its expression has not been documented in urine, we demonstrated that *MMP25* and *MMP26* are overexpressed in exosomal RNA, in the NanoString analysis (*MMP26)* (table 4.5) and the third generation sequencing (*MMP25)*. These finding are in agreement with several studies at cellular level in particular their correlation to gleason score (511,631).

Inflammation is known to induced carcinogenesis in several epithelial organs (632,633) as for example *Helicobacter Pylori* infection induced gastric cancer and hepatitis incuding hepatocellular carcinoma. Similarly infection induced stimuli are reported to cause prostate cancer through prolonged chronic inflammation that is the primary driver of this effect (447,449,634) by inducing tumour growth, angiogenesis and metastasis although the mechanism of this process is still not fully understood (3).
The PCPT (Prostate Cancer Prevention Trial) reported a link between inflammation and prostate cancer particularly high grade disease, where they showed that patients with chronic prostatitis has 1.79 time the odds in developing high grade disease (635). Other studies has

shown similar finding, for example, in a study on 71 patients on ADT (androgen depreviation therapy) (636) it was shown that tumour associated macrophage (TAM) infiltration detected on TRUS biopsy was associated with high grade disease, high clinical stage and in patients with biochemical failure (failed to respond to treatment with rising PSA), these macrophages are thought to be a part of inflammatory circuit that promote tumour progression. Similar finding was reported by other authors (637) (638). Another study demonstrated that serum elevation of IL6 is associated with aggressive prostate cancer and that it plays a role in the development of castration resistance in patients on ADT through activation of AR by ligand-independent mechanisms (639). Similarly on a mouse model it was shown that infection induced chronic inflammation in the prostate is associated with increased cell proliferation and reduced AR and *Hmebox1* expression (640). These chronic inflammation lead to acceleration of prostate cancer progression by conversion of basal cells into luminal cells and disruption of the basal cell layer (641). Thus multiple trials on immunotherapy for PCa has taken place some showing promising results such as the Sipuleucel-T trial which showed an overall survival advantage in castration resistant patients (642), although it thought that immunotherapy in the treatment of PCa in general may not be overall very useful as PCa is commonly diagnosed late in life, where age-related decline in immune response is common (643). Exososomes are known to promote tumour metastasis through several mechanisms, one of which is impairment of the immune function. Some studies, for example, determined that prostate cancer derived exosomes impair lymphocyte cytotoxic function and promote tumour excape by down-regulating the activating receptor NKG2D on natural killer cells and CD8+ T cells (644). To date prostate cancer derived exosome effect on the immune system and tumerogenicity is still under studied, nevertheless our third generation sequencing has identified numbers of genes that may have baring on immune including the downregulated genes; *HMBOX1*which is known for its inhibition of the NK cells activity (560), *ERMP1* that has been identified as lung tumour marker (645). Endoplasmic reticulum plays an important role in the immune response, their chaperones has been shown to have utility in anti-tumour vaccination when purified from tumour tissue (646) including ERMP1 (protein) (647), although it expression in prostate cancer exosomes has not been previously reported. Other down regulated genes includes *KNTC1*. Up regulated genes, include *PRR5L* (CD56 NK cells.), *PLCB2* (Expressed by Blood cells, myeloid, monocytes, NKs, CD4, CD8 T-cells), *NLRP3* (Negative regulation of NF-kappaB transcription factor activity and interleukin-1 beta production), *CLIC2* (CD31), *CKAP2L* and

ABCB9 (CD71) and *RP9* (B cells) (as presented in table 4.17). The function of these genes has not been previously reported in prostate cancer and is an avenue for further research.

5.10 Future Work

To validate our data, further gene expression analysis is planned on another 400 samples, and an addition of a further 117 gene probes to the 50 genes used in the present analysis. These additional gene probes have been suggested by further literature research and personal experience of members of the Movember consortium. Future work will also include selected genes from the exosomal RNA Next Generation Sequencing data discussed above.

In addition to this, various whole urine aliquots and biological fractions extracted from the samples that I have collected are being analysed by members of the Movember GAP1 Urine Biomarker consortium. This is an international consortium examining urine biomarkers for prostate cancer, involving 11 teams in 7 different countries, and set up and run from UEA. Analyses will consist of Mass Spectrometry, ELISA for 11 different proteins, DNA-methylation analysis, metabolite analysis, and expression analysis for cell and exosomal RNA. Collation of the data and its meta-analysis will be undertaken later this year. These analyses will look for the best combination of markers within urine for PCa diagnosis and prognosis, optimising the opportunities of producing a successful urine test for PCa.

The initial study period has been extended, and sample collection extended to 1200 samples in order to improve the chance of statistically significant results. As well as the urine samples collected from the NNUH, further samples are being collected from 5 other cohorts from 4 different nations. This should enable data to be validated in samples from different populations with a variety of diet and life styles. I have also arranged ethical approval for extended patient follow up (up to 5 years).

In order to ascertain whether the exosomal transcript expression data truly represents the prostate as a whole and can outperform TRUS biopsy by sampling the multiple cancer foci, a comparison of the final histology results in patients undergoing radical prostatectomy is planned. Further work should collect the prostate gland volume as documented by a histopathologist, along with the percentage of benign and cancer tissue and their volumes, and positions within the prostate. This could then be compared to the exosomal RNA expression signatures to identify whether, for example, benign prostate hyperplasia has any effect, and whether urine biomarker sampling is influenced by tumour size and position, and

location in the Transition zone.

A series of 125 exosomal RNA samples obtained from active surveillance patients at the Royal Marsden Hospital is available for study at UEA. Many of the patients have had serial urine sample collections (DRE as per PCA3 protocol), and these samples could be studied to identify how accurate our gene set is in identifying cancer progression.

Clinical follow up is critical to proper disease anlaysis, and the UEA patients, especially those treated with hormone manipulation should be followed up for longer periods (up to 5 years) with serial urine samples to assess the sensitivity of the gene set in predicting late progression. Clinical follow up for the benign/no-evidence of PCa men, and patients with raised PSA and negative first biopsies will be included in the analyses. A proportion of the latter will have second biopsies in which approximately 20% will be found positive for PCa. These samples will be extremely useful to confirm the specificity of our gene set for PCa detection and to assess whether there is any influence of histologically identified inflammatory changes and HG-PIN to the results.

We will also consider using a secondary validation method for a subset by RT-PCR.

5.11 Conclusions

The aim of this project was i) to discover a robust set of molecular markers with a superior sensitivity and specificity for identifying prostate cancer, superior that is, to the currently clinically available tests (PSA, *PCA3*); ii) to be able to differentiate between aggressive and non aggressive disease; iii) to predict which tumours will become aggressive.

There is a clinical need for a safe screening test for PCa so that patients with aggressive disease can be offered further investigations and treatment, and those with indolent disease can be reassured and possibly monitored with further urine testing, avoiding invasive biopsies as required under current practice.

A valid set of new biomarkers could revolutionise screening, prognosis, post-treatment monitoring, and even prediction of response to treatment, so that patients would be offered the best treatment modality earlier on in their journey with PCa. This would enable clinicians to optimise prognosis and prevent unnecessary treatments for patients with indolent disease, thus preventing exposing them to unnecessary side effect.

The data that I have strongly indicates that exosomal RNA is a great source of such biomarkers. I have demonstrated the presence of PCa specific transcripts such as *TMPRSS2/ERG, PCA3 and DLX1* in urine exosomal RNA. Preliminary analysis has found clinical structure in the data, and High-risk patients can be identified in unsupervised analysis. Most importantly, response to therapy is reflected in even this limited number of tested exosomal biomarkers. Implementation of further probes selected from the exosomal RNA sequence data could improve this system even further.

Appendix 1

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Urine and blood tests for detection of Prostate Cancer

Dear Sir

As you will shortly be attending our urology outpatient clinic, I would like to take this opportunity to invite you to participate in a research study, which we are conducting in the urology department at the Norfolk and Norwich University Hospital NHS Trust in combination with the University of East Anglia looking at urine and blood tests for prostate disease.

I would be grateful if you could take the time to read the information sheet provided with this letter, which outlines the study's aims. Further details will be provided on the day of your visit and any further questions you would like to ask to help you decide whether or not to take part can be answered at that time or by contacting me directly beforehand using the contact details on the enclosed information sheet.

Yours sincerely

Mr Marcelino Yazbek Hanna
Chief investigator, MD student and clinical fellow in urology.
Norfolk and Norwich University Hospital

Appendix 2

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Information Sheet

What is the purpose of the study?
Currently the only test widely available on the NHS to screen Men for prostate cancer is the serum PSA (prostatic specific antigen) blood test. This test is not sensitive or specific for prostate cancer which means the level of PSA may rise due to several other conditions including benign enlargement of the prostate, infection and several other reasons, leading to potentially unnecessary prostate biopsies which carry significant risk to patients health, such as severe life threatening infections (sepsis), inflammation, bleeding and retention of urine.
The purpose of this study is to look in urine and blood for prostate cancer markers in an attempt to find a better test for cancer than the one currently available (PSA, as a blood test). Some previous studies suggest that this might be possible as prostate tissue including cancer cells and microvesicles (small pockets containing cellular material such as RNA, DNA and protein that cells exchange for communication) are shed in the urine and blood after prostate examination, and by testing these for potential cancer markers there is a possibility of finding a new test to diagnose prostate cancer. This study will be undertaken by clinicians at the Norfolk and Norwich University Hospital (NNUH) and investigators at the University of East Anglia (UEA).

Why have I been invited?
To achieve our aim we need to collect and test urine and blood from patients with and without prostate cancer, so a comparison can be made to evaluate the sensitivity of the potential marker in question.
Now as you are aware your Doctor has referred you for a urological assessment in our clinics, this may or may not be due to a prostate concern, but as we need urine and blood from patients with and without cancer of the prostate you have been considered for the study.

Do I have to take part?
Participation is optional. This is a pilot study to help develop new tests and as such will not benefit you, but may help men in a similar situation to you in the future.
It is your decision whether to take part or not. When you attend the clinic we will describe the study and go through this information sheet with you and any other questions you wish to ask. If you wish to take part, we will then ask you to sign a form. You are free to withdraw at any time, without giving a reason. A decision not to participate would not affect the standard of care you receive.

What will happen to me if I take part?

After you have read this information sheet, and gone through it with your consulting doctor on the day of your outpatient clinic and all your questions been answered. If you decide to take part you will have to sign a consent form, which will also be explained to you in detail. You will then have your routine consultation and examination including internal examination, which is part of your routine examination in this clinic. For the purpose of this study the prostate examination will be systematic rather than random and will include 3 swipes on each side to enhance cell shedding into the urine, at the end of the clinic you will be asked to provide us with a urine sample, which will be used for the purpose of the research. This would then be sent off to a laboratory and analysed for cells and biomolecules such as DNA, RNA, protein, and metabolites for evidence of tumour cells. The data would be anonymised so that people who would be analysing the urine sample would not be able to identify any patients who have supplied a urine sample.

In addition, and only with your consent, you will be asked to provide us with some blood which will be used for the same purposes as for the urine (as described above).

Will I be paid any expenses?
Participation for the study is voluntary and as such you will not be paid any reimbursements.

What are the possible disadvantages and risks of taking part?
There are no potential disadvantages or expected side-effects by taking part in the study as all it will involve for you will be the supply of urine and blood samples after a physical examination however if you are worried about any issues please feel free to discuss it with the doctor who would be taking your consent.

What are the possible benefits of taking part?
This is an experimental study and as such will not have any obvious immediate benefit for you however the data that we would gather may help us in the future to better diagnose the patients suspected of prostate cancer and avoid unnecessary prostate biopsies.

Will my taking part in the study be kept confidential?
All the details about you will be kept confidential and will only be accessible by the research team. It is very likely that the results of the study will be published or presented in the future in medical journals, however the data will be anonymised so that your confidentiality is not breached.

What will happen if I don't want to carry on with the study?
Participation for the study is completely voluntary and as such you may withdraw from the study at any point without the need to give a reason. This will not affect your routine care or any care that you would receive before, during or after your hospital stay. In case of your withdrawal from the study we will ask you if the data collected from your urine sample could be still used or if you wish us to destroy it, and we will act upon your decision.

Who is organising and funding the research?
The urology department and the University of East Anglia are jointly carrying out the research, however a third party may also provide funds (A third party may be a big organisation such as the Big C who fund cancer research in Norfolk and Waveney). Ethical approval has been received for this study.

What will happen to the samples and information at the end of the study and how long will they be kept?
Urine and blood samples will be used as a source of markers and genetic materials, some of which will be used in the above research, and the rest will be kept to be used at a later date in future research projects. Samples including DNA, RNA and protein may be stored for up to 20 years after the end date of this project. The anonymised information collected from this study will be stored for up to twenty years for use in future studies designed to improve patient care and treatment. Samples and anonymised information may be shared with other collaborating laboratories for further specialised analysis in the UK or in other countries.

Links with other organisations
If you agree, we may send stored material or products derived from it to other approved laboratories or companies in the UK or abroad to support their research programmes. We are not, however, allowed to sell tissue or other samples in order to make any financial profit. We will release tissue or other samples to laboratories or companies only if they work to appropriate ethical and scientific standards.

Who has reviewed the study?
The study design and protocol has been reviewed by the research and development department at the Norfolk and Norwich University Hospital, Public and Patient Involvement in Research (PPIRes) and the Norfolk Research Ethics Committee.

If you need further information please contact:
Chief Investigator and Clinical fellow in Urology
Norfolk and Norwich University Hospital
Mr Marcelino Yazbek Hanna on
Phone: 07886302762
E-mail: marcelino.yazbekhanna@nnuh.nhs.uk
Post: Norfolk and Norwich University Hospital NHS Trust,
Urology Department,
Colney Lane,
Norwich,
NR4 7UY

Appendix 3

Norfolk and Norwich University Hospitals NHS

NHS Foundation Trust

Affix an addressograph label here or complete the following details:

Patient's name……..

Date of birth……

Hospital no. ……..

ADULT INFORMED CONSENT
For a Research Study

Diagnostic urine and blood biomarkers for prostate cancer

1. I confirm that I have read and understand the information sheet (version 4 dated 06/08/2012) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that an internal examination will be done as described in the information sheet (Version 4 dated 06/08/2012). ☐
4. I understand that the urine sample I provide will be used for the analysis of tumour cells, DNA, RNA, protein and metabolites. ☐
5. I agree to provide blood samples for the purpose of this research, and I understand that it will be used for DNA, RNA protein, metabolite and cell analysis. ☐
6. Anonymised information about my case may be kept on a secure database for up to twenty years after the end date of this study for use in future research studies to improve patient care. ☐
7. Anonymised information may be passed on to persons outside the NNUH and UEA in connection with research and may be published with any research findings. ☐
8. I understand that urine, blood and biological extracts of my samples will be kept for up to twenty years after the end date of this study and may be used in other future studies designed to improve patient care. ☐
9. The samples may be transferred to other approved laboratories and/or companies, which may be in the UK or abroad, in properly approved research programmes. ☐
10. I agree to take part in the above study. ☐

Signed _____(Patient) _____ Date _____

I have explained the research and have answered such questions as the patient has asked.

Signed _____ Clinical Investigator _____ Date _____

Appendix 4

International Prostate Symptom Score (I-PSS)

Patient Name: Date:	Not At All	Less Than 1 Time In 5	Less Than Half The Time	About Half The Time	More Than Half The Time	Almost Always	YOUR SCORE
1. Incomplete Emptying Over the past month, how often have you had a sensation of not emptying your bladder completely after you finish urinating?	0	1	2	3	4	5	
2. Frequency Over the past month, how often have you had to urinate again less than two hours after you have finished urinating?	0	1	2	3	4	5	
3. Intermittency Over the past month, how often have you found you stopped and started again several times when you urinated?	0	1	2	3	4	5	
4. Urgency Over the past month, how often have you found it difficult to postpone urination?	0	1	2	3	4	5	
5. Weak Stream Over the last month, how often have you had a weak urinary stream?	0	1	2	3	4	5	
6. Straining Over the past month, how often have you had to push or strain to begin urination?	0	1	2	3	4	5	
	None	Once	Twice	3 times	4 times	5 or more	YOUR SCORE
7. Nocturia Over the past month how many times did you most typically get up each night to urinate from the time you went to bed until the time you got up in the morning?	0	1	2	3	4	5	
Total I-PSS Score							

Quality of Life due to Urinary Symptoms	Delighted	Pleased	Mostly satisfied	Mixed	Mostly unhappy	Unhappy	Terrible
If you were to spend the rest of your life with your urinary condition just the way it is now, how would you feel about that?	0	1	2	3	4	5	6

The I-PSS is based on the answers to seven questions concerning urinary symptoms. Each question is assigned points from 0 to 5 indicating increasing severity of the particular symptom. The total score can therefore range from 0 to 35 (asymptomatic to very symptomatic).

Although there are presently no standard recommendations into grading patients with mild, moderate or severe symptoms, patients can be tentatively classified as follows: **0 - 7 = mildly symptomatic; 8 - 19 = moderately symptomatic; 20 - 35 = severely symptomatic.**

The International Consensus Committee (ICC) recommends the use of only a single question to assess the patient's quality of life. The answers to this question range from "delighted" to "terrible" or 0 to 6. Although this single question may or may not capture the global impact of BPH symptoms on quality of life, it may serve as a valuable starting point for doctor-patient conversation.

Appendix 5

PPIRes
Public and Patient Involvement in Research

NHS
Norfolk

PPIRes panel member comments

30th January 2012

Ref: N11/11/24

Title of Research: Diagnostic Urine Biomarkers for prostate cancer.

Researcher: Marcelino Yazbek-Hanna, Clinical fellow, Urology Department, Norfolk and Norwich University Hospital

Deadline for return of comments: Friday 10th February 2012

PANEL MEMBER NAME:

Review of: - Protocol, Consent Form and Information Sheet.

PLEASE FEEL FREE TO WRITE ON THE DOCUMENTS AND RETURN TOO

Questions to help reviewers as follows:

Do the panel have any comments or suggestions for how the study can be improved/amended?

From what I have read of the whole study. It has been very well presented and thought out and consideration has been given not to offend anyone, with the way the study has been brought together. I do not think anything can be done to make it any better.

Appendix 6

Proforma of the clinical information gathetered from all the patients

Clinical Information	Urine	Social History	Treatment and follow up	Histology Finding	Radiology Finding
Lab Number	Pre DRE urine volume	Alcohol consumption in unit per week	Radiotherapy	Gleason Major	TNM staging MRI
Date of presentation	PostDRE urine volume	Smoking history (Sig/day and period of smoking)	Hormone manipulation	Gleason Minor	Bone Scan results
Etnicity	Urine Color (Yellow, Orange, Red , Pale, Dark)	Ex smokers (How long are they smoking free)	Radical prostatectomy	Gleason Score	
Age	Speed of processing	Ocupation	Follow up PSA after treatment	Number of positive cores vs number of total cores on each lobe	
Reason for PSA Check	Method of processing (Ie Centrifuged immediately, Placed on wet ice, dry ice etc)		Further management (ie change in hormone manipulation and chemotherapy)	Perineural invasion	
Symptoms (LUTS, Haematuria, Bone pain, focal neurology, Hx of BPH and treatment for it)	How soon was the sample given after DRE			Neurovascular invasion	
IPSS score	Dip stick Finding			TRUS finding	
PSA at presentation	Nitrites			Volume of cancer % in positive cores	
Previous PSA	Leucocytes			Prostate Volume in gram	
Blood Results (UE,FBC,LFT)	Proteine			Post prostatectomy histological results	
Past Medical History Medications	Blood Ketone				
Treatment with 5 alpha reductase DRE Finding	PH				

Bibliography

1. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA: A Cancer Journal for Clinicians. 2010; 60:277–300.

2. Tseng C-H. Prostate cancer mortality in Taiwanese men: Increasing age-standardized trend in general population and increased risk in diabetic men. Ann Med. 2011; 43:142–50.

3. UK CR. Prostate cancer incidence statistics. cancerresearchukorg. Cancer Research UK. (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer/incidence)

4. Ilic D, O'Connor D, Green S, Wilt T. Screening for prostate cancer. Cochrane Database Syst Rev. 2006:CD004720.

5. Screening for Prostate Cancer: A Review of the Evidence for the U.S. Preventive Services Task Force. 2011 ;1–22.

6. Lemaitre L, Puech P, Poncelet E, Bouyé S, Leroy X, Biserte J, et al. Dynamic contrast-enhanced MRI of anterior prostate cancer: morphometric assessment and correlation with radical prostatectomy findings. Eur Radiol. 2009; 19: 470–80.

7. Bouyé S, Potiron E, Puech P, Leroy X, Lemaitre L, Villers A. Transition zone and anterior stromal prostate cancers: zone of origin and intraprostatic patterns of spread at histopathology. Prostate. 2009; 69: 105–13.

8. Scattoni V, Zlotta A, Montironi R, Schulman C, Rigatti P, Montorsi F. Extended and Saturation Prostatic Biopsy in the Diagnosis and Characterisation of Prostate Cancer: A Critical Analysis of the Literature. European Urology. 2007; 52: 1309–22.

9. Cancer Statistics Registrations, England (Series MB1). Office for National Statistics. (http://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancerregistrationstatisticsengland/previousReleases)

10. National cancer intelligence network, Prostate Cancer Incidence, Mortality and Survival Rates in the United Kingdom. Prostate cancer: 2008; 1–146.

11. Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. International Journal of Cancer; 20001; 85; 60–67.

12. Potosky AL, Kessler L, Gridley G, Brown CC, Horm JW. Rise in prostatic cancer incidence associated with increased use of transurethral resection. J Natl

Cancer Inst. 1990 ;82 :1624–8.

13. 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;108: 1402–8.

14. Haas GP, Delongchamps N, Brawley OW, Wang CY, la Roza de G. The worldwide epidemiology of prostate cancer: perspectives from autopsy studies. Can J Urol. 2008;15:3866–71.

15. Thompson IM, Ankerst DP, Chi C, Goodman PJ, Tangen CM, Lucia MS, et al. Assessing prostate cancer risk: results from the Prostate Cancer Prevention Trial. J Natl Cancer Inst. 2006;98:529–34.

16. Dai WS, Kuller LH, LaPorte RE, Gutai JP, Falvo-Gerard L, Caggiula A. The epidemiology of plasma testosterone levels in middle-aged men. American Journal of Epidemiology. 1981;114:804–16.

17. Prehn RT. On the prevention and therapy of prostate cancer by androgen administration. Cancer Research. 1999;59:4161–4.

18. Zeegers MPA, Jellema A, Ostrer H. Empiric risk of prostate carcinoma for relatives of patients with prostate carcinoma. Cancer. 2003;97:1894–903.

19. MR S, RD C, JJ F, RJ B, GR N. Familial patterns of prostate cancer: a case-control analysis. JURO. 1991;146:1305–7.

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

21. Johns LE, Houlston RS. A systematic review and meta-analysis of familial prostate cancer risk. Br J Urol. 2003;91:789–94.

22. Bratt O. Hereditary prostate cancer: clinical aspects. JURO. 2002;168:906–13.

23. Eeles RA, Durocher F, Edwards S, Teare D, Badzioch M, Hamoudi R, et al. Linkage Analysis of Chromosome 1q Markers in 136 Prostate Cancer Families. The American Journal of Human Genetics. 1998;62:653–8.

24. Nam RK, Zhang WW, Loblaw DA, Klotz LH, Trachtenberg J, Jewett MAS, et al. A genome-wide association screen identifies regions on chromosomes 1q25 and 7p21 as risk loci for sporadic prostate cancer. Prostate Cancer Prostatic Dis. 2008;11:241–6.

25. Smith JR, Freije D, Carpten JD, Gronberg H, Xu J, Isaacs SD, et al. Major Susceptibility Locus for Prostate Cancer on Chromosome 1 Suggested by a Genome-Wide Search. Science. 1996;274:1371–4.

26. 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;366:141–9.

27. Sigurdsson S, Thorlacius S, Tomasson J, Tryggvadottir L, Benediktsdottir K, Eyfjörd JE, et al. BRCA2 mutation in Icelandic prostate cancer patients. J Mol Med. 1997;75:758–61.

28. Rosen EM, Fan S, Goldberg ID. BRCA1 and prostate cancer. Cancer Invest. 2001;19(4):396–412.

29. Breast Cancer Linkage Consortium. Cancer risks in BRCA2 mutation carriers. J Natl Cancer Inst. 1999;91:1310–6.

30. Johannsson O, Loman N, Möller T, Kristoffersson U, Borg A, Olsson H. Incidence of malignant tumours in relatives of BRCA1 and BRCA2 germline mutation carriers. Eur J Cancer. 1999;35:1248–57.

31. Edwards SM, Kote-Jarai Z, Meitz J, Hamoudi R, Hope Q, Osin P, et al. Two percent of men with early-onset prostate cancer harbor germline mutations in the BRCA2 gene. Am J Hum Genet. 2003;72:1–12.

32. Olama Al AA, Kote-Jarai Z, Giles GG, Guy M, Morrison J, Severi G, et al. Multiple loci on 8q24 associated with prostate cancer susceptibility. Nature Genetics. Nature Publishing Group; 2009;41:1058–60.

33. Eeles RA, Kote-Jarai Z, Olama Al AA, Giles GG, Guy M, Severi G, et al. Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. Nature Genetics. Nature Publishing Group; 2009;41:1116–21.

34. Szulkin R, Whitington T, Eklund M, Aly M, Eeles RA, Easton D, et al. Prediction of individual genetic risk to prostate cancer using a polygenic score. Prostate. 2015;75:1467–74.

35. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet. 2000;16:168–74.

36. Van Der Cruijsen-Koeter IW, Wildhagen MF, De Koning HJ, Schröder FH. The value of current diagnostic tests in prostate cancer screening. BJU International 2001;88:458–66.

37. Mettlin C, Lee F, Drago J, Murphy GP, The Investigators of the American Cancer Society National Prostate Cancer Detection Project. The American cancer society national prostate cancer detection project. Findings on the detection of early prostate cancer in 2425 men. Cancer J. 1991;67:2949–58.

38. Smith RA, Cokkinides V, Brawley OW. Cancer screening in the United States, 2009: A review of current American Cancer Society guidelines and issues in cancer screening. CA: A Cancer Journal for Clinicians. 2009;59:27–41.

39. COOK LS, GOLDOFT M, SCHWARTZ SM, Weiss NS. Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants. J Urol. 1999;161:152-5.

40. Sato T. Chronic diseases suspected to arise through the enhanced activity of growth hormone by excessive intake of animal protein, 1~ 5. 公衆衛生院研究報告. 1963;12.

41. Waltz P, Chodick G. International comparisons of prostate cancer mortality rates with dietary practices and sunlight levels. InUrologic Oncology. 2007;25:85–5.

42. Allen NE, Key TJ, Appleby PN, Travis RC, Roddam AW, Rinaldi S, Egevad L, Rohrmann S, Linseisen J, Pischon T, Boeing H. Serum IGF-I and IGFBP-3 concentrations and prostate cancer risk: results from the European Prospective Investigation into Cancer and Nutrition (EPIC). Cancer Epidemiology, Biomarkers, and Prevention. 2007

43. 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. 2004;363:1346–53.

44. Wu J, Yu E. Insulin-like growth factor receptor-1 (IGF-IR) as a target for prostate cancer therapy. Cancer Metastasis Rev. 2014;33:607-17.

45. Giovannucci E. Dietary influences of 1,25(OH)2 vitamin D in relation to prostate cancer: a hypothesis. Cancer Causes Control. 1998;9:567–82.

46. 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 Journal of the National Cancer Institute. 1993;85:1571–9.

47. Traka M, Gasper AV, Melchini A, Bacon JR, Needs PW, Frost V, et al. Broccoli consumption interacts with GSTM1 to perturb oncogenic signalling pathways in the prostate. PLoS ONE. 2008;3:e2568.

48. G D Honda LBRKRSGVGBEH. Vasectomy, cigarette smoking, and age at first sexual intercourse as risk factors for prostate cancer in middle-aged men. Br J Cancer(1988),57,326-331. Nature Publishing Group; 1988;57:326–6.

49. Flier JS, Underhill LH, Zetter BR. The Cellular Basis of Site-Specific Tumor Metastasis. N Engl J Med. 1990;322:605–12.

50. JE M. Normal histology of the prostate. Am J Surg Pathol. 1988 Aug 1;12(8):619–33.

51. Shannon BA, Mc Neal JE, Cohen RJ. Transition zone carcinoma of the prostate gland. Pathology. 2003;35:467–71.

52. Cohen RJ, Shannon BA, Phillips M, Moorin RE, Wheeler TM, Garrett KL. Central Zone Carcinoma of the Prostate Gland: A Distinct Tumor Type With Poor Prognostic Features. The Journal of Urology. 2008;179:1762–7.

53. Epstein JI. Epstein: Pathology of adenocarcinoma of the prostate. Campbell's Urology. 1998.

54. Blackwood JK, Williamson SC, Greaves LC, Wilson L, Rigas AC, Sandher R, et al. In situ lineage tracking of human prostatic epithelial stem cell fate reveals a common clonal origin for basal and luminal cells. J Pathol. 2011 30;225:181–8.

55. Maitland NJ, Collins A. A tumour stem cell hypothesis for the origins of prostate cancer. BJU Int. 2005;96:1219–23.

56. Gleason DF. Histologic grading of prostate cancer: A perspective. Human Pathology. 1992;23:273–9.

57. Montironi R, Mazzuccheli R, Scarpelli M, Lopez-Beltran A, Fellegara G, Algaba F. Gleason grading of prostate cancer in needle biopsies or radical prostatectomy specimens: contemporary approach, current clinical significance and sources of pathology discrepancies. BJU Int. 2005;95:1146–52.

58. Villers A, McNeal JE, Redwine EA, Freiha FS, Stamey TA. The role of perineural space invasion in the local spread of prostatic adenocarcinoma. The Journal of urology. 1989;142:763-8.

59. Graham SD, Bostwick DG, Hoisaeter A, Abrahamsson P, Algaba F, di Sant Agnese A, et al. Panel III: Report of the committee on staging and pathology. Cancer; 1992;70:359–61.

60. Häggman MJ, Macoska JA, Wojno KJ, Oesterling JE. The relationship between prostatic intraepithelial neoplasia and prostate cancer: critical issues. The Journal of urology. 1997;158:12–22.

61. Bostwick DG, Brawer MK. Prostatic intra-epithelial neoplasia and early invasion in prostate cancer. Cancer. 1987;59:788–94.

62. McNeal JE, Villers A, Redwine EA, Freiha FS, Stamey TA. Microcarcinoma in the prostate: its association with duct-acinar dysplasia. Human Pathology. 1991;22:644–52.

63. Bostwick DG. Prostatic intraepithelial neoplasia (PIN): Current concepts. J Cell Biochem. 1992;50:10–9.

64. Qian J, Wollan P, Bostwick DG. The extent and multicentricity of high-grade prostatic intraepithelial neoplasia in clinically localized prostatic adenocarcinoma. Human Pathology. Elsevier; 1997;28:143–8.

65. Montironi R, Galluzzi CM, Diamanti L, Taborro R, Scarpelli M, Pisani E. Prostatic intra-epithelial neoplasia. Qualitative and quantitative analyses of the blood capillary architecture on thin tissue sections. Pathol Research and Practice. 1993;189:542–8.

66. Stearns ME, Wang M. Type IV collagenase (M(r) 72,000) expression in human prostate: benign and malignant tissue. Cancer Research. 1993;53:878–83.

67. Kovi J, Mostofi FK, Heshmat MY, Enterline JP. Large acinar atypical hyperplasia and carcinoma of the prostate. Cancer.1988;61:555–61.

68. Troncoso P, Babaian RJ, Ro JY, Grignon D, Von Eschenbach AC, Ayala AG. Prostatic intraepithelial neoplasia and invasive prostatic adenocarcinoma in cystoprostatectomy specimens. Urology. 1989;34:52-6.

69. Clark J, Attard G, Jhavar S, Flohr P, Reid A, De-Bono J, et al. Complex patterns of ETS gene alteration arise during cancer development in the human prostate. Oncogene. 2007;27:1993–2003.

70. Emmert-Buck MR, Vocke CD, Pozzatti RO, Duray PH, Jennings SB, Florence CD, et al. Allelic Loss on Chromosome 8p12–21 in Microdissected Prostatic Intraepithelial Neoplasia. Cancer Res 1995;55:2959-62.

71. Kim NW, Hruszkewycz AM. Telomerase activity modulation in the prevention of prostate cancer. Urology. 2001;57:148-53.

72. Ge K, Minhas F, Duhadaway J, Mao N-C, Wilson D, Buccafusca R, et al. Loss of heterozygosity and tumor suppressor activity ofBin1 in prostate carcinoma. Int J Cancer. 2000;86:155–61.

73. Bostwick DG, Qian J. High-grade prostatic intraepithelial neoplasia. Modern pathology. 2004;17:360-79.

74. Sakr WA, Haas GP, Cassin BF, Pontes JE, Crissman JD. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. The Journal of urology. 1993;150:379-85.

75. Bostwick DG, Norlén BJ, Denis L. Prostatic intraepithelial neoplasia: the preinvasive stage of prostate cancer: overview of the prostate committee report. Scandinavian Journal of Urology and Nephrology. 2000;34:1-2.

76. Wael Sakr A, Billis A, Ekman P, Wilt T, Bostwick DG. Epidemiology of high-grade prostatic intraepithelial neoplasia. Scandinavian Journal of Urology and Nephrology. 2000;34:11-8.

77. Sakr WA, Grignon DJ, Haas GP, Heilbrun LK, Pontes JE, Crissman JD. Age and racial distribution of prostatic intraepithelial neoplasia. European urology. 1995;30:138-44.

78. Sakr WA, Grignon DJ, Haas GP. Pathology of premalignant lesions and carcinoma of the prostate in African-American men. InSeminars in urologic oncology 1998;4:214-220.

79. Oh WK, Hurwitz M, D'Amico AV, Richie JP, Kantoff PW. Diagnosis and Staging of Prostate Cancer. The Keio Journal of Medicine.1988;37;10-23.

80. Johansson E, Bill-Axelson A, Holmberg L, Onelöv E, Johansson J-E, Steineck G. Time, Symptom Burden, Androgen Deprivation, and Self-Assessed Quality of Life after Radical Prostatectomy or Watchful Waiting: The Randomized Scandinavian Prostate Cancer Group Study Number 4 (SPCG-4) Clinical Trial. European Urology. 2009;55:422–32.

81. 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;367:203–13.

82. Elgamal AA, Van Poppel HP, Van de Voorde WM, Van Dorpe JA, Oyen RH, Baert LV. Impalpable invisible stage T1c prostate cancer: characteristics and clinical relevance in 100 radical prostatectomy specimens--a different view. JURO. 1997;157:244–50.

83. Holmberg L, Bill-Axelson A, Helgesen F, Salo JO, Folmerz P, Häggman M, et al. A randomized trial comparing radical prostatectomy with watchful waiting in early prostate cancer. N Engl J Med. 2002;347:781–9.

84. Heidenreich A, Bellmunt J, Bolla M, Joniau S, Mason M, Matveev V, et al. EAU Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Treatment of Clinically Localised Disease. European Urology. 2011;59:61–71.

85. Generator NEW. Prostate cancer: diagnosis and treatment. NICE guidelines. 2014;:1–45.

86. Epstein JI, Steinberg GD. The significance of low-grade prostate cancer on needle biopsy. A radical prostatectomy study of tumor grade, volume, and stage of the biopsied and multifocal tumor. Cancer. 1990;66:1927–32.

87. Prostate Cancer. EAU Guidlines for prostate cancer. 2013 Mar 6;:1–154.

88. Bott SRJ, Birtle AJ, Taylor CJ, Kirby RS. Prostate cancer management: (1) an update on localised disease. Postgraduate Medical Journal. 2003;79:575–80.

89. Johansson J-E, Andersson S-O. Deferred treatment in localized prostatic cancer. Acta Oncol. 1991;30:221–3.

90. Graversen PH, Nielsen KT, Gasser TC, Corle DK, Madsen PO. Radical prostatectomy versus expectant primary treatment in stages I and II prostatic cancer. A fifteen-year follow-up. Urology. 1990;36:493–8.

91. Schroder FH, Van den Ouden D, Davidson P. The role of surgery in the cure of prostatic carcinoma. Eur Urol Update Series. 1992;1:18-23.

92. Bill-Axelson A, Holmberg L, Ruutu M, Garmo H, Stark JR, Busch C, et al. Radical prostatectomy versus watchful waiting in early prostate cancer. N Engl J Med. 2011;364:1708–17.

93. Hodgson D, Warde P, Gospodarowicz M. The management of locally advanced prostate cancer. Urology. 1998;4:3–12.

94. Boccon-Gibod L, Bertaccini A, Bono AV, Dev SB, Höltl W, Mottet N, Tunn U, Zamboglou N. Management of locally advanced prostate cancer: a European consensus. International journal of clinical practice. 2003;57:187-94.

95. Bolla M, Collette L, Blank L, Warde P, Dubois JB, Mirimanoff R-O, et al. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. The Lancet. 2002;360:103–8.

96. Johnstone PAS, Ward KC, Goodman M, Assikis V, Petros JA. Radical prostatectomy for clinical T4 prostate cancer. Cancer. 2006;106:2603–9.

97. Huggins C. Endocrine-Induced Regression of Cancers. Science. 1967;156:1050–4.

98. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer. 2001;1:34–45.

99. NA. Studies on Prostatic Cancer. The Endocrinologist. 2005 Nov;15(6):335–9.

100. Edwards J, Bartlett JMS. The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: Androgen-receptor cofactors and bypass pathways. BJU Int. 2005;95:1327–35.

101. Fizazi K, Higano CS, Nelson JB, Gleave M. Phase III, randomized, placebo-controlled study of docetaxel in combination with zibotentan in patients with metastatic castration-resistant prostate cancer. Journal of Clinical Oncology 2013;31:1740-7.

102. Foley R, Marignol L, Keane JP, Lynch TH, Hollywood D. Androgen hypersensitivity in prostate cancer: Molecular perspectives on androgen deprivation therapy strategies. Prostate. 2010;71:550–7.

103. Gregory CW, Johnson RT, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Research. 2001;61:2892–8.

104. Gelfanov VM. Transformation of interleukin-3-dependent cells without participation of Stat5/bcl-xL: cooperation of akt with raf/erk leads to p65 nuclear factor kappaB-mediated antiapoptosis involving c-IAP2. Blood. 2001;98:2508–17.

105. Wise SC, Burmeister LA, Zhou XF, Bubulya A, Oberfield JL, Birrer MJ, Shemshedini L. Identification of domains of c-Jun mediating androgen receptor transactivation. Oncogene. 1998;16:2001-10.

106. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. New England Journal of Medicine. 1995;332:1393-8.

107. Tilley WD, Buchanan G, Hickey TE, Bentel JM. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res. 1996;2:277–85.

108. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler

W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ. Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. Journal of Clinical Oncology. 2003;21:2673-8.

109. Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. New England Journal of Medicine. 2004;351:1488-90.

110. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. Journal of cellular biochemistry. 2004;91:483-190.

111. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nature medicine. 1999;5:280-5.

112. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, et al. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem. 2002;277:29304–14.

113. Loberg RD, Logothetis CJ, Keller ET, Pienta KJ. Pathogenesis and treatment of prostate cancer bone metastases: targeting the lethal phenotype. Journal of Clinical Oncology. 2005;23:8232–41.

114. Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Research. 2001;61:4315–9.

115. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. Constitutive Activation of the Ras/Mitogen-activated Protein Kinase Signaling Pathway Promotes Androgen Hypersensitivity in LNCaP Prostate Cancer Cells. Cancer Res 2003;63:1981-9.

116. Bell WC, Myers RB, Hosein TO, Oelschlager DK, Grizzle WE. The response of extracellular signal-regulated kinase (ERK) to androgen-induced proliferation in the androgen-sensitive prostate cancer cell line, LNCaP. Biotechnic & histochemistry. 2003;78:11-6.

117. Fu Z, Smith PC, Zhang L, Rubin MA, Dunn RL, Yao Z, Keller ET. Effects of raf kinase inhibitor protein expression on suppression of prostate cancer metastasis. Journal of the National Cancer Institute. 2003;95:878-89.

118. Debes JD, Tindall DJ. Mechanisms of Androgen-Refractory Prostate Cancer. N Engl J Med. 2004;351:1488–90.

119. Sjoberg DD. Toward a Smarter Prostate Cancer Screening Program. European Urology. 2015;68:598–9.

120. Balk SP, Ko YJ, Bubley GJ. Biology of prostate-specific antigen. Urology. 2003;62:27–33.

121. Schieferstein G. Prostate-specific antigen (PSA) in human seminal plasma. Archives of andrology. 1999;42:193-7.

122. Killian CS, Corral DA, Kawinski E, Constantine RI. Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. Biochemical and Biophysical Research Communications. 1993;192:940–7.

123. Bok RA, Small EJ. Bloodborne biomolecular markers in prostate cancer development and progression. Nat Rev Cancer. 2002;2:918–26.

124. Melia J. Part 1: The burden of prostate cancer, its natural history, information on the outcome of screening and estimates of ad hoc screening with particular reference to England and Wales. BJU Int. 2005;3:4–15.

125. Adami H-O, Rothman K, Baron J. Ethics of a prostate cancer screening trial. The Lancet. 1994;343:958–60.

126. Johansson JE, Holmberg L, Johansson S, Bergström R, Adami HO. Fifteen-year survival in prostate cancer: a prospective, population-based study in Sweden. Jama. 1997;277:467-71.

127. Walsh PC. Prostate cancer kills: strategy to reduce deaths. Urology 1994;44:463-6.

128. Harmon MH, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15-36.

129. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia D, Church TR, et al. Mortality results from a randomized prostate-cancer screening trial. N Engl J Med. 2009;360:1310–9.

130. Schröder FH, Hugosson J, Roobol MJ, Tammela TLJ, Ciatto S, Nelen V, et al. Screening and prostate-cancer mortality in a randomized European study. N Engl J Med. 2009;360:1320–8.

131. Catalona WJ, Partin AW, Slawin KM, Brawer MK, Flanigan RC, Patel A, et al. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. JAMA. 1998;279:1542–7.

132. Stephan C, Lein M, Jung K, Schnorr D, Loening SA. The influence of prostate volume on the ratio of free to total prostate specific antigen in serum of patients with prostate carcinoma and benign prostate hyperplasia. Cancer. 1997;79:104–9.

133. Rabbani F, Stroumbakis N, Kava BR, Cookson MS, Fair WR. Incidence and clinical significance of false-negative sextant prostate biopsies. The Journal of urology. 1998;159:1247-50.

134. Norberg M, Egevad L, Holmberg L, Sparén P, Norlén BJ, Busch C. The sextant protocol for ultrasound-guided core biopsies of the prostate underestimates the presence of cancer. Urology. 1997;50:562–6.

135. Presti JC, O'Dowd GJ, Miller MC, Mattu R, Veltri RW. Extended peripheral

zone biopsy schemes increase cancer detection rates and minimize variance in prostate specific antigen and age related cancer rates: results of a community multi-practice study. The Journal of Urology. 2003;169:125–9.

136. Algaba AF, Chivite DL, Santaularia SJ, Oliver SA. Evidence of the radical prostatectomy Gleason score in the biopsy Gleason score. Actas urologicas espanolas. 2004;28:21-6.

137. Babaian RJ, Troncoso P, Bhadkamkar VA, Johnston DA. Analysis of clinicopathologic factors predicting outcome after radical prostatectomy. Cancer. 2001;91:1414–22.

138. Guillonneau B, El-Fettouh H, BAUMERT H, Cathelineau X, Doublet JD, Fromont G, Vallancien G. Laparoscopic radical prostatectomy: oncological evaluation after 1,000 cases at Montsouris Institute. The Journal of urology. 2003;169:1261-6.

139. Hricak H, White S, Vigneron D, Kurhanewicz J, Kosco A, Levin D, et al. Carcinoma of the prostate gland: MR imaging with pelvic phased-array coils versus integrated endorectal--pelvic phased-array coils. Radiology. 1994;193:703–9.

140. Reinsberg SA, Payne GS, Riches SF, Ashley S, Brewster JM, Morgan VA, et al. Combined Use of Diffusion-Weighted MRI and 1H MR Spectroscopy to Increase Accuracy in Prostate Cancer Detection. American Journal of Roentgenology. 2007;188:91–8.

141. Sawyers CL. The cancer biomarker problem. Nature. 2008;452:548–52.

142. Laxman B, Morris DS, Yu J, Siddiqui J, Cao J, Mehra R, et al. A First-Generation Multiplex Biomarker Analysis of Urine for the Early Detection of Prostate Cancer. Cancer Research. 2008;68:645–9.

143. Prensner JR, Iyer MK, Balbin OA. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. Nature Biotechnology. 2011;29:742-9.

144. Berger MF, Lawrence MS, Demichelis F, Drier Y, Cibulskis K, Sivachenko AY, et al. The genomic complexity of primary human prostate cancer. Nature. 2011;470:214–20.

145. Bussemakers MJG, van Bokhoven A, Verhaegh GW, Smit FP, Karthaus HFM, Schalken JA, et al. DD3:: A New Prostate-specific Gene, Highly Overexpressed in Prostate Cancer. Cancer Res 1999;59:5975-9.

146. de Kok JB, Verhaegh GW, Roelofs RW, Hessels D. DD3PCA3, a very sensitive and specific marker to detect prostate tumors. Cancer Research. 2002;62:2695-8.

147. Hessels D, Schalken JA. The use of PCA3 in the diagnosis of prostate cancer. Nature Publishing Group. Nature Publishing Group; 2009;6:255–61.

148. Roobol MJ, Schröder FH, van Leeuwen P, Wolters T, van den Bergh RCN, Van Leenders GJLH, et al. Performance of the Prostate Cancer Antigen 3 (PCA3) Gene and Prostate-Specific Antigen in Prescreened Men: Exploring the Value of PCA3 for a First-line Diagnostic Test. European Urology. 2010;58:475–81.

149. Haese A, la Taille de A, van Poppel H, Marberger M, Stenzl A, Mulders PFA, et al. Clinical Utility of the PCA3 Urine Assay in European Men Scheduled for Repeat Biopsy. European Urology. 2008;54:1081–8.

150. Xu J, Stolk JA, Zhang X, Silva SJ, Houghton RL, Matsumura M, et al. Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. Cancer Research. 2000;60:1677–82.

151. Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, et al. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. Cancer Research. 2001;61:4683–8.

152. Ferdinandusse S, Denis S, IJlst L, Dacremont G, Waterham HR, Wanders RJ. Subcellular localization and physiological role of alpha-methylacyl-CoA racemase. J Lipid Res. 2000;41:1890–6.

153. Chan JM, Stampfer MJ, Ma J, Gann PH, Gaziano JM, Giovannucci EL. Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study. The American journal of clinical nutrition. 2001;74:549-54.

154. Wanders RJ, Vreken P, Ferdinandusse S, Jansen GA, Waterham HR, van Roermund CW, et al. Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. Biochem Soc Trans. 2001;29:250–67.

155. Ockner RK, Kaikaus RM, Bass NM. Fatty-acid metabolism and the pathogenesis of hepatocellular carcinoma: review and hypothesis. Hepatology. 1993;18:669–76.

156. Tamatani T, Hattori K, Nakashiro K, Hayashi Y, Wu S, Klumpp D, et al. Neoplastic conversion of human urothelial cells in vitro by overexpression of H2O2-generating peroxisomal fatty acyl CoA oxidase. Int J Oncol. 1999;15:743–9.

157. Rubin MA, Zhou M, Dhanasekaran SM, Varambally S, Barrette TR, Sanda MG, et al. α-Methylacyl Coenzyme A Racemase as a Tissue Biomarker for Prostate Cancer. JAMA. American Medical Association; 2002;287:1662–70.

158. Rubin MA, Bismar TA, Andrén O, Mucci L, Kim R, Shen R, Ghosh D, Wei JT, Chinnaiyan AM, Adami HO, Kantoff PW. Decreased α-methylacyl CoA racemase expression in localized prostate cancer is associated with an increased rate of biochemical recurrence and cancer-specific death. Cancer Epidemiology and Prevention Biomarkers. 2005;14:1424-32.

159. Jiang Z, Fanger GR, Woda BA, Banner BF, Algate P, Dresser K, Xu J, Chu PG. Expression of α-methylacyl-CoA racemase (P504s) in various malignant neoplasms and normal tissues: a study of 761 cases. Human pathology.

2003;34:792-6.

160. Lin X, Tascilar M, Lee WH, Vles WJ, Lee BH, Veeraswamy R, et al. GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells. The American Journal of Pathology. 2001;159:1815–26.

161. Lee WH, Isaacs WB, Bova GS, Nelson WG. CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol Biomarkers Prev. 1997;6:443–50.

162. Yegnasubramanian S, Kowalski J, Gonzalgo ML, Zahurak M, Piantadosi S, Walsh PC, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Research. 2004;64:1975–86.

163. Goessl C, Müller M, Heicappell R, Krause H, Straub B, Schrader M, et al. DNA-based detection of prostate cancer in urine after prostatic massage. Urology. 2001;58:335–8.

164. Gonzalgo ML, Pavlovich CP, Lee SM, Nelson WG. Prostate cancer detection by GSTP1 methylation analysis of postbiopsy urine specimens. Clin Cancer Res. 2003;9:2673–7.

165. Cairns P, Esteller M, Herman JG, Schoenberg M, Jeronimo C, Sanchez-Cespedes M, et al. Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin Cancer Res. 2001;7:2727–30.

166. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001;61:3225-9.

167. Zong Y, Xin L, Goldstein AS, Lawson DA, Teitell MA, Witte ON. ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proc Natl Acad Sci USA. 2009;106:12465–70.

168. Tomlins SA. Recurrent Fusion of TMPRSS2 and ETS Transcription Factor Genes in Prostate Cancer. Science. 2005;310:644–8.

169. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, et al. TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. Oncogene. 2008;27:5348–53.

170. Prensner JR, Chinnaiyan AM. Oncogenic gene fusions in epithelial carcinomas. Current opinion in genetics & development. 2009;19:82-91.

171. Furusato B, Gao C-L, Ravindranath L, Chen Y, Cullen J, McLeod DG, et al. Mapping of TMPRSS2-ERG fusions in the context of multi-focal prostate cancer. Mod Pathol. 2008;21:67–75.

172. Perner S, Mosquera J-M, 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;31:882–8.

173. Carver BS, Tran J, Chen Z, Carracedo-Perez A, Alimonti A, Nardella C, Gopalan A, Scardino PT, Cordon-Cardo C, Gerald W, Pandolfi PP. ETS rearrangements and prostate cancer initiation. Nature. 2009;457:E1-.

174. Klezovitch O, Risk M, Coleman I, Lucas JM, Null M, True LD, et al. A causal role for ERG in neoplastic transformation of prostate epithelium. Proc Natl Acad Sci USA. 2008;105:2105–10.

175. Oikawa T, Yamada T. Molecular biology of the Ets family of transcription factors. Gene. 2003;303:11–34.

176. Yu J, Yu J, Mani R-S, Cao Q, Brenner CJ, Cao X, et al. An Integrated Network of Androgen Receptor, Polycomb, and TMPRSS2-ERG Gene Fusions in Prostate Cancer Progression. Cancer Cell. 2010;17:443–54.

177. Gupta SS, Iljin KK, Sara HH, Mpindi JPJ, Mirtti TT, Vainio PP, et al. FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Research. 2010;70:6735–45.

178. Brase JC, Johannes M, Mannsperger H, Fälth M, Metzger J, Kacprzyk LA, et al. TMPRSS2-ERG -specific transcriptional modulation is associated with prostate cancer biomarkers and TGF-β signaling. BMC Cancer. 2011;11:507–7.

179. King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, et al. Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. Nature Genetics. Nature Publishing Group; 2009;41:524–6.

180. King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, et al. Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. Nature Genetics. 2009;41:524–6.

181. Tomlins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, Rubin MA, et al. ETS Gene Fusions in Prostate Cancer: From Discovery to Daily Clinical Practice. European Urology. European Association of Urology; 2009;:1–12.

182. Mosquera J-M, Mehra R, Regan MM, Perner S, Genega EM, Bueti G, et al. Prevalence of TMPRSS2-ERG Fusion Prostate Cancer among Men Undergoing Prostate Biopsy in the United States. Clinical Cancer Research. 2009;15:4706–11.

183. Attard G, Swennenhuis JF, Olmos D, Reid AHM, Vickers E, A'Hern R, et al. Characterization of ERG, AR and PTEN Gene Status in Circulating Tumor Cells from Patients with Castration-Resistant Prostate Cancer. Cancer Research. 2009;69:2912–8.

184. Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, et al. Role of the TMPRSS2–ERG Gene Fusion in Prostate Cancer. NEO. 2008;10:177–88.

185. Iljin K, Wolf M, Edgren H, Gupta S, Kilpinen S, Skotheim RI, et al. TMPRSS2 Fusions with Oncogenic ETS Factors in Prostate Cancer Involve Unbalanced Genomic Rearrangements and Are Associated with HDAC1 and Epigenetic Reprogramming. Cancer Research. 2006;66:10242–6.

186. Guo CC, Zuo G, Cao D, Troncoso P, Czerniak BA. Prostate cancer of transition zone origin lacks TMPRSS2-ERG gene fusion. Mod Pathol. 2009;22:866–71.

187. Hessels D, Smit FP, Verhaegh GW, Witjes JA, Cornel EB, Schalken JA. Detection of TMPRSS2-ERG Fusion Transcripts and Prostate Cancer Antigen 3 in Urinary Sediments May Improve Diagnosis of Prostate Cancer. Clinical Cancer Research. 2007;13:5103–8.

188. Tomlins SA, Aubin SMJ, Siddiqui J, Lonigro RJ, Sefton-Miller L, Miick S, et al. Urine TMPRSS2:ERG Fusion Transcript Stratifies Prostate Cancer Risk in Men with Elevated Serum PSA. Science Translational Medicine. 2011;3:94ra72–2.

189. Demichelis F, Fall K, Perner S, Andrén O, Schmidt F, Setlur SR, et al. TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene. 2007;26:4596–9.

190. Hu Y, Dobi A, Sreenath T, Cook C, Tadase AY, Ravindranath L, et al. Delineation of TMPRSS2-ERG Splice Variants in Prostate Cancer. Clinical Cancer Research. 2008;14:4719–25.

191. Tomlins SA, Rhodes DR, Yu J, Varambally S, Mehra R, Perner S, et al. The Role of SPINK1 in ETS Rearrangement-Negative Prostate Cancers. Cancer Cell. 2008;13:519–28.

192. Barbieri CE, Baca SC, Lawrence MS, Demichelis F, Blattner M, Theurillat J-P, et al. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nature Genetics. 2012;44:685–9.

193. Attard G, Clark J, Ambroisine L, Fisher G, Kovacs G, Flohr P, et al. Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer. Oncogene. 2008;27:253–63.

194. Bismar TA, Yoshimoto M, Duan Q, Liu S, Sircar K, Squire JA. Interactions and relationships of PTEN, ERG, SPINK1 and AR in castration-resistant prostate cancer. Histopathology. 2012;60:645–52.

195. Reid AHM, Attard G, Ambroisine L, Fisher G, Kovacs G, Brewer D, et al. Molecular characterisation of ERG, ETV1 and PTEN gene loci identifies patients at low and high risk of death from prostate cancer. Br J Cancer. Nature Publishing Group; 2010;102:678–84.

196. Mosquera J-M, Beltran H, Park K, MacDonald TY, Robinson BD, Tagawa ST, et al. Concurrent AURKA and MYCN Gene Amplifications Are Harbingers of Lethal TreatmentRelated Neuroendocrine Prostate Cancer. Neoplasia. 2013;15:1–IN4.

197. Cooper CS, Campbell C, Jhavar S. Mechanisms of Disease: biomarkers and molecular targets from microarray gene expression studies in prostate cancer. Nat Clin Pract Urol. 2007;4:677–87.

198. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2:161-74.

199. Marchbank T, Freeman TC, Playford RJ. Human pancreatic secretory trypsin inhibitor. Distribution, actions and possible role in mucosal integrity and repair. Digestion. 1998;59:167–74.

200. Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. Nature Genetics. 2000;25:213–6.

201. Bhatia E, Choudhuri G, Sikora SS, Landt O, Kage A, Becker M, et al. Tropical calcific pancreatitis: strong association with SPINK1 trypsin inhibitor mutations. Gastroenterology. 2002;123:1020–5.

202. Paju A, Stenman U-H. Biochemistry and clinical role of trypsinogens and pancreatic secretory trypsin inhibitor. Crit Rev Clin Lab Sci. 2006;43:103–42.

203. Paju A, Hotakainen K, Cao Y, Laurila T, Gadaleanu V, Hemminki A, et al. Increased Expression of Tumor-Associated Trypsin Inhibitor, TATI, in Prostate Cancer and in Androgen-Independent 22Rv1 Cells. European Urology. 2007;52:1670–81.

204. Han B, Mehra R, Suleman K, Tomlins SA, Wang L, Singhal N, et al. Characterization of ETS gene aberrations in select histologic variants of prostate carcinoma. Mod Pathol. 2009;22:1176–85.

205. Sabaliauskaite R, Jarmalaite S, Petroska D, Dasevicius D, Laurinavicius A, Jankevicius F, et al. Combined analysis of TMPRSS2-ERG and TERT for improved prognosis of biochemical recurrence in prostate cancer. Genes Chromosomes Cancer. 2012;51:781-91.

206. Laxman B, Morris DS, Yu J, Siddiqui J, Cao J, Mehra R, et al. A First-Generation Multiplex Biomarker Analysis of Urine for the Early Detection of Prostate Cancer. Cancer Research. 2008;68:645–9.

207. Yusenko MV, Zubakov D, Kovacs G. Gene expression profiling of chromophobe renal cell carcinomas and renal oncocytomas by Affymetrix GeneChip using pooled and individual tumours. Int J Biol Sci. 2009;5:517–27.

208. Danila DC, Pantel K, Fleisher M, Scher HI. Circulating tumors cells as biomarkers: progress toward biomarker qualification. Cancer J. 2011;17:438–50.

209. Punnoose EA, Atwal SK, Spoerke JM, Savage H, Pandita A, Yeh RF, Pirzkall A, Fine BM, Amler LC, Chen DS, Lackner MR. Molecular biomarker analyses using circulating tumor cells. PloS one. 2010;5:e12517.

210. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Nature Publishing Group; 2009;100:1603–7.

211. Bai VU, Kaseb A, Tejwani S, Divine GW, Barrack ER, Menon M, et al. Identification of prostate cancer mRNA markers by averaged differential expression and their detection in biopsies, blood, and urine. Proceedings of the National Academy of Sciences. 2007;104:2343–8.

212. Downes MR, Byrne JC, Pennington SR, Dunn MJ, Fitzpatrick JM, Watson RWG. Urinary markers for prostate cancer. BJU Int. 2007;99:263–8.

213. Rak J. Microparticles in cancer. InSeminars in thrombosis and hemostasis. 2010;08;888-906.

214. Mathivanan S, Ji H, Simpson RJ. Exosomes: Extracellular organelles important in intercellular communication. Journal of Proteomics. 2010;73:1907–20.

215. György B, Módos K, Pállinger É, Pálóczi K. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. Blood. 2011;117:e39-48.

216. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, Functions, and Clinical Relevance of Extracellular Vesicles. Pharmacological Reviews. 2012;64):676–705.

217. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunol Lett. 2006;107:102-8.

218. van den Bemd G-JCM, Krijgsveld J, Luider TM, van Rijswijk AL, Demmers JAA, Jenster G. Mass spectrometric identification of human prostate cancer-derived proteins in serum of xenograft-bearing mice. Mol Cell Proteomics. 2006;5:1830–9.

219. Simons M, Raposo G. Exosomes – vesicular carriers for intercellular communication. Current Opinion in Cell Biology. 2009;21:575–81.

220. Van Niel G. Exosomes: A Common Pathway for a Specialized Function. Journal of Biochemistry. 2006;140:13–21.

221. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev. 2007;21:157–71.

222. Miranda KC, Bond DT, McKee M, Skog J, Păunescu TG, Da Silva N, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. Kidney International. 2010;78:191–9.

223. Jansen FH, Krijgsveld J, van Rijswijk A, van den Bemd G-J, van den Berg MS, van Weerden WM, et al. Exosomal secretion of cytoplasmic prostate cancer xenograft-derived proteins. Mol Cell Proteomics. 2009;8:1192–205.

224. Mears R, Craven RA, Hanrahan S, Totty N. Proteomic analysis of melanoma-

derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics 2004;4:4019-31.

225. Futter CE, White IJ. Annexins and endocytosis. Traffic 2007;8:951-8.

226. Wubbolts R, Leckie RS, Veenhuizen PTM, Schwarzmann G, Möbius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem. 2003;278:10963–72.

227. Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie. 2007;89:205–12.

228. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science. 2008;319:1244–7.

229. Chu Z, Witte DP, Qi X. Saposin C-LBPA interaction in late-endosomes/lysosomes. Exp Cell Res. 2005;303:300–7.

230. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer. 2005;113:752–60.

231. Barry OP, Praticò D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. Journal of Clinical Investigation. 1998;102:136–44.

232. Barry OP, FitzGerald GA. Mechanisms of cellular activation by platelet microparticles. Thromb Haemost 1999;82:794-800.

233. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.

234. Théry C, Boussac M, Véron P, Ricciardi-Castagnoli P, Raposo G, Garin J, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol. 2001;166:7309–18.

235. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9:581–93.

236. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98:1076-84.

237. Hakulinen J, Sankkila L, Sugiyama N, Lehti K, Keski-Oja J. Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. J Cell Biochem. 2008;105:1211–8.

238. Xiao D, Ohlendorf J, Chen Y, Taylor DD, Rai SN, Waigel S, et al. Identifying mRNA, microRNA and protein profiles of melanoma exosomes. PLoS ONE. 2012;7:e46874.

239. Wieckowski E, Whiteside TL. Human tumor-derived vs dendritic cell-derived exosomes have distinct biologic roles and molecular profiles. Immunol Res. 2006;36:247–54.

240. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10:1470–6.

241. Chen W, Wang J, Shao C, Liu S, Yu Y, Wang Q, et al. Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. Eur J Immunol. 2006;36:1598–607.

242. Mears R, Craven RA, Hanrahan S, Totty N, Upton C, Young SL, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics. 2004;4:4019–31.

243. Hwangbo C, Kim J, Lee JJ, Lee J-H. Activation of the integrin effector kinase focal adhesion kinase in cancer cells is regulated by crosstalk between protein kinase Calpha and the PDZ adapter protein mda-9/Syntenin. Cancer Research. 2010;70:1645–55.

244. Boukerche H, Su Z-Z, Prévot C, Sarkar D, Fisher PB. mda-9/Syntenin promotes metastasis in human melanoma cells by activating c-Src. Proceedings of the National Academy of Sciences. 2008;105:15914–9.

245. Dolo V, D'Ascenzo S, Giusti I, Millimaggi D, Taraboletti G, Pavan A. Shedding of membrane vesicles by tumor and endothelial cells. Ital J Anat Embryol. 2005;110:127–33.

246. Giusti I, D'Ascenzo S, Millimaggi D, Taraboletti G, Carta G, Franceschini N, et al. Cathepsin B mediates the pH-dependent proinvasive activity of tumor-shed microvesicles. Neoplasia. 2008;10:481–8.

247. Dolo V, Ginestra A, Ghersi G, Nagase H, Vittorelli ML. Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities. J Submicrosc Cytol Pathol. 1994;26:173–80.

248. Angelucci A, D'Ascenzo S, Festuccia C, Gravina GL, Bologna M, Dolo V, et al. Vesicle-associated urokinase plasminogen activator promotes invasion in prostate cancer cell lines. Clin Exp Metastasis. Kluwer Academic Publishers; 2000;18:163–70.

249. Taraboletti G, D'Ascenzo S, Giusti I, Marchetti D, Borsotti P, Millimaggi D, et al. Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. Nioplasia. 2006;8:96–103.

250. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. Endothelial

expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proceedings of the National Academy of Sciences. 2009;106:3794–9.

251. Yi M, Schnitzer JE. Impaired tumor growth, metastasis, angiogenesis and wound healing in annexin A1-null mice. Proceedings of the National Academy of Sciences. 2009;106:17886–91.

252. Rondepierre F, Bouchon B, Papon J, Bonnet-Duquennoy M, Kintossou R, Moins N, et al. Proteomic studies of B16 lines: involvement of annexin A1 in melanoma dissemination. Biochim Biophys Acta. 2009;1794:61–9.

253. Lokman NA, Ween MP, Oehler MK, Ricciardelli C. The role of annexin A2 in tumorigenesis and cancer progression. Cancer Microenviron. 2011;4:199–208.

254. Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012;18:883–91.

255. Goerdt S, Bhardwaj R, Sorg C. Inducible expression of MS-1 high-molecular-weight protein by endothelial cells of continuous origin and by dendritic cells/macrophages in vivo and in vitro. Am J Pathol. 1993;142:1409-22.

256. Bosserhoff AK. Novel biomarkers in malignant melanoma. Clinica Chimica. 2006; 367:28–35.

257. Yang NY, Pasquale EB, Owen LB, Ethell IM. The EphB4 receptor-tyrosine kinase promotes the migration of melanoma cells through Rho-mediated actin cytoskeleton reorganization. J Biol Chem. 2006;281:32574-86.

258. Yang N-Y, Lopez-Bergami P, Goydos JS, Yip D, Walker AM, Pasquale EB, et al. The EphB4 receptor promotes the growth of melanoma cells expressing the ephrin-B2 ligand. Pigment Cell Melanoma Res. 2010;23:684–7.

259. Hood JL, San RS, Wickline SA. Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis. Cancer Research. 2011;71:3792–801.

260. Park JE, Tan Sen H, Datta A, Lai RC, Zhang H, Meng W, et al. Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. Mol Cell Proteomics. 2010;9:1085–99.

261. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clin Cancer Res. 2005;11:1010–20.

262. Whitlow MB, Klein LM. Response of SCC-12F, a human squamous cell carcinoma cell line, to complement attack. J Invest Dermatol. 1997;109:39–45.

263. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-

released microvesicles as vehicles of immunosuppression. Cancer Research. 2007;67:2912–5.

264. Panagopoulos K, Cross-Knorr S, Dillard C, Pantazatos D, Del Tatto M, Mills D, et al. Reversal of chemosensitivity and induction of cell malignancy of a non-malignant prostate cancer cell line upon extracellular vesicle exposure. Mol Cancer. 2013;12:118–8.

265. Castellana D, Zobairi F, Martinez MC, Panaro MA, Mitolo V, Freyssinet J-M, et al. Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: a role for activated fibroblasts and CX3CL1-CX3CR1 axis. Cancer Research. 2009;69:785–93.

266. Itoh T, Ito Y, Ohtsuki Y, Ando M, Tsukamasa Y. Microvesicles released from hormone-refractory prostate cancer cells facilitate mouse pre-osteoblast differentiation. J Mol Histol. 2012;43:509-15.

267. Millimaggi D, Festuccia C, Angelucci A. Osteoblast-conditioned media stimulate membrane vesicle shedding in prostate cancer cells. Int J Oncol. 2006;28:909-14.

268. Mitchell PJ, Welton J, Staffurth J, Court J, Mason MD, Tabi Z, et al. Can urinary exosomes act as treatment response markers in prostate cancer? J Transl Med. 2009;7:4.

269. Schostak M, Schwall GP, Poznanović S, Groebe K, Müller M, Messinger D, et al. Annexin A3 in Urine: A Highly Specific Noninvasive Marker for Prostate Cancer Early Detection. The Journal of Urology. 2009;181:343–53.

270. Burden HP, Holmes CH, Persad R, Whittington K. Prostasomes--their effects on human male reproduction and fertility. Hum Reprod Update. 2006;12:283–92.

271. Uría JA, López-Otín C. Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res 2000;60:4745-51.

272. Zhao YG. Endometase/Matrilysin-2 in Human Breast Ductal Carcinoma in Situ and Its Inhibition by Tissue Inhibitors of Metalloproteinases-2 and -4: A Putative Role in the Initiation of Breast Cancer Invasion. Cancer Research. 2004;64:590–8.

273. Marchenko GN, Ratnikov BI, Rozanov DV, Godzik A, Deryugina EI, Strongin AY. Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem J. 2001;356:705–18.

274. Park HI, Turk BE, Gerkema FE, Cantley LC. Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. J Biol Chem. 2002;277:35168-75.

275. Park HI, Ni J, Gerkema FE, Liu D, Belozerov VE, Sang QX. Identification and

characterization of human endometase (Matrix metalloproteinase-26) from endometrial tumor. J Biol Chem. 2000;275:20540–4.

276. Zhao Y-G, Xiao A-Z, Newcomer RG, Park HI, Kang T, Chung LWK, et al. Activation of Pro-gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells. J Biol Chem. 2003;278:15056-64.

277. Lee S, Desai KK, Iczkowski KA, Newcomer RG, Wu KJ, Zhao Y-G, et al. Coordinated peak expression of MMP-26 and TIMP-4 in preinvasive human prostate tumor. Cell Res. 2006;16:750–8.

278. Riddick ACP, Shukla CJ, Pennington CJ, Bass R, Nuttall RK, Hogan A, et al. Identification of degradome components associated with prostate cancer progression by expression analysis of human prostatic tissues. Br J Cancer. 2005;92:2171–80.

279. López-Otín C, Overall CM. Protease degradomics: a new challenge for proteomics. Nat Rev Mol Cell Biol. 2002;3:509-19. 2002.

280. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. Journal of Cell Science. 2002;115:3719–27.

281. Li D. Peaking of MMP-26 and TIMP-4 marks invasive transition in prostate cancer. Cell Res. 2006;16:741–1.

282. Bister V, Skoog T, Virolainen S, Kiviluoto T, Puolakkainen P, Saarialho-Kere U. Increased expression of matrix metalloproteinases-21 and -26 and TIMP-4 in pancreatic adenocarcinoma. Mod Pathol. 2007;20:1128–40.

283. Tsuji A, Torres-Rosado A, Arai T, Le Beau MM, Lemons RS, Chou SH, et al. Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. J Biol Chem. 1991;266:16948–53.

284. Leytus SP, Loeb KR, Hagen FS, Kurachi K, Davie EW. A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. Biochemistry. 1988;27:1067–74.

285. Kazama Y, Hamamoto T, Foster DC, Kisiel W. Hepsin, a putative membrane-associated serine protease, activates human factor VII and initiates a pathway of blood coagulation on the cell surface leading to thrombin formation. J Biol Chem. 1995;270:66–72.

286. Torres-Rosado A, O'Shea KS, Tsuji A, Chou SH, Kurachi K. Hepsin, a putative cell-surface serine protease, is required for mammalian cell growth. Proc Natl Acad Sci USA. 1993;90:7181–5.

287. Vu TK, Liu RW, Haaksma CJ, Tomasek JJ, Howard EW. Identification and cloning of the membrane-associated serine protease, hepsin, from mouse preimplantation embryos. J Biol Chem. 1997;272:31315–20.

288. Chen Z, Fan Z, McNeal JE, Nolley R, Caldwell MC, Mahadevappa M, et al.

Hepsin and maspin are inversely expressed in laser capture microdissectioned prostate cancer. JURO. 2003;169:1316–9.

289. Stamey TA, Warrington JA, Caldwell MC, Chen Z, Fan Z, Mahadevappa M, et al. Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. JURO. 2001;166:2171–7.

290. Xuan J-A, Schneider D, Toy P, Lin R, Newton A, Zhu Y, et al. Antibodies neutralizing hepsin protease activity do not impact cell growth but inhibit invasion of prostate and ovarian tumor cells in culture. Cancer Research. 2006;66:3611–9.

291. Wu Q, Yu D, Post J, Halks-Miller M, Sadler JE, Morser J. Generation and characterization of mice deficient in hepsin, a hepatic transmembrane serine protease. Journal of Clinical Investigation. 1998;101:321–6.

292. Fromont GL, Chene L, Vidaud M, Vallancien G, Mangin P, Fournier G, et al. Differential expression of 37 selected genes in hormone-refractory prostate cancer using quantitative taqman real-time RT-PCR. Int J Cancer. 2005;114:174–81.

293. Klezovitch O, Chevillet J, Mirosevich J, Roberts RL, Matusik RJ, Vasioukhin V. Hepsin promotes prostate cancer progression and metastasis. Cancer Cell. 2004;6:185–95.

294. Moran P, Li W, Bin Fan, Vij R, Eigenbrot C, Kirchhofer D. Pro-urokinase-type plasminogen activator is a substrate for hepsin. J Biol Chem. 2006;281:30439–46.

295. Herter S, Piper DE, Aaron W, Gabriele T, Cutler G, Cao P, et al. Hepatocyte growth factor is a preferred in vitro substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. Biochem J. 2005;390:125–36.

296. Birchmeier C, Birchmeier W, Gherardi E, Woude GFV. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915–25.

297. Knudsen BS, Edlund M. Prostate cancer and the met hepatocyte growth factor receptor. Adv Cancer Res. 2004;91:31-67.

298. Podgorski I, Linebaugh BE, Sameni M, Jedeszko C, Bhagat S, Cher ML, et al. Bone Microenvironment Modulates Expression and Activity of Cathepsin B in Prostate Cancer. NEO. 2005;7:207–23.

299. JC K, H P, P R, G H, BR B. Plasminogen activator activity in bone metastases of prostatic carcinomas as compared to primary tumors. Invasion Metastasis. 1985;5:344–55.

300. Yamanaka K, Gohji K, Arakawa S, Kamidono S. Elevation of serum levels of urokinase-type plasminogen activator and its receptor is associated with disease progression and prognosis in patients with prostate cancer. The Prostate 1999;39:123-9.

301. Sheng S, Truong B, Fredrickson D, Wu R, Pardee AB, Sager R. Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. Proc Natl Acad Sci USA. 1998;95:499–504.

302. Bailey CM, Khalkhali-Ellis Z, Kondo S, Margaryan NV, Seftor REB, Wheaton WW, et al. Mammary serine protease inhibitor (Maspin) binds directly to interferon regulatory factor 6: identification of a novel serpin partnership. J Biol Chem. 2005;280:34210–7.

303. Endsley MP, Hu Y, Deng Y, He X, Warejcka DJ, Twining SS, et al. Maspin, the molecular bridge between the plasminogen activator system and beta1 integrin that facilitates cell adhesion. J Biol Chem. 2011;286:24599–607.

304. Bass R, Wagstaff L, Ravenhill L, Ellis V. Binding of extracellular maspin to beta1 integrins inhibits vascular smooth muscle cell migration. J Biol Chem. 2009;284:27712–20.

305. Blacque OE, Worrall DM. Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J Biol Chem. 2002;277:10783–8.

306. Yin S, Li X, Meng Y, Finley RL, Sakr W, Yang H, et al. Tumor-suppressive maspin regulates cell response to oxidative stress by direct interaction with glutathione S-transferase. J Biol Chem. 2005;280:34985–96.

307. Maspin expression inhibits osteolysis, tumor growth,and angiogenesis in a model of prostate cancerbone metastasis. Proc Natl Acad Sci U S A. 2003; 100: 7847–7852.

308. Sheng S, Carey J, Seftor EA, Dias L, Hendrix MJ, Sager R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci USA. 1996;93:11669–74.

309. Pierson CR, McGowen R, Grignon D, Sakr W, Dey J, Sheng S. Maspin is up-regulated in premalignant prostate epithelia. Prostate. 2002;53:255–62.

310. Thor A, Neveu M, Sheng S, Rafidi K, Seftor E, Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science. 1994;263:526-9.

311. Lonardo F, Li X, Siddiq F, Singh R, Al-Abbadi M, Pass HI, et al. Maspin nuclear localization is linked to favorable morphological features in pulmonary adenocarcinoma. Lung Cancer. 2006;51:31–9.

312. Sheng S. The promise and challenge toward the clinical application of maspin in cancer. Front Biosci (Landmark Ed). 2004;9:2733–45.

313. Lu Y, Yi Y, Liu P, Wen W, James M, Wang D, et al. Common human cancer genes discovered by integrated gene-expression analysis. PLoS ONE. 2007;2:e1149.

314. Kladney RD, Bulla GA, Guo L, Mason AL, Tollefson AE, Simon DJ, et al.

GP73, a novel Golgi-localized protein upregulated by viral infection. Gene. 2000;249:53–65.

315. Varambally S, Laxman B, Mehra R, Cao Q, Dhanasekaran SM, Tomlins SA, Granger J, Vellaichamy A, Sreekumar A, Yu J, Gu W. Golgi protein GOLM1 is a tissue and urine biomarker of prostate cancer. Neoplasia. 2008;10:1285IN35-94.

316. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, et al. Delineation of prognostic biomarkers in prostate cancer. Nature. 2001;412:822–6.

317. Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proceedings of the National Academy of Sciences. 2004;101:811–6.

318. Kristiansen G, Fritzsche FR, Wassermann K, Jäger C, Tölls A, Lein M, et al. GOLPH2 protein expression as a novel tissue biomarker for prostate cancer: implications for tissue-based diagnostics. Br J Cancer. 2008;99:939–48.

319. Strous GJ, van Kerkhof P, van Meer G, Rijnboutt S, Stoorvogel W. Differential effects of brefeldin A on transport of secretory and lysosomal proteins. J Biol Chem. 1993;268:2341–7.

320. Ivessa NEN, Gravotta DD, De Lemos-Chiarandini CC, Kreibich GG. Functional protein prenylation is required for the brefeldin A-dependent retrograde transport from the Golgi apparatus to the endoplasmic reticulum. J Biol Chem. 1997;272:20828–34.

321. Johnstone RM. Exosomes biological significance: A concise review. Blood Cells Mol Dis. 2006;36:315–21.

322. Gehring WJ, Hiromi Y. Homeotic genes and the homeobox. Annu Rev Genet 1986;20:147-73.

323. Wigle JT, Eisenstat DD. Homeobox genes in vertebrate forebrain development and disease. Clin Genet. 2008;73:212–26.

324. Stein S, Fritsch R, Lemaire L, Kessel M. Checklist: Vertebrate homeo□ genes. Mechanisms of development. 1996;55:91-108.

325. Song K, Wang Y, Sassoon D. Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation. Nature. 1992;360:477–81.

326. Waltregny D, Alami Y, Clausse N, de Leval J, Castronovo V. Overexpression of the homeobox gene HOXC8 in human prostate cancer correlates with loss of tumor differentiation. Prostate. 2002;50:162–9.

327. Miller GJ, Miller HL, van Bokhoven A, Lambert JR, Werahera PN, Schirripa O, et al. Aberrant HOXC expression accompanies the malignant phenotype in human prostate. Cancer Research. 2003;63:5879–88.

328. Ramachandran S, Liu P, Young AN, Yin-Goen Q, Lim SD, Laycock N, et al. Loss of HOXC6 expression induces apoptosis in prostate cancer cells. Oncogene. Nature Publishing Group; 2005;24:188–98.

329. Bannerman P, El-Deiry WS, Cohen P. IGFBP-3 mediates p53-induced apoptosis during serum starvation. Int J Oncol 2002;21:327-35.

330. Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, et al. PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. Oncogene. 1999;18:127–37.

331. Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology ofHOX genes and their role in cancer. J Pathol. 2005;205:154–71.

332. Harvey TJ, Hooper JD, Myers SA, Stephenson SA, Ashworth LK, Clements JA. Tissue-specific expression patterns and fine mapping of the human kallikrein (KLK) locus on proximal 19q13.4. J Biol Chem. 2000;275:37397–406.

333. Borgoño CA, Michael IP, DIAMANDIS EP. Human tissue kallikreins: physiologic roles and applications in cancer. Molecular Cancer Research. 2004;2:257–80.

334. Murtha P, Tindall DJ, Young CY. Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. Biochemistry. 1993;32:6459–64.

335. vgren JL, Valtonen-AndrÃ C, Marsal K, Lilja H, Lundwall A. Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. Journal of Andrology. 1999;20:348–55.

336. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab. 2000;11:54–60.

337. Solovyeva NI, Balayevskaya TO, Dilakyan EA, Topol LZ, Kisseliov FL. Plasminogen activators, kallikrein-like proteinase and type I and type IV collagenases at various stages of oncogenic transformation. Immunopharmacology. 1996;32:131–4.

338. Shang Z, Niu Y, Cai Q, Chen J, Tian J, Yeh S, et al. Human kallikrein 2 (KLK2) promotes prostate cancer cell growth via function as a modulator to promote the ARA70-enhanced androgen receptor transactivation. Tumour Biol. 2014;35:1881–90.

339. Haese A, Graefen M, Steuber T, Becker C. Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally-advanced prostate cancer in total PSA-levels below 10 ng/ml. The Prostate 2001;49:101-9. 2001.

340. Recker F, Kwiatkowski MK, Piironen T, Pettersson K. Human glandular

kallikrein as a tool to improve discrimination of poorly differentiated and non-organ-confined prostate cancer compared with prostate-specific antigen. Urology. 2000;55:481-5. 2000.

341. Stephan C, Jung K, Nakamura T. Serum human glandular kallikrein 2 (hK2) for distinguishing stage and grade of prostate cancer. Int J Urol. 2006;13:238-43. 2006.

342. Yousef GM, Obiezu CV, Luo LY, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. Cancer Research. 1999;59:4252–6.

343. Xi Z, Klokk TI, Korkmaz K, Kurys P, Elbi C, Risberg B, et al. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. Cancer Research. 2004;64:2365–70.

344. Dong Y, Bui LT, Odorico DM, Tan OL, Myers SA, Samaratunga H, et al. Compartmentalized expression of kallikrein 4 (KLK4/hK4) isoforms in prostate cancer: nuclear, cytoplasmic and secreted forms. Endocrine Related Cancer. 2005;12:875–89.

345. Veveris-Lowe TL. Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. Endocrine Related Cancer. 2005;12:631–43.

346. Klokk TI, Kilander A, Xi Z, Waehre H, Risberg B, Danielsen HE, et al. Kallikrein 4 Is a Proliferative Factor that Is Overexpressed in Prostate Cancer. Cancer Research. 2007;67:5221–30.

347. Merlo GR, Zerega B, Paleari L, Trombino S, Mantero S, Levi G. Multiple functions of Dlx genes. Int J Dev Biol. 2000;44:619–26.

348. Pascal LE, Vêncio RZ, Page LS, Liebeskind ES, Shadle CP, Troisch P, et al. Gene expression relationship between prostate cancer cells of Gleason 3, 4 and normal epithelial cells as revealed by cell type-specific transcriptomes. BMC Cancer. 2009;9:452.

349. Altintas DM, Allioli N, Decaussin M, de Bernard S, Ruffion A, Samarut J, et al. Differentially Expressed Androgen-Regulated Genes in Androgen-Sensitive Tissues Reveal Potential Biomarkers of Early Prostate Cancer. Migliaccio A, editor. PLoS ONE. 2013;8:e66278.

350. Cantile MM, Kisslinger AA, Cindolo LL, Schiavo GG, D'Antò VV, Franco RR, et al. cAMP induced modifications of HOX D gene expression in prostate cells allow the identification of a chromosomal area involved in vivo with neuroendocrine differentiation of human advanced prostate cancers. J Cell Physiol. 2005;205:202–10.

351. Loriot A, Boon T, De Smet C. Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer.

2003;105:371–6.

352. Reuter M, Chuma S, Tanaka T, Franz T, Stark A, Pillai RS. Loss of the Mili-interacting Tudor domain-containing protein-1 activates transposons and alters the Mili-associated small RNA profile. Nat Struct Mol Biol. 2009;16:639–46.

353. Kacprzyk LA, Laible M, Andrasiuk T, Brase JC, Börno ST, Fälth M, et al. ERG Induces Epigenetic Activation of Tudor Domain-Containing Protein 1 (TDRD1) in ERG Rearrangement-Positive Prostate Cancer. Chatterjee B, editor. PLoS ONE. 2013;8:e59976.

354. Clark JP, Cooper CS. ETS gene fusions in prostate cancer. Nature Reviews Urology. Nature Publishing Group; 2009;6:429–39.

355. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nat Rev Cancer 2008;8:497-511.

356. Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature. 2007;448:595–9.

357. Shin S, Kim TD, Jin F, van Deursen JM, Dehm SM, Tindall DJ, et al. Induction of Prostatic Intraepithelial Neoplasia and Modulation of Androgen Receptor by ETS Variant 1/ETS-Related Protein 81. Cancer Research. 2009;69:8102–10.

358. Boormans JL, Korsten H, Ziel-van der Made AJC, Van Leenders GJLH, de Vos CV, Jenster G, et al. Identification of TDRD1as a direct target gene of ERGin primary prostate cancer. Int J Cancer. 2013 133:335–45.

359. Jhavar S, Brewer D, Edwards S, Kote-Jarai Z, Attard G, Clark J, et al. Integration of ERG gene mapping and gene-expression profiling identifies distinct categories of human prostate cancer. BJU Int. 2009;103:1256–69.

360. Jhavar S, Reid A, Clark J, Kote-Jarai Z, Christmas T, Thompson A, et al. Detection of TMPRSS2-ERGTranslocations in Human Prostate Cancer by Expression Profiling Using GeneChip Human Exon 1.0 ST Arrays. The Journal of Molecular Diagnostics. 2008;10:50–7.

361. Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, et al. Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. NEO. 2012;14:600–11.

362. KRISTIANSEN G, Pilarsky C, Wissmann C, Kaiser S, Bruemmendorf T, Roepcke S, et al. Expression profiling of microdissected matched prostate cancer samples reveals CD166/MEMD and CD24 as new prognostic markers for patient survival. J Pathol. 2005;205:359–76.

363. Massie CE, Lynch A, Ramos-Montoya A, Boren J, Stark R, Fazli L, et al. The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. The EMBO Journal. Nature Publishing Group; 2011;30:2719–33.

364. Ashida S, Nakagawa H, Katagiri T, Furihata M, Iiizumi M, Anazawa Y,

Tsunoda T, Takata R, Kasahara K, Miki T, Fujioka T. Molecular Features of the Transition from Prostatic Intraepithelial Neoplasia (PIN) to Prostate Cancer. Cancer research. 2004;64:5963-72.

365. Karacosta LG, Foster BA, Azabdaftari G, Feliciano DM, Edelman AM. A Regulatory Feedback Loop Between Ca2+/Calmodulin-dependent Protein Kinase Kinase 2 (CaMKK2) and the Androgen Receptor in Prostate Cancer Progression. Journal of Biological Chemistry. 2012;287:24832–43.

366. Frigo DE, Howe MK, Wittmann BM, Brunner AM, Cushman I, Wang Q, et al. CaM kinase kinase beta-mediated activation of the growth regulatory kinase AMPK is required for androgen-dependent migration of prostate cancer cells. Cancer Research. 2011;71:528–37.

367. Shima T, Mizokami A, Miyagi T, Kawai K, Izumi K, Kumaki M, et al. Down-regulation of calcium/calmodulin-dependent protein kinase kinase 2 by androgen deprivation induces castration-resistant prostate cancer. Prostate. 2012;72:1789–801.

368. Natsumeda Y, Ohno S, Kawasaki H, Konno Y, Weber G, Suzuki K. Two distinct cDNAs for human IMP dehydrogenase. J Biol Chem. 1990;265:5292–5.

369. Jackson RC, Weber G, Morris HP. IMP dehydrogenase, an enzyme linked with proliferation and malignancy. Nature. 1975;256:331–3.

370. Weber G. Biochemical strategy of cancer cells and the design of chemotherapy: G. H. A. Clowes Memorial Lecture. Cancer Research. 1983;43:3466–92.

371. Allison AC, Eugui EM. Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection. Transplantation. 2005;80:181–90.

372. Han Z-D, Zhang Y-Q, He H-C, Dai Q-S, Qin G-Q, Chen J-H, et al. Identification of novel serological tumor markers for human prostate cancer using integrative transcriptome and proteome analysis. Med Oncol. 2012;29:2877–88.

373. Floryk D. Differentiation of Androgen-Independent Prostate Cancer PC-3 Cells Is Associated with Increased Nuclear Factor- B Activity. Cancer Research. 2005;65:11588–96.

374. Hååg P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. The Journal of Steroid Biochemistry and Molecular Biology. 2005;96:251–8.

375. Hara T, Miyazaki H, Lee A, Tran CP, REITER RE. Androgen Receptor and Invasion in Prostate Cancer. Cancer Research. 2008;68:1128–35.

376. Chen Y, Sawyers CL, Scher HI. Targeting the androgen receptor pathway in prostate cancer. Curr Opin Pharmacol. 2008;8:440–8.

377. Attar RM, Jure-Kunkel M, Balog A, Cvijic ME, Dell-John J, Rizzo CA, et al. Discovery of BMS-641988, a novel and potent inhibitor of androgen receptor signaling for the treatment of prostate cancer. Cancer Research. 2009;69:6522–30.

378. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science. 2009;324:787–90.

379. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, et al. The nuclear receptor superfamily: The second decade. Cell. 1995;83:835–9.

380. Comstock CES, Knudsen KE. The complex role of AR signaling after cytotoxic insult: implications for cell-cycle-based chemotherapeutics. Cell Cycle. 2007;6:1307–13.

381. Knudsen KE, Scher HI. Starving the Addiction: New Opportunities for Durable Suppression of AR Signaling in Prostate Cancer. Clinical Cancer Research. 2009;15:4792–8.

382. Zoubeidi A, Chi K, Gleave M. Targeting the Cytoprotective Chaperone, Clusterin, for Treatment of Advanced Cancer. Clinical Cancer Research. 2010;16:1088–93.

383. Andersen RJ, Mawji NR, Wang J, Wang G, Haile S, Myung J-K, et al. Regression of Castrate-Recurrent Prostate Cancer by a Small-Molecule Inhibitor of the Amino-Terminus Domain of the Androgen Receptor. Cancer Cell. 2010;17:535–46.

384. Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, et al. Ligand-Independent Androgen Receptor Variants Derived from Splicing of Cryptic Exons Signify Hormone-Refractory Prostate Cancer. Cancer Research. 2009;69:16–22.

385. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, et al. A Novel Androgen Receptor Splice Variant Is Up-regulated during Prostate Cancer Progression and Promotes Androgen Depletion-Resistant Growth. Cancer Research. 2009;69:2305–13.

386. Hu R, Isaacs WB, Luo J. A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. Prostate. 2011;71:1656–67.

387. Korkmaz KS, Elbi C, Korkmaz CG, Loda M, Hager GL, Saatcioglu F. Molecular cloning and characterization of STAMP1, a highly prostate-specific six transmembrane protein that is overexpressed in prostate cancer. J Biol Chem. 2002;277:36689–96.

388. Ohgami RS, Campagna DR, McDonald A, Fleming MD. The Steap proteins are metalloreductases. Blood. 2006;108:1388–94.

389. Korkmaz CG, Korkmaz KS, Kurys P, Elbi C, Wang L, Klokk TI, et al.

Molecular cloning and characterization of STAMP2, an androgen-regulated six transmembrane protein that is overexpressed in prostate cancer. Oncogene. 2005;24:4934–45.

390. Sanchez-Pulido L, Rojas AM, Valencia A. ACRATA: a novel electron transfer domain associated to apoptosis and cancer. BMC Cancer 2004;4:98.

391. Challita-Eid PMP, Morrison KK, Etessami SS, An ZZ, Morrison KJK, Perez-Villar JJJ, et al. Monoclonal antibodies to six-transmembrane epithelial antigen of the prostate-1 inhibit intercellular communication in vitro and growth of human tumor xenografts in vivo. Cancer Research. 2007;67:5798–805.

392. Maia C, Socorro S, Santos C. STEAP2 (six transmembrane epithelial antigen of the prostate 2). Atlas of Genetics and Cytogenetics in Oncology and Haematology. 2011;15:217-219.

393. Wellen KE, Fucho R, Gregor MF, Furuhashi M, Morgan C, Lindstad T, et al. Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. Cell. 2007;129:537–48.

394. Arner P, Stenson BM, Dungner E, Näslund E, Hoffstedt J, Ryden M, et al. Expression of six transmembrane protein of prostate 2 in human adipose tissue associates with adiposity and insulin resistance. Journal of Clinical Endocrinology & Metabolism. 2008;93:2249–54.

395. Tamura. STEAP4 regulates focal adhesion kinase activation and CpG motifs within STEAP4 promoter region are frequently methylated in DU145, human androgen-independent prostate cancer cells. Int J Mol Med. 2009;24.

396. Kadomatsu K, Muramatsu T. Midkine and pleiotrophin in neural development and cancer. Cancer Letters. 2004;204:127–43.

397. Muramatsu T. Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. Journal of Biochemistry. 2002;132:359–71.

398. Konishi NN, Nakamura MM, Nakaoka SS, Hiasa YY, Cho MM, Uemura HH, et al. Immunohistochemical analysis of midkine expression in human prostate carcinoma. Oncology. 1999;57:253–7.

399. You Z, Dong Y, Kong X, Beckett LA, Gandour-Edwards R, Melamed J. Midkine is a NF-kappaB-inducible gene that supports prostate cancer cell survival. BMC Med Genomics. 2008;1:6.

400. Takei Y, Kadomatsu K, Goto T, Muramatsu T. Combinational antitumor effect of siRNA against midkine and paclitaxel on growth of human prostate cancer xenografts. Cancer. 2006;107:864–73.

401. Yuan T-C, Veeramani S, Lin M-F. Neuroendocrine-like prostate cancer cells: neuroendocrine transdifferentiation of prostate adenocarcinoma cells. Endocrine Related Cancer. 2007;14:531–47.

402. Isaacs JT, Isaacs WB. Androgen receptor outwits prostate cancer drugs. Nat Med. 2004;10:26–7.

403. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med. 2003;10:33–9.

404. Nordin A, Wang W, Welén K, Damber J-E. Midkine is associated with neuroendocrine differentiation in castration-resistant prostate cancer. Prostate. 2012;73:657–67.

405. Holyoake A, O'Sullivan P, Pollock R, Best T, Watanabe J, Kajita Y, et al. Development of a Multiplex RNA Urine Test for the Detection and Stratification of Transitional Cell Carcinoma of the Bladder. Clinical Cancer Research. 2008;14:742–9.

406. Meeker AK. Telomeres and telomerase in prostatic intraepithelial neoplasia and prostate cancer biology. Urology. 2006;24:122–30.

407. Wierstra I, Alves J. FOXM1, a typical proliferation-associated transcription factor. Biol Chem. 2007;388:1257–74.

408. Laoukili J, Kooistra MRH, Brás A, Kauw J, Kerkhoven RM, Morrison A, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat Cell Biol. 2005;7:126–36.

409. Kasper S, Sheppard PC, Yan Y, Pettigrew N, Borowsky AD, Prins GS, et al. Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. Lab Invest. 1998;78:319-34.

410. Masumori N, Thomas TZ, Chaurand P, Case T, Paul M, Kasper S, et al. A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential. Cancer Research. 2001;61:2239–49.

411. Kalin TV, Wang I-C, Ackerson TJ, Major ML, Detrisac CJ, Kalinichenko VV, et al. Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Research. 2006;66:1712–20.

412. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell. 2002;13:1977–2000.

413. Mosley JD, Keri RA. Cell cycle correlated genes dictate the prognostic power of breast cancer gene lists. BMC medical genomics. 2008;1:11.

414. Van De Vijver MJ, He YD, Veer LV. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med. 2002;347:1999-2009.

415. Sotiriou C, Desmedt C. Gene expression profiling in breast cancer. Annals of Oncology. 2006;17:259–62.

416. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst. 2006;98:262–72.

417. 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. The Lancet Oncology. 2011;12:245–55.

418. Hörnberg E, Ylitalo EB, Crnalic S, Antti H, Stattin P, Widmark A, et al. Expression of Androgen Receptor Splice Variants in Prostate Cancer Bone Metastases is Associated with Castration-Resistance and Short Survival. Dent P, editor. PLoS ONE. 2011;6:e19059.

419. Roupret M, Hupertan V, Yates DR, Catto JWF, Rehman I, Meuth M, et al. Molecular Detection of Localized Prostate Cancer Using Quantitative Methylation-Specific PCR on Urinary Cells Obtained Following Prostate Massage. Clinical Cancer Research. 2007;13:1720–5.

420. Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, et al. Androgen Receptor Regulates a Distinct Transcription Program in Androgen-Independent Prostate Cancer. Cell. 2009;138:245–56.

421. Bubendorf L, Tapia C, Gasser TC, Casella R, Grunder B, Moch H, et al. Ki67 labeling index in core needle biopsies independently predicts tumor-specific survival in prostate cancer. Human Pathology. 1998;29:949–54.

422. Zellweger T, Günther S, Zlobec I, Savic S, Sauter G, Moch H, et al. Tumour growth fraction measured by immunohistochemical staining of Ki67 is an independent prognostic factor in preoperative prostate biopsies with small-volume or low-grade prostate cancer. Int J Cancer. 2009;124:2116–23.

423. Sebo TJ, Cheville JC, Riehle DL, Lohse CM, Pankratz VS, Myers RP, et al. Perineural invasion and MIB-1 positivity in addition to Gleason score are significant preoperative predictors of progression after radical retropubic prostatectomy for prostate cancer. Am J Surg Pathol. 2002;26:431–9.

424. Rubio J, Ramos D, López-Guerrero JA, Iborra I, Collado A, Solsona E, et al. Immunohistochemical expression of Ki-67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. European Urology. 2005;48:745–51.

425. Lens SMA, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer. 2010;10:825–41.

426. Clegg N, Ferguson C, True LD, Arnold H, Moorman A, Quinn JE, et al. Molecular characterization of prostatic small-cell neuroendocrine carcinoma. Prostate. 2003;55:55–64.

427. Aparicio A, Tzelepi V, Araujo JC, Guo CC, Liang S, Troncoso P, et al. Neuroendocrine prostate cancer xenografts with large-cell and small-cell features derived from a single patient's tumor: Morphological,

immunohistochemical, and gene expression profiles. Prostate. 2010;71:846–56.

428. Papandreou CN, Daliani DD, Thall PF, Tu S-M, Wang X, Reyes A, et al. Results of a phase II study with doxorubicin, etoposide, and cisplatin in patients with fully characterized small-cell carcinoma of the prostate. Journal of Clinical Oncology. 2002;20:3072–80.

429. Gorlov IP, Sircar K, Zhao H, Maity SN, Navone NM, Gorlova OY, et al. Prioritizing genes associated with prostate cancer development. BMC Cancer. 2010;10:599.

430. Wong P, Borst DE, Farber D, Danciger JS, Tenniswood M, Chader GJ, et al. Increased TRPM-2/clusterin mRNA levels during the time of retinal degeneration in mouse models of retinitis pigmentosa. Biochem Cell Biol. 1994;72:439–46.

431. Zellweger T, Miyake H, July LV, Akbari M, Kiyama S, Gleave ME. Chemosensitization of human renal cell cancer using antisense oligonucleotides targeting the antiapoptotic gene clusterin. NEO. 2001;3:360–7.

432. Gleave M, Miyake H, Zangemeister-Wittke U, Jansen B. Antisense therapy: current status in prostate cancer and other malignancies. Cancer Metastasis Rev. 2002;21:79–92.

433. Chen M, Wang K, Zhang L, Li C, Yang Y. The discovery of putative urine markers for the specific detection of prostate tumor by integrative mining of public genomic profiles. PLoS ONE. 2011;6:e28552–2.

434. Fleissner F, Goerzig Y, Haverich A, Thum T. Microvesicles as novel biomarkers and therapeutic targets in transplantation medicine. American Journal of Transplantation. 2012;12:289-97.

435. Davies H, Bignell GR, Cox C, Stephens P, Edkins S. Mutations of the BRAF gene in human cancer. Nature. 2002;417:949-954.

436. Palanisamy N, Ateeq B, Kalyana-Sundaram S, Pflueger D, Ramnarayanan K, Shankar S, et al. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. Nat Med. 2010;16:793–8.

437. Wang J, Kobayashi T, Floc'h N, Kinkade CW, Aytes A, Dankort D, et al. B-Raf Activation Cooperates with PTEN Loss to Drive c-Myc Expression in Advanced Prostate Cancer. Cancer Research. 2012;72:4765–76.

438. Hart GW, Housley MP, Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. Nature. 2007;446:1017–22.

439. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci. 1999;24:68–72.

440. Warburg O. On the origin of cancer cells. Science. 1956;123:309–14.

441. Marshall S. Role of insulin, adipocyte hormones, and nutrient-sensing pathways

in regulating fuel metabolism and energy homeostasis: a nutritional perspective of diabetes, obesity, and cancer. Sci. STKE. 2006;2006:re7-.

442. Yang WH, Kim JE, Nam HW, Ju JW, Kim HS, Kim YS, et al. Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability. Nat Cell Biol. 2006;8:1074–83.

443. Chou TY, Dang CV, Hart GW. Glycosylation of the c-Myc transactivation domain. Proc Natl Acad Sci USA. 1995;92:4417–21.

444. Park SY, Kim HS, Kim NH, Ji S, Cha SY, Kang JG, et al. Snail1 is stabilized by O-GlcNAc modification in hyperglycaemic condition. The EMBO Journal 2010 ;29:3787–96.

445. Lynch TP, Ferrer CM, Jackson SR, Shahriari KS, Vosseller K, Reginato MJ. Critical role of O-Linked β-N-acetylglucosamine transferase in prostate cancer invasion, angiogenesis, and metastasis. J Biol Chem. 2012;287:11070–81.

446. Giles GG. Epidemiological investigation of prostate cancer. Methods Mol Med. 2003;81:1–19.

447. Klein EA, Silverman R. Inflammation, infection, and prostate cancer. Current opinion in urology. 2008;3:315-319.

448. Roberts RO, Bergstralh EJ, Bass SE, Lieber MM, Jacobsen SJ. Prostatitis as a risk factor for prostate cancer. Epidemiology. 2004;15:93–9.

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

450. Lin J-F, Xu J, Tian H-Y, Gao X, Chen Q-X, Gu Q, et al. Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis. Int J Cancer. 2007;121:2596–605.

451. Zha S, Ferdinandusse S, Hicks JL, Denis S, Dunn TA, Wanders RJ, et al. Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer. Prostate. 2005;63:316–23.

452. Glatt H, Engelke CE, Pabel U, Teubner W, Jones AL, Coughtrie MW, et al. Sulfotransferases: genetics and role in toxicology. Toxicol Lett. 2000;112:341–8.

453. Shirai T, Cui L, Takahashi S, Futakuchi M, Asamoto M, Kato K, et al. Carcinogenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in the rat prostate and induction of invasive carcinomas by subsequent treatment with testosterone propionate. Cancer Letters. 1999;143:217–21.

454. Nowell S, Ratnasinghe DL, Ambrosone CB, Williams S, Teague-Ross T, Trimble L, et al. Association of SULT1A1 phenotype and genotype with prostate cancer risk in African-Americans and Caucasians. Cancer Epidemiol Biomarkers Prev. 2004;13:270–6.

455. Xu LL, Stackhouse BG, Florence K, Zhang W, Shanmugam N, Sesterhenn IA, et al. PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. Cancer Res. 2000;60:6568-72.

456. Xu LL, Sun C, Petrovics G, Makarem M, Furusato B, Zhang W, et al. Quantitative expression profile of PSGR in prostate cancer. Prostate Cancer Prostatic Dis. 2005;9:56–61.

457. Rodriguez M, Luo W, Weng J, Zeng L, Yi Z, Siwko S, et al. PSGR promotes prostatic intraepithelial neoplasia andprostate cancer xenograft growth through NF. Nature Publishing Group; 2014;3:e114–11.

458. Rigau M, Morote J, Mir MC, Ballesteros C, Ortega I, Sanchez A, Colás E, Garcia M, Ruiz A, Abal M, Planas J. PSGR and PCA3 as biomarkers for the detection of prostate cancer in urine. The Prostate. 2010;70:1760-7.

459. Rigau M, Ortega I, Mir MC, Ballesteros C, Garcia M, Llauradó M, et al. A Three-Gene panel on urine increases PSA specificity in the detection of prostate cancer. Prostate. 2011;71:1736–45.

460. Brindley DN, Pilquil C. Lipid phosphate phosphatases and signaling. J Lipid Res. 2009;50:225–30.

461. Swinnen JV, Heemers H, de Sande TV, Schrijver ED, Brusselmans K, Heyns W, et al. Androgens, lipogenesis and prostate cancer. The Journal of Steroid Biochemistry and Molecular Biology. 2004;92:273–9.

462. Waltering KK, Helenius MA, Sahu B, Manni V, Linja MJ, Janne OA, et al. Increased Expression of Androgen Receptor Sensitizes Prostate Cancer Cells to Low Levels of Androgens. Cancer Research. 2009;69:8141–9.

463. Carl-McGrath S, Lendeckel U, Ebert M, Röcken C. Ectopeptidases in tumour biology: a review. Histol Histopathol. 2006;21:1339–53.

464. Menrad A, Speicher D, Wacker J, Herlyn M. Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. Cancer Research. 1993;53:1450–5.

465. Ishii K, Usui S, Sugimura Y, Yoshida S, Hioki T, Tatematsu M, et al. Aminopeptidase N regulated by zinc in human prostate participates in tumor cell invasion. Int J Cancer. 2001;92:49–54.

466. Petrovic N, Schacke W, Gahagan JR, O'Conor CA, Winnicka B, Conway RE, et al. CD13/APN regulates endothelial invasion and filopodia formation. Blood. 2007;110:142–50.

467. Sørensen KD, Abildgaard MO, Haldrup C, Ulhøi BP, Kristensen H, Strand S, Parker C, Høyer S, Borre M, Ørntoft TF. Prognostic significance of aberrantly silenced ANPEP expression in prostate cancer. British journal of cancer. 2013;108:420-8.

468. Pinto JT, Suffoletto BP, Berzin TM, Qiao CH, Lin S, Tong WP, et al. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. Clin Cancer Res. 1996;2:1445–51.

469. Ross JS, Sheehan CE, Fisher HAG, Kaufman RP, Kaur P, Gray K, et al. Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer. Clin Cancer Res. 2003;9:6357–62.

470. Troyer JK, Beckett ML, Wright GL. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. Int J Cancer. 1995;62:552–8.

471. Bostwick DG, Pacelli A, Blute M, Roche P, Murphy GP. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. Cancer. 1998;82:2256–61.

472. Wright GL, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, Moriarty R. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. Urology. 1996;48:326-34.

473. Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, et al. Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. Prostate. 2003;54:249–57.

474. Zhang Y, Guo Z, Du T, Chen J, Wang W, Xu K, et al. Prostate specific membrane antigen (PSMA): a novel modulator of p38 for proliferation, migration, and survival in prostate cancer cells. Prostate. 2013 May 31;73(8):835–41.

475. Chang SS. Overview of prostate-specific membrane antigen. Reviews in Urology. MedReviews. 2004;6:S13.

476. Whitaker HC, Shiong LL, Kay JD, Gronberg H, Warren AY, Seipel A, et al. N-acetyl-L-aspartyl-L-glutamate peptidase-like 2 is overexpressed in cancer and promotes a pro-migratory and pro-metastatic phenotype. Oncogene. 2014;33:5274–87.

477. Park S-W, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, et al. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. Proc Natl Acad Sci USA. 2009;106:6950–5.

478. Zhao F, Edwards R, Dizon D, Afrasiabi K, Mastroianni JR, Geyfman M, et al. Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice. Dev Biol. 2010;338:270–9.

479. Zhang Y, Forootan SS, Liu D, Barraclough R, Foster CS, Rudland PS, et al. Increased expression of anterior gradient-2 is significantly associated with poor survival of prostate cancer patients. Prostate Cancer Prostatic Dis. 2007;10:293–300.

480. Maresh EL, Mah V, Alavi M, Horvath S, Bagryanova L, Liebeskind ES, et al. Differential expression of anterior gradient gene AGR2 in prostate cancer. BMC Cancer. 2010;10:680–0.

481. Bu H, Bormann S, Schäfer G, Horninger W, Massoner P, Neeb A, et al. The anterior gradient 2 (AGR2) gene is overexpressed in prostate cancer and may be useful as a urine sediment marker for prostate cancer detection. Prostate. 2010;71:575–87.

482. Chanda D, Lee JH, Sawant A, Hensel JA, Isayeva T, Reilly SD, et al. Anterior gradient protein-2 is a regulator of cellular adhesion in prostate cancer. PLoS ONE. 2014;9:e89940–0.

483. Hu Z, Gu Y, Han B, Zhang J, Li Z, Tian K, et al. Knockdown of AGR2 induces cellular senescence in prostate cancer cells. Carcinogenesis. 2012;33:1178–86.

484. Lin JH, Wu XR, Kreibich G, Sun TT. Precursor sequence, processing, and urothelium-specific expression of a major 15-kDa protein subunit of asymmetric unit membrane. J Biol Chem. 1994;269:1775–84.

485. Uroplakins Ia and Ib, Two Major DifferentiationProducts of Bladder Epithelium, Belong to aFamily of Four Transmembrane Domain (4TM) Proteins. Cell Biol 1994; 125: 171–182.

486. Wu XR, Manabe M, Yu J, Sun TT. Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. J Biol Chem. 1990;265:19170–9.

487. Lytle C, Xu JC, Biemesderfer D, Forbush B. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. Am J Physiol. 1995;269:1496–505.

488. Acuña R, Martínez-de-la-Maza L, Ponce-Coria J, Vázquez N, Ortal-Vite P, Pacheco-Alvarez D, et al. Rare mutations in SLC12A1 and SLC12A3 protect against hypertension by reducing the activity of renal salt cotransporters. J Hypertens. 2011;29:475–83.

489. Miranda KC, Bond DT, McKee M, Skog J, Păunescu TG, Da Silva N, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. Kidney International. 2010;78:191–9.

490. Chen C-L, Mahalingam D, Osmulski P, Jadhav RR, Wang C-M, Leach RJ, et al. Single-cell analysis of circulating tumor cells identifies cumulative expression patterns of EMT-related genes in metastatic prostate cancer. Prostate. 2012;73:813–26.

491. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, et al. Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. Molecular Cancer Research. 2011;9:997–1007.

492. Schmidt U, Fuessel S, Koch R, Baretton GB, Lohse A, Tomasetti S, et al.

Quantitative multi-gene expression profiling of primary prostate cancer. Prostate. 2006;66:1521–34.

493. Ohl F, Jung M, Xu C, Stephan C, Rabien A, Burkhardt M, Nitsche A, Kristiansen G, Loening SA, Radonić A, Jung K. Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization?. Journal of molecular medicine. 2005;83:1014-24.

494. De Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, et al. Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Invest. 2005;85:154–9.

495. Sikand K, Singh J, Ebron JS, Shukla GC. Housekeeping gene selection advisory: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are targets of miR-644a. PLoS ONE. 2012;7:e47510.

496. Kılıç Y, Çelebiler AÇ, Sakızlı M. Selecting housekeeping genes as references for the normalization of quantitative PCR data in breast cancer. Clin Transl Oncol. 2014;16:184–90.

497. Hessels D, Klein Gunnewiek JMT, van Oort I, Karthaus HFM, van Leenders GJL, van Balken B, et al. DD3PCA3-based Molecular Urine Analysis for the Diagnosis of Prostate Cancer. European Urology. 2003;44:8–16.

498. Groskopf J. APTIMA PCA3 Molecular Urine Test: Development of a Method to Aid in the Diagnosis of Prostate Cancer. Clinical Chemistry. 2006;52:1089–95.

499. 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. European Urology. European Association of Urology; 2014;65):534–42.

500. Van Gils MPMQ, Hessels D, van Hooij O, Jannink SA, Peelen WP, Hanssen SLJ, et al. The Time-Resolved Fluorescence-Based PCA3 Test on Urinary Sediments after Digital Rectal Examination; a Dutch Multicenter Validation of the Diagnostic Performance. Clinical Cancer Research. 2007;13:939–43.

501. Hendriks RJ, Dijkstra S, Jannink SA, Steffens MG, van Oort IM, Mulders PF, Schalken JA. Comparative analysis of prostate cancer specific biomarkers PCA3 and ERG in whole urine, urinary sediments and exosomes. Clinical Chemistry and Laboratory Medicine (CCLM). 2016;54:483-92.

502. Dijkstra S, Birker IL, Smit FP, Leyten GH, de Reijke TM, van Oort IM, Mulders PF, Jannink SA, Schalken JA. Prostate cancer biomarker profiles in urinary sediments and exosomes. The Journal of urology. 2014;191:1132-8.

503. Lipkowitz KB, Cundari TR, editors. Reviews in Computational Chemistry. Vol. 25. Hoboken, NJ, USA: 2007.

504. Karypis G, Han EH, Kumar V. Chameleon: Hierarchical clustering using dynamic modeling. Computer. 1999;32:68-75.

505. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nature Genetics. 2008;40:1413–5.

506. Sultan M, Schulz MH, Richard H, Magen A, Klingenhoff A, Scherf M, et al. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. Science. 2008;321:956–60.

507. Shendure J, Ji H. Next-generation DNA sequencing. Nat Biotechnol (2008) 26, 1135 - 1145. Nature Publishing Group; 2008;26:1135–45.

508. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.

509. Tombal B, Lecouvet F. Modern detection of prostate cancer's bone metastasis: is the bone scan era over?. Advances in urology. 2011.

510. Hiatt RA, Armstrong MA, Klatsky AL, Sidney S. Alcohol consumption, smoking, and other risk factors and prostate cancer in a large health plan cohort in California (United States). Cancer Causes Control. 1994;5:66–72.

511. Riddick ACP, Shukla CJ, Pennington CJ, Bass R, Nuttall RK, Hogan A, et al. Identification of degradome components associated with prostate cancer progression by expression analysis of human prostatic tissues. Br J Cancer. 2005;92:2171–80.

512. Rogers S, Girolami M, Campbell C, Breitling R. The latent process decomposition of cDNA microarray data sets. IEEE/ACM Trans Comput Biol Bioinform. 2005;2:143–56.

513. Stephens P, Edkins S, Davies H, Greenman C, Cox C, Hunter C, et al. A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. Nature Genetics. 2005;37:590–2.

514. Leyten GHJM, Hessels D, Smit FP, Jannink SA, de Jong H, Melchers WJG, et al. Identification of a Candidate Gene Panel for the Early Diagnosis of Prostate Cancer. Clin Cancer Res. 2015;21:3061–70.

515. Zhou L, Xia D, Zhu J, Chen Y, Chen G, Mo R, et al. Enhanced expression of IMPDH2 promotes metastasis and advanced tumor progression in patients with prostate cancer. Clin Transl Oncol. 2014;16:906–13.

516. Hussain M, Tangen CM, Higano C, Schelhammer PF, Faulkner J, Crawford ED, et al. Absolute prostate-specific antigen value after androgen deprivation is a strong independent predictor of survival in new metastatic prostate cancer: data from Southwest Oncology Group Trial 9346 (INT-0162). J Clin Oncol. 2006;24:3984–90.

517. Van Neste L, Hendriks RJ, Dijkstra S, Trooskens G, Cornel EB, Jannink SA, et al. Detection of High-grade Prostate Cancer Using a Urinary Molecular Biomarker–Based Risk Score. European Urology. European Association of Urology; 2016 20;:1–9.

518. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol. 2008;18:157–64.

519. Dai Z, Liu S, Marcucci G, Sadee W. 5-Aza-2'-deoxycytidine and depsipeptide synergistically induce expression of BIK (BCL2-interacting killer). Biochemical and Biophysical Research Communications. 2006;351:455–61.

520. Hur J, Chesnes J, Coser KR, Lee RS, Geck P, Isselbacher KJ, et al. The Bik BH3-only protein is induced in estrogen-starved and antiestrogen-exposed breast cancer cells and provokes apoptosis. Proc Natl Acad Sci USA. 2004;101:2351–6.

521. Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T. Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. Proc Natl Acad Sci USA. 2003;100:13994–9.

522. Xie X, Kong Y, Tang H, Yang L, Hsu JL, Hung M-C. Targeted BikDD expression kills androgen-dependent and castration-resistant prostate cancer cells. Molecular Cancer Therapeutics. 2014;13:1813–25.

523. Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. The EMBO Journal. 1997;16:2262–70.

524. Page G, Lödige I, Kögel D, Scheidtmann KH. AATF, a novel transcription factor that interacts with Dlk/ZIP kinase and interferes with apoptosis. FEBS Letters. 1999;462:187–91.

525. Campbell PA, Perez-Iratxeta C, Andrade-Navarro MA, Rudnicki MA. Oct4 targets regulatory nodes to modulate stem cell function. PLoS ONE. 2007;2:e553–3.

526. Huang C-N, Huang S-P, Pao J-B, Hour T-C, Chang T-Y, Lan Y-H, et al. Genetic polymorphisms in oestrogen receptor-binding sites affect clinical outcomes in patients with prostate cancer receiving androgen-deprivation therapy. J Intern Med. 2012;271:499–509.

527. Burgdorf S, Leister P, Scheidtmann KH. TSG101 interacts with apoptosis-antagonizing transcription factor and enhances androgen receptor-mediated transcription by promoting its monoubiquitination. J Biol Chem. 2004;279:17524–34.

528. Leister P, Burgdorf S, Scheidtmann KH. Apoptosis antagonizing transcription factor AATF is a novel coactivator of nuclear hormone receptors. Signal Transduction. 2003;3:17-25.

529. Louie MC, Revenko AS, Zou JX, Yao J, Chen H-W. Direct control of cell cycle gene expression by proto-oncogene product ACTR, and its autoregulation underlies its transforming activity. Molecular and Cellular Biology. 20060;26:3810–23.

530. Coutinho-Camillo CM, Salaorni S, Sarkis ÁS, Nagai MA. Differentially expressed genes in the prostate cancer cell line LNCaP after exposure to androgen and anti-androgen. Cancer Genetics and Cytogenetics. 2006;166:130–8.

531. Zhang H-H, Zhang Z-Y, Che C-L, Mei Y-F, Shi Y-Z. Array analysis for potential biomarker of gemcitabine identification in non-small cell lung cancer cell lines. Int J Clin Exp Pathol. 2013;6:1734–46.

532. Zeller C, Dai W, Steele NL, Siddiq A, Walley AJ, Wilhelm-Benartzi CSM, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. Oncogene. 2012;31:4567–76.

533. Fu J, Khaybullin R, Zhang Y, Xia A, Qi X. Gene expression profiling leads to discovery of correlation of matrix metalloproteinase 11 and heparanase 2 in breast cancer progression. BMC Cancer. 2015;15:473.

534. Wagner M, Suarez ER, Theodoro TR, Machado Filho CDAS, Gama MFM, Tardivo JP, et al. Methylene blue photodynamic therapy in malignant melanoma decreases expression of proliferating cell nuclear antigen and heparanases. Clin Exp Dermatol. 2012;37:527–33.

535. Lv B, Zhang B, Hu X-Y, Zeng Q-D. Heparanase regulates in vitro VEGF-C expression and its clinical significance to pancreatic ductal cell adenocarcinoma. Oncol Lett. 2016;11:1327–34.

536. Cortese R, Kwan A, Lalonde E, Bryzgunova O, Bondar A, Wu Y, et al. Epigenetic markers of prostate cancer in plasma circulating DNA. Human Molecular Genetics. 2012;21:3619–31.

537. Arvatz G, Shafat I, Levy-Adam F, Ilan N, Vlodavsky I. The heparanase system and tumor metastasis: is heparanase the seed and soil? Cancer Metastasis Rev. 2011;30:253–68.

538. Spinola M, Falvella FS, Colombo F, Sullivan JP, Shames DS, Girard L, et al. MFSD2A is a novel lung tumor suppressor gene modulating cell cycle and matrix attachment. Mol Cancer. 2010;9:62–2.

539. Lin S-L, Chiang A, Chang D, Ying S-Y. Loss of mir-146a function in hormone-refractory prostate cancer. RNA. 2008;14:417–24.

540. Baguma-Nibasheka M, Li AW, Murphy PR. The fibroblast growth factor-2 antisense gene inhibits nuclear accumulation of FGF-2 and delays cell cycle progression in C6 glioma cells. Mol Cell Endocrinol. 2007;267:127–36.

541. Giri D, Ropiquet F, Ittmann M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clin Cancer Res. 1999;5:1063–71.

542. Strefford JC, Lane TM, Hill A, LeRoux L, Foot NJ, Shipley J, et al. Molecular characterisation of the t(1;15)(p22;q22) translocation in the prostate cancer cell

line LNCaP. Cytogenet Genome Res. 2006;112:45–52.

543. Stolk JA, Jiang Y, Day CH, Klee JI, Zhang X, Dillon DC, et al. P704P, P712P, and P775P: A genomic cluster of prostate-specific genes. Prostate. 2004;60:214–26.

544. Arnaud L, Kelley LP, Helias V, Cartron J-P, Ballif BA. SMIM1 is a type II transmembrane phosphoprotein and displays the Vel blood group antigen at its carboxyl-terminus. FEBS Letters. 2015;589:3624–30.

545. Ozaki H, Matsuzaki H, Ando H, Kaji H, Nakanishi H, Ikehara Y, et al. Enhancement of metastatic ability by ectopic expression of ST6GalNAcI on a gastric cancer cell line in a mouse model. Clin Exp Metastasis. 2012;29:229–38.

546. Dong Z, Zhong Z, Yang L, Wang S, Gong Z. MicroRNA-31 inhibits cisplatin-induced apoptosis in non-small cell lung cancer cells by regulating the drug transporter ABCB9. Cancer Letters. 2014;343:249–57.

547. Medicine LEPDO, Molecular DO, Medicine HGBCO, Houston, Texas, Street UD, et al. Artificial Neural Network Analysis of DNAMicroarray-based Prostate Cancer Recurrence. 2005 IEEE Symposium on Computational Intelligence in Bioinformatics and Computational Biology.

548. Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, et al. The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? FEBS Letters. 2010;584:2093–101.

549. Chen J, Wang M, Zu L, Wang W, Li Y, Liu H. Establishment and characterization of a paired human non-small cell lung cancer cell lines from primary cancer and metastasis lymph node. Cancer Research. 2014.

550. Allen IC, TeKippe EM, Woodford R-MT, Uronis JM, Holl EK, Rogers AB, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. J Exp Med. 2010;207:1045–56.

551. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors. Nature medicine. 2009;15:1170-8.

552. Bruchard M, Mignot G, Derangère V, Chalmin F, Chevriaux A, Végran F, et al. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. Nat Med. 2012;19:57–64.

553. Wang Y, Kong H, Zeng X, Liu W, Wang Z, Yan X, et al. Activation of NLRP3 inflammasome enhances the proliferation and migration of A549 lung cancer cells. Oncol Rep. 2016;35:2053–64.

554. Chen AH, Tsau Y-W, Lin C-H. Novel methods to identify biologically relevant genes for leukemia and prostate cancer from gene expression profiles. BMC Genomics. 2010;11:274.

555. Thedieck K, Polak P, Kim ML, Molle KD, Cohen A, Jenö P, et al. PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. PLoS ONE. 2007;2:e1217.

556. Gan X, Wang J, Wang C, Sommer E, Kozasa T, Srinivasula S, et al. PRR5L degradation promotes mTORC2-mediated PKC-δ phosphorylation and cell migration downstream of Gα12. Nat Cell Biol. 2012;14:686–96.

557. Garcia-Rudaz C, Luna F, Tapia V, Kerr B, Colgin L, Galimi F, et al. Fxna, a novel gene differentially expressed in the rat ovary at the time of folliculogenesis, is required for normal ovarian histogenesis. Development. 2007;134:945–57.

558. Wu J, Liu S, Liu G, Dombkowski A, Abrams J, Martin-Trevino R, et al. Identification and functional analysis of 9p24 amplified genes in human breast cancer. Oncogene. 2012;31:333–41.

559. Grifantini R, Pileri P, Campagnoli S, Grandi A, Parri M, Pierleoni A, Nogarotto R, inventors; Externautics SPA, assignee. Breast Tumor Markers and Methods of Use Thereof. United States patent application US 13/503,430. 2010.

560. Feng X, Luo Z, Jiang S, Li F, Han X, Hu Y, et al. The telomere-associated homeobox-containing protein TAH1/HMBOX1 participates in telomere maintenance in ALT cells. Journal of Cell Science. 2013;126:3982–9.

561. Chen S, Saiyin H, Zeng X, Xi J, Liu X, Li X, et al. Isolation and functional analysis of human HMBOX1, a homeobox containing protein with transcriptional repressor activity. Cytogenet Genome Res. 2006;114:131–6.

562. Dai J, Wu L, Zhang C, Zheng X, Tian Z, Zhang J. Recombinant expression of a novel human transcriptional repressor HMBOX1 and preparation of anti-HMBOX1 monoclonal antibody. Cell Mol Immunol. 2009;6:261–8.

563. Wu L, Zhang C, Zhang J. HMBOX1 negatively regulates NK cell functions by suppressing the NKG2D/DAP10 signaling pathway. Cell Mol Immunol. 2011;8:433–40.

564. Stumpf CR, Moreno MV, Olshen AB, Taylor BS, Ruggero D. The translational landscape of the mammalian cell cycle. Molecular cell. 2013;52:574-82.

565. Hegemann B, Hutchins JR, Hudecz O, Novatchkova M, Rameseder J, Sykora MM, Liu S, Mazanek M, Lénárt P, Hériché JK, Poser I. Systematic phosphorylation analysis of human mitotic protein complexes. Science signaling. 2011;4:rs12.

566. Naqvi N, Li M, Yahiro E, Graham RM, Husain A. Insights into the characteristics of mammalian cardiomyocyte terminal differentiation shown through the study of mice with a dysfunctional c-kit. Pediatric cardiology. 2009;30:651-8.

567. Slattery ML, West DW. Smoking, alcohol, coffee, tea, caffeine, and theobromine: risk of prostate cancer in Utah (United States). Cancer Causes

Control. 1993;4:559–63.

568. Schoonen WM, Salinas CA, Kiemeney LA, Stanford JL. Alcohol consumption and risk of prostate cancer in middle□aged men. International journal of cancer. 2005;113:133-40.

569. De Stefani E, Fierro L, Barrios E, Ronco A. Tobacco, alcohol, diet and risk of prostate cancer. Tumori. 1995;81:315–20.

570. Taneja SS. Re: cigarette smoking is associated with an increased risk of biochemical disease recurrence, metastasis, castration-resistant prostate cancer, and mortality after radical prostatectomy: results from the SEARCH database. The Journal of Urology. 2014;191:1784.

571. Steinberger E, Kollmeier M, McBride S, Novak C, Pei X, Zelefsky MJ. Cigarette smoking during external beam radiation therapy for prostate cancer is associated with an increased risk of prostate cancer□specific mortality and treatment-related toxicity. BJU international. 2015;116:596-603.

572. Truong M, Yang B, Jarrard DF. Review ArticleToward the Detection of Prostate Cancer in Urine: A Critical Analysis. JURO. 2013;189:422–9.

573. van Gils MPMQ, Stenman UH, Schalken JA, Schröder FH, Luider TM, Lilja H, et al. Innovations in Serum and Urine Markers in Prostate Cancer. European Urology. 2005;48:1031–41.

574. Sanguedolce F, Cormio A, Brunelli M, D'Amuri A, Carrieri G, Bufo P, et al. Urine TMPRSS2: ERG Fusion Transcript as a Biomarker for Prostate Cancer: Literature Review. Clin Genitourin Cancer. 2016;14:117–21.

575. Tomlins SA, Aubin SMJ, Siddiqui J, Lonigro RJ, Sefton-Miller L, Miick S, et al. Urine TMPRSS2:ERG Fusion Transcript Stratifies Prostate Cancer Risk in Men with Elevated Serum PSA. Science Translational Medicine. 2011;3: 72–2.

576. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br J Cancer [Internet]. Nature Publishing Group. 2009;100:1603–7.

577. Duijvesz D, Luider T, Bangma CH, Jenster G. Exosomes as Biomarker Treasure Chests for Prostate Cancer. European Urology. European Association of Urology. 2011;59:823–31.

578. Rosell R, Wei J, Taron M. Circulating MicroRNA Signatures of Tumor-Derived Exosomes for Early Diagnosis of Non-Small-Cell Lung Cancer. Clin Lung Cancer. 2009;10:8–9.

579. Fedele C, Singh A, Zerlanko BJ, Iozzo RV, Languino LR. The αvβ6 integrin is transferred intercellularly via exosomes. Journal of Biological Chemistry. 2015;290:4545-51.

580. Corcoran C, Rani S, O'Brien K, O'Neill A, Prencipe M, Sheikh R, et al. Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. PLoS ONE. 2012;7:e50999.

581. Sokoll LJ, Ellis W, Lange P, Noteboom J, Elliott DJ, Deras IL, et al. A multicenter evaluation of the PCA3 molecular urine test: Pre-analytical effects, analytical performance, and diagnostic accuracy. Clinica Chimica Acta. 2008;389:1–6.

582. Franzen CA, Blackwell RH, Foreman KE, Kuo PC, Flanigan RC, Gupta GN. Urinary Exosomes: The Potential for Biomarker Utility, Intercellular Signaling and Therapeutics in Urological Malignancy. The Journal of Urology. 2016;195:1331–9.

583. Rodrigues G, Warde P, Pickles T, Crook J, Brundage M, Souhami L, et al. Pre-treatment risk stratification of prostate cancer patients: A critical review. Can Urol Assoc J. 2012;6):121–7.

584. Welch HG, Fisher ES, Gottlieb DJ, Barry MJ. Detection of Prostate Cancer via Biopsy in the Medicare-SEER Population During the PSA Era. JNCI Journal of the National Cancer Institute. 2007;99:1395–400.

585. Hong YM, Lai FC, Chon CH, McNeal JE, Presti JC. Impact of prior biopsy scheme on pathologic features of cancers detected on repeat biopsies. URO. 2004;22:7–10.

586. Bozeman CB, Carver BS, Eastham JA, Venable DD. Treatment of chronic prostatitis lowers serum prostate specific antigen. JURO. 2002;167:1723–6.

587. Puppo P. Repeated negative prostate biopsies with persistently elevated or rising PSA: a modern urologic dilemma. European Urology. 2007;52:639–41.

588. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ. Prevalence of prostate cancer among men with a prostate-specific antigen level≤ 4.0 ng per milliliter. New England Journal of Medicine. 2004;350:2239-46.

589. Catalona WJ, Smith DS, Ornstein DK. Prostate cancer detection in men with serum PSA concentrations of 2.6 to 4.0 ng/mL and benign prostate examination. Enhancement of specificity with free PSA measurements. JAMA. 1997;277:1452–5.

590. Candas B, Cusan L, Gomez JL, Diamond P, Suburu RE, Lévesque J, et al. Evaluation of prostatic specific antigen and digital rectal examination as screening tests for prostate cancer. Prostate. 2000;45:19–35.

591. 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. European Urology. 2011;59:407–14.

592. Quek S-I, Ho ME, Loprieno MA, Ellis WJ, Elliott N, Liu AY. A multiplex assay to measure RNA transcripts of prostate cancer in urine. PLoS ONE. 2012;7:e45656–6.

593. Huen N-Y, Pang AL-Y, Tucker JA, Lee T-L, Vergati M, Jochems C, et al. Up-regulation of proliferative and migratory genes in regulatory T cells from patients with metastatic castration-resistant prostate cancer. Int J Cancer. 2013;133:373–82.

594. Meng FJ, Shan A, Jin L, Young CYF. The expression of a variant prostate-specific antigen in human prostate. Cancer Epidemiol Biomarkers Prev. 2002;11:305–9.

595. Magklara A, Scorilas A, Stephan C, Kristiansen GO, Hauptmann S, Jung K, Diamandis EP. Decreased concentrations of prostate-specific antigen and human glandular kallikrein 2 in malignant versus nonmalignant prostatic tissue. Urology. 2000;56:527-32.

596. Rouzier C, Haudebourg J, Carpentier X, Valério L, Amiel J, Michiels J-F, et al. Detection of the TMPRSS2-ETS fusion gene in prostate carcinomas: retrospective analysis of 55 formalin-fixed and paraffin-embedded samples with clinical data. Cancer Genetics and Cytogenetics. 2008;183:21–7.

597. Abdul M, Hoosein N. Changes in beta-2 microglobulin expression in prostate cancer. Urol Oncol. 2000;5:168–72.

598. Cariello NF, Skopek TR. Analysis of mutations occurring at the human hprt locus. J Mol Biol. 1993;231:41–57.

599. Ichikawa T, Ichikawa Y, Isaacs JT. Genetic factors and suppression of metastatic ability of prostatic cancer. Cancer research. 1991;51:3788-92.

600. Ankerst DP, Hoefler J, Bock S, Goodman PJ, Vickers A, Hernandez J, et al. Prostate Cancer Prevention Trial Risk Calculator 2.0 for the Prediction of Low- vs High-grade Prostate Cancer. Urology. 2014;83:1362–8.

601. Hansen J, Auprich M, Ahyai SA, la Taille de A, van Poppel H, Marberger M, et al. Initial prostate biopsy: development and internal validation of a biopsy-specific nomogram based on the prostate cancer antigen 3 assay. European Urology. 2013;63:201–9.

602. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nat Rev Cancer. 2008;8:268–78.

603. Benson MC, Whang IS, Pantuck A, Ring K, Kaplan SA, Olsson CA, et al. Prostate specific antigen density: a means of distinguishing benign prostatic hypertrophy and prostate cancer. JURO. 1992;147:815–6.

604. Sfoungaristos S, Perimenis P. PSA density is superior than PSA and Gleason score for adverse pathologic features prediction in patients with clinically localized prostate cancer. Can Urol Assoc J. 2012;6:46–50.

605. Ohori M, Dunn JK, Scardino PT. Is prostate-specific antigen density more useful than prostate-specific antigen levels in the diagnosis of prostate cancer?. Urology. 1995;46:666-71.

606. Sammon JD, Abdollah F, Reznor G, Pucheril D, Choueiri TK, Hu JC, et al. Patterns of Declining Use and the Adverse Effect of Primary Androgen Deprivation on All-cause Mortality in Elderly Men with Prostate Cancer. European Urology. 2015;68:32–9.

607. Bienz M, Saad F. Androgen-deprivation therapy and bone loss in prostate cancer patients: a clinical review. Bonekey Rep. 2015;4:716–6.

608. Azvolinsky A. Androgen Deprivation Therapy-Linked Cardiovascular Disease Risk: Still Unresolved. J Natl Cancer Inst. 2015;107:–.

609. Saylor PJ, Smith MR. Metabolic Complications of Androgen Deprivation Therapy for Prostate Cancer. JURO. American Urological Association; 2009;181:1998–2008.

610. Ziehr DR, Chen M-H, Zhang D, Braccioforte MH, Moran BJ, Mahal BA, et al. Association of androgen-deprivation therapy with excess cardiac-specific mortality in men with prostate cancer. BJU Int. 2015;116:358–65.

611. Merrick GS, Wallner KE. Androgen deprivation therapy: a survival benefit or detriment in men with high-risk prostate cancer?. Oncology. 2010;24:790.

612. Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. Journal of Clinical Oncology. 2008;26:242–5.

613. Petrylak DP, Tangen CM, Hussain MHA, Lara PN, Jones JA, TAPLIN M-E, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med. 2004;351:1513–20.

614. Scher HI, Fizazi K, Saad F, TAPLIN M-E, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med. 2012;367:1187–97.

615. De Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman Jr OB, Saad F, Staffurth JN. Abiraterone and increased survival in metastatic prostate cancer. New England Journal of Medicine. 2011;364:1995-2005.

616. Dawson NA, Roesch EE. Sipuleucel-T and immunotherapy in the treatment of prostate cancer. Expert Opin Biol Ther. 2014;14:709–19.

617. Jocham D, Sommerauer M. Re: Prednisone Plus Cabazitaxel or Mitoxantrone for Metastatic Castration-Resistant Prostate Cancer Progressing After Docetaxel Treatment: A Randomized Open-Label Trial. European Urology. 2011;59:659–9.

618. Parker C, Nilsson S, Heinrich D, Helle SI, O'Sullivan JM, Fosså SD, et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. N Engl J Med. 2013;369:213–23.

619. Sarker D, Reid AHM, Yap TA, de Bono JS. Targeting the PI3K/AKT Pathway for the Treatment of Prostate Cancer. Clinical Cancer Research. 2009;15:4799–805.

620. Koreckij TD, Morgan TM. Clinical Study of Docetaxel, Prednisone and Bevacizunab in the Treatment of Hormone Refractory Prostate Cancer. Journal of International Translational Medicine. 2015.

621. Graff JN, Chamberlain ED. Sipuleucel-T in the treatment of prostate cancer: an evidence-based review of its place in therapy. Core Evidence. 2015;10:1–10.

622. Armstrong AJ, Eisenberger MA, Halabi S, Oudard S, Nanus DM, Petrylak DP, et al. Biomarkers in the Management and Treatment of Men with Metastatic Castration-Resistant Prostate Cancer. European Urology. 2012;61:549–59.

623. Grange C, Tapparo M, Collino F, Vitillo L, Damasco C, Deregibus MC, et al. Microvesicles Released from Human Renal Cancer Stem Cells Stimulate Angiogenesis and Formation of Lung Premetastatic Niche. Cancer Research. 2011;71:5346–56.

624. Mitchell S, Abel P, Ware M, Stamp G, Lalani E. Phenotypic and genotypic characterization of commonly used human prostatic cell lines. BJU Int. 2000;85:932–44.

625. Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a Novel Androgen Receptor Exon Generates a Constitutively Active Androgen Receptor that Mediates Prostate Cancer Therapy Resistance. Cancer Research. 2008;68:5469–77.

626. Sun S, Sprenger CCT, Vessella RL, Haugk K, Soriano K, Mostaghel EA, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest. 2010;120:2715–30.

627. Hu R, Isaacs WB, Luo J. A snapshot of the expression signature of androgen receptor splicing variants and their distinctive transcriptional activities. Prostate. 2011;71:1656–67.

628. Lu J, Lonergan PE, Nacusi LP, Wang L, Schmidt LJ, Sun Z, et al. The Cistrome and Gene Signature of Androgen Receptor Splice Variants in Castration Resistant Prostate Cancer Cells. The Journal of Urology. 2015;193:690–8.

629. Antonarakis ES, Lu C, Luber B, Wang H, Chen Y, Nakazawa M, et al. Androgen Receptor Splice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. JAMA Oncol. 2015;1:582.

630. Radisky DC, Bissell MJ. Matrix metalloproteinase-induced genomic instability. Curr Opin Genet Dev. 2006;16:45–50.

631. Sohail A, Sun Q, Zhao H, Bernardo MM, Cho J-A, Fridman R. MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer. Cancer Metastasis Rev. 2008;27:289–302.

632. Campisi J. 21: Cancer and ageing: Rival demons?. Eur J Cancer. 2014;50:S6–S7.

633. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. Proceedings of the National Academy of Sciences. 1995;92:5258-65.

634. Sutcliffe S, Platz EA. Inflammation and prostate cancer: A focus on infections. Curr Urol Rep. 2008;9:243–9.

635. Gurel B, Lucia MS, Thompson IM, Goodman PJ, Tangen CM, Kristal AR, et al. Chronic Inflammation in Benign Prostate Tissue Is Associated with High-Grade Prostate Cancer in the Placebo Arm of the Prostate Cancer Prevention Trial. Cancer Epidemiology Biomarkers & Prevention. 2014;23:847–56.

636. Nonomura N, Takayama H, Nakayama M, Nakai Y, Kawashima A, Mukai M, et al. Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer. BJU Int. 2010;107:1918–22.

637. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer and Metastasis Reviews. 2006;25:315-22.

638. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. Eur J Cancer. 2006;42:717–27.

639. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: the role of interleukin 6 (IL-6). BJU Int. 2014;113:986–92.

640. Shinohara DB, Vaghasia AM, Yu S-H, Mak TN, Brüggemann H, Nelson WG, et al. A mouse model of chronic prostatic inflammation using a human prostate cancer-derived isolate of Propionibacterium acnes. Prostate. 2013;73:1007–15.

641. Kwon O-J, Zhang L, Ittmann MM, Xin L. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin. Proceedings of the National Academy of Sciences. 2014;111:E592–E600.

642. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. New England Journal of Medicine. 2010;363:411-22.

643. Strasner A, Karin M. Immune Infiltration and Prostate Cancer. Frontiers in Oncology. 2015;5:128.

644. Lundholm M, Schröder M, Nagaeva O, Baranov V, Widmark A, Mincheva-Nilsson L, et al. Prostate Tumor-Derived Exosomes Down-Regulate NKG2D Expression on Natural Killer Cells and CD8+ T Cells: Mechanism of Immune Evasion. Busson P, editor. PLoS ONE. 2014;9:e108925.

645. Iwakawa R, Takenaka M, Kohno T, Shimada Y, Totoki Y, Shibata T, et al. Genome-wide identification of genes with amplification and/or fusion in small cell lung cancer. Genes Chromosom Cancer. 2013;52:802–16.

646. Graner MW, Lillehei KO, Katsanis E. Endoplasmic reticulum chaperones and their roles in the immunogenicity of cancer vaccines. Front Oncol. 2014;4:379–9.

647. Calderwood SK, Stevenson MA, Murshid A. Heat shock proteins, autoimmunity, and cancer treatment. Autoimmune diseases. 2012.