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MASTER OF SCIENCE THESIS

EVALUATION OF METABOLIC ENZYMES AS PREDICTIVE BIOMARKERS OF RISK FOR PROSTATE CANCER PROGRESSION

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Descriptive Note

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Lay Abstract

Currently, many patients with localized prostate cancer do not receive immediate therapy and are monitored within systematic active surveillance (AS) programs. The main aim of AS management is to prevent overtreatment and treatment-related complications in patients who would otherwise have a good quality of life despite dealing with prostate cancer. However, many of these patients, especially those with low intermediate-risk prostate cancer, have a significant risk for disease progression and metastasis. Additionally, there is a lack of promising tissue biomarkers to predict the risk for progression in AS patients at the time of initial diagnosis. Research showed that metabolism dysregulation is an essential hallmark of cancer progression, including prostate cancer. In this pilot study, we examined whether the expression of enzymes involved in lipid, glucose and protein metabolism could have value as biomarkers of risk for prostate cancer progression in patients managed with AS. The expression of five metabolic enzymes (ACLY, ACC, GLUT1, AMACR and PSMA) was examined in tumor and benign regions of diagnostic biopsies of the prostate obtained from men managed with AS. Our early results suggest that the expression of enzymes of protein (PSMA) and glucose (GLUT1) metabolism may have value as biomarkers of risk for prostate cancer progression and should be investigated further in systematic studies.

Abstract

Currently, many patients with early-stage localized prostate cancer (PrCa) (D'Amico: low risk or low-intermediate risk) do not receive immediate therapy but are monitored within systematic AS programs. Prospective trials showed rates of stage reclassification and progression to the treatment of 20–40% over 2–5 years. However, in certain patients, PrCa progresses rapidly to an advanced stage that requires combined modality therapies, which carry increased risk for toxicity and poor outcomes. There is a need to identify biomarkers that can predict the risk for disease progression in this population. Research showed that dysregulation of metabolism is an important hallmark of cancer progression. Here, we pursued a pilot investigation of enzymes of de novo lipogenesis [ATP-citrate lyase (ACLY), Acetyl-CoA Carboxylase (ACC)], lipid oxidation [a-Methylacyl-CoA Racemase (AMACR)], glucose uptake [facilitative glucose transporter 1 (GLUT1)], and folate – glutamate metabolism (PSMA: prostate-specific membrane antigen) as potential biomarkers of PrCa progression in AS patients. With ethics approval from the Hamilton Integrated Research Ethics Board (HiREB), 40 AS patients were accrued prospectively from the Niagara Health System PrCa diagnostic program clinics and were asked to donate their biopsy tissue. 28 patients progressed on repeat biopsies at 12 or 24 months after initial diagnosis and were included in the "Progressed" group, and 12 did not who were included in the "Non-Progressed" group. Baseline diagnostic prostate core biopsy tissues of both groups were evaluated with H&E and immunohistochemistry (IHC) staining for ACLY, ACC, GLUT1, AMACR and PSMA expression (quantified by H-score). H-scores were evaluated in benign and malignant components (epithelial cells) and were compared

between the two groups of patients. We observed statistically significant increased GLUT1 expression in malignant epithelial cells of the progressed group compared to the non-progressed group. Also, we found statistically significant increased PSMA expression in the benign epithelial cells of the progressed group compared to the non-progressed group. Further, our results demonstrated a statistically significant increase in ACLY and ACC expression in malignant epithelial cells compared to benign epithelial cells in the progressed group, while AMACR was detected solely in the malignant component. Overall, the results of this pilot study are consistent with the notion of induction of glycolytic metabolism, de novo lipogenesis and increased PSMA expression associated with the risk for PrCa progression. The levels of expression of PSMA within benign epithelial cells and GLUT1 within malignant epithelial cells may have value as predictive markers of risk for PrCa progression in AS patients. Future studies should investigate this concept systematically in larger AS cohorts.

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List of Abbreviations

ACC: Acetyl-CoA Carboxylase

ACLY: ATP-Citrate Lyase

ADT: Androgen Deprivation Therapy

AFP: Alpha-Foetoprotein

AJCC: American Joint Committee on Cancer

ALDH1A1: Aldehyde Dehydrogenase 1 Family, Member A1

ALL: Acute Lymphoid leukemia

AMACR: Alpha-methylacyl-CoA Racemase

AML: Acute Myeloid Leukemia

AMP: Adenosine Monophosphate

AMPK: AMP-Activated Kinase

APC: Adenomatous Polyposis Coli

AR: Androgen Receptor

ARA70: Androgen Receptor-Associated protein 70

AREs: Androgen Response Elements

AS: Active Surveillance

AST: Aspartate Transaminase

ATM: Ataxia-Telangiectasia

ATP: Adenosine Triphosphate

ATR: Ataxia Telangiectasia and Rad3-Related Protein

Bcl-2: B-cell Lymphoma 2

BCR: Biochemical Recurrence

BPH: Benign Prostatic Hyperplasia

BRCA-1: Breast Cancer gene 1

BRCA-2: Breast Cancer gene 2

bRFS: Biochemical Recurrence -Free Survival

CA 19-9: Cancer Antigen 19-9

CA125: Cancer Antigen 125

CA15-3: Cancer Antigen 15-3

CD95: Cluster of Differentiation 95

CD147: cluster of differentiation 147

CDKIs: Cyclin-Dependent Kinase Inhibitors

CDKs: Cyclins Dependent Kinases

CEA: Carcinoembryonic Antigen

CHK1: Checkpoint Kinase 1

CHK2: Checkpoint Kinase 2

CK5/6: Cytokeratin 5/6

CML: Chronic Myelogenous Leukemia

CNBx: Core Needle Biopsy

CO2: Carbon dioxide

COX-2: Cyclooxygenase 2

CRPC: Castration-Resistant Prostate Cancer

CSCs: Cancer Stem Cells

CT: Computed Tomography

CTCs: Circulating Tumor Cells

DBD: DNA Binding Domain

DDR: DNA Damage Repair

DHT: Dihydrotestosterone

DLX1: Distal-Less Homebox 1

DNA: Deoxyribonucleic Acid

DNL: De Novo Lipogenesis

DRE: Digital Rectal Examination

EAU: European Association of Urology

EBRT: External Beam Radiation Therapy

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked Immunosorbent Assay

ER: Estrogen Receptor

ERBB2: Erb-B2 Receptor Tyrosine Kinase 2

ERG: ETS-Related Gene

ERK1/2: Extracellular Signal-Regulated Kinases 1/2

ETS: Erythroblast Transformation Specific

FVIII: Factor VIII

FADH2: Flavin Adenine Dinucleotide

FASN: Fatty Acid Synthase

FDG-PET: Fluorodeoxyglucose Positron Emission Tomography

FISH: Fluorescence In Situ Hybridization

FNA: Fine Needle Aspiration

FDA: Food and Drug Administration

FOXA1: Forkhead Box A1

fPSA: Free PSMA

G6P: Glucose 6 Phosphate

GAB1: GRB2-Associated-Binding protein 1

GATA2: GATA Binding Protein 2

GLUT1: Glucose Transporter type 1

GLUT12: Glucose Transporter type 12

GLUT4: Glucose Transporter type 4

GnRH: Gonadotropin-Releasing Hormone

GP: Glycogen Phosphorylase

GRB2: Growth factor Receptor-Bound protein 2

GSTP1: Glutathione S Transferase pi 1

GTP: Guanosine Triphosphate

GUROC: Genitourinary Radiation Oncologists of Canada

Gy: Gray

H&E: Hematoxylin and Eosin

HCC: Hepatocellular Carcinomas

HCG: Human Chorionic Gonadotrophin

HDR: High-Dose-Rate

HER2: Human Epidermal Growth Factor Receptor 2

HIFU: High-Intensity Focused Ultrasound

HiREB: Hamilton Integrated Research Ethics Board

HK2: Hexokinase 2

HMGR: Hydroxy Methylglutaryl Coenzyme A Reductase

HOXC6: Homeobox C6

HSPs: Heat Shock Proteins

HR: High Risk

H-Score: Histologic Score

IGRT: Image Guided Radiation Therapy

IHC: Immunohistochemistry

IMRT: Intensity-Modulated Radiation Therapy

IR: Intermediate Risk

IRFs: Intermediate Risk Factors

IRS: Immunoreactive Score

ISUP: International Society of Urological Pathology

JH: John Hopkins

kDa: kilo Dalton

LBD: Ligand Binding Domain

LDR: Low-Dose-Rate

LH-RH: Luteinizing Hormone–Releasing Hormone

LIR: Low Intermediate Risk

LKB1: Liver Kinase B1

LR: Low Risk

MAPK: Mitogen-Activated Protein Kinase

mCRPC: Metastatic Castration-Resistant Prostate Cancer

MDS: Myelodysplastic Syndromes

mGluR1: Metabotropic Glutamate Receptor Group 1

MiPS: Michigan Prostate Score

MLCs: Multi Leaf Collimators

mRNA: Messenger Ribonucleic Acid

MSKCC: Memorial Sloan Kettering Cancer Centre

mTOR: Mammalian Target of Rapamycin

mTORC1: Mammalian Target of Rapamycin Complex 1

NADH2: Nicotinamide-Adenine Dinucleotide

NCCN: National Comprehensive Cancer Network

ng/ml: Nano Gram per Milliliter

NHS: Niagara Health System

NKX3.1: NK3 Homeobox 1

NP: Non-Progressed

NTD: N-terminal domain

P: Progressed

P2PSA: 2proPSA

P63: Protein 63

PBT: Proton Beam Therapy

PCA3: Prostate Cancer Antigen 3

PDAP: Prostate Diagnostic Assessment Program

PHI: Prostate Health Index

PI3K: Phosphoinositide-3-Kinase

PIN: Prostatic Intraepithelial Neoplasia

PR: Progesterone Receptor

PrCa :Prostate Cancer

PRIAS: Prostate Cancer Research International Active Surveillance

PSA: Prostate-Specific Antigen

PSAD: PSA Density

PSAP: Prostate-Specific Acid Phosphatase

PSMA: Prostate-Specific Membrane Antigen

PTEN: Phosphatase and Tensin Homolog

PUMA: p53-Up-Regulated Modulator of Apoptosis

RASSF1A: ras Association Domain of the Familial Protein 1 Isoform A

RB1: Retinoblastoma Protein 1

RFLP: Restriction Fragment Length Polymorphism

RM: Royal Marsden

RNA: Ribonucleic Acid

RP: Radical Prostatectomy

RT: Radiation Therapy

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SBRT: Stereotactic Body Radiation Therapy

SEM: Standard Error of Means

SGLTs: Sodium–Glucose Co-Transporters

SPDEF: SAM Pointed Domain-Containing Ets Transcription Factor

SREBPs: Sterol-Response Element Binding Proteins

TGFβ: Transforming Growth Factor Beta

TMPRSS2: Transmembrane Serine Protease 2

TNFR1: Tumor Necrosis Factor Receptor 1

TP53: Tumor Protein 53

TPO Ab: Thyroid Peroxidase Antibodies

tPSA: Total PSMA

TSC2: Tuberous Sclerosis Complex 2

TURP: Transurethral Resection of the Prostate

TXNIP: Thioredoxin-Interacting Protein

UCSF: University of California, San Francisco

UICC: Union for International Cancer Control

UoM: University of Miami

UoTPM: University of Toronto (Princess Margaret)

UoTSB: University of Toronto (Sunnybrook)

VHR: Very High Risk

VMAT: Volumetric-Modulated Arc Therapy

WW: Watchful Waiting

μm: Micrometer

18G: 18-Gauge

34βE12: 34BetaE12

3D-CRT: Three-Dimensional Conformal Radiation Therapy

Declaration of Academic Achievement

Contributions to concepts and design of research: Elham Ahmadi, Dr. Theos Tsakiridis, Dr. Gregory Steinberg, and Dr. Monalisa Sur

Contributions to data interpretation: Elham Ahmadi, Dr. Theos Tsakiridis, Dr. Gregory Steinberg, Dr. Monalisa Sur, Simon Wang.

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Chapter 1- Background

1.1 Introduction

1.1.1 The prostate gland (Normal anatomy and histology)

The prostate is a pear-shaped glandular organ which weighs up to 20 g in the healthy adult male and needs androgenic hormones synthesized in the testis for differentiation and growth through a poorly understood mesenchymal-epithelial interaction (Cunha, 1994).

The prostate is divided into the anterior fibromuscular stroma and three zones as proposed by McNeal: peripheral zone, transition zone, central zone (McNeal, 1968, 1972, 1984; McNeal JE, 2015).

The transition zone covers the urethra in the mid portion of the prostate and is the anatomic region involved in benign prostatic hyperplasia. The central zone is like an inverted pyramid in the base of the prostate and contains the ejaculatory ducts. The central zone glands may show a particular morphology revealing more deeply eosinophilic cytoplasm and/or more complex architecture like papillary infoldings or epithelial bridges. The peripheral zone wraps the transition zone and extends caudally to make the most of the apex. Finally, the anterior tissues consist of smooth and skeletal muscle and adipose tissue in the extraprostatic component. A fibromuscular layer covers the prostate, usually called a “capsule,” which is not a well-defined anatomic structure (Ayala et al., 1989).

The prostate gland is traditionally divided into acini and ducts, the latter subdivided into large and peripheral ducts. Both acini and ducts contain luminal secretory cells, a surrounding outer layer of basal cells, and scattered neuroendocrine cells (which are almost inconspicuous). The secretory cells, which are on the luminal side of the gland, secrete various products into the seminal fluid. Histologically, these cells make an undulating luminal surface and have pale cytoplasm that contains tiny closely packed vacuoles that may be difficult to identify including prostate-specific acid phosphatase (PSAP) and prostate-specific antigen (PSA) which can be identified by immunohistochemistry evaluation for diagnostic purpose because of their organ-related specificity as well as prostate-specific membrane antigen (PSMA) (Gelmann et al., 2003; Gurel et al., 2010; Wright et al., 1995).

Morphologically, basal cells often are revealed as small inconspicuous, almost pyknotic nuclei at the outer side of the secretory cells which express high-molecular weight cytokeratin (e.g., 34βE12 and CK5/6) and p63, which are very helpful in the differential diagnosis between well differentiated carcinomas (basal cells are absent) and benign conditions that mimic carcinoma (they are generally present, even in a patchy discontinuous fashion) (Brawer et al., 1985).

Basal cells do not express PSA or PSAP, but it has been demonstrated that they have strong immunoreactivity for androgen receptors focally (Bonkhoff & Remberger, 1993; Chodak et al., 1992).

The neuroendocrine cells express chromogranin A and B, secretogranin II, and various peptide hormones such as somatostatin, calcitonin, and bombesin (di Sant'Agnese et al., 1985; Schmid et al., 1994).

The prostatic stroma has significant content of smooth muscle fibers. Prostatic stromal cells have been found to contain androgen receptors. Peripheral nerve bundles are evenly distributed in the apex, mid gland, and base, which have importance because of the high frequency which prostate adenocarcinomas involve the loose connective space that surrounds them. The prostatic lymphatic drainage goes into the pelvic lymph nodes and the retroperitoneal chain.

1.1.2 Prostate cancer

1.1.2.1 General features

Prostate cancer is men's second most common solid tumor and the fifth cause of cancer mortality worldwide. The new cases of PrCa were estimated to be over 1 414 000 worldwide in 2020. (Gandaglia et al., 2021).

The general probability is 1 in 8 in all men. Prostate cancer prevalence at autopsy is up to 80% by age 80 years, while the clinical incidence is much lower, revealing that most men die because of other causes rather than prostate carcinoma (Sakr et al., 1996). The causes of prostate cancer are not very well known, despite its remarkable incidence and prevalence.

More than 95% of prostatic malignancies are adenocarcinomas which are usually clinically silent. The diagnosis may be made in the following clinical scenarios: (1) routine screening with digital rectal examination revealing a nodular or diffusely enlarged prostate; high serum PSA level; or imaging and biopsies are positive for malignancy; (2) incidental carcinoma in Transurethral resection of the prostate (TURP) samples; (3) metastatic adenocarcinoma of unknown primary; and (4) prostate carcinoma presenting as a rectal mass (rare).

1.1.2.2 Epidemiology

Prostate cancer causes a higher risk for American men, especially African American men. After age 40, the incidence increases quickly. Autopsy studies have shown a high latent (clinically occult) prostate cancer rate in men without clinical cancer. Prostatic adenocarcinoma incidence is much higher in men of African ancestry (100 per 100,000) compared to men of European ancestry (70.1 per 100,000), and the world's highest mortality rate from prostatic adenocarcinoma belongs to men of African ancestry in the United States (Bostwick et al., 2004). The prevalence of latent cancer is similar in different geographic and ethnic groups despite extensive variation in the incidence of clinically apparent cancer. The incidence is low in American Indians, Hispanics, and Asians, but high in American men of African and European ancestry.

Prostate cancer mortality rate is different from country to country. The United States has a high rate, especially among African Americans, while China and Japan have a low rate.

International differences in mortality could be a result of differences not only in the basic risk for the development of prostate cancer but also of differences in survival or reporting bias. The use of the serum PSA test has led to successful early detection of prostate cancer, but physicians are unable to accurately distinguish the patients who will progress and who will not.

1.1.2.3 Risk factors

Risk factors can be classified as endogenous or exogenous, although some factors are not exclusively one or the other (e.g., race, aging, and oxidative stress). Various endogenous risk factors for prostate cancer have been reported, including family history, hormones, race, aging and oxidative stress. Exogenous risk factors include but are not limited to diet, endocrine disrupting chemicals, and occupation (Bostwick et al., 2004). The risk factors for prostate cancer are summarized in Table 1.1.

Table 1.1 - Risk Factors for Prostate Cancer

Family history
Diet
Fat
Cadmium
Zinc
Obesity
Alcohol
Hormones
Smoking
Sexual activity
Early sexual activity
Multiple sexual partners
Occupational exposure
Agricultural fertilizers and pesticides
Rubber Ionizing
radiation
Venereal diseases
Herpesvirus type 2
Cytomegalovirus
Vasectomy
Benign prostatic hyperplasia
Prostatic intraepithelial neoplasia
Atypical small acinar proliferation

1.2 Molecular alterations associated with prostate cancer

PrCa growth and progression involve extensive energy metabolism and protein synthesis alterations, promoting proliferation and survival. Various genetic and molecular alterations result in the transformation of the normal prostatic epithelial cell into cancerous cells, including but not limited to alterations in the AR; alterations in tumor suppressor genes like (TP53, RB1), oncogenes and Cyclin Dependent TY Inhibitors (CDKIs); DNA methylation; chromosomal alterations and rearrangements; changes in PTEN and PI3K / mTOR pathways; apoptosis defect; and epigenetic mechanisms. Prostate cancer development is multifactorial, and probably a combination of these mechanisms is involved (Perdomo et al., 2018).

1.2.1 Androgens receptor

The AR gene is located on the X chromosome. The resulting protein is a 110- kDa protein composed of three main functional domains. The first domain is the N-terminal domain (NTD), the second domain is the DNA binding domain (DBD), and the third domain is the C-terminal ligand binding domain (LBD) (Tan et al., 2015).

The AR has a transcription factor role. Dimerization of ARs happens in the nucleus, which allows them to bind to androgen response elements (AREs) in promoter regions of

target genes to promote their transcription. Target genes are PSA, transmembrane serine protease 2 (TMPRSS2) and other genes responsible for prostate cell growth and survival (Green et al., 2012).

The DBD domain helps binding of the AR to promoter and enhancer regions of the target genes. This allows the NTD and LBD to have their functions and promote the transcription of those genes (Tan et al., 2015).

Combined effects of the AR and growth factor receptors, such as the Epidermal Growth Factor Receptor (EGFR), stimulate normal prostate cells and PrCa cell growth. ARs work as transcription factors for various genes involved in cell growth. Testosterone is converted into dihydrotestosterone (DHT) in cells. It subsequently binds to AR in the cytoplasm leading to its translocation to the nucleus, where several coactivators such as ARA70 and the P160 coactivators bind to it. These coactivators play roles in histone acetylation and chromatin remodeling. ARs also affect several transcription factors (like FOXA1, GATA2 and Oct1) to regulate gene expression (Takayama et al., 2013).

Growth factors can modulate AR activity. EGFR signaling pathway enhances ARs function; for example, a tyrosine kinase involved in EGFR activation named Src phosphorylates ARs at tyrosine 534, resulting in the translocation of ARs to the nucleus and subsequent increase in transcriptional function. Moreover, extracellular signal-regulated kinases 1/2 (ERK1/2), involved in the mitogen-activated protein kinase (MAPK) pathways, is suggested to enhance AR activity (Takayama et al., 2013).

1.2.2 Tumor suppressor genes

Tumor suppressor genes control cell growth, so they have importance in the normal cell cycle, DNA repair and cell signaling. The loss of the function of both alleles of a tumor suppressor gene results in cancer development, the two best characterized suppressor genes are the retinoblastoma gene (RB1) and the TP53 gene (Perdomo et al., 2018).

1.2.2.1 Tumor suppressor TP53

TP53 is a tumor suppressor gene that responds to cellular damage. It signals a stop to the cell cycle or results in damage repair pathways, but if repair is impossible, apoptosis will happen. In general, p53 regulates DNA damage repair (DDR), cell cycle progression, and apoptosis. When sensing stress, p53 stabilizes the genome and suppresses tumorigenesis using DDR responses, cell-cycle regulation, apoptosis, and other anti-tumour activities (Vousden & Lane, 2007). Bcl-2 mediates apoptosis induced by TP53 through an intrinsic pathway. Moreover, various tumor suppressive pathways have an association with TP53, like the response to DNA damage, cell senescence and apoptosis, so we can expect mutated TP53 in cancer frequently (Gonzalgo M, 2016; Oda et al., 2000).

AMP-activated kinase (AMPK) activation, like other kinases such as ATM, CHK1, ataxia telangiectasia and Rad3-related protein (ATR) and CHK2 have roles in p53 activation.

ATM and ATR phosphorylate p53 which results in stabilization of P53 and final DNA-damage response (Park et al., 2009).

p53 activation leads to activation of cyclin dependent kinase inhibitor P21 and subsequent G1 cell cycle arrest. Moreover, p53 can disrupt the cyclin B1/CDC2 complex's function, resulting in G2/M cell cycle arrest. p53 can also induce apoptosis through several mechanisms, for example, p53 interaction with p53-up-regulated modulator of apoptosis (PUMA) to induce apoptosis (Park et al., 2009).

In PrCa, p53 is usually silenced by mutations in the DNA or metabolic alterations. p53 loss has correlation with PrCa progression, with 20% of overall PrCa patients having p53 silenced but increased to 37% in metastatic PrCa and 73% in metastatic castration resistant PrCa (Hamid et al., 2019).

1.2.2.2 Retinoblastoma protein (RB1)

RB1 gene is important during the cell division process in the late G1 phase by controlling the R-point, which is a decisive point. Thus, the inactivation of RB1 pathways leads to cell proliferation and has been reported in at least 30% of bladder and prostate cancers. It has also rarely been associated with renal carcinoma (Kubota et al., 1995; Logothetis et al., 1992).

1.2.3 Oncogenes

Oncogenes have an association with cell proliferation. They are the mutated form of normal genes (proto-oncogenes) like MYC and MET. MYC plays a role in cell proliferation. This amplified gene is frequently found in prostate cancer (PrCa), and its expression has an association with PrCa cell immortalization (Gil et al., 2005).

There are 3 mechanisms by which a proto-oncogene can become an activated oncogene: 1) mutation, 2) gene amplification, and 3) chromosomal rearrangement. An example of the third mechanism is the famous translocation that results in the fusion of the TMPRSS2 gene with the ERG oncogene in a high percentage of PrCa patients (Tomlins et al., 2005).

1.2.4 Cyclin-dependent kinase inhibitors

Cyclins and cyclin-dependent kinases (CDKs) are responsible for events during cell division. They phosphorylate substrates which have a role in a specific activity in each phase. In contrast, cyclin-dependent kinase inhibitors (CDKIs) bind directly to CDKs and inhibit them (Sherr & Roberts, 1995).

There are two families of CDKIs: the Cip / Kip family, including the CDKN1A (p21), CDKN1B (p27) and CDKN1C (p57) proteins, and the INK4 family (which inhibits CDK4), including the INK4B (p15), INK4A (p16), INK4C (p18) and INK4D (p19) proteins. The p16 protein binds to CDKs 4 and 6 and inhibits their interaction with cyclin

D1; normally, active CDK4 and 6 regulate cell proliferation through G1 phase via the phosphorylation of RB1 (Gonzalgo M, 2016; Serrano et al., 1993).

Hypermethylation of p16 has been seen in 60% of prostate cancer cases, although inactivated p15 has been seen rarely (Herman et al., 1995).

Decreased p27 has correlation with decreased overall survival and disease-free survival after radical prostatectomy and in mice, its absence has association with prostatic hyperplasia (Cordon-Cardo et al., 1998; Freedland et al., 2003).

1.2.5 Methylation of DNA

Methylation changes the genetic function, resulting in the inactivation of the gene without alterations in the DNA sequence. Glutathione S transferases play roles in detoxifying xenobiotics that promote the nucleophilic attack of decreased glutathione in dangerous electrophilic compounds. The most prevalent somatic change detected in PrCa is the extensive methylation of the CpG island at the glutathione S transferase pi (GSTP1) locus (Jerónimo et al., 2001), which has been reported in up to 90% of PrCa and in 70% of prostatic intraepithelial neoplasia (PIN), which has an association with poor clinical outcomes (Maruyama et al., 2002). However, it can be detected in normal or hyperplastic tissue (Jerónimo et al., 2001).

The gene for the ras association domain of the familial protein 1 isoform A (RASSF1A) is a tumor suppressor gene methylated in 60–70% of prostate carcinomas. This

methylation rate is higher in high-grade tumors compared to less aggressive tumors (Kuzmin et al., 2002; Liu et al., 2002).

1.2.6 DNA damage and repair

Cancer is developed because of different gene changes, leading to alterations in tumor suppressor genes and oncogenes. To fight these changes, there are various defense mechanisms like free radicals such as vitamin C, alpha tocopherol, carotenoids and protective enzymes such as glutathione transferase. Also, associations between the polymorphisms of these genes and the risk of biochemical recurrence in PrCa patients have been reported (Nock et al., 2009). In DNA damage response (DDR), various genes with specific functions are involved like repairs of base cleavage, nucleotide cleavage, double helix rupture and imbalance (Gonzalgo M, 2016).

1.2.7 Chromosomal abnormalities

Deletions, gains and amplification of chromosomal segments are commonly found in PrCa. The most frequently changed chromosomes in PrCa are 8, 13, 7, 10, 16, 6 and 17, as well as gains or amplification of parts of the X chromosome and losses of the Y (Schulz et al., 2003).

1.2.8 Recurrent genetic rearrangements in PrCa

Recurrent gene fusions have been reported between the androgen-regulated gene TMPRSS2 and ERG, a member of the ETS family (Kumar-Sinha et al., 2008). More complex types of translocations lead to the other fusions (Perner, Mosquera, et al., 2007; Tomlins et al., 2005), which can be identified with reverse transcription polymerase chain reaction (RT-PCR) or by multi-colour fluorescence in situ hybridization (FISH). These markers have been evaluated in urine and blood in several clinical studies, as well as evaluation of expression of the ERG protein by immunostaining (Chaux et al., 2011; Falzarano et al., 2011; Park et al., 2010). Some authors consider the TMPRSS2-ERG fusion status a possible diagnostic marker, even with a potential prognostic value during patient follow up (Fine et al., 2010; Leyten et al., 2014). Some studies demonstrated that some fusions are single events or events that occur in only one patient by using next generation RNA sequencing technic, which reveals that our knowledge about PrCa is very little (Leyten et al., 2014).

1.2.9 PTEN and PI3K/mTOR

The phosphoinositide-3-kinase (PI3K)/AKT/mTOR pathway normally controls cell growth, metabolism, survival, and angiogenesis, which are highly relevant to carcinogenesis (Pflueger et al., 2011; Yang et al., 2019).

PI3K consists of two subunits: P85 as the regulatory subunit and P110 as the catalytic subunit. GRB2 binds to the intracellular C-terminus of EGFR, GRB2-associated-binding protein 1 (GAB1), which leads to phosphorylation of the P85 subunit of PI3K and subsequent Akt translocation to the plasma membrane and its phosphorylation. Activated Akt phosphorylate Tuberous Sclerosis Complex 2 (TSC2) and inactivation of mTORC1 and subsequent protein synthesis suppression. In cancer cells, Akt is involved in increasing glucose uptake by upregulating glucose transporters which leads to aerobic glycolysis and the Warburg effect (Wee & Wang, 2017).

Phosphatase and tensin homolog (PTEN) is a tumor suppressor which regulates the PI3K/AKT/mTOR pathway by inhibiting the phosphorylating signal from PI3K to AKT (Hennessy et al., 2005). Double deletion of PTEN leads to increased protein synthesis, proliferation, cell cycle progression, and apoptosis inhibition (Chen et al., 2018; Zadra et al., 2010). In mice models, homozygous deletion of PTEN resulted in PrCa development and PrCa progression (Wang et al., 2003). So, it is logical that PrCa patients usually have PTEN loss, which is associated with earlier biochemical relapse, metastasis, resistance to castration, ERG gene fusions, and the accumulation of nuclear TP53 (Netto, 2015). Studies have shown PTEN loss rate is higher in progressed PrCa patients compared to non-progressed PrCa patients (Jamaspishvili et al., 2018).

1.2.10 Association with telomeres

An association has been reported between telomere length and prostate cancer, as short telomere length has an association with decreased overall survival and increased biochemical recurrence. Some studies demonstrated that cancer that arises from these areas could result in higher genotype and phenotype heterogeneity and are more aggressive (Fordyce et al., 2005). Also, it has been reported that patients with short telomeres have up to 14 times more risk of death than patients with long telomeres (Heaphy et al., 2013).

1.2.11 Apoptosis

High levels of apoptosis have been reported in both PIN and PrCa, although it is lower than other malignancies. Although, both increased and decreased apoptosis rates have been reported in patients with castration-resistant prostate cancer (Koivisto et al., 1997).

There are intrinsic and extrinsic pathways for apoptosis. The intrinsic pathway controls many forms of stress, which can be originated from unrepaired DNA or the lack of signals from the cell surface (like hormones or diminished growth factors).

Major components of the intrinsic pathway are Mitochondria and the Bcl-2 family. There are 12 pro-apoptotic proteins in the Bcl-2 family, including Bax, Bak, Bok, Bik, Bas, Bid and Bim, as well as six pro-survival proteins, including Bcl-2, Bcl-XL, Bcl-W and Mcl1 (Adams & Cory, 1998).

The primary function of each protein in the family is to increase the mitochondrial membrane permeability (Kroemer & Reed, 2000) and subsequent release of cytochrome c into the cytoplasm, which binds to Apaf-1 proteins and makes the apoptosome complex leading to Caspase-9 activation and subsequent activation of the entire cascade. External signals from surface receptors called ‘death receptors,’ like tumor necrosis factor receptor 1 (TNFR1), CD95 and Fas receptor, result in apoptosis extrinsic pathway activation.

1.3 AMP-activated Kinase (AMPK) as an Energy Stress Sensor

AMPK senses the cell's energy status and maintains energy homeostasis by controlling several cellular processes. AMPK consists of three different subunits (Steinberg & Carling, 2019).

The alpha subunit is the catalytic subunit, while the betta and gamma subunits are regulatory. When the cell has low energy (the AMP to ATP ratio is high), AMPK is activated. The primary upstream kinase is liver kinase B1 (LKB1) (Shackelford & Shaw, 2009), which is needed for ultimate AMPK activation. AMPK activity can then be enhanced up to 100-fold, which results in changes in carbohydrates, fatty acids, cholesterol and amino acid metabolism, mitochondrial biogenesis and cell growth (Steinberg & Carling, 2019).

1.3.1 AMPK Regulation of Carbohydrate Metabolism

AMPK controls carbohydrate metabolism through several pathways, from glucose uptake to storage. It stimulates glucose uptake by translocating glucose transporter type 4 (GLUT4) and Glucose transporter type 1 (GLUT1) from an intracellular location to the plasma membrane. This step is achieved by several pathways (Kurth-Kraczek et al., 1999).

AMPK is suggested to activate and increase cell surface expression of GLUT1, in cells that express mainly GLUT1 (Fryer et al., 2002). Moreover, AMPK directly phosphorylates thioredoxin-interacting protein (TXNIP), which results in its degradation. TXNIP suppresses glucose uptake directly through two main mechanisms. The first mechanism involves the direct binding of TXNIP to the glucose transporter GLUT1, which induces GLUT1 internalization. The second mechanism consists of a reduction of GLUT1 mRNA expression. Therefore, TXNIP degradation increases GLUT1 plasma membrane localization and mRNA expression (Steinberg & Carling, 2019; Wu et al., 2013). The first critical step in glycolysis is the conversion of glucose to glucose 6 phosphate (G6P) by hexokinase in the cytoplasm because it causes glucose trapping within cells (TeSlaa & Teitell, 2014).

AMPK not only stimulates glycolysis to enhance the ATP concentration in the cell but also suppresses glycogen synthesis by the inhibitory phosphorylation of glycogen synthase to make glucose more available for glycolysis (Jeon, 2016). Although, this inhibition was not consistent. Also, AMPK stimulates glycogenolysis by glycogen phosphorylase (GP) activation (Jeon, 2016).

1.3.2 AMPK Regulation of Fatty Acids and Cholesterol Synthesis

Low cellular energy (high AMP to ATP ratio) results in AMPK-dependent suppression of fatty acid and cholesterol synthesis, leading to low lipid storage and promoting fatty acid

oxidation to help the cell maintain its intracellular ATP levels. Acetyl-CoA is involved in the Synthesis of cholesterol and fatty acids (Steinberg & Carling, 2019). In the cholesterol synthesis pathway, AMPK inhibits 3- hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the rate-limiting enzyme in the mevalonate pathway, which results in lower serum and liver cholesterol (Loh et al., 2019). Furthermore, AMPK phosphorylates and inhibits ACC enzyme (both ACC1 and ACC2 isoforms), leading to decreased acetyl-CoA conversion to malonyl-CoA (the first step in fatty acid synthesis) (Fullerton et al., 2013).

Furthermore, AMPK can decrease fatty acid and cholesterol synthesis by inhibiting transcription factors called sterol-response element binding proteins (SREBPs). SREBP 1a and SREBP 1c are fatty acid transcription factors, while SREBP 2 is a cholesterol transcription factor (Eberlé et al., 2004). AMPK phosphorylates SREBP1c and SREBP2, which leads to their inhibition (Steinberg & Carling, 2019).

1.4 Diagnosis

A combination of diagnostic tools is used for prostate cancer diagnosis, usually beginning with a serum prostate-specific antigen (PSA) test and a digital rectal exam (DRE) feeling for abnormalities in the prostate. Additional tests will be applied to confirm the diagnosis in suspicious cases, such as transrectal ultrasound, magnetic resonance imaging, and prostate biopsies. The most conclusive diagnostic test is the prostate biopsy, with pathologists directly evaluating prostate tissue for PrCa, but it is also the most invasive. It is usually done after other diagnostic tests indicate a likelihood of PrCa (Litwin & Tan, 2017).

However, limitations in prostate biopsies have been documented, with the diagnostic test missing 21-28% of PrCa and miss-scoring 14-17% of PrCa (Bjurlin et al., 2013).

Diagnostic biomarkers, such as 4Kscore and ConfirmMDx, are used to narrow the margin of error even more.

1.4.1 Tissue methods of detection

<u>Needle Core Biopsy:</u>

The use of transrectal thin needles in 1980 and serum PSA test resulted in early detection of prostate cancer. In recent years the false-negative rate decreased from 25% to 11%, and there was a quality improvement in the obtained tissue sample (little or no

compression artifact at the lateral edges of the specimens). Also, the 18-gauge (18G) needle lets multiple biopsies (average about 10-12 cores) of the prostate with very little discomfort, particularly with topical anesthetics such as lidocaine.

Fine Needle Aspiration:

Interest in FNA in the United States is low because the needle core biopsy application is available. The sensitivity in the diagnosis of prostatic adenocarcinoma is the same in both technics, and both are limited by small sample size; It is best to consider CNBx and FNA as complementary techniques.

Transurethral Resection (TURP):

The regions of the prostate sampled by TURP and transrectal needle biopsy tend to be different. TURP specimens usually consist of tissue from the transition zone, urethra, periurethral area, bladder neck, and anterior fibromuscular stroma. Occasionally, TURP specimens may also contain small portions of seminal vesicle tissue. Radical prostatectomies performed after TURP show that the resection does not usually include tissue from the central or peripheral zones, and not all of the transition zone is removed. Most needle biopsies consist only of tissue from the peripheral zone, rarely including central or transition zones.

Radical Prostatectomy:

There are two major surgical approaches to prostatectomy. The most popular approach in the United States is retropubic prostatectomy, allowing lymph node biopsy and staging

with frozen section. The second surgical approach, perineal prostatectomy, does not enable lymph node biopsy or staging during surgery. Other techniques include nerve-sparing prostatectomy, robotic prostatectomy, and laparoscopic prostatectomy, which have high popularity.

1.4.2 Microscopic pathology

Microscopically, most prostatic adenocarcinomas are acinar type, composed of small glands arranged in one or more patterns. Diagnosis is based on both architectural and cellular atypia and maybe IHC studies can help for confirmation of the diagnosis in suspicious cases.

Architectural atypia can be appreciated at low- to medium-power magnification revealing glands with variation in size, shape, and spacing. Suspicious foci consist of usually small or medium-sized glands with irregular or elongated contours that stand in contrast with the smooth contours of normal prostatic glands. Variable glandular size has value, particularly when there are small, irregular, abortive glands with primitive lumens. Comparison with adjacent benign glands has value always. The stroma frequently contains young collagen that appears lightly eosinophilic. The desmoplastic reaction is an uncommon feature in prostate cancer which is an unreliable finding when assessed in isolation.

Cytologic atypia includes nuclear and nucleolar enlargement, which are important for the diagnosis of malignancy. Enlarged nuclei are typically present in most malignant cells, and enlarged nucleoli are present in many. “Prominent” nucleoli define as at least 1.25 to 1.50 μm in diameter, although the ratio of nucleolus to nucleus and comparison with adjacent benign glands have the greatest importance.

The basal cell layer is absent in adenocarcinoma, an important finding that may be difficult to appreciate in routine H&E-stained slides. Compressed stromal fibroblasts may mimic basal cells, but they are usually focal. A basal cell layer is present in benign glands, while carcinoma entirely lacks a basal cell layer. In suspicious cases, evaluation of the basal cell layer presence or absence by IHC staining for high-molecular-weight Cytokeratin (e.g., clone 34βE12) and p63 is beneficial.

There are various morphologic types of prostatic carcinoma which probably most of them are variants of acinar adenocarcinoma, including Prostate adenocarcinoma with neuroendocrine differentiation (Paneth cell–like neuroendocrine differentiation, well-differentiated neuroendocrine tumor or carcinoid tumor, Small cell carcinoma, large cell neuroendocrine carcinoma), Mucinous (mucin-secreting) adenocarcinoma, Signet ring carcinoma, Adenosquamous carcinoma, Squamous cell carcinoma, Adenoid basal cell tumor, Basal cell carcinoma and adenoid cystic carcinoma, Lymphoepithelioma-like carcinoma and Sarcomatoid carcinoma.

PIN:

Prostatic intraepithelial neoplasia (formerly described as ductal–acinar dysplasia) is a range of Intraepithelial proliferation along pre-existing ducts and acini with atypia. Low grade PIN is at the lower end of this spectrum, which is recommended not to be reported in pathology reports (since variability in diagnosis exists even among experts (Epstein, 2009). It has also been shown that low-grade PIN is a relatively common finding in young male patients (Sakr et al., 1993). However, it is often associated with high grade PIN and maybe with elevated PSA level.

There is high-grade PIN (Formerly known as severe dysplasia, PIN 2 / PIN 3 and carcinoma in situ) at the higher end of this spectrum, diagnosed and reported in modern practice since it is a Putative precursor of prostatic adenocarcinoma.

Histopathologic features of high-grade PIN in low magnification include glands with a more basophilic appearance than surrounding ones due to the high nuclear to cytoplasmic ratio, nuclear crowding, and more amphophilic cytoplasm. Defining feature is large nuclei with prominent nucleoli. A statistical association between high-grade PIN and prostatic carcinoma has been reported in several studies; for example, high grade PIN has been found in 59%–100% of radical prostatectomy specimens. Also, in prostate tissues revealing both PIN and adenocarcinoma, both lesions have high consistency in the DNA ploidy pattern (Baretton et al., 1994; Crissman et al., 1993; Weinberg & Weidner, 1993).

1.4.3 Grading

One of the strongest prognostic factors in prostatic adenocarcinoma is histologic grade. Various grading systems have been reported since the pioneering work of Brodersmore in

1920. Since 1999, Gleason grading has been the international standard for prostate cancer grading and is the most routinely used by pathologists worldwide (Bostwick et al., 2000).

Gleason grading is based on the glandular architectural differentiation and the growth pattern of the tumor as evaluated on low to medium power magnification. Gleason score is the sum of the 2 most prevalent scores: primary (graded from 1 to 5) and secondary (graded similarly), with different rules for biopsy and prostatectomy. If the tumor has the same pattern (for example, only a "primary" pattern), the number is multiplied by 2 to have the final score (Allsbrook et al., 1999). In a needle biopsy, the Gleason score is composed of the Most prevalent pattern as primary and any amount of the worst pattern as secondary scores. In radical prostatectomy, if a minor pattern constitutes < 5%, the pattern should be mentioned as a minor (tertiary) pattern; any higher grade minor pattern ≥ 5% should be incorporated into the Gleason score as the secondary pattern (Sauter et al., 2018). But in biopsies, the minor (<5%) high-grade pattern is incorporated into the Gleason score. Gleason grades 1 and 2 are not clinically meaningful in modern pathology practice and are no longer recommended for use since cancer with those scores has an outcome like score 3, so the grading system effectively begins at Gleason score 3 + 3 = 6. Moreover, pure score 3 cancer almost never metastasizes and is reasonable to treat by active surveillance, which has raised the question about whether it should even be labelled cancer (Epstein et al., 2016; Iczkowski & La Rosa, 2014).

Under the 2014 criteria, Gleason pattern 3 is defined as well-formed glands with central lumina. (Epstein et al., 2016). Cribriform glands are no longer allowed in the Gleason pattern 3. Gleason pattern 4 is a heterogeneous pattern revealing cribriform,

glomerulations, “fused glands,” and poorly formed glands. Pattern 5 consists of sheets, central comedonecrosis and single cell infiltration. Other variants, such as pseudohyperplastic, atrophic, or “PIN-like” carcinoma, are graded as usual but are typically Gleason score 3 + 3 = 6.

As active surveillance has become more popular, the importance of distinguishing Gleason score 3 + 3 = 6 from 3 + 4 = 7 carcinomas has become more prominent. In the past, this was the threshold for determining aggressive cancer, which is now under question as more men with 3 + 4 = 7 carcinomas are managed with active surveillance at some centers. The threshold for identifying the presence of focal score 4 cancer differs between pathologists with low reproducibility for this decision (McKenney et al., 2011).

So, the International Society of Urological Pathology (ISUP) has proposed a conservative approach: difficult borderline cases should be graded “down” to prevent the potential for overtreatment (Epstein et al., 2016).

The Grade Group system has recently been improved to report the Gleason score categories better (Epstein et al., 2016). The Grade Groups range from 1 to 5; for example, low-risk cancers (Gleason score 3 + 3 = 6) are considered Grade Group 1. Moreover, this grading system avoids collapsing Gleason score 3 + 4 = 7 and 4 + 3 = 7 with each other, which are identified as Grade Groups 2 and 3, respectively. Histologic grading has correlation with PSAP and PSA levels (Humphrey et al., 1993; Pretlow et al., 1985), clinical and pathologic staging (Cantrell et al., 1981; Partin et al., 1993), the incidence of lymph node and bone metastases (Fan & Peng, 1983; Zincke et al., 1982), survival rate,

and response to therapy (Utz & Farrow, 1969). 2014 modified Gleason grading and grade group comparison with corresponding histologic features are summarized in Table 1.2.

Table 1.2 - 2014 modified Gleason grading and Grade Group comparison

Grade Group	Gleason Score	Histologic Features
1	≤3 + 3 = 6	Only individual discrete well-formed glands
2	3 + 4 = 7	Predominantly well-formed glands with lesser component of poorly formed glands, fused glands, glomerations, or cribriform glands
3	4 + 3 = 7	Predominantly poorly formed glands, fused glands, glomerations, or cribriform glands with lesser component of well-formed glands (if >5%) (a)
4	4 + 4 = 8 3 + 5 = 8 5 + 3 = 8	Only poorly formed glands, fused glands, glomerations, or cribriform glands(a) Predominantly well-formed glands with lesser component of sheets, cribriform glands with comedonecrosis, or single cells Predominantly sheets, cribriform glands with comedonecrosis, or single cells with lesser component of well-formed glands (if >5%) (a)
5	≥4 + 5 = 9	Only sheets, cribriform glands with comedonecrosis, or single cells (a)

(a) A lower-grade component, when less than 5% of the carcinoma, is not incorporated into the grade.

1.4.4 TNM staging, AJCC 8th edition

The international gold standard for staging cancers is the TNM staging system. There are two versions of this system: the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) (Edge & Compton, 2010; JAMES D.Brierley 2017). The 8th edition (TNM8), the latest revision of both classifications, was published in 2016. Clinical staging is essential in evaluating prostate cancer spread because the pathological stage would be available only after radical prostatectomy surgery (Varma et al., 2019). T as tumor, N as node and M as metastasis are contributing in TNM staging. See Table 1.3 for TNM staging.

Table 1.3 - Prostate cancer TNM staging, AJCC 8th edition

TNM	Description
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor which is not palpable
T1a	Tumor is incidental histologic finding (≤ 5% of tissue resected)
T1b	Tumor incidental histologic finding (> 5% of tissue resected)
T1c	Tumor identified by needle biopsy, found in one or both sides, but not palpable
T2	Tumor palpable and confined within the prostate
T2a	Tumor ≤ 50% of one lobe
T2b	Tumor > 50% of one lobe (not both lobes)
T2c	Tumor present in both lobes
T3	Extraprostatic tumor that is not fixed and does not invade adjacent structures
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle
T4	Tumor is fixed or invades adjacent structures other than the seminal vesicles, such as external sphincter, rectum, bladder, levator muscle and/or pelvic wall
Nx	Regional lymph nodes cannot be assessed
N0	No positive regional lymph nodes
N1	Metastasis in regional lymph node(s) (Regional lymph nodes = periprostatic, pelvic, hypogastric, obturator, internal iliac, external iliac, sacral)
M0	No distant metastasis
M1	Existence of distant metastasis
M1a	Non-regional lymph node(s) (Example: aortic, common iliac, deep / superficial inguinal, retroperitoneal)
M1b	Bone(s)
M1c	Other sites with or without bone disease

1.5 Risk stratification of prostate cancer patients

Risk stratification systems have multiple aims, including helping with decision-making about treatment, designing clinical trial stratification options or facilitating communication between physicians or organizations. Risk stratification systems are routinely used to define patients suitable for active surveillance program (Rodrigues et al., 2012).

Multiple factors have been routinely used to evaluate the patient's outcome related to treatment which are usually clinical (overall survival, disease-free survival, metastasis-free survival), surgical (rates of extracapsular disease, seminal vesicle involvement, positive margins and lymph node positivity) or biochemical (PSA biochemical-free failure). Pre-treatment PSA, clinical stage and Gleason score have been demonstrated to have independent predictive value of various combinations of prostate cancer treatment in the non-metastatic patients (Sutcliffe et al., 2009).

Since pre-treatment PSA, Gleason score and clinical stage have confirmed prognostic value, multiple prostate cancer risk stratification systems have been established based on these criteria. In 1998, D'Amico and colleagues first suggested a three-group risk stratification system to predict post-treatment biochemical failure after radical prostatectomy and external-beam radiotherapy (D'Amico et al., 1998). In this system, non-metastatic patients are divided into 3 groups: low-, intermediate-, and high-risk based on initial PSA, clinical stage and Gleason score. Table 1.4 shows D'Amico risk classification.

Table 1.4 - D'Amico risk classification

Risk Category	**PSA level**	**Gleason Score**	**Clinical T stage**
Low (must have all criteria)	≤10 ng/m	≤6	T1-T2a
Intermediate (must have all criteria if not low risk)	≤20 ng/ml	7	T1/T2
High (one is sufficient)	≥20 ng/ml	8-10	T3a-T4

In 2001, the Genitourinary Radiation Oncologists of Canada (GUROC) published the results of a meeting about risk stratification (Lukka et al., 2001). An agreement for prostate cancer risk stratification was made at this meeting based on the available articles on clinical risk factors related to the biochemical failure. However, there were differences in the exact definitions of risk categorization.

Multiple risk stratification systems have been proposed by various cancer and urological organizations, including the National Comprehensive Cancer Network (NCCN, USA) (Mohler et al., 2010), which is summarized in Table 1.5.

Table 1.5 - Initial risk stratification for clinically localized PrCa, NCCN guideline (version 3.2022)

<table>
<tr><th>Risk Group</th><th colspan="3">Clinical/Pathologic Features</th></tr>
<tr><td>Very low</td><td colspan="3">It has all the following:
CT1c
Grade group 1
PSA<10 ng/ml
Fewer than 3 prostate biopsy fragments/cores positive;< or equal 50%cancer in each fragment/core
PSA density<0.15 ng/ml/g</td></tr>
<tr><td>Low</td><td colspan="3">It has all the following but does not qualify for very low risk:
cT1-cT2a
Grade group 1
PSA<10 ng/ml</td></tr>
<tr><td rowspan="2">Intermediate</td><td rowspan="2">It has all the following:
No high-risk group features
No very high-risk group feature
Has 1 or more intermediate risk factors (IRFs):
cT2b-cT2c
Grade group 2 or 3
PSA 10-20 ng/ml</td><td>Favorable (Low) intermediate</td><td>It has all of the following:
1 IRF
Grade group 1 or 2
<50% biopsy cores are positive (e.g., <6 of 12 cores)</td></tr>
<tr><td>Unfavorable intermediate</td><td>Has 1 or more of the following:
2 or 3 IRFs
Grade group 3
Equal or more than 50% biopsy cores are positive</td></tr>
<tr><td>High</td><td colspan="3">It has no very high-risk feature and has exactly one high risk feature:
cT3a Or
Grade group 4 or 5 OR
PSA>20 ng/ml</td></tr>
<tr><td>Very high</td><td colspan="3">Has at least one of the following:
cT3b-cT4
Primary Gleason pattern 5
2 or 3 high risk features
>4 cores with grade group 4 or 5</td></tr>
</table>

1.6 Diagnostic vs prognostic vs predictive biomarkers

There are various types of biomarkers, including diagnostic, prognostic, and predictive markers. Diagnostic biomarkers are used to confirm disease diagnosis. Prognostic biomarkers

informs about a probable disease outcome (like recurrence, progression or death) irrespective of treatment, while predictive biomarkers help optimize treatments (Shaw et al., 2015). In Table 1.6, there are multiple established diagnostic and prognostic markers but less predictive markers. Therefore, new predictive markers need to be stablished for earlier detection of risk for PrCa progression than existing predictive biomarkers.

Table 1.6 - Existing biomarkers for PrCa. PrCa biomarkers include diagnostic, prognostic, and predictive markers, with predictive markers having the least number of established biomarkers.

Biomarker	Biomarker Use	Type	Description
Prostate-specific antigen (PSA)	Before initial biopsy	Circulating, protein, diagnostic	Measure level of PSA in serum. Used for consideration of initial biopsy.
ExoDX Prostate IntelliScore	Before initial biopsy	Urine, RNA expression, diagnostic/prognostic	Exosomal RNA expression of SPDEF, ERG, and PCA3
Apifiny	Initial biopsy	Circulating, protein, diagnostic	8 PrCa-specific autoantibodies. Used for consideration of initial biopsy.
SelectMDx	Before/Initial biopsy	Urine, mRNA expression, diagnostic/prognostic	mRNA expression of DLX1 and HOXC6. Used to predict the chance of PrCa on biopsy and severity of the disease. Used for consideration of initial biopsy and repeat biopsies for patients with previous negative biopsies.
Prostate health index (PHI)	Before/Initial biopsy	Circulating, protein, prostate/prognostic	Levels of fPSA, tPSA, and p2PSA isoform. Used to predict the risk of aggressive PrCa.

Progensa (PCA3)	Before/Repeat biopsy	Urine, long non-coding RNA, diagnostic	PCA3 gene detects long non-coding RNA. Used for consideration of repeat biopsies in patients with previous negative biopsies.
ConfirmMDx	Initial/Repeat biopsy	Tissue, DNA methylation, diagnostic	Detect DNA hypermethylation in tumour suppressor genes. Predict the correctness of negative biopsies to reduce the number of repeat biopsies.
4KScore	Before/Initial/ Repeat biopsy	Circulating, protein, diagnostic/prognostic	Levels of tPSA, fPSA, intact PSA, and human kallikrein-related peptidase 2. Used to predict the risk of aggressive PrCa in patients considering initial and repeat biopsies in patients with previous negative biopsies.
Michigan Prostate Score (MiPS)	Before/Initial/ Repeat biopsy	Circulating/urine, protein/mRNA expression, diagnostic/prognostic	PSA level (circulating), PCA3 mRNA (urine), and TMPRSS2: ERG mRNA (urine). Used for consideration of initial or repeat biopsy in patients with previous negative biopsies.
AMACR	Initial/Repeat	Tissue, protein, diagnostic	Used in combination with high-molecular-weight cytokeratin (34βE12 or CK5/6) or p63 (or a combination of the 2). Used to support PrCa diagnosis.
NKX3.1	Initial/Repeat	Tissue, protein, diagnostic	Loss of expression indicates PrCa. Used to support PrCa diagnosis.
PSMA	Initial/Repeat biopsy, after RP	Tissue, protein, diagnostic/prognostic	High expression of PSMA correlated with a high risk of recurrence. Used to support PrCa diagnosis.
PTEN/TMPRSS2: ERG (Metamark)	After biopsy	Tissue, protein, prognostic	Molecular assay for presence/absence of PTEN and TMPRSS2: ERG. Used to predict PrCa aggressiveness binarily (presence/absence is the indicator).
Oncotype DX	Initial/After biopsy	Tissue, mRNA expression, predictive	mRNA expression of reference genes and tumorigenesis genes. Used to predict PrCa severity and determine the best course of treatment (AS vs treatment).
ProMark	Initial/After biopsy	Tissue, protein, prognostic	Measure 8 proteins related to tumorigenesis through quantitative fluorescence. Used to predict the risk of high-grade PrCa and metastasis.
Prolaris	Initial/After biopsy, after RP	Tissue, mRNA expression, predictive	Measure expression of cell cycle progression genes and reference genes. Used to determine the best treatment method (AS vs RP/RT).
Decipher	Initial/After biopsy, after RP	Tissue, mRNA, prognostic	Measure expression of genes involved in tumorigenesis. Used to predict the chance of metastasis, PrCa-specific mortality, and high-grade PrCa.

1.7 Management of prostate cancer

Management of localized PrCa includes 1) active treatments, such as radical prostatectomy, external radiation therapy, brachytherapy, focal therapies (e.g., cryotherapy or high-intensity focused ultrasound [HIFU]), androgen deprivation and anti-androgen therapy, as well as 2) active surveillance or watchful waiting (Whitmore, 1994).

Hormonal treatments including estrogens, luteinizing hormone–releasing hormone (LH-RH) analogues, and antiandrogens have mostly replaced orchiectomy as a palliative option in the locally advanced and metastatic tumor, especially to help patients with severe pain particularly associated with skeletal disease (Daneshgari & Crawford, 1993; Kinsella et al., 2018; Labrie, 1991; Mcleod; Samson et al., 2002).

Systemic chemotherapy generally has had a nonsignificant role in hormone-refractory metastatic PrCa, although newer modalities are now available, including chemotherapeutic agents (e.g., docetaxel and cabazitaxel) and next-generation hormonal therapies (e.g., abiraterone and enzalutamide) (Ryan et al., 2013; Scher et al., 2012; Scher et al., 2016; Wozniak et al., 1993).

Many treatment options for PrCa are used in conjunction to maximize the treatment effect and minimize patient discomfort. The treatment options available for PrCa patients are summarized in Table 1.7.

Table 1.7 - Treatment options available for PrCa patients depending on grade group. Lower grade PrCa patients have less intensive treatment options, while higher grade PrCa patients require more urgent treatment options. AS = active surveillance, WW = watchful waiting, RP = radical prostatectomy, RT = radiation therapy, ADT = androgen deprivation therapy.

Grade Group	Gleason Score	Risk Group	Definition	Likely Outcome	Treatment Options
1	≤ 6 (3+3)	Low	Stage T1-T2a, PSA level < 10 ng/mL	Very slow growing and very slow to spread	AS, WW, RP, RT
2	7 (3+4)	Low-intermediate	Stage T2a-T2b, PSA 10-20 ng/mL	Slow growing and slow to spread	
3	7 (4+3)	Intermediate	Stage T2b-T2c, PSA 10-20 ng/mL	Slow growing and slow to spread but faster than grade group 2	WW, RP, RT, ADT
4	8 (4+4, 3+5, 5+3)	High risk	Stage T3a, PSA > 20 ng/mL	Quick growing and quick to spread	
5	9, 10 (4+5, 5+4, 5+5)	Very high risk	Stage T3b-T4, primary Gleason pattern of 5	Quick growing and quick to spread but faster than group 4	

1.7.1 Surgery

1.7.1.1 Radical prostatectomy

Radical prostatectomy is an appropriate option for fit younger patients who have low surgical risks and early localized PrCa (Sooriakumaran et al., 2014).

Radical prostatectomy is a treatment option for localized PrCa in healthy patients in all PrCa groups with a life expectancy of another 10 years but younger than 75 years old. The purpose is to remove all the tissue involved by PrCa to stop PrCa proliferation and spreading. More treatment can be applied after prostatectomy with specific pathologic findings. Radical prostatectomy may also involve pelvic lymph node dissection (Heidenreich et al., 2014).

Since most low-risk patients are now managed with active surveillance and surgery does not play an established role in high-risk patients, prostatectomy is mainly applied in intermediate risk patients. Radical prostatectomy has a few types. In Retropubic radical prostatectomy, the prostate gland and lymph nodes are removed, while in perineal radical prostatectomy, lymph nodes are not removed, which is a shorter and less painful procedure (Lepor, 2005). In addition, radical prostatectomy with the assistance of a robotic system can be approached, which is less invasive with lower complications (Finkelstein et al., 2010).

1.7.1.2 Surgery side effects

Radical prostatectomy has several side effects, including but not limited to damage to surrounding organs, anesthesia complications, bleeding, urinary incontinence, and erectile dysfunction. Despite surgical approaches to spare the nerves responsible for erections called the nerve-sparing approach, erectile dysfunction occurs because prostate cancers are more extensive than expected, and nerve sparing cannot be achieved without compromising cancer control (Lepor, 2005).

1.7.2 Radiation therapy

Radiation therapy inhibits cancer growth through induction of DNA damage, which is significantly higher in cancer cells compared to normal cells and results in inhibition of tumor cell growth, cell cycle arrest and induction of cancer cell death.

Radiotherapy (RT) is a treatment option for localized prostate cancer in all PrCa groups. It is suitable for PrCa patients older than 70 because RT doesn't lead to age-related surgical complications. RT aims to kill PrCa cells with ionizing radiation by focusing the rays on the PrCa to prevent off-target effects. Although RT is less invasive than other treatment options, it has both acute and late side effects, such as the development of surrounding organ inflammation (acute radiation proctitis and cystitis) and long-term fibrosis, respectively. After a radical prostatectomy, adjuvant RT is often used to reduce PrCa recurrence (Heidenreich et al., 2014).

1.7.2.1 External beam radiation therapy (EBRT)

Dose-escalated radiotherapy is the standard treatment for prostate cancer patients. Current NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) for Prostate Cancer recommend delivery of 75.6 to 81.0 Gy total cumulative dose using conventional 1.8- to 2.0-Gy daily fractions or treatments (Mohler et al., 2010).

NCCN recommendation is developed after multiple randomized trials revealing improved cancer treatment with dose-escalation compared with the standard dose of radiotherapy (≤70 Gy) (Pollack et al., 2002; Zietman et al., 2010; Zietman et al., 2005).

By developing treatment techniques that tailor high-dose radiation to the target and minimize dose to adjacent organs, and the development of IGRT (image-guided radiation therapy), Safe delivery of dose-escalated radiotherapy has been made possible, which increases treatment localization accuracy (Pugh et al., 2013).

A linear accelerator is used to deliver radiotherapy in the form of high energy photons. Modern accelerators regulate EBRT in terms of photon energy, shape and timing of photon beams to have maximum tumor targeting and final tumor control, which can be achieved when cancer cells receive optimal radiation dose with minimal or negligible radiation dose to surrounding healthy tissues. Three dimensional models and other techniques are used to achieve this goal (Zaorsky, Harrison, et al., 2013).

1.7.2.2 Three-dimensional conformal radiation therapy (3D-CRT)

In 3DCRT, CT imaging designs radiation fields that aim at the patient from specific angles to converge on the prostate (Pugh et al., 2013). Different beams are entered from different angles to the prostate, reducing scattering and the damage to surrounding healthy tissues (Dal Pra & Souhami, 2016).

Intensity-modulated radiation therapy (IMRT) is the most common technique currently used to deliver EBRT (Pugh et al., 2013) and is an advanced 3DCRT technique widely adopted for prostate cancer treatment in the 2000s (Jacobs et al., 2012).

In IMRT, the same CT-based imaging is used to design the target and the adjacent organs, Although the delivery technique is different to shape the radiation field. During radiation, mobile metal blocks (called multi leaf collimators [MLCs]) move in and out of the radiation beam to regulate the intensity of the dose administered to an area through the radiation portal, which leads to a heterogeneous dose distribution (Pugh et al., 2013).

Volumetric-modulated arc therapy (VMAT) is an advanced IMRT in which modulated beams of RT are delivered continuously around the patient (target) in Arcs. VMAT is significantly replacing IMRT because it leads to higher conformality and speed of treatment delivery (Zaorsky, Harrison, et al., 2013).

Stereotactic Body Radiation Therapy (SBRT): Patient immobilization, imaging techniques and high precision accelerators are used in SBRT to deliver higher doses of radiation to a specific site which mainly improves dose drop off into surrounding normal

tissues. Because SBRT is a highly precise technique, doses per fraction are significantly increased safely (up to 20 Gy per fraction). This leads to a shorter time of treatment delivery and, frequently, a lower overall radiotherapy dose (Zaorsky, Harrison, et al., 2013).

Proton Beam Radiation Therapy:

Proton beam therapy (PBT) is progressively used for prostate cancer treatment. PBT uses accelerated protons to deliver radiation doses. Protons have distinct physical properties different from the high-energy x-rays (photons) produced in linear accelerators for IMRT. The advantage of PBT is the reduced unwanted radiation dose, which may reduce acute and chronic radiation toxicity (Pugh et al., 2013).

Although Proton therapy may lead to higher conformality of radiotherapy, the PBT equipment is significantly more costly, making this treatment challenging to implement (Patel et al., 2014).

1.7.2.3 Conventional fractionation – Dose escalated radiotherapy (RT)

PrCa standard RT is delivered in many fractions. Conventional fractionation composed of fractions of 1.8-2 Gy of RT applied 39 to 40 times for a total of 78-80 Gy for 8 weeks (5 daily fractions per week) (Zaorsky, Ohri, et al., 2013). This treatment was recommended

due to the resistance of PrCa to RT and was developed following clinical trials revealing that dose escalated RT is needed for improved PrCa local control (Morgan et al., 2019).

1.7.2.4 Hypofractionation

PrCa cells have a high degree of radiation resistance, a slow cell cycle and relatively higher sensitivity to larger doses of RT per fraction. Based on these radiobiological models, delivery of a lower overall dose of RT delivered in larger daily fractions over a shorter period of time can lead to similar tumor control as prolonged courses of radiotherapy of a higher overall dose (Morgan et al., 2019). Recent studies demonstrated that PrCa could be safely and effectively radiated in larger fraction sizes (up to 3.1 Gy per fraction) and fewer fractions overall, called hypofractionation (Mangoni et al., 2014).

1.7.2.5 Brachytherapy

Brachytherapy is the most conformal radiation delivery method with the minimum amount of surrounding healthy tissue exposure. In this method, a radioactive source is inserted into the prostate gland to deliver radiation directly to the prostate (Pugh et al., 2013).

The radioactive source has lower energy that does not travel long distances into tissues, so it has the advantage of not delivering a high dose of RT to surrounding healthy tissues (Skowronek, 2017).

This method is more convenient than EBRT for PrCa patients (because it can be done in a single outpatient visit). The disadvantages include the dependence on practitioner skill level, acute prostate edema following multiple needles placed into the prostate for inserting the radiation sources, the need for spinal or general anesthesia, and the concern about possible undertreating cancer spread beyond the planned treatment area (D'Amico et al., 2009).

Prostate brachytherapy can be achieved in 2 forms: permanent low-dose-rate (LDR) radiation seeds or a temporary high-dose-rate (HDR) radiation source.

1.7.2.6 Radiation therapy side effects

Intestinal or urinary bladder toxicity, fatigue and erectile dysfunction are some of the significant side effects of PrCa RT. Rectal radiation toxicity (radiation proctitis) might result in rectal leakage and diarrhea, which usually gets better after radiation but can persist. Radiation-induced inflammation of the bladder (radiation cystitis) might lead to hematuria and dysuria. Chronic side effects of radiation include but are not limited to urinary frequency and erectile dysfunction. However, precise RT techniques reduce side effects (Michaelson et al., 2008).

1.7.3 Androgen deprivation therapy (ADT)

Hormone therapy (androgen deprivation therapy (ADT) is indicated in intermediate-risk (IR), high risk (HR), and very high risk (VHR) PrCa patients. It involves regulating or blocking hormones participating in PrCa growth. ADT examples include luteinizing hormone-releasing hormone (LHRH) agonist, LHRH antagonist, anti-androgen, or an orchiectomy (Heidenreich et al., 2014). The disadvantages of ADT include the risk of epithelial-mesenchymal transition in cancer cells, leading to cancer spread; neuroendocrine differentiation leading to a resistant and aggressive form of PrCa; and developing into castration-resistant PrCa, a self-sufficient and highly proliferative state of PrCa. ADT is still an effective and valuable treatment option for locally advanced PrCa, and recurrent PrCa, in conjunction with RT and neoadjuvant therapy (Nouri et al., 2017).

1.7.3.1 Castration-resistant prostate cancer (CRPC)

Eventually, all PrCa patients will develop castration-resistant prostate cancer (CRPC), usually within a few months to 2–3 years following initiation of ADT. The mechanisms of progression to CRPC state have been unclear; however, androgens and the androgen receptors (AR) have been reported as essential drivers of CRPC in recent years. Various resistance mechanisms have been described, including reactivation of AR (via AR amplification, mutations, or variants), activation of AR via aberrant pathways, and intratumoral or alternative androgen production. Enzalutamide and abiraterone acetate (agents approved for treating CRPC) target a part of these resistance mechanisms. Although, resistance occurs over time against these agents (Tilki et al., 2016).

Apalutamide and Darolutamide have been recently approved by Food and Drug Administration (FDA) for CRPC patients (Rice et al., 2019), while several other AR inhibitors are currently in development for the treatment of CRPC like ODM-201, a new-generation AR inhibitor (Fizazi et al., 2015).

1.7.3.2 Classification of ADT and side effects

In 1941, Huggins et al. reported the favourable effect of androgen deprivation therapy (ADT) via orchidectomy or oestrogens in metastatic prostate cancer patients (Heidenreich et al., 2008; Huggins & Hodges, 1972; Huggins C, 1941). After that, the gold standard for ADT in advanced PrCa was orchidectomy (Heidenreich et al., 2008)**.** Oestrogens (most commonly diethylstilboestrol) was used as a medical alternative to surgical castration until the late 1960s and 1970s (Crawford, 2004). The use of oestrogen has been limited because of an increased risk of adverse cardiovascular events, as confirmed by the Veterans Administration Cooperative Urological Research Group studies (Anderson, 2003; Byar & Corle, 1988).

Anti-androgenic agents were proposed in the late 1960s and early 1970s. They compete with androgens for binding sites on androgen receptors in the prostate cell nucleus, promoting apoptosis and inhibiting the PrCa cell growth (Crawford, 2004). The most extensively studied non-steroidal anti-androgen is Bicalutamide. European Association of Urology (EAU) guidelines consider non-steroidal anti-androgen monotherapy (e.g., bicalutamide) as an alternative to castration in patients with locally advanced diseases

(Heidenreich et al., 2008). Common complications include gynecomastia, breast pain, loss of libido, erectile dysfunction, cardiovascular toxicity and hepatotoxicity (Anderson, 2003).

Another alternative to surgical castration is LHRH agonists, which were suggested in the 1980s and are a mainstay of ADT for advanced PrCa (Heidenreich et al., 2008). Although, LHRH agonist agents have multiple disadvantages. The first stimulation of LHRH receptors results in testosterone surge, delaying the achievement of castrate testosterone levels for around 2–4 weeks which may have an association with clinical flare effects in advanced disease (e.g., bone pain, spinal cord compression, ureteral obstruction and possibly death) (Thompson, 2001; Waxman et al., 1985).

The flare phenomenon related to LHRH agonists raised the need for establishing alternative agents, like GnRH antagonists. These compounds bind directly to and block GnRH receptors without triggering the initial testosterone surge and flare related to agonists (van Poppel & Nilsson, 2008).

1.7.4 Chemotherapy

Chemotherapy treatment for prostate cancer has been developed dramatically. Previous studies reported that several chemotherapy regimens are mainly palliative and hardly lead to long-lasting or significant responses. However, recent studies suggested docetaxel is the first chemotherapy agent that has an important role in better overall survival in

metastatic castration-resistant prostate cancer (mCRPC). Although, combination chemotherapy or any other chemotherapy agents added to docetaxel don't have an additive effect. Since the Docetaxel agent in the metastatic hormone-sensitive patients improved overall survival, secondary endpoints of prostate-specific antigen (PSA), and time to recurrence, the standard treatment of newly diagnosed de novo metastatic PrCa has been changed. Promising results in locally advanced PrCa and high-risk PrCa have been reported in both the neoadjuvant and adjuvant settings (Nader et al., 2018).

Docetaxel is a taxane derivative which works by binding to microtubules and preventing androgen receptor nuclear translocation and causing apoptosis through B-cell lymphoma (Bcl-2) phosphorylation (Pienta, 2001).

Other chemotherapy agents used less frequently in PrCa are Paclitaxel, Mitoxantrone, Estramustine, Doxorubicin, Epirubicin and vinorelbine (Nader et al., 2018).

1.7.4.1 Chemotherapy adverse effects

The mechanism of antineoplastic activity can explain chemotherapy toxicity. Bone marrow suppression and hair loss are examples of chemotherapy agents affecting normal tissues. Allergic reactions, nephrotoxicity, and hepatotoxicity are complications that are not explicitly related to the drug's mechanism of action (Beer & Bubalo, 2004).

Commonly used chemotherapy agent complications include but are not limited to alopecia, diarrhea, myalgia, nausea and vomiting, neutropenia, headache, infertility,

myelosuppression, anorexia, mucositis, urine discoloration, breast tenderness or enlargement, edema, dyspnea, elevated AST, decreased libido, anemia, cough, hepatic dysfunction, hypotension, leukopenia, constipation, metallic taste and stomatitis (Beer & Bubalo, 2004).

1.7.5 Active surveillance

Active Surveillance (AS) includes close monitoring of tumor progression through serial digital rectal examinations, serum PSA tests, three to four times a year and annual re-biopsies. If there is evidence of tumor progression, active treatment will start (Kinsella et al., 2018).

PrCa is slow-growing and localized to the prostate in many cases. Patients presenting with slow-growing and localized PrCa are managed with an active surveillance program. Patients managed with AS program include low-risk (LR) (Gleason scores ≤ 6 and PSA < 10 ng/mL), and low-intermediate risk (LIR) patients (Gleason score of 3 + 4 = 7 and PSA of 10 – 20 ng/mL) (Klotz et al., 2015)

The main aim of active surveillance (AS) is to prevent overtreatment by selecting low-risk and low intermediate-risk prostate cancer patients and actively monitoring them until definitive treatment is needed (Komisarenko et al., 2018).

Typical PrCa progression while on AS would lead to reclassification of LR into LIR or LIR into HIR disease. However, studies showed that almost 25–30% of LR/LIR PrCa patients will eventually be reclassified to high-grade PrCa on repeat biopsy (Hu et al., 2014; Ouzzane et al., 2015), described as “wolf in sheep’s clothing.” This implies that these patients would probably have benefited from earlier definitive treatment and have been followed with AS program initially but later undergo delayed treatment and eventually miss the opportunity for cure. There is a chance of grade progression from

Gleason 3 to 4 or 5, although it is not very common. Unfortunately, it occurs. In most cases, grade progression occurs in high volume Gleason 6 cancers (Inoue et al., 2014; Komisarenko, Wong, et al., 2016).

The application of AS differs extensively between institutions regarding inclusion criteria and follow-up protocol. The most remarkable differences include the maximum accepted Gleason score, T-stage and prostate-specific antigen (PSA) levels.

The results of 8 significant AS studies by different institutes are available, which include: the University of California, San Francisco (UCSF), University of Toronto (Sunnybrook cohort) (UoTSB), Toronto (Princess Margaret cohort) (UoTPM), Memorial Sloan Kettering Cancer Centre (MSKCC), Prostate Cancer Research International Active Surveillance (PRIAS), University of Miami (UoM), John Hopkins (JH), and Royal Marsden (RM). Approximately 10,000 PrCa patients are involved in these eight institutional cohorts of AS. The most significant limitation of these cohorts is the length of follow-up.

In AS study by Welty and Carroll et al. at the University of California, San Francisco (UCSF), the inclusion criteria were PSA 10 ng/mL or less, clinical stage T1/2, biopsy Gleason grade 3+3 or less, 33% or less positive cores and 50% or less tumor in any single core. At a median follow-up of 60 months, no deaths were reported due to PrCa. Metastatic disease developed in 1 patient (0.12%). Five-year overall survival was 98%, treatment-free survival was 60%, and biochemical recurrence -free survival (bRFS) was

40%. The median time to treatment and reclassification was 25 months and 17 months, respectively (Welty et al., 2015).

In the AS study at the University of Toronto (Sunnybrook), the inclusion criteria were Gleason score <6 and PSA<10 ng/mL and to patients older than age 70 years with PSA <15 ng/mL or Gleason score <3+4, between 1995 and 1999. Since 2000, the inclusion criteria were restricted to low-risk patients (Gleason score <6 and PSA<10 ng/mL) or patients with favorable intermediate-risk disease (PSA 10 to 20 ng/mL and/or Gleason score 3+4) with significant comorbidities and a life expectancy of less than 10 years. The median follow-up time was 6.4 years. 15.0% of patients died, and only 1.5% of deaths were from PrCa. The 10- and 15-year cancer-specific survival rates were 98.1% and 94.3%, respectively. 1.3% of patients developed metastasis. The treatment-free survival rate was 75.7%, 63.5%, and 55.0% At 5, 10, and 15 years, respectively. The relative risk of death by non-prostate causes was 9.2 times that of PrCa (Choo et al., 2002; Klotz, 2005; Klotz et al., 2015; Klotz et al., 2010).

Many studies reported that LIR patients, compared to LR patients, have a much greater risk for PrCa progression within the next 5-15 years. As mentioned before, Klotz et al., in AS study at the University of Toronto (Sunnybrook) (UoTSB), reported that AS patients, including both LR and LIR patients, had a disease progression rate of 25–45% at 5-15 years (Klotz et al., 2015).

In AS study by Finelli et al. at the University of Toronto (Princess Margaret Cancer Centre-PMCC) (UoTPM), the eligibility criteria were PSA ≤10, clinical stage ≤ T2a,

Gleason score ≤6, number of positive core numbers ≤3, no single core >50% involved and age ≤75 years. The median follow-up time was 56 months. Overall, 2.4% died, while PrCa was only the cause of death in 0.2% of patients. Metastasis happened in 0.6% of patients. Five-year overall survival, cancer-specific, and metastasis-free survival were 96.8%, 100% and 99.7%, respectively. The reclassification rates were 28% and 40% at 5 and 10 years, and cumulative treatment rates were 21% and 26% at 5 and 10 years. Only 2 patients (0.2%) died of PrCa, and 7 patients developed metastasis (Finelli et al., 2011; Komisarenko, Timilshina, et al., 2016; Satkunasivam et al., 2013; Wong, Fleshner, et al., 2013; Wong et al., 2014; Wong, Trottier, et al., 2013).

In AS study by Eastham et al. at the Memorial Sloan Kettering Cancer Centre (MSKCC), the inclusion criteria were clinical stage ≤ T2a, PSA <10 ng/mL, and low-risk features on initial prostate biopsy: Three or fewer cores involved, no single core with ≥50% maximum involvement of cancer, and no Gleason grade >3 present in the specimen. The 2- and 5-year progression-free probability was 80% and 60%, respectively (with PSA included in progression criteria). When PSA was excluded from progression criteria, the 2 and 5-year progression-free probability was 91% and 76%, respectively (Berglund et al., 2008).

The Prostate Cancer Research International Active Surveillance (PRIAS) is an international AS study which includes more than 100 centres in 17 countries all over the world. Inclusion criteria are clinical stage T1/T2, PSA ≤10 ng/mL, PSA density <0.2 ng/mL per milliliter, one or two positive biopsy cores, and Gleason score ≤6.

2,494 patients were involved and followed for a median of 1.6 years. During follow-up, 28% of patients had grade reclassification, and 21.1% received treatment. Active therapy-free survival for 2 years was 77.3%. The disease-specific survival rate was 100%.

In total, 75.6% of patients continued AS, 21.1% had treatment, 1.7% didn't participate in follow-up, 0.8% switched to watchful waiting because of comorbidity, and 0.7% died of causes other than PrCa (Bul et al., 2013).

The Inclusion criteria for AS study by Soloway et al. at the University of Miami (UoM) were a Gleason score of ≤6, a serum PSA level of ≤15 ng/mL, clinical stage ≤T2, low-volume disease and >12 months of follow-up. The mean follow-up was 45.3 months. The treatment-free probability rate was 85% at 5 years, and no patient died from PrCa (Soloway et al., 2008).

In AS study of the John Hopkins (JH) University by Carter et al., eligibility criteria were Gleason score ≤6, PSA <10 ng/ml, clinical stage T1c, ≤50% of any cores and ≤3 cores involved, PSA density (PSAD) <0.15 ng/mL/g. The median follow-up time was 5 years. Overall, cancer-specific and metastasis-free survival rates were 93%, 99.9%, and 99.4%, respectively, at 10 years and 69%, 99.9%, and 99.4%, respectively, at 15 years. The cumulative incidence of grade reclassification was 26% and 31% at 10 and 15 years, respectively; the cumulative incidence of curative intervention was 50% and 57% at 10 and 15 years, respectively. The median treatment-free survival was 8.5 years (Scott et al., 2017).

In the Royal Marsden (RM) AS study by Parker et al., the inclusion criteria were age 50–80 years, fit for radical treatment, stage T1/T2 disease, PSA <15 ng/mL, Gleason score ≤3+3, and percent positive biopsy cores ≤50%.

73% of patients were still on AS at 22 months, and 5% switched to watchful waiting. In 2013, they reported satisfactory medium-term outcomes for AS in selected localized PrCa patients. Approximately 70% didn't receive treatment within 5 years of diagnosis, and the adverse histology and treatment-free probability rate was 22% at 5 years. The results of deferred treatment were comparable with immediate treatment (Selvadurai et al., 2013).

Recently, our group contacted a retrospective review of AS patients that were diagnosed through the Niagara Health System (NHS), Prostate Diagnostic Assessment Program (PDAP), Ontario, Canada, which offers a centralized diagnostic program for all Niagara patients. Patients diagnosed by the NHS-PDAP service that are placed on AS are being followed at regional urology office practices and return to NHS-PDAP for repeat biopsies at 12 and 24 months. Unlike previous studies, in this cohort, we observed a high (40%) overall rate of disease progression/reclassification and treatment utilization as well as a significant (>14%) rate of progression to unfavourable intermediate or high-risk disease requiring combined modality treatments (Mesci et al., Prostate Cancer Patients on Active Surveillance in Niagara Health System, Ontario, Canada: Real World Data on Patterns of Progression and Treatment).

It is confirmed that death among AS patients is most commonly due to diseases other than PrCa like cardiovascular disease. In the recent studies with a median follow-up of 9

years, the risk of non-PrCa death was 10 times higher than PrCa mortality (Komisarenko et al., 2018).

As previously mentioned, the length of follow-up is the most significant limitation of prospective AS studies. In the most recent studies, the median follow-up ranges from less than 1 year to about 8.5 years (Komisarenko et al., 2018).

The approach of Toronto studies was more liberal since they included all low-risk and selected low intermediate risk (Gleason 7 or PSA >10) patients (Klotz et al., 2015) detecting a mortality rate of 5% for 15 years. Most metastatic cases were LIR patients (Gleason 7 at diagnosis). The rate of metastasis for LIR patients was 3.75 times higher than LR patients at 15 years. The LIR patients (Gleason 7 patients) in particular were at risk; these patients had a 20% or greater metastasis rate at 15 years (Klotz et al., 2015).

In contrast to the Toronto group, the John Hopkins group (JH) had a restrictive approach, offering surveillance only to patients with restricted inclusion criteria. They reported a mortality rate of 0.5% at 15 years (Komisarenko et al., 2018).

The results of the described AS cohorts by different institutes are summarized in Table 1.8.

Table1.8 - Summary of results of some large AS cohort studies by different institutes

Institution	Eligibility criteria	Median follow up (months)	Overall survival	PrCa mortality	Treatment free survival	Biochemical recurrence
University of Toronto (Sunnybrook)	GS ≤6, PSA ≤10 ng/mL, cT1c, <3 cores positive, <50% of any cores involved (Or if >70 years, GS ≤3+4, PSA <15 ng/mL)	102	68% at 120 months	5% at 180 months	70% at 60 months	53% at 60 months
University of Toronto (Princess Margaret)	GS ≤6, PSA <10 ng/mL, cT1–T2a, ≤3 cores positive, <50% of any cores involved	56	3.2% at 56 months	<0.02% at 56 months	27% at 56 months	NA
John Hopkins	GS ≤6, PSA <10 ng/mL, cT1c, ≤50% of any cores and ≤3 cores involved, PSAD <0.15 ng/mL/g	60	69% at 180 months	0.1% at 180 months	59% at 60 months	9.4% recurrence at 24 months
Royal Marsden	GS ≤7 (3+4), if ≥65 years old), PSA levels <15 ng/mL, cT1c–T2a, ≤50% of any cores involved	68	9% at 96 months	2% at 96 months	70% at 60 months	15% at 60 months
Prostate Cancer Research International Active Surveillance	GS ≤6, PSA ≤10 ng/mL, cT1c–T2a, PSAD <0.2 ng/mL/g, ≤2 cores involved	18	87% at 48 months	NA	77 at 24 months	NA
University of California, San Francisco	GS ≤6, PSA <10 ng/mL, cT1c–T2a, <33% of total cores	43	NA	NA	67% at 60 months	<1% at 36 months
Memorial Sloan Kettering Cancer Centre	GS ≤6, PSA <10 ng/mL, cT1–T2a, ≤3 cores positive	22	NA	NA	NA	NA
University of Miami	GS ≤6, PSA ≤10 ng/mL, cT1c–T2a, ≤20% of any cores involved, ≤2 cores involved	31	NA	NA	85.7% at 60 months	NA

Based on various AS cohorts, it can be concluded that there are two types of patients in AS program, some patients who will probably have PrCa progression and patients who can live a good quality of life despite having PrCa. Therefore, there is a need to develop biomarkers for predicting PrCa progression to discriminate between these 2 groups within the AS patient population.

Watchful waiting (WW) is another option for PrCa patients who do not want the side effects of interventions or treatment cannot be applied due to other medical conditions. WW is like active surveillance but is less stressful, including fewer tests and watching for changes in symptoms (Chapple et al., 2002; Chodak, 1994).

1.8 Role of cancer metabolism

In recent years, extensive research revealed that metabolism is an essential hallmark of cancer progression. Early-stage PrCa cells do not extensively depend on glucose metabolism like many other carcinomas until they progress. Instead, PrCa cells show only increased lipid metabolism at the early stages, which provides the building blocks for growth, secondary signals to drive carcinogenesis, and energy for PrCa cell survival (Cutruzzolà et al., 2017; Liang & Mulholland, 2014). Several studies reported that metabolic conditions, like obesity, diabetes, and high-fat diets, are associated with PrCa development, progression, and recurrence (Bairati et al., 1998; Chen et al., 2018; Scaglia et al., 2021). PrCa has enhanced fatty acid uptake, so metabolic conditions and a high-fat diet will probably lead to PrCa progression (Zadra et al., 2013).

1.8.1 Carbohydrate metabolism: glycolysis

Glucose is the major source of energy in most healthy cells. It is catabolized through a chain of reactions to produce ATP, which lets the cell have its biological function, growth and proliferation (Dashty, 2013).

Two types of transporters exist that have a role in glucose transport to the cell. The first type is sodium–glucose co-transporters (SGLTs), and the second type is facilitated diffusion glucose transporters (GLUTs). Fourteen types of GLUTs are present in human cells with different affinities and located in various organs. GLUTs transport glucose

through the plasma membrane by diffusion. Therefore, they do not need energy to function. Some types of GLUTs are regulated by hormones. For example, GLUT4 is stimulated by insulin, which results in increased glucose transportation 10-20 times more (Navale & Paranjape, 2016).

When glucose enters the cell, it is metabolized in a chain of reactions that leads to ATP generation, which is the primary energy source for various biological functions. The first step is glycolysis which the six-carbon glucose molecule is broken down into two three-carbon pyruvates. This reaction occurs in the cytoplasm and does not need oxygen. The product of Anaerobic glycolysis is 2 ATP and two lactic acid molecules. In comparison, the outcome of aerobic glycolysis is 2 ATP, two pyruvate molecules and two NADH2 molecules, which can then be used to generate more ATP. Then pyruvate undergoes decarboxylation in the mitochondria through an oxidative carboxylation reaction catalyzed by PDC, which results in one molecule of acetyl-CoA and one NADH2 production. This is a transition step between glycolysis which take place in the cytosol, and the Krebs cycle, which occurs in the mitochondria. The two pyruvate molecules are metabolized in the Krebs cycle to release their energy. The Krebs cycle yields 3 NADH2, 4 CO2, 1 FADH2, 2 CoA and 2 GTP per one pyruvate molecule. The coenzymes NADH2 and FADH2 are then used in the oxidative reaction (Dashty, 2013). They use the energy provided by the hydrogen ions to synthesize ATP from ATP and Pi (Bonora et al., 2012). In normal prostate epithelial cells, aerobic glycolysis is the main energy source because citrate should be accumulated for secretion into prostatic fluid, preventing oxidative phosphorylation as an energy source. To accumulate citrate, m-aconitase, a mitochondrial enzyme which converts

citrate to isocitrate in the Krebs cycle is inhibited by zinc (Eidelman et al., 2017). Zinc is in high concentrations in normal prostate epithelial cells because zinc should also be secreted into the seminal fluid (Costello & Franklin, 1998). In PrCa cells, silencing of zinc transporters occurs, which results in oxidative phosphorylation reactivation (Eidelman et al., 2017; Franz et al., 2013). During the conversion of normal epithelial cells to PrCa, Zinc transporters are silenced because zinc plays a role in apoptotic control by interfering with cytochrome c in the electron transport chain, resulting in a caspase cascade (Franz et al., 2013). The reactivation of oxidative phosphorylation provides the high energy required by the uncontrolled proliferation of PrCa. During PrCa progression, glucose reliance is identified, known as the Warburg effect, which means aerobic glycolysis returns as a source of energy (Eidelman et al., 2017). This observation requires enhanced glucose transporters and enzymes involved in glycolysis. The significant amount of resources dedicated to proliferation and growth forces progressed PrCa cells to look for alternative energy sources, leading to aerobic glycolysis and catabolic β-oxidation.

The first rate-limiting step for glycolysis is achieved by GLUTs which transport glucose across the plasma membrane. Metabolic and hormonal signals can regulate GLUT transporters, and various transcription factors can enhance glucose uptake by overexpressing or translocating GLUTs on the cell membrane. Moreover, in hypoxic or nutrient deprivation conditions, overexpression of at least one of the isoforms of GLUT transporters (predominantly GLUT1) occurs in tumoral cells (Hu et al., 2013), which makes cancer cells more resistant to apoptosis (Zhao et al., 2008).

Because GLUT1 overexpression has been identified in various tumors, it has been the most studied glucose transporter in tumors, including PrCa. Although higher GLUT1 levels were also identified in non-tumor tissues (Reinicke et al., 2012), it seems that GLUT1 expression has an association with tumor aggressiveness and poor prognosis since GLUT1 is usually overexpressed in poorly differentiated tumours (Stewart et al., 2008; Yu et al., 2017). Studies have shown that overexpression of GLUT1 has an association with unfavourable overall survival in multiple cancer types, considering its role in tumour progression (Wang et al., 2017; Yu et al., 2017).

Although GLUTs overexpression is reported in various cancers (Blayney et al., 2018; Koh et al., 2017; Sun et al., 2016), they have not been extensively studied as relevant biomarkers in PrCa because glucose metabolism in PrCa is not like other carcinomas (Cutruzzolà et al., 2017; Gonzalez-Menendez et al., 2018). However, GLUT1 overexpression has been reported in advanced PrCa (Stewart et al., 2008; Vaz et al., 2012). Meziou et al demonstrated that GLUT1 immunohistochemical expression has an association with tumour aggressiveness. They showed that GLUT1 expression was related to poor prognostic clinicopathological features. Moreover, earlier biochemical recurrence and castration resistance and metastasis were reported in patients with GLUT1 overexpression, revealing that high-risk PrCa patients with enhanced glucose metabolism have more aggressive tumor (Meziou et al., 2020).

Other studies demonstrated an association between GLUT1 expression and recurrence after radical prostatectomy (Qu et al., 2016). GLUT1, GLUT12 and HK2 are direct targets of androgen receptors in PrCa cells which is essential for PrCa cell growth (Wang et al., 2014;

Xiao et al., 2018), while the highest levels of GLUT1 expression are found in androgen independent PrCa cells (Vaz et al., 2012).

1.8.2 Lipid metabolism: DNL

De novo lipogenesis is a minor pathway in most human cells, excluding hepatic, breast and adipose tissues (Weiss et al., 1986). Dietary fatty acids usually control lipogenesis. Circulating dietary fatty acids suppresses de novo lipogenesis in normal cells. But cancer cells are not very sensitive to this inhibition and actively produce fatty acids despite having an inhibitory level of circulating fatty acids.

De novo lipogenesis (DNL) products are long and complex fatty acid chains used to build the cell membrane, receptors and secondary signals (Scaglia et al., 2021; Zadra et al., 2013). The first substrate of DNL is Citrate which is transported out of the mitochondria by citrate transport proteins from the Krebs cycle. ATP-citrate lyase (ACLY) converts citrate to acetyl-CoA, which is subsequently converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) (Wu et al., 2014). In a rate-limiting reaction catalyzed by fatty acid synthase (FASN), malonyl-CoA and acetyl-CoA are converted to palmitate. Palmitate is used by numerous lipogenesis pathways, contributing to biosynthesis, radical oxygen species buffering, β-oxidation, and cancer signalling (Flavin et al., 2011; Sena & Denmeade, 2021; Wu et al., 2014; Zadra et al., 2014).

Enhanced expression of many lipogenic enzymes in prostate cancer cells has been reported in many studies, usually as a result of stimulation by oncogenic signaling pathways such as PI3K/AKT and HER2 (Suburu & Chen, 2012) as well as activation and nuclear localization of Akt/PKB in clinical tumor samples (Van de Sande et al., 2005). Androgens have been reported to increase lipogenic enzyme activity (Swinnen et al., 1997).

A survey of the gene expression database (named Oncomine, which analyzed the published data from multiple separate studies) (Rhodes et al., 2004) reported that mRNAs encoding lipogenic enzymes [ATP citrate lyase (ACLY), acetyl-CoA carboxylase α (ACC), fatty acid synthase (FASN)], are increased in prostate cancer, as well as the transcription factor, sterol regulatory binding protein 1 (SREBP1), that regulates expression of fatty acid and cholesterol synthesizing enzymes. This survey (Oncomine) reported that ACLY mRNA expression significantly increased in 50% and decreased in 9% of studies; ACC mRNA increased in 67% and decreased in 5% of studies; FASN mRNA increased in 54% and decreased in 14% of studies; Expression of SREBP1 mRNA is increased in 36% and decreased in 9% studies. The importance of cholesterol metabolism has also recently been reconfirmed. Increased cholesteryl ester accumulation in cellular lipid droplets was related to prostate cancer aggression (Yue et al., 2014).

1.8.3 Catabolic beta oxidation

Although a significant part of lipid metabolism dysregulation in cancer is lipogenesis and increased expression of lipogenic enzymes, fatty acid oxidation also supports the malignant phenotype (Carracedo et al., 2013).

α-Methylacyl-CoA racemase (AMACR) is a peroxisomal and mitochondrial enzyme which plays a role in the β-oxidation of branched fatty acid. AMACR is needed to catalyze the interconversion of the R- and S-isomers, both in the peroxisome and the mitochondria, which is necessary before oxidation.

Increased fatty acid oxidation in prostate cancer has been confirmed extensively. Multiple studies revealed the consistent and specific overexpression of AMACR in prostate cancer versus normal prostate epithelial cells (Jiang et al., 2001; Kumar-Sinha et al., 2004; Luo et al., 2002; Rubin et al., 2002). Also, Sequence variants of AMACR have been reported to be linked to prostate cancer risk. AMACR enzymatic activity is consistently increased in prostate cancer tissue specimens (Kumar-Sinha et al., 2004). All these data reveal an activated β-oxidative pathway in prostate cancer, which provides ATP as an energy source.

A survey of mRNA expression data at Oncomine (Rhodes et al., 2004) revealed that AMACR is increased in 80% of studies and decreased in none. AMACR is a well-known diagnostic biomarker that also seems to have functional significance. Moreover, other studies demonstrated that downregulation of AMACR expression via siRNA treatment in prostate cancer cells results in inhibition of proliferation (Zha et al., 2003), indicating that AMACR might be a potential target for prostate cancer therapy (Carnell et al., 2013).

During PrCa progression, the catabolic β-oxidation process is used more to generate more energy (Evans, 2003). Long-chain lipids generated from DNL are oxidized and produce more ATP than other energy generation processes but also use more oxygen, which may be in short supply in progressed PrCa.

1.8.4 The role of PSMA in prostate cancer

Prostate specific membrane antigen (PSMA) was first reported in 1987 and subsequently described as a trans membranous glycoprotein with hydrolytic activity (Horoszewicz et al., 1987; Pinto et al., 1996). PSMA is expressed in normal, benign and malignant prostate epithelial cells, including intraepithelial neoplasia and metastatic tissues (Troyer et al., 1995; Wright et al., 1995). Particularly, PSMA expression is significantly higher in primary PrCa compared to benign glands and considerably higher in lymph nodes and distant metastases compared to primary tumors (Queisser et al., 2015). Its sensitivity and specificity are 84% and 95% for PrCa, respectively (Bravaccini et al., 2018). In rare cases, PSMA can be expressed in normal tissue of the adrenal gland, bladder, breast, esophagus, fallopian tube, kidney, large intestine, liver (canalicular membranes), ovary, small intestine, spinal cord, stomach and testis and less frequently in tumor cells and neovasculature of various epithelial and mesenchymal tumor types (Mhawech-Fauceglia et al., 2007) and neovasculature of physiologic regenerative and reparative conditions (Gordon et al., 2008). Despite its expression by multiple types of malignancies, PSMA is

still considered to be relatively sensitive and highly specific for PrCa (Mhawech-Fauceglia et al., 2007).

There are several clinical implications for PSMA in managing PrCa (Lütje et al., 2017). After biochemical recurrence following therapy, a 68Ga-PSMA PET/CT can be applied to find local and distant cancer sites. PSMA plays a role as the target for the radiotracer (Han et al., 2018; Rauscher et al., 2018). Also, during salvage surgery for recurrent PrCa, it has been suggested that intravenous 99mTc-PSMA can intraoperatively improve the detection rate of metastatic lesions using a gamma probe known as radio-guided surgery (Maurer et al., 2019). PSMA ligands can also target PSMA for a radioligand therapy, such as Lutetium-177. This therapy is recommended as the last treatment option for metastatic castration resistant PrCa (Fendler et al., 2017; Heck et al., 2017).

Despite the remarkable and exciting role of PSMA in imaging and therapy, its biological function in prostate cancer is not very well known. Although previous studies demonstrated that PSMA presents an advantage to prostate cancer cells through the metabolism and conversion of polyglutamated folates to folic acid and its additional effect on proinflammatory cytokines expression, the definite biological function of PSMA in prostate cancer is not very clear (Kaittanis et al., 2018).

In radical prostatectomy specimens, a correlation of PSMA overexpression with an unfavourable biochemical recurrence free survival rate has already been reported (Bravaccini et al., 2018; Minner et al., 2011; Perner, Hofer, et al., 2007), as well as a significant correlation between PSMA expression and Gleason grades in prostate biopsies

and radical prostatectomy specimens (Hupe et al., 2018) indicating a potential prognostic value for PSMA in prostate biopsy as well. Interestingly, studies evaluating the potential predictive value of PSMA for PrCa outcome have been done using radical prostatectomy specimens at the time of treatment; in these cases, the PSMA level does not affect treatment decision-making (Hupe et al., 2018).

PSMA has folate hydrolase activity, leading to the release of glutamate from the enzyme substrates (Palamiuc & Emerling, 2018). In a study by Kaittanis et al, they evaluated the folate hydrolase activity, biological function and potential therapeutic value of PSMA in prostate cancer. They reported a strong positive correlation between PSMA expression, disease aggressiveness, and phosphorylation of the AKT target in localized prostate cancer patients. Therefore, they proposed that PSMA has a significant role in regulating signalling pathways involved in prostate cancer pathogenesis, particularly the PI3K–AKT–mTOR pathway.

This study revealed the role of PSMA in the activation of AKT and subsequently enhanced phosphorylation of downstream targets, 4EBP1 and S6, in the absence of any known intrinsic signals. Significantly, PSMA enzymatic activity leads to glutamate release and subsequent AKT activation. Glutamate alone is reported to activate AKT signalling. In addition, they proposed that PSMA activates PI3K signalling through phosphorylation of p110β, independent of PTEN status. They also evaluated the molecular mechanisms involved in PI3K regulation following PSMA activation, revealing that PSMA colocalizes with and activates the metabotropic glutamate receptor group 1 (mGluR1). In the absence

of mGluR1, neither PSMA activation nor glutamate supplementation was sufficient to induce p110β phosphorylation and activate AKT.

Furthermore, they demonstrate that inhibiting PSMA, mGluR1, or p110β equally suppresses AKT signaling. Their data establish a direct relationship between the AR pathway and PSMA enzymatic activity. The work presented by Kaittanis et al enhances our understanding of the mechanism behind the activation of the PI3K pathway in prostate cancer, by uncovering PSMA as a modulator of this major driver of tumor growth transition to CRPC (Castration Resistant Prostate Cancer) (Kaittanis et al., 2018).

1.9 Metabolic enzymes as predictive biomarkers of risk of PrCa progression

Metabolic changes are early signs of cancer progression, including prostate cancer: glycolysis, DNL, and β-oxidation play important roles in prostate cancer growth and progression (Scaglia et al., 2021; Xiao et al., 2018). Lipid metabolism is involved in PrCa development and progression (Zadra et al., 2013). Therefore, all three pathways must be investigated for potential biomarkers for predicting PrCa progression in PrCa patients. It is reasonable to examine enzymes of the metabolic pathways discussed, include ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), glucose transporter 1 (GLUT1), and α-methylacyl-CoA racemase (AMACR). Further, the work discussed above indicated that PSMA should also be examined in human prostate cancer tissue as a potential biomarker of PrCa progression. Such studies could enable in the future metabolic enzyme-based risk stratification of PrCa patients with localized disease at the initial diagnosis.

1.10 Hypothesis

"Enzymes involved in prostate cancer metabolism may have value as biomarkers predicting risk for prostate cancer progression in patients managed with AS."

This hypothesis is based on the notion that metabolic alterations participate in early molecular events that support prostate cancer progression. In that case, enzymes involved in prostate cancer metabolism may have value as biomarkers to predict the risk of prostate cancer progression because it can be expected that differences exist in the expression of metabolic enzymes between patients where the prostate neoplasia is progressing to advanced and those that the disease does not progress.

1.11 Aims

To test the above hypothesis, the research work included in this thesis involved a pilot study with the following aims:

1) Analyze whether enzymes of cellular metabolism are differentially expressed in the human prostate cancer tumors compared to benign prostate epithelial cells

2) Examine whether there is differential expression of metabolic enzymes amongst benign or malignant prostate tissues in patients that progressed while managed with AS vs those that did not progress.

Chapter 2 - Methodology

This is a pilot study which aims to lay the foundation for future prospective analysis of metabolic enzymes as predictive biomarkers. If such studies are positive, biomarker tests can be developed for AS patients at risk for PrCa progression, to determine whether treatment intervention is necessary early, or it can be deferred without risk for the patient long-term outcomes. Positive results may also lead to usage in ongoing and planned drug intervention trials for the required analyses.

Goal: Our goal is to develop an inexpensive tissue biomarker that can be used in most clinical facilities, analyzed with standard pathological techniques, and has a high predictive value for the risk of prostate cancer progression in patients on AS programs.

We investigated ACLY, ACC, GLUT1, AMACR and PSMA for their potential predictive values for PrCa progression because these enzymes have critical roles in PrCa metabolism.

2.1 Patient population

Accrual and specimen collection:

With ethics approval from the Hamilton integrated Research Ethics Board (HiREB), we pursued a prospective accrual of patients through the Niagara Health System (NHS) prostate diagnostic assessment program (PDAP), which was managed with active

surveillance (AS) to contact a pilot biomarker study. Patients were approached for accrual at the time of return to NHS-PDAP for re-biopsy 12 or 24 months after entry into the AS program. Over 24 months (2018 – 2020), 40 consecutive AS patients were accrued to this study who donated their biopsy tissue and were allowed access to their clinical information. Twelve of these patients had no biochemical (PSA), clinical (DRE), or pathological signs of PrCa disease progression. They were selected for the control (non-progressed) group, and another 28 PrCa patients progressed biochemically or pathologically and went to active treatment and were defined as progressed group.

After informed consent based on NHS REB guidelines, baseline and repeat (12 or 24 months after initial diagnosis) prostate core needle biopsy paraffin blocks and their clinical information, including pathology reports, were collected from pathology NHS hospital, pathology departments and clinical information systems. All patients had a baseline diagnosis of Gleason Score 3+3=6 (grade group 1) or Score 3+4=7 (grade group 2) adenocarcinoma, acinar type and serum PSA level < 20 ng/ml. Progression was defined as a higher Gleason Score or serum PSA level > 20 ng/ml or any clinical indication of progression 12 or 24 months after initial diagnosis. In the first phase of analysis, **only baseline biopsy tissues were examined**.

The tissues were examined with H&E staining to categorize patient samples suitable for immunohistochemistry (IHC) staining and analysis by H-score. Baseline blocks were used to assess the potential biomarkers' predictive value by comparing the results to the patient's outcome: whether the prostate cancer progressed or not.

2.2 First step: Sectioning and H&E staining

Formalin-fixed and paraffin-embedded blocks of Prostate core biopsies were serially sectioned into 5 µm thickness, and then they were left to dry overnight at room temperature. The first section of prostate core needle biopsies was H&E stained to evaluate whether the tissue is satisfactory for following step analysis by IHC staining or not. 34 out of 40 patients had enough tissue satisfactory for pathologic evaluation and scoring (26 patients in progressed group and 8 patients in the non-progressed group).

For H&E staining, slides were deparaffinized, rehydrated, and stained with hematoxylin (Modified Mayer's solution) and eosin Y solution. H&E staining kit (catalogue #ab245880) was purchased from Abcam (Cambridge, UK). After staining, the slides were mounted and left overnight to dry.

Each patient had at least 1 malignant paraffin block and at most 6 malignant paraffin blocks on their baseline biopsies. The average number of malignant blocks was 2.9 blocks per each patient. If a patient had multiple malignant blocks/cores, the 3 blocks with higher tumor percentage/ involvement were selected for IHC staining and scoring.

2.3 Second step: IHC staining

Before IHC staining on prostate core needle biopsy, the technic and all the reagents were first validated on radical prostatectomy tissue samples to ensure the technic and all the reagents were working properly. Also, dilution of primary antibodies was optimized on radical prostatectomy tissue samples first. Negative and positive controls (internal and/or external) were included in every round of IHC staining for quality assurance. Few representative images for IHC validation are presented in Figure 2.1, 2.2, 2.3, 2.4 and 2.5.

Figure 2.1 - Example of AMACR IHC staining validation on (A) radical prostatectomy tissue and (B) prostate core biopsy. A) Note the AMACR expression in malignant glands, while the entrapped benign glands between infiltrative malignant glands are negative for AMACR. B) The same pattern of AMACR expression in prostate core biopsy reveals AMACR expression in tumoral focus, while non-malignant epithelial cells are negative for AMACR. (100x magnification)

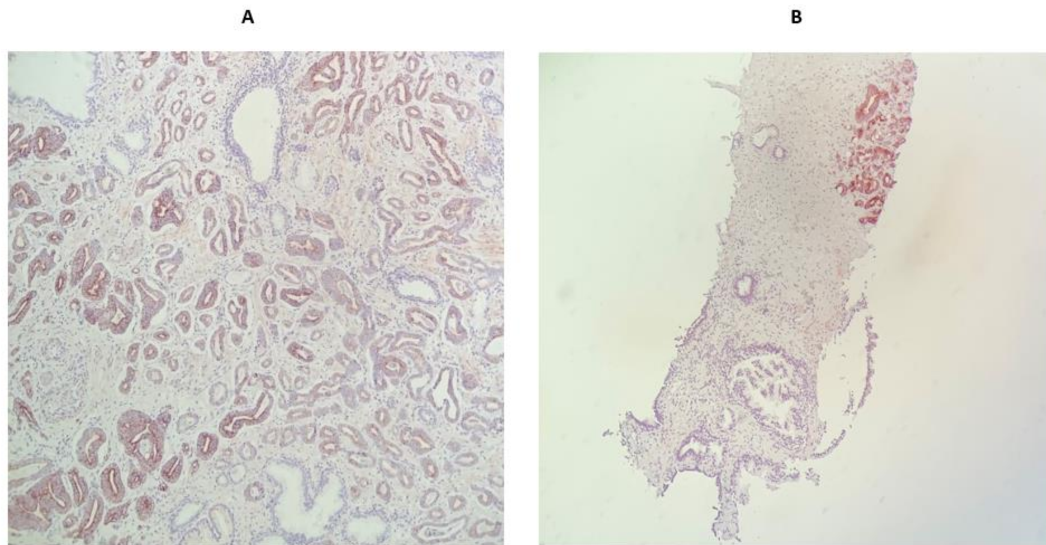


Figure 2.2 - Example of PSMA IHC validation on prostate core biopsy. Note the PSMA-positive prostate adenocarcinoma, while the epithelial cells in the tiny fragment of rectal mucosa (adjacent to prostate core biopsy) are negative for PSMA. (A: 100x, B: 400x)

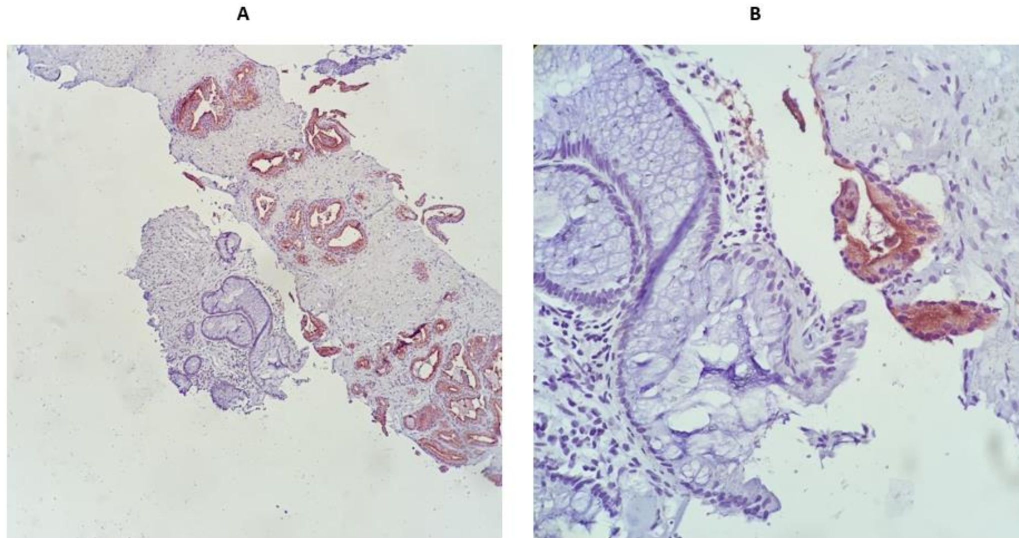


Figure 2.3 - Example of GLUT1 IHC staining validation on prostate core biopsy. Intravascular and scattered RBCs are showing GLUT1 expression. (400x).

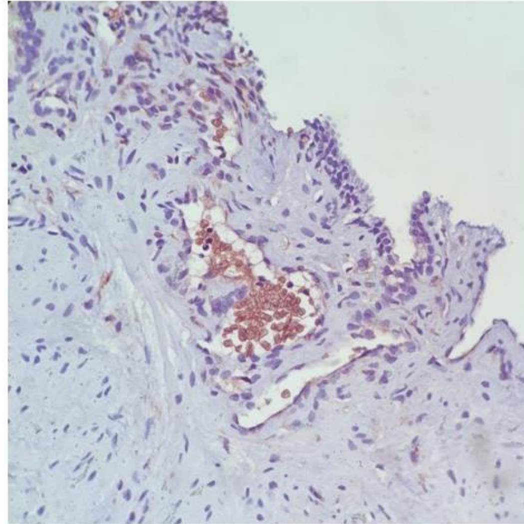


Figure 2.4 - Example of ACLY IHC staining validation on fibroadipose tissue. Note the ACLY expression in adipocytes while surrounding fibroconnective tissues, including vascular components, are negative for ACLY. (400x).

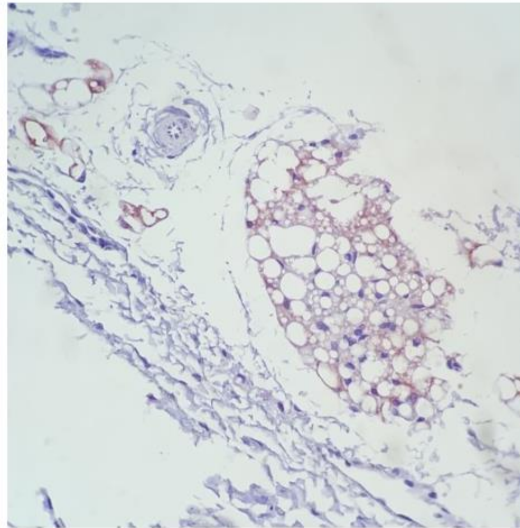
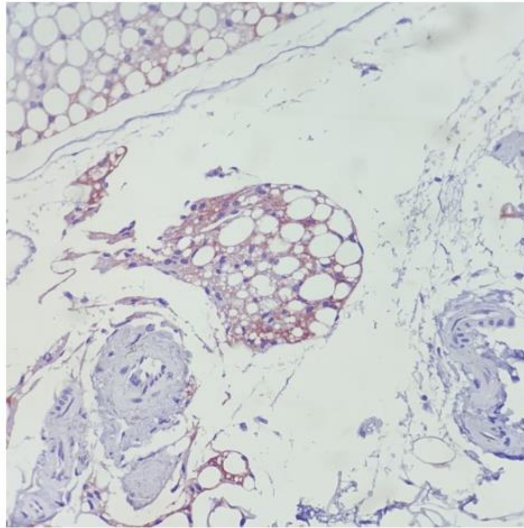


Figure 2.5 - Example of ACC IHC staining validation on fibroadipose tissue. Note the ACC expression in adipocytes while surrounding fibroconnective tissues, including vascular components, are negative for ACC. (400x).



For IHC staining, tissue sections were deparaffinized and rehydrated in xylenes and ethanol, followed by endogenous peroxidase removal and heat antigen retrieval in citrate buffer with PH 6 (Sigma-Aldrich#C9999). Tissues were blocked in 10% normal goat serum (Vector laboratories#S-1000-20) for 2 hours and incubated overnight at 4°C with either non-specific (negative control) serum or primary antibodies for ACLY, ACC, GLUT1, AMACR and PSMA, followed by 1:500 biotinylated goat-anti-rabbit IgG secondary antibody (vector laboratories#BA-1000) and 1:50 streptavidin peroxidase (vector laboratories#SA-5004), and developed using Nova Red kit (vector laboratories#SK-4800).

The following timings were used for NovaRED staining: 3 minutes for GLUT1, ACC, ACLY and PSMA and 6 minutes for AMACR. After counterstaining with Hematoxylin (Abcam#245880), the slides were mounted and left to dry overnight.

Table 2.1 includes the company, catalogue number for each antibody and the dilution used. All standard chemicals were purchased from Fisher Scientific (Toronto, ON), Sigma Aldrich (Oakville, ON), Bio Rad (Mississauga, ON).

Table 2.1 - Antibodies used for IHC staining

Primary antibody	**Company**	**Catalogue number**	**Dilution**	**Clonality**
GLUT1	Abcam	115730	1/500	Rabbit monoclonal
ACLY	Abcam	40793	1/200	Rabbit monoclonal
ACC	Cell Signaling (NEB)	3662	1/50	Rabbit polyclonal
AMACR	ThermoFisher (Invitrogen)	14576	1/100	Rabbit monoclonal
PSMA	Cell Signaling (NEB)	12815	1/100	Rabbit monoclonal

2.4 Third step: H-scoring

Semiquantitative scoring systems are extensively used to transform the subjective expression of IHC-marker into quantitative data by pathologists, which can be evaluated by statistical tests to reach conclusions.

There are usually multiple parameters in most semi-quantitative scoring systems, which are separately quantified and finally combined in a total and final score. Then, the average scores of the different groups can be compared by statistical tests (Klopfleisch, 2013).

These parameters should be selected based on the study hypothesis and the morphological features of expression of IHC markers evaluated in the study. The “golden standard” in IHC scoring is defined for the evaluation of only 3 markers so far: Her2/neu, estrogen (ER), and progesterone (PR) in breast cancer that have established guidelines (Fitzgibbons et al., 2014).

There are no standard scoring systems for most IHC markers, and researchers design a specific scoring system for each marker, which might be the best way to evaluate the study hypothesis.

Three examples of commonly used combined scoring systems are Allred-score (Koerdt et al., 2014), immunoreactive score (IRS) (Remmele & Stegner, 1987) and H-score (McCarty et al., 1985), which are frequently used for IHC evaluation of progesterone and estrogen receptors in breast cancer patients and are considered as “gold standard” in IHC evaluation.

Leading associations and organizations extensively accept and recommend these systems (Bartley et al., 2014; Cagle et al., 2014; Fitzgibbons et al., 2014; Torlakovic et al., 2010).

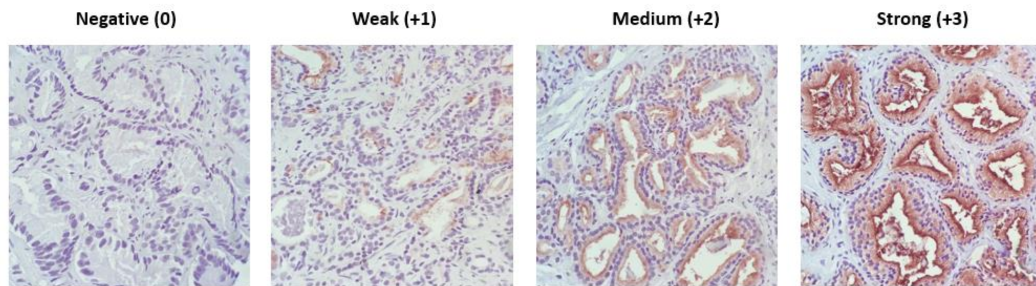
In this study, we selected the H-Score system, which is determined by multiplication of the percentage of cells with staining intensity ordinal value (0 for no staining, 1 for weak staining (yellow colour), 2 for medium staining (light brown colour), 3 for strong staining (red-dark brown colour)), which ranges from 0 to 300 possible values (McCarty et al., 1985).

Calculating the H-score as follows:

H-score = (% weak staining) (1) + (% medium staining) (2) + (% heavy staining) (3)

Benign glands/epithelium and malignant glands were identified and scored separately. Atrophic glands/epithelium and high-grade PIN (Prostatic intraepithelial neoplasia) were excluded from scoring. H-Scoring was performed in a blind fashion by using an Olympus BX-40 microscope with no previous knowledge of the patient group. Figure 2.6 reveals few representative images of different staining intensities for PSMA IHC staining.

Figure 2.6 - Representative images of different staining intensities for PSMA (400x)



2.5 Final step: Statistical analysis

The H-scores for GLUT1, ACC and ACLY and PSMA were averaged and statistically compared between non-progressed and progressed groups in both benign and malignant epithelial cells. The average H-score for AMACR was only scored in malignant component since we do not expect to see AMACR expression in benign glands (except in rare cases of PIN and other prostate cancer mimickers).

Values are reported as means with bars indicating the standard error of means (SEM) using GraphPad Prism 8. Several tests were used to test significant differences between groups, such as independent t-test.

If multiple (more than 1) malignant cores/blocks for a patient were available, the 2 or 3 blocks with higher tumor percentage/ involvement were selected for IHC staining and scoring to increase the scoring accuracy, especially in the presence of marker expression heterogeneity and the average score for each marker per each patient was taken.

Chapter 3 - Results

3.1 GLUT1 results

GLUT1 expression was higher in malignant epithelial cells compared to benign epithelial cells in both non-progressed and progressed groups, but this increased expression was not statistically significant (p-value: 0.73 and 0.12, respectively) (figure 3.1-A, B). Furthermore, GLUT1 expression was higher in the progressed group's benign epithelial cells than in the non-progressed group, but this increased expression was not statistically significant (p-value: 0.12) (figure 3.1 C).

Interestingly, GLUT1 expression was statistically significantly higher in malignant epithelial cells of the progressed group compared to the non-progressed group (P-Value <0.05) (Figure 3.1 D). These changes in GLUT1 expression are visualized in figures 3.2 and 3.3.

Figure 3.1 - The average expression of GLUT1 in the non-progressed group, progressed group, benign and malignant epithelial cells. A, B) There is an insignificant increase in average GLUT1 expression in malignant epithelial cells compared to benign epithelial cells in both non-progressed and progressed groups, and C) when comparing non-progressed and progressed groups in benign epithelial cells. D) There is a significantly increased expression of GLUT1 in malignant epithelial cells of the progressed group compared to the non-progressed group. Error bars are standard errors of the mean (SEM).

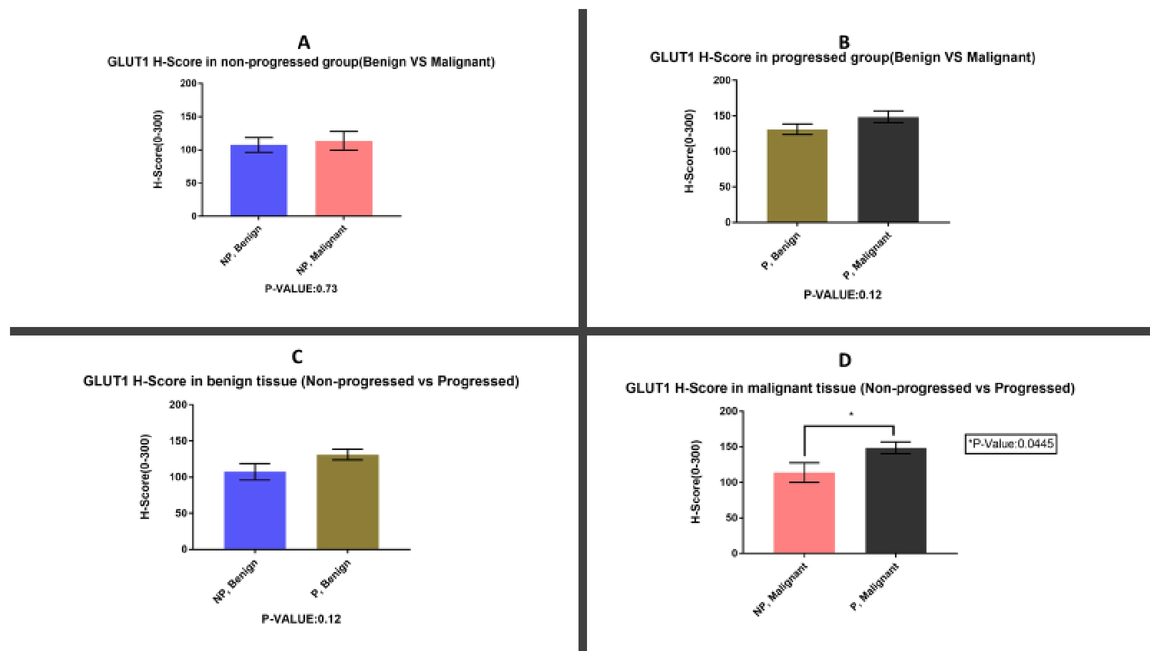


Figure 3.2 - Representative H&E and IHC staining of GLUT1 in benign and malignant epithelial cells in non-progressed and progressed groups. Benign epithelial cells visually show lower expression than malignant epithelial cells. GLUT1 expression is higher in the progressed group compared to the non-progressed group in both benign and malignant glands but is not statistically significant.

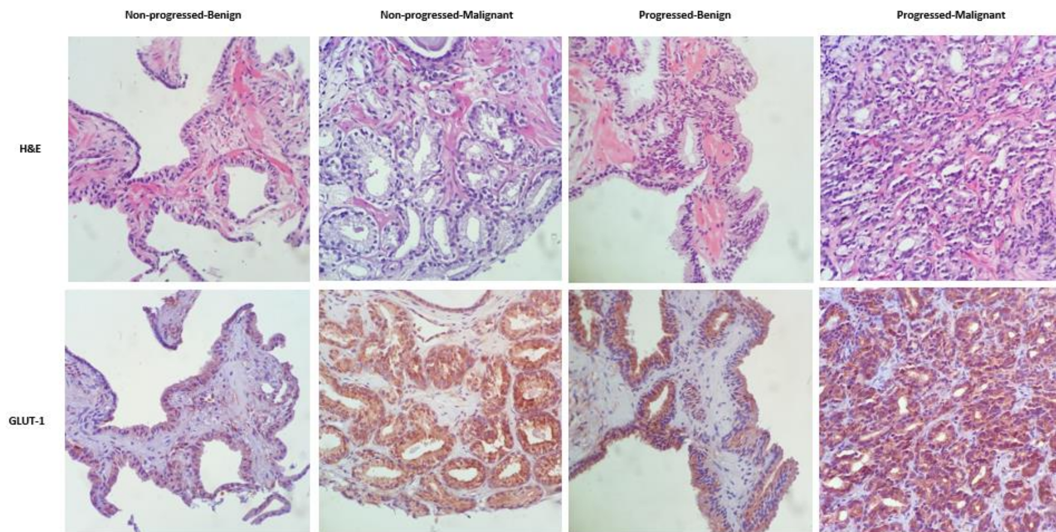
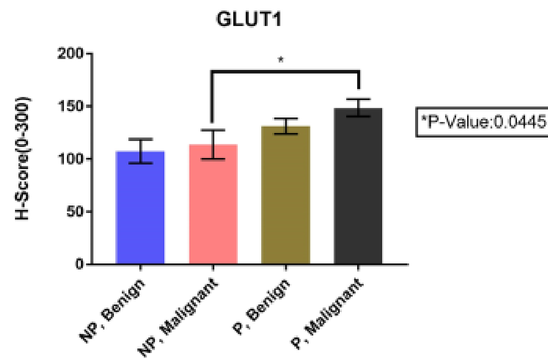


Figure 3.3 - Average GLUT1 expression of benign and malignant tissues in non-progressed and progressed groups. Average GLUT1 expression in non-progressed benign tissue is less than in progressed benign tissue. Average GLUT1 expression in non-progressed malignant tissue is less than in progressed malignant tissue, too. Error bars are SEM.



3.2 ACLY results

ACLY expression was higher in malignant epithelial cells compared to benign epithelial cells in both non-progressed and progressed groups, which were statistically insignificant in the non-progressed group and statistically significant in the progressed group (p-value: 0.55 and <0.05, respectively) (figure 3.4 A, B). But no significant difference was observed between the non-progressed and progressed groups in both benign and malignant epithelial cells (p-value: 0.68 and 0.99, respectively) (figure 3.4 C, D). These changes in ACLY expression are visualized in Figures 3.5 and 3.6.

Figure 3.4 - The average expression of ACLY in the non-progressed group, progressed group, benign epithelial cells and malignant epithelial cells. A, B) There is an insignificant increase in average ACLY expression in malignant epithelial cells compared to benign epithelial cells in the non-progressed group and a statistically significant increase in the progressed group. C, D) Also no significant difference when comparing non-progressed and progressed groups in both benign and malignant epithelial cells. Error bars are standard errors of the mean (SEM).

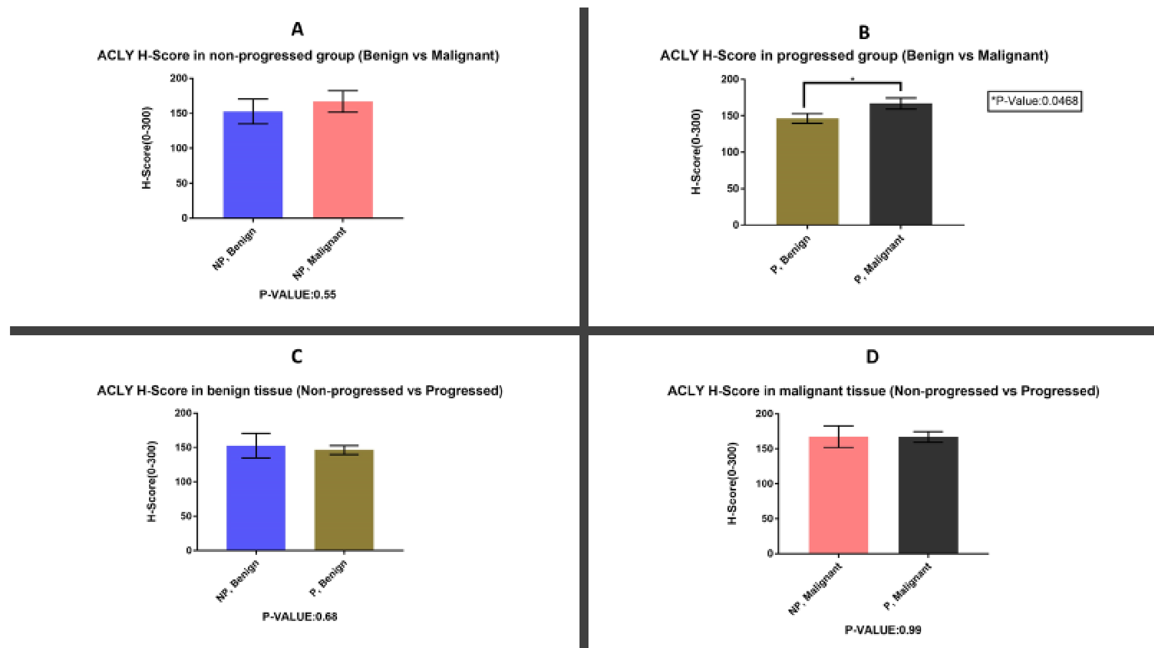


Figure 3.5 - Representative H&E and IHC staining of ACLY in benign and malignant epithelial cells of non-progressed and progressed groups. Benign epithelial cells revealing lower expression than malignant glands. No visible differences were observed between non-progressed and progressed groups in both benign and malignant epithelial cells. Benign glands are marked as "B," and the rest are malignant glands.

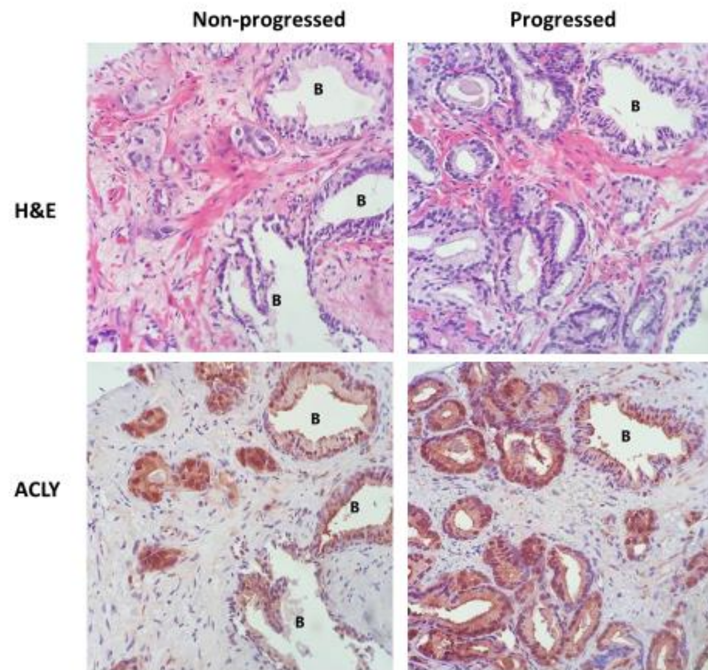
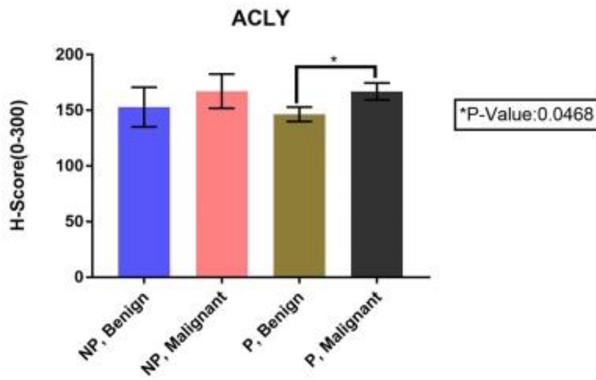


Figure 3.6 - Average ACLY expression of benign and malignant epithelial cells in non-progressed and progressed groups. Average ACLY expression in progressed malignant epithelial cells is significantly higher than in progressed benign epithelial cells. Error bars are SEM.



3.3 ACC results

ACC expression was higher in malignant epithelial cells compared to benign epithelial cells in the non-progressed group, but this increased expression was not statistically significant (p-value: 0.16) (figure 3.7A).

Moreover, ACC expression was statistically significantly higher in malignant epithelial cells compared to benign epithelial cells in progressed groups (p-value <0.0001) (3.7B). But no significant difference was observed between the non-progressed and progressed groups in both benign and malignant epithelial cells (p-value: 0.85 and 0.84, respectively) (figure 3.7 C, D). These changes in ACC expression are visualized in figures 3.8 and 3.9.

Figure 3.7 - The average expression of ACC in the non-progressed group, progressed group, benign epithelial cells and malignant epithelial cells. A) An insignificant increase in malignant epithelial cells compared to benign epithelial cells in the non-progressed group. B) A significant increase in malignant epithelial cells compared to benign epithelial cells in the progressed group. C, D) There is no significant difference between non-progressed and progressed groups in both benign and malignant epithelial cells. Error bars are SEM.

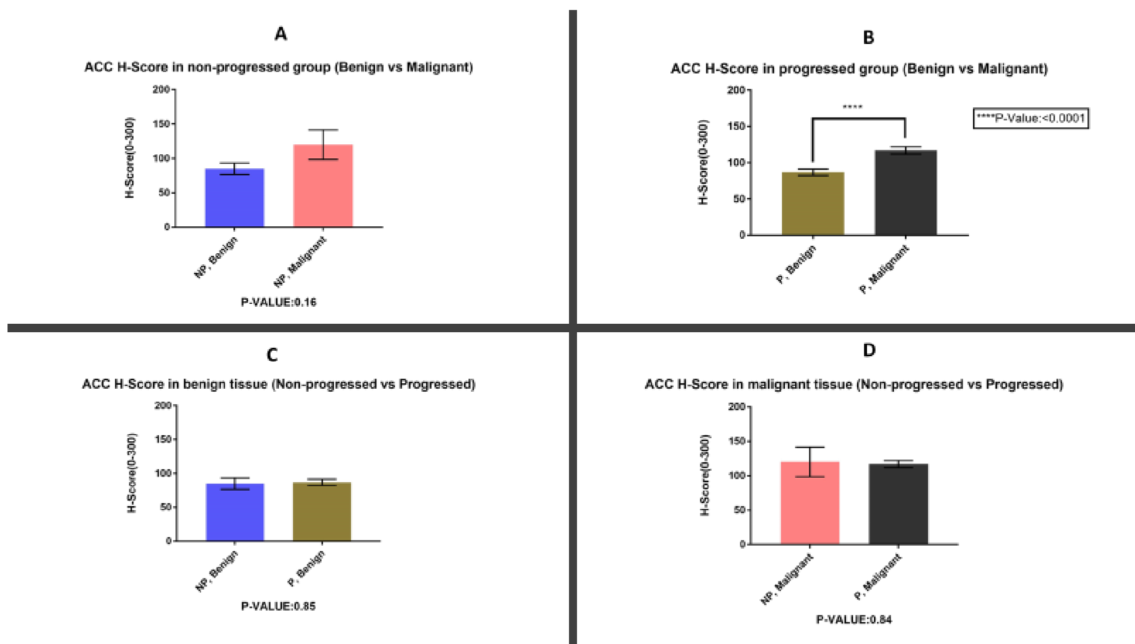


Figure 3.8 - Representative H&E and IHC staining of ACC in benign and malignant epithelial cells in non-progressed and progressed groups. Benign epithelial cells reveal lower expression than malignant epithelial cells. No visible differences were observed between non-progressed and progressed groups. Benign glands are marked as "B," and the rest are malignant glands.

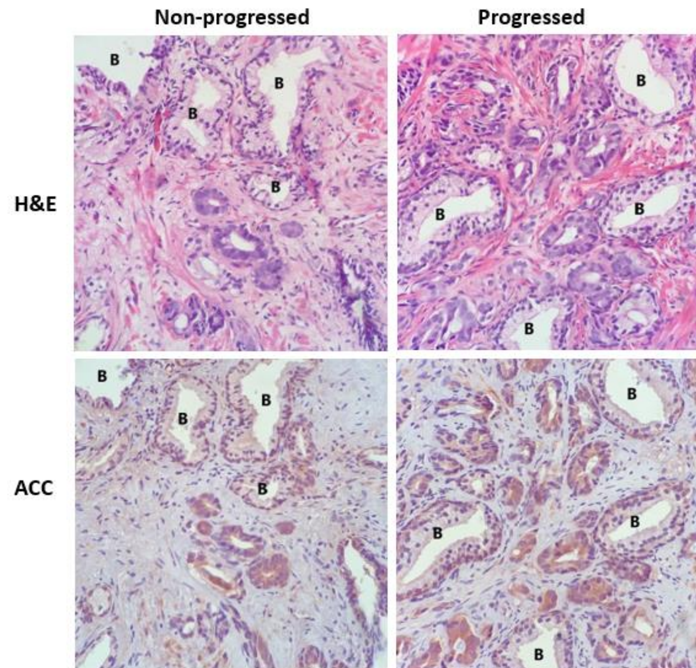
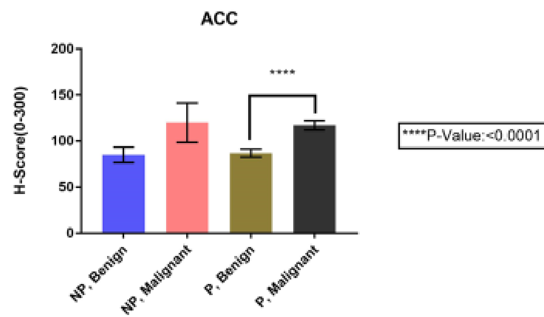


Figure 3.9 - Average ACC expression of benign and malignant epithelial cells in non-progressed and progressed groups. Average ACC expression in malignant epithelial cells is statistically significantly higher than benign epithelial cells in the progressed group. Error bars are SEM.



3.4 AMACR results

Since AMACR is an established diagnostic biomarker with mostly expression in malignant glands, average AMACR expression was compared only between the non-progressed and progressed groups in malignant epithelial cells. However, there was no significant difference between the non-progressed and progressed groups (p-value: 0.58, figure 3.10). These changes in AMACR expression are visualized in figure 3.11.

Figure 3.10 - The average expression of AMACR in non-progressed and progressed groups. There is no significant difference between the non-progressed and progressed groups. Error bars are SEM.

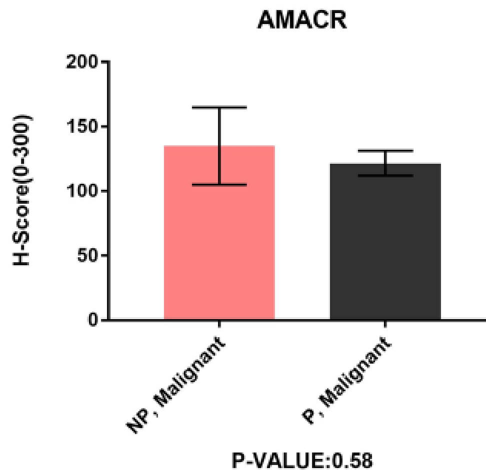
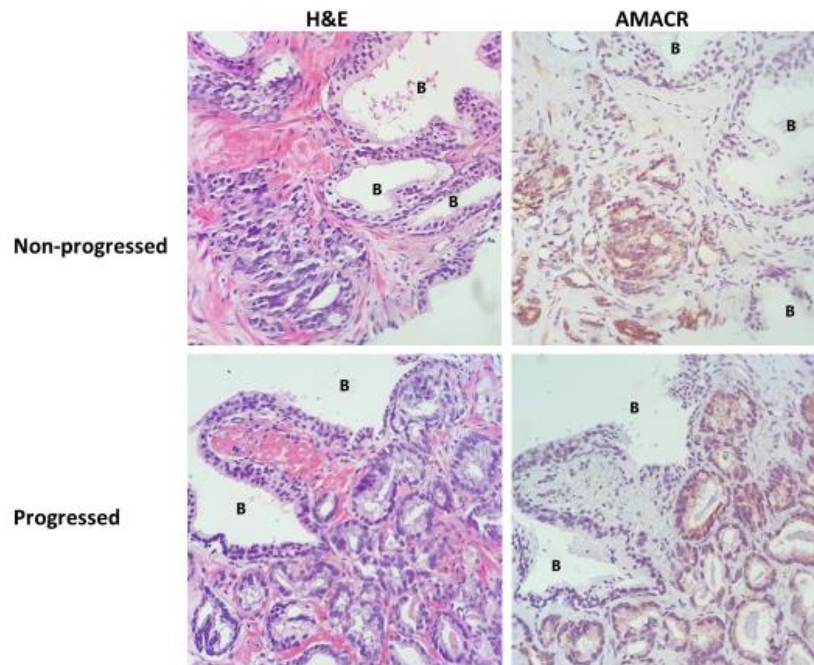


Figure 3.11 - Representative H&E and IHC staining of AMACR in non-progressed and progressed groups. No visible differences were observed in malignant epithelial cells between non-progressed and progressed groups. Benign glands/epithelium are marked as "B," and the rest are malignant glands.



3.5 PSMA results

PSMA expression was statistically significantly higher in benign epithelial cells of the progressed group compared to the non-progressed group (p-value <0.05) (figures 3.12 and 3.13). Moreover, PSMA expression was higher in the progressed group's malignant epithelial cells than in the non-progressed group, but this increased expression was not statistically significant (p-value: 0.17) (figures 3.14 and 3.15). Additionally, no significant differences in malignant epithelial cells compared to benign epithelial cells were identified in both non-progressed and progressed groups (p-value: 0.40 and 0.62, respectively) (figures 3.16, 3.17, 3.18 and 3.19). These changes in PSMA expression are finalized in figure 3.20.

Figure 3.12 - The average expression of PSMA in the benign epithelial cells of non-progressed and progressed groups. This graph shows a significant increase in benign epithelial cells of the progressed group compared to the non-progressed group. Error bars are SEM.

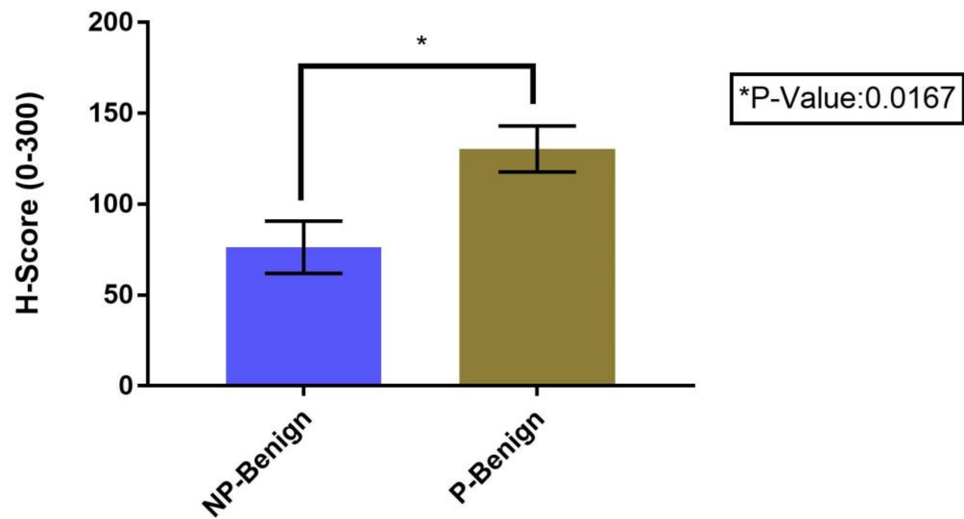


Figure 3.13 - Representative H&E and PSMA IHC staining of benign epithelial cells in non-progressed and progressed groups. This image reveals the stronger PSMA expression in the progressed group's benign epithelial cells compared to the non-progressed group's benign epithelial cells.

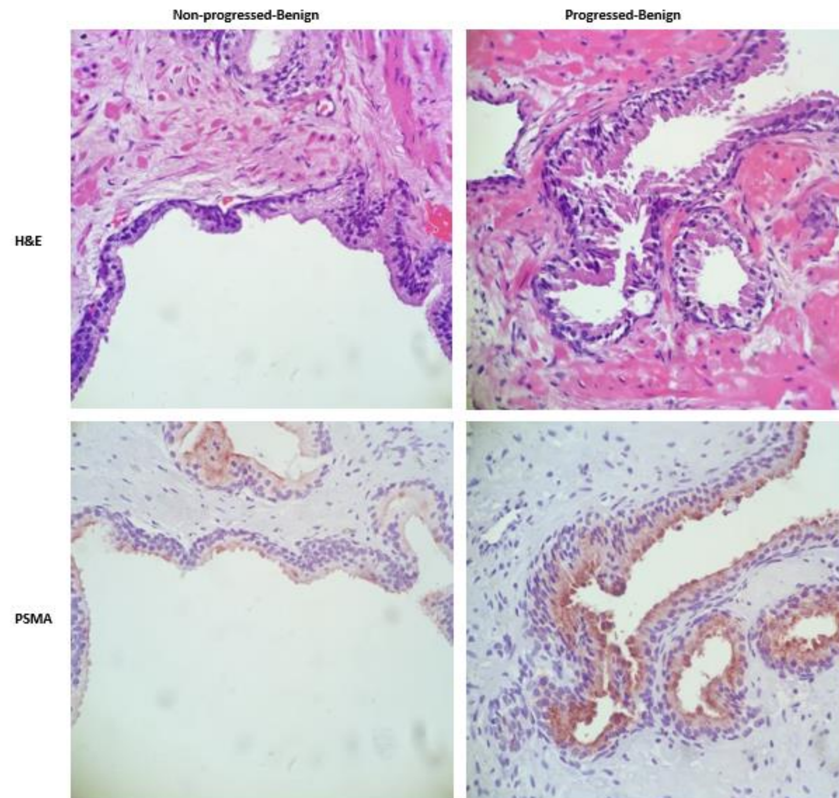


Figure 3.14 - The average expression of PSMA in the malignant epithelial cells of non-progressed and progressed groups. An insignificant increase in malignant epithelial cells of the progressed group compared to the non-progressed group. Error bars are SEM.

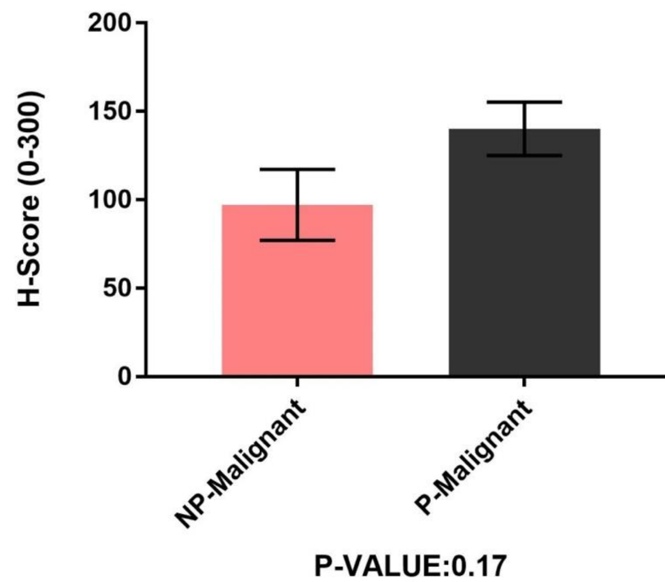


Figure 3.15 - Representative H&E and PSMA IHC staining of malignant epithelial cells in non-progressed and progressed groups. This image reveals the stronger PSMA expression in the progressed group's malignant epithelial cells compared to the non-progressed group's malignant cells.

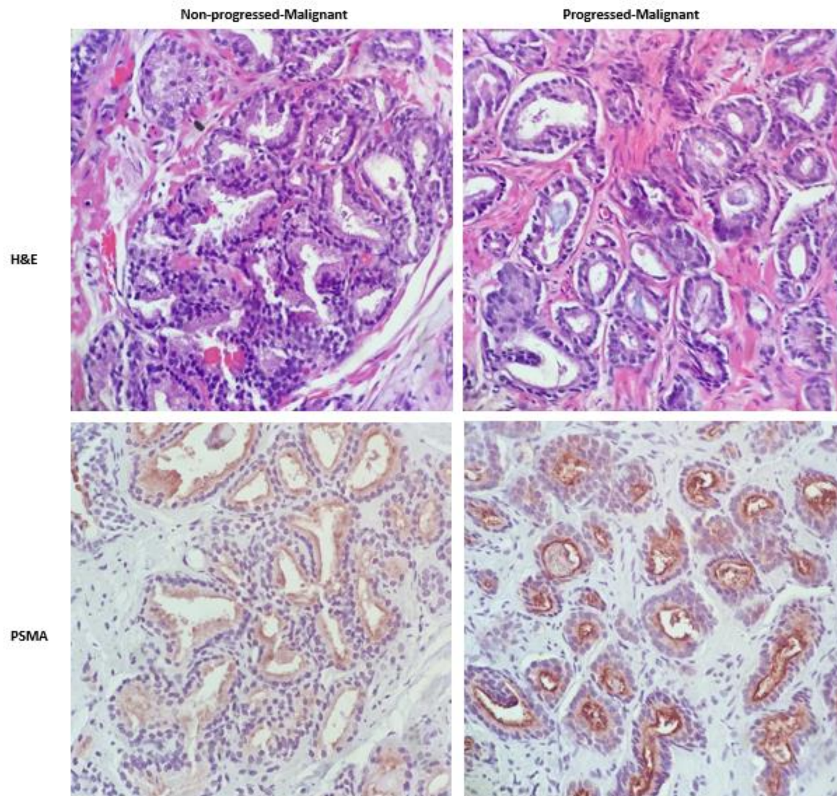


Figure 3.16 - The average expression of PSMA in the benign and malignant epithelial cells of non-progressed group. No significant difference in malignant epithelial cells compared to benign epithelial cells in non-progressed group. Error bars are SEM.

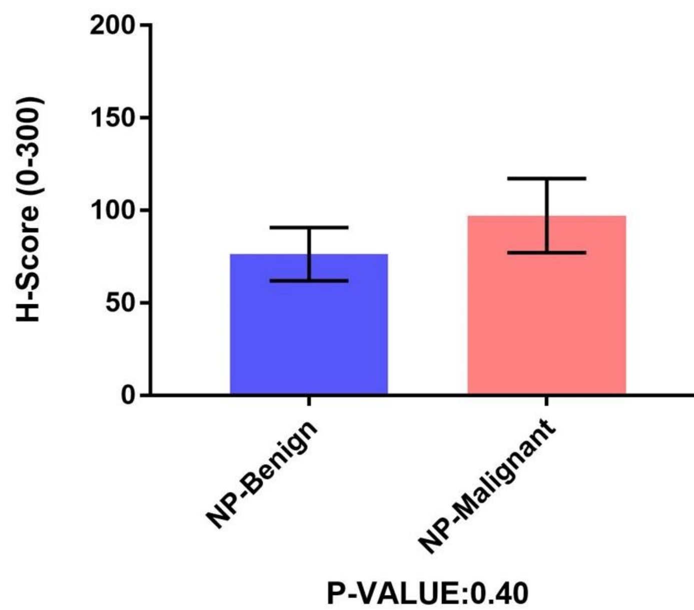


Figure 3.17 - Representative H&E and PSMA IHC staining of benign and malignant epithelial cells in non-progressed group. No significant difference in malignant epithelial cells compared to benign epithelial cells in non-progressed group.

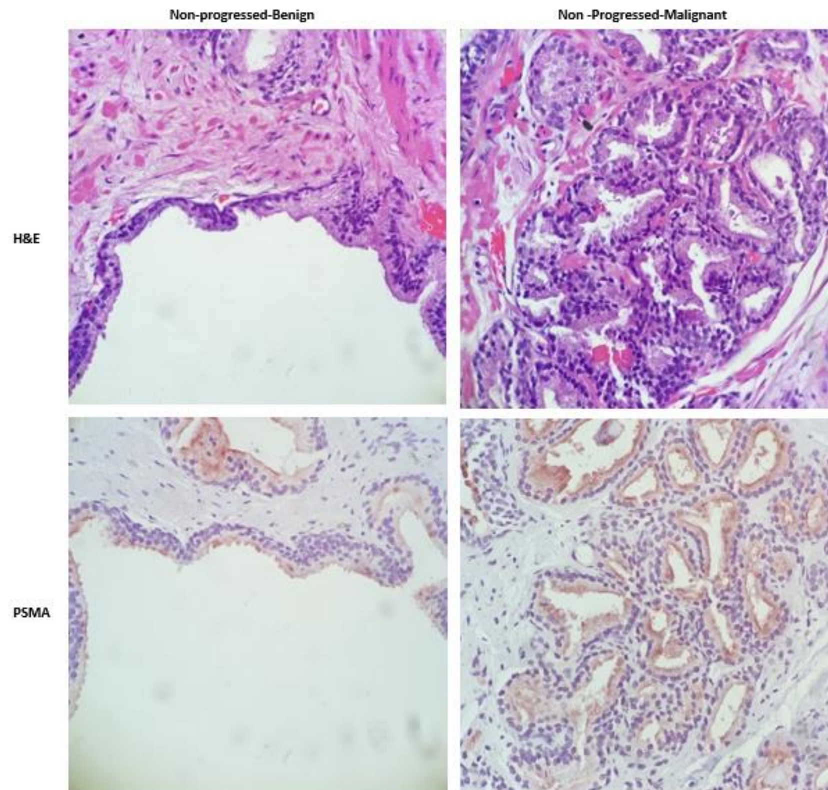


Figure 3.18 - The average expression of PSMA in the benign and malignant epithelial cells of progressed group. No significant difference in malignant epithelial cells compared to benign epithelial cells in progressed group. Error bars are SEM.

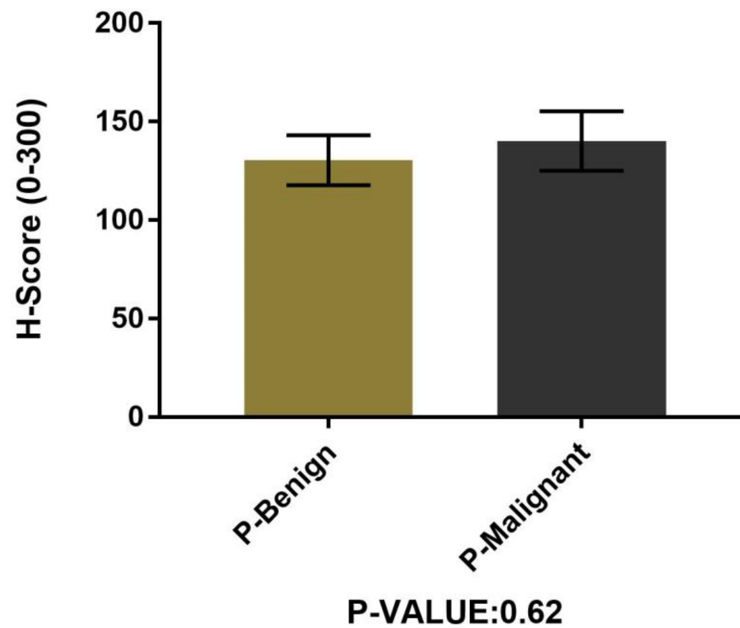


Figure 3.19 - Representative H&E and PSMA IHC staining of benign and malignant epithelial cells in progressed group. No significant difference in malignant epithelial cells compared to benign epithelial cells in progressed group.

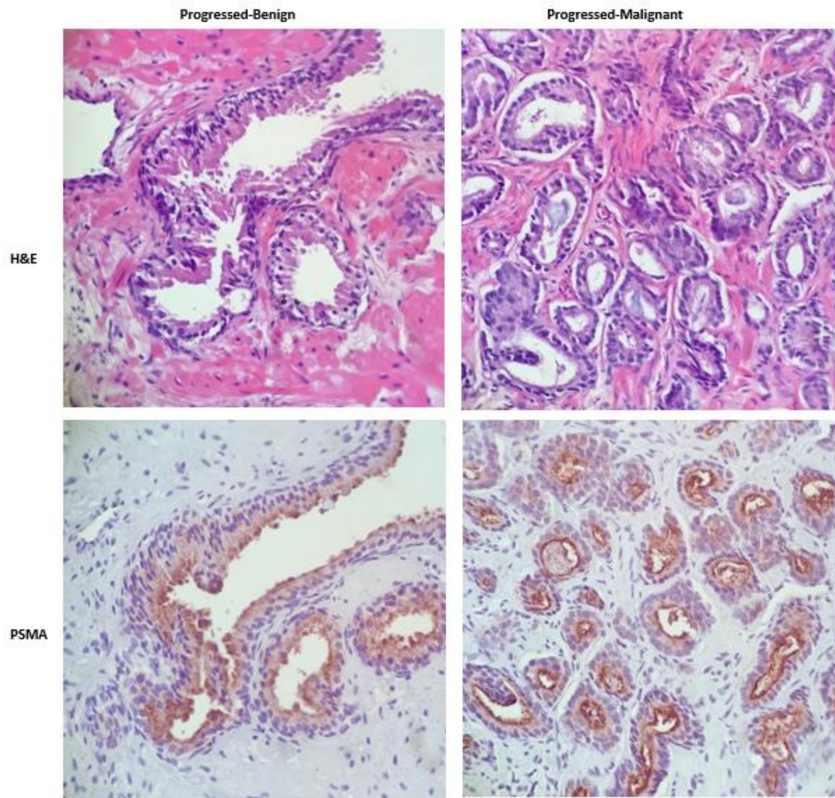
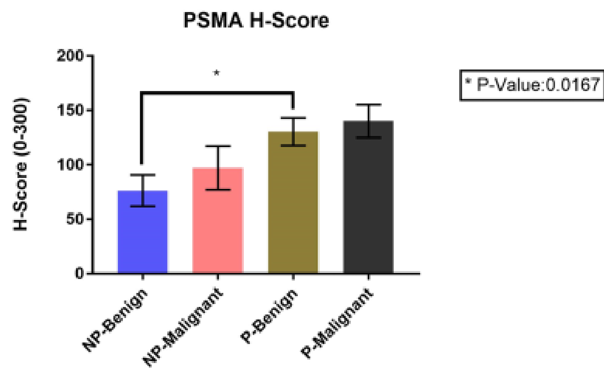


Figure 3.20 - Average PSMA expression of benign and malignant epithelial cells in non-progressed and progressed groups. Average PSMA expression in progressed benign epithelial cells is higher than in non-progressed benign epithelial cells. Error bars are SEM.



Chapter 4 - Discussion

Recently, interest in cancer biomarkers has been enhanced significantly to improve cancer management by increasing the detection rate and treatment efficacy. Extensive research has enabled the examination of many potential biomarkers to develop new biomarkers. Cancer biomarkers include many biochemical entities (Bhatt et al., 2010), such as metabolic enzymes like the ones we evaluated in this study.

Some important and frequently used traditional cancer biomarkers that are applied in routine practice as diagnostic and prognostic cancer biomarkers are summarized in Table 4.1 (Bhatt et al., 2010).

Table 4.1 - Some traditional cancer biomarkers with diagnostic and prognostic applications

Biomarker	Tumor	Application	Sample type/Method of detection
Cancer antigen (biomolecules) based biomarkers:			
Prostate specific antigen (PSA)	Prostate cancer	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 125 (CA125)	Ovarian cancers Fallopian tube cancer	Diagnostic and prognostic	Serum/ Immunoassay
Alpha-foetoprotein (AFP)	Hepatocellular carcinomas (HCC)	Diagnostic and prognostic	Serum/ Immunoassay
Cancer antigen 15-3 (CA15-3)	Breast cancer	Diagnostic and prognostic	Serum/ ELISA, Lymph node/ IHC, Bone marrow/ IHC
BRCA-1, BRCA-2	Breast cancer	Diagnostic	Tumor samples/ RT-PCR
Cancer antigen 19-9 (CA 19-9)	Pancreatic cancer Bladder cancer	Diagnostic and prognostic	Serum/ ELISA Urine/ ELISA
Carcinoembryonic antigen (CEA)	Colorectal cancer	Diagnostic and prognostic	Serum/ ELISA
Thyroglobulin (Tg)	Papillary and follicular thyroid cancer	Diagnostic and prognostic	Serum/ ELISA or IHC with TPO Ab
Human chorionic gonadotrophin (hCG)	Germ cell tumors (ovarian and testicular)	Diagnostic	Serum/ ELISA
TGFβ	Malignant tumors	Diagnostic and prognostic	Serum/ ELISA
Heat shock proteins (HSPs) Hsp27; Hsp70	Gastric, prostate carcinoma, osteosarcomas, uterine, cervical, and bladder carcinoma	Diagnostic and prognostic	Serum/ ELISA
Metabolic biomarker:			
Glucose metabolism	All cancers, general	Diagnostic, prognostic and therapeutic	Imaging/ FDG-PET scan/Tumor sample
Genetic biomarkers:			
APC gene	Adenocarcinoma, squamous cell carcinoma of the stomach, pancreas, thyroid and ovary	Diagnostic and prognostic	Blood, Tumor sample/ RFLP of chromosome 5q21-22, Methylation status of APC gene
Genetic translocations like Philadelphia chromosome, Bcl2 and other gene translocation fusion product	AML, ALL, CML, MDS and Burkitt's lymphoma	Diagnostic	Bone marrow or peripheral blood/ FISH
Cells as biomarkers:			
Cancer stem cells (CSCs)	AML, melanoma, brain tumor, breast cancer, prostate cancer	Diagnostic, prognostic and therapeutic	Tumor sample/ Immunocytometry
Circulating tumor cells (CTCs)	Metastatic breast cancer, etc.	Diagnostic and prognostic	Blood/ Immunocytometry

One of the most widely studied biomarkers in prostate cancer is Prostate Specific Antigen (PSA), produced by both normal and neoplastic prostate epithelial cells and plays roles in the initiation, growth, invasion and metastasis of prostate cancer. A serum PSA test is considered the most effective test currently available for the early detection of prostate cancer and close follow up the patients in active surveillance program. A small amount of PSA is present in the serum of normal men and is usually increased in the presence of prostate cancer and other prostate disorders. This marker has a few limitations. For example, prostate cancer can also be present in the complete absence of an increased PSA level, which has a correlation with poor differentiation and poor prognosis as well as being negative in some types of prostate cancers, such as prostate basal cell carcinoma, squamous cell carcinoma and sarcomatous elements of carcinosarcoma. Additionally, PSA expression is androgen-dependent and less sensitive in older men. Obesity has been proposed to decrease serum PSA levels. PSA levels can be falsely low because of medications like 5-alpha reductase inhibitors and Herbal mixture. Moreover, PSA Can be falsely elevated in settings unrelated to carcinoma like postsurgical conditions (i.e., cystoscopy), benign prostatic nodular hyperplasia (BPH), prostatitis (infectious, granulomatous...), infarction, irritation and post-ejaculation. On the other hand, the serum PSA test has limitations as a biomarker for evaluating treatment response. An increase in serum level not correlating with tumor regression following radiotherapy has been identified in some cases.

In the systematic review and meta-analysis of published articles on immunohistochemistry-based prognostic biomarkers on prostate cancer by Zhao et al., only a few markers revealed some promising independent prognostic value. In this study, they analyzed Ninety-three prognostic biomarkers from 92 high-quality cohort studies and found that only a few biomarkers may have predictive value for predicting the outcome of prostate cancer patients, including Ki-67, Bcl-2, CD147, COX-2, ALDH1A1, and FVIII (Zhao et al., 2014).

These limitations of PSA as a main biomarker in prostate cancer patients managed with AS program and the absence of any well-established and promising tissue biomarkers for predicting the risk of prostate cancer progression at the time of initial diagnosis led us to evaluate metabolic enzymes as potential predictive biomarkers of risk for prostate cancer progression because metabolic alterations participate in early molecular events that support prostate cancer progression. We expect to see the different expressions of metabolic enzymes between patients where the prostate neoplasia progresses to advanced and those where the disease does not progress. Our aims in this pilot study were to analyze the differential expression of metabolic enzymes between benign and malignant prostate epithelial cells and to evaluate the differential expression of metabolic enzymes amongst benign or malignant prostate tissues in patients that progressed while managed with AS vs those that did not progress.

We investigated ACLY, ACC, GLUT1, AMACR and PSMA for their potential predictive values for PrCa progression because these enzymes have critical roles in PrCa metabolism.

4.1 GLUT1

Few studies have investigated the correlation between GLUT1 expression and prognosis in some cancers like breast carcinoma and oral squamous cell carcinoma (Wang et al., 2017), hepatocellular carcinoma (Sun et al., 2016), and esophageal adenocarcinoma (Blayney et al., 2018), but the conclusions are still controversial. In general, increased expression of GLUT1 was associated with unfavourable overall survival and poorer disease-free survival in different tumors. Additionally, overexpression of GLUT1 has a correlation with poorly differentiated tumors, positive lymph nodes, metastasis and larger tumor size, which suggests that GLUT1 may have potential predictive value in various cancers (Yu et al., 2017).

Previous studies involving IHC stains for GLUT1 relating to malignant glands are few, but existing papers have similar findings. Studies by Gasinska et al. and Luczynska et al. found GLUT1 expression correlating to the tumour grade, but not significantly (p-value: 0.143 and 0.110, respectively) (Gasinska et al., 2020; Luczynska et al., 2012).

In our study, the insignificant increase in the average GLUT1 expression between the benign and malignant epithelial cells in the non-progressed and progressed groups indicates some enhancement in GLUT1 expression in malignant epithelial cells compared to benign epithelial cells. Any increase in GLUT1 enhancement between benign and malignant epithelial cells can be attributed to increased energy demand for uncontrolled proliferation and the beginning stages of glucose reliance.

The insignificant increase in the average GLUT1 expression between the non-progressed and progressed groups in benign epithelial cells is inconclusive to GLUT1's predictive value. Still, the increase hints at some potential predictive value. However, it was interesting that GLUT1 expression in benign epithelial cells of the progressed group was higher than in benign epithelial cells of the non-progressed group, which may illustrate early trends for metabolic alterations in the tissues of patients at risk for progression. This observation may also indicate priorly benign glands may be the ones that progress rapidly to invasive metastatic PrCa. Indeed, it has been observed in more differentiated glands with lower Gleason scores eventually progressing into metastatic PrCa (De Marzo et al., 2004). Many more samples need to be evaluated to establish a clear trend and assess whether investigating benign glands is a possible avenue for predicting PrCa progression.

The significant increased GLUT1 expression in malignant epithelial cells of the progressed group compared to malignant epithelial cells of the non-progressed group suggests the beginning stages of metabolic alterations in the malignant epithelial cells of patients at risk for progression, revealing early evidence of glucose reliance and Warburg effect which is expected in progressed malignant epithelial cells.

All the prostate core biopsies stained for evaluation for predictiveness were from the baseline prostate biopsy, so this observation is consistent with current research and can be confirmed more conclusively by IHC staining of repeat biopsies because an increase in glucose reliance is not observed until PrCa has progressed and the Warburg effect can be observed (Eidelman et al., 2017). By evaluating repeat prostate biopsies, we expect to

identify GLUT1 expression patterns more clearly and potentially establish a trend for GLUT1 expression and PrCa progression.

4.2 ACLY

Few studies investigated the ACLY expression in some cancers, revealing distinctive elevation of ACLY expression and activity in lung, prostate, bladder, breast, liver, stomach, and colon tumors. For example, Chen et al. reported that ACLY mRNA and protein expression (evaluated by IHC) was significantly enhanced in the breast cancer tissues compared to normal tissues, and ACLY overexpression had an association with ER status, PR status, tumor size, TNM stage, lymph node invasion and worse tumor relapse-free survival (RFS) of breast cancer patients, as well as resistance to docetaxel (Chen et al., 2020).

In human lung adenocarcinoma, the expression of phosphorylated ACLY correlated with stage, differentiation grade, and poor prognosis. Thus, overexpression and activation of ACLY were proved to be a statistically significant negative prognostic factor for some cancers, including lung, prostate, bladder, breast, liver, stomach, and colon cancer (Zaidi et al., 2012).

Although there are no previous studies analyzing the expression of ACLY during the clinical progression of PrCa, it is known that ACLY is upregulated in PrCa for DNL in response to the resource demand of PrCa (Singh et al., 2002; Wallace et al., 2008; Welsh

et al., 2001). The significant increase in average ACLY expression in malignant epithelial cells compared to benign epithelial cells in the progressed group indicates an enhancement in ACLY expression in malignant epithelial cells compared to benign epithelial cells. ACLY's increase in expression clearly shows that DNL in malignant cells is enhanced. This observation is expected because ACLY catalyzes downstream substrates for DNL, which is needed to sustain the resource demand by PrCa proliferation (Wu et al., 2014; Zadra et al., 2013). In PrCa progression, DNL is even more upregulated, so higher expression of ACLY is expected (Singh et al., 2002; Wallace et al., 2008; Welsh et al., 2001). It would be interesting to observe whether DNL is further enhanced in progressed PrCa by IHC staining for ACLY in repeat biopsies and establish a clear trend for developing a predictive biomarker.

The insignificant increase in ACLY expression in malignant epithelial cells compared to benign epithelial cells of the non-progressed group is compatible with increased DNL in malignant cells, which is needed to provide resources demand for PrCa proliferation.

The absence of difference in average ACLY expression in the progressed group compared to non-progressed groups in both benign and malignant epithelial cells is inconclusive to ACLY's predictive value. Further studies with a larger sample size are needed for a definite conclusion.

4.3 ACC

Most of the studies in the context of **ACC** approached this marker as a target for cancer therapy rather than biomarkers, like in non-small cell lung carcinoma (Svensson et al., 2016), hepatocellular carcinoma (Lally et al., 2019) and thyroid cancer (Hyoung kim, 2008)**.**

Very few investigations have been done for ACC in the context of PrCa progression and even less involving IHC analysis. Previous research investigating potential lipogenic markers found that ACC levels quantified by Raman micro-spectroscopy are correlated with signs of aggressive PrCa, such as oncogenes MAPK and ERBB2 (O'Malley et al., 2017).

In our study, the significant increase in average ACC expression between benign and malignant epithelial cells in the progressed group reaffirms that DNL is enhanced in malignant epithelial cells (Wu et al., 2014; Zadra et al., 2013). As the second and the rate-limiting step of DNL, ACC is essential for synthesizing lipids and its enhancement, along with ACLY enhancement, in malignant epithelial cells, confirming that DNL is active. IHC staining of repeat biopsies for ACC would help establish a trend for ACC expression to develop predictive biomarkers. ACC is expected to increase with PrCa progression due to increased resource demand.

The insignificant increase in ACC expression in malignant epithelial cells compared to benign epithelial cells of the non-progressed group is compatible with upregulated lipogenesis in malignant cells, which is needed to provide resources demand for PrCa proliferation.

When comparing the non-progressed to the progressed group in both benign and malignant epithelial cells, there is no significant difference, which was also observed in ACLY and is inconclusive with ACC's predictive value. To ascertain the cause of these findings, more studies need to be conducted with more samples.

4.4 AMACR

Although AMACR is mainly Used to aid in the diagnosis of prostatic adenocarcinoma, it can be expressed in various tumor types, and it has been shown that AMACR overexpression might represent an adverse prognostic factor in different types of tumors, such as gastric adenocarcinoma, hepatocellular carcinoma, gallbladder carcinoma, nasopharyngeal carcinoma, gastrointestinal stromal tumor and myxofibrosarcoma (He et al., 2018; Lee et al., 2014; Xu et al., 2014).

In prostate cancer, AMACR overexpression was reported to have a significant association with disease progression, ERG gene rearrangement, positive surgical margins, and marginal association with PSA biochemical recurrence (BCR). Patients with high AMACR/ERG-positive may be at higher risk for disease progression (Box et al., 2016).

Previous studies have shown that AMACR expression is increased in higher grade prostate cancers than in lower grade prostate cancers (Fu et al., 2021).

The absence of AMACR expression in benign epithelial cells reaffirms its diagnostic value and that malignant epithelial cells have enhanced lipid metabolism. The presence of

AMACR expression strongly suggests using β-oxidation as an energy source. This indicates that β-oxidation is likely in use in conjunction with oxidative phosphorylation in PrCa. To confirm the use of β-oxidation in PrCa, another enzyme in the β-oxidation pathway can be probed in future studies, such as acetyl-CoA dehydrogenase (Thorpe & Kim, 1995).

In this study, the insignificant difference between the non-progressed and progressed groups indicates that AMACR has a low predictive value. A larger dataset is required to validate these implications.

4.5 PSMA

Multiple studies involving IHC stains for PSMA report similar findings to ours. These studies reveal that PSMA expression correlates with the PrCa stage and Gleason grade (Bostwick et al., 1998). The increase in both expression and enzymatic activity of PSMA in aggressive PrCa points to a selective advantage imparted on cells that express PSMA, thereby contributing to the development and progression of PrCa (Yao et al., 2008). Increased PSMA expression is an independent predictor of PrCa recurrence (Ross et al., 2003; Yao et al., 2008). In other studies, it has been demonstrated that PSMA mRNA expression was 3-fold less in the normal prostate compared to the primary prostate tumors; however, this difference was not statistically significant. A trend of increasing PSMA expression between the normal prostate and tumors with increasing Gleason score has been observed. The expression of PSMA was 2-fold and 5-fold higher in the lymph node

metastases compared to prostate tumors and normal prostate, respectively (not statistically significant) (Schmittgen et al., 2003).

In our study, the insignificant increase in the average PSMA expression in malignant epithelial cells compared to benign epithelial cells in both non-progressed and progressed groups indicates an enhancement in PSMA expression in malignant epithelial cells compared to benign epithelial cells. IHC staining of repeat biopsies of PSMA will more conclusively determine PSMA expression patterns and potentially establish a trend for PSMA expression and PrCa progression. A significant increase in PSMA expression is expected in progressed malignant glands. However, here we made exciting observations that PSMA expression in benign glands of baseline biopsies of patients that eventually progressed was significantly higher than in benign epithelial cells of baseline biopsies of patients that did not finally progress. This observation may indicate benign glands that may develop into invasive PrCa, which has been observed in more differentiated glands with lower Gleason scores, eventually progress into aggressive PrCa (De Marzo et al., 2004).

Moreover, we found insignificantly higher PSMA expression in malignant epithelial cells of the progressed group compared to the non-progressed group, which is compatible with our expectation that PSMA expression correlates with PrCa progression. Clearly, a larger number of patients and tissues need to be evaluated to establish a clear trend and assess whether analysis of benign glands is a possible avenue for assessment of risk for PrCa progression.

Limitations and future directions

This study had its limitations and challenges. It represents a pilot and early analysis of metabolic enzymes as predictive biomarkers of risk for prostate cancer progression in patients managed with active surveillance.

The main limitation of this study was related to the patient population, including the limited number of patients (especially those categorized as the non-progressed group). In this study, we accessed clinicopathologic data and prostate core tissue biopsies of only 40 patients. This number decreased to 34 final patients with satisfactory tissue after primary sectioning and H&E staining for subsequent IHC staining and scoring (26 patients in progressed group and 8 patients in the non-progressed group). More evaluation with a more extensive data set and patient population is required to validate the results of this pilot study.

Another limitation was the presence of a small focus of adenocarcinoma in a few patients who finally were identified as satisfactory for evaluation in this study, which limits the accuracy of H-Scoring. This problem was mainly related to the relatively small volume of prostate tissues obtained by the core biopsy technic and the previous sectioning for diagnostic purposes. However, this is a fairly common finding in the pathology field, which results in diagnostic difficulties and leads to the need for more evaluation by IHC staining to confirm the diagnosis. To increase the amount of evaluable tissues, 3 blocks with the

highest tumoral involvement percentage were selected for each patient. Despite our attempts, the low amount of evaluable tissue was sometimes problematic. To decrease the effect of this problem, we decided to evaluate more tissue blocks per patient to increase our study power, especially for the PSMA marker, which demonstrated promising preliminary results.

In this phase of the study, which constitutes this thesis, we only evaluated the baseline prostate tissue biopsies due to limited time and resources, which resulted in potential findings and outcomes restrictions. Analyzing patients' repeat biopsies can help to verify our primary outcomes.

Although the H-Score quantification is considered one of the gold standard systems in IHC evaluation, its limitations include its subjective quality, which leads to interobserver and intraobserver variability. To prevent and at least diminish intraobserver variability, we tried to score each marker for all patients in one day. Another limitation of the H-scoring system is that the staining intensity is quantified as an ordinal value (0, +1, +2, +3), which has lower precision than the continuous value. To improve the ability and precision of the scoring, our next step would be a digital scoring system like image J or HALO system. This study evaluated the difference in the mentioned metabolic enzymes expression at the protein level. Future analysis of the RNA expression (like RNAscope) can help to validate our results.

Conclusion

PrCa has a highly variable disease course, resulting in a major clinical challenge for patients' classification into risk groups for individual treatment decisions. This reveals the need to establish new predictive biomarkers for PrCa progression, especially for AS patients. This study aimed to assess the potential predictive value of these markers when assessed on biopsies at the time of initial diagnosis, i.e., prior therapy decision. The long-term goal of this study is to consider prostate biopsy during decision-making for risk stratification of PrCa management.

In this small pilot study, we concluded that GLUT1, ACLY, and ACC had enhanced expression in malignant epithelial cells, indicating that glycolysis and DNL are more active in malignant epithelial cells than in benign epithelial cells. Also, we identified that PSMA had increased expression in malignant epithelial cells compared to benign epithelial cells, revealing its oncogenic signalling role in malignant epithelial cells.

Moreover, we noticed increased expression of GLUT1 in malignant epithelial cells with disease progression, indicating that this marker may have some value as a predictive biomarker.

Further, our results show a potential predictive value of PSMA expression at the time of initial diagnostic biopsy for risk for PrCa progression. A higher PSMA expression in benign epithelial cells of the progressed group than in the non-progressed group suggests the potential predictive value of PSMA for PrCa progression on biopsy specimens. The

difference in PSMA expression between the non-progressed and progressed groups in benign epithelial cells may be an area of interest to investigate because prostate cancer progression can occur in sites other than the malignant gland, which indicates that benign glands or glands with lower Gleason scores that appear benign may become metastatic (De Marzo et al., 2004). Other studies have already confirmed the predictive potential of PSMA on radical prostatectomy specimens. Furthermore, our study suggests its predictive value for PrCa progression on baseline prostate biopsies. This needs to be confirmed with larger data sets in the future.

As a pilot study, preliminary observations only reveal a snippet of the larger picture and require a larger dataset and more advanced statistical analysis to achieve any conclusive findings. Further studies will reveal more conclusive data for the predictiveness of GLUT1, ACLY, ACC, AMACR and PSMA.

Plans to build on this study's findings include expanding the patient population, analyzing PrCa patients' repeat biopsies, and analyzing RNA expression data from consenting patients to help validate results. This pilot study accomplished its goal of creating a foundation for investigating potential predictive biomarkers for patients on active surveillance by gathering preliminary data on a few metabolic enzymes of interest.

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