

Identification of Genomic Biomarkers for Improving Risk Stratification of Low-
and Intermediate-Risk Prostate Cancer Patients

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ABSTRACT

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide. Currently, the treatment decisions are solely based on clinicopathological features, which are not adequately powered to distinguish indolent from aggressive disease. Thus, there is a need for clinically applicable biomarkers that could aid the decision-making process, especially in low- and intermediate-risk patients, who show heterogeneity in clinical outcome. DNA copy number alteration (CNA) signatures can predict biochemical recurrence (BCR). Thus, we hypothesize that CNAs relevant to aggressive PCa can be retrieved from the primary tumors and be used to predict risk of BCR. Since current methods do not have the multiplexing capability to assess CNA signatures or are not compatible with low-quantity and quality of DNA obtained from formalin-fixed paraffin embedded (FFPE) biopsy samples, we aim to develop an assay that can evaluate several clinically relevant CNA in FFPE PCa tissues. We thus developed an assay using multiplex ligation-dependent probe amplification (MLPA) and rigorously validate and optimize it for CNA detection in PCa tumors, showing the average accuracy of 90% compared to fluorescent *in situ* hybridization and 97% compared to droplet digital TaqMan polymerase chain reaction (PCR). We used our developed MLPA assay to profile the CNAs of 433 low- and intermediate-risk PCa patients. A CNA classifier capable of predicting BCR independent of clinicopathological features was developed. The prognostic value of our CNA-classifier was further validated in additional 541 low- and intermediate-risk PCa patients from four published datasets, including a biopsy dataset. Our CNA classifier was able to further stratify these low- and intermediate-risk patients into favorable and unfavorable outcome groups in Kaplan-Meier analysis. We further improved the prognostic efficacy of this CNA-classifier by including clinicopathological features. The CNA-clinical classifiers showed improved risk stratification and prognostic efficacy over the standard clinical model in all tested datasets and showed higher 3-years and 5-years area under the curve (AUC) compared to the standard clinical model. Our novel assay is inexpensive, high-throughput, highly reproducible and can accurately predict the risk of BCR and showing potential to improve the clinical management of patients with low- and intermediate-risk PCa by providing more accurate prognosis at the time of diagnosis.

RÉSUMÉ

Le cancer de la prostate (CaP) est le 2^{ème} cancer le plus fréquemment diagnostiqué chez les hommes dans le monde. Actuellement, les décisions thérapeutiques reposent uniquement sur des caractéristiques clinicopathologiques, qui ne sont pas suffisamment puissantes pour distinguer les cancers indolents des agressifs. Il est donc nécessaire de disposer de biomarqueurs aidant lors du processus de décision clinique, en particulier chez les patients de risque faible et intermédiaire et qui présentent une grande hétérogénéité quant à l'évolution du CaP. Les signatures moléculaires basées sur l'altération du nombre de copies de gènes (CNAs) peuvent prédire la récurrence biochimique (RBch) des patients. Ainsi, nous émettons l'hypothèse que les CNAs pertinentes au CaP agressif peuvent être identifiées à partir des tumeurs primitives et utilisées pour prédire le risque de RBch. Étant donné que les méthodes actuelles ne permettent pas d'évaluer ces signatures ou ne sont pas compatibles avec la faible quantité et qualité de l'ADN extrait de biopsies de CaP enrobées dans la paraffine (FFPE), nous visons à mettre au point un test qui permettra d'évaluer plusieurs CNAs cliniquement significatives à partir de telles tumeurs. Nous avons développé un test basé sur l'amplification de multiple sondes dépendantes de leur ligation (MLPA) et l'avons rigoureusement validé et optimisé; il détecte les CNAs dans les tumeurs prostatiques avec une précision de 90% par rapport à l'hybridation in situ et de 97% par rapport à la réaction de polymérase en chaîne, type « gouttelettes digitales » (ddPCR)-TaqMan. Notre test MLPA a permis d'établir le profil de CNAs de 433 patients à risque faible ou intermédiaire de progression. Nous avons défini un classificateur-CNA capable de prédire la RBch, indépendamment des caractéristiques clinicopathologiques des patients. Sa valeur prédictive a été validée chez 541 patients de risque faible et intermédiaire, dont les données de CNAs sont publiées, incluant celles dérivées de biopsies. Notre classificateur-CNA a permis de stratifier davantage les patients en groupes de pronostic favorable et défavorable lors d'analyses Kaplan-Meier. Nous avons en outre augmenté son efficacité pronostique en incluant les caractéristiques clinicopathologiques. Nos classificateurs-CNA cliniques améliorent la stratification du risque et l'efficacité pronostique par rapport au modèle clinique seul, et ce, dans toutes les cohortes analysées et montrant une aire sous la courbe (AUC) supérieure à 3 ans et à 5 ans par rapport au modèle clinique standard. Ce nouveau test, peu coûteux, à haut débit, hautement reproductible et prédisant avec précision le risque de RBch, montre ainsi un grand potentiel afin d'améliorer la gestion clinique des patients en fournissant un pronostic plus précis au moment du diagnostic.

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As Ralph Waldo Emerson said, “*Bad times have a scientific value. These are occasions a good learner would not miss*”. Accordingly, I have learned a great deal through this complex and taxing journey. The value of proper scientific research, candid communication, proper scientific design, teamwork and attention to details are among many valuable lessons that I will carry with me.

First, I would like to thank my supervisor Dr. Jacques Lapointe who under his supervision I gained invaluable knowledge both in life and scientific research.

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I started this acknowledgment section with a quote from Ralph Waldo Emerson and it is only suitable to conclude it by another quote from him:

“Do not go where the path may lead, go instead where there is no path and leave a trail”.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Various microarray and next-generation sequencing studies have revealed the landscape of genomic alterations in PCa and used detailed patients' clinical outcomes data to delineate the effect of genomic alterations in initiation and progression of PCa. These data show that copy number alterations (CNAs) are dominant genomic alterations in PCa and thus various CNAs were identified and are linked to tumor initiation and progression. However, despite the prognostic value of these biomarkers, they are yet to be used in a clinical setting to improve prognosis and decision-making process for patients. Thus, my aim in this study was to identify the relevant CNAs in prognosis of PCa and designed an assay that can be used in the clinical setting to improve risk stratification and the decision-making process for low- and intermediate-risk patients.

The original contribution made by my research in the field of Uro-Oncology and genomic biomarker assays are as follows:

For Manuscript one:

- Identification of 10 CNA cytobands relevant to tumor biology of PCa to be used in multiplex ligation-dependent probe amplification (MLPA) assay.
- Identification of 9 CNA-quiet cytobands that can be used as references in PCa genomic studies
- Development of a protocol to design a MLPA probe mix using synthetic oligonucleotides.

- Design and development of a MLPA-based assay that can accurately detect CNAs of 29 different loci with 50 ng of DNA extracted from formalin-fixed paraffin-embedded (FFPE) prostate tumor blocks.
- Development of an analysis approach that can accurately detect CNA in low grade tumors with various cancer cellularity, with an accuracy of 90% compared with FISH and 97% compared to droplet digital TaqMan PCR.

For Manuscript two:

- Improving the MLPA assay by adding four additional genes and providing a CNA profile of 14 genes in a total of 433 PCa patients, including samples from two areas of the tumor.
- Development of quality control (QC) criteria for high-throughput analysis of MLPA data.
- Development and validation of a CNA-classifier that can stratify patients with aggressive disease in the low- and intermediate-risk PCa, retaining its prognostic efficacy in heterogeneous samples.
- Development and validation of a CNA-clinical classifier that can better predict aggressive disease compared to standard clinicopathological model alone.

Overall, my thesis resulted in the development of a highly reproducible and accurate genomic assay that can provide prognosis in low- and intermediate-risk PCa patients using only 50 ng of FFPE extracted DNA with a cost of 7.90\$ CAD per reaction. This assay and the analysis approach are optimized for high-

throughput clinical use and can provide accurate prognosis at the time of diagnosis to aid the decision-making process for a heterogeneous population of PCa patients.

CONTRIBUTION OF AUTHORS

Selection of CNA biomarkers were done by WE and Dr. Lapointe, who previously studied copy number alterations in PCa. Selection of reference genes, experimental design, scoring FISH, development of the assay, optimization, development of quality-control, and analysis approaches were done by WE under the supervision of Dr. Lapointe. All generated data*, statistical analysis and development of biomarker classifiers were done by WE under the supervision of Dr. Lapointe. All manuscripts, Tables and Figures were prepared by WE, under the supervision of Dr. Lapointe and Dr. Chevalier.

*Capillary electrophoresis and droplet digital TaqMan PCR were paid for service methods offered by the Genomics platform of the Institute for Research in Immunology and Cancer (IRIC) at the Université de Montréal.

Co-authors contributions are listed below.

Karl-Philippe Guérard: DNA and RNA extraction of all samples of the McGill PARSE MLPA cohort. Also, participated in performing independent MLPA experiments for optimization and calculation of the error-rate of the developed MLPA assay.

Shaghayegh Rouzbeh: Performed DNA and RNA extraction of a subset of samples used in the development of the assay.

Yogesh M Bramhecha: Provided independent FISH score validation for samples used in the development of the MLPA analysis approach.

Eleonora Scarlata: Provided pathological review, identified cancer foci to be used in the study, and assistance in the preparation of patient's samples used in the development of the assay and PARSE MLPA cohort.

Luci Hamel: Collected and organized the clinical data of patients on the McGill site used in this study.

Fadi Brimo: Provided pathological review of patient's tumor samples used in the PARSE MLPA cohort and identified cancer foci to be used in the study.

Palak Patel: Provided assistant in the preparation of TMA used for validation of MLPA assay and performed DNA and RNA extraction of Queen's University samples used in the PARSE MLPA cohort.

Armen G Aprikian: Provided access to the McGill cohort used in this study.

Anna YW Lee: Assisted in the batching of samples in the PARSE MLPA cohort.

David M Berman: Provided access to samples used in this study. Instrumental in the overall design of the PRONTO project which included the CNA profiling of PARSE MLPA cohort.

John MS Bartlett: Provided access to reference DNA. Instrumental in the overall design of the PRONTO project which included the CNA profiling of PARSE MLPA cohort.

Simone Chevalier: Co-supervisor and provided samples used in the development, validation and application of the assay.

Jacques Lapointe: Supervisor.

LIST OF ABBREVIATIONS

ADT: Androgen Deprivation Therapy

AJCC: American Joint Committee on
Cancer

AMACR: A-Methylacyl-CoA Racemase

AR: Androgen Receptor

Array-CGH: Array Comparative Genomic
Hybridization

AUA: American Urological Association

BAC: Bacterial Artificial Chromosome

BCR: Biochemical Recurrence

BER: Base Excision Repair

BPH: Benign Prostatic Hyperplasia

CDK: Cyclin-Dependent Kinase

CHD1: Chromodomain Helicase DNA
Binding Protein 1

C-Index: Concordance Index

CK: Cytokeratin

CNA: Copy Number Alteration

CRPC: Castration Resistant Prostate Cancer

cT: Clinical TNM Stage

DHT: Dihydrotestosterone

DRE: Digital Rectal Exam

DSB: Double Stranded Break

EAU: European Association of Urology

ERG: V-Ets Erythroblastosis Virus E26
Oncogene Homolog

ERSPC: European Randomized Study for
Reducing Prostate Cancer

ESMO: European Society for Medical
Oncology

ETS: E26 Transformation-Specific

FAK: Focal Adhesion Kinase

FDA: Food and Drug Administration

FISH: Fluorescence *In Situ* Hybridization

GABARAPL2: GABA Type A Receptor–
Associated Protein Like 2

GG: Gleason Group	NCCN: National Comprehensive Cancer Network
GS: Gleason Score	
<i>GTF2H2</i> : General Transcription Factor IIH	NER: Nucleotide-Excision Repair
GUROC: Genitourinary Radiation Oncologists of Canada	NICE: National Institute for Health and Clinical Excellence
HGPIN: High-Grade Intraepithelial Neoplasia	<i>NKX3-1</i> : Nk3 Homeobox 1
HR: Homologous Recombination	PARP: Poly (ADP-Ribose) Polymerase
IHC: Immunohistochemistry	PCa: Prostate Cancer
IL-1: Interleukin 1	PCA3: Prostate Cancer Antigen 3
<i>KLK3</i> : Kallikrein-3	<i>PDPK1</i> : 3-Phosphoinositide Dependent Protein Kinase 1
LH-RH: Luteinizing Hormone-Releasing Hormone	<i>PDZD2</i> : PDZ Domain-Containing Protein 2
LUTS: Lower Urinary Tract Symptoms	PI3K: Phosphatase-3-Kinase
<i>MAP3K7</i> : Mitogen-Activated Protein Kinase Kinase Kinase 7	PIN: Intraepithelial Neoplasia
mCRPC: Metastatic Castration Resistant Prostate Cancer	PIP2: Phosphatidylinositol 4,5-Bisphosphate
MLPA: Multiplex Ligation-Dependent Probe Amplification	PIP3: Phosphatidylinositol 3,4,5-Trisphosphate
	PKC: Protein Kinase C

PLCO: Prostate, Lung, Colorectal, And

Ovarian Cancer Screening Trial

PRIAS: Prostate Cancer Research

International Active Surveillance

PRL3: Tyrosine Phosphatase 4A3

ProtecT: Prostate Testing for Cancer and
Treatment

PSA: Prostate Specific Antigen

PSCA: Prostate Stem Cell Antigen

PSMA: Prostate-Specific Membrane
Antigen

pT: Pathological TNM Stage

PTEN: Phosphatase and Tensin Homolog

QC: Quality Control

qPCR: Quantitative PCR

RBI: Rb Transcriptional Corepressor 1

ROS: Reactive Oxygen Species

RP: Radical Prostatectomy

RSK: P90 Ribosomal Protein S6 Kinase

S6K: P70 Ribosomal S6 Kinase

SGK: Serum Glucocorticoid-Dependent
Kinase

shRNA: Short Hairpin RNA

siRNA: Small Interfering RNA

SNP: Single Nucleotide Polymorphism

SPCG-4: Scandinavian Prostate Cancer
Group Study Number 4

SSB: Single-Strand Breaks

Tak1: TGF- β Activated Kinase-1

TMA: Tissue Microarray

TMPRSS2: Transmembrane Serine Protease
Isoform 2

TNF: Tumor Necrosis Factor

TP53: Transcriptional Factor P53

TRAIL: TNF-Related Apoptosis-Inducing
Ligand

TRUS: Transrectal Ultrasound

TURP: Transurethral Resection of The
Prostate

UCSF: University of California, San
Francisco

UICC: Union for International Cancer
Control

USPTF: US Preventive Task Force

1. CHAPTER 1 - LITERATURE REVIEW

1.1. OVERVIEW OF THE PROSTATE

1.1.1. Anatomy

The prostate gland is a part of the male reproductive system and is located in the subperitoneal space, in front of the rectum, just below the bladder and above the urogenital diaphragm and surrounds the urethra as it exits the bladder. This organ is generally described as “walnut-shaped” and weighs approximately twenty grams in a young adult, with a length of three and width of two centimeters [1, 2] (Figure 1).

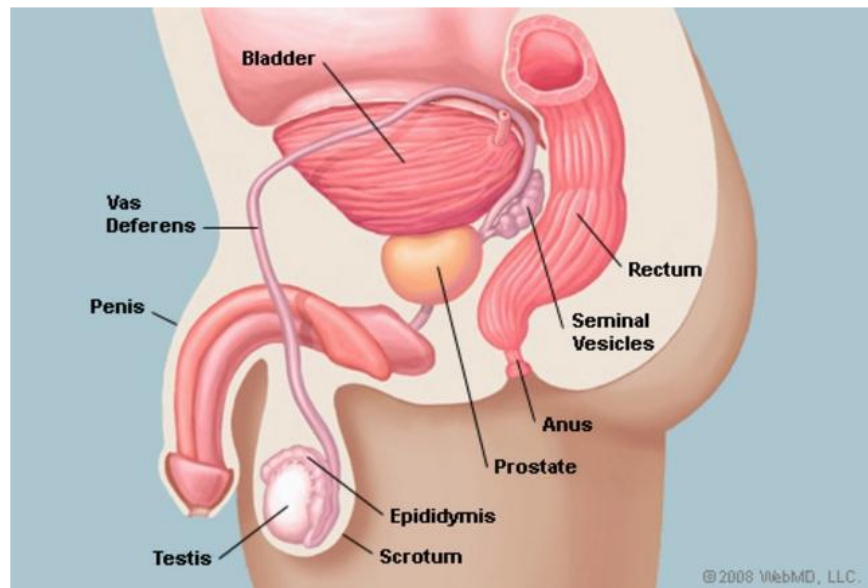


Figure 1: Anatomy of the human pelvis and location of the prostate gland.

(reproduced from WebMD, LLC: <https://www.webmd.com/urinary-incontinence-oab/picture-of-the-prostate#1>).

The prostate has four surfaces. The anterior surface (facies anterior) is narrow and convex and is separated from the pubic symphysis by extraperitoneal fat of the retropubic space. The posterior surface is broad, triangular in shape and is separated

from the rectum by the rectovesical fascia. The posterior surface is divided into a smaller upper part (median lobe) and larger lower part (lateral lobes) by a horizontal groove. The two inferior-lateral surfaces join the anterior surface and rests on the levator ani fascia above the urogenital diaphragm [3] (Figure 2).

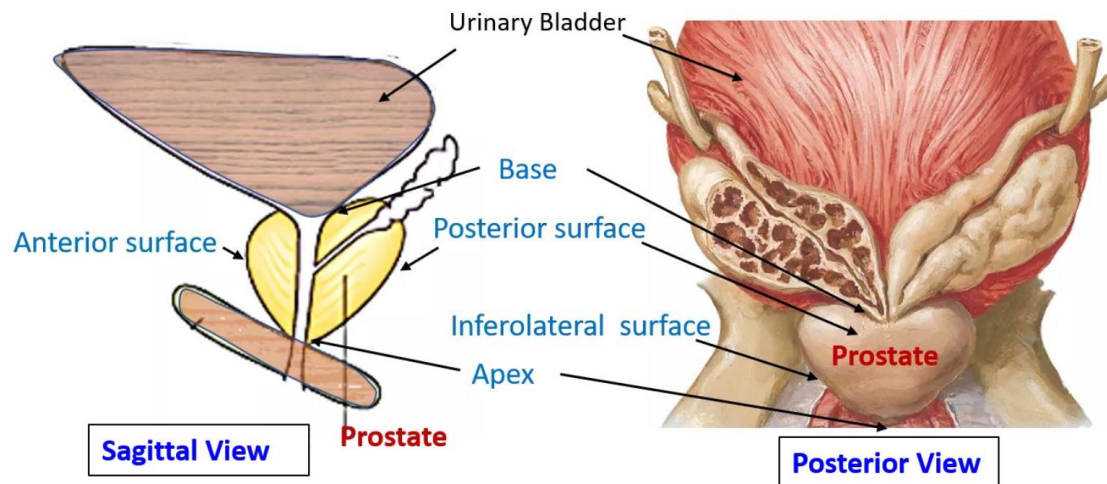


Figure 2: Anatomical features of the prostate gland.

(Reproduced from Anatomy questions and answers: <http://www.anatomyqa.com/anatomy/pelvis-and-perineum/prostate-gland-anatomy/>)

The base of the prostate is located at the top of the organ and is attached to the bladder and the apex is located at the lower part of the prostate and is attached to the superior fascia of the urogenital diaphragm [2].

1.1.1.1. Lobes of the prostate

Although the prostate is a single mass organ, it can be divided into four lobes based on the position of the urethra and the ejaculatory ducts coming from the vas deferens. The anterior lobe is located in front of the urethra and is mainly composed of fibromuscular tissue and contains few glands. The median lobe is between the ejaculatory ducts and

the urethra. The posterior lobe lies behind the urethra and the median lobe, below the ejaculatory ducts. The lateral lobes, which are the main mass of the prostate gland, are on each side of the urethra and comprise most of the glandular tissue [4] (Figure 3).

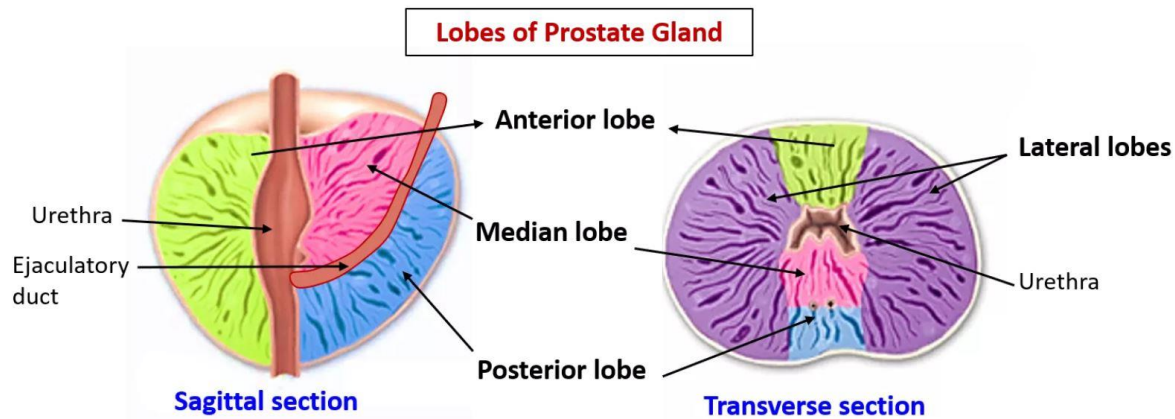


Figure 3: Lobes of the prostate.

(Reproduced from Anatomy questions and answers: <http://www.anatomyqa.com/anatomy/pelvis-and-perineum/prostate-gland-anatomy/>)

1.1.1.2. Zones of the prostate

According to the embryonic origin, histology, biological function and susceptibility to pathological disorders, the prostate gland is composed of three separate zones: transition, central, and peripheral. The transition zone accounts for 10% of the prostatic glandular tissue and hosts 20% of the adenocarcinomas, the most common form of PCa. The transition zone consists of submucosal glands with a mesodermal origin and are mostly prone to benign prostatic hyperplasia (BPH). The area surrounding the ejaculatory ducts is defined as the central zone and is also has a mesodermal origin [5]. This zone consists of 25% of the glandular tissue and is less susceptible to adenocarcinomas. Only 1-5% of prostate tumors arise from the central zone. The

largest zone is the peripheral zone, which has an endoderm origin and consists of long branching glands. This zone covers the posterior and lateral lobes of the prostate. The peripheral zone includes approximately 70% glandular tissue and 30% of fibromuscular stroma. About 70% of adenocarcinomas are found in this zone [2, 4] (Figure 4).

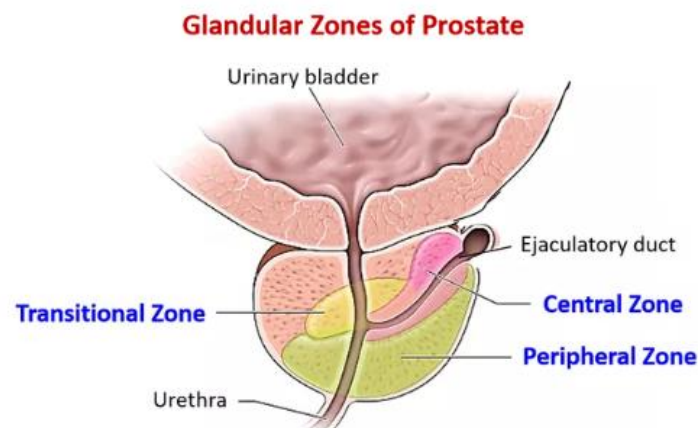


Figure 4: Zones of the prostate.

(Reproduced from Anatomy questions and answers: <http://www.anatomyqa.com/anatomy/pelvis-and-perineum/prostate-gland-anatomy/>)

1.1.2. Prostate development

In mouse and similarly in human, the embryonic prostate organogenesis can be separated into four stages (Figure 5). In the first stage, prostate development starts following signals induced directly or indirectly by androgenic hormones and results in commitment of cells from urogenital sinus into the epithelium to form the prostate. In the second stage, paracrine androgen signaling from the urogenital sinus mesenchyme stimulates cells of the urogenital sinus epithelium to outgrow and form buds and a system of ducts that form epithelial cords, which will surround the urogenital sinus

mesenchyme. A proper function of the androgen receptor (AR) is vital for this process. Next, in the third stage, outgrow of epithelium ducts will result in branching that forms the mature ductal network and prostatic zones. In the fourth stage, the epithelial cords undergo differentiation and form canals of ductal lumen with a glandular epithelium [1].

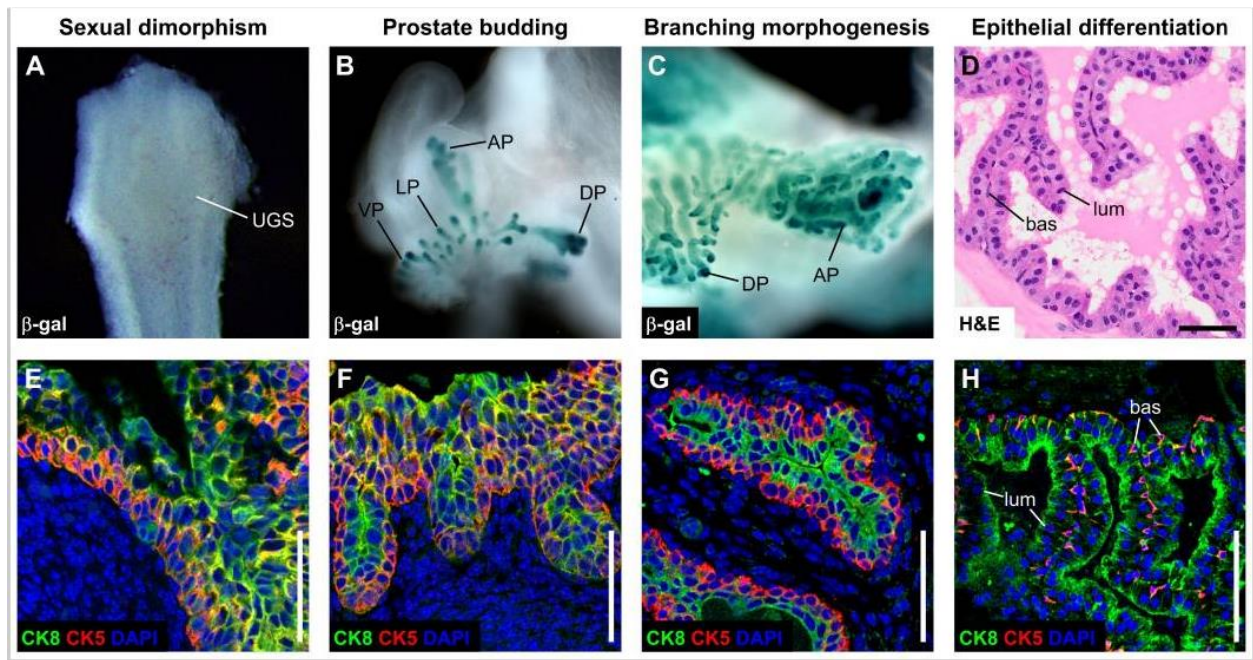


Figure 5: Stages of prostate organogenesis

Images of mouse urogenital sinus (UGS) stained for β -galactosidase (β -gal) activity, at 16.5 days post-coitum (A), 2 days postnatal (B) 14 days postnatal (C). (D) Hematoxylin and Eosin (H&E) staining of the adult mouse prostatic duct. (E, F) Immunofluorescence staining for cytokeratin 5 (CK5) and cytokeratin 8 (CK8) in the mouse prostate epithelium, at 16.5 days post-coitum (E), 2 days postnatal (F), 14 days postnatal (G) and 8 weeks old (H). AP: anterior prostate, VP: ventral prostate, LP: lateral prostate and DP dorsal prostate. Reproduced from [6].

The mature prostate (post-puberty) in young adults is formed of several epithelial and mesenchymal cell types with different morphology and function. The epithelial acini are formed of two cell layers protected by a basement membrane and from a lumen where the secreted products are emptied at the time of ejaculation. The luminal epithelial cells are prostate specific and fully differentiated. They are elongated and cylindrical and are positive for CKs 8 and 18, AR and prostate specific antigen (PSA). The cells of the basal layer are comprised of basal cells, which are smaller and unlike the luminal cells are non-secretory. They express CK5, CK14 and p63 and have low or undetectable expression levels of AR. The basal cell layer also contains a small subpopulation of cells that have both luminal and basal characteristics and referred to as intermediate cells. Another subset of rare cells is referred to as neuroendocrine and appear between and underneath the luminal cells and have dendritic like processes. These cells can secrete neuropeptides and other hormones and are suggested to have a role in prostatic growth and homeostatic regulation of mature differentiated glands [7].

Several different cell populations exist in the mesenchymal or stromal compartment. Smooth muscle cells help with the release of the prostatic fluids into the ejaculatory ducts by their contractile activity. Fibroblasts contribute to the prostate function with secretion of extracellular matrix components such as proteoglycans, fibrillar proteins, and glycoproteins that form a structural support and provide growth factors. This compartment also includes blood vessels, nerves and immune cells which contribute to the survival and function of other cell types and the regulation of rare stem cells located in the basal layer of acini [7].

1.1.3. Prostate function

The mature prostate produces secretions that make up one-third of the semen's volume and contains zinc, citric acid, calcium, phosphates, sugars and prostatic enzymes that are essential for healthy and motile spermatozoa. One of the most well-known enzymes of the prostatic fluid is PSA, which is produced by the luminal cells. This serine protease lyses the semenogelin, the main component of the human semen coagulum, resulting in semen liquefaction and initiation of sperm motility within the uterus, helping the fertilization of the ovum in fallopian tubes [8-11].

During ejaculation, sperm cells reach the prostate ejaculatory ducts via the vas deferens and mix with the prostatic fluid and other secretions coming from accessory glands. At this time, the prostate will contract to close the sphincter between the bladder, pushing the semen through the urethra [1].

1.1.4. Common diseases of the prostate

The prostate gland is a common site of diseases in adult men. Prostatitis is an inflammation of the prostate that can be due to bacterial or nonbacterial infections. Prostatitis causes discomfort and pain of the pelvic region and may be treated by antibiotics and other modalities to eradicate the cause of infection; otherwise, sustainment of infection and inflammation are known as chronic prostatitis or chronic pelvic pain syndrome and could increase the risk of PCa [12, 13].

The chronic inflammation of the prostate is often associated with atrophic epithelial lesions, which despite expected quiescence of prostate cells then exhibit a higher proliferation index and a lower apoptotic rate, compared to the normal epithelium [14].

These lesions are defined as proliferative inflammatory atrophy (PIA) and include glands with a basal cell layer and variably compressed secretory cells. Most of the PIA lesions are located in the peripheral zone where the majority of tumors are found. The proliferative nature of these lesions suggests a correlation between PIA and cancer initiation [15]. Furthermore, morphological studies of PIA lesions show a transition between atrophic glands and carcinoma or high-grade intraepithelial neoplasia (HGPIN). Furthermore, secretory cells in PIA lesions show molecular changes similar to that of cancer cells [16].

The most common disease observed in aging men (>50 years of age) is BPH, which is a result of progressive hyperplastic growth of the prostatic epithelium, stroma, or both, in the periurethral transition zone of the prostate. This enlargement of the prostate compresses the urethra and is associated with lower urinary tract symptoms (LUTS). These symptoms include increased frequency of urination, urinary incontinence, nocturia, weakened urinary stream and difficulty to void. BPH is considered to be the fourth most common disease in men over 50 years old and approximately 80% of men over 80 years of age will have BPH, although, not all men will have symptoms that result in LUTS [17, 18].

HGPIN is the replacement of the normal luminal cells with neoplastic cells, while the basement membrane remains intact. HGPIN is considered to be a likely precursor of PCa [19, 20].

PCa is identified by uncontrolled proliferation of epithelial cells, dedifferentiation and loss of acinar architecture. PCa is histologically confirmed by loss the basal cell differentiation CK markers and unrecognized basal cell membrane in the gland.

Prostatic tumors are mainly slow growing, nevertheless, PCa remains a lethal disease and a common cause of cancer-related mortality in men [2, 20, 21].

1.2. PROSTATE CANCER

Primary and localized PCa is mostly asymptomatic and remains a clinically insignificant disease in a large proportion of newly diagnosed patients [22]. However, PCa tumors have potential to progress and develop a deadly metastatic disease [23]. Progression of primary tumors may lead to an increase size of the prostate, blocking the urethra and causing pain, discomfort and LUTS [22]. If left untreated, the cancer could further progress beyond the prostatic capsule and invade nearby organs such as seminal vesicles and bladder. The most common site of local spread of the tumor, is the adjoining lymph nodes [24]. The locally advanced PCa can further progress to a lethal metastatic disease [25]. Most common site of metastasis in PCa is bone. Other organs such as liver, lung and brain may also be affected [26]. Due to the lack of treatments for the metastatic PCa and the long time needed for the progression of the disease which could be more than 15 years, radical treatments such as surgical removal of prostate is routinely carried out in most patients with long life expectancy [27].

1.2.1. Epidemiology

In men, worldwide, PCa is the second most prevalent cancer with 1.3 million new diagnosis in 2018 and the fifth leading cause of cancer related mortality with 359,000 cases [28]. PCa shows significant geographical variations in both mortality and incidence rates [29]. In the Americas, Northern and Western Europe, Australia/New Zealand, and much of Sub-Saharan Africa, PCa is the most frequently diagnosed cancer among men. Moreover, in Sub-Saharan Africa and the Caribbean, it is also the

leading cause of cancer-related mortality. Although in western countries, incidence rates are higher, the mortality rates are lower than what is seen in the Sub-Saharan Africa and the Caribbean regions [28]. Overall, the odds of being diagnosed with PCa by the age of 79 in countries with low-middle socio-demographic index is one in 47 and in countries with high socio-demographic index the odds are one in six [29, 30].

In Canada, in 2017, there were 21,300 new diagnoses and 4,100 PCa cancer-specific deaths. This means that one in every seven Canadian men will be diagnosed with PCa in their lifetime and one in every 29 men will die from the disease [31].

1.2.2. Risk factors

1.2.2.1. Age

Although PCa is a frequent disease, there is little information on its aetiology. Based on current studies, age is one of the strongest risk factors. PCa is generally diagnosed in men in their 60s and rarely affects men under 50 years of age [32].

1.2.2.2. Family history

Men with family members affected by PCa show twice the risk of being diagnosed and of having more family members affected by the disease and increases in the risk of being diagnosed at a younger age [33-35]. However, although having a family history increases the risk of being diagnosed with PCa, the severity of the disease is the same, and shows the same prognosis as men affected by non-familial PCa [36-39].

1.2.2.3. Ethnicity

Studies show that men with African descent living in the United States have higher rate of PCa compared to Caucasian men living in the same country, suggesting a role of ethnicity in the disease [40, 41]. Indeed, men with African descent have the highest incidence rates of PCa and are susceptible to the disease at younger ages and show more aggressive disease compared to other ethnic groups [32, 42, 43].

1.2.2.4. Diet and environmental factors

Several studies have shown an association between diet and PCa [44-47]. A prospective study indicated that the body mass index (BMI) of greater than 27.8 is an independent predictor of being diagnosed with PCa [44]. A recent study at McGill University Health Centre on 1,813 patients with PCa treated with RP also revealed significant association between a higher BMI (continuous variable) and the risk for locally advanced, high-risk PCa in a multivariate analysis [48].

Other studies of PCa incidence on Asian men born in the United States compared to Asian men living in their native countries show higher rates of the disease in the United States [49-51]. Furthermore, other studies show a higher PCa incidence in Asian men immigrated to the USA compared to men in Asia [52, 53], hence suggesting a role of diet and environmental factors in the development of the disease.

1.2.3. Diagnosis of PCa

PCa diagnostic tools include measurement of serum PSA levels, digital rectal exam (DRE) and transrectal ultrasound (TRUS)-guided biopsies [54].

1.2.3.1. PSA

The PSA protein is encoded by the kallikrein-3 (*KLK3*) gene. This glycoprotein is secreted by the prostate gland luminal epithelium and it was first discovered by Richard Ablin in the 1970s [55]. PSA has a serine protease activity and liquefies the semen and dissolves cervical mucus helping sperm motility [56]. The clinical utility of PSA in early diagnosis was reported by Catalona *et al.* in 1991 [57] and showed improved sensitivity in detection of PCa when used in combination with DRE. The United States Food and Drug Administration (FDA) approved PSA testing in combination with DRE for screening of PCa in 1994 and defined the cut-off point of up to 4 ng/ml as the normal limit. However, blood PSA levels can rise due to variety of other conditions that affect prostate such as prostatitis, BPH, prostatic massage, prostate biopsies and surgeries [58, 59], resulting in lack of PSA testing specificity for diagnosis of PCa.

Table 1: Risk of PCa diagnosis with different serum PSA levels

PSA level (ng/ml)	% Risk of PCa
0 – 0.5	6.6
0.6 – 1	10.1
1.1 – 2	17
2.1 – 3	23.9
3.1 – 4	26.9

Adapted from [54].

Moreover, studies have shown that men with PSA levels of below 4 ng/ml could have up to 26.9% risk of PCa (Table 1) [54]. Furthermore, about 20% of men with PCa have PSA levels lower than 4 ng/ml [59-62].

A multi-centric European Randomized Study of Screening for Prostate Cancer (ERSPC) with 19,970 participants had indicated that in men with PSA levels higher than 4 ng/ml, 75.9% had negative biopsies [63]. The study also suggested that 1,055 men need to be screened and 37 treated for PCa to prevent one cancer specific death.

The most optimistic result of PSA testing was obtained by the Göteborg trial with 20,000 men, which also included a subset of men of the ERSPC study. Results showed that to prevent one PCa specific death, 293 men (95% confidence interval 177 - 799) would need to be screened and 13 men would need to be treated [64].

Other studies have shown that PSA testing alone has a sensitivity of 20 – 40% and specificity of 70 – 90% with a positive predictive value of 25 – 40% [61, 65]. Data from meta-analyses of randomized clinical trials showed that PSA screening will not have a significant impact on mortality in non-selected PCa patients and is associated with frequent harm and un-necessary treatments [66, 67]. Nevertheless, despite unsatisfactory specificity, the low-cost, availability and non-invasiveness of test has made PSA part of a routine PCa screening [68-70] and has contributed in significant reduction of age-adjusted PCa related mortality since 1990s [59, 71].

1.2.3.2. Digital Rectal Exam

DRE is referred to the palpation of the posterior portion of prostate through the rectum by the physician. During the examination, the physician assesses the presence of

nodules at the surface of the organ, which is normally smooth; the examination also gives information on the size and shape of the prostate [72]. DRE can detect PCa independent of a serum PSA test in about 18% of patients [73] and DRE in patients with a serum PSA level of more than 2 ng/ml has a positive predictive value of 5–30% [74]. Sensitivity, specificity and positive predictive value of DRE in detection of PCa in a meta-analysis in men from 39 to 92 years was calculated to be 53.2%, 83.6% and 17.8%, respectively [75]. However, the clinical efficacy of DRE is highly variable and dependent on the location of tumor nodules and experience of the physician [76, 77]. Moreover, the test cannot differentiate between BPH and PCa or aggressiveness of the disease [78]. Therefore, due to high false-positive rates and lack of sufficient evidence, routing application of DRE to screen for PCa in the primary care setting is not recommended [79].

1.2.3.3. Transrectal ultrasound (TRUS)-guided biopsies

The first clinical use of TRUS was for the evaluation of rectal pathology [80, 81]. Takahashi *et al.* [82] described the use of TRUS for evaluation of prostate abnormalities in 1963. However, due to the low quality of images at the time [82, 83], the clinical application of the TRUS in PCa diagnosis was postponed until 1967 [84]. With advances in the ultrasound technology and ability to obtain higher quality images delineating the hypoechoic areas in the prostate, TRUS became a standard diagnostic tool for urologists in the mid 1980s [85].

Currently, TRUS-guided needle biopsy has become the standard for obtaining prostate tissue samples for histopathological examination and confirmation of cancer by the pathologist [86, 87]. The decision to proceed with biopsy is determined on the bases of

serum PSA value and suspicious DRE. TRUS-guided biopsy allows visualization of the prostate with the ability to accurately direct the biopsy needle to the regions of interest and taking multiple samples from the tissue. There are different schemes for taking biopsy samples (Figure 6).

Currently, Magnetic Resonance Imaging (MRI) provides higher resolution images and brings more precision in obtaining guided biopsy samples of high-grade cancers compared to TRUS [88-90].

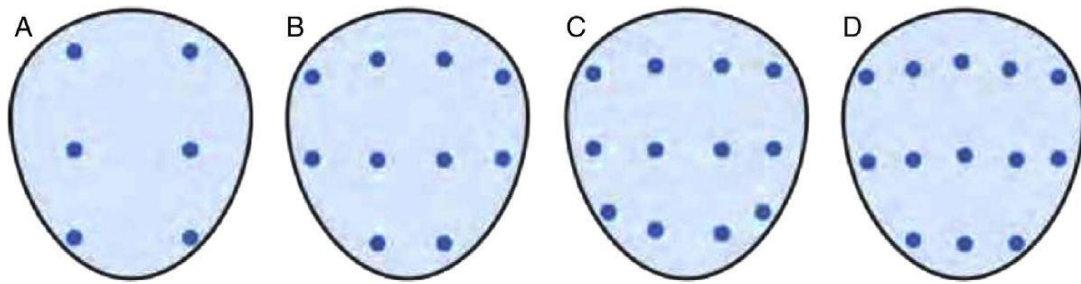


Figure 6: Schematic representation of different PCa biopsy techniques

(A) Sextant biopsy. (B) 10-core biopsy. (C) 12-core or double sextant. (D) 13-core biopsy. (Reproduced from [87])

In chronological order of reviewing different biopsy schemes; first the sextant method was described by Hodge *et al.* [91] in 1989; they described a random systematic method of taking biopsy samples from six different sites of prostate: the apex, middle and base of each prostate lobe, in addition to any hypoechoic lesions. This technique resulted in 9% increase in cancer detection compared to targeted- hypoechoic biopsy sampling. Then in 1995, Eskew *et al.* [92] described a 13-sampling protocol with two

cores from the far lateral region on each side and three centralized cores in addition to the sextant method. They reported that 35% of detected cancers were in additional regions. In 1998, Levine *et al.* [93] described a bi-sextant biopsy scheme with a 10% increase in cancer detection compared to the sextant model in 137 men with high PSA levels or abnormal DRE. In 2000 Presti *et al.* [94] improved the original sextant biopsy protocol by taking a total of 10 biopsy samples from two extra regions laterally on each side, at the base and middle of the gland. They reported that this technique results in 20% more cancer detection compared to the sextant method in a cohort of 483 men.

Later, systematic reviews showed that the number of biopsy cores should be between 8 and 12, as taking more than 12 samples do not provide statistically better diagnosis [95]. Other factors such as the size and volume of the prostate also influence the number of biopsy samples that are taken during the procedure.

1.2.4. Prognostic Tools of PCa

1.2.4.1. Gleason score (GS)

The Gleason grading system was first introduced by Dr. Donald Gleason in 1966 [96, 97]. As of today, the Gleason score (GS) remained as one of the most important prognostic indicators of the disease. This grading system is simple and reproducible, thus frequently used for prognosis. Pathological findings of prostatic tumors help the physicians in decision-making, such as considering an active surveillance or definitive treatment options, namely radical prostatectomy (RP) or radiation therapy, for localized or organ-confined disease. GS has shown to predict biochemical recurrence (BCR) or local and distant metastasis after therapy and PCa specific mortality [98-102]. However, due to sampling issues and small foci obtained from biopsy specimens,

accurate determination of GS is difficult and biopsy GS are often underestimated and do not represent the actual grade of the tumor [103].

The Gleason grading system (Figure 7) assigns scores ranging from 1 to 5 solely based on the architectural features and histological patterns of the cancerous glands, representing the levels of cell differentiation.

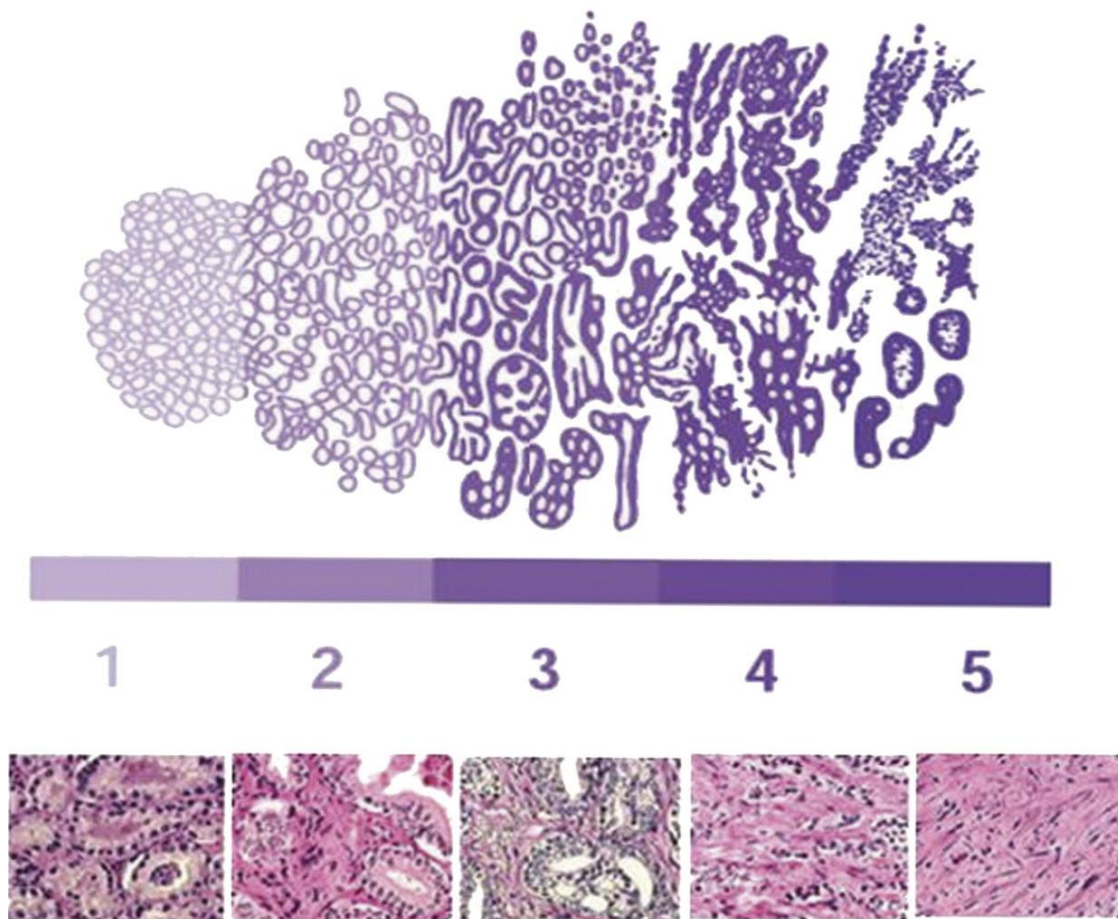


Figure 7: Gleason patterns schematics and H&E staining.

Numbers refer to Gleason pattern. Above are the original Gleason drawings of each grade and below are the corresponding stained micrographs of tumor foci for each pattern [104].

Since tumor heterogeneity is one of the characteristics of PCa, the GS is reported as the sum of two most prevalent carcinoma patterns seen in the tissue. Technically, the GS ranges from 2 (1+1), which represents well-formed individual glands composed of differentiated cells representing luminal structures and shows the best prognosis to 10 (5+5), which shows lack of cell differentiation with no glandular formation and has the poorest prognosis (Figure 7). Overall, GS show a strong correlation with the size of the tumor, clinical stage, metastatic disease and survival rate [105]. The most prevalent pattern is reported first and then followed by the second most prevalent Gleason pattern. For example, if the most prevalent pattern seen is Gleason pattern 3 and the second most prevalent pattern is 4, the GS is reported as 3+4=7.

Gleason patterns 1 and 2 are defined as circumscribed well-formed and separated glands with small and uniform cells with no infiltration into the stroma (pattern one) or focal peripheral infiltration between or around benign glands (grade two) (Figure 7). In 2000, Epstein [106] published an editorial paper suggesting that GS 2 to 4 should never be reported on needle biopsy samples. This argument was due to low level of reproducibility of the diagnosis among pathologists, in 2005 and again in 2014. Accordingly, the International Society of Urological Pathology (ISUP) recommended against reporting GS of less than 6 on prostate needle biopsy samples [107, 108].

Gleason 3 is the most common pattern seen in primary tumor samples. This pattern is defined as having infiltrating cancer cells in between well-formed glands composed of malignant cells. Such glands, with branching and without cribriform morphology are recognized as Gleason pattern 3 under the 2014 ISUP guideline [108] (Figure 7).

Gleason pattern 4 has the most diverse morphology and is defined as poorly formed and fused glands, with cribriform or glomeruloid features (Figure 8). It consists of diffused clear cells infiltrating among fused-glandular and branching tumors [108, 109].

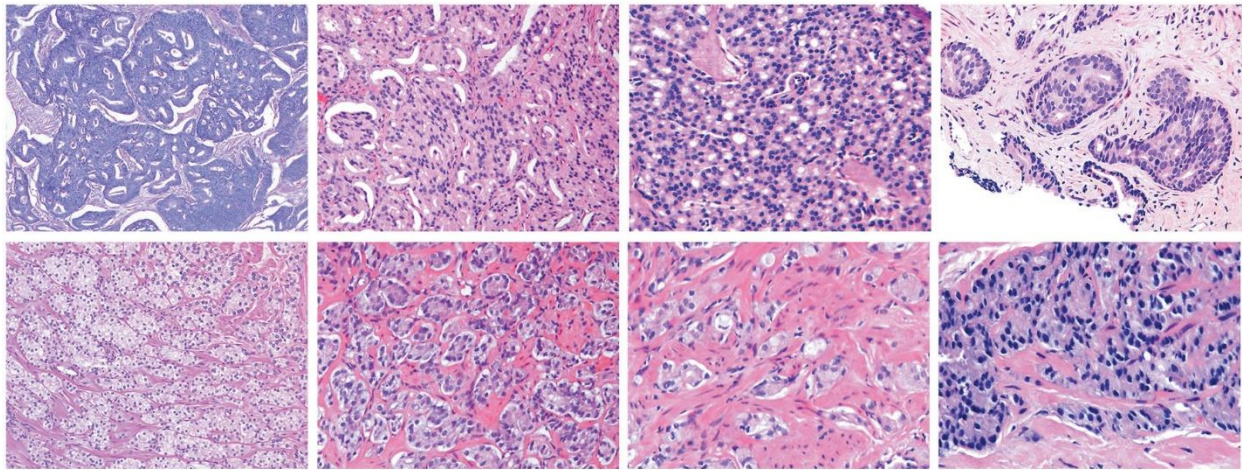


Figure 8: Gleason Pattern 4.

Top row from left to right: Large irregular cribriform glands with well-delineated lumen; Irregular cribriform glands with slit-like lumen, glomeruloid structures, and fused glands; Irregular cribriform glands with small round lumen; Small round cribriform glands.

Bottom row from left to right: Poorly formed glands with peripherally arranged nuclei; Small poorly formed glands with low fibroblast content; Small poorly formed glands; Fused poorly formed glands. (Reproduced from: The Johns Hopkins University. <http://pathology.jhu.edu/ProstateCancer/NewGradingSystem.cfm>)

Distinction between Gleason pattern 3 and 4 has a significant impact on the prognosis and the therapeutic decision-making process. Upgrade from Gleason pattern 3 to pattern 4 often results in more aggressive treatment approaches that are irreversible and may not be necessary and could disqualify patients from active surveillance (described

in detail later). Thus, in case of a doubt or borderline morphology between the Gleason pattern 3 and 4, it is advised to report the lower grade that could result in a better prognosis [98]. This approach most likely will not harm patients that are put on active surveillance as the treatment decision could be modified if deemed necessary.

Gleason pattern 5 lacks glandular formation and is represented as solid sheets of tumor cells (Figure 6). Based on the 2014 ISUP guidelines, nests of cells with rosette-like structures but no definitive lumen also should be considered to represent Gleason pattern five [108, 110]. The key features of each Gleason pattern are summarized in Table 2.

Table 2: Key characteristics of Gleason patterns

Gleason pattern	Key feature
Gleason pattern three	Well-formed individual glands
Gleason pattern four	Fused/poorly formed/cribriform/glomeruloid glands
Gleason pattern five	Comedonecrosis or no gland formation

Note that the Gleason patterns 1 and 2 are no longer reported; the lowest considered pattern is 3. Adapted from [111].

New Gleason grading system

With the new modifications of the Gleason grading system, Gleason pattern 3 is the lowest considered pattern, which effectively eliminates the GS of 3 to 5. This results in GS 6 to be the lowest reported score on a scale of 1 to 10. In an effort to avoid confusion for patients that might incorrectly assume that GS 6, which has a favorable prognosis, is a moderately aggressive cancer, a new Gleason grading system has been

introduced. Moreover, for most therapeutic purposes, GS is classified into three tiers of GS 6, 7 and 8-10. However, studies have shown that GS 3+4 vs. 4+3 and GS 8 vs. 9-10 have different prognosis [109]. Therefore, to overcome these limitations, in 2013 Pierorazio *et al.* [112] have proposed a new five-tier grading system that was adopted by ISUP and World Health Organization in 2014 and has been implemented into the 2016 edition of the genitourinary pathology blue book [113]. In this grading system which is referred to as Gleason grade grouping, GS 2 to 6 are considered Gleason group (GG) 1, GS 3+4=7 is GG 2, GS 4+3=7 is GG 3, GS 8 is considered as GG 4 and GS 9 to 10 are defined as GG 5. This new GG grading system showed an improved correlation with the BCR risk (Figure 9) in a meta-analysis of over 19,000 patients treated with RP in five different institutions worldwide. Relative risk of progression or recurrence-free progression (RFP) for GGs 2, 3, 4 and 5 compared to GG 1 were 2.6, 8.5, 16.8, and 29.3, respectively, and the 5-year BCR free survival rate for GGs 1 to 5 were 97.5%, 93.1%, 78.1%, 63.6%, and 48.9%, respectively [114].

The new Gleason grading system has been validated in several studies and showed improvement over the previous GS system and better reflects patient's prognosis [112, 115-121]. However, this system does not address the clinical relevance of the tertiary pattern, which is the third most prevalent pattern composing more than 5% of the tumor and shown to have prognostic value [122]. The tertiary pattern will likely add further improvement in the future [123].

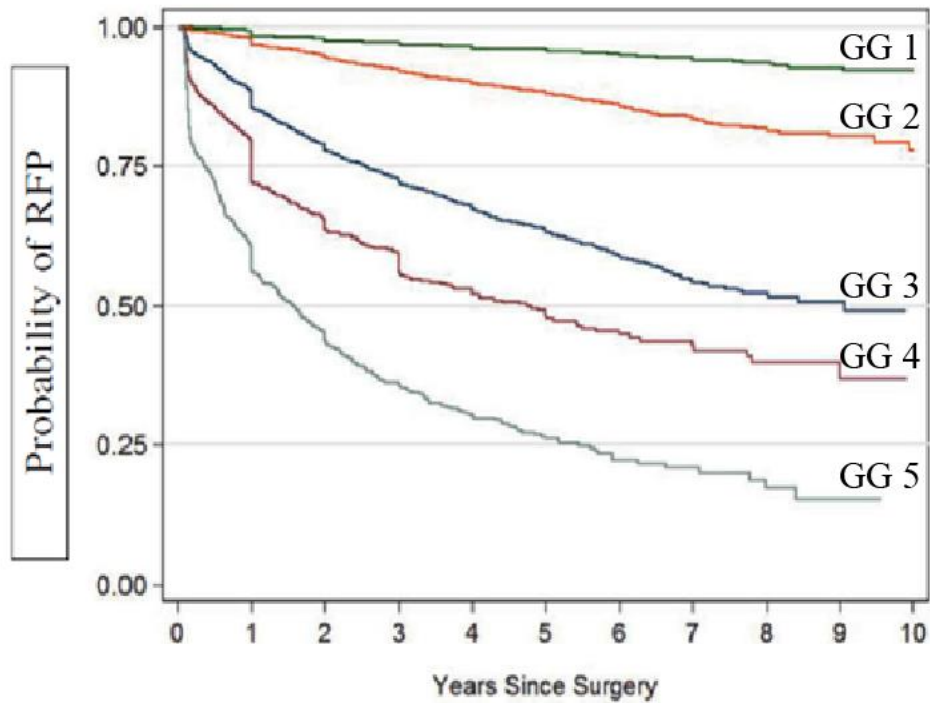


Figure 9: Clinical significance of the Gleason grade groups

Prognostic value of different GGs in patients following radical prostatectomy. RFP: recurrence-free progression. Reproduced from [114].

1.2.4.2. Tumor, Lymph Node and Metastases (TNM) Staging

Accurate and uniform staging of tumors is necessary for prognosis, therapeutic decision-making, prediction of response to a certain treatment and also exchange of data among different institutions for research purposes. The T (tumor extent), N (lymph node invasion), and M (presence or absence of metastasis) was accepted as a unified method for PCa staging in 1992 by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) [124]. Both clinical staging (cT) and pathological staging (pT) of PCa are used for prognosis. Clinical

staging is done with the information available before treatment, such as DRE, TRUS or other imaging modalities and biopsy sampling, whereas pathological staging is done using information obtained histologically on tumors spreading within the prostate, the capsule and the surrounding tissues [125].

TNM staging is used with combination of tumor grade and PSA serum levels to provide guidance for therapeutic decision-making. Criteria for clinical and pathological TNM staging are presented in Table 3 [126].

Table 3: Definitions of American Joint Committee on Cancer TNM Criteria

	Category	Criteria
Clinical stage (cT)	cTX	Primary tumor cannot be assessed
	cT0	No evidence of primary tumor
	cT1	Clinically inapparent tumor that is not palpable
	cT1a	Tumor incidental histologic finding in 5% or less of tissue resected
	cT1b	Tumor incidental histologic finding in more than 5% of tissue resected
	cT1c	Tumor identified by needle biopsy found in one or both sides, but not palpable
	cT2	Tumor is palpable and confined within prostate
	cT2a	Tumor involves one-half of one side or less
	cT2b	Tumor involves more than one-half of one side but not both sides
	cT2c	Tumor involves both sides
	cT3	Extraprostatic tumor that is not fixed or does not invade adjacent structures
	cT3a	Extraprostatic extension (unilateral or bilateral)
	cT3b	Tumor invades seminal vesicle(s)
	cT4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathological stage (pT)	pT2	Organ confined
	pT3	Extraprostatic extension
	pT3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
	pT3b	Tumor invades seminal vesicle(s)
	pT4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
N	NX	Regional lymph nodes were not assessed
	N0	No positive regional lymph nodes
	N1	Metastases in regional lymph node(s)
M	M0	No distant metastasis
	M1	Distant metastasis
	M1a	Nonregional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s) with or without bone disease

TNM staging criteria Adapted from Eight Edition of Cancer Staging manual of AJCC [126].

1.2.5. Risk stratification models

Risk stratification models are routinely used in various clinical settings by the physicians to provide a more accurate prognosis of the disease for patients. These models use pre-treatment parameters to develop a mathematical model that can predict the probability of an outcome [127]. For PCa, these models rely on pre-treatment prognostic features such as serum PSA levels, biopsy GS and clinical TNM staging. One of the first reported risk stratification models was by D'Amico *et al.* in 1998 [128], which stratifies patients into three groups of low, intermediate and high risk based on pre-treatment PSA levels, cT stage (based on 1992 AJCC definition) and biopsy GS for prediction of BCR after RP or radiotherapy. Low risk patients are defined as having the cT stage of T1 or T2a, PSA of ≤ 10 ng/ml and GS of ≤ 6 ; intermediate risk groups are patients with T2b, and/or PSA of 10 – 20 ng/ml and/or GS of 7 (3+4 and 4+3); and the high risk group are patients with stage $\geq$ cT2c or PSA of > 20 or GS of ≥ 8 .

The Genitourinary Radiation Oncologists of Canada (GUROC) also divide patients into three risk groups based on its 2001 report [129] which is slightly different than the D'Amico model. The low risk group is defined as cT1/cT2a, PSA ≤ 10 ng/ml and GS ≤ 6 ; patients with cT1 to cT2, PSA ≤ 20 ng/ml and GS ≤ 7 are classified as having intermediate risk; and high risk group are patients with either cT3 to cT4, PSA > 20 ng/ml or GS of ≥ 8 . The cT stages were based on the AJCC 1997 guidelines.

Several other organizations have also defined risk groups for prediction of PCa outcome (Table 4). The National Institute for Health and Clinical Excellence (NICE, UK) has similar classification as the Canadian Consensus Classification System,

whereas the American Urological Association (AUA) and European Association of Urology (EAU) have adapted the D'Amico's risk classification [130-134].

Table 4: Pre-treatment risk stratification models

Institution/organization	Low risk	Intermediate risk	High risk
Harvard (D'Amico) AUA EAU	cT1-cT2a and GS ≤ 6 and PSA ≤ 10	cT2b and/or GS =7 and/or PSA >10-20 and not low-risk	$\geq$ cT2c or PSA >20 or GS 8-10
GUROC* NICE	cT1-cT2a and GS ≤ 6 and PSA ≤ 10	cT1-cT2 and/or GS ≤ 7 and/or PSA ≤ 20 and not low risk	$\geq$ cT3a or PSA >20 or GS 8-10
CAPSURE*	cT1-cT2a and GS ≤ 6 and PSA ≤ 10	cT2b and/or GS =7 and/or PSA >10-20 and not low-risk	cT3-4 or PSA >20 or GS 8-10
NCCN	cT1-cT2a and GS 2-6 and PSA ≤ 10 and not very low-risk very-low risk category: cT1c and GS ≤ 6 and PSA <10 and fewer than 3 biopsy cores positive and $\leq 50\%$ cancer in each core	cT2b or cT2c and/or GS =7 and/or PSA >10-20 and not low risk	cT3a or PSA >20 or GS 8-10 and not very high risk very high-risk category: T3b-4
ESMO	cT1-cT2a and GS ≤ 6 and PSA <10	Not high risk and not low risk	cT3-4 or PSA >20 or GS 8-10

AUA: American Urological Association; EAU: European Association of Urology; GUROC: Genitourinary Radiation Oncologists of Canada; NICE: National Institute for Health and Clinical Excellence; CAPSURE: UCSF Cancer of the Prostate Strategic Urologic Research Endeavour; NCCN: National Comprehensive Cancer Network; ESMO: European Society for Medical Oncology. *Uses the 1997 TNM staging system. Adapted from [127].

1.2.6. Common treatment options

Most of newly diagnosed PCa patients have local disease (80%) or regional disease (12%). Patients with metastasis at the time of diagnosis comprise of less than 5% of all

new cases [135, 136]. The results of the Prostate Cancer Outcomes Study (prospective cohort of men with localized prostate cancer) [137] showed that depending of the risk category, the 10-years risk of PCa-specific death is between 3-18%. Moreover, results of randomized clinical trials suggest the risk of death from other causes is higher than the risk of PCa-specific death [138, 139].

Patients with localized PCa are often managed with surgery, radiotherapy or expectant management, which is defined as close disease monitoring without undergoing definitive treatment. It mostly consists of watchful waiting and active surveillance [140]. The former approach aims to treat patients' symptoms with palliative intent, whereas active surveillance aims at close monitoring of the disease via routine examination and assessment of clinicopathological features such as serum PSA levels and biopsy samples with an intent to proceed with therapeutic approaches at the sign of progression [141].

Selection of the therapeutic approach depends on the risk of progression, patient's overall health and life expectancy, and preference.

1.2.6.1. Active surveillance

Active surveillance is gaining in popularity for low risk PCa patients (as per risk stratification models described in Table 4) as a viable substitute to over-treatment of the disease [142]. For instance, a study showed that during a 10-year period more than 90% of low-risk PCa patients were treated with radical therapies [143]. Such overtreatments of PCa often have negative impact on the patient's quality of life. As most prostate tumors grow slowly and the risk of PCa-specific death is generally low,

patients and their physician may choose to postpone radical treatment approaches and associated comorbidities until there are signs of progression, therefore the benefits of treatment outweigh side-effects [144].

Currently, one of the main issues with active surveillance is the lack of unified criteria for patient's selection, thus most of data now is based on institutional standards [145]. Most active surveillance protocols take into account clinicopathological parameters such as cT stage, PSA levels, biopsy GG, number of positive biopsy cores, the amount of cancer per sample and patient's life expectancy [145, 146]. Moreover, during active surveillance, patients are usually monitored every 3 to 6 months by PSA measurements and DRE and if necessary, biopsy every 1 or 2 years. If the disease progresses, according to the patients condition and preference, definitive therapeutic approaches will be evaluated and applied [144].

Several studies have assessed the usefulness of active surveillance and reported that risk of metastasis and PCa-specific death is low, ranging between 0 to 6.1% (Table 5) [147-151]. For example, in a randomized Prostate Testing for Cancer and Treatment (ProtecT) trial, 1,643 patients with localized PCa were divided into three groups, active surveillance (n=545), RP (n=553) and radiotherapy (n=545). After 10 years, only 8 of patients in the active surveillance group (1.5%) have died due to PCa. This was similar to other groups, with 5 (0.9%) patients in the RP group and 4 (0.7%) patients in the radiotherapy group had died of the disease [151]. Although, it should be mentioned that about half of the patients in the active surveillance group have shown signs of progression and received treatments, however, patients in this group showed better quality of life [152].

Overall results of various trials (Table 5) indicate that active surveillance should be recommended for management of low-risk disease [140, 153, 154]. Nevertheless, compared to healthy individuals, PCa patients under active surveillance may show reduced sexual potency, erectile dysfunction, and urinary incontinence, moreover, the psychological impact for cancer patients not receiving treatment has been shown to negatively impact the quality of life in men under active surveillance [155]. However, despite increased anxiety, 5-years quality of life in men under active surveillance is similar to patients treated with RP [155].

A recent study published by the Prostate Cancer Research International Active Surveillance (PRIAS) [156] on 5,302 men showed 52% and 73% of patients had discontinued active surveillance at 5 years and 10 years, respectively, and received definitive treatments. In third of patients who received RP, the pathological results revealed low-risk disease (GS 3+3 and pT2) and favorable outcome. The decision to upgrade the treatment protocol was solely based on biopsy GS and clinical staging. These results showed that although active surveillance is a viable approach for low-risk patients, better prognostic markers are needed for the selection criteria. Accordingly, such biomarkers allow well fit patients with indolent disease to be offered active surveillance while patients that might have aggressive tumors and a higher probability of progression would undergo radical treatments without losing the therapeutic window.

Table 5: Protocols and Outcomes of Active Surveillance Cohorts

	University of Toronto	University of California, San Francisco	Johns Hopkins University	Göteborg Screening Trial	ProtecT Active Monitoring Group
Source	Klotz et al, 2015 [147]	Welty et al, 2015 [148]	Tosoian et al, 2015 [149]	Godtman et al, 2016 [150]	Hamdy et al, 2016 [151]
No. of participants	993	810	1298	474	545
Median follow-up, months	77	60	60	96	120
Entry criteria	From 1995-1999: GS ≤ 6 and PSA level ≤ 10 ng/ml; GS $\leq 3 + 4$ and PSA level ≤ 15 ng/ml if age > 70 year. Since 2000: GS ≤ 6 and PSA level ≤ 10 ng/ml; GS $\leq 3 + 4$ and PSA level 10-20 ng/ml if life expectancy < 10 years	GS ≤ 6 , PSA level ≤ 10 ng/ml, clinical stage $\leq T2c$, $\leq 33\%$ of positive biopsy cores, and $\leq 50\%$ cancer in each biopsy core	GS ≤ 6 , PSA density < 0.15 ng/ml, clinical stage $\leq T1c$, ≤ 2 positive biopsy cores, and, $\leq 50\%$ cancer in each biopsy core For older men: GS ≤ 6 , clinical stage $\leq T2a$, and PSA level < 10 ng/ml	Gleason score ≤ 7 , PSA level < 20 ng/ml, and clinical stage $\leq T2c$ (78% had GS of 6, clinical stage of T1, and PSA level < 10 ng/ml)	PSA level < 20 ng/ml, and clinically localized disease (77% had GS of 6, 90% had PSA level ≤ 10 ng/ml, and 75% had clinical stage of T1c)
Monitoring protocol	PSA test every 3 months for 2 y and then every 6 months, prostate biopsy within 1 y and then every 3-4 y until age 80 years	PSA test every 3 months, transrectal ultrasound every 6 months, prostate biopsy within 1 y and then every 1-2 year thereafter	PSA test or digital rectal examination every 6 months, prostate biopsy annually	PSA test and clinical examination every 3-6 months (every 12 months in older men), prostate biopsy if cancer < 2 mm and then when progression suspected or every 2-3 year	PSA test every 3 months for 1 y and then every 6-12 months, repeated prostate biopsy not required
Treatment threshold	PSA doubling time < 3 years until 2008, biopsy reclassification, clinical progression	Primary biopsy reclassification, secondary anxiety, CAPRA risk reclassification, or clinical progression	Biopsy reclassification	PSA progression, biopsy reclassification, clinical progression	Increase of 50% in PSA triggered a review of treatment
Surveillance outcomes, No. (%)					
Definitive treatment	267 (27)	348 (43)	471 (36)	202 (43)	291 (53)
Metastasis	28 (2.8)	1 (0.1)	5 (0.4)	7 (1.5)	33 (6.1)
PCa-specific mortality	15 (1.5)	0	2 (0.1)	6 (1.3)	8 (1.5)

CAPRA: Cancer of the Prostate Risk Assessment. Adapted from:[157].

1.2.6.2. Radical prostatectomy (RP)

Surgery or RP is the most common definitive treatment option for localized PCa [158]. It involves the removal of the entire prostate and surrounding tissues such as seminal vesicles and (depending on the hospital and the surgeon) pelvic lymph nodes [159]. The purpose is to eliminate the entire organ-confined cancer and prevent the spreading of the disease. Although RP is an effective approach, it is associated with serious side-effects such as erectile dysfunction and urinary incontinence [160-162], which significantly reduce quality of life. Robotic assisted surgery and new surgical methods that allow preservation of cavernous nerves have reduced such side-effects [163].

In the Prostate Cancer Intervention versus Observation Trial (PIVOT) [164], 731 patients were randomized to receive either RP or watchful waiting. Results showed that patients with PSA levels higher than 10 ng/ml showed less all-cause mortality (48.4% vs. 61.6%, respectively; $p = 0.02$) and PCa-specific death (5.6% vs. 12.8%; $p = 0.02$) after RP.

Results of the Scandinavian Prostate Cancer Group Study Number 4 (SPCG-4) [165] that compared RP to watchful waiting in 695 PCa patients indicated that RP gains more benefit over watchful waiting over time in patients with palpable tumor ($cT \geq 2$). The number of patients needed to treat with RP to avoid one death declined from 20 to 8 men at 10 to 18 years post-treatment.

In the ProtecT trial [151, 152], the effects of RP and radiotherapy were compared to active surveillance. Results showed reduced risk of progression (8.3% vs. 20.6% for

RP; and 8.4% vs. 20.6% for radiotherapy, $p < 0.001$) and metastasis (2.4% vs. 6.1% for RP; and 2.9% vs. 6.1 for radiotherapy, $p = 0.004$).

While RP remains an effective treatment option for more aggressive cancers, about 40% of patients will experience BCR [166]. The successful removal of the prostate usually results in a significant reduction of serum PSA levels that eventually become undetectable in a majority of patients. BCR after RP is defined as two consecutive measurements of serum PSA of more than 0.2 ng/ml [138, 167, 168]. Since the prostate is removed during surgery, the rise in serum PSA is likely results from local and distant spreading of tumor cells to secondary sites, although often not detectable with current imaging means. However, incomplete removal of the prostate (positive surgical margins), may also result in detectable PSA levels in the blood after RP.

1.2.6.3. Radiotherapy

External beam radiotherapy is a method of delivering radiation to target tumor cells within the prostate. This treatment method is less invasive than RP and has a long history in treating organ-confined and locally advanced PCa.

Effect of radiation is best defined by the deposition of charged particles (ionizing radiation) energy into cells and tissues they pass through [169]. Most of the damage caused by radiation is at the level of cell DNA, thereby blocking the division of proliferating cancer cells [170]. Hence, depending on the proliferation rate, normal cells may also be affected by radiation. Thus, it is important to minimize damage to normal cells adjacent to cancer cells in the path of radiation. Due to the loss of normal function and defective DNA repair pathways, cancer cells are generally less efficient in

repairing damages caused by mutations compared to normal cells [171]. Therefore, radiotherapy is an effective approach to treat cancer. Furthermore, radiotherapy can be offered in combination to other therapeutic approaches such as after surgery (adjuvant or salvage), hormonal therapies, or chemotherapy to maximize their effects on cancer cells.

Radiotherapy had significantly improved since the 1990s, which translated into reduced side-effects. A majority of these advances have applied to PCa treatment [172]. Three-dimensional conformal prostate radiotherapy shows less toxicity and allows to precisely target intraprostatic tumor foci with increased radiation dose [173]. Fractionating radiation also allows the delivery of the radiation dose in several sessions, minimizing toxicity and increasing the probability to eradicate cancer cells [174, 175]. Intensity-modulated radiation therapy allows delivering radiation beams with various intensities to the area to better conform to the irregular shape of the organ, thus reducing radiation to surrounding tissues and subsequent urinary and bowel toxicity [176, 177].

Previous meta-analysis studies have indicated more therapeutic benefits of surgery over radiotherapy [178]. However, ProtecT was the first randomized trial that compared the therapeutic effect of RP to radiotherapy [179]. The study showed no significant difference in overall or PCa-specific mortality or metastasis between radiotherapy or RP. Moreover, the radiotherapy group showed significantly better urinary control and sexual function compared to the RP group, which on the other hand had less nocturia and bowel dysfunction [151, 152].

Various clinical trials showed benefits of adjuvant radiotherapy (radiotherapy after RP) in reducing the rate of BCR in patients with adverse pathology (i.e. pT3) [180, 181]. However, adjuvant radiotherapy may be associated with side-effects and increasing complications [182].

1.2.6.4. Androgen deprivation therapy and other therapeutic options

Androgen deprivation therapy (ADT) is the first-line systemic treatment option for locally advanced and metastatic PCa. The main purpose of ADT is suppression of testosterone which can be achieved by either surgical (orchiectomy) or medical castration [183]. Due to significant psychological impact of surgery caused by the removal of testicles, and similar clinical benefits to the medical castration, orchiectomy is rarely used as an ADT in Western countries [184]. Current strategies for ADT involve inhibition of androgen synthesis using Luteinizing Hormone-Releasing Hormone (LH-RH) agonists or antagonists.

LH-RH agonists yield to an initial release of luteinizing hormone by the pituitary gland, causing an initial rise of serum testosterone within the first 72 hours to one week of administration. Continuous stimulation leads to the down-regulation of gonadal luteinizing hormone receptors and decrease of the testosterone production to the castration range after 1 month [185]. This initial increase of testosterone may cause a temporary worsening of symptoms known as flare phenomenon. However, most patients improve within a few weeks without the need for major interventions [186].

LH-RH antagonists were developed and introduced more recently in the clinical management of PCa. They bind and block the LH-RH receptors on pituitary

gonadotropin-producing cells. This does not lead to the initial release of luteinizing hormone or follicle-stimulating hormone thus, avoiding the flare phenomenon seen with LH-RH agonists. Although long term suppression of serum testosterone can be achieved by both approaches, LH-RH antagonists cause less side-effects and provide faster decline in testosterone levels [185]. Anti-androgens may be administered jointly to these agonists or antagonists to obtain a more complete inhibitory effect of residual androgens on tumor growth, by blocking androgen binding to the AR and preventing its signalling and transcriptional activity in tumor cell nuclei [187].

Overall, ADT is associated with toxicity and adverse effects such as metabolic changes, sexual dysfunction, hot flashes, decreased bone mineral density, cardiac morbidity and cognitive dysfunction [188, 189]. All patients respond to ADT, however the response is transient and almost all of them develop resistance, referred to as castration-resistant PCa (CRPC) and become metastatic (mCRPC) afterwards, with a median survival of 18 months [190]. The next line of therapy is taxane-based with docetaxel improving overall survival of mCRPC patients [191, 192]. After positive results in the phase III TROPIC study, cabazitaxel was approved by the FDA as a treatment option for mCRPC in patients progressing after docetaxel-based therapy [193]. Similarly, most patients develop resistance to cabazitaxel as well. Consequently, more therapeutic options such as abiraterone and enzalutamide have been approved and implemented in then patient's therapeutic regimen. Phase III clinical studies revealed that these drugs improve survival and prolong time to disease progression (Table 6) [191-198].

Table 6: Overview of current therapies and key phase III clinical trials

Drug name and approved indication	Study name	Patient population and treatment arms	OS benefit, months	PFS benefit, months
Docetaxel mCRPC	TAX 327 [191]	mCRPC: docetaxel (n= 335) ^a vs. mitoxantrone (n=337)	18.9 vs. 16.5 (p=0.009)	-
	SWOG 9916 [192]	mCRPC: docetaxel+ estramustine (n=338) vs. mitoxantrone (n=336)	17.5 vs. 15.6 (p =0.02)	TTP 6.3 vs. 3.2 (p <0.001)
Cabazitaxel mCRPC (post-docetaxel)	FIRSTANA [198]	mCRPC (chemotherapy-naïve): cabazitaxel (n=388) ^b vs. docetaxel (n=391)	25.2 vs. 24.3 (p =0.757)	5.1 vs. 5.3 (p =0.804)
	TROPIC [193]	mCRPC (post-docetaxel): cabazitaxel (n=378) vs. mitoxantrone (n=377)	15.1 vs. 12.7 (p <0.0001)	2.8 vs. 1.4 (p <0.0001)
Abiraterone mCRPC	COU-AA-302 [195]	mCRPC (chemotherapy naïve): abiraterone (n=546) vs. placebo (n=542)	34.7 vs. 30.3 (p =0.003)	rPFS 16.5 vs. 8.3 (p <0.001)
	COU-AA-301 [194]	mCRPC (post-docetaxel): abiraterone (n=797) vs. placebo (n=398)	15.8 vs. 11.2 (p <0.001)	rPFS 5.6 vs. 3.6 (p <0.001)
Enzalutamide mCRPC	PREVAIL [196]	mCRPC (chemotherapy naïve): enzalutamide (n=872) vs. placebo (n=845)	NR (82% at 18 months) vs. 31.0 (p <0.001)	rPFS 65% vs. 14% at 12 months (p <0.001)
	AFFIRM [197]	mCRPC (post-docetaxel): enzalutamide (n=800) vs. placebo (n=399)	18.4 vs. 13.6 (p <0.001)	rPFS 8.3 vs. 2.9 (p <0.001)

^aData presented for the study arm where patients received docetaxel every 3 weeks. This study had an additional arm with weekly docetaxel administration (not shown).

^bData presented for the study arm where patients received cabazitaxel 25 mg/m². This study had an additional arm with cabazitaxel 20 mg/m² (not shown), which did not show different results from the 25 mg/m² arm. PFS: progression-free survival, cPFS: clinical PFS, rPFS: radiographic PFS, NR: not reached, NS: not statistically significant, OS: overall survival, TTP: time to progression. Adapted from [199].

Since abiraterone inhibits steroid synthesis and androgen precursors in the adrenals, while enzalutamide affects AR signalling, their function does not overlap and can be used as a second line systemic therapy in mCRPC patients who have failed ADT and other AR-based therapies.

Currently there is no cure for mCRPC and patients eventually develop resistance to these drugs as well.

1.3. BIOMARKERS OF PCa

PCa is a heterogenous disease and its course or trajectory varies in different patients. While most patients have indolent disease, a subset of patients could progress rapidly and develop an aggressive disease. The period between diagnosis and treatment or death could be long, and patients need to be routinely monitored for signs of progression. This is especially important as more patients are being diagnosed with low- and intermediate-risk disease and nowadays are considered for active surveillance. Thus, diagnostic biomarkers that could identify aggressive subtype from indolent disease are required to select patients that would benefit from definitive treatments, such as RP and radiotherapy. Accordingly, molecular biomarkers have been identified in patients tumors and bodily fluids such as blood and urine and are used at different stages, from screening and diagnosis to post-treatment monitoring and assessing response to particular treatments or recurrence of the disease [200]. Below is a short description of known and promising biomarkers of PCa that can be assessed in the urine (PCA3 and ETS-TMPRSS fusion), blood (PSA and PSCA), and primary tumor tissues (PSMA, AMACR, and CNA) of patients.

1.3.1. PSA

PSA is expressed by luminal cells of the normal prostate as well as well differentiated tumor cells. It has been long used as a biomarker of PCa when present in the blood. It is currently used in all stages of the disease, from screening and risk determination to assessing response to treatments or disease progression. However, its application in diagnosis comes with several limitations. PSA cannot discriminate between the indolent and aggressive disease especially at values below 10 ng/ml [201]. Moreover, it can be detected in the blood of most patients with other prostate diseases such as prostatitis, BPH or HGPIN [202]. These limitations make the use of PSA controversial as a screening tool for PCa.

The European Randomized Study for Reducing Prostate Cancer (ERSPC) assessed the efficacy of PSA as a screening tool for PCa on 162,387 men [203, 204]. The study had two arms, the PSA screening group consisted from 72,890 men and a control group with 89,353 men. The PCa screening was offered once every three years and the cut-off was set at 3 ng/ml. After 13 years of follow-up, the results showed that to prevent 1 mortality, 781 men need to be screened and 27 patients detected. The conclusion was that PSA screening does not reduce PCa-specific mortality and is associated with disease over-diagnosis and over-treatment.

Similarly, the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial [205] which assessed the use of PSA in the standard of care setting in 38,343 men in prevention of PCa-specific mortality, failed to show a screening benefit. Based on these results the US Preventive Task Force (USPTF) recommended against routine use

of PSA as a screening tool for PCa and suggested that men should be notified about benefits and harms of PSA screening [206].

1.3.2. Prostate cancer antigen 3 (PCA3)

Prostate cancer antigen 3 (PCA3) is a non-coding RNA expressed in prostate tumors and was first reported by Bussemakers *et al.* in 1999 [207]. Despite that the function of PCA3 is unknown, it has been reported to be expressed in more than 95% of primary and metastasis cases [58, 208]. Because it is not expressed in normal prostate, and has a low expression in BPH yet high in cancer, it is considered as a specific PCa marker [207]. Unlike PSA, expression of PCA3 is not related to the prostate volume and even in instances where the serum PSA levels are low, urinary levels of PCA3 are indicative of PCa [209, 210]. Thus, its efficacy in detection of cancer was assessed in the urine of men with serum PSA values of above 2.5 ng/ml after DRE [211]. The assay showed a sensitivity of 69% and a specificity of 79% in predicting a positive biopsy.

The ProgenSA PCA3 urine test, which is an *in vitro* amplification assay for possible detection of PCa after a negative biopsy result and/or negative PSA level and/or DRE results was approved by FDA in 2012 [212]. However, the cut-off points of the assay are not established, and more studies are required to define an appropriate threshold and accurate criteria to prevent over-diagnosis.

1.3.3. Prostate stem cell antigen (PSCA)

Prostate stem cell antigen (PSCA) was discovered in gene-expression analysis of LAPC-4 (human PCa cell line) xenograft [213]. PSCA is a cell surface glycosyl phosphatidylinositol-anchored glycoprotein that is expressed in PCa [213]. This protein

shows a weak homology with stem cell antigen type-2 (SCA-2), a cell surface marker expressed on immature lymphocyte, which resulted in its name. Hence, PCSA is not a stem cell marker and is not exclusively expressed in the prostate or PCa [214]. Its function is not yet been identified, however, studies using homologous proteins suggest a role in T cell activation and proliferation [215] as well as cytokine and growth factor response [216].

Nonetheless, the expression of PSCA is associated with PCa, higher GS, stage, disease progression and metastasis [217-221]. Furthermore, PSCA expression has been validated in prostate tumor tissues by immunohistochemistry (IHC) [217-220] and its mRNA levels has been detected in the blood of PCa patients [221].

In a study that assessed the expression of PCSA in men with BPH undergoing transurethral resection of the prostate (TURP), only 32.3% of patients (93 out of 288) tested positive; however, out of those with positive PCSA expression, 23.7% (22 out of 93) developed PCa compared to 1% of PCa patients (2 out of 195) with negative PCSA expression [222].

Application of PCSA as a prognosis biomarker needs further validation and studies, including development of better and more accurate methods for detection and quantification of PSCA at the mRNA or protein level in the blood [223].

1.3.4. Prostate-specific membrane antigen (PSMA)

Prostate-specific membrane antigen (PSMA) also known as folate hydrolase I or glutamate carboxypeptidase II is a type II transmembrane protein which was identified by patients' antibodies recognizing LNCaP cell line. The protein was then identified in

1987 by Horoszewicz *et al.* [224] as a surface protein in the luminal epithelial cells of prostate and also in the serum of PCa patients. Various studies have been conducted to assess the prognosis value of PSMA in PCa diagnosis [224-236]. Higher PSMA expression in the sera and prostate tissue is associated with GS and stage, thereby indicating its usefulness in a clinical setting [231, 232, 235-237]. However, other studies failed to confirm prognostic benefits of PSMA in the sera over PSA [226, 234].

Currently, PSMA antibodies labeled with Indium-111 are used in immunoscintigraphic imaging tests commonly known as ProstaScint™ to more precisely detect residual PCa disease and metastasis [238]. Multiple studies have confirmed the benefits of combined use of ProstaScint™ and PSA serum levels for determining disease recurrence and location of residual disease [239, 240]. Moreover, use of radioactive PSMA ligands are now making their way in Nuclear Medicine for earlier detection of metastases [241, 242], whereas PSMA antibody-drug conjugates with toxins and radioactive particles or bispecific T-cell engager (BiTE) are being explored in treatment of PCa and of metastatic disease [243-252].

1.3.5. α -Methylacyl-CoA racemase (AMACR)

The enzyme α -Methylacyl-CoA racemase (AMACR) catalyzes the conversion of branched-chain fatty acids from R-stereoisomers to S-stereoisomers and also participates in the peroxisomal β -oxidation of branched-chain fatty acids [253-255]. Several studies have showed overexpression of AMACR in PCa [254, 256, 257].

Efficacy of AMACR expression in detection of PCa was assessed in a multi-institutional study in 807 PCa tissue samples [258]. Results showed that IHC staining

for AMACR can detect PCa with sensitivity of 97% and specificity of 92%. Thus, IHC staining for AMACR is now used as routine practice for detection of PCa in various institutions [259]. Overexpression of AMACR has shown to be prognostic and is associated with higher probability of BCR and PCa-specific mortality [260]. Studies have also showed detection of AMACR in serum [261], prostatic secretions [262] and urine of PCa patients [263].

1.3.6. E26 transformation-specific (ETS) gene fusion

In almost 50% of all Caucasians diagnosed with PCa, a genetic fusion between a member of the ETS family of transcription factors and an androgen-regulated gene is seen in primary tumors [264, 265]. The frequent occurrence of gene-fusions near AR binding sites is clonal and due to the consistent activation of AR signaling, genotoxic stress, and recruitment of specific stress-induced enzymatic processes that cause double-stranded breaks [266]. The fusion between ERG (v-ets erythroblastosis virus E26 oncogene homolog) and the androgen-regulated transmembrane serine protease isoform 2 (TMPRSS2) has been detected in almost half of all PCa patients and results in ERG over-expression [267, 268]. Although detection of these fusions is highly specific to PCa, there are conflicted results regarding their utility as prognostic biomarkers [208, 267, 268].

A prospective study of 1,180 RP patients with a median follow-up of 12.6 years showed that 49% had ERG over-expression [268]. However, no association between the fusion and BCR, metastasis, PCa-specific death or overall death was found. Further meta-analysis of 5,074 patients also showed that TMPRSS2-ERG was not associated with outcome [268]. Moreover, in another study by FitzGerald *et al.* [269] no

relationship was found between TMPRSS2–ERG gene fusion and worse disease-specific survival. However, in a study by Kulda *et al.* [270] in 108 PCa patients, a significant correlation between higher TMPRSS2–ERG expression and shorter disease-specific survival was found. Similar results in a smaller cohort were also reported by Font-Tello *et al.* [271].

To overcome these conflicting results and develop a urine-based diagnostic assay, the TMPRSS2–ERG fusion was combined with other biomarkers, such as PCA3 and PSA [272, 273]. Addition of TMPRSS2–ERG fusion to the PCA3 assay increased sensitivity for PCa diagnosis from 68% to 76% [274]. Combining TMPRSS2–ERG, PCA3 and serum PSA levels has increased accuracy in predicting high grade disease at biopsy [273].

1.3.7. DNA copy number alteration (CNA)

DNA copy number alterations (CNAs) are genomic alterations consisting of deletion or gain of DNA segments ranging from one kilobase to several megabases that could influence the function of several genes and regulatory elements [275]. Genome-wide analysis of prostate tumors resulted in the identification of recurrent alterations in different chromosomes that often result in arm-level deletions or gains that indeed affect various oncogenes and tumor suppressors [138, 167, 276-279]. Meta-analysis of sequencing studies of prostate tumors revealed that frequencies of single nucleotide mutations are surprisingly low, whereas, large-scale CNAs occur with high frequencies, hence suggesting that the development and progression of the disease is primarily due to an accumulation of such genomic events, namely deletions and gains [138, 279, 280]. Moreover, a recent study [281] on localized PCa, which sequenced

more than 290 tumors revealed that CNAs are the predominant type of mutations in PCa and proposed that tumors are commonly initiated by a low amount of point mutations and recurrent genomic deletions which increase as the disease progresses and copy number gains become more frequent. This was further confirmed by several genome-wide analyses of prostate tumors that indicate more frequent deletions than gains in the PCa CNA landscape [138, 167, 278, 279, 282].

The prognostic value of CNA [138, 167, 278-282] has been established by assessing specific deletions and gains or calculating the overall CNA burden which is a surrogate for genomic instability [282-284]. Although, the CNA burden is predictive of BCR [282], its utility as a prognostic biomarker in low- and intermediate-risk localized PCa remains limited. Single nucleotide mutations and CNAs accumulate as the cancer progresses, thus the CNA burden in early stages is often lower than in advanced cancers [285]. Nonetheless, recurrent deletions and gains remain prognostic indicators of early disease.

1.4. CNAs RELEVANT TO PROSTATE TUMOR BIOLOGY

Assaying alterations of specific tumor suppressors, oncogenes and key regulators of cellular pathways that derive tumorigenesis and affect tumor cells behaviour can have more prognostic value than CNA burden in early stages of the disease [286-292]. Such deletions and gains are likely to cause more aggressive phenotypes and result in fast disease progression and poor outcome. Thus, assessment of these recurrent CNAs at the time of diagnosis could be valuable in patients risk classification, provide a more accurate prognosis and aid in the selection of more appropriate options for disease

management. Below, the description of candidate genes residing in recurrent CNAs, and play a role in prostate tumor cell biology studied in this thesis is provided.

1.4.1. *RWDD3* (1p21.3)

RWDD3 is known as RWD-domain-containing sumoylation enhancer or RSUME and is located on the 1p21.3. *RWDD3* has high expression in various organs, including pituitary, cerebellum, heart, kidney, liver, pancreas, stomach, adrenal gland, prostate and spleen [293]. The protein plays a key role in cancer by interacting with Hypoxia-inducible factor (HIF)-1 α , von Hippel–Lindau protein, I κ B and glucocorticoid receptor [294-296].

RWDD3 is involved in post-translational modifications of proteins using ubiquitin-related modifier (SUMO), which is also known as sumoylation, a regulatory mechanism of protein function and cellular localization, such as intracellular transport, mitochondrial dynamics, DNA repair, cell cycle and replication [297-301].

The sumoylation reaction is carried out at different steps of enzymatic cascades [298, 302, 303] and can be reversed by members of the SENP family of proteins, which release SUMO from proteins [299, 303]. The balance of binding and removal of SUMO polypeptides from specific proteins has significant impact on cellular pathways and functions [304, 305].

RWDD3 enhances the sumoylation of specific proteins by direct interaction with the E2 SUMO conjugase protein, UBC9. *RWDD3* performs this function by the formation of the UBC9-SUMO-1 thioester, and also transfers the SUMO polypeptide from the thioester to a specific substrates [294, 306, 307].

Of interest, the dysregulation of sumoylation by upregulation of the UBC9 enzyme was reported in metastatic PCa [308]. More specifically, UBC9 sumoylation of Flotillin-1 results in epithelial-to-mesenchymal transition, a cell phenotype characterizing cancer progression [309]. Although this study did not directly investigate the role of *RWDD3*, previous studies have shown its direct interaction with UBC9 in cell line models [307].

Various studies have shown 1p21.3 is a common site of CNA in PCa [279, 310, 311]. For instance, Array Comparative Genomic Hybridization (Array-CGH) performed on 20 prostate samples from African American patients and 21 Caucasian Americans matched by Gleason grade and tumor stage revealed that the 1p21 region more frequently undergoes copy number gain in Caucasian Americans while deletion is more common in African Americans [311]. A meta-analysis of array-CGH data on 821 prostate tumors across 41 different studies comparing frequencies of CNAs in advanced versus localized disease revealed that deletion of 1p21 region is significantly associated with advanced disease, with a frequency of 14.9% deletion across patients with advanced PCa [280]. Furthermore, survival analysis using significant analysis of microarray (SAM) on 29 primary tumor samples of advanced cases [279] showed that deletion of 1p21 region is significantly associated with a lower probability of disease-free survival. Functional studies are lacking to directly illustrate the role of *RWDD3* in PCa. Nonetheless, the deletion of 1p21.3 region in advanced disease remains associated with poor outcome.

1.4.2. *PDZD2* (5p13.3)

The PDZ domain-containing protein 2 (*PDZD2*), which is also known as PIN-1 [312], PAPIN [313], activated in PCa (AIPC) [314] and PDZ domain-containing protein3

(PDZD3) is coded by the *PDZD2* gene residing at the 5p13.3 loci. Its transcription and translation produce a 301 kDa intracellular protein with six-PDZ domains which is localized in the endoplasmic reticulum and in periphery of the nucleus [315]. *PDZD2* is expressed in multiple human tissues including heart, brain, liver, spleen, lung, pancreas, kidney and prostate. Moreover, its overexpression was reported in some cancers such as PCa [314, 316, 317]. The PDZ domains are mainly associated with protein-protein interactions, protein folding, interaction with ion-channel transmembrane receptors and intracellular signaling [318, 319]. However, the exact function of PDZD2 in humans is still unknown [320].

Several reports suggest a possible role of *PDZD2* early in the prostate tumorigenesis [321] and its progression to form bone metastases [322]. The up-regulation of the protein was first reported in primary cultures of PCa cells, HGPIN, and primary tumors [321]. The IHC study of tissue microarray (TMA) of 313 prostates, including 91 benign prostates, 46 atrophic prostates, 18 HGPIN, and 158 primary PCa showed *PDZD2* overexpression in 83% of HGPIN and 75% of tumors compared to only 3% of high expression in normal prostate tissue. All staining were cytoplasmic without membrane localization. The lack of correlation between high *PDZD2* expression and stage, grade or pre-operative PSA levels led to the conclusion that *PDZD2* is closely associated with the initiation or early promotion of prostate tumorigenesis [321].

Studies showed that this protein has a post-translational modification, which involves cleavage via a caspase-dependent mechanism and producing a secreted PDZD2 form (or sPDZD2) [317], which has a role in the PCa pathogenesis [323]. The sPDZD2 protein can induce a concentration dependent cell-proliferation arrest in PCa cell lines,

such as PC-3, DU145 and 22Rv1, and immortalized prostate cell line RWPE-1, while inducing apoptosis in LNCaP cells [320]. The anti-proliferating effect is due to a prolonged G0/G1 phase, slowing the entry of cells into the S-phase. Further studies [323] showed similar anti-proliferative effect of sPDZD2 in the breast cancer cell line, MCF-7. Different mechanisms of action were observed in these cell lines. In DU145, sPDZD2 upregulates p21 and p53, while only upregulation of p53, but not p21 was detected in MCF-7. Knockdown siRNA studies showed that p53 knockdown reduces the effect of exogenous sPDZD2 expression on cell cycle arrest in both DU145 and MCF-7. Knockdown of p21 completely reversed the effect of sPDZD2 in DU145, while no significant effect was seen in MCF-7 cell line. These results suggest both p53-dependent and p53-independent mechanisms of action for PDZD2 and sPDZD2 that cause cell reprogramming and initiation of cell dormancy linked to bone metastases in prostate and breast cancers. [322]. Authors argue that bone metastasis is initiated by disseminated tumor cells (DTCs) entered into a state of cellular dormancy. The dormant state allows resistance to conventional chemotherapeutic agents and prevents eradication of DTCs from bones using current drug therapies. They suggest that autocrine secretion of PDZD2 could induce a transcriptional reprogramming via p53 that initiates quiescence or senescence programs. The induction or maintenance of dormancy allows tumor cells residing in bones to escape the immune system and conventional therapies. However, the mechanism of tumor cell residency in bones and the relevance of autocrine sPDZD2 secretion to cellular dormancy have not yet been described *in vivo*.

Several studies reported the gain of the 5p13.3 region in PCa [138, 324, 325], which is consistent with reports on upregulation of *PDZD2*. Furthermore, a study on CNAs in hereditary PCa revealed that *PDZD2* gain is highly associated with hereditary PCa among Finnish families [325]. In this study, the CNA profile of 105 PCa patients and 37 unaffected relatives was assessed using genome-wide SNP-arrays. Four CNAs that showed significant difference among patients affected with PCa and unaffected controls were further validated using real-time qPCR in an additional 189 hereditary PCa patients and 476 male controls. *PDZD2* duplication was shown to be one of the four genes identified in this study as significantly different among PCa patients and controls. Further analysis showed that unlike the other three CNA candidates, gain of *PDZD2* was not in Hardy-Weinberg equilibrium, either in patients or in controls. Results indicated that frequency of *PDZD2* gain was double in patients compared to the control group and the majority of duplication carries were homozygous, suggesting selection of this CNA in hereditary PCa.

Despite various studies on the *PDZD2* role in PCa cells and gain of the 5p13.3 region where the *PDZD2* gene resides, its exact mechanism in prostate tumor pathogenesis has not yet been revealed, as both tumor suppressor [320] and oncogenic [314] functions are attributed to the protein.

1.4.3. *GTF2H2* (5q13.2)

General Transcription Factor IIH is a part of 500 kb inverted duplication on chromosome 5q13. This duplicated region contains at least four genes and repetitive elements, which makes it prone to rearrangements and deletions [326]. This gene encodes the 44 kDa subunit of RNA polymerase II transcription initiation factor IIH,

involved in basal transcription and nucleotide excision repair. The protein is also known as TFIIH, P44 or BTF3. *GTF2H2* is composed of two protein complexes. The core complex contains XPD, p62, p53, p44, p34 and p8; the CDK-activating kinase complex CAK is composed of MAT1, cyclin H and cdk7 kinase. The catalytic XPD complex has DNA helicase activity and functions to open the DNA at the promoter region [327]. The cdk7 subunit has kinase activity and phosphorylates the C-terminal of RNA polymerase II [328] to allow initiation of transcription and promoter clearance.

According to Lee *et al.* [329], *GTF2H2* directly interacts with AR in PCa cell lines. In AR transfected PC-3 and LNCaP under physiological conditions, *GTF2H2* enhances AR transactivation in a ligand-dependent manner, and in a ligand-independent manner in AR transfected DU145. Using co-immunoprecipitation methods in LNCaP cell line, AR was shown to directly interact with both the core and the catalytic complexes. Overexpression of the catalytic subunit, CAK, increased AR transcriptional activity in the presence of the ligand dihydrotestosterone (DHT) in PC-3 (co-transfected with AR) and LNCaP cell lines up to 5-folds, and up to 3-folds in absence of DHT in AR transfected DU145 cell line.

GTF2H2 also plays a role in the AR post-translational modification by serine phosphorylation. Chymkowitch *et al.* [330] reported that *GTF2H2* phosphorylates AR at the position of S515 via the cdk7 subunit. The interaction of *GTF2H2* with AR and its phosphorylation is specific and not universal and is required for accurate AR transcriptional activity on AR-targeted genes. The authors further showed that phosphorylation of AR via cdk7 subunit of *GTF2H2* will result in ubiquitination of AR and recruitment of proteasome machinery at the promoter site. Disrupting the AR S515

phosphorylation using mutated copy of *cdk7* or silencing *GTF2H2* resulted in increased AR half-life after ligand interaction and impeded DHT-mediated PSA expression. Moreover, the disruption of AR phosphorylation via *cdk7* knockdown changed the proteasome recruitment machinery and resulted in more mono-ubiquitination rather than poly-ubiquitination and accumulation of AR on the PSA promoter. Although the exact effect of change in the mono- vs. poly- ubiquitination was not studied, the authors indicated the non-proteolytic activities of the ubiquitin-proteasome pathway could have a role in AR-mediated transcription [331, 332]. This is supported by the fact that the interaction of 26S subunit of proteasome with *GTF2H2* did not result in proteolysis [333, 334].

Interaction of *GTF2H2* with AR could affect the transcription of AR-targeted genes in two different manners, firstly, the *cdk7* subunit phosphorylates the AR and modulates its transcription and half-life, and secondly, interaction of AR with *GTF2H2* enhances the phosphorylation of C-terminal domain of RNA polymerase II and facilitates the elongation phase of the transcription process.

Another mechanism of interest is the *GTF2H2* implication as a part of the nucleotide-excision repair (NER) pathway [335], which is involved in repairing a broad spectrum of DNA damages, such as cross-links, oxidative damages and UV-induced photoproducts. Malfunction of NER pathway has been known to play a role in multiple cancers including lung, skin, head and neck, breast and prostate [336-349]. Defects in the NER pathway could result in accumulation of mutations in PCa cells and contribute to the pathogenesis and increased risk of the disease [350].

Deletion of 5q13.2 is frequently observed in PCa including in organ-confined and aggressive disease [351-353]. Overall, this common deletion site appears to contribute to the biology and progression of PCa via multiple pathways that require further validation.

1.4.4. *CHD1* (5q15-q21.2)

Chromodomain helicase DNA binding protein 1 or CHD1 is a part of ATP-dependent chromatin remodeling factors that contains SNF2-like helicase domain regulating chromatin assembly [354, 355], activating transcription by binding to H3K4me3 and recruiting elongation factors involved replication and DNA repair [356-358]. Furthermore, CHD1 functions to maintain chromatin structure during transcription [359] and is important for correct positioning of nucleosomes and proper initiation of transcription in yeast [360-362]. Previous studies have shown that deletion of *CHD1* increases the ratio of heterochromatin to euchromatin and could cause embryonic lethality [363]. Additionally, CHD1 plays an important role in DNA damage repair, especially double stranded breaks (DSB) [364]. DSBs are the most frequent causes of genomic instability and formation of tumors, which are often repaired by homologous recombination (HR) [23]. The importance of CHD1 in HR-mediated repair of DNA damages and of the effects of its deletion in PCa biology was illustrated by Kari *et al.* [365]. In their study, the authors also reported that CHD1 is recruited to the chromatin break site and initiates the assembly of the DNA repair machinery.

The *CHD1* locus is one of the most frequently altered genes in PCa. Tumors with this deletion are associated with poor prognosis [366-368]. Huang *et al.* [369] have performed an array-CGH study on 86 primary tumors and reported that 17% of cases

harbor the *CHD1* deletion. They further performed siRNA knockdown of *CHD1* in prostate cancer epithelial cell lines and reported an increased invasiveness and clonogenicity. The high frequency of the deletion was further confirmed by a Meta-analysis that reported 42.9% of all PCa cases harbour a *CHD1* deletion [370]. This deletion is also associated with increased chromosomal instability, which is a recognized hallmark of cancer [371].

The homozygous deletion of *CHD1* is also considered as a distinct subtype associated with CRPC [276]. Studies in mice have shown that homozygous deletion of *CHD1* in the prostate epithelium results in intraepithelial neoplasia (PIN) but not PCa [372]. Moreover, the *CHD1*-deleted prostate epithelium showed increased sensitivity to double-stranded breaks caused by radiation. It is known that AR activity could cause DNA double strand breaks [266] and also influence repair after ionizing radiation in PCa [373-376]. These findings support that *CHD1*-deleted tumors are susceptible to double-strand DNA breaks and could respond to poly (ADP-ribose) polymerase (PARP) inhibitors that target DNA repair pathways. Accordingly, a study showed sensitivity to PARP inhibitors in organoids generated from PCa tissues of mCRPC patients with homozygous *CHD1* deletion and that had progressed after receiving castration, radical prostate radiotherapy, abiraterone and docetaxel. Xenograft animal models with homozygous *CHD1* deletions also showed a better response rate to PARP inhibitors and DNA damaging agents compared to tumors with normal *CHD1* [372].

1.4.5. *MAP3K7* (6q15)

The Mitogen-Activated Protein Kinase Kinase Kinase (*MAP3K*)7 gene encodes the TGF- β activated kinase-1 (Tak1) protein. Tak1 is an important part of multiple

signaling pathways, such as interleukin 1 (IL-1) [377], Toll receptor [378], TNF-related apoptosis-inducing ligand (TRAIL) [379] and Tumor Necrosis Factor (TNF) [380]. Moreover, *MAP3K7* regulates the Wnt/ β -catenin pathway by phosphorylation of the nemo-like kinase [381]. This protein involvement in multiple pathways and the vital regulatory function of Tak1, along with the frequent deletion of the gene in cancer, suggest a tumor suppressor role.

Deletion of the 6q14 to 6q21 region is commonly seen in PCa. Strategies such as high-resolution single nucleotide polymorphism arrays, fluorescence *in situ* hybridization (FISH) and qPCR led to the mapping of the minimal deletion region, an 817 kilobases, which included five genes [382]. In their study population of 95 PCa tumors, 32% showed deletion of the 6q15 region. This minimal deletion region was consistent with the deletion region observed in LNCaP and other previous reports on PCa [383-386]. RNA expression analysis of the five genes in the region revealed that only *MAP3K7* correlates with the CNA status, whereas the other four genes were either not expressed in the prostate or their expression did not differ between deleted and normal samples. These findings were validated by IHC using anti-Tak1 antibody, which showed a significant correlation between the 6q15 deletion and expression of Tak1 [387]. The deletion of *MAP3K7* was also significantly associated with GS and the correlation was stronger than the four other genes studied [387].

Functional assays were performed to further understand the impact of *MAP3K7* deletion in PCa [388], by knocking down Tak1 expression in murine prostate stem cells using short hairpin RNA (shRNA). *In vitro* loss of Tak1 expression resulted in increased cell viability, proliferation, migration and invasion. Engraftment of these

Tak1-attenuated cells under mouse renal capsules resulted in development of PIN consistent with the PCa-like phenotype and invasive carcinoma. Further analysis revealed that loss of Tak1 expression results in decreased activity of *c-jun*-NH₂-kinase and p38, which in turn leads to an increased proliferation. Under normal conditions and after TNF α stimulation, Tak1 activates JNK/p38 and NF κ B and regulates transcription of genes involved in survival, motility, inflammation and apoptosis [378, 380, 389].

MAP3K7 deletion in PCa was also investigated using FISH on 2,289 PCa [366]. Results showed 18.5% of all cases have heterozygous deletion at the 6q15 region. Deletion in this region was significantly associated with Gleason score, advanced tumor stage, lymph node metastasis and early BCR. However, the analysis did not show the deletion of *MAP3K7* as an independent prognostic factor. *In vitro*, the *Tak1* overexpression in DU145 and BPH-1 (prostate epithelial cell line) cells resulted in growth inhibition, while Tak1 knockdown did not significantly affect cell growth.

Furthermore, the meta-analysis of genomic studies revealed that 46.7% of all PCa cases harbor this deletion [370]. Overall, the *MAP3K7* gene deletion in the 6q15 loci shows significance relevance to the PCa outcome. Thus, the deletion of *MAP3K7* would be a viable biomarker for risk stratification of patients.

1.4.6. *WRN* (8p12)

WRN gene encodes Werner Syndrome RecQ Like Helicase or WRN protein, which is also known as RECQL2 or RECQ3. This protein is a member of RecQ family of DNA helicase and plays a vital role in maintenance of the genome stability, DNA repair, replication, transcription and telomere maintenance. Defects in this gene are the cause

of Werner syndrome, an autosomal recessive disorder characterized by accelerated aging and an elevated cancer risk [390]. WRN functions as a part of DNA repair machinery and mutations in these genes lead to significant genome instability and increased mutations at other loci [391]. Specifically, patients with Werner syndrome display high amount of genomic recombination and elevated frequency of large chromosomal deletions [392], which is also a characteristic of the PCa genomic mutational landscape (Figure 10).

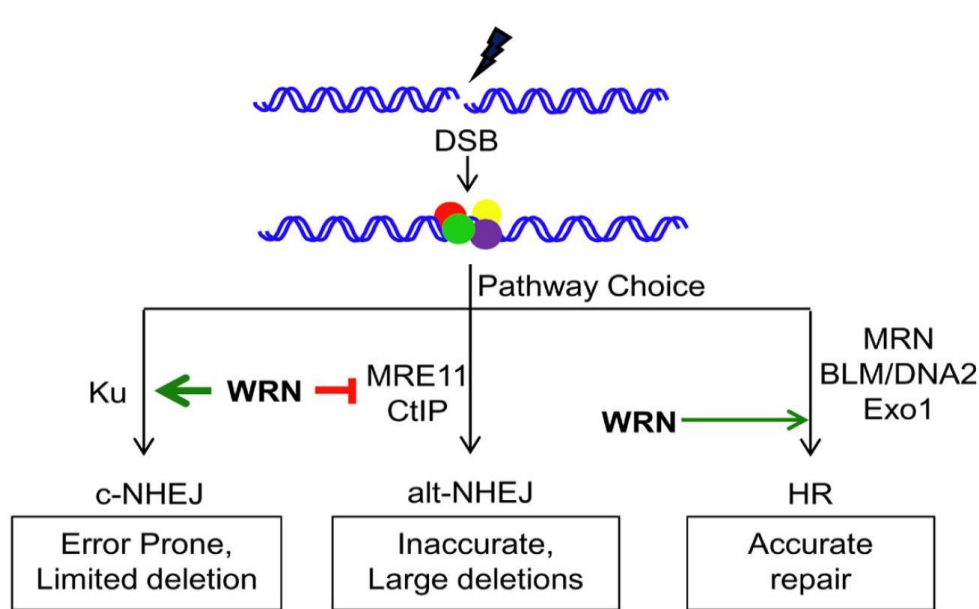


Figure 10: Mechanism of WRN in DNA repair pathways

DSBs generated by extrinsic and intrinsic factors are recognized by the sensor proteins Ku70/Ku80, WRN, MRN, and PARP1 to mediate repair. DSBs are repaired via classical/canonical non-homologous end joining (c-NHEJ), alternative (alt)-NHEJ, and homologous recombination (HR) pathways. WRN promotes Ku-dependent c-NHEJ with its catalytic activities and strongly inhibits alt-NHEJ with its non-catalytic activities. WRN suppresses the recruitment and downstream functions of MRE11 and CtIP to inhibit alt-NHEJ. During S/G2 phases of the cell cycle, WRN promotes HR. Accurate repair of DSBs is required for genome stability without loss of genetic information. Reproduced from [393].

Accordingly, the WRN network of protein-protein interactions involve pathways that control cellular response to DNA damage and replication (Table 7)

Table 7: Evidence on the roles of WRN in cellular response to DNA damage

Function	Reference
Translocation of WRN to the replication/repair site upon blockage of replication by hydroxyurea	[394]
WRN co-purifies with DNA replication complexes and interacts with PCNA and topoisomerase I	[395]
The single stranded DNA binding protein, RPA, interacts with WRN and enhances helicase activity	[396]
Werner syndrome cells show prolonged S-phase	[397]
Werner syndrome cells are hypersensitive to oxidative DNA damages and DNA stand breaks	[398]
WRN has functional interactions with DNA polymerase δ and is involved in DNA replication and repair	[399]

Furthermore, various studies have shown a direct interaction between WRN and p53, the tumor suppressor that is essential for maintenance of genomic stability [400, 401]. This is also shown in Werner syndrome cells that have reduced p53-mediated apoptosis, while overexpression of WRN in these cells can rescue this defect [400]. In normal cells, the p53-WRN complex can recognise abnormal DNA structures and damages, which lead to cell cycle arrest and initiation of DNA repair systems or induction of apoptosis. The DNA repair can take place via two different pathways that requires WRN function. First, WRN is able to directly restart replication by removing the damaged DNA or Okazaki fragments on the lagging strand via its 3'→5'

exonuclease activity. This has been shown by Shen *et al.* [402], with WRN exonuclease activity being essential for degradation of DNA structural abnormalities, suggesting that both helicase and exonuclease activities are required for DNA repair. This was further confirmed by Cooper *et al.* [403]. In the second pathway, WRN can repair the blocked replication fork by homologous recombination events. WRN can facilitate translocation of Holiday junctions by disassociating recombination intermediates and preventing abnormal recombination events [394].

WRN also directly interacts with DNA polymerase δ . However, this interaction is not required for normal DNA replication, rather it functions at the site of DNA damage or abnormal DNA secondary structures. It is required for the restart of blocked DNA replication and when the replication machinery has been detached from DNA [404].

Thus, the disruption of WRN activity can cause significant genome instability and increases the risk of various cancers [405-407], including PCa [280, 408-411]. Indeed, deletion of 8p arm (8p11-p24) is the most frequent alteration seen in PCa by meta-analysis studies [280, 370]. Frequency of this deletion is reported to be almost 62% in all PCa cases and increases to 90% in advanced disease [370]. The mapping of this deletion region was done using FISH and PCR in 14 PCa cell lines and xenograft models to the 8p12 region. Deletion of *WRN* was then confirmed in PCa xenografts [410]. Other studies further confirmed this results and deletion of this gene in PCa [408, 412].

1.4.7. *NKX3-1* (8p21.2)

NK3 Homeobox 1 or *NKX3-1* belongs to a subfamily of NK homeobox genes that play important functions in cell fate and organogenesis in a variety of species [413]. *NKX3-1* is mainly expressed in the prostate and involved in prostate development [413]. Developmental studies in mice have shown that expression of *NKX3-1* is the earliest known marker of prostate formation and it precedes expression of AR [413]. Functional studies showed that loss of *NKX3-1* during development leads to defects in the ductal morphology, cell differentiation and secretion of prostatic proteins and, most importantly, causes PIN which is a known precursor lesion of PCa [414].

NKX3-1 has DNA-binding activity and recognises the canonical “TAAT” sites [415, 416]. However, endogenous binding sites within the promoter or enhancer regions of downstream target genes have not yet been conclusively identified, although it has been proposed that the murine *AR* gene contains a functional *NKX3-1* binding site [417]. In addition to DNA-binding properties, the *NKX3-1* protein interacts with various other proteins. For example, *NKX3-1* interacts with serum response factor (SRF) to enhance binding to the DNA serum response element and activate transcription [418]. Furthermore, *NKX3-1* directly interacts with the MADS box of SRF via its homeodomain and can do this in absence of DNA [419].

NKX3-1 mainly functions as a transcriptional repressor, either by interacting with Gro/TLE corepressors or by repressing transcriptional activators such as Prostate-Derived Ets Factor (PDEF) [420]. PDEF activates the expression of *KLK3* gene, which encodes PSA, while *NKX3-1* can repress this activity [420, 421]. Moreover, *NKX3-1*

can also interact with the SP-1 transcription regulatory protein and represses the expression of PSA. [422].

In vivo studies using *NKX3-1*-deleted mice revealed defects in the production of prostatic secretory proteins and reduced ductal branching [413]. Most importantly, both homozygous and heterozygous *NKX3-1*-deleted mice showed these defects suggesting that deletion of one copy is sufficient for adverse effects on the prostate. As these studies show, *NKX3-1* is required for production of prostatic secondary proteins, maintenance of normal differentiated state of prostate epithelium and control of cell proliferation.

One of the consequences of *NKX3-1* deletion observed in animal studies is the development of prostatic hyperplasia and dysplasia that increases with advancing age in men [413, 414, 423-425]. Additionally, the prostatic luminal epithelium of these mice showed significantly higher proliferation rate compared to normal mice [414, 426]. Furthermore, the serial transplantation of prostatic tissue from the *NKX3-1*-deleted mice resulted in increased dysplastic histopathological alterations, with progression to cancer [409].

Another mechanism in which *NKX3-1* prevents cancer initiation or progression is by protecting the genome from oxidative damages [427]. Gene expression profiling revealed that loss of *NKX3-1* leads to deregulation of several antioxidant and prooxidant enzymes. This is consistent with the observation of PIN in the *NKX3-1* deleted mice, as formation of PIN is significantly associated with increased oxidative damages to the DNA [428]. *NKX3-1* directly interacts and activates topoisomerase I

which enhances its binding to the DNA. This interaction enhances cellular DNA repair, thus loss of *NKX3-1* predisposes PCa cells to further DNA damages [429].

Array-CGH studies of prostate tumors from a cohort of low- and intermediate-risk PCa patients treated with image-guided radiotherapy showed that *NKX3-1* haploinsufficiency is strongly associated with higher rate of genomic instability, as shown by higher rate of CNAs in the genome. [430]. Patients with *NKX3-1* deletion showed a higher rate of BCR after treatment. Further studies using post-treatment biopsy samples and cell line showed that *NKX3-1* deletion might be associated with resistance to radiotherapy due higher CNA rates. Along the same line, IHC studies on 57 PCa samples using anti-*NKX3-1* antibody showed that loss of *NKX3-1* expression is highly associated with advanced stage and hormone-refractory disease [431].

As mentioned above, the deletion of the 8p arm is the most frequent alteration in PCa and is commonly associated with adverse disease and poor prognosis. *NKX3-1* is one of the most recognized tumor suppressor gene in this region, which is prostate specific and involved in variety of functions, including prostate development and pathogenesis, cell growth and differentiation and response to DNA damages. Deletion of *NKX3-1* is one of the characteristics of PCa and is considered as a biomarker for prognosis.

1.4.8. *MYC* (8q24.21)

MYC is a well-recognized proto-oncogene and encodes the C-MYC protein, a nuclear phosphoprotein acting as a transcription factor, and thus playing a role in cell cycle progression, apoptosis and cellular transformation. C-MYC forms a heterodimer with the transcription factor MAX and binds to the E-box DNA consensus sequence

and regulates the transcription of specific target genes [432]. These target genes are induced in virtually all signal transduction pathways known to be altered in cancer, including, tyrosine kinase growth factor receptors, NF- κ B and β -catenin [433-437].

Gene expression profiling studies in clinical samples have shown that *MYC* is overexpressed in a majority of primary tumors [438-444]. The 8q24 region where the *MYC* gene is located is a frequent site of amplification in PCa and is associated with advanced and recurrent disease [445-447]. Gain of the 8q24 region was first identified using FISH and chromosome microdissection on 44 prostatectomy samples. The results showed gain in 9% of cases, however, among cases with advanced PCa, the frequency of gain was 75%. Further array-CGH study on 9 cases with recurrent disease revealed 8q24 gain in 8 cases [448].

There are a few genes in the 8q24 minimal gain region that could be responsible for the adverse effects and poor outcome in PCa patients. These genes include: the tyrosine phosphatase 4A3 (PRL3), which is associated with risk of metastasis in several cancers [449]; focal adhesion kinase (FAK) that regulates several cellular pathways involved in proliferation and migration, and its expression is increased in PCa compared with normal tissues [450, 451]; the squalene epoxidase (SQLE) which is involved in the synthesis of cholesterol and precursors of steroid hormones. High expression of SQLE is associated with both 8q amplification and poor prognosis in breast cancer [450].

To find out which gene is mainly responsible for the adverse effect seen by the gain of 8q24 region, both FISH and IHC studies were performed on TMA constructed from 325 PCa tissues [452]. The TMA included primary prostate tissues from 121 PCa patients with disease relapse matched with 121 tissues from PCa patients without

relapse, in addition to prostate tissues from 55 CRPC and 28 metastatic cases. Results indicated that 29% of cases showed gain in the 8q24 region, which was significantly associated with disease stage and GS. Moreover, 53% of metastatic cases and 35% of CRPC cases showed gain of the 8q24 region, while this gain was seen in 22% of patients with local disease. IHC results showed that only expression of C-MYC was associated with GS and disease progression, and most importantly, only expression of C-MYC was associated with the 8q24 gain. Additionally, tumor cells displayed a higher proliferation index in cases with the 8q24 gain significantly associated with higher probability of BCR.

The observation that gain of the 8q24 region is mostly seen in cases with late stage disease and aggressive PCa supports that C-MYC is associated with disease stage and metastasis [445, 453-460].

The fact that *MYC* overexpression is critical for the development of PCa was shown by a single step overexpression of the gene in primary cells obtained from benign prostate tissue, which immortalized the cells [461]. This was in part a result of upregulation of hTERT, which maintained telomerase activity and also the ability of these cells to bypass the Rb/p16 checkpoint. The transformed cells were also able to form colonies *in vitro*, which confirms cell transformation. Similar experiments of *MYC* overexpression in isolated prostatic epithelial cells also immortalized cells in a single-step transduction and cells were then able to form tumors in tissue recombination studies and also when injected under the mouse renal capsule [462]. Several mouse models were also developed by overexpression of *MYC*, which resulted in the development of PIN and PCa [463-465].

All these results indicate the significance of C-MYC in PCa pathogenesis. Furthermore, given the frequency of 8q24 gain and its association with poor outcome, the assessment of *MYC* amplification in this region could be a valid biomarker to predict patient outcome [458, 459].

1.4.9. *PTEN* (10q23.31)

The first evidence of a highly frequent deletion at the 10q23 region in PCa was reported in 1995 [466], the deletion being observed in 62% of all cases, a finding confirmed afterwards by additional studies [467-469]. Phosphatase and Tensin homolog or PTEN, a gene located on 10q23.31 region, was identified in 1997 by two separate teams [470, 471] as a tumor suppressor mutated in samples from patients with various cancers, including PCa, glioblastoma, breast, and kidney.

PTEN is a tyrosine and serine/threonine phosphatase that dephosphorylates the 3rd position of the inositol ring of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of phosphoinositide 3-kinase (PI3K) pathway, to regenerate phosphatidylinositol 4,5-bisphosphate (PIP2) [472, 473]. This results in a negative regulation of the PI3K/AKT/mTOR pathway [276]. The activation of the AKT protein kinase by PIP3 is known to regulate multiple downstream target proteins, such as BAD, CASP3, CASP9, MDM2, mTOR, FKHR10, FOXO3A, p27 and JNK. These will impact multiple pathways involved in apoptosis and cell-cycle progression (Figure 11) [474].

Further evidence on the tumor suppressor function of PTEN was obtained by studies showing that *PTEN* expression in PCa models can hinder cell cycle progression by

inducing a G1 arrest, inhibition of cell migration and induction of apoptosis [475]. Thus, loss of function or deletion of the *PTEN* gene leads to constitutive activation of the PI3K/AKT pathway that enhances cell proliferation and tumor angiogenesis and inhibits apoptosis [476-478].

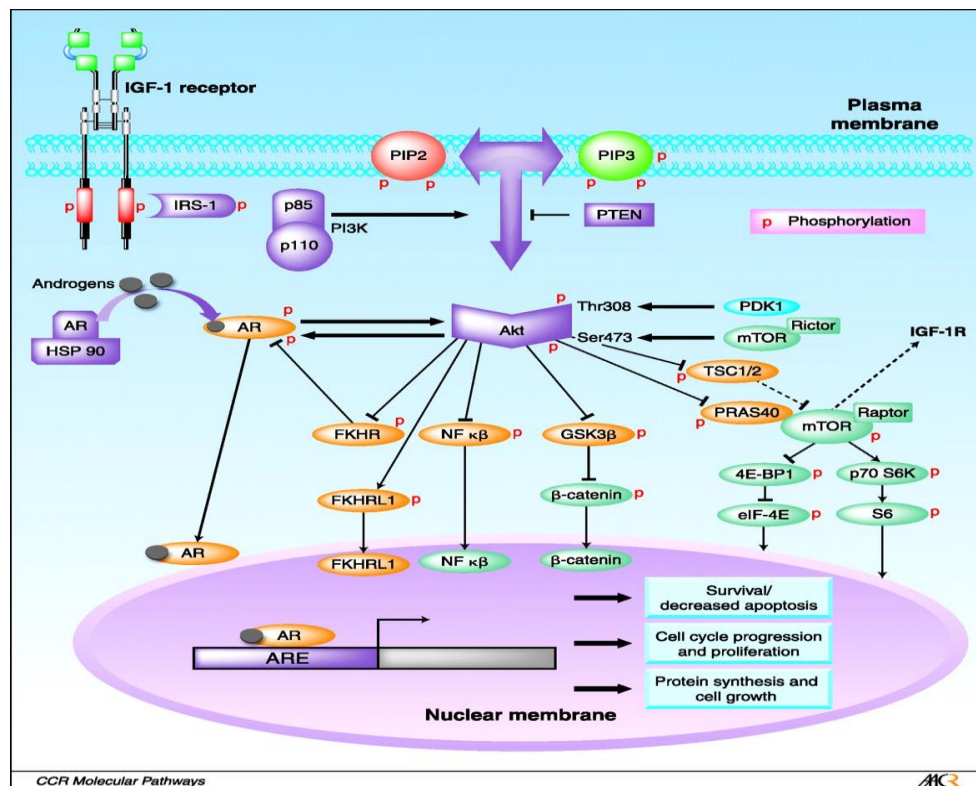


Figure 11: PTEN in the PI3K/AKT pathway and downstream signaling

Deletion or loss of *PTEN* function removed the inhibition from the AKT pathways which affects variety of cellular functions. Activation of the PI3K pathway leads to AKT phosphorylation, triggering a downstream cascade of events that are likely to interact with AR transcriptional activity. These include interaction of the AR with FKHR and FKHRL1 transcription factors, crosstalk of AR and AKT with NF κβ; regulation of AR via coactivator Wnt/β- catenin, and activation of AR via the mTOR pathway. Reproduced from [479].

Various studies using animal models with *PTEN* haploinsufficiency revealed that even small changes in *PTEN* expression could have significant impact on cell fate [480-485]. For instance, mice with a single copy deletion of the *PTEN* gene develop tumors in various organs, a finding which suggests that the PTEN protein functions in a dose-dependent manner [480]. Furthermore, mice with a single copy of hypomorphic allele, which expresses a half of the amount of PTEN protein expressed by a wild-type copy, or a single wild-type copy show prostate anomalies compatible with cancer initiation and progression [482]. Furthermore, homozygous deletion of *PTEN* causes a rapid development of HGPIN and invasive PCa in mice at 3 months of age [276, 482-487]. On the other hand, overexpression of *PTEN* in mice that spontaneously develop PCa results in resistance to cancer development [484, 485]. These findings illustrate the significance of *PTEN* in the control of prostate cell fate and even a small decrease in expression results in PCa.

In 16%-41% of all PCa clinical studies, *PTEN* is often deleted or rearranged, while point mutations are rare [138, 139, 488]. For example, a sequencing analysis of the *PTEN* gene in more than 300 primary tumor samples showed 15% copy number loss, whereas, the frequency of point mutations was only 2.4% [276].

The *PTEN* gene deletion is recorded in various FISH studies, performed on primary prostate tumors. The frequency of deletion was 40% in a cohort of 330 cases [489], a finding in agreement with 41% heterozygous deletion reported in our lab (by Choucair *et al.* [490]), which showed a higher rate of BCR and lower AR signaling in *PTEN*-deleted patients. Association of *PTEN* deletion with higher probability of BCR was also reported in a cohort of 612 patients, which showed a correlation with GS [491].

Furthermore, *PTEN* deletion was associated with more aggressive disease as 77% of cases in a cohort of 59 CRPC patients showed this CNA. [492].

PTEN deletion was found in a larger RP cohort of primary tumors from 4,699 patients, at a frequency of 20% [493], and was significantly associated with GS, disease stage, metastasis and higher risk of BCR. Altogether, these data of a significant correlation between *PTEN* deletion and PCa poor outcome [494-496] strongly point to the utility of *PTEN* deletion as a biomarker for PCa prognosis.

1.4.10. *CDKN1B* (12p13.1)

CDKN1B, also known as p27 or KIP1, is a cyclin-dependent kinase (CDK) inhibitor that binds and inhibits the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls cell cycle progression at the G1 phase. Phosphorylation of *CDKN1B* by CDK leads to its ubiquitination and degradation, which is required for the cellular transition from quiescence to the proliferative state [497, 498]. Thus, *CDKN1B* is a negative regulator of the cell cycle and has tumor suppressor functions. Loss of *CDKN1B* function has been reported in a variety of cancers, including breast [499], colon [500], lung [501], and prostate [502-507].

The gene is located at the 12p13 region, and deletion was reported in about 28% of all PCa cases by two meta-analysis genomic studies [280, 370]. There is an enrichment of the 12p13 deletion in advanced cases and nearly 57% of patients with metastatic and advanced disease have this deletion. To identify the specific genes located in this region, representational difference analysis was performed on metastatic PCa xenografts and normal samples [508, 509]. Results showed that 50% of metastatic PCa

tumors harbor deletion at the 12p13 region and the minimal region of deletion contained two genes, ETV6/tel and *CDKN1B*. While involvement of ETV6/tel has not been shown in PCa, loss of *CDKN1B* expression has been reported by several studies [510-515].

For instance, an IHC study showed a complete loss of *CDKN1B* expression in 67.5% of primary tumors (n=40) and 40% of unmatched metastases (n=5) [510]. Furthermore, reduced *CDKN1B* expression was significantly associated with higher cell proliferation and higher tumor grade. These results were confirmed by another study on a cohort of 138 RP specimens and low *CDKN1B* expression was associated with a higher GS and pathologic stage [511]. Additional IHC studies revealed that the decreased expression of *CDKN1B* was an independent predictor of BCR (n=86 RP samples) [515], significantly associated with higher risk of early relapse after treatment [504, 516], disease-free survival and overall survival (n=96) [513].

FISH analysis on more than 3,700 primary prostate tumor samples was performed using a probe targeting *CDKN1B* [517]. The *CDKN1B* deletion was observed in 13.7% of all cases. This deletion was significantly associated with higher pre-treatment PSA levels, higher GS, advanced tumor stage, lymph node metastasis and higher risk of BCR. A higher rate of cell proliferation was evidenced in tumors with *CDKN1B* deletion and overall, the deletion was enriched in patients with invasive disease.

Taken together, the loss of *CDKN1B* tumor suppressor function is a frequent event in PCa correlating with more aggressive tumor features and patient outcome.

1.4.11. *RB1* (13q14.2)

RB or retinoblastoma protein is also known as Transcriptional Corepressor 1 or RB1. The protein is encoded by *RB1* gene, which is the first identified tumor suppressor gene [518]. The *RB1* gene was first mapped on chromosome 13.q14 [519] through cytogenetic studies. The RB1 protein has a significant role in the regulation of cell cycle, resulting from its interaction with transcription factors of the E2F family (Figure 12) [520-522].

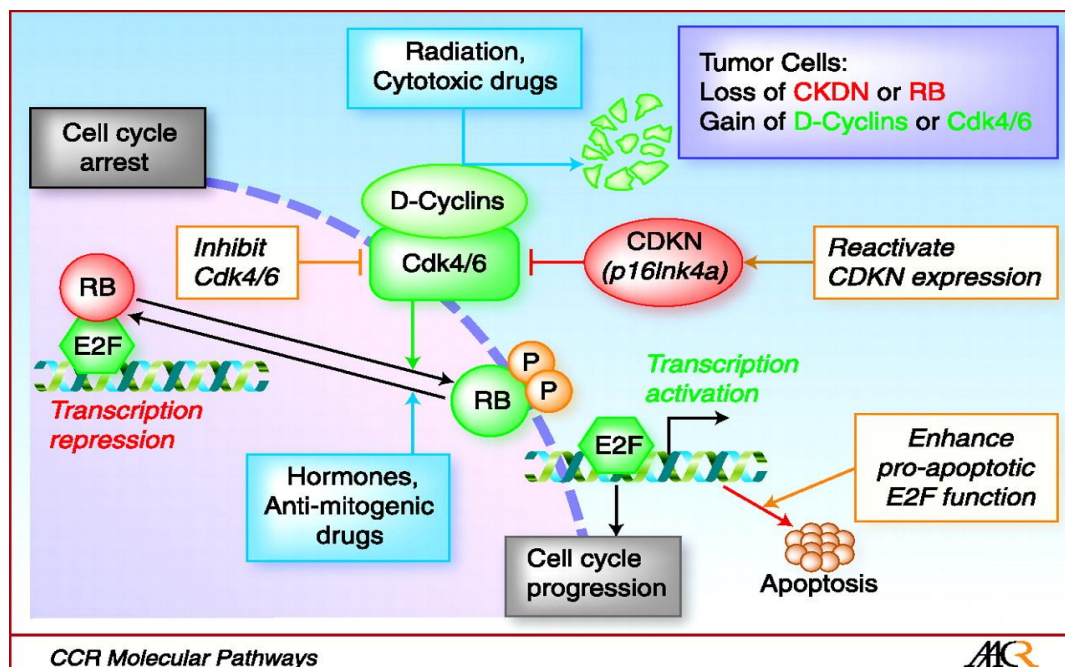


Figure 12: RB1 pathway alterations in cancer

The components of the RB-pathway, i.e., RB, E2F, d-type cyclins, Cdk4/6, p16Ink4a (CDKN2a), and their functional interactions, are depicted in the diagram. Genetic and epigenetic alternations in the RB-pathway are consistently detected in the majority of sporadic human cancers. The status of the RB-pathway affects tumor cell responses to radiation and genotoxic drugs, which cause cell cycle arrest through the degradation of cyclin D1 and the consequent RB dephosphorylation. The status of the RB-pathway also affects tumor cell responses to hormone and other therapeutic strategies that block mitogenic signaling. Defects in the RB-pathway cause deregulated E2F activity, which stimulates gene expression to promote G1/S transition and apoptosis. Reproduced from [523].

RB1 binds to E2F proteins and either inhibits the recruitment of transcriptional co-activators or recruits transcriptional co-repressors to the promoter regions of gene involved in progression to the S-phase and preceding to cell division, thus blocking the expression of these genes and halting the G1/S cell cycle transition.

In a normal cell cycle, after mitogen stimulation, CDK4, CDK6 and CDK2 become activated and can then proceed to phosphorylate RB1 [524-526]; this will result in the release of the E2F transcription factors and recruitment of co-activators to transcribe genes that allow cells to progress through the G1/S-phase [527]. The negative regulation of the cell cycle through RB interaction with E2F is considered as the main mechanism for RB1 to exert its tumor suppressor function. However, RB1 is known to interact with more than 200 proteins, such as histone acetyltransferases (HATs), deacetylases (HDACs), SWI/SNF chromatin remodelers (SMARCA2, SMARCA4), and DNA repair factors (BRCA1, CtIP, RPA) and several other proteins involved in multiple pathways beyond cell cycle control [528]. The RB1 protein is considered as a platform for multiple protein contacts [520] and, in addition to being a transcription repressor of E2Fs, it functions as a chromatin-associated protein [529].

However, previous studies show that tumor initiation in *RB1*-deleted cells requires functional E2Fs, indicating that the main tumor suppression activity of RB1 is through its interaction with E2F [530]. While the control of cell cycle is the main result of this interaction, several proteins involved in apoptosis, cell differentiation and stem cell biology, cell adhesion are also known to be affected by RB1 function [531-534].

Furthermore, RB1 plays a role in maintaining genome stability [535]. Indeed, RB1 is recruited at the site of DNA DSBs and its loss impacts HR and the DNA repair machinery [536]. It participates in TopBP1-E2F1-RB-BRG1 complex at the DSB site and functions to protect E2F1 from proteolytic degradation, thus allowing proper HR and DNA repair [536-539]. This was further proven by the fact that *RB1*-deficient cells have increased genomic instability and are susceptible to radiation-induced DNA damage and chemotherapeutic agents, such as PARP inhibitors, which is consistent with a defect in the HR pathway [540].

The deletion of 13q12-q31 is a frequent event in PCa and based on meta-analyses, it is observed in 45% of all primary and 90% of advanced tumors [370]. A loss of heterozygosity (LOH) at 13q14 performed in 51 HGPIN, 21 cases of non-symptomatic, 31 cases of indolent, and 102 cases of PCa with clinical significance showed the deletion in 0%, 38%, 56% and 49% of cases, respectively [541]. Authors concluded that the deletion of the 13q14 region is associated with tumor initiation and is an important event in progression of PCa.

Complementary studies by restriction fragment length polymorphism, followed by Southern blotting and hybridisation showed the *RB1* deletion in 60% of the 40 studied PCa samples [542]. A decrease expression of the RB1 protein was detected by IHC in samples with deletion at the 13q14 region. However, there was no association between the deletion status, disease stage and grade. Similarly, in another study, the deletion of this region was seen in 50% of primary tumor samples (n=36) [516]. However, since the expression analysis of RB1 did not correlate with the deletion status, authors concluded that the 13q14 deletion is an early and non-random event in PCa. Moreover,

they stated that other tumor suppressor genes than *RB1* might be the target of the 13q14 deletion and PCa tumorigenesis.

The deletion of 13q14 was recently assessed by FISH on 7,375 primary prostate tumors [543] and 21% of cases were harboring the deletion. One of the two FISH probes required for a call for deletion specifically targeted the *RB1* gene. The analysis with this probe showed a significant correlation between deletion at 13q14 and pre-treatment features such as higher PSA levels, high GS, advanced tumor stage, and lymph node metastasis. Deletion of this region was also associated with higher risk of BCR. Furthermore, multivariate analysis revealed that deletion of 13q14 region is an independent predictor of BCR irrespective of pre-treatment parameters.

Collectively, the above observations on the deletion of the 13q14 region indicate a frequent event of 18% to 72% in prostate tumors of all stages [516, 544-551] supporting a role in tumor initiation. As *RB1*, one of the main tumor suppressor genes in this region is involved in maintenance of genomic stability, the deletion of 13q14 can lead to poor prognosis, which is indeed evident by enrichment of this deletion in advanced cases [370].

1.4.12. *PDPK1* (16p13.3)

The 3-Phosphoinositide Dependent Protein Kinase 1 (*PDPK1*) gene encodes the PDK1 protein, which is a member of AGC protein kinases [552]. PDK1 was discovered by its ability to phosphorylate AKT and activate the PI3K pathway (Figure 10) [553].

Furthermore, PDK1 can phosphorylate various other proteins, such as p70 ribosomal S6 kinase (S6K) leading to activation of the protein synthesis machinery and cell

growth [554-558]. Other kinases, including serum glucocorticoid-dependent kinase (SGK), p90 ribosomal protein S6 kinase (RSK) and protein kinase C (PKC) are also known to be direct targets of PDK1, which phosphorylates specific serine/threonine residues of their activation loop [559]. For this reason, PDK1 is known as “master regulator” of AGC kinase signal transduction and regulates main pathways controlling cell survival, proliferation, and motility [559]. *In vivo* study of the PDK1 function revealed that *PDPK1* is an essential gene and mice with homozygous deletion will not survive past the embryonic stage [560]. Animal study using hypomorphic allele of *PDPK1* showed mice with a 40-50% decreased body size compared to wild-type mice; however, no significant changes in the activation of AKT, S6K and RSK were observed. Further analysis indicated that this decrease is due to smaller cell size rather than reduction in cell number [560]. The cross breeding of mice harboring the hypomorphic allele of *PDPK1* with *PTEN* heterozygous mice resulted in offspring with a lesser frequency in tumor development and increased *PTEN*/PI3K/AKT pathway, suggesting an oncogenic function of PDK1 [561]. Indeed, overexpression and gain in the copy number of *PDPK1* has been reported in various cancers including gastric, leukaemia, ovarian breast and prostate [562-568]. Furthermore, overexpression of PDK1 is sufficient to transform mammary epithelial cells *in vitro* [569].

Abnormal PI3K signaling and de-regulation of *PTEN* is shown to be strong indicators of metastasis and poor patient outcome in many cancers [570, 571]. The role of PDK1 in PCa has been described by the host lab, Choucair *et al.* [572], where knocking down PDK1 in PCa cell lines reduced migration. Further, exogenous expression of PDK1 rescued motility. Copy number analysis of *PDPK1* in 75 samples from PCa patients,

including 10 lymph node metastases and their matched primary tumors, 9 prostate tumor samples from CRPC patients, and 46 additional primary tumor specimens showed gain in 50% of metastases and 60% of their matched primary tumors, 33% of CRPC samples, and 20% of primary tumors. Gain of *PDPK1* was significantly associated with pre-treatment PSA levels and higher GS.

In a recent study in our lab by Bramhecha *et al.* [286], FISH analysis performed on 267 RP specimens showed 42% gain in primary tumors. Gain of *PDPK1* was highly associated with pre-treatment PSA levels, higher GS and higher pathological stage. Gain of *PDPK1* was shown to be an independent predictor of BCR. Remarkably, the prognostic value of *PDPK1* gain was also observed in low- and intermediate-risk patients ($GS \leq 7$) with $PSA \leq 10$ ng/ml. Furthermore, gain of *PDPK1* was a predictor of metastasis and aggressive disease. These observations indicate a strong prognostic value of *PDPK1* gain in PCa. Since the detection of *PDPK1* gain in primary tumors can be an indication of poor outcome and aggressive disease, this gene represents a robust biomarker for the risk stratification of patients.

1.4.13. *GABARAPL2* (16q23.1)

The GABA Type A Receptor Associated Protein Like 2 (*GABARAPL2*) is located at 16q23.1, a frequent deletion site in PCa. *GABARAPL2* belongs to LC3/GABARAP protein family involved in autophagy and regulation of intracellular trafficking [573]. More specifically, these proteins function to maintain cell homeostasis and energy levels through directing lysosomal degradation of old/damaged organelles (e.g., mitochondria), protein aggregates, and pathogens [574, 575]. Metabolic stress, such as insufficient glucose or amino acids and hypoxia, causes an upregulation of the

autophagy process in order to sustain cellular energy level and provide building blocks for essential cellular processes [576, 577].

Cancer cells require more energy for sustaining their rapid growth, thus, the main energy producing organelle of the cell, mitochondria, usually display upregulated metabolism and an increased number [578]. On the other hand, increased mitochondrial metabolism leads to the formation of by-products such as reactive oxygen species (ROS) causing DNA damages and mutations [579]. Therefore, dysregulation in maintenance of a healthy population of mitochondria causes an accumulation of oxidative stress and DNA damage leading to decreases in DNA stability. Regulation of mitochondria number is an autophagy-dependent process, called mitophagy, which requires the function of LC3/GABARAP family of proteins [573] co-localized with depolarized mitochondria during basal, starvation, and apoptotic conditions [580-582]. Deficiency in these proteins lead to reduced autophagic flux, including mitophagy, and results in an accumulation of damaged mitochondria and oxidative stress [582].

GABARAPL2 plays a specific function in mitophagy by interacting with FUN14 domain-containing (FUNDC)-1, an integral mitochondrial outer-membrane protein, and the target of ubiquitination that results in mitophagy. Interaction of GABARAPL2 with FUNDC-1 during hypoxia results in increase colocalization and interaction of FUNDC1 and other GAPBARAP family of proteins and eventually degradation of mitochondria [583].

The tumor suppressor gene function of GABARAP family has been reported. For instance, in breast cancer primary tumors and cell lines, the mRNA and protein level of

GABARAP is decreased, and exogenous expression of *GABRAP* gene decreases tumor growth rate [584]. Furthermore, this tumor suppressor function was confirmed by injection of GABARAP-proficient cells in GABARAP-knockout mice, which resulted in reduction of tumor growth [585].

In PCa, genome-wide CNA analysis of 34 surgical CRPC specimens and 5 xenografts, with matched transcriptomic profiling of 25 specimens, revealed that *GABRAPL2* expression was significantly reduced in CRPC samples. Furthermore, deletion of *GABRAPL2* was significantly associated with CRPC and transcript expression levels [586]. Study of 16q23-24 CNA in 32 cases showed a 75% frequency of deletion in primary tumors, and 52% of lymph nodes and all brain metastases [587]. This led to the conclusion that deletion of 16q23-24 region in PCa is significantly associated with metastasis. This is consistent with FISH analysis of the 16q deletion observed in 50% of cases in a series of 30 tumors. Further mapping of the deletion site showed that the minimal alteration region is 16q23.1 [588]. Moreover, the deletion of 16q23 was found in 32% of patients with localized disease compared to 80% of patients with metastasis; thus, it was concluded that the deletion was associated with metastasis and invasiveness in PCa. [589]. Further FISH analysis of the 16q23 region in 7,400 cases revealed that 21% of patients harbored this deletion [590]. Deletion of 16q23 was linked to high GS, higher proliferation index, advanced tumor stage, lymph node metastasis and invasive disease. The meta-analysis of genomic studies on the deletion of 16q23 refers to an occurrence in 18% - 52% of all PCa cases [280, 370], with a frequency reaching 89% in advanced cases [370]. Therefore, the deletion of 16q23

region together with the *GABARAPL2* could serve as a biomarker of advanced disease and poor outcome.

1.1.14. *TP53* (17p13.1)

The gene encoding the transcriptional factor P53 or *TP53* is one of the most recognized tumor suppressor genes and its protein product plays a vital role in cell cycle control and homeostasis [591]. Cellular stress, DNA damage or oncogenic transformation result in activation of P53 and the transcription of series of genes involved in cell cycle arrest, DNA repair, or in cases of irreparable damages, apoptosis [592]. The fact that P53 is involved in other pathways like senescence, angiogenesis, autophagy, metabolic regulation, development, and stem cell biology, illustrates the complexity and significance of the P53 protein in cell fate [593, 594]. Many of the P53-mediated effects are achieved by the functions of genes that are up or down regulated by P53. These P53-responsive genes include *WAF1* that functions as a cell cycle checkpoint control, *BAX* that controls apoptosis, thrombospondins 1 and 2, and *VEGF* which regulate ROS levels and angiogenesis. Other genes, such as *MDM2*, control P53 expression and function through a feedback regulation [595].

In PCa, P53 acts as a negative regulator of PSA expression [596]. This was shown by cDNA microarray and transient expression analysis resulting in a four-fold increase of PSA expression when suppressing wild-type P53 activity in the LNCaP cell line. Due to the significant impact of P53 in cellular function and regulation, it is not surprising to find *TP53* inactivating mutations in more than 50% of human cancers [592]. Rate of single nucleotide mutations in PCa is generally low, however, *TP53* still represents one the highest mutated gene [597]. In a study of 218 PCa samples, only two primary

tumors showed *TP53* missense mutations, while 24% of cases showed deletion of the gene [138]. Consistent with this result, in the TCGA study [276] on the molecular taxonomy of prostate cancer, only 7% of primary tumors out of 333 samples showed a *TP53* mutation, while 31% harbored copy number deletion of the gene. Deletion or inactivation of *TP53* or abnormal *TP53* expression in PCa have been reported by various types of studies. FISH analysis on 37 RP samples from patients who died from the disease and 26 matched patients responding to therapy revealed that 31% of samples harbored the 17p13 deletion and loss of *TP53* was observed in 46% of patients with poor outcome compared to 15% of patients with favorable outcome [598]. IHC analysis in 150 primary tumors showed altered expression in 13% of samples, and abnormal expression was strongly associated with disease stage [599]. A more complete study [600] included analyzed *TP53* status in PCa using FISH, IHC and gene sequencing and again, *TP53* showed low number of single nucleotide mutations while genomic copy loss was the common mechanism of P53 inactivation. A high correlation was observed between FISH and IHC results and loss of P53 function by either method supported a significant association with poor outcome. Out of 7,961 analyzed cases, 15% showed deletion of *TP53* by FISH. Deletion was associated with higher Gleason grade and risk of BCR, metastasis and advanced stage. The consensus is that the *TP53* deletion in primary tumors is associated with increase risk of disease progression and poor outcome.

An overview of genes studied in this thesis is presented in Table 8.

Table 8: List and functions of candidate genes under study in thesis

Genes	Locus	Functions in PCa	CNA
<i>RWDD3</i>	1p21.3	• Maintenance of genome stability • PCa progression • Early BCR	Deletion
<i>PDZD2</i>	5p13.3	• Early PCa tumorigenesis • Progression of bone metastasis • Hereditary PCa	Gain
<i>GTF2H2</i>	5q13.2	• Maintenance of genome stability • Phosphorylation of AR for accurate transactivation	Deletion
<i>CHD1</i>	5q15-q21.1	• Maintenance of genome stability • Progression to CRPC	Deletion
<i>MAP3K7</i>	6q15	• Early PCa tumorigenesis • Increases cancer cell proliferation	Deletion
<i>WRN</i>	8p12	• Maintenance of genome stability	Deletion
<i>NKX3-1</i>	8p21.2	• Early PCa tumorigenesis • Maintenance of genome stability • Early BCR	Deletion
<i>MYC</i>	8q24.21	• PCa tumorigenesis • PCa progression • Early BCR	Gain
<i>PTEN</i>	10q23.31	• PCa tumorigenesis • PCa progression • Early BCR	Deletion
<i>CDKN1B</i>	12p13.1	• PCa progression • Early BCR	Deletion
<i>RB1</i>	13q14.2	• PCa tumorigenesis • Maintenance of genome stability	Deletion
<i>PDPK1</i>	16p13.3	• PCa progression • PCa metastasis • Early BCR	Gain
<i>GABARAPL2</i>	16q23.1	• PCa progression • PCa metastasis • Early BCR	Deletion
<i>TP53</i>	17p13.1	• PCa tumorigenesis • PCa progression • Early BCR	Deletion

1.5. CNA DETECTION METHODS

Initially, techniques such as Southern blot hybridization, FISH and microsatellite scanning provided the standard and optimum methods for identifying copy number of specific DNA segments. However, these methods lacked scalability [601]. A major advantage of FISH compared to other methods is the sole contribution of cancer cells to the copy number data and therefore the lack of influence from cellular elements found in the stroma. However, resolution is commonly more than 20 kb, which does not allow detection of microdeletion and copy number changes at exon level. FISH is also labor-intensive, requires well-trained or highly skilled personnel and is difficult to adapt to the high-throughput settings of most hospital-based pathology facilities. Furthermore, due to limited multiplex capability, multiple FISH need to be performed for analysis of several regions [602].

New techniques, such as array-based approaches and sequencing are more suitable for whole genome scanning and finding new CNAs, while quantitative and semi-quantitative PCR-based assays are used for locus-specific examination of the genome. Quantitative array-based methods allow robust analysis of copy number at whole-genome level. Different platforms exist that employ genomic DNA, cDNA clones, PCR products or oligonucleotides. Furthermore, different hybridization techniques such as competitive hybridization of two DNA sources or single-source hybridization can be used.

Comparative genome hybridization (or CGH) assess copy number variations between two differentially labelled tests and reference genomes [603]. To detect CNA, the fluorescence ratios between the test and reference sample are assessed, which can then

be translated into gains or deletions. In the array-CGH, the DNA is hybridized to specific DNA sequences spotted on precise locations on a solid surface. This approach increases the specificity and the resolution of the assay [604]. Alternatively, cDNA clones [605], bacterial artificial chromosome (BAC) clones or specific synthetic oligonucleotides can be used [606-614].

Single nucleotide polymorphism (SNP) chips are also routinely used for CNA analysis [615-621]. The main difference between SNP chips and array-CGH is that the SNP chips do not directly compare the hybridization between two DNA sources. Each the test and reference genomes are rather hybridized separately, and the intensity of hybridization are then compared [601].

DNA copy number can also be assessed using sequencing methods and *in silico* analysis. The advantage of sequencing is its ability to detect practically any type of variation, even down to the single nucleotide. This provides an advantage over array-based methods, where the resolution is dependent on the density of probes spotted on the array. However, due to the current high cost of generating full sequence coverage, the use of sequencing for analysis of large sample sets from the clinic are not feasible. Detection of copy number analysis in sequencing data is usually done by assessing read depth of sequenced genomic fragments. The regions of the assembly with greater read depth are presented in multiple copies and, regions with lower read depth are represented with a lower copy number in the genome.

Quantitative methods are effective techniques for assessing CNA in a loci specific manner. This allows assessment of regions associated with a specific disease and pathology in a cost effective and high throughput means.

qPCR is a quantitative technique that assesses the amplification rate of a specific loci. The amplification rate in the PCR reaction is proportional to the copy number of the assessed region. Monitoring the reaction in the real-time allows to determine the start of exponential phase and the copy number before the plateau phase.

It is also necessary to use control regions that do not undergo copy number variation to control for technical variations, such as difference in the DNA input or reaction conditions. Although, current qPCR methods are suitable for assessing CNAs [622, 623], they generally do not have a multiplexing capability to assess several regions.

Probe-based multiplex assays such as Multiplex Amplifiable Probe Hybridisation (MAPH) [624, 625] and Multiplex Ligation-dependent Probe Amplification (MLPA) [626-628] allow differences in copy number to be detected based on quantification of probes-specific target regions in a multiplex manner. In MAPH, the test DNA is bound to a nylon filter after denaturation, the specific probes are then added and amplified. In the design stage, each probe is given a specific length and a universal binding sequence that allows amplification of all probes with fluorescently labelled primers and their subsequent separation based on their size using gel electrophoresis. This approach allows assessment of up to 50 different loci in a single reaction [629].

MLPA is like MAPH; however, it is performed in solution. Moreover, in MLPA, each sequence is targeted by two probes that are designed to hybridize adjacent to each other at the target region. After the ligation the step, a contiguous probe molecule is created. Each probe contains a sequence that serves as a template for universal fluorescently labelled primers in a subsequent amplification step. Such PCR amplified probes can then be separated by electrophoresis, where the fluorescent intensity can be measured

and expressed proportionally to the initial copy number of the target sequence. In the initial protocol for MLPA, each probe was cloned in a vector. A more recent advance [630] has demonstrated that synthesised probes work equally well, but there is a limit to the size of probes that can be produced. MLPA can be used to assess the CNA of up to 50 different loci in a single reaction. Once the probe mix has been designed and optimized, it can work reproducibly in screening large cohorts of clinical samples [628].

2. CHAPTER 2 - RATIONALE, HYPOTHESIS, OBJECTIVES AND METHODS

2.1. RATIONALE

PCa is a highly heterogeneous disease with a wide range of clinical outcome. With the widespread PSA screening, most patients are diagnosed with low- to intermediate-risk disease [631]. Currently, pre-treatment clinicopathological features such as pre-treatment PSA levels, clinical stage and biopsy GS are used for risk-stratification of patients and therapeutic decision-making. For this purpose, various risk stratification systems have been developed that can predict the risk of aggressive disease with good accuracy [129-131, 632, 633]. However, significant clinical heterogeneity is still observed in these risk groups, which leads to over-treatment of patients with insignificant and slow-growing tumors and under-treatment of patients with potentially aggressive disease. This clinical heterogeneity is mostly seen in the intermediate-risk category, where current clinicopathological risk stratification modalities fail to provide accurate risk assessment [634]. Thus, there is an unmet need for clinically applicable biomarker assays that can improve risk stratification of this growing population of patients, which represents the main group requiring curative therapies. Furthermore, the new assay can be used to assess the association of selected CNAs with response to different therapeutic approaches (i.e. radical prostatectomy, radiotherapy and ADT) and to develop predictive CNA signatures. A recent study also suggest that patients with defects in the DNA repair machinery might respond to other systemic therapies such as PARP inhibitors [635]. Accordingly, an increase in the percentage of genes with CNAs and/or deletion of genes involved in maintenance of genomic stability might indicate a better response to such therapeutic drugs.

Several next generation sequencing studies with deep coverage have collectively demonstrated a low rate of single nucleotide mutations in PCa, which is significantly lower in comparison to other cancers [636]. More interestingly, the rate of single nucleotide mutations between localized and advanced disease does not increase significantly [488].

These genome-wide analyses of primary tumors of PCa patients in different stages of the disease have also demonstrated a significant correlation between CNAs and initiation of cancer and progression [138, 276, 282, 310], with the rate of CNAs exceeding single nucleotide mutations. Moreover, the rate and the landscape of CNA differ between localized indolent and aggressive disease [280, 370]. There is an increasing body of evidence showing the prognostic power of such genomic alterations, and consequently, several candidate CNAs have been introduced and validated as biomarkers [287, 289, 292, 366, 452, 517, 543, 545, 600]. Meta-analysis studies have shown that the frequency of several CNAs such as 5q15-q21, 6q15, 8p12-24, 8q24, 10q23.1, 12p13.1, 16p13.3, 16q23.1 and 17p13.1 significantly increases in advanced PCa [280, 370]. Moreover, the rate of CNAs per se is prognostic of aggressive cancer. While higher number of CNAs as a side-effect of genome instability is prognostic, studies also show the prognostic power of CNAs in certain cytobands for patients with low- and intermediate-risk disease [70, 281, 637]. In early stages, CNAs in oncogenes and tumor suppressor genes can alter the biology of cancer cells. They may also be a precursor of genomic instability that is an indicative of aggressive disease. The prognostic efficacy of CNAs in predicting aggressive PCa has been extensively validated, but the clinical utility of these genomic biomarkers has been

hindered, mainly due to lack of suitable assays [285]. Current CNA detection methods do not have the multiplexing capability to assess CNA signatures or are not compatible with low-quantity and quality of DNA obtained from FFPE biopsy samples. As mentioned above, the routinely used FISH assays in the clinic can only assess up to four cytobands in a single reaction. Other genomic assays such as qPCR face the same issue and do not have multiplexing capability to assess sufficient CNAs for accurate prognosis. Microarray and sequencing techniques require large amount of DNA, often not compatible with FFPE extracted DNA and are not cost-effective for routine clinical applications [285]. New assays that can accurately assess multiple CNAs relevant to distinguish cases at risk of progression using the low-quality and quantity of DNA that can be extracted from biopsies is needed in the clinic to improve risk stratification of patients and help in the therapeutic decision-making process of active surveillance vs. definitive therapies. Comparison of different CNA detection methods reveals that multiplex ligation-dependent probe amplification (MLPA) is compatible with low-quality and quantity of DNA obtained from FFPE samples, has a low cost and is compatible with clinical setting as it does not require sophisticated instruments or analysis approaches. MLPA was thus chosen for development of the assay to detect CNAs in PCa. Previous studies show that MLPA assays using synthetic oligonucleotide probes can include up to 20-30 probes per assay [630, 638]. Therefore, we performed a literature review and identified recurrent CNAs showing prognostic value in PCa that were included in the assay. We then analyzed the genes in the minimal region of alterations and selected 14 genes showing relevance to tumor biology. We further identified 10 CNA-quiet regions to serve as references. We further

improved the assay to include up to 38 probes, targeting each gene of interest by two probes for more accuracy.

2.2. HYPOTHESIS

Based on the available data and state of knowledge in the PCa field, we hypothesize that:

- 1) Certain CNAs can be identified in primary tumors of low- and intermediate-risk PCa patients and be used for prediction of aggressive disease.
- 2) Combination of certain CNAs, which affect tumor biology can predict aggressive disease, independently of clinicopathological features.
- 3) Combination of a CNA-based risk classifier and clinicopathological features can improve risk stratification in low- and intermediate-risk categories of PCa patients.

2.3. OBJECTIVES

- 1) To identify recurrent CNAs using literature review and select relevant genes in the minimal alteration regions which affect the prostate tumor cell biology and are useful for disease prognosis.
- 2) To design an assay that can accurately assess the candidate genes in low-quality and quantity of DNA extracted from tumor foci of FFPE blocks from primary tumors of RP PCa patients, compatible with prostate samples obtained at biopsy and suitable for routine clinical application.
- 3) To develop a CNA-based classifier that can predict disease outcome in low- and intermediate-risk PCa patients.

- 4) To develop a risk stratification model with combined CNA and clinicopathological features that improves the current risk stratification systems in low- and intermediate-risk PCa patients.

2.4. PROPOSED METHODOLOGY

A detailed description of methodology used in the thesis is provided in each manuscript. A summary of experimental design to achieve the above-mentioned objective is presented below.

Selection of CNA assessment method: Selection of the suitable assay for CNA assessment in clinical PCa specimens was done by comparison of most-common techniques suited with routine clinical application, including, cost per assay, compatibility with low-quality and quantity DNA input and multiplexing capability. MLPA was chosen as the most suitable technique to achieve the objectives of the study.

Candidate genes: The selection of candidate genes in the minimal alteration regions of frequent CNA in PCa was done through a literature review and previous research-based evidence in the host lab. Since MLPA synthetic probe mixes can target 10-15 regions, we focused on well-known CNAs and genes that showed prognostic value in previous studies. Recurrent CNAs in PCa were first identified in meta-analysis and genome-wide studies and overlapped to select most common CNAs. Minimal region of alteration was then identified by overlapping the available data. A total of 14 genes showing relevance to prognosis and tumor biology were selected to design the final MLPA probe mix and develop the assay.

Test and reference probes: The design of probes was achieved using MLPA designer® software, followed by an assessment of all probe-probe interactions to avoid false-positive and unspecific results. All probes were synthesized and purified using standard desalting by Integrated DNA Technologies (IDT) and received at a 4 nmole scale. The performance of the assay and probes was assessed using normal healthy human genome from commercial sources and other FFPE human tissues (after approval by the MUHC Ethic committee), such as normal kidney obtained from the McGill University Health Centre Pathology Department and normal breast lymph node obtained from Ontario Institute for Cancer Research.

FISH: To test the CNA detection accuracy, we used 18 FFPE clinical samples from 15 PCa patients provided by the Queen's University Pathology Department. TMAs representing these samples were used for FISH assessment of the candidate CNAs in the tissues to provide a reference for performance of the designed assay. For FISH analysis of the candidate genes, various BAC and commercial probes were tested on the normal metaphase spread of human peripheral blood lymphocytes obtained from McGill Genetics department. Centromere (for chromosomes 1, 6, 8, 10, and 12), telomere (chromosome 13) targeting commercial FISH probes, BAC targeting 16qh and 5p12 were used as reference FISH probes. Probes showing strong and specific signals were chosen for assessment of CNAs in TMA and 5µm thick FFPE tissue sections.

DNA extraction for CNA assessment: DNA was extracted from three 0.6 mm tissue cores punched in the immediately adjacent area of tissue punched previously used to

build a TMA of primary tumors. This DNA was used to develop and test the performance of the designed MLPA assay.

Development of the MLPA assay: The performance of probes was evaluated by repeated assays on normal and PCa FFPE sample extracted genomes to calculate variations in probe performance and obtain a range of size in which synthetic probes can produce an accurate, reliable and reproducible signal. Error-rate of the assay was calculated by repeated MLPA experiments of 96 repeats of normal commercially available genome testing variables, such as effect of PCR plates, PCR plate caps, evaporation during the PCR reaction, thermal cycler and experimenter. The accuracy of the MLPA in CNA detection was determined by comparing various cut-offs and analysis approaches to determine the most accurate strategy to analyze results.

Validation of the assay: The developed analysis approach of the 10 genes was validated using an independent set of PCa FFPE clinical samples obtained from the MUHC Pathology department and using FISH results as a reference. FISH was done on whole-tissue sections of FFPE blocks that were used for obtaining 0.6 mm tissue punches for DNA extraction and MLPA assays. Further validation of the MLPA probe performance was done by performing droplet digital TaqMan PCR on a subset of samples in the validation sample set.

CNA profiling and analysis of the RP PCa cohort: An additional series of probes targeting 4 more genes was included and validated. The optimized MLPA assay and analysis approach were used to profile the CNA in the 14 targeted genes in a cohort of 433 low- and intermediate-risk PCa patients based on the RP GS 6 (3+3) and GS 7 (3+4 and 4+3). The RP cohort was established from 253 patients from the MUHC and

180 patients from Queen's University. To assess intra-tumor heterogeneity, prostate tissue FFPE blocks of most patients were sampled twice. Sample A was taken in a tumor foci of the highest GS of the patient and sample B was taken from a tumor foci of the lowest GS. In case of GS 6=3+3, assignment of sample A or B was done randomly. In total, 361 patients had a sample A and 342 had sample B, while 292 patients had both samples available.

Correlation of CNAs with clinicopathological features with continuous variables such as pre-operative PSA was done using non-parametric Mann-Whitney U test. For categorical variables such as Gleason group or Gleason pattern, χ^2 test was used. Univariate Cox proportional hazard and Kaplan-Meier analysis was used to assess the correlation of CNAs with BCR. Difference in the CNA between A and B samples of the same patients were assessed using Wilcoxon matched-pairs signed rank test.

Development and validation of the CNA classifier: The development of a CNA-based classifier was done using multivariate stepwise backward Cox proportional hazard modeling providing list of genes at each step, which their prognostic value was tested to finally select 6 genes defining our classifier. Performance of the CNA classifier was assessed using calibration curves and Harrell's C-index.

To validate our CNA classifier and compare the results of this study to previously published studies on RP datasets such as Cambridge [167], MSKCC [138] and CPC [281] along with Toronto biopsy dataset [278], patients in the high Gleason Group (≥ 4 or Gleason score ≥ 8) and distant disease (T4) were removed to keep only low- and intermediate-risk patients. The prognostic value of the model for detection of BCR, was assessed using univariate and multivariate Cox proportional hazard analysis, C-

index and Kaplan Meier analysis. BCR was defined as two consecutive measurements of PSA level of more than 0.2 ng/ml in post-radical prostatectomy cohorts (PARSE MLPA cohort, MSKCC, Cambridge, and CPC) or increase of more than 2 ng/ml above the post-radiation nadir value for the Toronto biopsy cohort. Comparing the goodness of fit between models was done using likelihood ratio test. The prognostic value of the developed models in short-term (3 years) and long-term (5 years) was calculated using area under the receiver operator curve (AUC) and positive and negative predictive values.

3. CHAPTER 3 – MANUSCRIPT ONE

Design and development of a fully synthetic MLPA based probe mix for detection of copy number alterations in prostate cancer formalin-fixed paraffin embedded tissue samples

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3.1. Abstract

In men, prostate cancer (PCa) is the second most commonly diagnosed cancer worldwide and is one of the leading causes of cancer-related mortality. Current standard clinicopathological parameters such as PSA, biopsy Gleason score and clinical staging fail to accurately differentiate between the clinically insignificant and aggressive disease. DNA copy number alterations (CNAs) are promising biomarkers to predict PCa outcome. However, fluorescence *in situ* hybridization (FISH), the standard CNA detection method, cannot assess CNA signatures due to low multiplexing capabilities. Thus, new approaches that can assess multiple CNAs in small amount of genomic DNA extracted from formalin fixed paraffin embedded (FFPE) biopsy samples are needed.

Multiplex ligation-dependent probe amplification (MLPA) is the most common PCR based CNA detection method compatible with low- quality and quantity of DNA. Due to the lack of PCa specific MLPA probe mixes, we have designed MLPA probes targeting CNAs frequently seen in PCa and tested their performance on genomic DNA extracts from PCa cell lines, along with normal and cancer FFPE radical prostatectomy (RP) specimens. The performance of the designed assay was compared to FISH and validated by droplet digital TaqMan PCR. Our probes were able to detect previously reported CNAs in PCa cell lines. In RP-FFPE samples compared to FISH, our probes showed a median accuracy of 89% in the test sample set and 90% in the validation sample set. Compared to droplet digital TaqMan PCR, MLPA showed an accuracy of 97%. These findings demonstrate that our PCa probe mix can accurately identify CNAs in multiple genes using low quality and quantities of DNA extracted from clinical FFPE samples.

3.2. Introduction

Other than non-melanoma skin cancer, prostate cancer (PCa) is the most commonly diagnosed malignancy in North America and a leading cause of cancer-related mortality among men [1, 2]. PCa has a heterogeneous clinical outcome ranging from an indolent disease to a deadly metastatic cancer. Standard treatments for localized PCa consist of radical prostatectomy (RP) or radiotherapy, which are often associated with significant adverse side effects. Active surveillance, a close monitoring of the disease with intent for therapeutic interventions at first sign of progression, has become a viable option for patients with low-risk PCa [3]. The key challenge in the management of PCa is to distinguish between an indolent disease and clinically significant tumors. Current prognostic parameters such as pre-operative PSA levels, clinical staging and Gleason grading of biopsy specimens cannot accurately predict individual clinical outcome [4], which consequently leads to overtreatment of clinically insignificant diseases and under-treatment of aggressive cancers with metastatic potential [5]. Better prognostic indicators would allow an immediate treatment of patients with potentially life-threatening cancer and active surveillance of patients that would not benefit from immediate or aggressive treatments.

DNA copy number alterations (CNAs) are genomic alterations consisting of deletion or gain of genomic DNA segments ranging from one kilobase to several megabases that may influence the function of several genes and regulatory elements [6] and impact disease progression [7, 8]. In PCa, CNAs are more common than single nucleotide alterations [9, 10] and their frequency increases in advanced cancers [9-12]. Gain of oncogenes such as *MYC* and loss of tumor suppressors such as *PTEN* and *TP53* are

examples of CNAs that are associated with disease progression and may serve as prognostic biomarkers in PCa [13-19]. Furthermore, studies suggested that the assessment of a combination of multiple CNAs in PCa patients improves risk stratification [14, 20-22]. The gold standard method for detection of CNAs in clinical tissue samples is fluorescence *in situ* hybridization (FISH) [23]. However, FISH sensitivity and resolution are probe dependent and implementation for high throughput analysis has been limited by the cost of reagents, labor and the small number of CNAs that can be assessed in a single assay [24]. Given the small amount of tissue obtained from prostate biopsy, there is an unmet need for new multiplex assays to apply CNA biomarker signatures in the clinical setting.

Multiplex ligation-dependent probe amplification (MLPA) is the most common multiplex PCR-based CNA detection method [24]. MLPA probes consist of two half-probes, flanked by universal primer binding sites, which hybridize next to each other to a specific DNA sequence target (CNA region). Upon ligation of the two half probes, which can only occur if the DNA target sequence is present in the sample, the complete probe is PCR amplified using fluorescently labeled primers. By addition of a stuffer sequence at the design step, each MLPA probe is given a unique size and fluorescent PCR products corresponding to CNAs can be separated and quantified by capillary electrophoresis. MLPA can assess up to 50 different loci in a single reaction with a resolution of 30-60 bp and is compatible with low amount (50 ng) of fragmented DNA extracted from formalin fixed paraffin embedded (FFPE) biopsy samples. This assay is low-cost, and results can be generated within two days using a multi-well format suitable for high throughput analysis. MLPA has been used to detect simultaneously

multiple CNAs in a variety of cancers [25, 26]. However, there is still no PCa-specific probe mix available to assess multiple PCa relevant CNAs. In this study, we report the design, validation and application of a PCa specific MLPA probe mix that can be used on FFPE samples to assess ten CNAs relevant to this disease.

3.3. Materials and Methods

Cell lines

Human prostate cancer cell lines PC-3, DU145, and LNCaP were purchased from American Type Culture Collection (ATCC) and the LAPC4 cell line was obtained from Dr. Robert Reiter, Department of Urology, University of California, Los Angeles. All cell lines were cultured in RPMI 1640 media (Hyclone, GE Life Sciences) supplemented with 10% fetal bovine serum (Wisent Bio Products), 1% penicillin-streptomycin, and 2 mM L-Glutamine (Gibco).

Prostate tissue samples

This study was approved by the Research Ethics Board of McGill University Health Centre (Quebec, Canada, BDM-10-115) and amended to include samples from Kingston General Hospital affiliated to Queen's University, Ontario, Canada. The written informed consent of participants was obtained at their respective institutions. All RP FFPE specimens were histologically reviewed by pathologists to identify the cancer area and assign the final Gleason grade, according to the latest International Society of Urological Pathology/World Health Organization recommendations [27]. The samples from Queen's University were used to develop the method and hereafter will be referred to as the test sample set (n=18: 15 cancers and 3 matched benign areas, Table 1). Tissue cores of 0.6 mm were harvested from the same RP FFPE block to

extract DNA for MLPA (3 cores) and to build a tissue microarray (TMA) used for FISH analyses (3 cores). For the method validation, an independent set of RP FFPE tissue specimens (n=20, Table 1) collected at the McGill University Health Centre were cored (3 x 0.6 mm, 2-3mm in length) for DNA extraction in two areas representing the highest (A sample) and lowest (B sample) Gleason grade patterns identified by the pathologist. When only one Gleason grade pattern was represented, the two sampled areas were assigned to A or B randomly. The FISH on the validation samples was then performed on tissue sections of the same blocks used for DNA extraction.

DNA extraction

DNA from cell lines was extracted using Qiagen DNeasy Blood & Tissue Kit. Two million cells grown in monolayers were detached using trypsin (Gibco) and washed twice with PBS before DNA extraction according to manufacturer instructions. For FFPE tissue samples, the DNA was extracted from three 0.6 mm cores with a modified protocol of the AllPrep DNA/RNA FFPE Kit (Qiagen) as we previously described [28].

Normal reference DNA samples

Commercially available normal female genomic DNA (Promega), hereafter referred to as fresh DNA, DNA extracted from FFPE pathology punches of normal kidney (McGill University Health Centre) and normal breast lymph node (Ontario Institute for Cancer Research) were used as reference samples for MLPA experiments.

The fresh male genomic DNA (Promega) was only used for the dilution of the PC-3 genomic DNA for assessment of the CNA detection limit.

MLPA probe selection, design, and synthesis

Due to technical limitations in synthesis of long oligonucleotides, synthetic MLPA probe mix can include 20-30 probes [29-31]. Thus, to build a PCa specific MLPA probe mix and considering the number of synthetic probes that can be included, we performed a literature review and selected ten most relevant genes (loci) that undergo CNA in PCa and are known to be associated with clinical outcome or have the potential to improve patient risk stratification. These include the known oncogene *MYC* (8q24.21) [13, 17, 19], the tumor suppressors *PTEN* (10q23.31) [14, 16, 18, 32], *TP53* (17p13.1) [15, 17, 33], *CDKN1B* (12p13.1) [34, 35], and *RB1* (13q14.2) [11, 36], genes in loci associated with metastasis such as *GABARAPL2* (16q23.1) [11, 21] and *PDPK1* (16p13.3) [37, 38], and genes associated with the previously described molecular PCa subtypes [39] *CHD1* (5q15-q21.1) [11, 40, 41], *MAP3K7* (6q15) [11, 40, 42] and *NKX3-1* (8p21.2) [11] (Table 2).

MLPA analysis also requires internal reference probes targeting loci that are less likely to undergo CNA for normalization and data analysis. In most synthetic MLPA probe mixes, a commercially available reference mix named P200 from MRC-Holland is used for this purpose. The P200 reference mix is unsuitable for PCa since it contains probes that target loci commonly deleted in PCa, such as 21q22 (TMPRSS2-ERG fusion) [43-45], 10q22, 5q31 and 16q24 [11, 12]. Therefore, three publicly available PCa CNA datasets [11, 12, 22] were analyzed to select genes least affected by CNA (<5%) and were used to design nine reference probes for the new MLPA assay (Table 2).

MLPA probes were designed using MLPA Designer® 7.91 software (PREMIER Biosoft), according to the synthetic probe design guidelines for MLPA assay provided by MRC-Holland (www.mrc-holland.com). All genomic sequences were obtained from the NCBI Reference

Sequences (RefSeq) database. Twenty probes were designed for each exon of the selected genes. The GC content was chosen to be between 40% and 65% and the T_m was $72 \pm 5^\circ\text{C}$ for all probes. The assay was designed to work with DNA fragments shorter than 200 bp usually extracted from FFPE samples [28] and therefore the combined hybridization sequences of the 5' half-probe and 3' half-probe of all probes were kept under 200 nucleotides.

The top-ranking probes based on GC content, T_m , hairpin ΔG and probes terminal nucleotide sequences were assessed for probe-probe interactions. Probes showing interactions stronger than $-3 \Delta G$ were replaced with the next ranking exon probes available. In instances that exon probes could not be selected, intron probes were designed.

Two probes for each CNA gene targeting two different genomic regions and one probe per reference gene were selected in the final probe mix. Stuffer sequences as previously reported by Zhi and Hatchwell [46], were added between the hybridization sequence and the universal primer binding sequences of each half-probe to give a unique length to the final probe with at least three nucleotides differences between consecutive probes. The size of the complete MLPA probes (ligated 5' and 3' half-probes including stuffer sequences) were kept under 260 nucleotides, since longer synthetic probes did not yield reproducible results (data not shown). Specificity of each probe was assessed by BLAT (<https://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). The presence of known PCa mutations and SNPs within the probe target sequence was ruled out using Ensembl Variation database [47] and PCa SNP data downloaded from cBioPortal website, respectively [48, 49].

All probes were synthesized and purified using standard desalting by Integrated DNA Technologies (IDT) and received at 4 nmole scale. The 3' half-probes were 5' phosphorylated. Probes specifications including the coordinates of their target sequences according to the 2013

UCSC Genome Browser assembly (hg38) are available in Table 2. All probes were dissolved and diluted in TE₁ buffer (10mM Tris-HCl, 1 mM EDTA; pH 8.0). Synthetic probe mix was constructed by adding 0.8 µl of 1 µM concentration of each half-probe and 1:25 dilution of the CF004-A1 control fragments (Q+D-control fragments, Lot: A1-0715, MRC-Holland), allowing assessment of DNA quantity and quality of MLPA reactions. Final volume of the probe mix was then adjusted to 600 µl using TE₁ buffer. The newly designed probe mix was named PCa probe mix.

MLPA reaction and analysis

MLPA reaction was done according to the MLPA General Protocol (One-Tube) using MLPA kit EK1-FAM (MRC-Holland). Components of the kit and buffers are available in MLPA General Protocol (MDP version-007; Issued on 01 March 2019) at the manufacturer website. Unless otherwise specified, each experiment included duplicate reactions for each sample and four repeats of each reference sample.

Briefly, for each reaction, 50 ng of DNA in 5 µl of TE_{0.1} buffer (10mM Tris-HCl; 0.1 mM EDTA; pH 8.2) was used. After initial denaturation at 98°C for 5 min, the hybridization mix (1.5 µl of PCa probe mix and 1.5 µl of SALSA MLPA buffer per reaction; as per manufacturer instructions) was added at room temperature. After denaturation at 95°C for 1 min, the hybridization was done at 60°C for 16 h. The ligation mix (25 µl of ddH₂O, 3 µl of Ligase buffer A, 3 µl of Ligase buffer B and 1 µl of SALSA Ligase-65 per reaction) were then added at 54°C followed by incubation at 54°C for 15 min and then 98°C for 5 min. The polymerase mix (7.5 µl of ddH₂O, 2 µl of PCR primer mix and 0.5 µl of SALSA polymerase per reaction) was added at room temperature and the PCR reaction was carried out for 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min followed by a final extension of 20 min at 72°C. For all reactions,

a Bio-Rad *MYCycler* thermal cycler and Bio-Rad PCR plates (Cat. No. 2239441) and caps (Cat. No. TCS0803) were used. For detection of the error-rate of the assay, another thermal cycler (T100 Bio-Rad), different PCR plates (Cat. No. AB0600, ThermoFisher), caps (Cat. No. AB0265, ThermoFisher), mineral PCR oil (Vapor-Lock, Qiagen) and a different reference probe mix (P200 – B1, Lot: B1-1215, MRC-Holland) were also used.

Capillary electrophoresis was done by the Genomics platform of the Institute for Research in Immunology and Cancer (IRIC), Université de Montréal using GeneScan™ 500 LIZ™ dye Size Standard molecular weight marker (ThermoFisher) and ABI 3730 DNA analyzer equipped with G5 filter set.

The Coffalyser software (Version 140721.1958) was used for fragment and comparative analyses of CNAs. Unless otherwise specified, the default settings of the software were selected. The manual probe recognition method was applied. For intra sample normalization, the median value of the test probe over each of the reference probes and for the inter sample normalization the average value of the normalized probe signal in test sample over each of the reference samples were used. The classic and the P.I.N.P.2 analysis protocol were used for CNA analysis of cell lines and FFPE samples, respectively.

Four repeats of fresh healthy female genome were used as a reference population in the analysis of cell lines and four repeats of each reference sample (fresh female genome, DNA extracted from normal FFPE kidney and breast lymph node reference samples) were used as reference population for analysis of FFPE samples. For testing further analysis approaches, probe ratios, standard deviations and CNA calls based on cutoff points (probe ratios below 0.65 are considered deletion and above 1.3 are considered as gain) and CNA calls based on the 95% confidence intervals of probes (as described below) were exported to Microsoft Excel.

For the 95% confidence interval approach, gain or deletion calls were assigned for each probe according to the Coffalyser software if this value in the test samples was respectively above or below what it showed in reference samples. If the 95% confidence intervals in the sample and the reference overlapped, normal copy number were assigned.

Reactions showing high standard deviation (>10%) in more than four probes were considered to fail the quality control (QC) and were repeated (if sufficient DNA was available) or removed from the analysis.

For comparison with existing datasets, the probe ratios resulting from the MLPA analysis of the four PCa cell lines were log2 transformed to compare with two CNA datasets generated by array-CGH (Array comparative genomic hybridization): Zhao et al [50] and the cancer cell line encyclopedia (CCLE) [51]. Pearson correlation coefficient between all data points were calculated.

Fluorescence *in situ* hybridization

The *TP53* CNA (17p13.1) was assessed using *TP53* / CEP 17 FISH probe kit from Abbott Molecular.

The following BAC clones mapping to the remaining nine target genes were labeled with Spectrum Orange dUTP (Enzo Life Science) using Nick Translation kit (Abbot Molecular) and used for FISH: RP11-813D1 (5q15-5q21.1; *CHD1*), RP1-154G14 (6q15; *MAP3K7*), RP11-325C22 (8p21.2; *NKX3-1*), RP11-440N18 (8q24.21; *MYC*), CTD-2557P6 (10q23.31; *PTEN*), RP11-180M15 (12p13.2-p13.1; *CDKN1B*), RP11-893E5 (13q14.2; *RB1*), RP11-16C11 (16q23.1; *GABARAPL2*), and RP11-20I23 (16p13.3, *PDPK1*).

As control probes for each chromosome we used: Centromere Spectrum Green CEP6, CEP8, CEP10, and CEP12 (all from Abbot Molecular), green 13qtr subtelomere probe (Cytocell) as well as RP11-530D2 (5p12) and pHuR-195 (16qh) labeled with Spectrum Green dUTP.

The *PTEN* CNA in cell lines was detected using the XT *PTEN*/GRID1 probe (Metasystems™).

Specificity of FISH probes was confirmed on normal metaphase chromosome preparations (Molecular Genetics Laboratory, McGill University Health Centre). Metaphase spreads of PC-3 and LAPC4 cells were prepared for FISH hybridization. Briefly, cells were grown in standard culture medium described above supplemented with 1 µg/ml of Colcemid (Invitrogen) for three hours and then washed with PBS and detached using Trypsin (Gibco). After a centrifugation step at 1000 g for 5 minutes, 5 million cells were resuspended in 2 ml of 0.56% KCl solution and incubated at room temperature for 5 minutes. Cells were re-centrifuged (1000 g, 5 min) and fixed in 0.5 ml of methanol:glacial acetic acid (3:1) solution before being spread on slides and air dried. Slides were then pretreated by incubation in 70% formamide and saline-sodium citrate (SSC) solution (0.3 M sodium chloride and 30 mM trisodium citrate, pH 7.0) at 75°C for 5 minutes followed by dehydration in ethanol series of 70%, 85% and 100% one minute each. The test and control FISH probes along with the target DNA were codenatured at 73°C for 6 minutes and left to hybridize overnight at 37°C using the ThermoBrite System (Abbott Molecular). Post-hybridization washes were performed in 2X SSC and 0.3% NP-40/0.4X SSC at 73°C for 2 minutes and 1 minute, respectively, followed by a 30-second incubation at room temperature in 2X SSC. Dual-color tissue FISH was performed on 5µm sections of TMAs or whole tissue blocks as we previously reported [38].

FISH data analysis

To evaluate copy number status, fluorescent signals were counted in 100 non-overlapping interphase nuclei for each case (as identified on corresponding H&E staining for tissues) counterstained with ProLong® Diamond antifade reagent with DAPI (Life Technology), to delineate nuclei. Deletion was defined as $\geq 15\%$ of nuclei containing one or no test locus signal and by the presence of two control signals as we previously reported (13). A gene was considered homozygously deleted if $\geq 15\%$ of nuclei had no test locus signal and two control signals. A gain was defined as present at a threshold of $\geq 15\%$ of nuclei containing three or more test locus signals and by the presence of two control signals. Images were acquired with an Olympus IX-81 inverted microscope at 96X magnification, using Image-Pro Plus 7.0 software (Media Cybernetics).

Droplet digital TaqMan PCR

Droplet digital PCR for CNA analysis of *PTEN* and *PDPK1* was carried out using TaqMan probes targeting the same regions assessed by the MLPA probes. For *PTEN* gene, probe (5'-/6-FAM/AGAAAGCTTACAGTTGGGCCCTGT/Iowa Black®/-3') and primers (forward: 5'-TCTGTCGCCATGGCTTATTC-3', reverse: 5'-CACCAAGACCCTGTCTCAAA-3') were designed to target exon 9 and for the *PDPK1* gene, probe (5'/6-FAM/CGTGTACGGAGTTCCACTTTCCATGA/Iowa Black®/-3') and primers (forward: 5'-AGCAGCTTACATGTCTGAAGTTA -3', reverse: 5'-TGTTCAAGAGGAGCTACAAAGG-3') were designed to target the intron 10 region. For inter-sample normalization, a probe targeting *AGO1* (1p34.3) (5'-HEX/ CAAGTCCAGTGACCACACTCCCAG/ Iowa Black®-3', forward primer: 5'-GAAGATGATGCTCAACATTGATGG-3', reverse primer: 5'-AGAGCTGGGAGGGATGAG-3') was used. Test and control probes were respectively labeled

with 6-FAM (Fluorescein amidite) and HEX (Hexachloro-Fluorescein) dyes (Integrated DNA Technologies). DNA extracted from normal kidney (FFPE tissue) was used as the reference sample. Fresh female genome and PC-3 cell line genomes were used as controls.

The assay was carried out by the Genomics platform of IRIC, Université de Montréal according to Bio-Rad TaqMan qPCR instructions (Bio-Rad) with some modifications as described below. Amplification was performed in a 20 µl multiplex reaction containing 6 ng of purified DNA, 500 nM of primers and 250 nM of probes, 2X ddPCR Supermix for probes (no UTP) and 5 unit/reaction XhoI enzyme (New England Biolabs). Samples were subjected to droplet generation by an automated droplet generator. End-point PCR was performed with cycling steps as follows: initially an enzyme activation at 95°C for 10 min followed by 50 cycles of denaturation, annealing and extension (each cycle at 95°C for 30 s; 58°C for 1 min; 72°C for 30sec; 2.5°C/sec ramp rate) and finally enzyme deactivation at 98° C for 10 min. Droplets were read on droplet reader QX200 (Bio-Rad) and data were analyzed using QuantaSoft™ Software (Bio-Rad) which determines the numbers of positive and negative droplets for each fluorophore in each sample. The fraction of positive droplets was then fitted to a Poisson distribution in QuantaSoft™ Software to determine the absolute copy number in units of copies/µl.

Average ratios of duplicate reactions were used for normalization. Intra sample normalization was done by calculating the ratio of the test probe over *AGO1* reference probe and inter sample normalization was done by calculating the normalized probe ratio in the test sample over the normalized probe ratio obtained from the kidney reference sample. Cutoff for gains (>1.2) and deletion (<0.8) corresponded to two standard deviations above and below the average ratio obtained from samples (fresh female DNA and tumor DNA from FFPE blocks) with no gains and deletions based on MLPA and FISH assays.

3.4. Results

Assessment of the performance of designed MLPA probes in presence of normal genome

MLPA was selected as a suitable assay to measure CNAs from small amounts of genomic DNA that can be extracted from FFPE tumor samples in a multiplex format (Figure 1a). A custom-made MLPA probe mix including nine control probes targeting CNA quiet loci was designed to simultaneously determine the CNA status of ten genes relevant to PCa prognostication (Table 2). To assess potential probe-probe interactions, a MLPA reaction was carried out in absence of DNA (water) and showed no peaks in the region corresponding to the designed probes (101 to 232 bp), confirming the absence of false-positive signals (Figure 1b). As expected, strong quantity control Q-DNA probes peaks at 64, 70, 76, and 82 bp, indicated the absence of DNA contamination and probe-probe interactions in an otherwise successful PCR reaction.

In presence of normal genomic DNA, each probe generated one specific fluorescence peak within the acceptable range of 100 to 3500 relative fluorescent units (RFU) that appeared on capillary electrophoresis 2-4 nucleotides smaller in average than the designed length, which is consistent with the results obtained by Stern et al [30] and likely explainable by the negative charge-to-mass ratio of the dye [52] (Figure 1c). Consistent with normal copy number values, the probe ratios in the fresh normal genomic DNA (4 repeats of both male and female) and FFPE (6 repeats of both normal kidney and normal breast lymph node) were respectively between 0.94 and 1.04 and between 0.9 and 1.07 with less than 5% standard deviation (Figure 1d, Table S1). The type 1 error-rate of the new assay was assessed in six separate experiments of MLPA (96 repeats each) using the PCa test probes mix with the designed reference probes or the commercial P200 – B1 reference probe mix on fresh normal genomic DNA. Each experiment assessed potential sources of variation due to the thermal cycler, the experimenter, the PCR

plates, the caps and the presence of a mineral oil overlay to minimize evaporation. On average across the six experiments, the false CNA call was very low representing 3.65% (95% confidence interval: 2.77% - 4.52%) of the reactions or 0.89% of the probes in each experiment (total of 2,784 probes; 95% confidence interval: 0.46% - 1.31%). The error rate was independent of probes or any of the assessed parameters mentioned above and appeared to be intrinsic to the assay.

Detection of CNAs in PCa cell lines by MLPA

The PCa probe mix was next applied to fresh genomic DNA extracted from PCa cell lines (PC-3, DU145, LNCaP, and LAPC4). The Pearson correlation between the probe ratios of duplicate reactions was 0.985 (95% confidence interval: 0.978 – 0.989, $p < 0.0001$). Reliability of the assay was assessed by repeating MLPA performed on PC-3 genome in ten separate experiments which showed consistent CNA calls (Fleiss' Kappa= 0.79, 95% confidence interval: 0.72 – 0.85, $p < 0.0001$).

Overall positive Pearson coefficient of correlation was seen when comparing the log₂ transformed probe ratios generated from MLPA analysis of the four cell lines and those obtained from the two published array-CGH datasets of Zhao et al [50] and the cancer cell line encyclopedia (CCLE) [51] in all probes, with the exception of *CHD1* intron 1 probe (Table 3). Moreover, the correlation coefficients between the MLPA ratios obtained from probes targeting the same gene were all above 0.7, with the exception of *PDPK1* (0.56).

Considering the standard cutoff for gain and deletion, MLPA detected previously reported CNAs such as *MYC* gain, *PTEN* homozygous deletion, and *NKX3-1* (exon 2c probe) hemizygous deletion in PC-3 cells as well as deletions of *CDKN1B* and *CHD1* (intron 1 probe) in LAPC4 cells (Figure 2a). Of note, the *NKX3-1* and *CHD1* deletions were respectively identified in PC-3

and LAPC4 cells by both probes targeting each of these genes when considering the 95% confidence interval. Moreover, the previously reported hemizygous deletion of *TP53* in PC-3 cells [53] is called by the confidence interval approach, but completely missed when considering the fixed cutoff. These observations suggested that the sensitivity of the MLPA assay might increase by using the 95% confidence interval instead of a fixed cutoff for calling the CNAs.

Intriguingly, ratios of probes targeting exon 9 of *PTEN* and *NKX3-1* suggested a single copy gain of these genes in LAPC4 (Figure 2a, right panel). To confirm our observations, the commercially available *PTEN* probe mix targeting exons 1 to 9 of the gene and flanking genes at 10q23.31 (MRC Holland) was applied on both cell lines. As expected and in agreement with the literature [54], a deletion from exons 3 to 9 of *PTEN* was detected in PC-3 (Figure 2b, left panel), and one copy gain (exons 1-9) was observed in LAPC4 (Figure 2b, right panel), confirming the results obtained by our designed PCa probe mix.

Further validation was performed by FISH analysis on PC-3 and LAPC4 metaphase spreads with XT *PTEN*/GRID1 del probe (Metasystems™) that targets exon 4 to the upstream of *PTEN* gene (orange), intron 3 of the *WAPAL* gene to the intron 3 of *GRID1* (green) distal to *PTEN* gene at 10q23.1, and chromosome 10 centromere probe (aqua) as reference. In PC-3 cells, an average of 3.6 green and 4.6 aqua signals were detected per nucleus with no orange signal, suggesting a *PTEN* deletion with a chromosome 10 polysomy (Figure 2c). In LAPC4, the 4.3 average orange and green signals suggested a gain at 10q region including both the *PTEN* and *GRID1* genes and a chromosome 10 polysomy with an average of 3.3 aqua signal per nucleus (Figure 2c).

FISH in PC-3 using *MYC* targeting probes showed an amplification with an average of 12.1 (orange) signals along with a 2.9 (green) signals for chromosome 8 per nucleus, suggesting a polysomy (Figure 2c). In LAPC4, FISH showed a chromosome 8 polysomy with an average of

3.9 signals per nucleus for both test and control probes (Figure 2c). For both genes and cell lines, the ratio of CNA probes over control probes were similar in MLPA and FISH. As illustrated with the LAPC4 cells, MLPA, like other ratio-based CNA assessment method, can detect genomic imbalances, but balance ploidies result in normal probe ratios (undetected).

Assessment of the CNA detection limit

Prostate tumor tissues are highly heterogeneous and often include benign glandular and stromal cells alongside cancer cells. Thus, genomic DNA extracted from such sample consists of cancer cell DNA diluted to various degrees with benign cell DNA. To estimate the minimum content of cancer cell DNA to accurately call CNAs with MLPA, a series of samples containing increasing percentages of PC-3 DNA (0-100%) over normal human male genomic DNA was used as a template for MLPA. Fresh female genome was used as the reference sample. When using pre-defined cutoffs to call CNAs, amplification of *MYC*, deletion of *PTEN* and *NKX3-1*, and one copy gain of *CHD1* (exon 35 probe) could be accurately detected when the PC-3 genome was at least at 10%, 60%, 90%, and 80%, respectively (Figure 3a-d). However, if the CNA calls are made based on the 95% confidence interval, all deletions and gains can be detected when the percentage of PC-3 genome is as low as 30% while *MYC* amplification is still detectable at 10% (Figure 3a-d). Since tumor samples could have variable tumor content with different levels of CNAs, a highly sensitive approach is required to accurately detect these alterations. This is especially important in prognostic assays on low-grade tumors that often have lower tumor contents and low CNA levels. The 95% confidence interval approach would thus appear more suitable to call CNAs in clinical samples.

Detection of CNAs using the MLPA designed probe mix in FFPE prostate tumor samples

MLPA was performed in duplicate reactions on all samples of the test sample set. To account for the range of variability expected in FFPE clinical samples and to match their DNA quality, we opted to include two FFPE extracted DNA references (normal breast and kidney) in addition to the fresh genomic DNA. All reactions passed the QC and were included in the analysis. In parallel, FISH for the genes assessed by MLPA was performed on all corresponding samples represented in a TMA.

Since each gene is targeted by two probes in our PCa probe mix and duplicate reactions were performed for each sample, we considered various combinations of one or two probes in one or both replicates, to make the final CNA call for a given sample. To explore different normalization schemes for determining the CNA calls per reaction, we first normalized all reactions against the average ratio of the three DNA reference samples. In the second normalization scheme, we normalized all reactions against each of the three DNA reference samples separately, yielding three CNA data points per replicate reaction. We further explored whether conventional cutoff points, or the 95% confidence interval were best suited to call the CNA in each normalization scheme. Similar to the results obtained with cell lines, a lack of sensitivity of cutoff points in detection of CNAs called by FISH was evident (Table S2, approaches 1 to 8). Based on the 95% confidence interval, the sensitivity of assays remained low when the data were normalized against the average value of the three reference samples (Tables S2, approaches 9 to 12). However, sensitivity was improved when the dataset was normalized against each reference sample separately and the alteration was detected in at least two of the three separately reference-normalized datasets (Table S2, approaches 13-16). MLPA CNA calls based on the 95% confidence interval in all samples when data is normalized against each of the

reference sample separately are presented in Figure 4a. Optimal results were obtained with approach 14, yielding a median sensitivity of 80%, specificity of 93% and accuracy of 89% (Table S2). With this approach, the data is normalized against each of the three reference DNA samples separately. CNA in a gene was thus defined when both duplicate reactions show the same CNA in at least one of the probes targeting the gene and in at least two of the three normalizations. Final CNA calls by MLPA based on approach 14 and the corresponding CNA call by FISH for all samples in the test sample set is shown in Figure 4b. An example of MLPA probe ratio profile is shown in Figure 4c for sample (7-C) in which the deletions of *NKX3-1*, *PTEN* and *RB1* were also called by FISH (Figure 4d).

As illustrated in Figure 4b, the three benign samples were CNA quiet. While one of the benign samples (6-B) did not show any CNA, sample 1-B showed *GABRAPL2* deletion which was not seen in the corresponding tumor (1-C). The third benign sample (10-B) showed *RB1* deletion which was also detected in the corresponding cancer sample 10-C. However, no CNAs were detected by FISH in the benign samples.

In cancer samples, heterogeneity was evident among surveyed genes, ranging from none in sample 2-C to six in sample 13-C as measured by MLPA (Figure 4b). The highest CNA frequency were for *RB1* (9/15, 60% in both MLPA and FISH) and *NKX3-1* (7/15, 47% in both MLPA and FISH), whereas the lowest CNA frequency was for *PDPK1* (no CNA was detected with either method) and *TP53* (1/15, 7% in both methods).

To validate our analysis approach, we used the same experimental design and performed MLPA on genomic DNA extracted from 20 RP-FFPE independent cases in the validation sample set. Each case was represented by two samples, A and B corresponding to the highest and the lowest

Gleason grade pattern, respectively. All samples passed the QC and were included in the analysis.

Given our laboratory interest in *PTEN* and *PDPK1* as prognostic biomarkers in PCa patients [55, 56], we performed FISH for these two genes on full sections of the FFPE blocks of this sample set targeting the area where tissue cores were harvested for DNA extraction. We also performed TaqMan PCR assays to survey *PTEN* and *PDPK1* CNAs in 17 samples with the same DNA extracts used for MLPA (Table S3). Figure 5a shows the CNA profile of the final call for MLPA, FISH and TaqMan. An example of the MLPA profile for sample 23-C along with FISH and TaqMan data for *PTEN* and *PDPK1* are presented in Figures 5b and 5c. All three methods showed the deletion of *PTEN* and gain of *PDPK1*. In this validation sample set, deletions of *NKX3-1* (25/40, 63%) and *RB1* (21/40, 53%) were the most frequent CNAs detected by MLPA, while the gain of *PDPK1* (5/40, 13%) was the least common, similarly to what was observed in the test set (Figure 5a).

Compared to FISH, the sensitivity and specificity of MLPA to call a *PTEN* deletion were respectively 85% and 100%. MLPA was able to detect a *PDPK1* gain with a sensitivity of 100% and a specificity of 92%. Compared to TaqMan, MLPA showed a sensitivity of 100% and a specificity of 83% in detecting *PTEN* deletion and a sensitivity of 67% and a specificity of 93% in detection of *PDPK1* gain. However, if we consider the MLPA CNA calls based on the *PDPK1* intron 10 probe, which assesses the same region targeted by the ddTaqMan PCR *PDPK1* probe, the sensitivity and specificity to detect the *PDPK1* gain reach 100% and 93% respectively.

The impact of sampling was investigated by comparing the CNA patterns of tumor samples A and B taken from the same FFPE prostate block of each case. No significant differences in CNA calls was observed between samples A and B (McNemar's test $p > 0.4$, Table S4).

3.5. Discussion

A detailed molecular classification of PCa and its associated prognostic value rely on the detection of multiple genomic alterations. Diagnostic tests to survey multiple CNAs should be amenable to small amount and suboptimal quality of DNA obtained in standard clinical samples such as prostate biopsies. MLPA has a significant advantage over array-CGH and DNA sequencing which require large amounts of DNA and often are not compatible with low-quality DNA obtained from FFPE biopsy specimens. Furthermore, genome-wide analysis is often not necessary in a clinical setting when assessment of relevant biomarkers is needed. FISH, the routine CNA detection method used in the clinical setting, is labor intensive and cannot be used for high-throughput assessment of CNA signatures. The Oncoscan microarray system [57], a variation of the array-CGH method, as well targeted next generation sequencing [58] are additional approaches to assess CNAs in small amounts of DNA extracted from FFPE tissue samples. Hence, the MLPA method appeared highly promising as it offers competitive advantages over the above-mentioned methods. It is relatively inexpensive and can be performed in a single tube. Beside a Thermal cycler, the only equipment needed is a capillary electrophoresis system. Thus, we selected MLPA in order to simultaneously measure multiple CNAs in a same reaction using DNA extracted from FFPE tissue samples. MLPA can fill the gap between research and clinical application of CNA signatures by providing a simple, high-throughput method compatible with low quantities and qualities of DNA in prostate biopsies.

Here we designed a synthetic probe mix specific for CNA detection in PCa targeting ten genes commonly altered and nine reference genes, less likely to be affected by CNA. Our custom-made PCa specific probe mix was capable of accurately detecting CNAs in various samples with low error-rate. Using synthetic probes significantly reduces the cost and time associated with development of MLPA probe mix. One potential shortcoming of using synthetic probes could be the errors in the coupling efficiency of the synthesis process which results in generation of a small percentage of longer probes not having the full designed length. Although these incomplete half-probes could compete in the hybridization to the target sequence, they may not be able to ligate and form a complete MLPA probe that can be amplified in the subsequent PCR reaction. This might result in low performance of long probes and lower fluorescent signal, as we noticed but without affecting the performance of probes. Hence, all of our designed probes showed an accurate CNA detection with high reproducibility. The high coupling efficiency, at 99.5% of long synthetic probes (Ultrasizer® DNA oligonucleotides, IDT) might have overcome this limitation. Our observation is in agreement with findings of Stern et al [30], who found no difference in the performance of long synthetic MLPA probes compared to short probes. According to the authors, this may be explained by excess amounts of full-length probes compared to the partially synthesized probes and by hybridization of the full-length probes to the target sequence in much higher efficiency and strength than partially synthesized probes. Using a DNA synthetic approach with higher coupling efficiency, we were able to increase the length of synthetic probes in MLPA from 156 nucleotides reported by Stern et al [30] to 232 nucleotides. The performance of these probes was validated by the reproducibility of results we obtained and the low standard deviation of all probes in our probe mix when using both fresh and FFPE extracted DNA. Our findings are in agreement with reports on commercially available probe

mixes [30]. Based on our experience, we conclude that synthetic MLPA probes of a length up to 250 nucleotides can be used to generate highly reproducible results.

We further improved and customized our probe mix by designing nine different reference probes based on reported PCa datasets. The rationale was to account for possible genomic instability of cancer cells that could result in random CNAs and the incompatibility of commercially available reference probes mixes such as the P200 for PCa. Furthermore, and as we and others have observed [30], a higher number of reference probes allows for better normalization, higher reproducibility of results and smaller standard deviation across different samples, less than 5% compared to less than 10% in other reported MLPA studies [30, 59]. Designing customized reference probes with an average distance of 4 nucleotides apart has also allowed us to include more test probes in our PCa-specific probe mix compared to P200 probes, which does not allow inclusion of more than 17 probes as they have an average of 6 nucleotides apart. Furthermore, in our design process, for more accurate CNA detection, we used two probes per gene to assay each of the chosen ten target genes. The rationale stems from previous studies showing that MLPA probes targeting the same regions could yield different results [59, 60]. Bendavid et al [61] used two different MLPA probe mix kits targeting same regions and showed that not all alterations can be captured by both kits. Alterations that were only seen by one of the kits were further confirmed by qPCR. Altogether, this supports that more than one probe per target region may allow better CNA detection, especially in tumor samples. The use of duplicate reactions in our experimental design was advantageous to reduce false-positive results and yielded better reproducibility. We reasoned that results would be reliable if the CNA was detected in both replicates. Further analysis confirmed high sensitivity and specificity of MLPA duplicate reactions in detection of CNAs compared to FISH data. In addition, a high correlation between

duplicate reactions was observed in all experiments. We also demonstrated that MLPA assays performed in various experimental conditions yield a low error-rate, on average 0.89% of probes used in each experiment yielded false-positive results which is lower than the reported 1.7% using commercially available probe mixes [61].

To investigate the accuracy of CNA detection by our designed probe mix, we compared the CNA results obtained from MLPA to published array-CGH CNA results of PCa cell lines. Although these studies have used a different technique with different set of probes with a lower resolution than MLPA, our results are consistent with previous reports [59] and show a high correlation between MLPA and array-CGH data. On average we showed Pearson correlation coefficients of 0.68 with Zhao et al [50] and 0.75 with data available on CCLE (cancer cell line encyclopedia). The lowest observed correlation was for the *CHD1* Intron 1 probe (Pearson correlation of -0.013 for Zhao et al [50] and -0.89 for CCLE). *CHD1* is a large gene (74,569 bp) that expands between two cytobands (5q15-5q21.1). To cover both cytobands, we designed one probe to target the first intron in the 5q21.1 cytoband and the other probe targets exon 35 on the 5q15 cytoband. Zhao et al [50] used a cDNA microarray which does not include intron probes and the *CHD1* probes on the microarrays targets the exon 35 which could explain the low correlation with the intron 1 probe, while exon 35 probe shows a positive correlation. The probe used for determination of *CHD1* CNA in CCLE database maps to intron 25, which is closer to the exon 35 than the intron 1 probe and showed a correlation of 0.90 with the MLPA exon 35 probe.

For a better measurement of MLPA probes performance and to circumvent differences in part linked to analysis of different parts of the gene or tumor areas, we validated MLPA results via ddPCR TaqMan assays performed on the same DNA extracts as used for MLPA and with probes targeting the same regions. We observed a high concordance of results between MLPA and

TaqMan as well. This further showed the robustness of the designed assay. It is worth emphasizing that TaqMan has limited multiplexing capability and, in this case, only one reference probe was used, whereas in the designed MLPA assay we used 9 different reference probes, thereby allowing a more accurate normalization.

While these results indicate that MLPA is capable of detecting copy number imbalances in homogeneous samples such as cell lines, the accurate detection of genomic alterations in heterogeneous samples such as prostate primary tumors is more challenging. This is not specific to MLPA. Indeed, most genomic assays that use DNA extracted from heterogeneous tumors face the same limitations. This was reported by Schwab et al [59] to explain discrepancies observed between MLPA and FISH. The main reason for such discrepancies is intrinsic to the prostate per se and the heterogeneous tumor content used for genomic DNA extraction which contain tumor DNA mixed with varying proportions of DNA from surrounding non-malignant cells in the stroma and in benign glands. Furthermore, when there is a low percentage of tumor cells with CNA in samples, due to the same dilution effect, genomic assays are not capable of accurately detecting alterations. To overcome this limitation, we used 95% confidence interval for each probe within the reference sample population and test samples to detect CNAs. This approach has the advantage of calculating the range of variations seen for each loci in reference samples and detect alterations outside what is normally seen in healthy tissues, thus providing a more accurate measurement of CNAs. Thus, using the confidence interval approach, we were able to capture CNAs when the PC-3 genome was diluted up to 30%. This method of analysis of MLPA data on the test clinical samples resulted in a median sensitivity of 80% and specificity of 93% compared to the conventional cutoff points approach with the sensitivity of 14% and specificity of 100%. Even when analyzing cell lines, we noticed that the previously reported CNAs such as

deletion of *TP53* in PC-3 was completely missed or called only by one of the two probes for the deletion of *NKX3-1* in PC-3 and *CHD1* in LAPC4 when using cutoff points. However, when using the 95% confidence interval, all these CNAs were readily identified which further supports the use of this approach over the fix cutoff points.

When comparing the results of MLPA and FISH in our test and validation samples, we observed good concordance between the two types of assays. Yet discrepancies were also observed in some prostate samples with MLPA revealing CNAs but not FISH and *vice versa*. The former could be explained by the higher resolution of MLPA compared to FISH while the latter could be due to the dilution effect. Including more probes to target each gene could allow better mapping of the CNA region and increase the sensitivity of the assay. However, since MLPA can include limited number of probes, that would result in assessment of smaller number of genes.

We also assessed CNA frequencies in different Gleason grade patterns of the same samples in our small validation sample set and did not observe significant differences, thereby suggesting negligible intra-tumor CNA heterogeneity in the assessed genes in primary PCa. A more robust study with larger sample size that would include a wider range of Gleason patterns is needed to confirm these results.

Taken together, we have designed and rigorously tested an MLPA based assay using a fully synthetic probe mix. We used DNA extracted from three 0.6 mm punches (2-3 mm length) from FFPE clinical samples to simulate needle biopsies, as obtained before diagnosis. The assay was capable of detecting CNAs with high sensitivity and specificity compared to FISH. This demonstrates compatibility and applicability of the assay on clinical biopsy samples for the detection of most common CNAs, thereby opening the door to a better prognosis based on CNA profiles. Furthermore, the cost is low, our estimate being 7.90 \$CAD (6\$ USD) per reaction

which includes the cost of probe synthesis, lab materials, MLPA kits, and capillary electrophoresis. MLPA does not require other sophisticated instruments or analysis approaches. The effectiveness of our designed probe mix and analysis approach, were validated using a small number of clinical PCa samples. Further validation on a larger cohort with adequate clinical follow-up data is necessary to determine the impact of these CNAs and utility of the assay for prognosis. This assay shows potential for clinical application, and by identifying CNAs relevant to poor prognosis could aid clinicians and patients in decision-making in view of an optimal management of the disease.

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3.8. Tables

Manuscript 1, Table 1: List and characteristics of samples used in this study

ID	Type of core	Reviewed Gleason Score (Gleason group) of cases	% of cancer cells	Sample set
1-C	Cancer	4+5 (5)	85	Test
1-B	Benign	N/A	0	Test
2-C	Cancer	5+4 (5)	85	Test
3-C	Cancer	4+3 (3)	50	Test
4-C	Cancer	3+4 (2)	85	Test
5-C	Cancer	4+5 (5)	90	Test
6-C	Cancer	4+3 (3)	70	Test
6-B	Benign	N/A	0	Test
7-C	Cancer	4+5 (5)	70	Test
8-C	Cancer	3+4 (2)	75	Test
9-C	Cancer	3+4 (2)	85	Test
10-C	Cancer	4+3 (3)	90	Test
10-B	Benign	N/A	0	Test
12-C	Cancer	4+3 (3)	90	Test
13-C	Cancer	4+3 (3)	85	Test
14-C	Cancer	4+5 (5)	80	Test
16-C	Cancer	5+4 (5)	80	Test
18-C	Cancer	4+5 (5)	80	Test
19-C	Cancer	4+3 (3)	95	Validation
20-C	Cancer	4+4 (4)	90	Validation
21-C	Cancer	4+5 (5)	90	Validation
22-C	Cancer	4+5 (5)	90	Validation
23-C	Cancer	4+4 (4)	75	Validation
24-C	Cancer	4+3 (3)	75	Validation
25-C	Cancer	4+4 (4)	90	Validation
26-C	Cancer	4+5 (5)	85	Validation
27-C	Cancer	4+3 (3)	90	Validation
28-C	Cancer	3+4 (2)	70	Validation
29-C	Cancer	4+5 (5)	85	Validation
30-C	Cancer	4+3 (3)	80	Validation
31-C	Cancer	3+4 (2)	80	Validation
32-C	Cancer	3+4 (2)	65	Validation
33-C	Cancer	4+5 (5)	90	Validation
34-C	Cancer	3+4 (2)	80	Validation
35-C	Cancer	4+3 (3)	70	Validation
36-C	Cancer	4+3 (3)	80	Validation
37-C	Cancer	4+3 (3)	90	Validation
38-C	Cancer	4+3 (3)	80	Validation

Manuscript 1, Table 2: List and characteristics of designed PCa specific MLPA probe mix

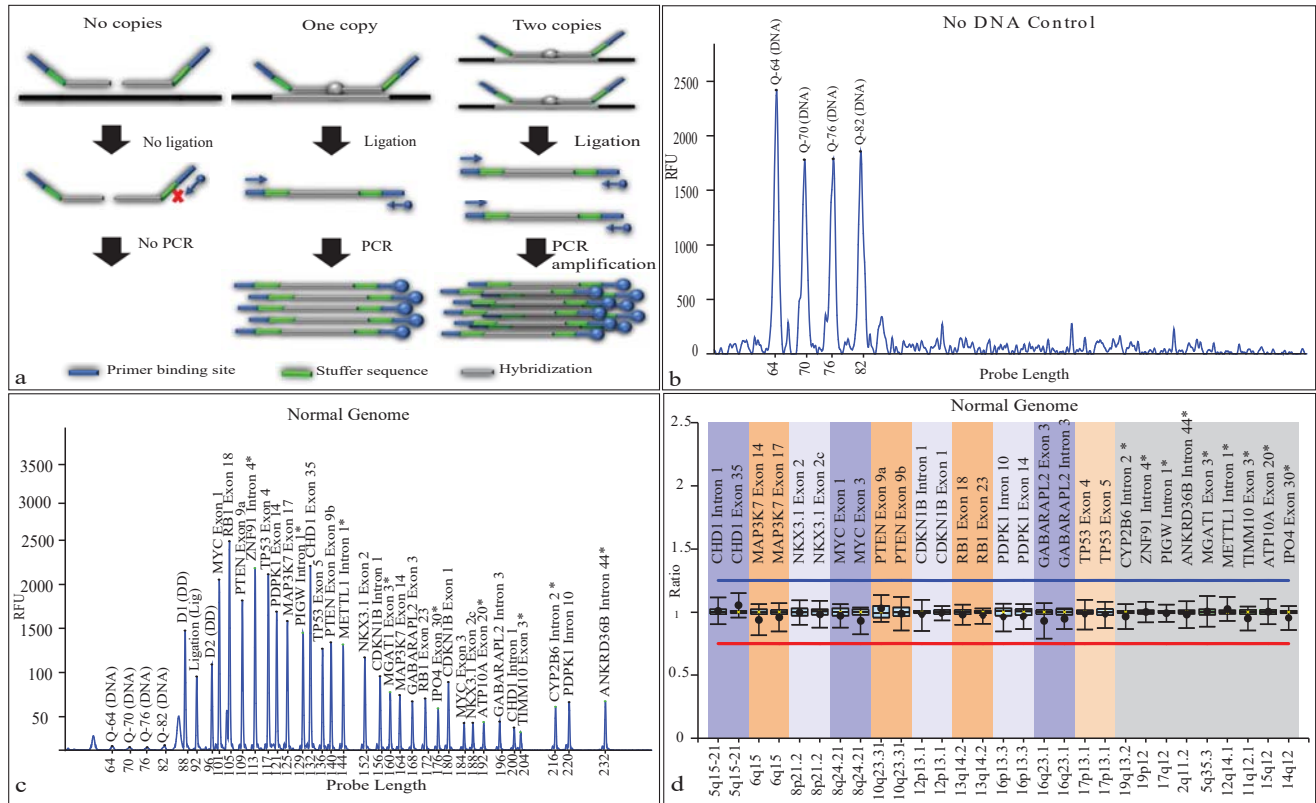
Name	Function	Cytoband	Hybridization sequence coordinates (hg38)	Probe Length
<i>CHD1</i> Intron 1	CNA	05q15	98927383-98927458	200
<i>CHD1</i> Exon 35	CNA	05q21.1	98856501-98856587	132
<i>MAP3K7</i> Exon 17	CNA	06q15	90516154-90516215	125
<i>MAP3K7</i> Exon 14	CNA	06q15	90523700-90523761	164
<i>NKX3-1</i> Exon 2c	CNA	08p21.2	23679195-23679260	188
<i>NKX3-1</i> Exon 2	CNA	08p21.2	23679829-23679894	152
<i>MYC</i> Exon 1	CNA	08q24.21	127736564-127736621	101
<i>MYC</i> Exon 3	CNA	08q24.21	127740610-127740671	184
<i>PTEN</i> Exon 9a	CNA	10q23.31	87966936-87966999	109
<i>PTEN</i> Exon 9b	CNA	10q23.31	87968061-87968130	140
<i>CDKN1B</i> Intron 1	CNA	12p13.1	12714777-12714852	156
<i>CDKN1B</i> Exon 1	CNA	12p13.1	12717331-12717394	180
<i>RBI</i> Exon 18	CNA	13q14.2	48453031-48453090	105
<i>RBI</i> Exon 23	CNA	13q14.2	48465212-48465273	172
<i>PDPK1</i> Exon 14	CNA	16p13.3	2597685-2597741	121
<i>PDPK1</i> Intron 10	CNA	16p13.3	2584302-2584368	220
<i>GABARAPL2</i> Exon 3	CNA	16q23.1	75568131-75568188	168
<i>GABARAPL2</i> Intron 3	CNA	16q23.1	75573764-75573839	196
<i>TP53</i> Exon 5	CNA	17p13.1	7674901-7674962	136
<i>TP53</i> Exon 4	CNA	17p13.1	7675285-7675346	117
<i>ANKRD36B</i> Intron 44	Reference	02q11.2	97495557-97495632	232
<i>MGAT1</i> Exon 3	Reference	05q35.3	180790824-180790885	160
<i>TIMM10</i> Exon 3	Reference	11q12.1	57528778-57528839	204
<i>METTL1</i> Intron 1	Reference	12q14.1	57774661-57774736	144
<i>IPO4</i> Exon 30	Reference	14q12	24180336-24180397	176
<i>ATP10A</i> Exon 20	Reference	15q12	25680143-25680204	192
<i>PIGW</i> Intron 1	Reference	17q12	36533015-36533090	129
<i>ZNF91</i> Intron 4	Reference	19p12	23350707-23350774	113
<i>CYP2B6</i> Intron 2	Reference	19q13.2	41004294-41004359	216

Manuscript 1, Table 3: Correlation of log2 transformed probe ratios between PCa specific MLPA probe mix, and array-CGH studies

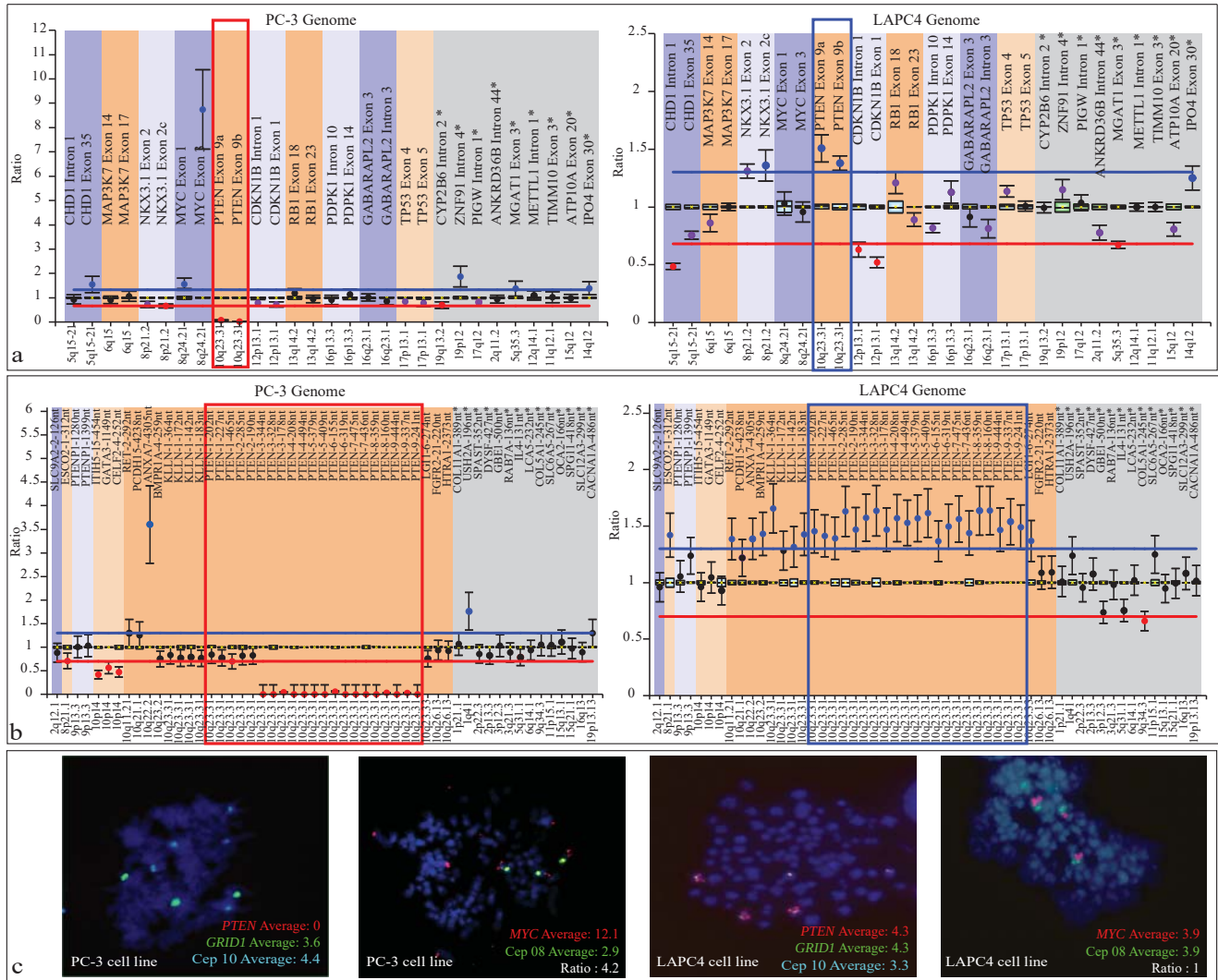
Gene	Method	Study/Probe	Log2 transformed probe ratio				Pearson Correlation with Zhao et al	Pearson Correlation with CCLE	Pearson Correlation between MLPA probes
			PC-3	DU145	LNCaP	LAPC4			
<i>CHD1</i>	Array-CGH	Zhao et al (exon 35)	0.81	-0.42	-1.07	-0.48			
		CCLE	0.49	0.17	0.09	N/A	0.99		
	MLPA	<i>CHD1</i> Intron 1	-0.12	0.03	0.27	-0.96	-0.13	-0.89	
		<i>CHD1</i> Exon 35	0.44	0.07	0.16	-0.43	0.50	0.91	0.78
<i>MAP3K7</i>	Array-CGH	Zhao et al (exon 4)	-0.67	-0.35	-1.40	-0.45			
		CCLE	-0.50	-0.29	-1.21	N/A	1.00		
	MLPA	<i>MAP3K7</i> Exon 14	-0.18	-0.54	-0.55	-0.20	0.40	0.32	
		<i>MAP3K7</i> Exon 17	-0.07	-0.55	-0.48	-0.02	0.30	0.16	0.98
<i>NKX3-1</i>	Array-CGH	Zhao et al (exon 2)	-0.40	-0.30	0.17	-0.06			
		CCLE	-0.47	0.12	0.09	N/A	0.61		
	MLPA	<i>NKX3-1</i> Exon 2	-0.56	0.04	0.19	0.43	0.73	0.97	
		<i>NKX3-1</i> Exon 2c	-0.73	0.19	0.28	0.27	0.72	0.99	0.95
<i>MYC</i>	Array-CGH	Zhao et al (exon 3)	0.69	-0.10	-0.01	-0.17			
		CCLE	0.95	0.32	0.09	N/A	0.93		
	MLPA	<i>MYC</i> Exon 1	0.84	0.23	0.17	0.29	0.95	0.98	
		<i>MYC</i> Exon 3	3.14	0.19	-0.14	-0.20	0.98	0.99	0.98
<i>PTEN</i>	Array-CGH	Zhao et al (exon 1)	-0.36	-0.03	-0.55	0.05			
		CCLE	-2.40	0.15	-0.91	N/A	0.54		
	MLPA	<i>PTEN</i> Exon 9a	-3.74	-0.12	-2.65	0.59	0.87	0.95	
		<i>PTEN</i> Exon 9b	-5.64	0.01	-0.50	0.47	0.44	0.94	0.82
<i>CDKN1B</i>	Array-CGH	Zhao et al (exon 3)	-0.29	-0.54	-0.13	-0.73			
		CCLE	-0.51	-0.29	0.06	N/A	0.49		
	MLPA	<i>CDKN1B</i> Intron 1	-0.47	-0.34	0.19	-0.75	0.84	0.98	
		<i>CDKN1B</i> Exon 1	-0.55	-0.53	0.09	-0.92	0.89	0.93	0.99
<i>RB1</i>	Array-CGH	Zhao et al (exon 27)	0.23	0.15	0.15	0.29			
		CCLE	-0.05	-0.31	-0.23	N/A	0.96		
	MLPA	<i>RB1</i> Exon 18	0.03	-0.22	-0.14	0.30	0.99	1.00	
		<i>RB1</i> Exon 23	-0.14	-0.67	-0.35	-0.18	0.75	0.95	0.77
<i>PDPK1</i>	Array-CGH	Zhao et al (exon 14)	0.34	0.12	0.27	0.16			
		CCLE	-0.53	-0.35	0.07	N/A	-0.09		
	MLPA	<i>PDPK1</i> Intron 10	-0.11	-0.22	0.17	-0.24	0.59	0.83	
		<i>PDPK1</i> Exon 14	0.04	-0.32	0.15	0.08	0.60	0.45	0.56
<i>GABARA PL2</i>	Array-CGH	Zhao et al (exon 4)	-0.10	0.02	-0.07	0.05			
		CCLE	-0.50	0.10	-0.04	N/A	0.87		
	MLPA	<i>GABARA PL2</i> Exon 3	-0.04	0.09	-0.07	-0.14	0.01	0.59	
		<i>GABARA PL2</i> Intron 3	-0.31	0.21	-0.03	-0.30	0.26	0.97	0.83
<i>TP53</i>	Array-CGH	Zhao et al (exon 17)	0.01	0.52	0.31	0.22			
		CCLE	-0.50	0.16	0.08	N/A	0.95		
	MLPA	<i>TP53</i> Exon 4	-0.43	0.24	0.05	0.20	0.85	0.99	
		<i>TP53</i> Exon 5	-0.41	0.35	0.05	0.06	0.98	0.96	0.94

* For MLPA probes, except for DU145 cell line which is represented by one replicate, average probe ratios obtained from duplicate reaction of cell lines were used.

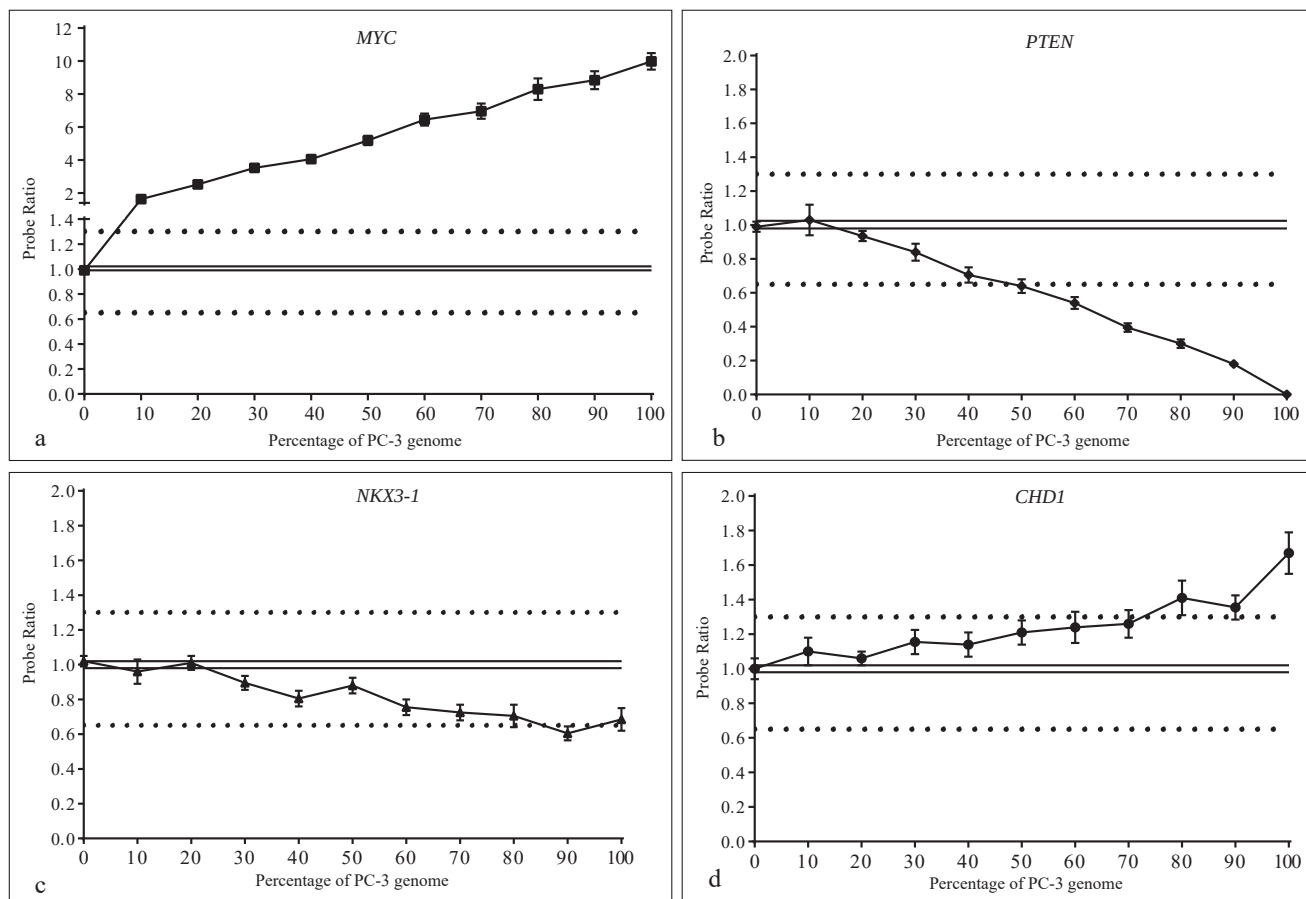
3.9. Figures



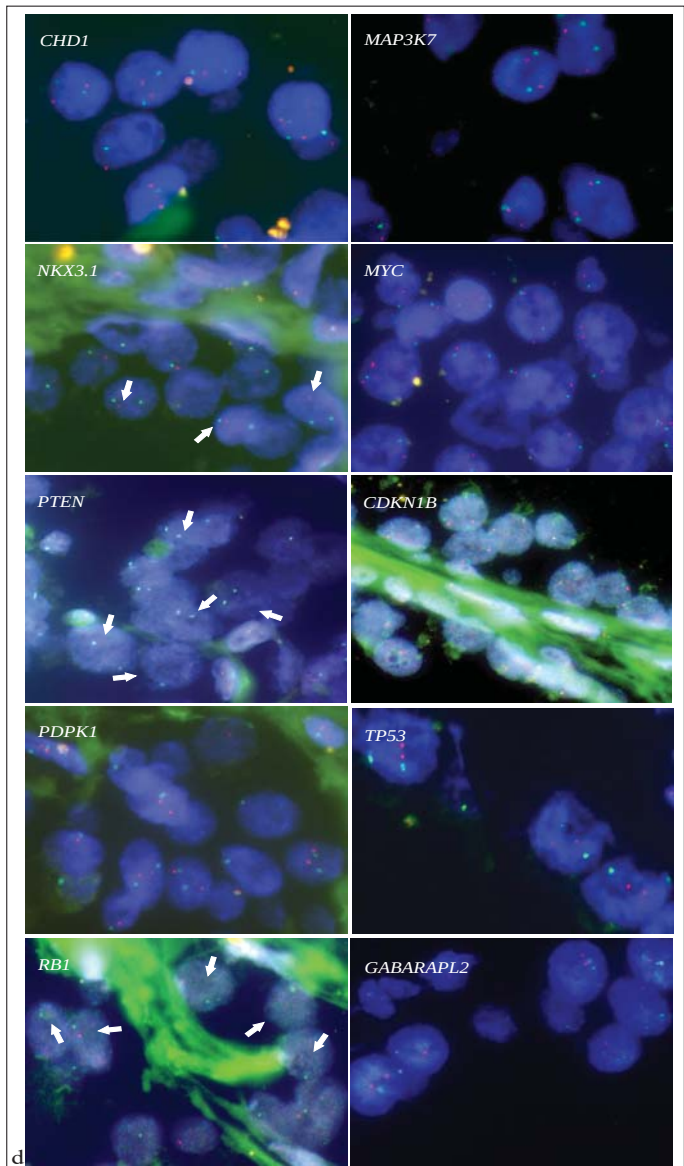
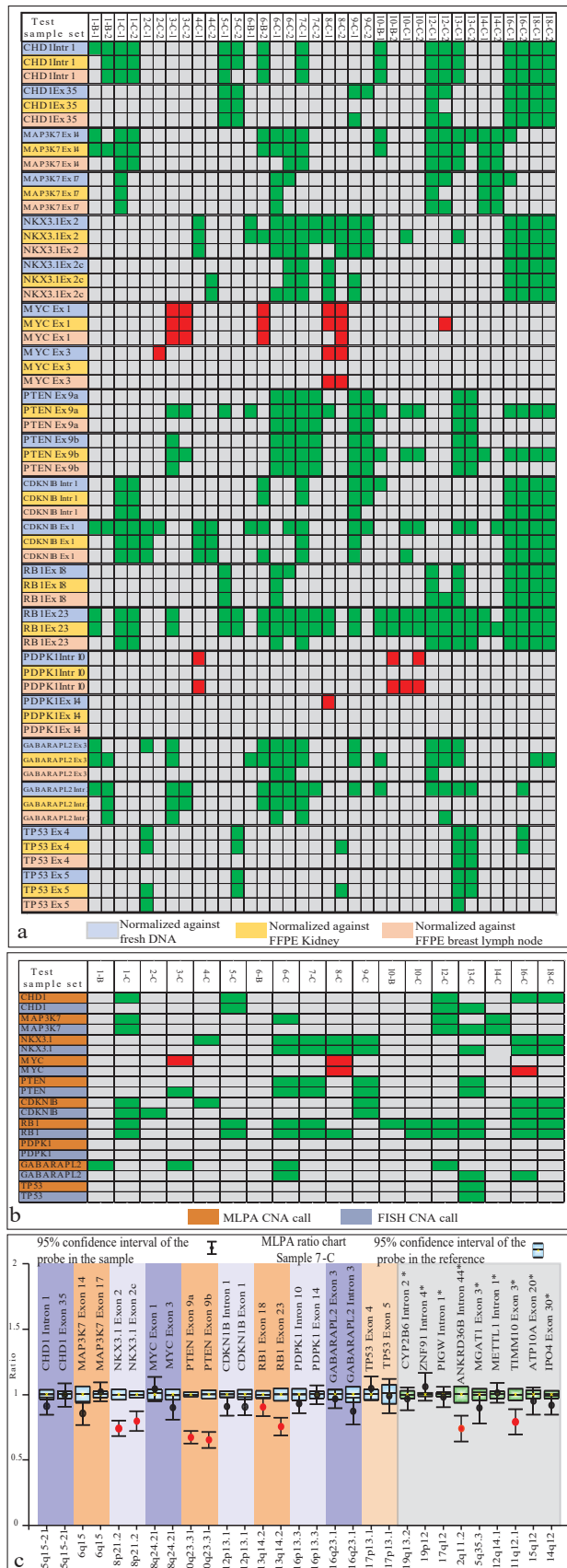
Manuscript 1, Figure 1: Performance of the newly designed MLPA probes in presence of normal genomic DNA. a: Schematic representation of MLPA reaction. In presence of the target sequence, the two MLPA half-probes anneal to the DNA next to each other. After a ligation step, the complete probes are amplified using fluorescently labeled primers. The amount of fluorescent signal is correlated with the copy number of the target sequence. **b:** Electropherogram of PCa probe mix in no-DNA reaction. Relative fluorescent units (RFU) are shown for all probes ordered from left to right according to their length. The probes are identified at the top of their respective peak. **c:** Electropherogram of PCa probe mix as in **b**, but in presence of normal genome. Reference probes are marked by a star (*) at the top of the probe name. **d:** MLPA profile of normalized probe ratios in normal genome. The upper blue line is the cut-off for gain and the red line is the cut-off for deletions set as 1.3 and 0.65, respectively, as described in MLPA guidelines.



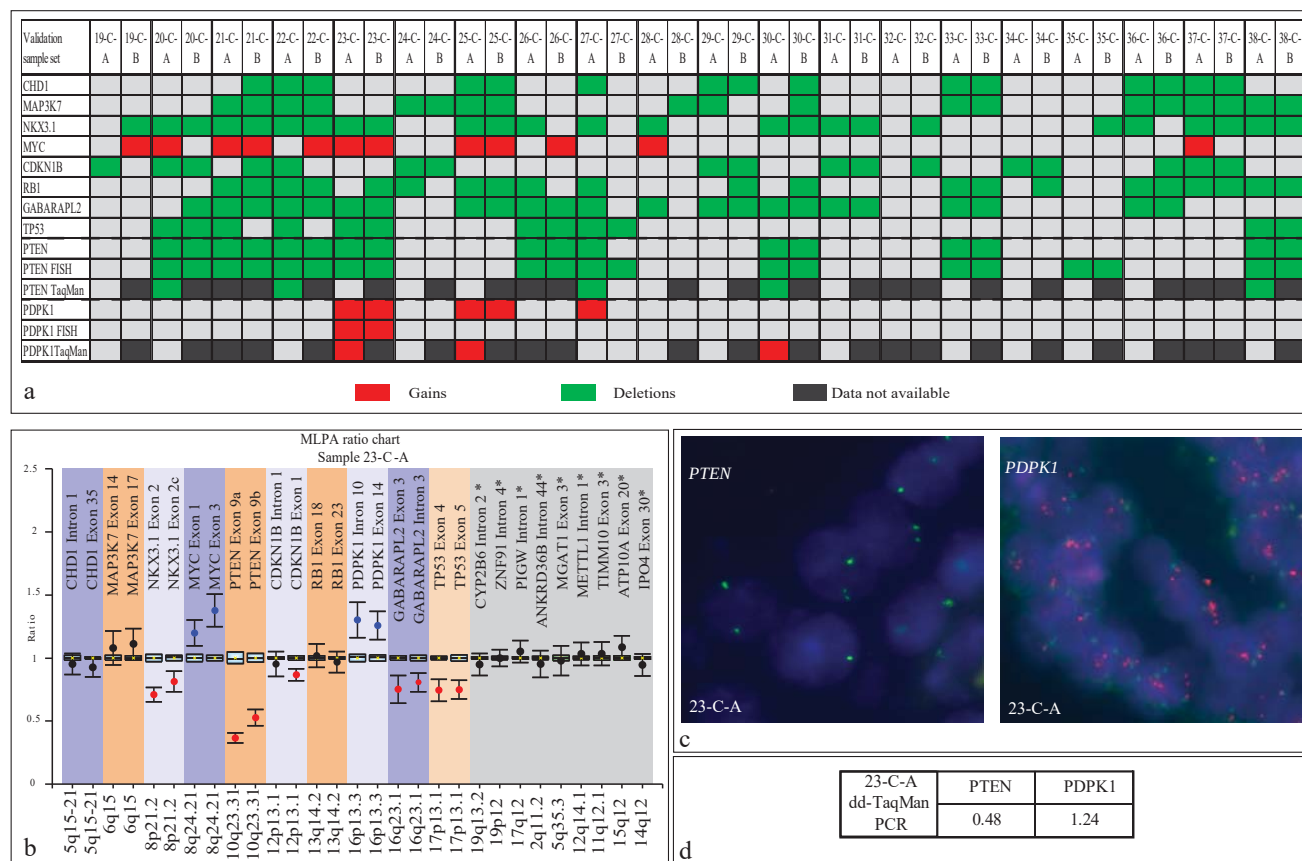
Manuscript 1, Figure 2: Detection of CNAs in PC-3 and LAPC4 cell lines by MLPA and FISH. In MLPA ratio charts, the probe values shown by a blue color correspond to gain based on the standard cut-off point of above 1.3 and probe values with red color show deletion based on the cut-off point of below 0.65. Probes values in purple color show gain or deletion based on the 95% confidence interval only. **a.** MLPA results of PC-3 (left panel) and LAPC4 (right panel) cell lines using the designed PCa probe mix. The red and the blue box highlights respectively the *PTEN* deletion in PC-3 and the *PTEN* gain in LAPC4. **b.** As in a, but showing the MLPA profiles of PC-3 and LAPC4 cell lines using commercial *PTEN* probe mix (P225-*PTEN*, D2-0315, MRC-Holland). **c.** FISH results of *PTEN* and *MYC* on metaphase spreads of PC-3 and LAPC4 cell lines. The *PTEN* FISH probe targets three regions of chromosome 10. The orange signal corresponds to the *PTEN* gene, the green signal represents the *GRID1* gene upstream of the *PTEN* gene and outside of the deletion region while the aqua signal is the centromere control. The *MYC* fluorescent signal is orange and the centromere control for chromosome 8 is green. The average number of signals detected and the test probe over control probe counts ratio is indicated on each FISH picture (96x magnification).



Manuscript 1, Figure 3: Improving the CNA detection limit of MLPA by using 95% confidence interval of probes. The probe ratio value plotted against the percentage of PC-3 genome is shown for *MYC* (a), *PTEN* (b), *NKX3-1* (c) and *CHD1* (d). The dashed lines show the standard cut-off points for gains and deletions based on probe ratios at 1.3 and 0.65, respectively. The solid lines show the cut-off points for gains and deletions based on 95% confidence interval of the probes in the reference DNA.



Manuscript 1, Figure 4: CNA profiles of the test sample set by MLPA and FISH. a. MLPA results of the test sample set. Duplicate reactions were done on each sample and data for each probe are normalized against each of the three reference DNA samples separately. In sample names, numbers refer to the patients ID, followed by C (for cancer) or B for (benign) and the replicate number. Deletions are indicated in green and gains in red. **b.** Final MLPA and FISH CNA calls in the test sample set. **c.** Example of MLPA results for one sample (7-C) which shows deletion in *NKX3-1*, *PTEN* and *RB1*. Probes in red are positive for CNA according the 95% confidence interval method. **d.** FISH results for sample 7-C showing gene specific signal in orange and control signal in green for the 10 loci assessed. Hemizygous deletions in *NKX3-1* and *RB1* and homozygous deletions *PTEN* genes are indicated with arrows.



Manuscript 1, Figure 5: CNA calls by MLPA, FISH and TaqMan in the validation sample set. a. Final MLPA CNA call for all samples in the validation sample set. FISH and MLPA CNA calls are also provided for *PTEN* and *PDPK1* genes. In sample names, numbers refer to the patients ID, followed by C (for cancer), A (the highest Gleason pattern area of the tumor), or B (the lowest Gleason pattern of the tumor). **b.** Example of MLPA profile of sample 23-C-A of the validation cohort showing deletions in *NKX3-1*, *PTEN*, *GABARAPL2* and *TP53* and gain of *MYC* and *PDPK1*. Probes in red or blue are positive for deletion or gain, respectively according to the 95% confidence interval method. **c.** Example of FISH results of sample 23-C-A confirming the *PTEN* deletion and *PDPK1* gain reported by MLPA. Gene specific signals are shown in orange and the control signals in green. **d.** Example of ddTaqMan PCR result of sample 23-C-A confirming *PTEN* deletion and *PDPK1* gain. Final ratios are shown with a cut-off for deletions set at 0.8 and at 1.2 for gains corresponding to two standard deviations below and above average normal values obtained in normal samples.

3.10. Supplementary Tables

Manuscript 1, Table S1: Performance of PCa specific MLPA probe mix

Performance of PCa specific MLPA probe mix assessed by standard deviations, mean and the range of probe ratios in normal fresh and FFPE extracted DNA

Probe	Fresh DNA *				FFPE extracted DNA **			
	Mean	Standard Deviation	Max	Min	Mean	Standard Deviation	Max	Min
<i>CHD1</i> Intron 1	1	0.02	1.04	0.98	1	0.02	1.04	0.97
<i>CHD1</i> Exon 35	1	0.01	1.01	0.98	1	0.01	1.02	0.98
<i>MAP3K7</i> Exon 14	1	0.01	1.01	0.98	1	0.01	1.03	0.98
<i>MAP3K7</i> Exon 17	1	0.01	1.02	0.99	1	0.02	1.02	0.96
<i>NKX3-1</i> Exon 2c	1	0.01	1.02	0.99	1	0.01	1.02	0.98
<i>NKX3-1</i> Exon 2	1	0.01	1.01	0.99	1	0.03	1.03	0.91
<i>MYC</i> Exon 1	1	0.01	1.02	0.98	1	0.01	1.02	0.98
<i>MYC</i> Exon 3	1	0.01	1.01	0.99	1	0.02	1.03	0.97
<i>PTEN</i> Exon 9a	1	0.01	1.01	0.98	1	0.03	1.04	0.95
<i>PTEN</i> Exon 9b	1	0.01	1.03	0.98	1	0.02	1.03	0.97
<i>CDKN1B</i> Intron 1	1	0.01	1.01	0.96	1	0.03	1.03	0.92
<i>CDKN1B</i> Exon 1	1	0.01	1.02	0.98	1	0.03	1.07	0.94
<i>RB1</i> Exon 18	1	0.01	1.01	0.99	1	0.01	1.02	0.98
<i>RB1</i> Exon 23	1	0.01	1.02	0.99	1	0.02	1.04	0.95
<i>PDPK1</i> Intron 10	1	0.01	1.02	0.99	1	0.02	1.05	0.95
<i>PDPK1</i> Exon 14	1	0	1.01	0.99	1	0.02	1.04	0.96
<i>GABARAPL2</i> Exon 3	1	0.01	1.01	0.99	1	0.02	1.02	0.97
<i>GABARAPL2</i> Intron 3	1	0.01	1.02	0.98	1	0.02	1.04	0.96
<i>TP53</i> Exon 4	1	0.01	1.02	0.98	1	0.01	1.01	0.98
<i>TP53</i> Exon 5	1	0.01	1.03	0.98	1	0.01	1.03	0.98
<i>CYP2B6</i> Intron 2	1	0.02	1.02	0.94	1	0.03	1.03	0.9
<i>ZNF91</i> Intron 4	1	0.01	1.02	0.97	1	0.01	1.03	0.99
<i>PIGW</i> Intron 1	1	0.01	1.02	0.98	1	0.01	1.03	0.96
<i>ANKRD36B</i> Intron 44	1	0.01	1.01	0.98	1	0.02	1.03	0.96
<i>MGAT1</i> Exon 3	1	0.01	1.02	0.98	1	0.02	1.04	0.97
<i>METTL1</i> Intron 1	1	0.01	1.01	0.99	1	0.02	1.03	0.96
<i>TIMM10</i> Exon 3	1	0.01	1.01	0.99	1	0.02	1.03	0.95
<i>ATP10A</i> Exon 20	1	0.01	1.02	0.96	1	0.02	1.05	0.96
<i>IPO4</i> Exon 30	1	0.01	1.02	0.98	1	0.02	1.05	0.94
* Four repeats of both male and female fresh DNA								
** Six repeats of both normal kidney and normal breast lymph node FFPE extracted DNA								

Manuscript 1, Table S2: Sensitivity, specificity and accuracy of final CNA calls according to the different criteria and normalization methods

	CHD1	MAP3K7	NKX3-1	MYC	PTEN	CDKN1B	RB1	PDPK1	GABARAPL2	TP53	Median	25% Percentile	75% Percentile
Final CNA call is made when the alteration is detected in the dataset normalized against the average of the three references based on the probe cut-off according to the following criteria:													
Approach 1 : Either probes targeting the gene is altered in either replicates													
Sensitivity	67%	50%	14%	50%	80%	60%	50%		0%	0%	50%	7%	64%
Specificity	80%	100%	100%	88%	100%	100%	100%	94%	100%	100%	100%	93%	100%
Accuracy	78%	89%	67%	83%	94%	89%	72%	94%	83%	94%	86%	77%	94%
Approach 2 : Either probes targeting the gene is altered in both replicates													
Sensitivity	67%	0%	0%	50%	60%	60%	30%		0%	0%	30%	0%	60%
Specificity	80%	100%	100%	94%	100%	100%	100%	100%	100%	100%	100%	99%	100%
Accuracy	78%	78%	61%	89%	89%	89%	61%	100%	83%	94%	86%	74%	90%
Approach 3 : Both probes targeting the gene are altered in either replicates													
Sensitivity	0%	0%	0%	50%	60%	0%	0%		0%	0%	0%	0%	25%
Specificity	93%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	78%	78%	61%	94%	89%	72%	44%	100%	83%	94%	81%	69%	94%
Approach 4 : Both probes targeting the gene are altered in both replicates													
Sensitivity	0%	0%	0%	0%	60%	0%	0%		0%	0%	0%	0%	0%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	83%	78%	61%	89%	89%	72%	44%	100%	83%	94%	83%	69%	90%
Final CNA call is made when the alteration is detected in at least two of the three reference-normalized datasets based on the probe cut-off according to the following criteria:													
Approach 5 : Either probes targeting the gene is altered in either replicates													
Sensitivity	67%	50%	29%	50%	80%	60%	60%		0%	0%	50%	15%	64%
Specificity	80%	100%	100%	94%	100%	100%	100%	94%	100%	100%	100%	94%	100%
Accuracy	78%	89%	72%	89%	94%	89%	78%	94%	83%	94%	89%	78%	94%
Approach 6 : Either probes targeting the gene is altered in both replicates													
Sensitivity	67%	0%	14%	0%	60%	60%	20%		0%	0%	14%	0%	60%
Specificity	80%	100%	100%	94%	100%	100%	100%	100%	100%	100%	100%	99%	100%
Accuracy	78%	78%	67%	83%	89%	89%	56%	100%	83%	94%	83%	75%	90%
Approach 7 : Both probes targeting the gene are altered in either replicates													
Sensitivity	0%	0%	0%	50%	60%	0%	10%		0%	0%	0%	0%	30%
Specificity	87%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	72%	78%	61%	94%	89%	72%	50%	100%	83%	94%	81%	69%	94%
Approach 8 : Both probes targeting the gene are altered in both replicates													
Sensitivity	0%	0%	0%	0%	60%	0%	0%		0%	0%	0%	0%	0%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	83%	78%	61%	89%	89%	72%	44%	100%	83%	94%	83%	69%	90%
Final CNA call is made when the alteration is detected in the dataset normalized against the average of the three references based on the 95% confidence interval according to the following criteria:													
Approach 9 : Either probes targeting the gene is altered in either replicates													
Sensitivity	67%	50%	14%	50%	80%	60%	50%		0%	0%	50%	7%	64%
Specificity	80%	100%	100%	88%	100%	100%	100%	94%	100%	100%	100%	93%	100%
Accuracy	78%	89%	67%	83%	94%	89%	72%	94%	83%	94%	86%	77%	94%
Approach 10 : Either probes targeting the gene is altered in both replicates													
Sensitivity	67%	0%	0%	50%	60%	60%	30%		0%	0%	30%	0%	60%
Specificity	80%	100%	100%	94%	100%	100%	100%	100%	100%	100%	100%	99%	100%
Accuracy	78%	78%	61%	89%	89%	89%	61%	100%	83%	94%	86%	74%	90%
Approach 11 : Both probes targeting the gene are altered in either replicates													
Sensitivity	0%	0%	0%	50%	60%	0%	0%		0%	0%	0%	0%	25%
Specificity	93%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	78%	78%	61%	94%	89%	72%	44%	100%	83%	94%	81%	69%	94%
Approach 12 : Both probes targeting the gene are altered in both replicates													
Sensitivity	0%	0%	0%	0%	60%	0%	0%		0%	0%	0%	0%	0%
Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	83%	78%	61%	89%	89%	72%	44%	100%	83%	94%	83%	69%	90%
Final CNA called is made when the alteration is detected in at least two of the three reference-normalized datasets based on the 95% confidence interval according to the following criteria:													
Approach 13 : Either probes targeting the gene is altered in either replicates													
Sensitivity	100%	100%	86%	50%	100%	100%	100%		67%	100%	100%	77%	100%
Specificity	47%	64%	82%	88%	100%	69%	25%	83%	60%	82%	76%	57%	84%
Accuracy	56%	72%	83%	83%	100%	78%	67%	83%	61%	83%	81%	66%	83%
Approach 14 : Either probes targeting the gene is altered in both replicates													
Sensitivity	67%	75%	86%	50%	80%	80%	90%		33%	100%	80%	59%	88%
Specificity	80%	93%	91%	94%	100%	92%	88%	100%	80%	100%	93%	86%	100%
Accuracy	78%	89%	89%	89%	94%	89%	89%	100%	72%	100%	89%	86%	96%
Approach 15 : Both probes targeting the gene are altered in either replicates													
Sensitivity	67%	75%	86%	50%	80%	80%	60%		33%	100%	75%	55%	83%
Specificity	87%	93%	100%	100%	100%	85%	100%	100%	73%	88%	97%	87%	100%
Accuracy	83%	89%	94%	94%	94%	83%	78%	100%	67%	89%	89%	82%	94%
Approach 16 : Both probes targeting the gene are altered in both replicates													
Sensitivity	67%	50%	29%	50%	80%	60%	20%		33%	100%	50%	31%	74%
Specificity	87%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Accuracy	83%	89%	72%	94%	94%	89%	56%	100%	89%	100%	89%	80%	96%

Manuscript 1, Table S3: CNA calls by droplet digital TaqMan PCR on a subset of samples from the validation sample set.

Sample	Target gene	Probe	Average Fluorescent read	SE	Average fluorescence intensity of test probe/ average fluorescence intensity of reference probe (AGO1)	Normalized probe ratio of the test sample / Normalized probe ratio of the reference sample (FFPE Kidney)	Copy number
19-C-A	PDPK1	PDPK1 Int10	302	4.24	2.12	0.93	Normal
		AGO1	143	4.95			
	PTEN	PTEN Exon9	172	7.07	1.12	0.92	Normal
		AGO1	154	11.31			
20-C-A	PDPK1	PDPK1 Int10	71	2.33	2.07	0.90	Normal
		AGO1	34	2.69			
	PTEN	PTEN Exon9	8	0.42	0.22	0.18	Deletion
		AGO1	36	3.82			
22-C-A	PDPK1	PDPK1 Int10	128	0.71	2.29	1.00	Normal
		AGO1	56	0.28			
	PTEN	PTEN Exon9	45	2.33	0.82	0.67	Deletion
		AGO1	55	1.48			
23-C-A	PDPK1	PDPK1 Int10	80	2.12	2.83	1.24	Gain
		AGO1	28	1.13			
	PTEN	PTEN Exon9	18	0.85	0.59	0.48	Deletion
		AGO1	30	0.78			
24-C-A	PDPK1	PDPK1 Int10	335	7.78	2.23	0.98	Normal
		AGO1	150	1.41			
	PTEN	PTEN Exon9	179	16.26	1.14	0.94	Normal
		AGO1	157	12.73			
25-C-A	PDPK1	PDPK1 Int10	176	3.54	2.99	1.31	Gain
		AGO1	59	0.35			
	PTEN	PTEN Exon9	81	1.70	1.31	1.08	Normal
		AGO1	62	0.71			
27-C-A	PDPK1	PDPK1 Int10	48	2.62	2.50	1.10	Normal
		AGO1	19	1.63			
	PTEN	PTEN Exon9	10	0.28	0.49	0.41	Deletion
		AGO1	20	2.55			
27-C-B	PDPK1	PDPK1 Int10	52	4.03	2.30	1.01	Normal
		AGO1	23	4.31			
	PTEN	PTEN Exon9	22	0.42	1.13	0.94	Normal
		AGO1	20	0.99			
28-C-A	PDPK1	PDPK1 Int10	11	1.34	2.53	1.11	Normal
		AGO1	4	1.13			
	PTEN	PTEN Exon9	6	0.35	1.23	1.01	Normal
		AGO1	5	1.13			
29-C-A	PDPK1	PDPK1 Int10	49	0.57	2.55	1.12	Normal
		AGO1	19	1.41			
	PTEN	PTEN Exon9	24	2.47	1.16	0.95	Normal
		AGO1	21	3.32			
30-C-A	PDPK1	PDPK1 Int10	126	0.00	2.91	1.28	Gain
		AGO1	43	2.47			
	PTEN	PTEN Exon9	24	3.68	0.53	0.44	Deletion
		AGO1	45	2.76			
31-C-A	PDPK1	PDPK1 Int10	342	12.73	2.27	1.00	Normal
		AGO1	151	4.95			
	PTEN	PTEN Exon9	175	17.68	1.14	0.94	Normal
		AGO1	153	7.78			
33-C-A	PDPK1	PDPK1 Int10	76	2.26	1.81	0.79	Deletion
		AGO1	42	3.75			
	PTEN	PTEN Exon9	43	6.43	1.01	0.83	Normal
		AGO1	43	0.49			
34-C-A	PDPK1	PDPK1 Int10	154	9.19	2.23	0.98	Normal
		AGO1	69	6.22			
	PTEN	PTEN Exon9	101	3.54	1.38	1.14	Normal
		AGO1	73	2.55			
35-C-A	PDPK1	PDPK1 Int10	130	5.66	2.41	1.06	Normal
		AGO1	54	0.99			
	PTEN	PTEN Exon9	67	0.71	1.14	0.94	Normal
		AGO1	59	1.06			
36-C-A	PDPK1	PDPK1 Int10	76	1.77	2.58	1.13	Normal
		AGO1	30	2.12			
	PTEN	PTEN Exon9	49	2.83	1.60	1.32	Gain
		AGO1	31	0.21			
38-C-A	PDPK1	PDPK1 Int10	66	4.38	1.94	0.85	Normal
		AGO1	34	3.04			
	PTEN	PTEN Exon9	23	1.84	0.66	0.55	Deletion
		AGO1	34	0.00			
FFPE Kidney	PDPK1	PDPK1 Int10	197	12.73	2.28	1.00	Normal
		AGO1	86	4.67			
	PTEN	PTEN Exon9	103	1.41	1.21	1.00	Normal
		AGO1	85	0.28			
Fresh healthy genome	PDPK1	PDPK1 Int10	316	24.04	1.96	0.86	Normal
		AGO1	161	4.24			
	PTEN	PTEN Exon9	156	5.66	1.03	0.85	Normal
		AGO1	152	0.00			
PC-3 cell line	PDPK1	PDPK1 Int10	340	5.66	1.70	0.75	Deletion
		AGO1	200	2.12			
	PTEN	PTEN Exon9	0	0.00	0.00	0.00	Deletion
		AGO1	191	21.92			

Manuscript 1, Table S4: Statistical difference between the MLPA CNA call of samples A and B of the validation data set

	McNemar Test (<i>p</i> value)
<i>CHD1</i>	1
<i>MAP3K7</i>	1
<i>NKX3-1</i>	1
<i>MYC</i>	0.68
<i>PTEN</i>	1
<i>CDKN1B</i>	1
<i>RB1</i>	1
<i>PDPK1</i>	1
<i>GABARAPL2</i>	1
<i>TP53</i>	0.48

RATIONALE FOR CHAPTER 4 (MANUSCRIPT TWO)

In chapter 3 (manuscript one), we conducted a literature search which led to the identification of 10 CNAs relevant to prostate tumor biology and known to correlate with disease outcome. We next used these CNAs to design, develop and optimize a novel PCa-specific MLPA assay. We showed that our approach allows accurate detection of CNAs in human PCa cell lines and heterogeneous tumor samples compared to FISH as routinely used in the clinic. Validation of the assay was achieved on a second set of PCa samples using FISH and droplet digital PCR as complementary methods. Our novel assay has several advantages over FISH, as it allows the high-throughput assessment of CNA signatures in small quantity and quality of FFPE extracted DNA usually obtained from biopsy samples.

About 80% of patients diagnosed with PCa have a local disease of low- and intermediate-risk (Gleason scores 6 and 7). However, significant clinical heterogeneity is observed among patients within these recognized risk groups. Accordingly in Chapter 4 (manuscript two), we applied our newly designed PCa probe mix, upgraded to test 4 additional genes, to DNA extracts from prostate tissues of a large cohort of patients with Gleason scores 6 and 7. The simultaneous assessment of these CNAs showed a significant association with clinicopathological features. Further analysis led to the development of a CNA classifier that better stratifies this population of patients and differentially predicts biochemical recurrence. Our newly developed CNA classifier was validated using genomic datasets of three additional cohorts of radical prostatectomy patients along with a cohort of men who received radiotherapy and from whom DNA from prostate biopsies had been analyzed for CNAs. The superiority of our classifier, also enhanced by combining clinicopathological characteristics, further closes the gap between the research and clinic. Moreover, this novel and accurate prognostic tool is expected to aid the decision-making process in low- and intermediate-risk PCa patients.

4. CHAPTER 4 – MANUSCRIPT TWO

Copy number alteration classifier as a prognostic tool for risk stratification of patients with low- and intermediate- risk prostate cancer

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Running title: CNA classifier in low- and intermediate risk prostate cancer

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4.1. Abstract

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide and has a heterogeneous clinical outcome. Currently used clinicopathological features cannot precisely distinguish aggressive from indolent disease and orient treatment selection. Thus, there is a need for clinically applicable biomarkers that could aid the decision-making process, particularly for patients with low- and intermediate- risk disease. Since DNA copy number alterations (CNAs) can predict biochemical recurrence (BCR), we optimized our previous multiplex ligation-dependent probe amplification (MLPA) specific PCa probe mix and used it to develop a CNA classifier for prediction of BCR. We expanded the probe mix to target 14 genes and applied it in patients with low- and intermediate- risk PCa. We profiled the CNAs of 433 patients who underwent radical prostatectomy (RP) at McGill and Queen's University Health Centres. Our results indicate that samples with a higher percentage of CNAs show significantly higher rate of pre-surgery PSA levels, Gleason score and BCR. A CNA classifier for prediction of BCR was developed using Cox proportional hazard modeling. Our CNA classifier was predictive of BCR independently of clinicopathological features in multivariate analysis. The prognostic value of our model was further validated in 541 PCa patients with low- and intermediate- risk disease from four published datasets. We further improved the prognostic efficacy of this CNA classifier by including pre-treatment and post-treatment clinicopathological features. The CNA-clinical classifiers showed improved risk stratification and prognostic efficacy over the clinical model in all tested datasets. Altogether our novel assay predicting outcome has the potential to improve the clinical management of patients with low- and intermediate-risk disease.

4.2. Introduction

In men, prostate cancer (PCa) is the second most frequently diagnosed cancer worldwide with 1.3 million new diagnoses, and the fifth cause of cancer-related mortality with 359,000 deaths in 2018 [1]. Compared to other types of cancer, PCa is recognized by long survival rates [2] and slow-growing tumors with low potential of spreading to other organs [3], thus most men will likely not progress and experience aggressive symptoms in their lifetime or die from PCa. Meanwhile, reports suggest that 23-42% of diagnosed cases in developed countries are due to overdiagnosis [3]. This has been further proven by clinical trial showing that in order to prevent one prostate cancer-specific death, on average, 8.69 (95% confidence interval/CI 5.5-19.3) patients must undergo aggressive treatments [4]. Currently, treatment decisions are based on clinical and pathological features such as prostate-specific antigen (PSA) serum levels, tumor stage, and biopsy Gleason scores. Patients with biopsy Gleason scores (GS) 3+3, and in some instances, 3+4 (Gleason groups/GG ≤ 2) and PSA of less than 15 ng/ml and local disease have been offered active surveillance which allows close monitoring of the disease [5, 6]. Patients with more advanced disease are treated using more aggressive procedures such as radical prostatectomy (RP) or radiotherapy, often coupled to androgen deprivation therapy [7].

Nowadays, the widespread screening of PCa through monitoring PSA levels and needle biopsies result in new diagnoses that mostly have local and indolent disease [8, 9]. Unfortunately, current diagnostic tools are not adequately powered to select patients that would most benefit from active surveillance from patients that would require immediate curative treatments [10]. Thus, there is an unmet need for clinically applicable biomarkers that would aid the decision-making process, especially in the increasing population of patients with localized disease and low- and intermediate-risk of progression.

Molecular and genetic markers have improved risk stratification and provided more precision with treatment decisions in other types of cancer, such as breast [11] and leukemia [12]. In PCa, gene expression signatures have been developed and related tests are now being introduced into the clinic for risk classification [13, 14]. However, further prospective studies are needed to confirm their added benefit to patient's disease management. DNA copy number alteration (CNA) signatures have not yet been used in the clinical setting. There are ample data suggesting the usefulness of CNA signatures or individual alterations in the prognosis of PCa [15-18]. Moreover, it has been shown that CNA signatures can be predictive of biochemical recurrence (BCR) after RP in low- and intermediate-risk PCa (Gleason scores/GS ≤ 7) [19] which may identify potential candidates for active surveillance.

Previous studies have shown that CNAs associated with aggressive PCa can be retrieved in tumor biopsies and are indicative of early relapse after treatment and fast progression [19, 20]. They could therefore help the decision making process [21]. Therefore, including genomic biomarkers in the management of men undergoing biopsies for PCa would likely allow a more accurate risk stratification and patient classification. As a result, it would be possible to prevent overtreatment of cases with clinically insignificant disease and provide more evidence to treat patients with more aggressive approaches at time of diagnosis and thereby increasing the likelihood of success in curing the disease.

The clinical application of CNA signatures remains limited due to the complexity, cost and incompatibility of common CNA detection methods, such as array-comparative genomic hybridization (CGH) or sequencing with low quality and quantity of DNA obtained from formalin-fixed paraffin embedded (FFPE) biopsy samples. Simple and more cost-effective approaches are needed to facilitate the integration of genomic classifiers into the routine clinical

setting. Multiplex ligation-dependent probe amplification (MLPA) is considered as the gold standard PCR-based method for DNA copy number quantification [22]. MLPA is compatible with DNA extracted from FFPE samples and only requires 50 ng of DNA per reaction. MLPA can provide a cost-effective approach for simple, fast and accurate copy number read of up to 50 different loci in a single reaction. We previously developed and optimized a MLPA-based assay for profiling 10 most common CNAs in PCa including 5q15-21.1 (*CHD1*), 6q15 (*MAP3K7*), 8p21.2 (*NKX3-1*), 8q24.21 (*MYC*), 10q23.31 (*PTEN*), 12p13.1 (*CDKN1B*), 13q14.2 (*RB1*), 16p13.3 (*PDPK1*), 16q23.1 (*GABARAPL2*) and 17p13.1 (*TP53*) (Chapter 3, Manuscript one). Here we describe its expansion by including four additional PCa relevant genes identified in the Stanford dataset [15] and confirmed in previously published studies [23, 24]. These new genes include; *RWDD3* a gene located at 1p21.3, a locus deleted in PCa [25, 26] and involved in DNA repair machinery, hypoxia-inducible factor (HIF)-1a and cellular transport pathways [27, 28]; *PDZD2* at 5p13.3 that undergoes copy number gain and could be involved in early-stages of prostate tumorigenesis [29, 30]; *GTF2H2* one of the regulators of DNA excision repair pathway [31] and AR transcriptional activity [32] located at 5q13.3 and which deletion is associated with disease stage [20, 33]; and *WRN* a gene involved in the maintenance of genomic stability [34, 35] residing at 8p12, a frequent deletion site in PCa [15, 36]. Furthermore, we included an additional reference probe targeting the *DHRS4L2* in a CNA-quiet region of 14q11.2.

We use this expanded PCa specific probe mix on a RP cohort of patients with low- and intermediate- risk disease. We introduced and validated a CNA classifier, which predicts BCR independently of clinicopathological features. We further improved the prognostic efficiency of our CNA classifier by including clinicopathological features and validated its performance in a

dataset of patients with biopsies collected prior to radiation therapy. We show improved prognostic indexes of our CNA classifiers over the clinical model.

4.3. Materials and Methods

Patients and samples

For expansion of the PCa specific probe mix we used the previously extracted DNA from an independent set of PCa clinical samples obtained from Queen's University (n=13) to develop the MLPA assay (Chapter 3, Manuscript one).

For developing the CNA classifier, we used a cohort consisting of 433 patients treated by RP between 1996 and 2013, at McGill University and Queen's University Health Centres, with prostate tissues collected by pathology departments of the respective institutions and hereafter referred to as PARSE MLPA cohort. The inclusion criteria were patients with low- and intermediate-risk disease based on the GS of ≤ 7 of the RP tissue, according to the latest International Society of Urological Pathology/World Health Organization recommendations [37], with no prior treatments. The ethical approval for the use of all samples and clinical data was granted by the Research Ethics Board of McGill University Health Centre (Quebec, Canada, BDM-10-115), amended to include samples from Kingston General Hospital affiliated to Queen's University, Ontario, Canada.

Patient's tumors in the PARSE MLPA cohort are represented by two samples; sample A, taken from the highest Gleason pattern and sample B, taken from the lowest Gleason pattern. In the case of GS 6= 3+3, samples were assigned to A or B randomly. Details of the cohort and clinical attributes are provided in Table 1.

Genomic DNA extraction

Three cores of 0.6 mm in diameter and 2-3 mm in length, pre-identified by pathologists (FB, DMB) on corresponding sections, were punched from two tumor foci in FFPE prostate blocks, representing samples A and B. Genomic DNA of each sample was extracted using a modified protocol of Qiagen Allprep DNA/RNA extraction kit as we described before [38]. PC-3 cell line DNA was extracted from 2 million cells using Qiagen DNeasy Blood & Tissue DNA extraction kit. All extracted DNAs were dissolved in water and quantified via Qubit 2.0 Fluorometer (Life Technologies) and diluted in TE buffer (10mM Tris-HCl; 0.1 mM EDTA; pH 8.2) prior to use.

Expansion of the PCa MLPA probe mix

We improved our previously described (Chapter 3, Manuscript one) by targeting four more genes with two probes each and the addition of another reference probe. These additional target genes include *RWDD3*, *PDZD2*, *GTF2H2* and *WRN* and the new reference gene, *DHRS4L2*. All probes were designed using MLPA designer® software according to our previously described criteria (Chapter 3, Manuscript one). Probes were designed to target regions on the gene that do not contain common SNP and mutation sites in PCa according to the Ensembl Variation database [39] and information available on cBioPortal website, respectively [40, 41]. Probe-probe interactions were assessed to ensure the newly designed probes are compatible with the previous PCa specific probe mix. Probes were synthesized at 4 nmole scale and purified using standard desalting by Integrated DNA Technologies (IDT). The 3' half-probes were 5' phosphorylated.

The complete PCa specific probe mix targets 14 genes along 10 reference probes targeting CNA-quiet cytobands are listed in Table S1.

Fluorescence *in situ* hybridization

We assessed the sensitivity and specificity of the newly designed probes as described previously using fluorescence *in situ* hybridization (FISH) as a reference assay on a tissue microarray representing cores from regions adjacent to those used for DNA extraction and MLPA assay development (Chapter 3, Manuscript one). BAC clones RP11-335D10 mapping to 1p21.3, RP11-437P15 mapping to 5p13.3, RP11-195E2 mapping to 5q13.2 and RP11-363L24 mapping to 8p12 were labeled with spectrum Orange dUTP (Enzo Life Science) using Nick Translation kit (Abbot Molecular) and used for CNA assessment of *RWDD3*, *PDZD2*, *GTF2H2* and *WRN*, respectively. Spectrum green labeled chromosome 1 subtelomere specific probe (Cytocell), RP11-530D2 BAC (5p12) and CEP8 (Abbot Molecular) were used as control reference probes. Images were acquired using Olympus IX-81 inverted microscope at 96X magnification and Image-Pro Plus 7.0 software (Media Cybernetics).

DNA copy number assessment by MLPA

Our expanded PCa specific MLPA probe mix along with the optimized MLPA assay were used for CNA profiling. For reference samples, normal control DNA consisted of a commercially available healthy female genome (Promega) along with DNA extracted from FFPE tissues, kidney (provided by McGill University Health Centre, Pathology department) and breast lymph node (provided by Ontario Institute for Cancer Research). The PC-3 cell line genome served as a positive control and a no-DNA reaction as a negative control.

MLPA was done on all samples in duplicate in a total of 22 batches. Gleason scores and BCR status were harmonized across all 22 batches to minimize batch effects. A and B samples of each patient were included in the same batch. MLPA was performed on 50 ng of DNA per reaction as described before (Chapter 3, Manuscript one) and according to the manufacturer guidelines using MLPA One-Tube general protocol and EK5-FAM kit. Briefly, the initial denaturation was done

at 98°C for 5 min. Then the hybridization mix was added at room temperature, followed by denaturation step at 95°C for 1 min and hybridization step at 60°C for 16 h. The ligation mix was then added at 54°C followed by incubation at 54°C for 15 min and then 98°C for 5 min. The PCR reaction was carried after the addition of polymerase mix, for 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by a final extension of 20 min at 72°C. Capillary electrophoresis was done by the Genomics platform of the Institute for Research in Immunology and Cancer (IRIC), Université de Montréal.

MLPA Data processing

Before data processing, quality control (QC) was performed on the data and low-quality reactions were repeated whenever possible or removed. Low quality was defined as standard deviation (SD) of more than 0.1 in more than four probes or absence of fluorescent signal in the reaction. Raw data generated by MLPA were processed using Coffalyser software (Version 140721.1958) and the P.I.N.P.2 protocol. The median value of the test probe over each of the reference probes was used for intra sample normalization and the average value of the normalized probe signal in test sample over each of the reference samples were used for the inter sample normalization. If the 95% CI value of the probe in the test samples were above or below what it showed in the reference samples, gain or deletion calls were respectively made, otherwise the normal copy number was assigned to the probe. Data were normalized as described above against each of the three reference DNA sample populations separately. CNA data were then exported to Microsoft Excel for further analysis. Deletion or gain on gene level was assigned to the sample when both probes targeting the gene in both replicates reported deletion or gain, respectively in at least two of the three normalizations, otherwise, the copy number of the gene

was considered as normal (Chapter 3, Manuscript one). If both A and B samples of the patient were available, the sum of CNAs in both samples were considered as the patients CNA profile.

Statistical analysis

Statistical analysis was done using R version 3.5.2, IBM SPSS version 25 or GraphPad Prism version 6. For comparison between groups for continuous variables, non-parametric Mann-Whitney *U* test or Wilcoxon matched-pairs signed rank test were used and for categorical variables, the χ^2 test was performed. Survival package (version 2.43) and log-rank test were used for survival analysis. Cox modeling (stepwise backward method) and calculation of Harrell's C-index was performed using rms package (version 5.1). In all statistical tests, *p* values of less than 0.05 were considered as statistically significant.

To validate the CNA classifier and compare the results of our study cohort to previously published array-CGH studies, we only included patients with GS ≤ 7 (GG ≤ 3) and local disease ($\leq pT3$) from MSKCC dataset (FFPE radical prostatectomy samples) [23], Toronto dataset (flash-frozen pre-treatment biopsy samples) [24], Cambridge dataset (flash-frozen radical prostatectomy samples) [42], and CPC dataset (FFPE radical prostatectomy samples) [43] to represent patients with low- and intermediate- risk disease similarly to our cohort. Clinical characteristics of cases in the cohort are presented in Table S2.

The primary endpoint of this study was prediction of BCR defined as two consecutive measurements of PSA level of more than 0.2 ng/ml in post-RP cohorts (PARSE MLPA, MSKCC, Cambridge, and CPC) or increase of more than 2 ng/ml of PSA level above the post-radiation nadir value for the Toronto cohort. Unless otherwise stated, through the study GS 4+3 vs. $\leq 3+4$ and stage (in pre-treatment setting T2 vs. T1 and in post-treatment setting T3 vs. T2)

were used as binary variables whereas pre-treatment PSA levels were used as a continuous variable.

The prognostic effect of the model for detection of BCR was assessed using C-index, univariate and multivariate Cox proportional hazard analysis adjusting for RP GS, pathological stage and pre-treatment PSA. Comparing the goodness of fit between models was done using likelihood ratio test. We also assessed the prognostic value of the developed models at 3 years and 5 years using area under the receiver operator curve (AUC) (pROC package, version 1.14.0). To assess the prognostic value of the model at different time points, patients that did not have follow-up data before or had BCR after the tested time points were filtered out. Cutoff points on the linear predictor assessed by multivariate Cox proportional hazard model for prediction of risk groups, Kaplan-Meier analysis and prognostic indexes were calculated using cutpointr package (version 0.7.6) and maximize metric method.

4.4. Results

Expansion of PCa MLPA probe mix

Sensitivity and specificity of the newly added probes, *RWDD3*, *PDZD2*, *GTF2H2* and *WRN* in the detection of CNAs were evaluated in 13 clinical PCa samples previously used for the development of the assay (Chapter 3, Manuscript one). Results of CNA calls based on MLPA and FISH for each sample are illustrated in Figure 1a. The deletion of *RWDD3* was detected in one sample with a sensitivity and specificity of 100%. *PDZD2* targeting probes showed specificity of 100%, (no gains or deletions were observed in this sample-set by both FISH and MLPA). Deletion of *GTF2H2* was detected with the sensitivity of 80% and specificity of 75% and observed in 6 patients. CNA of *WRN* was detected with 80% sensitivity and 88% specificity,

showing deletion in 5 patients (Figure 1a). Examples of these specific CNAs are also shown in the profiles of two patients and confirmed by FISH (Figures 1b and 1c).

DNA copy number analysis of PARSE MLPA cohort

Overall, out of 1,674 reactions, 89.7% of samples passed the QC. Of those that failed, 0.7% (12 reactions) was due to lack of fluorescent signal and 9.6% (160 reactions) was due to high SD of the probes. Out of the 459 patients, 433 (94.3%) passed the QC (both replicates of A or B samples of the patient passed the QC) and were included in the subsequent analysis.

Correlation in CNA call between the two probes targeting the same gene reveal positive values for all probes except for *PDPK1*. Thus, the performance of *PDPK1* probes were further compared to the previously reported FISH results of a subset of patients in PARSE MLPA cohort [44] and showed lack of sensitivity of the exon 14 targeting probe. Thus, this probe was removed from subsequent analyses.

Pearson correlation between the CNA call of duplicate reactions was 0.73 ($p < 0.0001$). Reproducibility of the assay over all 22 batches was 0.74 (95% CI 0.72-0.74, $p < 0.0001$), as assessed using Fleiss' Kappa interclass correlation obtained from CNA calls of PC-3 positive controls included in each batch.

Similarly to previous reports [23, 24, 42, 43], the most frequent CNAs (Figure 2a) were deletions of *NKX3-1* (8p21.2) at 35%, followed by *WRN* (8p12) at 30%. After the 8p arm deletion, the most frequent CNAs were deletion of *GTF2H2* (5q13.2) at 28% and deletion of *CDKN1B* (13p13.1) at 25%. The lowest CNA frequency was gains of *PDZD2* (5q13.3) and *PDPK1* (16p13.3) at 1% and 4%, respectively. Overall, there was no statistical difference (p : 0.0979,

ANOVA) in the obtained CNA frequencies of the assessed genes in our cohort compared to previously reports used in this study [23, 24, 42, 43].

Of interest, we had access to the mRNA expression levels (by NanoString technology, unpublished data) of 10 of the genes assessed by our developed MLPA assay. Results in Figure S1 confirmed that DNA copy number results for most genes were paralleled by corresponding change in RNA expression level in both A samples and B samples. These findings support that the observed CNA translates into changes in transcripts.

In our data set, 23% of samples showed no CNA in the assessed genes. This value is within the range of samples without CNA for the same 14 genes in GS 6 and 7 (GG 1-3) patients of other cohorts, with 45% for the Cambridge, 30% for the MSKCC, 29% for Toronto, and 23% for the CPC datasets. The distribution of number of CNAs per patient (Figure 2b) in PARSE MLPA cohort ranged from no and only one CNA detected in 24% and 21% of the cohort, respectively to maximum of 10 CNAs detected in only one patient.

Copy number alterations correlate with clinicopathological features

The overall analysis of CNA frequency by Kaplan-Meier presented in Figure 2c show that patients without CNA in the studied 14 genes do not show higher risk of BCR compared to those with CNAs. Nonetheless, patients with 2 or more CNAs in these genes had significantly higher probability of BCR (Figure 2d). Furthermore, and as indicated in Table 2, the percentage of CNAs in patients showed to be an independent predictor of BCR in univariate and multivariate analyses, after adjusting for pre-treatment PSA levels, surgical Gleason score and pathology stage. Moreover, patients with a higher percentage of genes affected by CNAs had significantly higher pre-surgery PSA levels ($p < 0.0001$, Wilcoxon matched-pairs signed rank test), Gleason

score ($p < 0.0001$, Kruskal-Wallis test), and BCR at 5 years ($p < 0.0024$, Mann Whitney U test), while no correlation with stage was observed.

Gene by gene analysis revealed association between CNAs and certain clinicopathological features. For instance, Figure S2 shows that higher pre-treatment PSA levels in patients were associated with prostate tumors harboring deletions in *NKX3-1*, *CDKN1B*, and *GABARAPL2* compared to samples without these CNAs. Similarly, higher Gleason groups were significantly associated with deletions in *MAP3K7*, *WRN*, *PTEN*, *GABARAPL2* or gain in *MYC* (Figure S3). These findings strongly support that CNAs in specific genes included in our selection have clinical relevance.

Developing a classifier based on CNAs for prediction of outcome

To investigate the prognostic value of specific CNAs in prediction of risk for BCR, univariate Cox proportional hazard was performed. Results are presented in Figure 3a and show that deletion of *RWDD3*, gain in *PDPK1*, deletions of *PTEN*, *TP53*, *NKX3-1*, *WRN* were associated with a higher probability of BCR, while gain of *MYC* and deletion of *CDKN1B* showed borderline p values of 0.055 and 0.053, respectively. This was further confirmed by Kaplan-Meier analysis presented in Figure S4.

To develop a CNA classifier that can best predict BCR, stepwise backward Cox proportional hazard was applied on our 14 gene set and combination of genes showing the highest C-index was selected to be included in the classifier. The combination of six CNAs, deletions in *RWDD3*, *WRN*, *PTEN*, *TP53* and gains in *MYC* and *PDPK1*, was selected to develop an optimal CNA classifier. This CNA classifier was able to predict BCR with a C-index of 0.68. In a multivariate analysis presented in Table 3 and after adjusting for GS, pathological stage and pre-treatment

PSA, the model retained its significance (Wald test, p : 0.001) with a hazard ratio of 2.12. Moreover, ROC analyses (Figures 3b and 3c) showed an AUC of 0.68 at 3-years and 0.66 at 5-years. BCR-free survival analysis shown in Figure 3d indicate that the CNA-classifier can further stratify GS 6-7 patients with low- and intermediate-risk disease into two distinct groups with favorable and unfavorable outcomes. We next compared the performance of our classifier with the percentage of CNAs *per se* expressed as a continuous variable. The multivariate analyses revealed the superiority of the 6-gene CNA classifier over percentage of CNA (Table S3). Furthermore, AUC analyses (Figure S5) comparing the CNA classifier to both percentage of CNAs and *PTEN* deletion, a well-known prognostic marker in PCa, showed higher prognostic value for the CNA classifier at both 3- and 5-years (0.68 vs. 0.63 vs. 0.58 and 0.66 vs. 0.63 vs. 0.56, respectively). Our findings indicate that the CNA of 6 genes related to tumor biology shows a stronger prognostic value over the percentage of CNAs in any other genes assessed or a single CNA (*PTEN* deletion).

Effect of tumor heterogeneity on the performance of the CNA classifier

Within the perspective of patient management, we investigated the impact of sampling on the performance of the CNA classifier. Therefore, we rebuilt the CNA profile of the cohort by taking the CNA results of A or B samples randomly for patients that have both samples A and B and BCR information available ($n=292$). This was repeated 10 times to create 10 variations of the cohort where each time the CNA profile of each patient was randomly taken from the A or B sample. Results in Figure 4a indicate that the CNA classifier showed similar C-index in all cohorts randomly assembled from the CNA of the A or B samples with no statistical difference (ANOVA, p : 0.99). These findings suggest that sampling different area of the tumor with Gleason grade 3 or 4 did not significantly impact the prognostic value of the classifier.

The C-index and hazard ratio of the CNA classifier were further calculated in A and B samples separately. When the sum of CNAs in both samples A and B were considered as the patients CNA (Figure 4b), the C-index was 0.69 (hazard ratio of 2.85). In the same patients, the A samples had a C-index of 0.68 (hazard ratio of 2.74) and B samples also showed a C-index of 0.68 (hazard ratio of 2.67). Similarly, ROC analysis showed that at 3-years when both samples were considered, the AUC was 0.70, while AUCs were 0.66 and 0.64 for the A and B samples respectively. At 5-years, AUCs were 0.68 for the sum of CNA on both samples and 0.64 for both A and B samples (Figures 4c to 4h). As shown by Kaplan-Meier curves in Figures 4i to 4k, the CNA classifier was able to identify patients with favorable or unfavorable outcome when either the A or B sample was used. However, best risk stratification was seen when the sum of CNAs in both samples were considered as the patients CNA profile.

Validation of the CNA classifier in RP cohorts

The prediction accuracy of the CNA classifier was calculated using C-index in RP patients with GS of ≤ 7 of the MSKCC, Cambridge and CPC datasets. We observed (Figure S6) similar or higher C-index in all datasets compared to PARSE MLPA cohort. The highest value was Cambridge (0.74, 95% CI: 0.58-0.89, p 0.003), then MSKCC (0.69, 95% CI: 0.58-0.79, p 0.0001), and lastly CPC (0.66, 95% CI: 0.58-0.74, p 0.0001) cohorts. Multivariate analysis also revealed that the CNA-classifier predicts BCR independently of other clinicopathological features (Table 4) in all tested cohorts. In addition, Kaplan-Meier analysis showed classification of patients into favorable- and unfavorable groups in all tested datasets ($p < 0.05$) (Figure S7a-S7c). Altogether these results confirmed the efficacy of our CNA classifier in stratification of low- and intermediate-risk patients into favorable and unfavorable outcome groups.

Combining CNA classifier with post-treatment clinicopathological features

To evaluate if the addition of clinicopathological features available after treatment to the CNA classifier would improve the prediction of BCR after RP, a post-treatment model using RP GS, pathological stage and pre-treatment PSA levels was developed and combined with the CNA classifier. In the subset of patients in the PARSE MLPA cohort with all post-treatment clinical data available (n=406), combination of the CNA classifier with the post-treatment model showed higher C-index than either model alone (Table 5). Similarly, data in Table 6 showed superiority of the combined model in the CPC and MSKCC datasets with higher C-index than CNA-classifier or post-treatment model alone. However, in Cambridge dataset, while the CNA-post-treatment classifier showed higher C-index compared to the post-treatment model, the CNA classifier alone outperformed these two models (Table 6). Kaplan-Meier analysis also revealed patients' classification into favorable and unfavorable outcome groups, using the CNA-post-treatment model in all datasets (Figure 5a, 5d, 5g and 5j). In all tested datasets, the CNA-post-treatment classifier showed higher AUC compared to the post-treatment model alone at both 3- and 5-years in all cohorts, except for the PARSE MLPA cohort for which the post-treatment model showed an AUC of 0.78 vs. the 0.77 value observed from the CNA-post-treatment at 3-years (Figure 5b). Furthermore, likelihood ratio test confirmed the superiority of the CNA-post-treatment classifier over the post-treatment model alone (X^2 : 11.40, $p < 0.0001$).

Combining CNA classifier with pre-treatment clinicopathological features

To adopt this assay for prognosis purposes, and provide risk stratification to guide therapeutic decision making, we developed a pre-treatment classifier based on the information available at diagnosis, i.e. before treatment. Accordingly, biopsy Gleason group, clinical stage (cT) and pre-operative PSA levels were combined to develop a pre-clinical model, which was then combined to our CNA classifier. The likelihood ratio test indicated the superiority of the CNA-pre-

treatment classifier over the pre-treatment model alone (χ^2 : 4.57, $p < 0.05$). Moreover, in the subset of patients of the PARSE MLPA cohort with pre-treatment available data (n=252), the CNA-pre-treatment classifier (Table 7) showed improved C-index compared to the CNA classifier or the pre-treatment model alone.

As shown in Figure S8, the CNA-pre-treatment classifier further improved patients' classification in Kaplan-Meier analysis. Moreover, we validated the performance of the CNA-pre-treatment classifier over the pre-treatment model using Kaplan-Meier, 3-years and 5-years AUC (Figure S8) and multivariate analysis (Table S4) in all RP validation datasets.

Application of the developed classifiers on a biopsy cohort

In view of potential application at the time of diagnosis, we evaluated the performance of our developed classifiers in the Toronto cohort, in which the CNA measurements were done on biopsy samples obtained prior of patients' treatment with image-guided radiotherapy. Results available in Table 8 show that highest C-index of the CNA classifier was achieved in this biopsy cohort with the value of 0.74 and hazard ratio of 3.82. In addition, the CNA-classifier was an independent predictor of BCR after adjusting for pre-treatment PSA, clinical stage and biopsy GS (Table 8). Furthermore, Kaplan-Meier analysis (Figure 6a) indicates that our CNA-classifier can further stratify these low- and intermediate-risk patients into two distinct groups, with favorable and unfavorable outcomes and the CNA-pre-treatment classifier, further improved this risk stratification (Figure 6b). Finally, the AUC at both 3- and 5-years (Figures 6c and d) showed improved prognostic value for the CNA-Pre-treatment classifier at both 3- and 5-years over the pre-treatment model alone, with values of 0.93 vs.0.85 and 0.77 vs.0.65, respectively.

Collectively these findings strongly suggest that our CNA classifier, alone or combined to pre-treatment data at time of diagnosis, may be a valuable tool for the management of low- and intermediate-risk PCa patients.

4.5. Discussion

MLPA provides a fast, easy and cost-effective approach for the assessment of candidate CNAs in small quantities of nucleic acids extracted from fixed tissue samples. This is especially important in the assessment of specific genomic biomarkers in a clinical setting where the amount of biopsy specimens is limited, and full genome profiling is not required. We previously described the development of our PCa specific MLPA probe mix that can be used for high throughput CNA analysis of clinical samples. We further improved this probe mix by addition of probes targeting four more genes of relevance for PCa outcome. Here we used our developed PCa specific MLPA probe mix to profile the DNA copy number of 14 genes in cytobands with recurrent alterations in PCa in a large cohort of 433 patients with low- and intermediate-risk disease with GS of ≤ 7 treated by RP performed at two Canadian University Health Institutions. The CNA data were next used to develop a CNA classifier that improves risk prediction in this group of patients. We further validated the efficacy of our CNA classifier in 541 additional patients with similar clinicopathological features from four reported PCa datasets obtained from prostate primary tumors of RP cases (CPC, MSKCC, Cambridge) and biopsy specimens from patients who were next treated by radiation therapy (Toronto).

The frequency of CNAs detected by our method was within the range of previous reports on PCa patients, thereby supporting the accuracy of our assay and analysis approach. Approximately a quarter (23%) of our prostate tumor samples did not harbor CNA in the assessed genes, a finding

which is within the range found in other published four datasets we have analyzed. Furthermore, previous reports have also mentioned that about a third of the samples obtained from localized PCa tumors have no CNA, yet 30% of them develops BCR [17]. The rate of BCR in samples without CNA in our cohort was lower, about 12%. The robustness of our assay and analysis approach for detection of CNAs was reinforced by high correlation between DNA copy number results and mRNA expression level in both A and B samples (independently assessed via NanoString technology, unpublished). Since our nucleic acid extraction protocols allow simultaneous extraction of DNA and RNA, the high correlation of the CNA and RNA expression supports the accuracy and reliability of our new approach and strategy to identify CNAs in prostate tumors. It also suggests that alterations in specific genes in tumor cells are accompanied by changes in corresponding transcripts.

One of the advantages of the methodology that we adopted and optimized for this study is the use of two different areas from primary tumors per patient and the assessment of the effect of sampling different sites or tumor grades on risk prediction. This concern was raised in previous studies stating that the PCa intratumor heterogeneity could negatively impact the performance of genomic classifiers [17]. Results of our study suggest that the selected target genes show similar CNAs in both samples obtained from the same patient. Our CNA classifier showed no statistical difference in risk stratification of patients when only sample A or B were used (Wilcoxon matched-pairs rank test, p : 0.23). Moreover, when only A samples were used, 89% of patients and when only B samples were used, 85% of patients showed similar risk classification to when sum of both samples CNAs were used to define risk groups. Similarly, application of the CNA classifier on either A or B samples resulted in a similar C-index and hazard ratios. These results indicate that although considering the sum of CNA of both samples results in a better risk

stratification, our developed CNA classifier is still able to predict the patients risk group with a high accuracy when either the low-grade sample or the high-grade sample of the tumor is used. Altogether, these results indicate the robustness of our CNA classifier in predicting outcome in a clinical setting when biopsy samples are randomly taken from different tumor areas.

We observed that a higher percentage of genes affected by CNA in prostate tumors are highly correlated with a higher rate of pre-treatment PSA levels, BCR, and Gleason score. In multivariate analysis, the percentage of CNAs was predictive of patient outcome, independently of other clinicopathological features. This is in agreement with previous studies that concluded that the CNA burden is an independent predictor of outcome [19, 45]. However, in a multivariate analysis with the CNA classifier, percentage of CNAs lost its significance to predict BCR. Both 3-years and 5-years AUC also revealed that our CNA classifier has more prognostic value than the percentage of CNA. Furthermore, in multivariate analysis of percentage of genes affected by CNA and the CNA-classifier in other validation datasets, where all the genome was profiled, the CNA burden did not show to be superior to our CNA-classifier. More importantly, in the Toronto biopsy dataset, the CNA-classifier showed to be superior to the CNA burden. This indicates that although genomic instability, which results in more frequent CNAs, is a good predictor of BCR, CNA in genes or loci with biological relevance to tumor biology is a stronger predictor.

Our developed PCa CNA classifier was further validated on an additional 541 patients of low- and intermediate-risk disease with GS of ≤ 7 from previously published studies. Accordingly, we further improve the prognostic value of our classifier by the addition of clinicopathological features in both post-treatment and pre-treatment settings. These developed CNA-clinical classifiers showed improved patients risk stratification compared to clinicopathological features

alone. Therefore, they could have clinical significance and improve patients risk classification and aid the decision-making process. For example, data suggest that about 40% of patients who are treated with RP [46] will experience BCR. Adjuvant radiotherapy have been shown to reduce this risk and thus provides survival advantages for these patients [47]. However, radiation therapy after RP is significantly associated with genitourinary and rectal toxicities, urinary incontinence, and increased time to recover functional continence after surgery [48]. Our developed CNA-post-treatment classifier showed improved risk prediction of patients compared to the standard clinicopathological features available after surgery. This was confirmed by an increased in positive predictive value and AUC at both 3- and 5-years in all validation datasets. Thus, patients predicted to have unfavorable outcome by our CNA-post-treatment classifier could be eligible candidates for adjuvant radiotherapy.

Similarly, our CNA-pre-treatment classifier showed higher prognostic value at both 3- and 5-years compared to standard pre-treatment model available before treatment in all validation datasets. More importantly, the prognostic value of this CNA-pre-treatment classifier was superior to the pre-treatment model in the Toronto biopsy dataset at both tested time points. Collectively these findings demonstrate the performance of the CNA-based classifiers developed, tested and validated on RP cohorts and show its effectiveness on biopsy samples. These findings support the likelihood of clinical usefulness, particularly for the identification of patients that may benefit from more aggressive treatments at the time of diagnosis, while decreasing the overtreatment side effects for patients that may not benefit from immediate aggressive treatments. Furthermore, the higher prognostic efficiency of the developed classifiers over the standard clinicopathological features, strongly supports the usefulness of our assay as a

screening or monitoring tool for selecting the optimal management strategy for patients on active surveillance and having biopsy samples taken annually or bi-annually [49].

Our developed MLPA-based assay showed robustness, accuracy and could serve as a viable option for clinical application. Further validation in other multi-institutional cohorts and biopsy specimens are needed prior its implementation in the clinic. Furthermore, prospective studies will be required to evaluate the prognostic efficiency of our CNA classifiers. Since the designed MLPA based assay is compatible with FFPE extracted genome, only requires 50 ng of DNA (can be reduced to 40ng without impacting results), and is of low cost at 7.90\$ CAD (6\$ USD) per reaction (including MLPA kits, probe synthesis and laboratory materials), the potential for clinical utility is very promising. The cost per sample becomes 18.50\$ CAD (14.13\$ USD) in an optimal experimental design which implies each sample assayed in duplicate reactions (100 ng DNA), along with three reference DNA sample populations, a positive and a negative controls. This will allow testing a series of 41 samples per experiment in a 96-well plate. To our knowledge, the amount of DNA and the cost per reaction of this assay outperforms commercial genomic assays available so far. Overall, our assay is simple and can be routinely adopted into the clinical setting to provide more precision in the decision-making process and clinical management of patients with localized disease.

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4.8. Tables

Manuscript 2, Table 1: Clinical features of PARSE MLPA cohort

Clinicopathologic variables	Category	n (%)
Total number of patients	n	433
Patients with A samples	n*	380 (88%)
Patients with B samples	n*	358 (83%)
Patients with both samples	n*	305 (75%)
Age (years)	Median	60
	Min–max	43-77
Preoperative PSA (ng/mL)	n*	427 (99%)
	Mean ($\pm$ SD)	7.74 $\pm$ 5.18
	PSA < 10	350 (82%)
	PSA $\geq$ 10	77 (18%)
GS at biopsy	n*	298 (69%)
	GS 3+3	158 (53%)
	GS 3+4	112 (38%)
	GS 4+3	21 (7%)
	GS $\geq$ 8	7 (2%)
Clinical stage (c-stage)	n*	385 (89%)
	cT1	244 (63%)
	cT2	139 (36%)
	cT3	3 (1%)
GS at surgery	GS 3+3	130 (30%)
	GS 3+4	210 (49%)
	GS 4+3	93 (21%)
Pathologic stage (T-stage)	pT2	263 (61%)
	pT3	170 (39%)
Follow-up (months)	n*	412 (95%)
	Median (min–max)	68.55 (1-226)
BCR	n*	412 (95%)
	Positive	79 (19%)

* Number differs for each category

Percentage in each subcategory is calculated over total number of patients within the category

Manuscript 2, Table 2: Univariate and multivariate analysis of percentage of CNA with clinicopathological features

PARSE MLPA cohort n = 406	Univariate				Multivariate			
	Hazard ratio	95% CI		<i>p</i> value†	Hazard ratio	95% CI		<i>p</i> value†
		Lower	Upper			Lower	Upper	
Percentage of CNA	10.88	2.75	43.08	0.001	5.74	1.38	23.77	0.016
Pre-treatment PSA*	1.06	1.03	1.09	<0.0001	1.05	1.01	1.08	0.008
Pathologic stage (pT3 vs. pT2)	3.29	2.06	5.26	<0.0001	2.67	1.64	4.33	<0.0001
GS (4+3 vs. ≤3+4)	2.27	1.44	3.60	<0.0001	1.44	0.89	2.34	0.140

CI: Confidence interval

†Wald test

Manuscript 2, Table 3: Multivariate analysis of CNA classifier with clinicopathological features

PARSE MLPA cohort n=406	Univariate				Multivariate			
	Hazard ratio	95% CI		<i>p</i> value†	Hazard ratio	95% CI		<i>p</i> value†
		Lower	Upper			Lower	Upper	
CNA classifier*	2.76	1.87	4.08	<0.0001	2.12	1.42	3.18	<0.0001
Pre-treatment PSA*	1.06	1.03	1.09	<0.0001	1.05	1.01	1.08	0.006
Pathologic stage (pT3 vs. pT2)	3.29	2.06	5.26	<0.0001	2.49	1.53	4.06	<0.0001
GS (4+3 vs. ≤3+4)	2.27	1.44	3.60	<0.0001	1.39	0.85	2.25	0.187

* Continuous variable

CI: Confidence interval

†Wald test

Manuscript 2, Table 4: Multivariate analysis of CNA classifier with clinicopathological features in RP validation datasets

		Univariate				Multivariate			
		Hazard ratio	95% CI		<i>p</i> value†	Hazard ratio	95% CI		<i>p</i> value†
			Lower	Upper			Lower	Upper	
Cambridge (n= 104)	CNA classifier*	3.94	1.68	9.25	0.002	2.89	1.18	7.06	0.020
	Pre-treatment PSA*	1.04	0.93	1.17	0.481	1.06	0.94	1.20	0.320
	Pathology stage (pT3 vs. pT2)	1.23	0.40	3.81	0.724	0.80	0.25	2.53	0.698
	GS (4+3 vs.≤3+4)	5.22	1.91	14.26	0.001	5.15	1.67	15.89	0.004
MSKCC (n= 127)	CNA classifier*	2.82	1.69	4.72	<0.0001	2.26	1.19	4.29	0.013
	Pre-treatment PSA*	1.01	1.00	1.01	<0.0001	1.01	1.00	1.01	<0.0001
	Pathology stage (pT3 vs. pT2)	1.58	0.77	3.22	0.210	1.31	0.60	2.88	0.500
	GS (4+3 vs.≤3+4)	4.14	1.99	8.59	<0.0001	2.21	0.88	5.51	0.090
CPC (n= 135)	CNA classifier*	2.09	1.18	3.69	0.011	2.11	1.06	4.20	0.034
	Pre-treatment PSA*	1.03	0.97	1.11	0.357	1.00	0.92	1.09	0.999
	Pathology stage (pT3 vs. pT2)	4.42	1.84	10.65	0.001	3.94	1.62	9.61	0.003
	GS (4+3 vs.≤3+4)	2.19	0.99	4.83	0.052	1.63	0.73	3.67	0.236

* Continuous variable

CI: Confidence interval

[†]Wald test

Manuscript 2, Table 5: Univariate and multivariate analysis of CNA classifier, post-treatment model and CNA-post-treatment classifier

PARSE MLPA cohort n=406					Univariate				Multivariate			
	95% CI				95% CI				95% CI			
	C-Index	Lower	Upper	<i>p</i> value	Hazard ratio	Lower	Upper	<i>p</i> value	Hazard ratio	Lower	Upper	<i>p</i> value
CNA classifier	0.68	0.61	0.74	<0.0001	2.76	1.87	4.08	<0.0001	2.10	1.41	3.15	<0.0001
Post-treatment model	0.69	0.63	0.76	<0.0001	2.72	1.98	3.73	<0.0001	2.48	1.77	3.47	<0.0001
CNA-post-treatment classifier	0.71	0.65	0.77	<0.0001	2.72	2.06	3.58	<0.0001				

All variables are continuous risk scores generated from a Cox proportional hazard model
CI: Confidence interval

Manuscript 2, Table 6: C-Index, univariate and multivariate analysis of CNA-classifier, post-treatment model model and CNA-post-treatment model classifier in RP validation datasets

						Univariate				Multivariate			
						95% CI				95% CI			
						C-Index	Lower	Upper	p value	Hazard ratio	Lower	Upper	p value†
Cambridge (n=104)	CNA classifier	0.74	0.58	0.89	0.003	3.94	1.68	9.25	0.002	3.49	1.45	8.40	0.005
	Post-treatment model	0.65	0.48	0.83	0.090	2.09	0.74	5.95	0.166	1.63	0.54	4.86	0.384
	CNA-post-treatment classifier	0.68	0.54	0.83	0.015	3.09	1.34	7.14	0.008				
MSKCC (n=127)	CNA classifier	0.69	0.58	0.79	0.001	2.82	1.69	4.72	<0.0001	2.92	1.75	4.87	<0.0001
	Post-treatment model	0.69	0.60	0.79	<0.0001	1.21	1.11	1.31	<0.0001	1.23	1.13	1.34	<0.0001
	CNA-Post-treatment classifier	0.73	0.65	0.81	<0.0001	1.25	1.14	1.37	<0.0001				
CPC (n=135)	CNA classifier	0.70	0.60	0.80	<0.0001	2.09	1.18	3.69	0.011	2.07	1.10	3.90	0.025
	Post-treatment model	0.73	0.64	0.81	<0.0001	2.87	1.61	5.12	<0.0001	2.85	1.57	5.20	0.001
	CNA-Post-treatment classifier	0.76	0.68	0.84	<0.0001	2.97	1.76	5.02	<0.0001				

All variables are continuous risk scores generated from a Cox proportional hazard model

CI: Confidence interval

†Wald test

Manuscript 2, Table 7: Univariate and multivariate analysis of CNA classifier, pre-treatment model and CNA-pre-treatment classifier

PARSE MLPA cohort n=252					Univariate				Multivariate			
	95% CI			<i>p</i> value	95% CI			<i>p</i> value†	95% CI			<i>p</i> value†
	C-Index	Lower	Upper		Hazard ratio	Lower	Upper		Hazard ratio	Lower	Upper	
CNA classifier	0.62	0.52	0.72	0.025	2.04	1.18	3.54	0.011	1.93	1.10	3.38	0.022
Pre-treatment model	0.58	0.48	0.69	0.108	2.72	1.29	5.72	0.008	2.52	1.17	5.44	0.018
CNA-Pre-treatment classifier	0.63	0.54	0.72	0.006	2.72	1.54	4.80	0.001				

All variables are continuous risk scores generated from a Cox proportional hazard model

CI: Confidence interval

† Wald test

Manuscript 2, Table 8: C-index, univariate and multivariate analysis of CNA-classifier, pre-treatment model and CNA-pre-treatment classifier in Toronto biopsy dataset

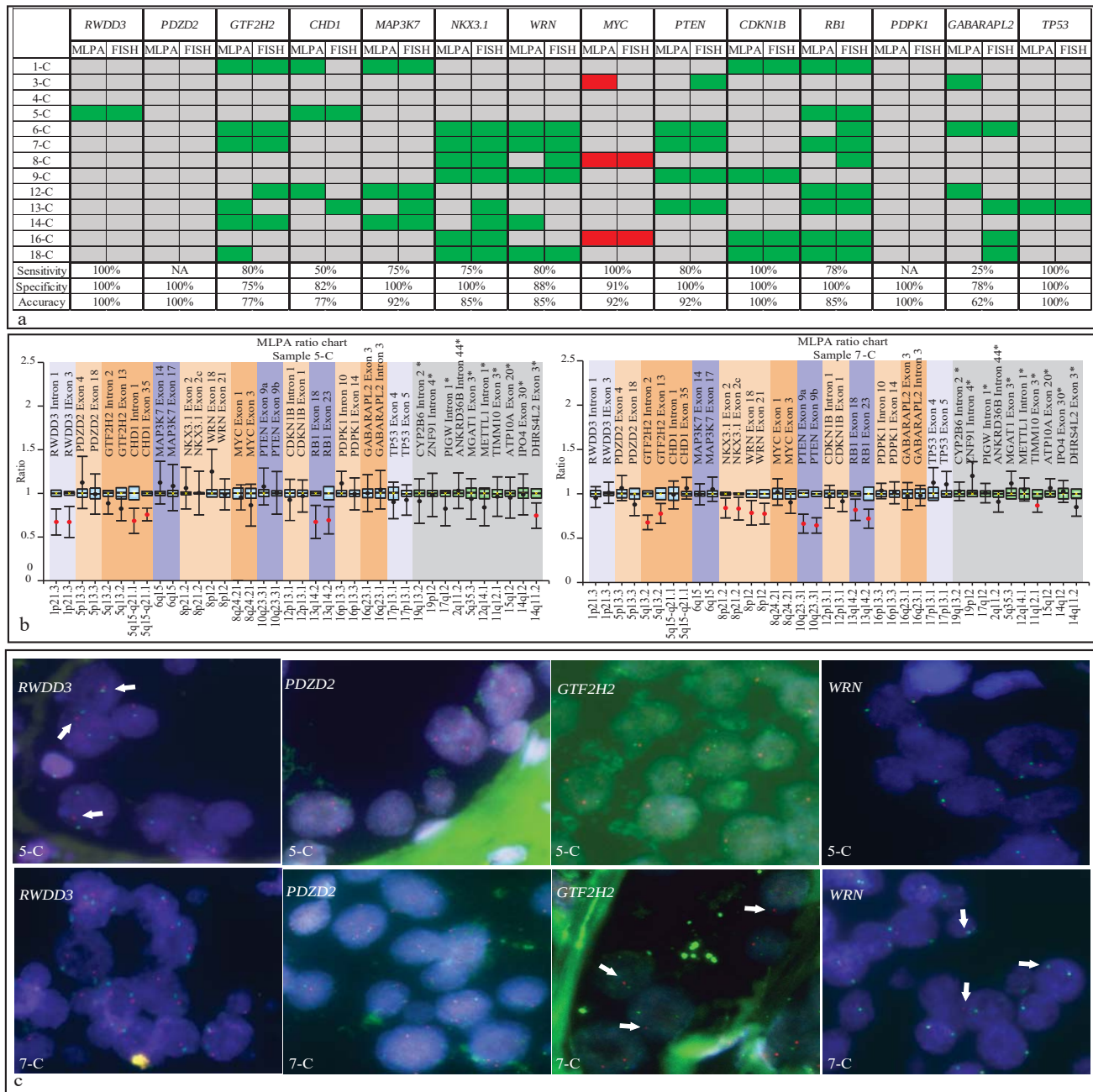
	Univariate				Multivariate			
	95% CI			p value	95% CI			p value†
	C-Index	Lower	Upper		Hazard ratio	Lower	Upper	
CNA classifier	0.74	0.67	0.82	<0.0001	3.82	2.20	6.65	<0.0001
Pre-treatment model	0.60	0.52	0.69	0.0136	4.02	1.68	9.64	0.0020
CNA-Pre-treatment classifier	0.69	0.61	0.77	<0.0001	4.71	2.63	8.45	<0.0001

All variables are continuous risk scores generated from a Cox proportional hazard model

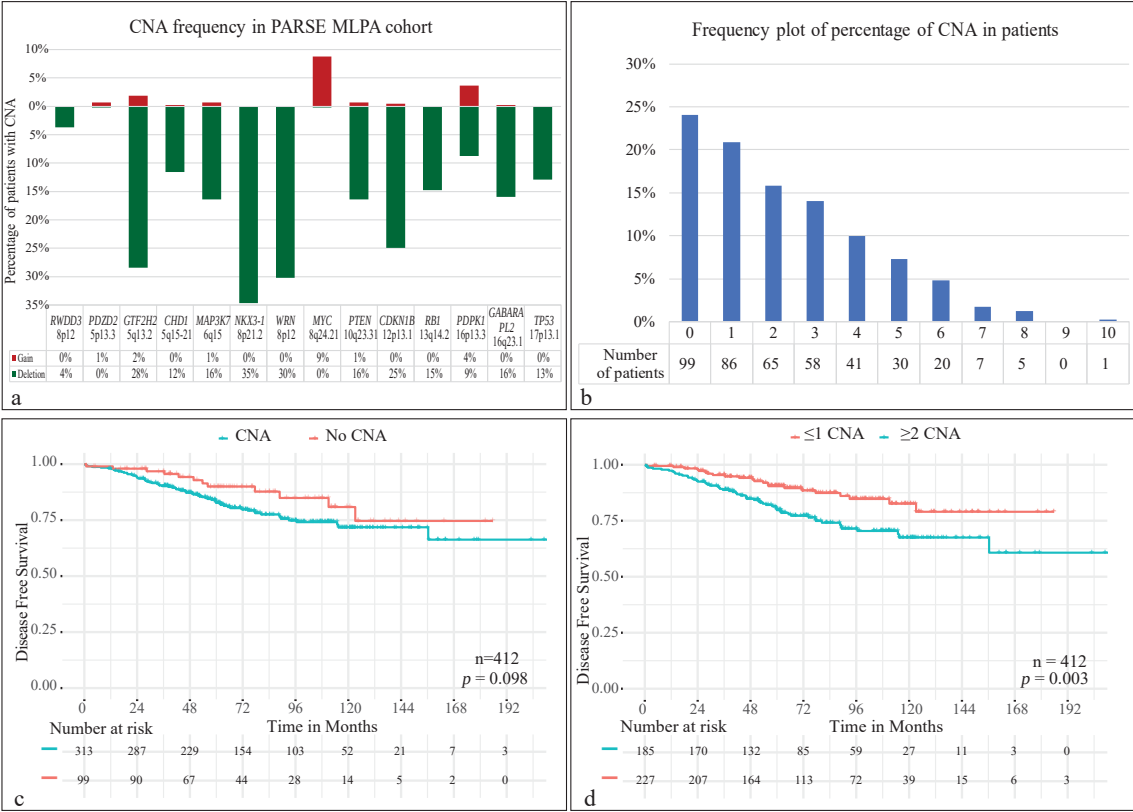
CI: Confidence interval

† Wald test

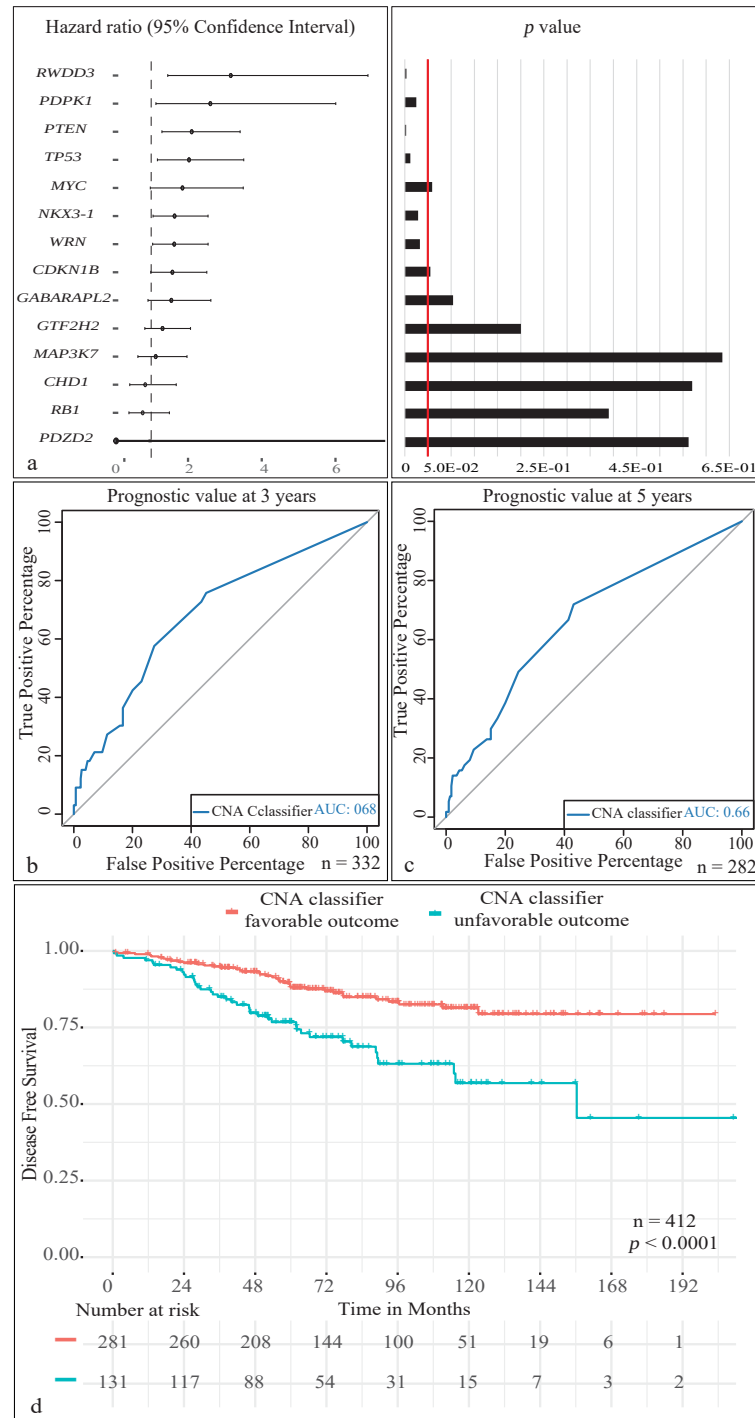
4.9. Figures



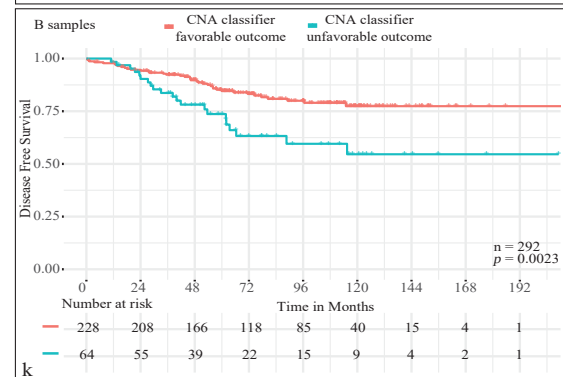
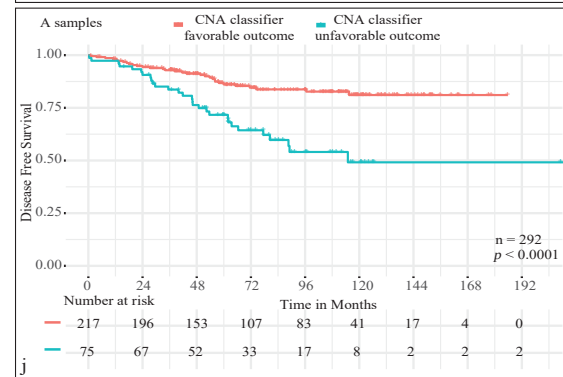
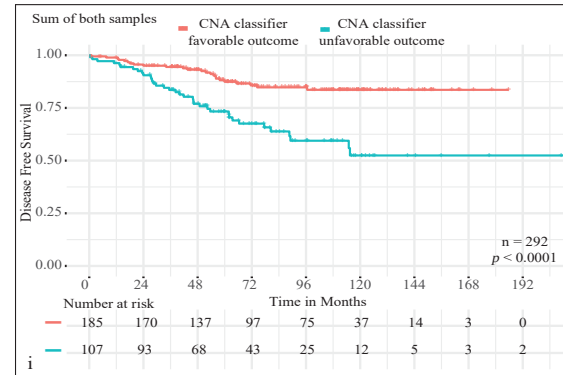
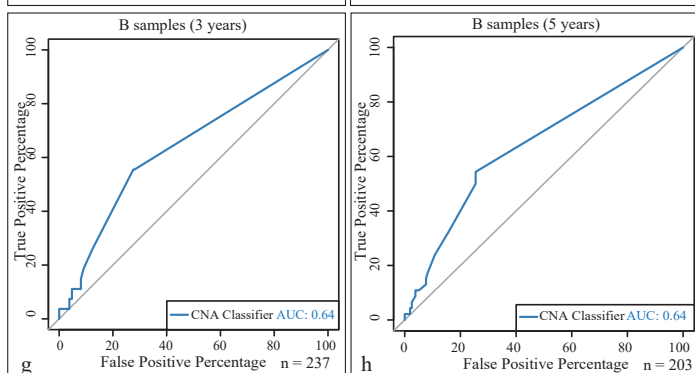
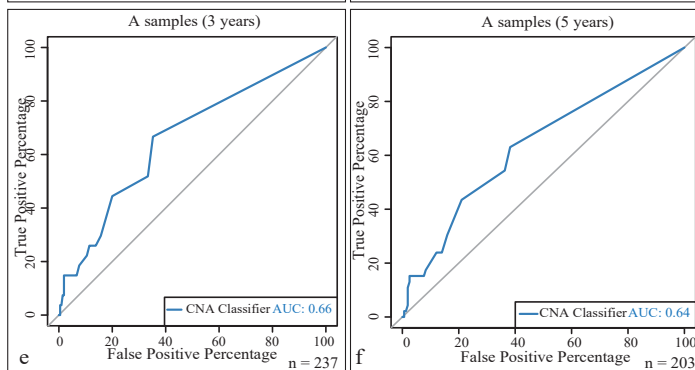
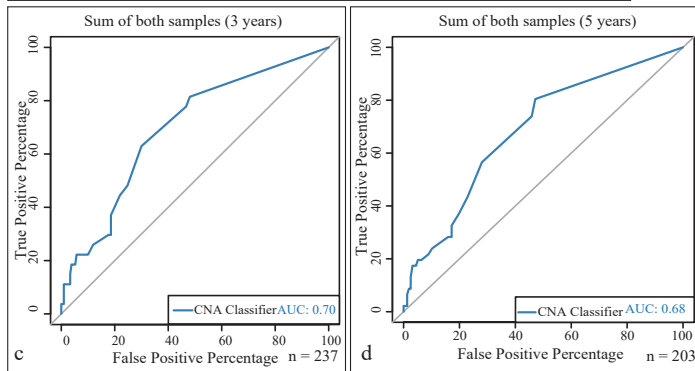
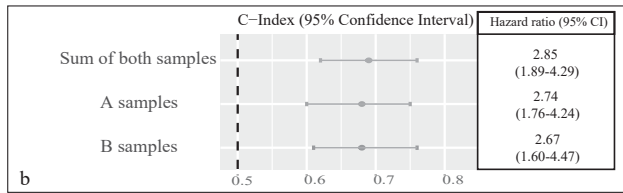
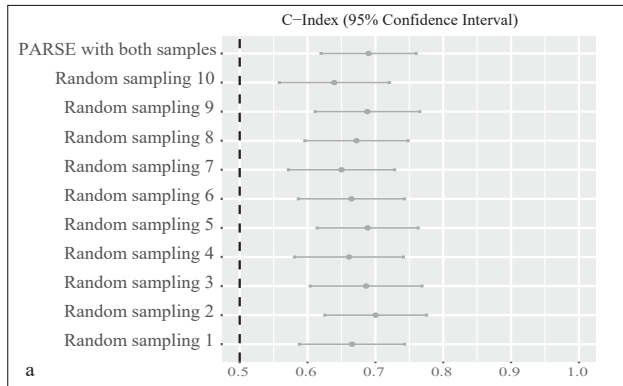
Manuscript 2, Figure 1: Expansion of the PCa specific MLPA probe mix. a. CNA calls based on MLPA and FISH on an independent set of RP PCa samples used for assay optimization described in our previous study (Ebrahimizadeh et al. Submitted 2019). Deletions are represented by green and gains are represented by red colors. FISH results of *CHD1*, *MAP3K7*, *NKX3-1*, *MYC*, *PTEN*, *CDKN1B*, *RBI*, *PDPK1*, *GABARAPL2* and *TP53* were obtained from our previous report (Ebrahimizadeh et al. Submitted 2019). **b.** MLPA ratio chart of samples; 5-C showing deletion in the newly added probe of *RWDD3* and previously reported and validated deletions in the *CHD1* and *RBI*. 7-C, showing deletion in the newly added probes of *GTF2H2* and *WRN*. Previous deletions such as *NKX3-1*, *PTEN* and *RBI* were reported and validated by FISH in the previous report. **c.** FISH results of newly assessed genes *RWDD3*, *PDZD2*, *GTF2H2* and *WRN* confirming the results obtained by MLPA on samples 7-C and 5-C. CNAs are indicated by white arrows. In all reactions, gene specific test probes are labeled with spectrum orange and the reference probes are labeled with spectrum green.



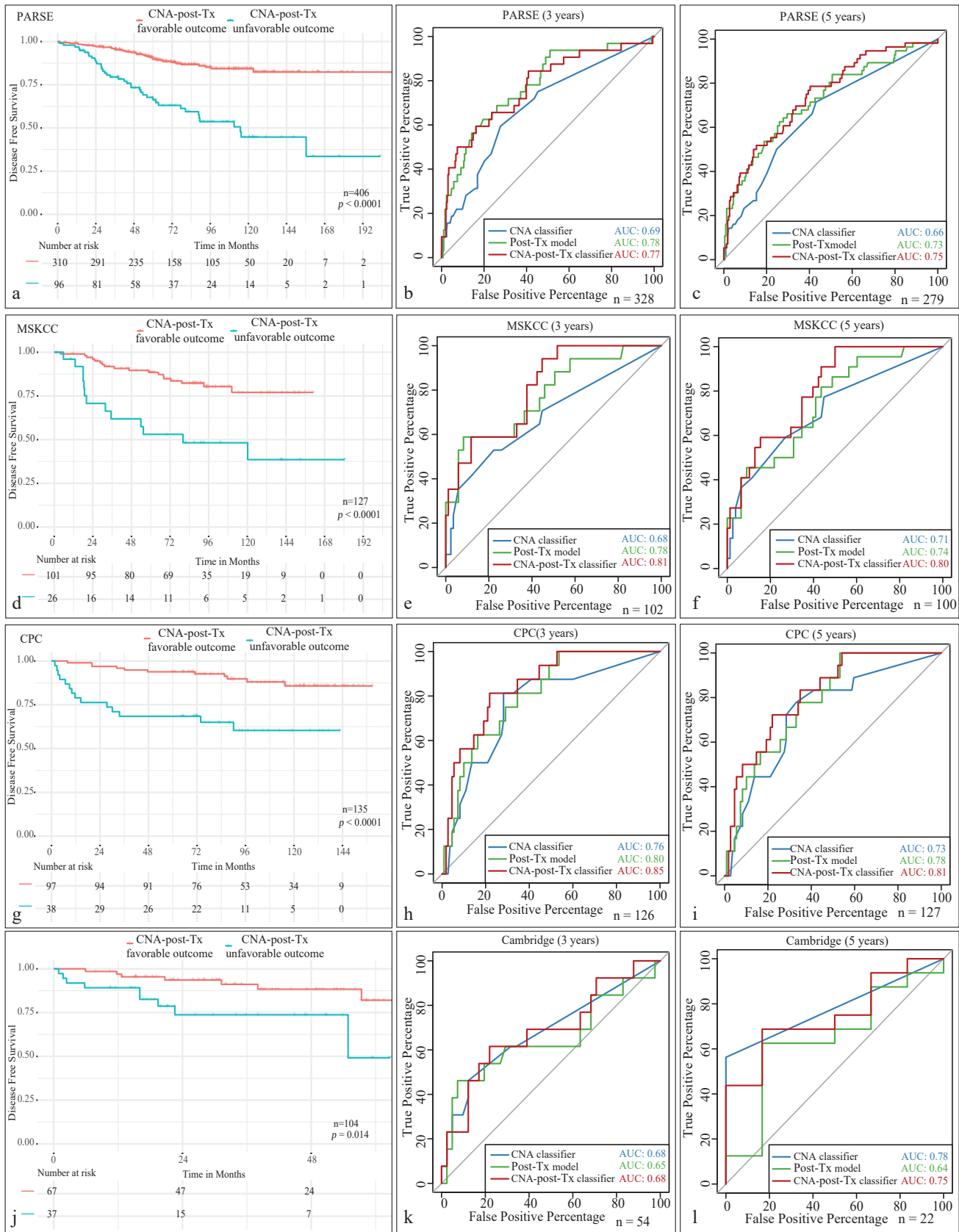
Manuscript 2, Figure 2: Copy number profile of the PARSE MLPA cohort. a. CNA frequency of assessed genes by MLPA in PARSE cohort. The most frequent CNAs are deletions of *NKX3-1* and *WRN* on the 8p arm. **b.** Frequency plot of percentage of CNAs in patients in PARSE MLPA cohort showing 23% of samples did not show CNA and 21% showed CNA in only one of the assessed genes. **c.** Kaplan-Meier analysis showing patients with no CNA vs. patients with CNA. **d.** Kaplan-Meier analysis of patients with two and more CNAs of the assessed gene vs one or no CNA. *p* value was assessed using log rank test.



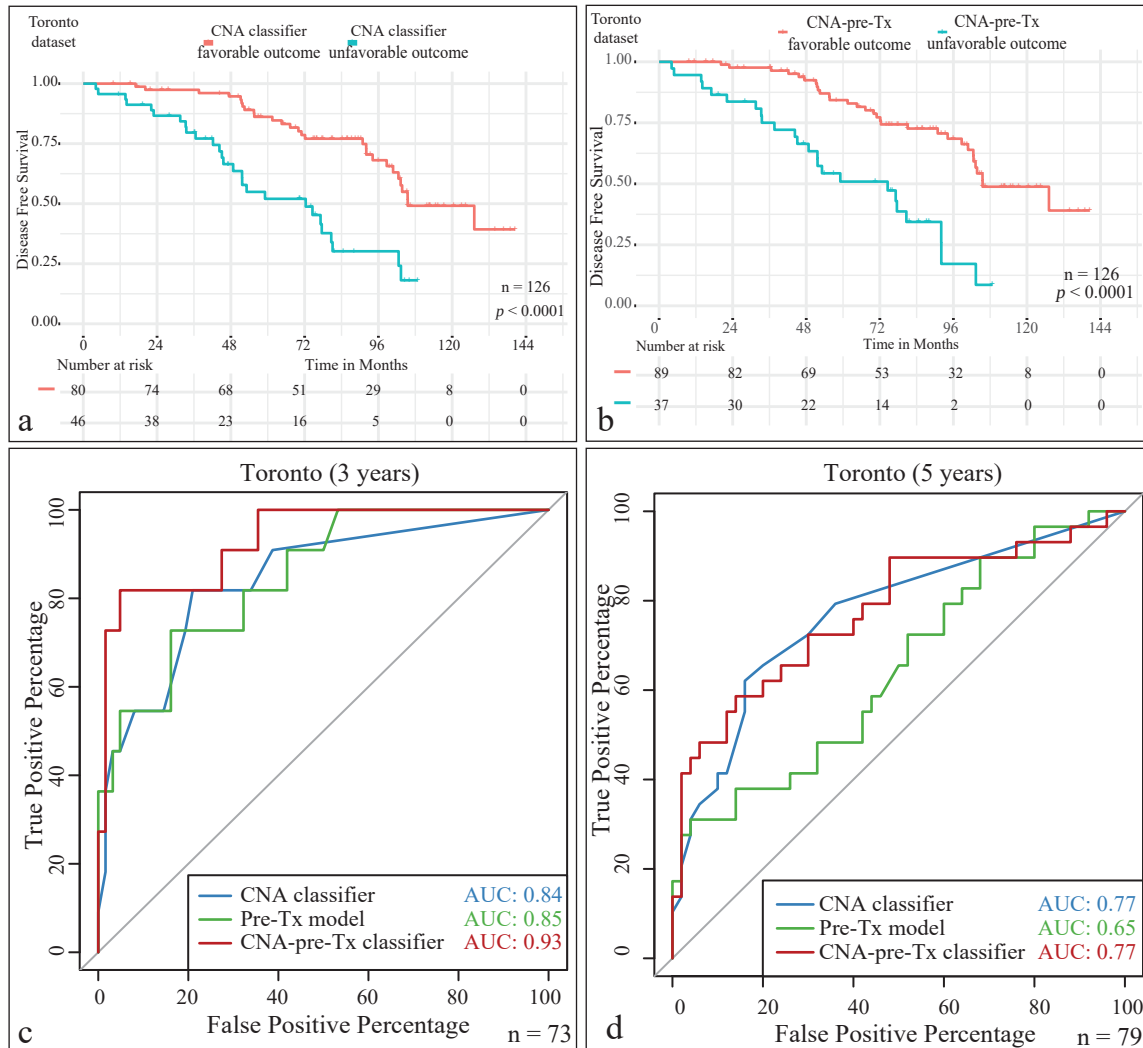
Manuscript 2, Figure 3: Association of specific CNAs with BCR and performance of the CNA classifier. **a.** Hazard ratio plot of 14 studied gene. **b.** ROC analysis of the CNA classifier for prediction of BCR at 3-years. p value <0.05. **c.** ROC analysis of the CNA classifier for prediction of BCR at 5-years. p value <0.05. **d.** Kaplan-Meier analysis of risk scores predicted by the CNA-classifier in PARSE MLPA cohort.



Manuscript 2, Figure 4: Effect of heterogeneity on the performance of the CNA-classifier. **a.** The CNA-classifier showed similar C-index in all variation of the cohort randomly created from either sample A or B. **b.** C-index and hazard ratio of the CNA classifier in PARSE MLPA cohort when sum of CNAs in both samples, or when sample A or B of the patient was used. **c.** ROC analysis when the sum of the CNA in both samples were used to assess patients risk for BCR at 3-years, **d.** and at 5-years. **e.** ROC analysis when sample A of patients where used to determine the risk of BCR at 3-years. **f.** and at 5-years. **g.** ROC analysis when sample B of patients where used to determine the risk of BCR at 3-years. **h.** and at 5-years. **i.** Kaplan-Meier analysis of the predicted risk scores by the CNA-classifier when the sum of sample A and B are used, **j.** when sample A of patients was considered. **k.** when the sample B of patients was considered.



Manuscript 2, Figure 5: Prognostic efficiency of the CNA-post-treatment(Tx) classifier in all tested RP datasets. CNA-post-treatment a. Kaplan-Meier analysis; b. 3-years; and c. 5-years AUC in PARSE MLPA cohort. d. Kaplan-Meier analysis; e. 3-years; and f. 5-years AUC in MSKCC dataset. g. Kaplan-Meier analysis; h. 3-years; and i. 5-years AUC in CPC dataset. j. Kaplan-Meier analysis; k. 3-years; and l. 5-years AUC in Cambridge dataset. *P* value for Kaplan-Meier analysis was obtained via log rank test.



Manuscript 2, Figure 6: Prognostic efficiency of CNA classifiers in Toronto dataset. a. Kaplan-Meier analysis of CNA classifier, **b.** CNA- pre-treatment(Tx) classifier **c.** 3-years and **d.** 5-years ROC analysis of risk scores.

4.10. Supplementary Tables

Manuscript 2, Table S1: Characteristics of PCa specific probe mix

Probe name	Function	Cytoband	Hybridization sequence coordinates (hg38)	Probe length
<i>RWDD3</i> Intron 1	CNA	1p21.3	95241234-95241307	208
<i>RWDD3</i> Exon 3	CNA	1p21.3	95244384-95244446	148
<i>PDZD2</i> Exon 4	CNA	5p13.3	31995632-31995695	212
<i>PDZD2</i> Exon 18	CNA	5p13.3	32074548-32074610	228
<i>GTF2H2</i> Intron 2	CNA	5q13.2	71083035-71083100	236
<i>GTF2H2</i> Exon 13	CNA	5q13.2	71042192-71042253	224
<i>CHD1</i> Intron 1	CNA	5q15	98927383-98927458	200
<i>CHD1</i> Exon 35	CNA	5q21.1	98856501-98856587	132
<i>MAP3K7</i> Exon 17	CNA	6q15	90516154-90516215	125
<i>MAP3K7</i> Exon 14	CNA	6q15	90523700-90523761	164
<i>WRN</i> Exon 18	CNA	8p12	31100856-31100933	244
<i>WRN</i> Exon 21	CNA	8p12	31120339-31120400	240
<i>NKX3-1</i> Exon 2c	CNA	8p21.2	23679195-23679260	188
<i>NKX3-1</i> Exon 2	CNA	8p21.2	23679829-23679894	152
<i>MYC</i> Exon 1	CNA	8q24.21	127736564-127736621	101
<i>MYC</i> Exon 3	CNA	8q24.21	127740610-127740671	184
<i>PTEN</i> Exon 9a	CNA	10q23.31	87966936-87966999	109
<i>PTEN</i> Exon 9b	CNA	10q23.31	87968061-87968130	140
<i>CDKN1B</i> Intron 1	CNA	12p13.1	12714777-12714852	156
<i>CDKN1B</i> Exon 1	CNA	12p13.1	12717331-12717394	180
<i>RB1</i> Exon 18	CNA	13q14.2	48453031-48453090	105
<i>RB1</i> Exon 23	CNA	13q14.2	48465212-48465273	172
<i>PDPK1</i> Exon 14	CNA	16p13.3	2597685-2597741	121
<i>PDPK1</i> Intron 10	CNA	16p13.3	2584302-2584368	220
<i>GABARAPL2</i> Exon 3	CNA	16q23.1	75568131-75568188	168
<i>GABARAPL2</i> Intron 3	CNA	16q23.1	75573764-75573839	196
<i>TP53</i> Exon 5	CNA	17p13.1	7674901-7674962	136
<i>TP53</i> Exon 4	CNA	17p13.1	7675285-7675346	117
<i>ANKRD36B</i> Intron 44	Reference	2q11.2	97495557-97495632	232
<i>MGAT1</i> Exon 3	Reference	5q35.3	180790824-180790885	160
<i>TIMM10</i> Exon 3	Reference	11q12.1	57528778-57528839	204
<i>METTL1</i> Intron 1	Reference	12q14.1	57774661-57774736	144
<i>IPO4</i> Exon 30	Reference	14q12	24180336-24180397	176
<i>DHRS4L2</i> Exon 3	Reference	14q11.2	23995055-23995116	248
<i>ATP10A</i> Exon 20	Reference	15q12	25680143-25680204	192
<i>PIGW</i> Intron 1	Reference	17q12	36533015-36533090	129
<i>ZNF91</i> Intron 4	Reference	19p12	23350707-23350774	113
<i>CYP2B6</i> Intron 2	Reference	19q13.2	41004294-41004359	216

Manuscript 2, Table S2: Clinical features of validation datasets

Clinicopathologic variables	MSKCC		CPC		Cambridge		Toronto®	
	Category	n (%)	Category	n (%)	Category	n (%)	Category	n (%)
Total number of Patients	n	127	n	184	n	104	n	126
Age (years)	Median	58	Median	63	Median	61	Median	72
	Min-max	37-73	Min-max	44-81	Min-max	41-73	Min-max	55-83
Preoperative PSA (ng/mL)	Mean (±SD)	15 (±62.6)	Mean (±SD)	8.2 (±4.9)	Mean (±SD)	8.7 (±3.8)	Mean (±SD)	8.3 (±3.8)
	PSA < 10	103 (81%)	PSA < 10	141 (77%)	PSA < 10	77 (74%)	PSA < 10	92 (73%)
	PSA ≥ 10	24 (19%)	PSA ≥ 10	43 (23%)	PSA ≥ 10	27 (26%)	PSA ≥ 10	34 (27%)
GS at biopsy	GS 3+3	75 (59%)	GS 3+3	36 (20%)	GS 3+3	27 (26%)	GS 3+3	31 (25%)
	GS 3+4	32 (25%)	GS 3+4	119 (65%)	GS 3+4	55 (53%)	GS 3+4	64 (51%)
	GS 4+3	12 (9%)	GS 4+3	27 (15%)	GS 4+3	15 (14%)	GS 4+3	31 (25%)
	GS ≥ 8	8 (6%)	GS ≥ 8	2 (1%)	GS ≥ 8	7 (7%)	GS ≥ 8	0 (0%)
Clinical stage (c-stage)	cT1	64 (50%)	cT1	90 (49%)	cT1	61 (59%)	cT1	45 (36%)
	cT2	59 (46%)	cT2	94 (51%)	cT2	30 (29%)	cT2	81 (64%)
	cT3	4 (3%)	cT3	0 (0%)	cT3	13 (13%)	cT3	0 (0%)
GS at surgery	GS 3+3	38 (30%)	GS 3+3	23 (13%)	GS 3+3	16 (15%)	GS 3+3	NA
	GS 3+4	64 (50%)	GS 3+4	108 (59%)	GS 3+4	69 (66%)	GS 3+4	NA
	GS 4+3	25 (20%)	GS 4+3	53 (29%)	GS 4+3	19 (18%)	GS 4+3	NA
Pathologic stage (T-stage)			n*	135 (73%)				
	pT2	84 (66%)	pT2	78 (58%)	pT2	30 (29%)	pT2	NA
	pT3	43 (34%)	pT3	57 (42%)	pT3	74 (71%)	pT3	NA
Follow-up (months)	Median (min-max)	87 (1.5-180.5)	Median (min-max)	94 (59-165.6)	Median (min-max)	31 (7.9-66.8)	Median (min-max)	75 (9.7-104.4)
BCR	Positive	31 (24%)	Positive	51 (28%)	Positive	16 (15%)	Positive	55 (44%)

* Number differs for each category

Percentage in each subcategory is calculated over total number of patients within the category

NA: Not applicable

® Treated by image guided radiotherapy

Manuscript 2, Table S3: Multivariate analysis of CNA classifier with percentage of CNA

PARSE MLPA cohort n=412	Univariate				Multivariate			
	Hazard ratio	95% CI		<i>p</i> value	Hazard ratio	95% CI		<i>p</i> value
		Lower	Upper			Lower	Upper	
CNA classifier*	2.72	1.84	4.01	<0.0001	2.69	1.45	5.01	0.002
Percentage of CNA*	11.12	2.85	43.43	0.001	1.04	0.13	8.30	0.969

* All continuous variable

CI: Confidence interval

Manuscript 2, Table S4: C-Index, univariate and multivariate analysis of CNA-classifier, pre-treatment model and CNA-pre-treatment classifier in RP validation datasets

		Univariate								Multivariate			
			95% CI				95% CI				95% CI		
		C-Index	Lower	Upper	<i>p</i> value	Hazard ratio	Lower	Upper	<i>p</i> value†	Hazard ratio	Lower	Upper	<i>p</i> value†
Cambridge (n=104)	CNA classifier	0.74	0.58	0.89	0.003	3.94	1.68	9.25	0.002	3.67	1.56	8.61	0.003
	Pre-treatment model	0.65	0.48	0.81	0.080	3.58	0.92	13.90	0.066	3.27	0.76	14.14	0.112
	CNA-Pre-treatment classifier	0.71	0.57	0.85	0.003	5.36	1.98	14.53	0.001				
MSKCC (n=127)	CNA classifier	0.69	0.58	0.79	0.001	2.82	1.69	4.72	<0.0001	3.21	1.90	5.41	<0.0001
	Pre-treatment model	0.67	0.56	0.77	0.002	1.16	1.09	1.25	<0.0001	1.19	1.11	1.28	<0.0001
	CNA-Pre-treatment classifier	0.78	0.71	0.85	<0.0001	1.19	1.11	1.28	<0.0001				
CPC (n=184)	CNA classifier	0.66	0.58	0.74	0.000	1.81	1.14	2.88	0.012	1.76	1.12	2.76	0.015
	Pre-treatment model	0.58	0.49	0.66	0.073	2.42	1.21	4.84	0.012	2.43	1.19	5.00	0.015
	CNA-Pre-treatment classifier	0.64	0.56	0.72	0.0005	2.46	1.48	4.09	<0.0001				

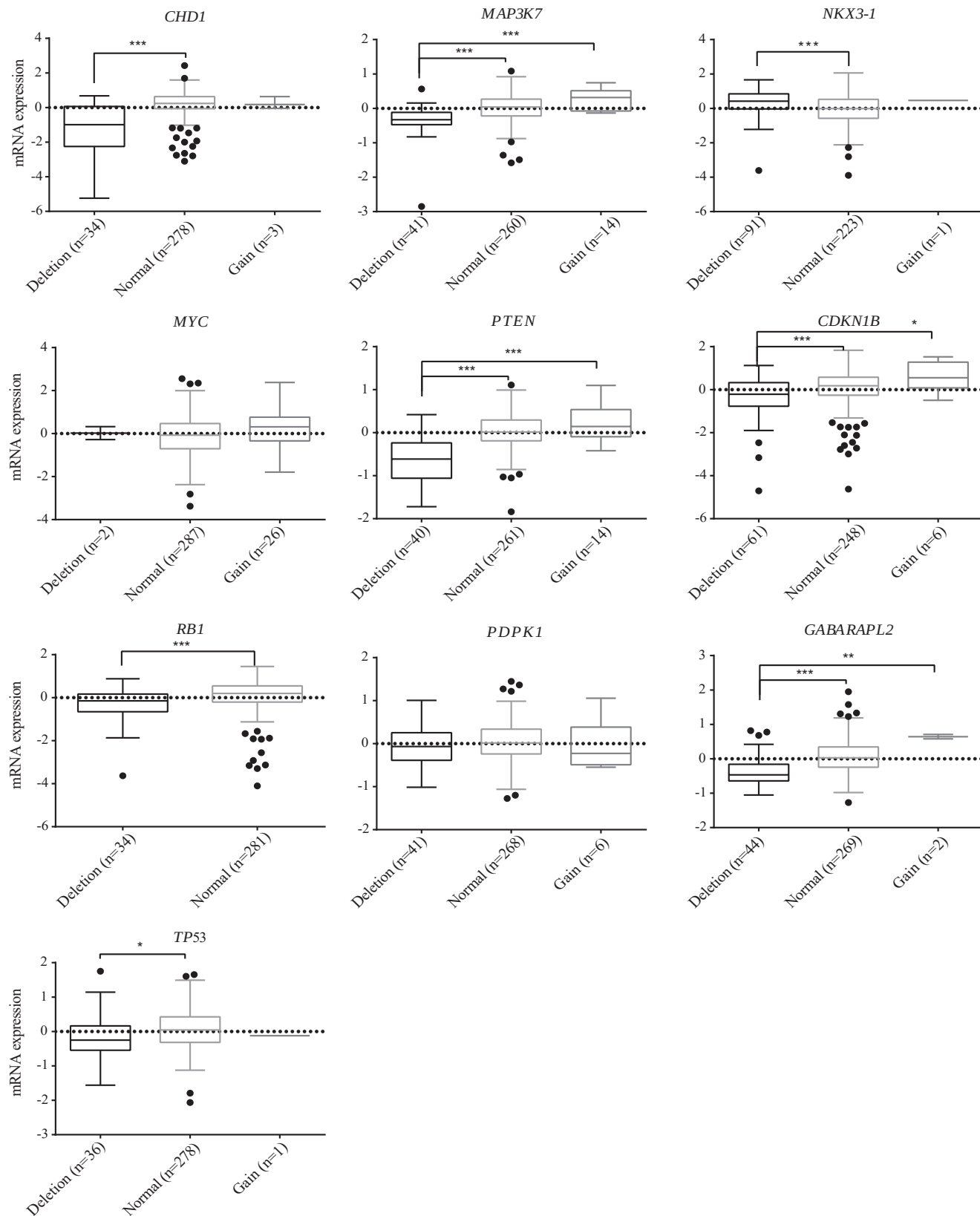
All variables are continuous risk scores generated from a Cox proportional hazard model

CI: Confidence interval

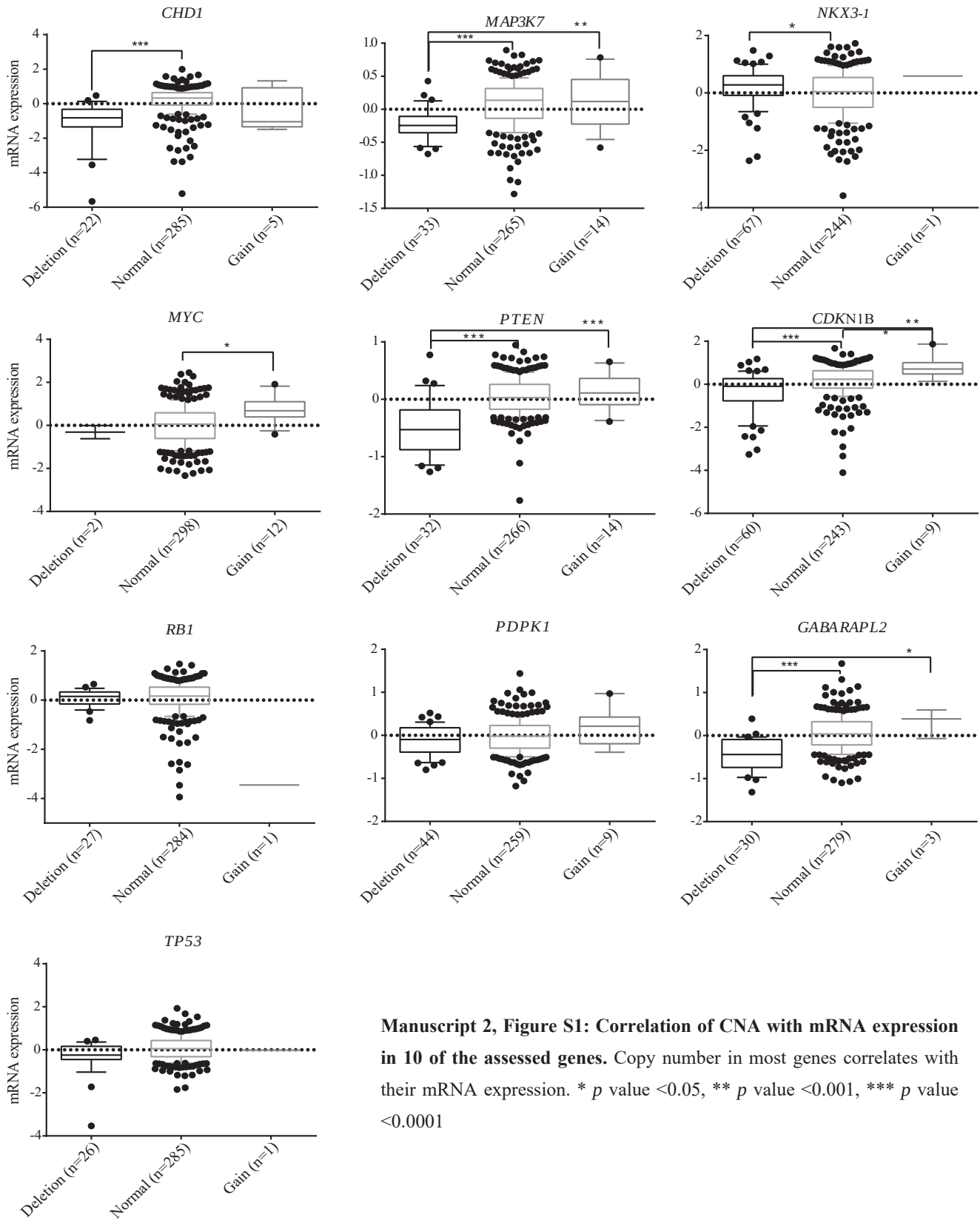
†Wald test

4.11. Supplementary Figures

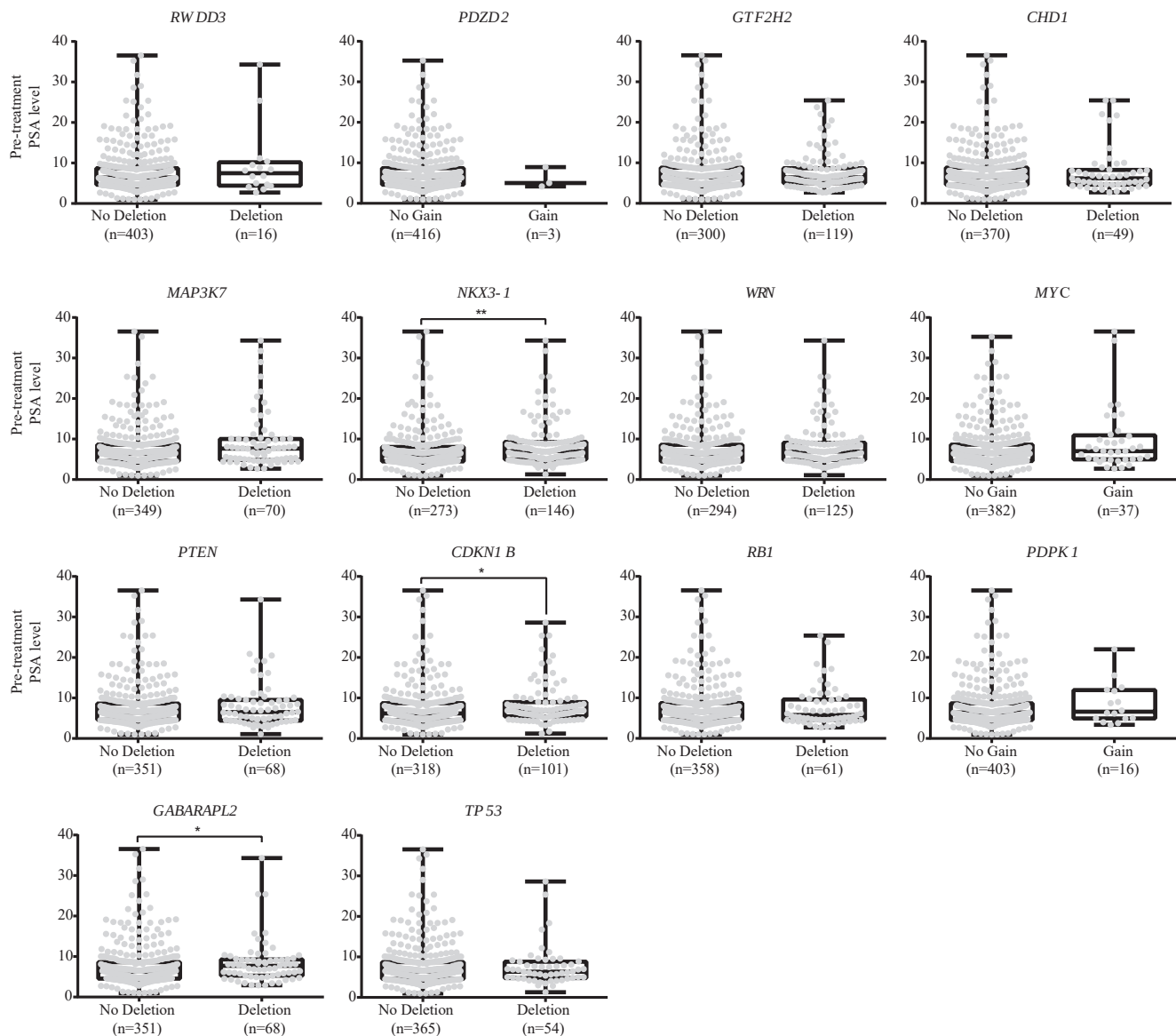
Correlation of CNA with mRNA expression in A samples



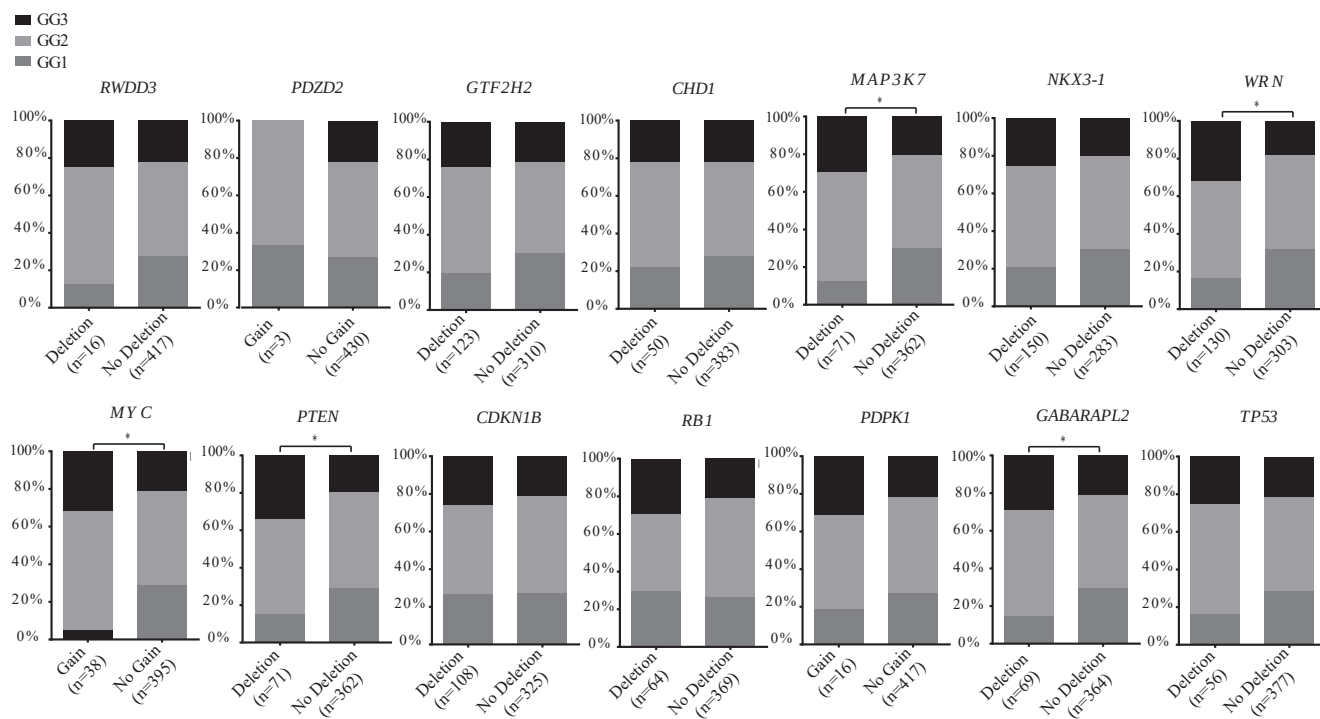
Correlation of CNA with mRNA expression in B samples



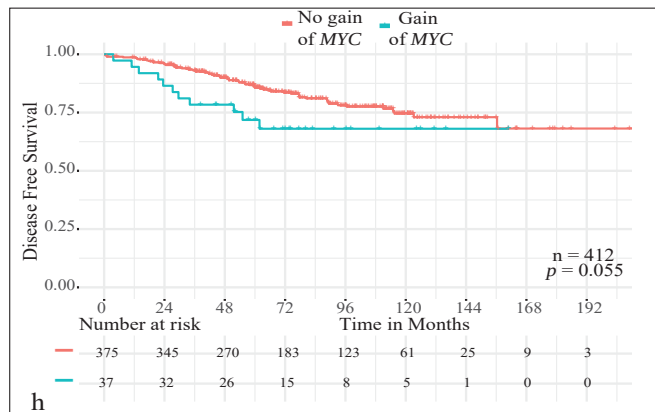
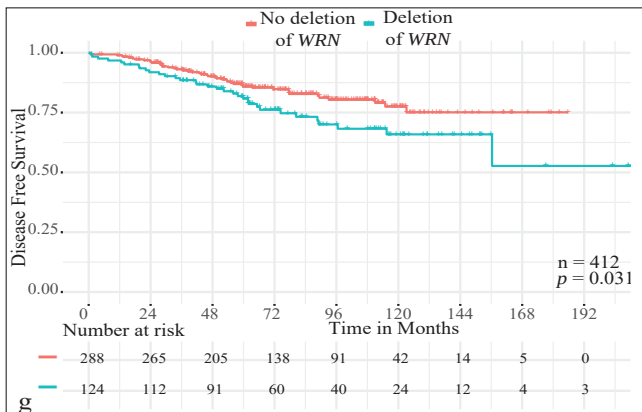
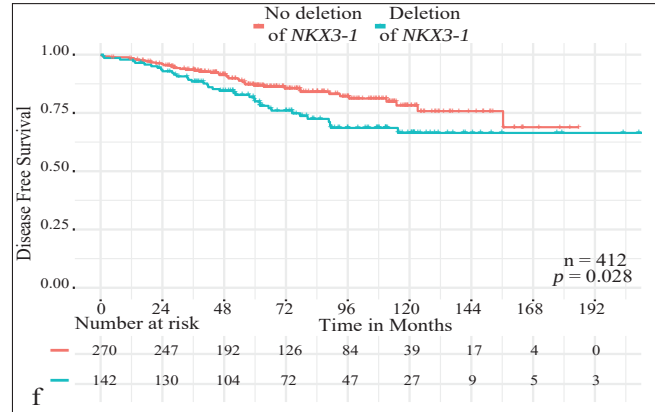
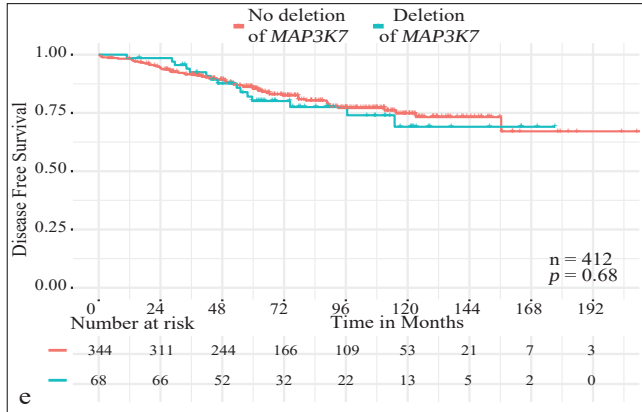
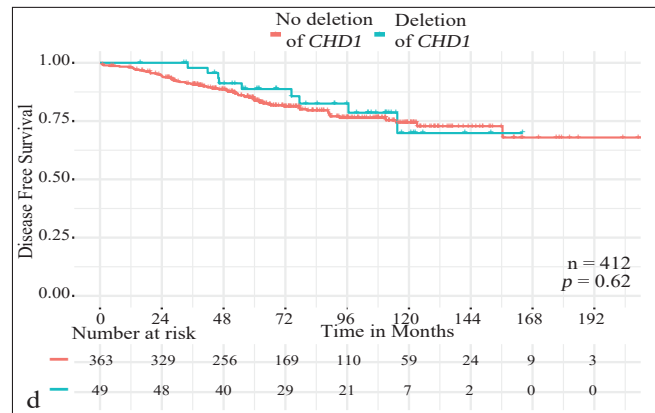
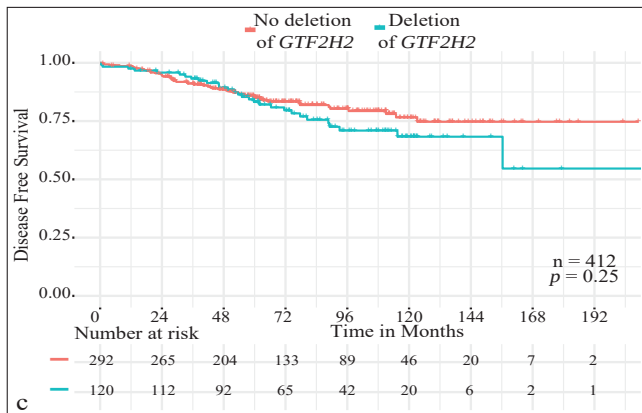
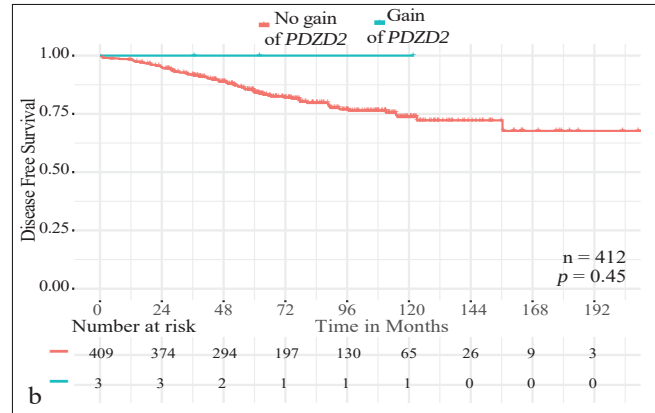
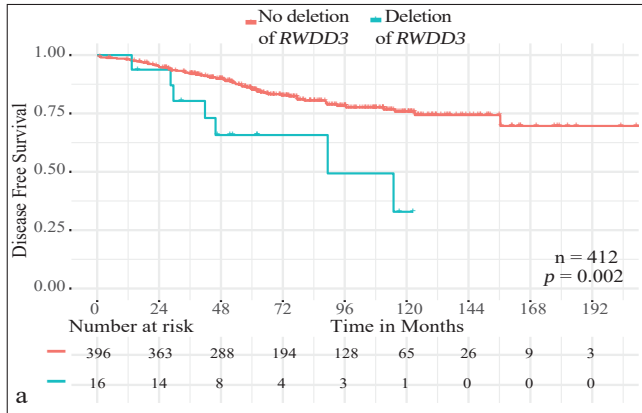
Manuscript 2, Figure S1: Correlation of CNA with mRNA expression in 10 of the assessed genes. Copy number in most genes correlates with their mRNA expression. * p value < 0.05, ** p value < 0.001, *** p value < 0.0001

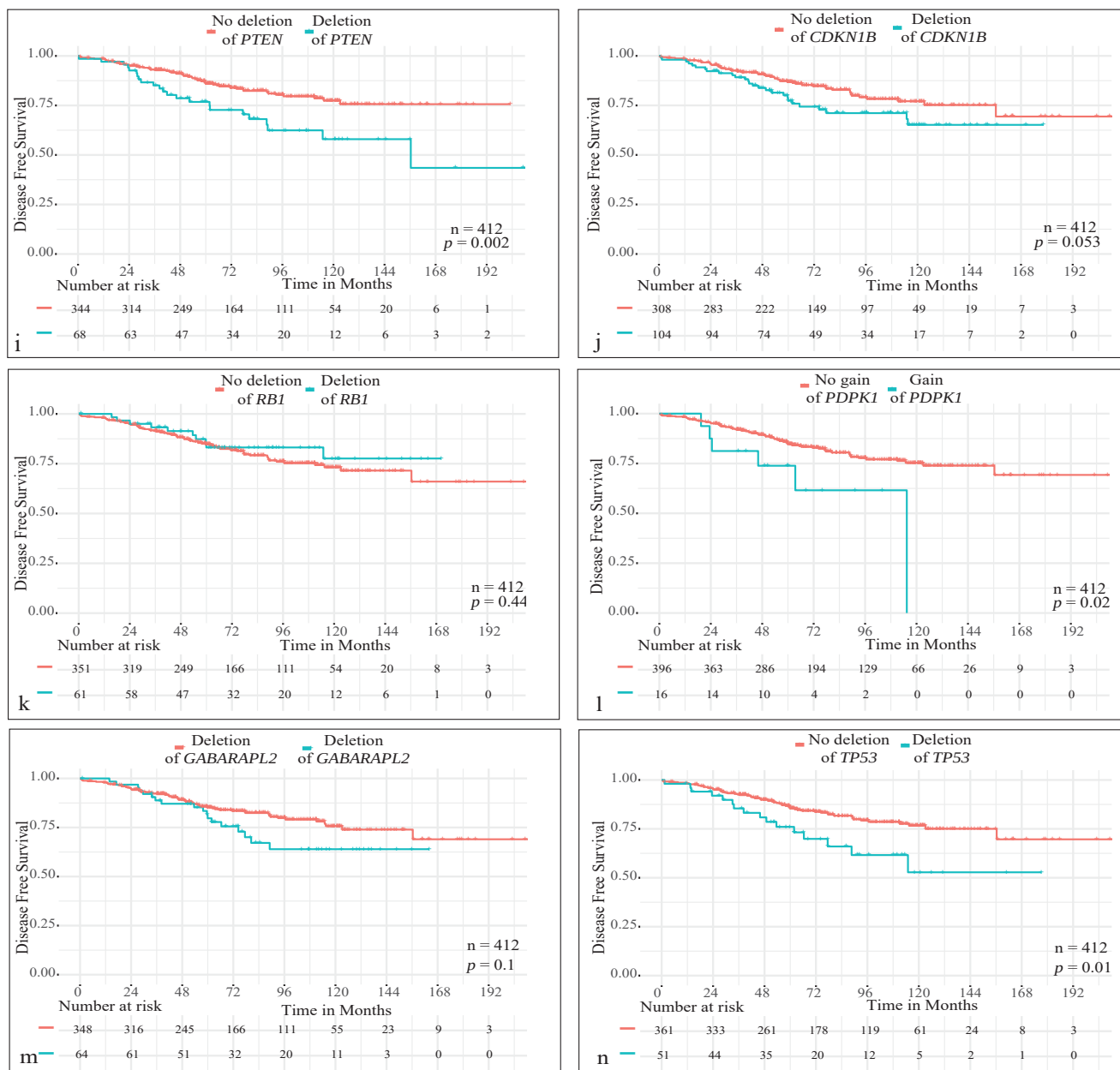


Manuscript 2, Figure S2: Association of CNA in the 14 genes with pre-treatment PSA levels. Deletions in *NKX3-1*, *CDKN1B*, and *GABARAPL2* genes was correlated with higher pre-surgery PSA values compared to samples without CNA (Mann Whitney U test, $p < 0.05$).

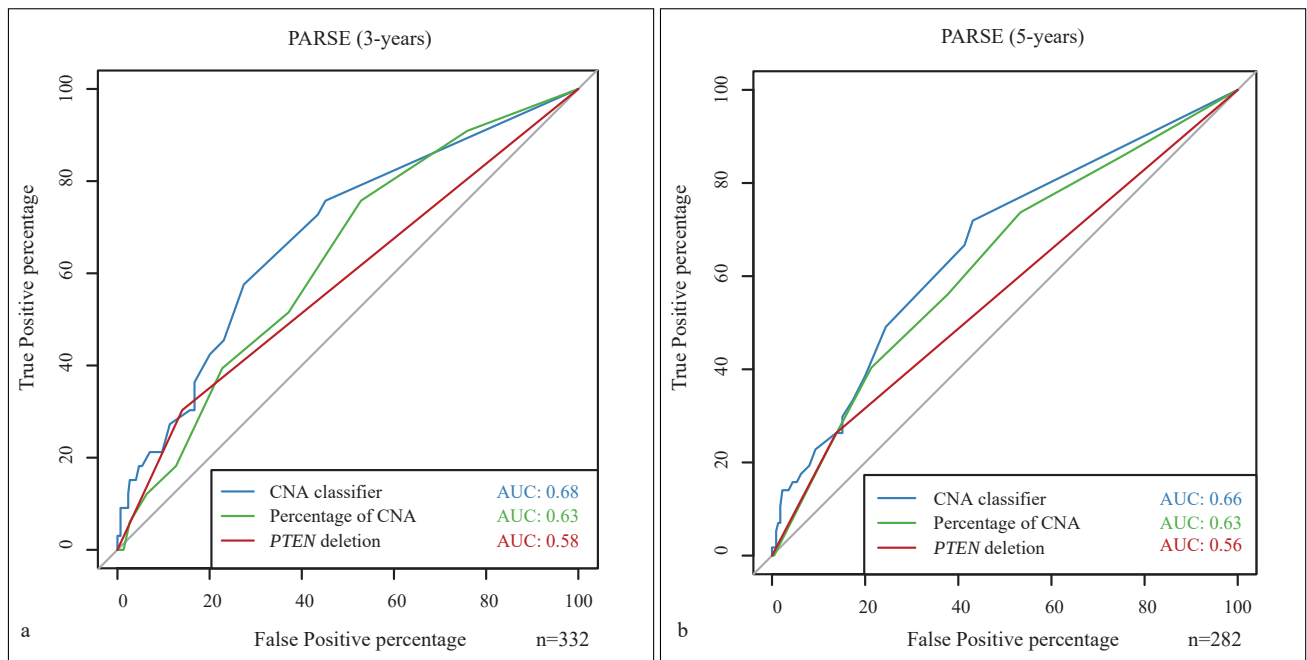


Manuscript 2, Figure S3: Association of CNA in the 14 genes with Gleason group. Deletions in *MAP3K7*, *WRN*, *PTEN*, *GABARAPL2* and gain in *MYC* is associated with higher Gleason groups (χ^2 , $p < 0.05$).

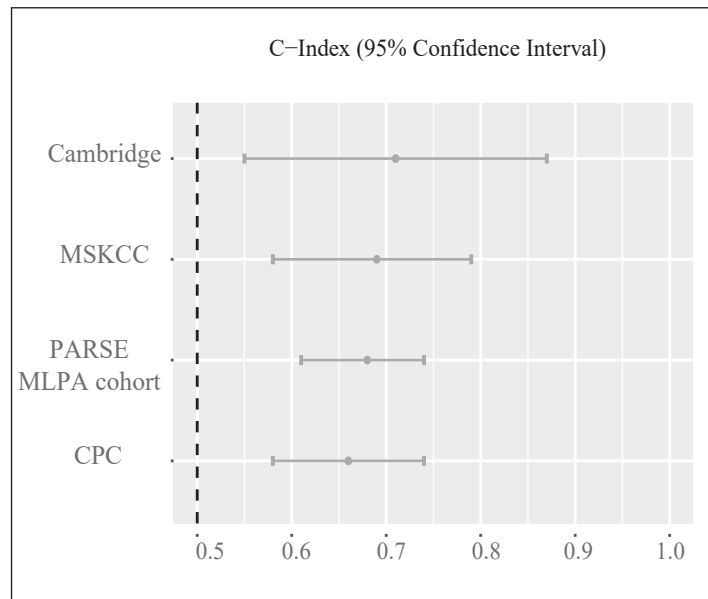




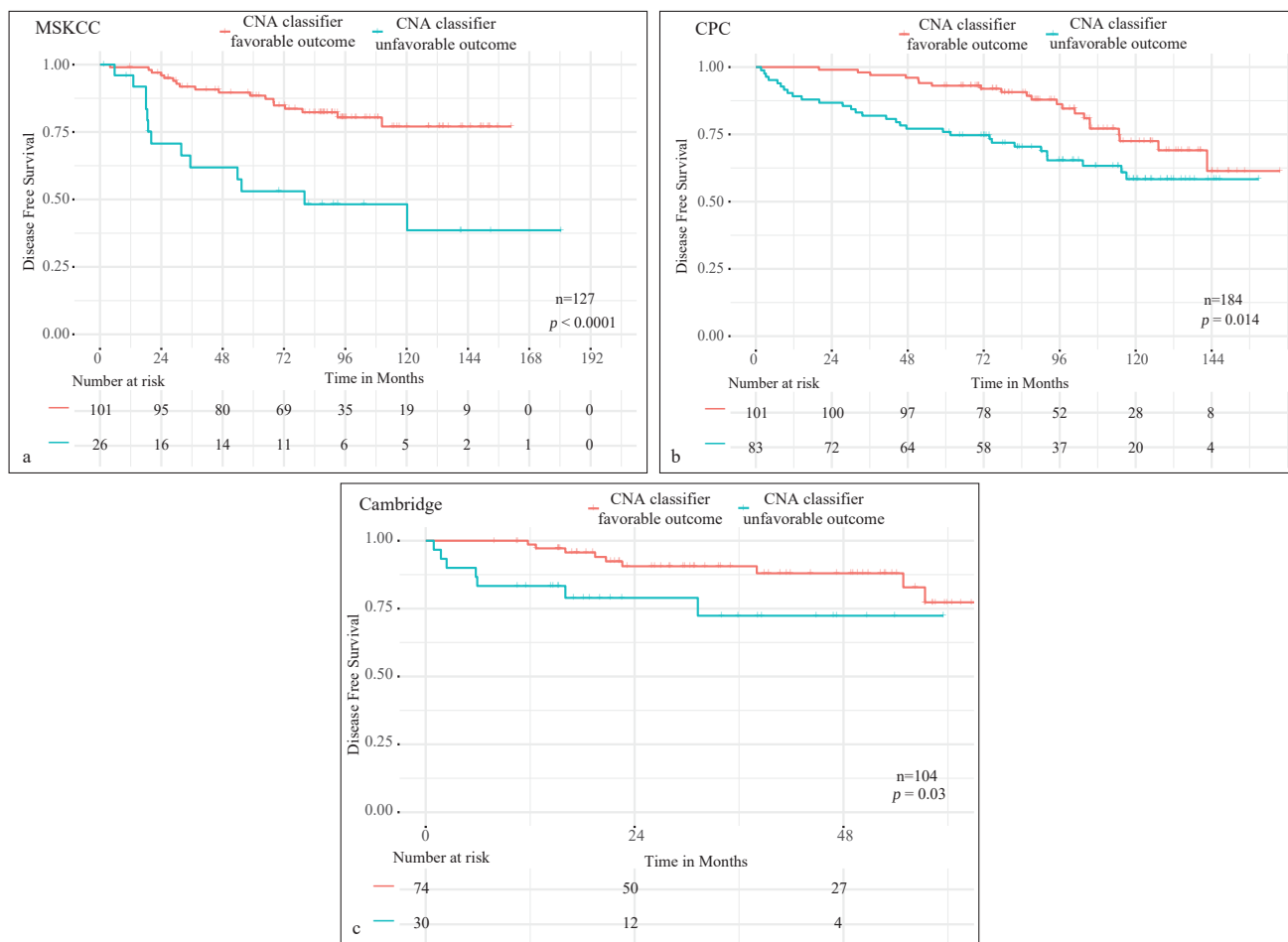
Manuscript 2, Figure S4: Association of CNA in the 14 genes with BCR using Kaplan-Meier analysis. Deletion of *RWDD3*, *WRN*, *NKX3-1*, *PTEN* and *TP53* and gain in *PDPK1* were associated with BCR. p value was assessed using log rank test.



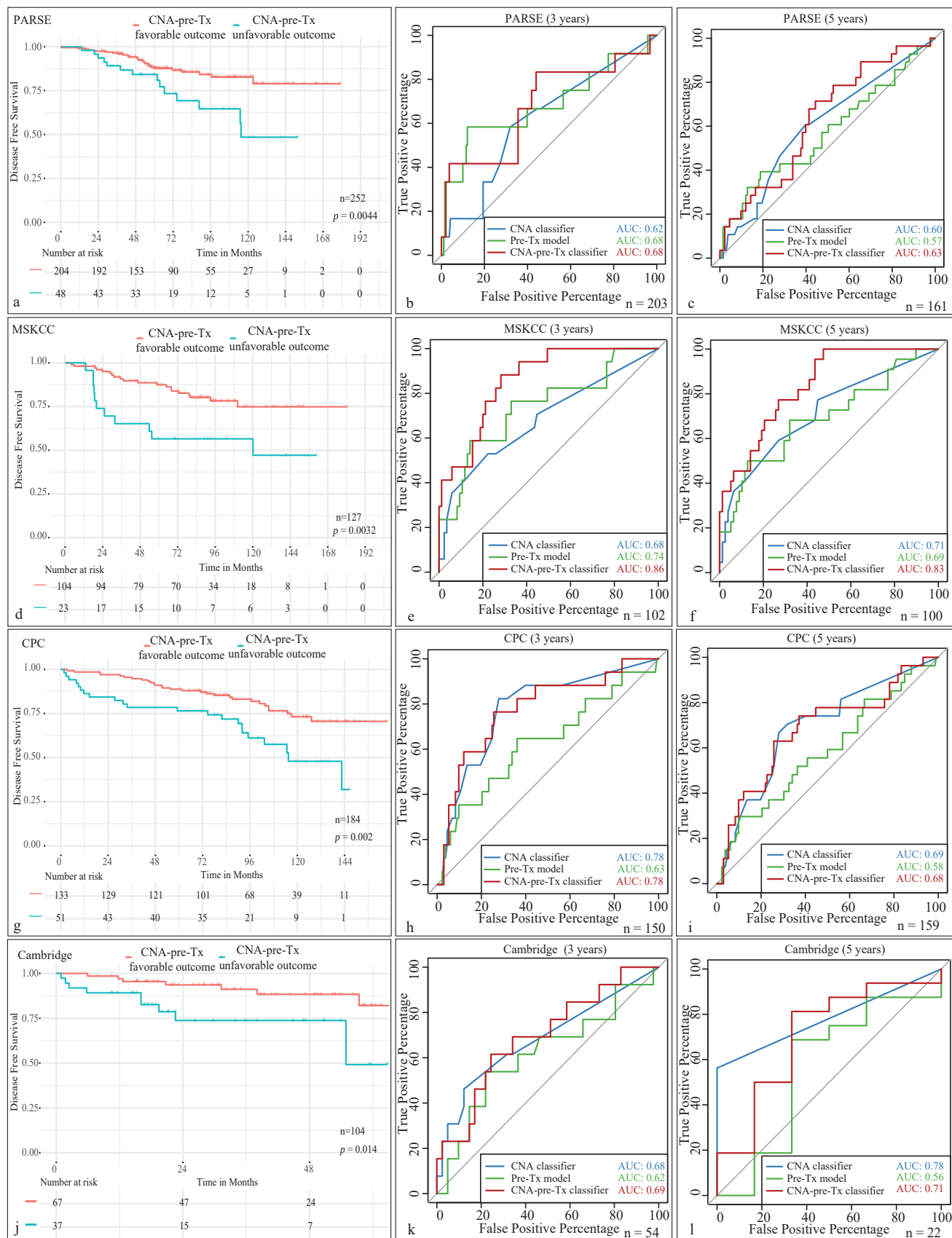
Manuscript 2, Figure S5: AUC of CNA-classifier, percentage of CNA and deletion of *PTEN* at 3- and 5-years. Results indicate that the CNA-classifier can predict BCR better than the percentage of CNA or deletion of *PTEN*.



Manuscript 2, Figure S6: C-index plot of the CNA-classifier in all RP validating cohorts.
The CNA-classifier shows similar or higher C-index in all validation datasets.



Manuscript 2, Figure S7: Kaplan-Meier analysis of the CNA-classifier in the RP validation datasets. a. MSKCC dataset, b. CPC dataset, c. Cambridge dataset.



Manuscript 2, Figure S8: Prognostic efficiency of the CNA-pre-treatment(Tx) classifier in all tested RP datasets. CNA-pre-treatment a. Kaplan-Meier analysis; b. 3-years; and c. 5-years AUC in PARSE MLPA cohort. d. Kaplan-Meier analysis; e. 3-years; and f. 5-years AUC in MSKCC dataset. g. Kaplan-Meier analysis; h. 3-years; and i. 5-years AUC in CPC dataset. j. Kaplan-Meier analysis; k. 3-years; and l. 5-years AUC in Cambridge dataset. *P* value of Kaplan-Meier analyses were calculated using log rank test.

5. CHAPTER 5 - OVERALL DISCUSSION AND CONCLUSION

Since the adoption of the PSA test as a routine part of screening in men, PCa detection has significantly increased. Although PSA testing has low sensitivity, it contributed to the early detection of the disease and about 81% of patients diagnosed with PCa have an early stage and localized disease [631]. However, PSA and other common prognostic tools such as clinical stage and biopsy GS cannot distinguish cases at risk of progression with likelihood of death from PCa from those with indolent disease that will not become clinically significant. Various risk stratification models have been developed using these pre-treatment criteria (Table 4), which can predict the risk of aggressive disease with a good reliability; however, significant heterogeneity still remains in these risk groups [129-131, 632, 633]. Because all patients in the same risk group are treated similarly, this intra-group heterogeneity has resulted in undertreatment of some patients in the intermediate-risk group and overtreatment of patients in the low-risk group [639-641]. In recent years, patients in the low-risk group are considered for active surveillance (discussed in section 1.2.6.1. Active surveillance) and patients in the high-risk group are treated with more aggressive approaches such as RP or radiotherapy. However, the management of patients in the intermediate-risk group which show the highest heterogeneity in the clinical outcome is a subject of controversy [634].

Several active surveillance clinical trials have shown the efficacy and favorable long-term outcome in low-risk PCa patients [147-149, 642-645]. For example, an active surveillance trial from the John Hopkins University including low and very low-risk patients showed PCa specific survival and metastasis-free survival rates of 99.9% and

99.4%, respectively after 15 years [149]. On the other hand, clinical trials with more heterogeneous cohorts that included intermediate-risk patients also reported favorable outcomes. In an active surveillance trial by the University of California, San Francisco where 559 PCa patients were followed from 1990 to 2013 (median follow-up of 5-years), 125 (22.4%) of patients had an intermediate-risk disease. Results of this trial showed no PCa specific death and only one case with metastasis [148]. Similarly, the results of the Toronto active surveillance trial [147] with 993 patients which 21% of whom had intermediate-risk disease showed 98% and 94% PCa specific survival rates at 10-year and 15-year, respectively. After 15 years, there were only 15 PCa specific deaths and 13 metastatic cases. Results of these two trials with heterogeneous populations that included a significant number of intermediate-risk patients showed excellent favorable outcome with PCa specific survival rates close to published results of immediate and definitive treatment cohorts [633, 646, 647]. While data obtained from several large clinical trials with heterogeneous cohorts demonstrated acceptable oncologic outcomes of active surveillance approach in intermediate-risk patients [147, 648-650], most patients showing Gleason pattern 4 (i.e. GS of 3+4 or 4+3 and higher) in the biopsy sample are disqualified from this popular disease management approach [634], which results in overtreatment and significant side-effects for the patient.

Variability in the clinical outcome of patients shows that current stratification systems do not account for the molecular heterogeneity of PCa. Thus far, genomic profiling has shown to be able to explain, in part, the heterogeneity seen in PCa standard risk groups and can help to provide more information which with addition to the patients clinicopathological features can improve risk stratification and provide a better

assessment of the disease status and help in clinical management such as selecting different therapeutic approaches for patients. For example, Prolaris test assesses the expression of 31 genes associated with cell-cycle progression in RP or biopsy samples and may aid the decision-making process between selecting active surveillance and definitive treatment options in low-risk PCa, and it may also suggest the use of adjuvant therapy in high-risk patients with adverse pathological features after surgery [651]. Oncotype Dx Genomic Prostate Score is a real-time PCR assay that can be performed on FFPE specimens obtained from biopsy to assess the expression of 12 cancer-related genes involved in androgen pathway, cellular organization, proliferation, and stromal response [652]. The continuous risk score generated by the assay can be further combined with clinical parameters such as age, PSA, clinical stage, and biopsy GS to predict high-grade (primary GS of 4 or any pattern of 5) and high-stage disease (pT3 or higher) at the time of RP [653]. Thus, the assay may be used to guide clinicians in the decision-making process for selection of patients for active surveillance versus therapeutic intervention [654, 655]. Other tests such as Decipher genomic classifier were designed to predict the risk of developing metastasis after surgery by assessing the expression of 22 RNAs involved in pathways related to cell proliferation, differentiation, motility, immune modulation, and AR signaling. The assay provides a continuous risk score, which can then be translated into low-, intermediate-, and high-risk Decipher categories based on predefined cut-offs [656, 657]. Decipher has shown to be an independent predictor of metastasis after adjusting for clinicopathological features [658]. Other studies also showed the value of Decipher to guide treatment decision making after RP in high-risk PCa patients to identify those that may benefit

from adjuvant radiotherapy [659]. These data show that genomic biomarkers can improve current risk stratification systems to provide a more accurate patient classification and disease management. However, the clinical utility of DNA-based prognostic biomarkers has lagged the RNA-based markers even though they hold some advantages such as robustness to degradation and remain stable in various physiological and environmental conditions. Although many studies have explored the application of DNA alterations for the prognosis of clinical outcome in PCa, none has been successfully adopted for clinical use [285]. Furthermore, various whole genomic studies suggest that CNAs occur at high frequencies in PCa, suggesting the initiation and progression of PCa is primarily due to chromosomal rearrangements. Consequently, various DNA biomarkers have been introduced that can predict the risk of BCR in PCa independent of the standard clinicopathological features [138, 278, 279, 282, 493, 660-662].

Several microarray and next-generation sequencing studies have revealed the composition of genetic alterations seen in PCa and characterized CNAs as a major component of its genomic landscape [138, 488, 597, 663-665]. These studies have shown the genetic characteristics of various PCa tumors at different grades and stages and increased our understanding toward the correlation of specific CNAs with adverse clinicopathological features [430, 452, 600, 666]. These studies enabled us to use these genetic alterations and design better molecular classifiers for more accurate patient risk stratification and personalized cancer care.

To provide a better risk stratification and improve the current standard risk groups, in this study we have first identified relevant genomic biomarkers associated with PCa

tumorigenesis and progression, then designed an assay that could assess these genomic biomarkers in a multiplex format in low-quality and quantities of DNA compatible with PCa biopsy sample and rigorously assessed its performance, reproducibility, and accuracy in clinical samples. We further developed an analysis approach that can accurately detect CNAs in tumor samples with low cellularity often seen in low-grade samples. We used this validated assay to profile a large cohort of PCa patients with low- and intermediate-risk disease based on their RP GS. We used the generated data to develop a CNA classifier that can further improve risk stratification in this heterogeneous population of patients and predict BCR independent from clinicopathological features. We validated the efficacy of this classifier in patients with similar low- and intermediate-risk PCa from four previously published cohorts. Our developed signature was also able to improve risk stratification of low- and intermediate-risk patients in a biopsy cohort. Addition of this CNA classifier to standard clinicopathological features further improved risk stratification of patients and showed higher AUC at both 3-years and 5-years compared to the standard clinical model. We also addressed the issue of intra-tumor heterogeneity in PCa and showed that our developed CNA classifier can distinguish between patients with a favorable outcome and those that show poor prognosis in both the Gleason pattern 3 sample and the Gleason pattern 4 sample of the same tumor.

To achieve those goals, we first selected CNAs that can be used for prediction of tumor behavior using literature review and focused on genes in the minimal alteration regions of frequent CNAs in PCa. We focused on well-known oncogenes, tumor suppressors and genes that impact tumor biology and regulate DNA damage repair, cell cycle, and

PI3K/AKT pathways that are often altered in PCa and are drivers of aggressive tumors [276, 277, 667]. Our selected genes have been shown by previous studies to be able to predict poor prognosis in PCa. Furthermore, a combination of multiple biomarkers provides a more robust and accurate prognosis. Studies have shown that concordant deletion of *MAP3K7* and *CHD1* promotes aggressive PCa. Suppression of *MAP3K7* and/or *CHD1* expression in mouse prostate epithelial progenitor/stem cells and tissue recombination experiments in vivo revealed that dual loss of *MAP3K7* and *CHD1* creates regions of HGPIN and carcinoma with significant disruption of normal prostatic lineage differentiation. Concordant loss of *MAP3K7* and *CHD1* results in a significant loss of AR, increased neuroendocrine differentiation, and neural differentiation [368]. Similarly, combined deletion of *MAP3K7* and *PTEN* in 3,845 PCa cases, showed significant association with BCR. The prognosis effect of this combined deletion was retained in multivariate analysis with preoperatively and postoperatively parameters [668]. Previous studies also have shown that loss of *NKX3-1* alone is not sufficient for initiation of PCa, however, combined deletions of *NKX3-1* and *PTEN* in mice show an increased incidence of HGPIN lesions and carcinoma in situ in the prostate by 6 months of age compared to the single mutants [669]. Kim et al. [670] showed that expression of *MYC* alone in the prostate of mice did not induce PCa, which was attributed to the lower expression of *MYC* and the mice strain. However, when the same mice were crossed with mice with prostate-specific *PTEN* deletion, they developed HGPIN and PCa. Likewise, Hubbard et al. [671] showed overexpression of *MYC* alone or loss of *PTEN* in mice does not progress beyond PIN, however, the combined loss of *PTEN* and overexpression of *MYC* developed a mouse model with

lethal adenocarcinoma with distant metastases and widespread genomic instability presented by genome-wide CNAs. In a separate study, co-overexpression of *MYC* and loss of *TP53* in the urogenital sinus cells of mice led to the development of PCa in 100% of mice while 95% showed metastasis [672]. Others showed that mice with *CDKN1B* deficiency do not develop PCa [673-675], however, simultaneous inactivation of one *PTEN* allele and one or both *CDKN1B* alleles results in increased cell proliferation and spontaneous development of PCa within 3 months in mice [676]. Moreover, in another study, expression analysis of *PTEN* and *CDKN1B* in 104 primary PCa tumors showed that combined loss of *PTEN* and *CDKN1B* is associated with elevated tumor cell proliferation, increased tumor diameter, seminal vesicle invasion, and increased pathological stage. Multivariate analysis also revealed that combined loss of *PTEN* and *CDKN1B* was independent predictors of BCR [677]. The studies in mice are corroborated by alterations detected in patients. For instance, patients with co-deletions of *GABARAPL2* and *PTEN* assessed by FISH in more than 7,400 prostate tissues, showed worse prognosis compared to patients with the *PTEN* or the *GABARAPL2* deletion alone. Multivariate analysis also revealed the prognostic value of *GABARAPL2/PTEN* deletion in prediction of outcome, independent from prognostic features [292]. In a recent study in the host lab, Bramhecha et al. [287] assessed the deletion status of *PTEN* and gain of *PDPK1* in 332 primary RP specimens using FISH. Combination of *PDPK1* gain and *PTEN* deletion improved BCR risk stratification and provided more accurate prognostic information. Kaplan-Meier analysis showed that *PDPK1* gain provides additional risk assessment in cases without *PTEN* deletion and vice versa, deletion of *PTEN* provided additional risk assessment in cases with no

PDPK1 gain. Other studies showed that combining multiple CNAs as a prognostic biomarker signature provides a stronger and more accurate prognosis. Assessment of the deletion of *TP53*, *PTEN*, and *RB1* using next-generation sequencing in 285 PCa cases showed that concurrent deletions of these tumor suppressor genes lead to an incremental increase in the risk of relapse. Increased number of CNA in these tumor suppressor genes was observed in cases with metastasis. Furthermore, patients with more CNAs showed a lower overall survival [677]. Similarly, *MYC* overexpression in tissue samples with concurrent deletion of *TP53* and *PTEN* in mouse models results in increased cell proliferation and metastasis. This was not observed in mice with only of *PTEN* or *TP53* deletion [678]. All these studies show that combining multiple CNAs provides better prognosis and more accurate prediction of tumor behavior. Furthermore, assessing multiple CNAs provides a more accurate representation of tumor genomic status and various pathways that can lead to tumor progression, metastasis, or possible susceptibility to certain therapies. Additionally, the combination of certain CNAs can have a synergic effect and lead to more aggressive tumors, thus increasing the risk of rapid progression and metastasis. This synergic effect can be seen in simultaneous deletion of *PTEN* and gain of *PDPK1* that both regulate the PI3K/AKT pathway. *PDPK1* is a positive regulator and its gain is known to activate this pathway [679]. Whereas, *PTEN* is a negative regulator and its deletion is associated with activation of PI3K/AKT pathway [680]. Overactivation of this pathway is a common molecular phenotype of PCa associated with poor prognosis. Activation of this pathway derive protein translation, growth, proliferation, metabolism, and cell migration giving survival advantages to cancer cells [681]. A recent study also

confirms that simultaneous alteration in these genes results in worse outcome compared to either alteration alone [682]. Another example would be the co-deletion of *WRN* and *TP53*. Studies have shown a direct interaction between these two tumor suppressors that is essential for maintenance of genomic stability [400, 401]. This is also shown in *WRN* mutated cells that have reduced p53-mediated apoptosis [400]. In normal cells, the p53-*WRN* complex recognizes abnormal DNA damages and cause cell cycle arrest and initiation of DNA repair systems or induction of apoptosis. Simultaneous deletion of these proteins may further impair the cellular checkpoints and lead to progression of cell cycle despite various DNA damages and lead to increase genomic instability, associated with poor outcome.

It is worth emphasizing that *PTEN*, *PDPK1*, *WRN* and *TP53* are a part of our CNA-classifier that uses combination of CNAs in these genes to assess the risk of patients for BCR independently from clinicopathological features. This further reinforces the use of combination of CNAs with biological relevance to tumor behaviour as a biomarker signature for poor outcome. We also included genes that are involved in various DNA repair pathways. Impairment of DNA damage repair pathway genes is an essential hallmark for the initiation and progression of cancer and is often observed in primary and localized tumors and even more, in metastatic PCa [683]. Inactivation of genes involved in the repair of the genome in early stages of tumorigenesis leads to genomic instability which is a known biomarker of aggressive tumors. CNA burden, defined as the percentage of the genome affected by CNAs, is shown to be an independent predictor of poor prognosis [276, 667, 683]. Tumor cells with higher genomic instability that is a result of dysfunctional DNA repair pathways accumulate more

CNAs leading to a higher risk of inactivation of tumor suppressor genes and activation of oncogenes resulting in a more aggressive disease. However, the CNA burden in early stages of the disease is often low. Studies show that metastatic samples have an average CNA burden of 32%, while primary tumors show a CNA burden of only 5% [282, 367]. Higher CNA burden is seen when tumor cells have accumulated a significant amount of CNAs and may already show aggressive features. Thus, rather than predicting tumor behavior, it identifies an already aggressive tumor. Furthermore, assessing the CNA burden requires whole-genome analysis, as CNA burden is presented as a fraction of CNAs compared to the whole genome. Whole-genome assessment methods such as array-CGH and whole-genome sequencing, although high throughput, are not suitable for clinical practice. In addition, assessment of the entire genome is often unnecessary. Therefore, inexpensive and robust methods with simple and effective analysis algorithms must be developed for common clinical practice until higher-cost technologies with more complex analyses is proven necessary for added clinical benefits [285]. Thus, instead of assessing CNA burden, which was not feasible due to the limitation of the selected technique allowing an assessment of up to 50 different loci, we focused on impairments of genes involved in DNA damage repair which promote genome instability and increase the rate of mutagenesis, leading to high CNA burden. Previous reports have shown that DNA damage repair pathways function through either single-strand breaks (SSBs) or DSBs repair [334, 335, 342, 348, 350, 373, 407]. SSBs are repaired via mismatch repair, NER, and base excision repair (BER) mechanisms, whereas, DSBs are mainly repaired by HR. In primary PCa, the collective genetic alterations of genes involved in these pathways were reported to

occur in 10–20% of cases [276, 667]. Therefore, in our gene panel, we selected *RWDD3* (1p21.3), *GTF2H2* (5p13.3), *CHD1* (5q15-q21.1) and *WRN* (8p12) that are involved in various DNA damage repair pathways (reviewed in section 1.4. CNAs relevant to prostate tumor biology). *RWDD3* regulates sumoylation which has pivotal roles in virtually all DNA repair mechanisms, including BER, non-homologous end joining, and HR [684, 685]. *GTF2H2* is one of the main regulators of the NER pathway and is involved in various steps of DNA damage repair [335]. *CHD1* is involved in chromatin remodeling and initiates the assembly of the DNA repair machinery in HR-mediated repair of DNA damage [354-358]. *WRN* regulates a network of proteins that respond to DNA damage and DNA replication. Furthermore, it is directly involved in the removal of mismatched and damaged DNA and can repair a blocked replication fork by HR events [394-398, 404]. All these genes are frequently deleted in PCa that leads to impairment of DNA damage repair machinery and increases genome instability. Thus, deletion of these genes in the early stages of tumorigenesis could predict high CNA burden. Moreover, two of these DNA damage repair regulators (*RWDD3* and *WRN*) are a part of our DNA classifier in virtue of the important role of these genes in driving aggressive disease. We further tested the efficacy of our CNA-classifier compared to the CNA burden in the validation datasets where the whole genome was assayed for CNAs. Multivariate analysis showed that CNA burden was not superior to our CNA-classifier; furthermore, in the Toronto biopsy dataset, our CNA-classifier outperformed the CNA burden. This shows that our developed assay and model can provide more accurate risk stratification than CNA-burden especially in

the early stages of the disease with a high throughput and a cost-effective manner that is more suited for routine clinical application.

Along the same line, recent studies show that tumors with impaired DNA damage repair pathways are susceptible to PARP inhibitors and about 20% of mCRPC patients show loss-of-function in DNA repair genes [139]. A recent phase II clinical trial in the assessment of the use of PARP inhibitors in PCa patients with advanced castration-resistant disease (TOPARP, NCT01682772) [635] showed high success rate with 16 out of 49 patients (33%) responding to the treatment. Further analysis showed a significant correlation between mutations or deletions in DNA repair genes and patients who responded to the treatment [635]. The success of this study has led to a second trial (TOPARP-B), wherein patients are prospectively selected based on the presence of biomarkers associated with DNA damage repair [683].

A limitation of our study was lack of patients with adjuvant therapies. Since all patients in our studied cohorts had low- and intermediate-risk disease based on the GS and they were all treated by RP, we were not able to assess the correlation of deletion in these genes with other therapeutic approaches. However, the inclusion of these genes in the probe mix enables us to potentially assess alterations in future studies and established new CNA signatures for prediction of response rate to such treatments. Another future application for the developed assay could be investigating the association of specific CNAs with a therapeutic response to develop predictive signatures to select patients for a particular treatment such as radiotherapy, RP, or addition of ADT.

Altogether, these data provide additional evidence that specific alterations in the early stages of tumorigenesis play a significant role in determining tumor behavior and predict aggressive disease. Thus, focusing on alterations in genes affecting the biology of the disease rather than overall genome instability and CNA burden, especially in early stages of the disease and in a clinical setting is more informative, feasible and accurate.

The current preferred clinical technique for assessment of CNAs is FISH, which does not permit simultaneous assessment of multiple CNAs. Thus, for targeted assessment of multiple genes, new genomic methods are needed. Furthermore, a genomic-based assay for routine clinical use must be cost-effective and currently due to the high costs of available high-throughput sequencing and array-based techniques, implementation of them in the everyday clinical practice is not feasible [284]. The other common CNA assessment method is TaqMan PCR which is compatible with low amounts of FFPE extracted DNA; however, it has limited multiplexing capability and is not suitable for simultaneous assessment of multiple genes. Comparison of different CNA assessment techniques shows that MLPA is a suitable method for this purpose. Furthermore, this technique is a PCR based method and does not require sophisticated instruments or analysis approaches. It is fast, cost-effective and because it can assess the copy number of up to 50 different loci in low amounts of FFPE extracted DNA, it can be easily adopted into the clinical setting and be used on DNA extracted from biopsy specimens. Therefore, we selected MLPA to develop our prognostic assay. Diagnostic or prognostic strength of every newly developed assay must be assessed. This includes precision, reproducibility, accuracy, sensitivity, specificity, and limit of detection. To

maximize clinical benefit, various sets of cut-off criteria and algorithms for clinical prediction must be developed to minimize the risks of underestimating cancer progression and vice versa [285]. To address these issues, we carefully assessed the performance of our designed assay against most common CNA detection methods and showed that it can assess multiple CNAs in low quality and quantities of DNA with high reproducibility, sensitivity, and specificity. We rigorously assessed the performance of each designed probes in both fresh and FFPE extracted DNA. We assessed the performance of the assay in various conditions to calculate the error rate. We tested the effect of various PCR-plates, caps, thermal cyclers, the experimenter and effect of sample evaporation during the experiment that could increase the salt concentration in the reaction. Results showed that on average only 0.89% of probes used in each experiment show false-positive results. This is much lower than other types of techniques and even other reported MLPA assays [686]. We further confirmed the accuracy of each probe in CNA detection against FISH.

Due to the various tumor cellularity seen in clinical samples, the cancer DNA is often diluted with DNA of other stromal compartments such as fibroblasts, endothelial and immune cells, thus accurate detection of CNAs requires the establishment of sensitive cut-offs. This is especially important in PCa, where most deletions are heterozygous, and gains are at one or two copy level [138, 167, 278, 279]. We tested various cut-offs including those routinely used in MLPA. We also developed a customized cut-off according to the tumor cellularity. However, the standard MLPA cut-offs did not provide an accurate representation of the CNA levels compared to FISH. While the customized cut-off points adjusted with the tumor cellularity of the samples performed

slightly better, it was not suitable for routine clinical use as in most cases the exact cellularity of the sample is unknown and is hard to assess in the three-dimensional tissue punch. Therefore, we developed another approach based on the range of variations seen in the normal tissues. We calculated the 95% confidence interval of each probe in three reference DNA sample populations and test samples. Thus, probe values in the test samples that are above or below the 95% confidence interval of the same probe in the normal tissues will represent gains and deletions respectively. We tested various analysis approaches and selected the one that can detect CNAs in various clinical samples, with different tumor cellularity with high sensitivity and specificity in both our test and validation sample sets compared to FISH. We further validated the performance and accuracy of our developed assay compared to dd-TaqMan PCR for two of the genes we have in our probe mix, *PTEN*, and *PDPK1*. These data show the high sensitivity, specificity, and accuracy of our assay compared to other commonly used molecular techniques. In addition, our assay has more multiplex capability and assesses 28 different loci (14 genes, targeted by 2 probes each). Furthermore, we use 10 different reference probes and three reference sample populations to normalize our data, thus we believe that our assay reports CNAs with more accuracy compared to these methods.

Clinical applicability of new prognostic assays requires assessments of their performance via AUC, Kaplan-Meier, positive and negative predictive values to show their advantage and utility compared to the current prognostic tools. Assessment of the prognostic criteria in large cohorts provides more accuracy and validity to the results, therefore, we tested the prognostic value of our designed assay in a large cohort of 433

low- and intermediate-risk patients treated with RP. Furthermore, this cohort was created from patients treated at two different institutions, thus it removes institutional biases such as differences in patient selection, treatment procedures and tissue processing that may exist among different centers. To address the issue of intra-tumor heterogeneity, patients in this cohort were represented with two samples, sample A was taken from the highest Gleason pattern and sample B was taken from the lowest Gleason pattern of the tumor. To increase the accuracy of our result all samples in the cohort were assayed in duplicates.

Overall our assay showed to be high throughput, highly reliable and reproducible. From a total of 2,075 reactions (433 patients with A and B samples, in duplicates in addition to the references and controls), 90% passed the QC criteria. Furthermore, a high correlation was also seen between the CNA calls of the two replicates reactions.

For most of the genes, our CNA calls based on our developed analysis approach was highly correlated with the mRNA expression levels of the same genes in both A and B samples assessed independently in another Center via NanoString technology. This shows the accuracy and robustness of our normalization and analysis approach. Furthermore, the CNA frequencies of the assessed genes in our cohort were similar to previous reports, which further supports the accuracy of our assay and designed analysis procedure.

We used our CNA data of PARSE MLPA cohort obtained via MLPA and developed a 6-genes CNA classifier that could improve risk classification of low- and intermediate-risk patients and predict the risk of BCR. We further validated the performance of this CNA classifier in four other previously published low- and intermediate-risk PCa

cohorts of 541 patients when combined. The CNA-classifier was an independent predictor of BCR when adjusted for other clinicopathological features, both pre- and post-treatment.

We further improved our CNA classifier by including the standard clinical prognostic values such as stage, Gleason group, and pre-treatment PSA levels and developed CNA-clinical classifiers that were able to predict BCR in 5-years better than the clinical model in all test and validation cohorts.

Another important aspect of validation of our prognostic model is its performance regardless of intra-tumor heterogeneity. In this study, we assayed each patient two times in the highest and lowest cancer grade within the tumor. PCa is highly heterogeneous and previous studies show that the performance of genomic models varies in different samples obtained from the same patients [687]. To assess this, we tested the prognostic capability of our model in the low-grade and high-grade samples separately and showed that this model can predict outcome in either sample. Our developed model could further stratify patients even in the low-grade tumors of this low- and intermediate-risk patients. To further show the efficacy of our model when samples are taken from the patients without prior knowledge of the tumor grade, we randomly took sample A or B from each patient and tested the prognostic capability of the model using C-index. We observed no statistical difference in the performance of the model in all ten different randomly assembled cohorts from A or B samples. This shows that even in cases with intra-tumor heterogeneity when biopsy samples are taken from different parts of the tumor, our model can predict the outcome with high accuracy, regardless of where in the tumor the samples are taken.

To further assess the clinical utility of our model, we tested its performance in Toronto biopsy cohort where CNA measurements were done in biopsy specimens rather than RP samples. Patients in the Toronto cohort were treated with image-guided radiotherapy instead of RP, thus the prognostic value of the model in different treatment options was also tested. Our CNA-classifier showed the best prognostic index in the Toronto biopsy cohort shown by the C-index, 3-year and 5-year AUC of the CNA- and CNA-pre-treatment classifier. All these data confirm and validate that our model can predict aggressive disease in low- and intermediate-risk patients in different treatment options and using biopsy or RP samples with high accuracy and better than current clinical models. In future studies, this probe mix can be further modified to only target these 6 genes included in the classifier with three or four probes each to increase the sensitivity of CNA detection. This may increase the accuracy of this assay.

One other important aspect of the clinical utility of genomic assays is the cost. Currently, most genomic screening assays have a high cost which prevents them from being routinely used in the clinical setting. The cost of our assay per reaction is currently 7.90\$ CAD (6\$ USD) and only requires 50 ng of DNA that can easily be obtained from the small needle biopsy. Using our developed methodology that uses duplicate reactions and three reference sample populations the cost of each sample per batch will increase to 18.50\$ CAD (14.17\$ USD). This is much more cost-effective compared to other technologies available now. Another recently published study [688], used NanoString technology, that is also compatible with FFPE extracted DNA. This assay cost 150\$ USD per sample and required 300 ng of DNA. Our developed assay is

more cost-effective, requires less amount of DNA and does not require sophisticated instruments, thus its translation into the clinical setting would be more feasible.

In this study, we aimed to close the gap between research and clinic, and achieved this by using the prognostic value of the CNAs in PCa to predict tumor behavior and provide more accurate risk-stratification of low- and intermediate-risk PCa patients that present the most heterogeneous clinical outcome and often over or under-treated. Our developed assay was able to successfully detect CNAs in FFPE clinical samples and our CNA classifiers were able to predict aggressive disease better than the currently used clinical model. Our CNA classifier can also predict aggressive disease in the biopsy specimens, and the assay is also compatible with low quality and quantities of DNA obtained from these samples. Thus, it can be easily applied on needle biopsy samples of patients to provide more accurate risk-stratification in low- and in intermediate-risk PCa patients at the time of diagnostic to improve the decision-making process for this highly heterogeneous population of patients.

6. CHAPTER 6 - REFERENCES

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