AMERICAN UNIVERSITY OF BEIRUT

# INVESTIGATING THE ANTINEOPLASTIC EFFECT OF NSAIDS (PIROXICAM) AND STATINS (ATORVASTATIN) ON PROSTATE CANCER: NOVEL THERAPEUTIC POTENTIAL

by
TAMARA AKRAM ABDUL SAMAD

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# ABSTRACT
# OF THE THESIS OF

Tamara Akram Abdul Samad for Master of Science
Major: Physiology

Title: Investigating the Antineoplastic Effect of NSAIDs (Piroxicam) and Statins (Atorvastatin) on Prostate Cancer: Novel Therapeutic Potential

Prostate cancer (PCa) is currently a challenging medical health issue worldwide. For the last 30+ years, PCa has been the most common cancer among men. Mortality due to metastatic castration-resistant PCa (CRPC) becomes problematic due to a high number of patients developing resistance to therapy. Therefore, elucidating new therapeutics to treat PCa is a top research priority.

Non-steroidal anti-inflammatory drugs (NSAIDs) and lipid-lowering drugs (statins) have shown to have anti-tumorigenic effects in many cancers. Consequently, we were interested in testing the anti-cancer properties of NSAIDs (Piroxicam-PXM) and statins (Atorvastatin-Ato), alone or in combination, on novel murine PCa cells (PLum-AD and PLum-AI). We employed *in vitro* assays to assess the effect of those drugs at a functional cellular level. We started by testing for cell proliferation and viability using MTT cell growth assay and trypan blue exclusion assay, respectively. Significant reduction of growth of cells and viability were observed exhibiting the sensitivity of PLum-AD and PLum-AI cells to those drugs, in a dose and time-dependent manner. Additionally, we have demonstrated, by using wound healing assay, that PXM and Ato inhibit cell migration, an important feature in malignancy. Treated cells failed to migrate and close up the wound compared to untreated cells. Furthermore, we assessed the potential of PXM and Ato in targeting cancer stem/progenitor cells. Using the sphere-formation assay, the number and size of spheres formed was reduced significantly when PLum-AD and PLum-AI cells were treated with PXM and Ato, suppressing their sphere-formation capacity.

In summary, our study illuminated the potential use of PXM and Ato in inhibiting cell proliferation, viability, migration, and stemness of PLum-AD and PLum-AI cell lines, promising a potential novel management of the disease.

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# ABBREVIATIONS

. O2-: Superoxide Anion

. OH: Hydroxyl Radical

2D: 2 Dimensional

3D: 3 Dimensional

AD: Androgen Dependent

ADP: Adenosine Diphosphate

AFS: Anterior Fibromuscular Stroma

AI: Androgen Independent

Akt: phosphatidylinositol 3-kinase (PI3K)/protein kinase B

ANOVA: Analysis of Variance

AR: Androgen Receptor

Ato: Atorvastatin

ATP: Adenosine Triphosphate

BMI1: B lymphoma Mo-MLV insertion region 1 homolog

BPH: Benign Prostatic Hyperplasia

BRCA: Breast Cancer

CAF: Cancer-associated Fibroblasts

CDK2: Cyclin-Dependent Kinase 2

CO2: Carbon Dioxide

COX: Cyclooxygenase

CRPC: Castrate-resistant prostate cancer

CSCs: Cancer Stem Cells

CXCR: Chemokine Receptor

DCs: Dendritic cells

DHT: Dihydrotestosterone

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular Matrix

ED: Ejaculatory ducts

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

EMT: Epithelial-Mesenchymal Transition

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

GTP: Guanosine Triphosphate

H2O2: Hydrogen Peroxide

HMG-CoA: 3-Hydroxy-3-Methylglutaryl-Coenzyme A

IFN-γ: Interferon-gamma

IL: Interleukins

IL1RA: Interleukin 1 Receptor Antagonist

MDSCS: Myeloid-Derived Suppressor Cells

MFB: Myofibroblast

MIC1: Microneme-associated protein 1

MSCs: Mesenchymal Stem Cells

MSR1: Macrophage Scavenger Receptor 1

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NF-κB: Nuclear Factor Kappa B

NK cells: Natural Killer cells

NO: Nitric Oxide

NSAIDS: Non-Steroidal Anti-Inflammatory Drugs

PARP: Poly Adenosine Diphosphate-Ribose Polymerase

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PCa: Prostate Cancer

PFS: Progression-Free Survival

Plum: Prostate Luminal

PSA: Prostate-specific antigen

PSMA: Prostate-Specific Membrane Antigen

PTGS: Prostaglandin-Endoperoxide Synthase

PXM: Piroxicam

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RT: Radiation Therapies

SEM: Scanning Electron Microscope

SMA: Smooth Muscle Actin

SMAD: fusion of Sma genes and the Drosophila Mad

STAT3: Signal Transducer and Activator of Transcription 3

SV: Seminal Vesicles

TAA: Tumor-associated antigens

TAMs: Tumor-Associated Macrophages

TGF: Transforming Growth Factor

THR 160: Threonine 160

TLR: Toll-Like Receptors

TNF: Tumor Necrosis Factor

VEGF: Vascular Endothelial Growth Factor

# CHAPTER I

# INTRODUCTION

## A. Overview of Prostate Cancer: Epidemiology and Etiology

Prostate cancer (PCa) is the second most common, and fifth most aggressive, cancer among men worldwide [1, 2]. One in seven men, in the US, have been estimated to receive a PCa diagnosis in their lifetime [2]. PCa accounts for 6.7% of men-related cancer deaths [2]. This disease is responsible for the death of around 375,000 men, with almost 1.4 million new cases in 2020, globally [1]. The epidemiology of prostate cancer varies, having Northern Europe, followed by the Caribbean with the highest incidence and mortality. On the other hand, the lowest reported incidence was in South-Central Asia **(Figure 1)** [3, 4]. The incidence of PCa is strongly related to age, where it dramatically increases after the age of 55 and is often found to be rare below the age of 40 [5].

While the precise etiology of PCa remains elusive, there are several identified risk factors that are involved in PCa; the best characterized are genetics, race, age, ethnicity and obesity [6]. Although several races are affected by this disease, African American descent is seen to be of highest risk [2]. Genetics also play a role in the development of PCa; an example is the BRCA1/2 mutations [2, 7]. Genetic studies in PCa, which looked at individual single-nucleotide polymorphisms or haplotypes, identified genes that contribute to the etiology of the disease. These genes are often involved in processes such as DNA reparation, inflammation, and cancer and steroid hormone metabolism [8].

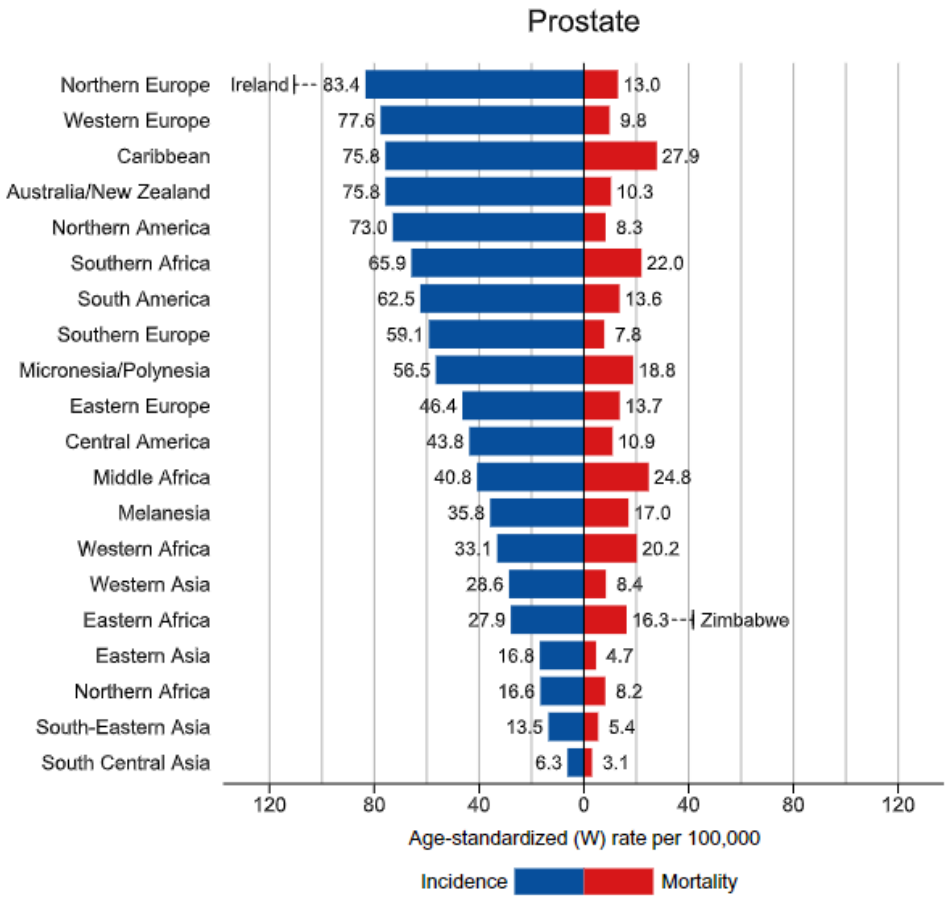


**Figure 1: Region- Specific Incidence and Mortality Age- Standardized Rates for Prostate Cancer in 2020.**
Rates are shown in descending order of the world (W) age- standardized incidence rate, and the highest national age- standardized rates for incidence and mortality are superimposed. Source: GLOBOCAN 2020.

## B. Prostate Gland: Anatomy and Physiology

The prostate is a male-specific gland located in the retroperitoneal space, the pelvis, and is associated with the urethra and neck of the bladder **(Figure 2)**. It sits adjacent to the bladder and rectum, partially surrounded by a thin capsule of collagen, elastin, and smooth muscle [9]. Its parenchyma can be divided into four distinct zones: the peripheral, central, transitional, and periurethral zones [10, 11]. Histologically, the prostate is a hormone-responsive glandular tissue that consists of a basal layer of cuboidal epithelial cells that is layered by columnar secretory cells. Each gland is separated by abundant

fibromuscular stroma [12]. The prostate gland cells are regulated by androgen hormones that statute the growth and survival of the cells. Castration, which abolishes androgens, leads to atrophy of the prostate [13]. Certain pathologies tend to take place in particular zones of the gland. Prostate cancers commonly occur in the peripheral zone while hyperplastic proliferative lesions commonly occur within the transitional zone. The three major pathologies that affect the prostate are inflammation (or prostatitis), benign prostatic hyperplasia (BPH), benign nodular (or prostate), and malignant prostate cancer [13].

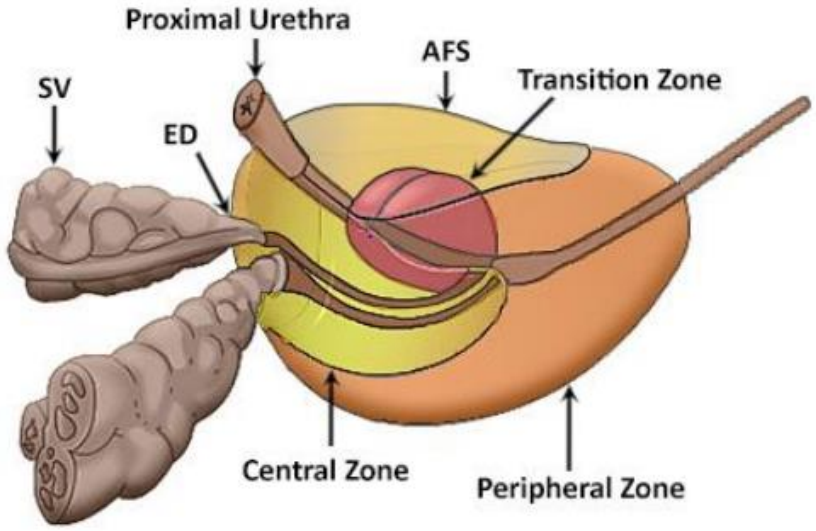


**Figure 2: Zonal anatomy of the prostate gland.**
ED: ejaculatory ducts; SV: seminal vesicles; AFS: anterior fibromuscular stroma. Adapted and modified from Bhavsar, A. and S. Verma, *Anatomic imaging of the prostate* [11].

The prostatic epithelial cells secrete an alkaline, white substance that contains minerals, sugars, and proteins that provide nourishment and protection to sperm cells. This secretion empties during ejaculation, passing through the glandular ducts into the prostatic urethra and undergo mixing with semen and secretions produced from the testes and epididymis and the seminal vesicles, respectively [11, 13]. The protein portion is

composed of proteolytic enzymes, prostatic acid phosphatase, prostate-specific antigen (PSA), and β-microseminoprotein. PSA is a glycoprotein that is secreted in prostatic epithelial cells and is involved in breaking down seminal proteins to allow the free movement of spermatozoa [14]. Testosterone, produced by Leydig cells and being the predominant male hormone, is metabolized by 5α-reductase in prostatic epithelial cells into the active hormone dihydrotestosterone (DHT) [13]. The physiology of the prostate gland is dependent on androgens as well [15]. This is applied to the developing prostate, and in the developed, functioning prostate; in other words, prostate epithelial cell survival relies on androgens. During development, mesenchymal cells express androgen receptors (AR) and regulate prostate size through epithelial cell growth and development [16, 17]. In the developed prostate, androgens are involved in stromal smooth muscle maintenance and epithelial differentiation and function [16, 17]. Hence, the expression of AR in the stromal and epithelial compartments are noted due to the stromal-epithelial cell interactions [16].

## C. Treatments

### *1. Non-Advanced Prostate Cancer*

There are several management options when it comes to treating non-advanced, i.e. localized, PCa. These include active surveillance, radical prostatectomy, interstitial brachytherapy, and radiation therapy [18]. Clinically localized PCa patients, with a life expectancy of >10 years, are often recommended to undergo radical prostatectomy. It has been observed that the likelihood of receiving this treatment decreases with increasing age. Effective and successful treatment is usually indicated through undetectable serum PSA levels, with PSA being a prostate-specific biomarker. Detectable PSA levels post-

removal indicates recurrence, or residual prostate cancer [18]. Target patients, for radical prostatectomy, are often men under the age of 70 with localized (organ-confined) prostate cancer. Other patients eligible for this procedure include patients that have benign prostatic hypertrophy, with irritating symptoms, and certain patients with high-risk potential of developing PCa [19]. However, the procedure can result in complications such as erectile dysfunction, incontinence, possible rehabilitation, or follow-up surgery for the treatment of these complications [19].

Recent radiation therapies (RT) comprise different options, each with high effectiveness and precision. This is via the use of high-energy accelerators that treat PCa with high doses of radiation. The surrounding tissue is at minimal risk from the high doses through the precise use of computerized three-dimensional planning [18]. It is well-suited for patients with localized prostate lesions. For patients with intermediate, or high-risk, prostate cancer, RT can be used with androgen deprivation as a standard approach which has shown to improve outcomes [19]. With some higher-risk patients, a combination of RT and brachytherapy have been shown to have a greater effect than RT alone [19].

Prostate brachytherapy techniques provide an effective treatment using real-time imaging and precise planning to implant radioactive sources [18]. Although very limited worldwide, proton beam therapy is a growing interest for prostate cancer treatment due to the protons' unique properties [18]. This option comes in two forms: low-dose-rate and high-dose-rate brachytherapy. The lose-dose consists of radioactive seeds implanted permanently into the prostate. It is often used, mono-therapeutically, for low- or intermediate-risk PCa patients. An option for high-risk PCa patients include using lose-dose brachytherapy in combination with radiation therapy [19]. The high-dose treatment, on the other hand, is administered through temporary catheters, containing the radioactive

sources, for around 10 minutes. This is often used with radiation therapy for intermediate- or high-risk disease [19].

### *2. Advanced Prostate Cancer*

Throughout disease-progression of PCa, there comes a stage where the disease has advanced to the extent where it reaches castration-resistance. Advanced PCa patients, especially if referred early, often undergo interdisciplinary care to receive optimal care to increase survival rate and quality of life. Recent therapeutic approaches involve androgen-production inhibition. Taxanes are diterpenes that target microtubules during mitosis and interphase of the cell cycle and stabilize the mitotic spindle. This halts cell proliferation leading to cell death. Their anti-cancer mechanisms are thought to be via anti-androgenic properties that can block nuclear translocation of the androgen receptor [20]. This method was found effective in treatments for slowing down the tumor's progressions by depriving it from androgens [21]. This method was also coupled with anti-androgens, i.e. bicalutamide, nilutamide, ketoconazole, and corticosteroids, to manipulate the hormonal balance [21].

a. <u>Chemotherapeutic Drug Therapy</u>

Mitoxantrone was an initial drug, used against PCa, to improve quality of life of patients. It is a type II topoisomerase inhibitor which is active in prostate tumors. Although found to have toxicity and some side effects, this drug is still often used to alleviate symptoms [20]. The cytotoxic drug was FDA-approved for its palliative care but played no effect in improving survival rates [21].

A drug that was used in treating PCa was Docetaxel. The microtubule inhibitor demonstrated its effectiveness in two important phase III trials resulting in an overall survival, symptoms, and quality of life improvement in CRPC patients [20, 21].

Another chemotherapeutic agent for metastatic CRPC treatment is Cabazitaxel, a semisynthetic taxane. The taxane has a low affinity for the ATP-dependent drug efflux pump, P-glycoprotein [22]. The tubulin-binding drug was set as a second-line treatment after docetaxel for its activity against docetaxel-resistant cancers by bypassing taxane resistance and exhibiting its antitumor mechanisms [21]. Several phase III trials exhibited its activity with a reduced toxicity profile compared to others.

Enzalutamide is an androgen receptor (AR) inhibitor that targets several steps of the AR signaling pathway at several steps and prevents androgen-binding to AR [22]. It first binds to the ligand-binding domain, competitively, of the AR which in turn prevents its translocation to the cell nucleus. This further prevents AR binding to DNA [21]. CRPC patients taking the drug, even with prior exposure to docetaxel, had an improved overall survival rate and a decrease in death risk, making it FDA-approved in 2012 [22].

Abiraterone acetate is a selective inhibitor of a key enzyme, involved in androgen synthesis, cytochrome P 17 [21]. The drug is able to hinder the production of androgens at the level of adrenal glands, testis, and the tumor [22]. The drug, combined with prednisone, was shown to have clinical advantages in patients that have, or haven't, taken docetaxel. Along with its high survival rate shown in clinical trials, the drug is overall well-tolerated with minimal side-effects [21].

b. Immunotherapy

Sipuleucel-T is an FDA-approved immunotherapy. The drug treats asymptomatic, or minimally symptomatic, castration-resistant patients that had no visceral metastasis [21, 22]. The approach is to induce a T-cell response by monoclonal bodies directly targeting TAA-expressing tumor cells. This will induce cell destructions via several pathways [23]. PBMCs, from the patient, are cultured with a recombinant human protein that comprises of prostatic acid phosphatase linked to granulocyte-macrophage colony-stimulating factor. Every two weeks, the PBMCs are re-injected to the patient three times [22]. The therapeutic approach proved effective in showing an increased overall survival rate in trials.

c. PARP Inhibitors

Polyadenosine diphosphate [ADP]-ribose polymerase (PARP) inhibitors are therapeutic techniques used in tackling ovarian and breast cancer. They were initially used on patients with underlying BRCA1/2 mutations and resisted previous treatments [23]. PARP is nuclear enzyme that assists in repairing single strand breaks in DNA. Cells with mutations, such that of BRCA1/2, are prone to further genomic damage and instability when attempted to be repaired by PARP. Almost 12% of advanced prostate cancer patients showed possession of germline defects in DNA damage repair genes [21]. Olaparib is a PARP inhibitor drug that was FDA-approved, in 2020, for its response rate in previously pretreated CRPC patients. Trials showed that these patients had higher progression-free survival (PFS) and a decline in PSA [21, 23]. The inhibition of androgen pathway proves the ability of having a synergic relationship with PARP inhibitors via increasing cell sensitivity to them. When this was tested with abiraterone acetate and

olaparib, there was a greater PFS response than abiraterone acetate alone [23]. There are still ongoing trials looking into the combination of PARP inhibitors with other variables and drugs to test their outcome.

d. Radiation Therapy

Radiotherapy is an option in the treatment of advanced PCa. Samarium-153, Phosphorus-32, and Strontium-89 are radioisotopes that have been used as monotherapy or in conjunction with chemotherapy for the treatment of bone cancers. These were used for palliative care without any survival benefit [21, 23]. As the former isotopes were beta-emitters, with possible effects of toxicity, Radium-223 is an alpha-emitting isotope. The irradiation of the calcium mimetic is less as it binds to microenvironments of sclerotic metastases, with less risk of complications [21]. Phase III trials demonstrated a gain in overall survival rate and toxicity reduction when the radiopharmaceutical was administered, however observing a low PSA response [21]. Furthermore, a promising radioisotope is the 177-Lutetium-PSMA-617. It comprises of admirable properties such as beta-emission, medium energy, small-tumor volume delivery, a half-life that is beneficial for anti-tumor effects, and others. Also, there was a high uptake and retention in prostate tumor cells with a lower uptake in normal cells expressing PSMA. Current ongoing trials show a promising response of PSA decline and PFS. The radiopharmaceutical is expected for FDA-approval for advanced PCa patients and high PSMA expression [23].

The cost of these various treatments comes at understanding the complex genome of PCa. The range of PCa progressive stages makes it challenging to utilize one specific

treatment. For this reason, trials and pharmaceutical manufacturing have been ongoing. The important aspect to take from this is that understanding the genomic complexity, and using advanced imaging for accurate diagnosis, is vital. With progression of medicine and understanding PCa, treatments are becoming loco-regional with better management of the disease.

## D. Tumor Microenvironment

### *1. An overview of PCa microenvironment*

When observing the environment of which malignant cells are situated in, there are several components that support the livelihood of these cells. Some of these components include fibroblasts and myofibroblasts, endothelial cells, pericytes, and mesenchymal stem cells (MSCs) [24, 25]. There are also many different immune cells such as innate, adaptive, and immune-suppressive cells that play a role in the tumorigenic environment [24].

### *2. Fibroblasts and Myofibroblasts*

The role of fibroblasts in tumorigenesis has often appeared paradoxical. These cells, mainly derived from mesenchymal tissue, are major constituents of the ECM. They are implicated in normal tissue development and physiology [26]. During a wound-healing event, fibroblasts are converted to myofibroblasts (MFB) via the secretions of fibronectin and transforming growth factor -β (TGF-β) [27]. MFBs are characterized by their co-expression of vimentin and α-smooth muscle actin (α-SMA) and their ability to contract granulation tissue for wound-closure [27]. During the last stages of wound-

healing, these cells are deactivated and undergo apoptosis when epithelialization is complete [27].

Tumorigenesis studies have identified that these cells play an interesting, double role. During early malignancy, healthy fibroblasts are involved in impeding its progression [28]. However, during late stages, these cells further promote tumor development. These fibroblasts are referred to as cancer-associated fibroblasts (CAF). These cells are found to excel in the stromal population [28]. Prostate adenocarcinoma cells, of low tumorigenic state, are promoted by the cell by sustaining them, eventually leading them to become castration-resistant and later osteogenic metastasis [29, 30]. There are cases where normal fibroblasts can suppress the conversion of malignancy in epithelial cells or turn non-invasive carcinoma into invasive, as seen in breast tissue [29, 31]. This effect may be due to the certain triggers during the oncogenic process of transforming normal epithelium to cancerous ones [32].

### *3. Endothelial Cells and Pericytes*

Angiogenesis, the formation of blood vessels, is a vital event that supports cancerous activity such as viability growth. It is no surprise that prostatic tumorigenesis would rely on the upregulation of angiogenesis [33]. Normal prostate tissue physiology comprises a regulated balance between pericytes, endothelial cells, and smooth muscle cells. Cancerous angiogenesis would involve abnormal formation of blood vessels, characterized by immaturity and leakiness [34]. More importantly, tumors are characterized by the increased density of new vessels [35]. Pro-angiogenic factors are increased when surrounding stromal cells and tumor cells interact and trigger angiogenic events [34]. Upon inspection of endothelial cells, it would come to notice

their important role in metastatic activity. They play a part in suppressing AR expression and transcriptional activity, making them an important component in tumor microenvironments. Their inhibition is proposed to block PCa progression [36].

Upon closer examination, the interplay of endothelial cells and pericytes is an important aspect for angiogenesis in PCa [35]. During immature development of vessels, in healthy or benign hyperplastic tissue, pericytes and endothelial cells appear to have a normal rounded, elongated phenotype. These cells appear to have a uniform basement membrane surrounding them with a condensed cytoplasm. Tumorous vessels appear to have an irregular phenotype consisting of a dilated cytoplasm with an abnormal vessel outline [35]. One important observation was the increased density of pericytes that was associated with forming endothelial cell sprouts, during early tumor angiogenesis. They become very active and have increased contact with endothelial cells. This is especially true around new vessel sprouts leading to maturation. Further characteristics include vascular remodeling and basement membrane matrix assembly with suggestive of a more migratory function [35].

### *4. Mesenchymal Stem Cells*

A component of the prostate tumor microenvironment is mesenchymal stem cells (MSCs). These are multipotent entities that have the ability to differentiate into many cells [37]. These include adipocytes, osteoblasts, fibroblasts, smooth muscle cells, and others. These cells are found in regions of inflammation after being recruited from bone marrow [37-40]. MSCs are important players of tissue regenerations and homeostasis [41, 42]. There is increasing evidence that MSCs are implicated in tumor progression via mechanisms. One of these is the suppression of the innate and adaptive immune

responses against tumor progression. Another mechanism would be pertaining to angiogenesis, tissue invasion and tumor growth and survival. In addition, these cells could also generate cancer-associated fibroblasts (CAFs) [38, 43-45]. Through this, cytokine networks would be established, thus creating a favorable tumor microenvironment and promoting progression and migration [44, 46, 47]. Due to their characteristics, their behavior is not entirely understood and still goes a long way to be studied entirely.

### *5. Tumor Epithelium and the Surrounding Stroma*

Upon a closer look within the microenvironment, there is a complex interplay between the tumor epithelium and the surrounding stroma. This stromal compartment consists of several important components such as the extracellular matrix, growth factors, cytokines, chemokines, enzymes, and many nonmalignant cells. These cells include lymphocytes, macrophages, and others, that promote tumor progression due to being activated by tumor-released cytokines [28]. Tumorigenesis, further, results from crosstalk, between the epithelial and stromal component, from mechanisms such as castrate-resistant growth factors release, angiogenesis, and ECM remodeling [25, 48]. Epithelial cells proliferation is done via an interplay between the ECM, stromal cells, and the epithelial cells themselves. Carcinogenic transformation of epithelial cells occur when the homeostatic crosstalk balance and the normal ultrastructure is dysregulated which further sustains cancer cell growth and malignancy [28].

### *6. Tumor-associated Immune Cells*

Tumor progression often involves the recruitment of immune cells. These cells are involved in a series of events that promote the disease in the tumor microenvironment. The role of these immune cells remains ambiguous and difficult to determine in specificity. This is because these immune cells can have carcinogenic [49, 50] or anti-carcinogenic behavior, depending on interactions, localization, and components of the microenvironment [51]. The full role of immune cells in the tumor environment is not fully understood as studies lack a complete characterization of the cells. Tumor-associated macrophages (TAMs) are also associated with prostate carcinogenesis [52], specifically the M2 macrophage subtype, but not many studies have been able to associate the full range of TAMs with PCa [53]. The innate immune cells consist of several cells such as granulocytes, dendritic cells, natural killer cells, mast cells, and macrophages. Mast cells produce pro-tumorigenic and anti-tumorigenic cytokines which have dynamic roles within the tumor microenvironment [52]. Studies have reported different observations of mast cells in PCa patients. A study reported increased amounts of intra-tumor infiltrated mast cells which was linked to lower Gleason-grade tissue and better prognosis [54, 55] while another study linked low amounts of mast cells to lower Gleason scores and longer progression-free survival times [56]. More studies are needed to fully understand their role in PCa. Macrophages play a role in PCa via their increased levels of macrophage colony-stimulating factor and colony-stimulating factor-1 receptor in tumor and stromal cells within metastatic primary tumors [57]. Macrophages could also secrete proteases, such as cathepsin K and S, that degrade the extracellular matrix and promote prostate carcinogenesis [57]. Moreover, cathepsin S was found to be upregulated in metastatic PCa patients and mice [57]. T-

lymphocytes are often linked to good prognosis due to having anti-tumorigenic behavior [52]. B-lymphocytes play a role in PCa by progressing castration-resistant cancer cells via the activation of STAT3 and BMI1, a proto-oncogene [58].

## E. Inflammation

### *1. The Role of Inflammation*

Inflammation has been found to play an important role in the development of cancer and is one of its hallmarks [59]. Epidemiological studies have found that acute and chronic inflammation predisposes individuals to higher risks of cancer through the generation of a microenvironment that supports the proliferation of cancer cells, i.e. multiplication, migration, and metastasis [60]. In adults, 20% of all cancer is caused by chronic inflammation [61, 62]. Inflammation can result from exposures to different infections and injuries purposed to clear the area from pathogens and debris and repair the tissue via a cascade of events [24]. Pathogenesis results from a deviation of this cycle and becomes an uncontrolled or unchecked cycle, lingering the inflammatory response. This, in turn, produces an abnormal amount of reactive oxygen species (ROS), chemokines and cytokines, and growth factors. This gives way to cellular or genetic damage, via irregular cell proliferation or gene alteration, and increases the risk of tumorigenesis [24]. Biopsy studies have shown inflammation to be a marker in many prostatic diseases, i.e. benign prostatic hyperplasia, prostatitis, inflammatory atrophies, and prostate cancer [63-65]. A widely accepted precursor to prostatic adenocarcinoma often points to neoplastic behavior. With prostatic intraepithelial neoplasia, epithelial cells present abnormal structures surrounding inflammatory cells [63-65]. Proliferative atrophy, although not a common precursor, has the potential to develop into prostate

cancer under chronic inflammatory conditions [66, 67]. Inflammatory conditions arise from several potential factors. Some include hormonal play, i.e. estrogen, dietary changes, urine reflux, and bacterial, or viral, infections [68]. Another PCa risk factor includes mutations or variants of inflammatory genes; TLR (toll-like receptors), MIC1 (Microneme-associated protein 1), MSR1 (macrophage scavenger receptor 1), and IL1RA (Interleukin 1 Receptor Antagonist) [69-72]. Single nucleotide polymorphisms of TLR4, for example, were found to be associated with risk of early prostate cancer. However, this was also found to be correlated with ethnicity [69, 70].

### *2. Mechanisms of Inflammation-Induced Tumorigenesis*

Neoplastic development can be initiated via an inflamed environment. This is done through altering cells within the local environment from a benign state to a malignant phenotype. Alterations include angiogenesis, cellular and tissue remodeling, and metastasis [24]. Having an environment filled with proinflammatory cytokines and inflammatory cells, neoplastic transformation is induced via several methods [24].

#### a. Generation of Reactive Oxygen and Nitrogen Species (ROS and RNS)

Inflammatory cells, such as neutrophils and macrophages, often synthesize several toxic molecules to eradicate foreign particles during infections. Such molecules include reactive oxygen species (ROS), reactive nitrogen species (RNS), and hydrogen peroxide (H2O2). Other hazardous compounds involve the hydroxyl radical (•OH), nitric oxide (NO), organic peroxides, singlet oxygen and the superoxide anion (•O2-) [73]. These compounds are synthesized via NADPH oxidase, an extracellular membrane-bound enzyme complex, and release ROS into the microenvironment [24].

Within the inflamed environment, the host loses the ability to process the produced oxidized agents balancedly. Enzymes and antioxidant molecules, which play a role in detoxifying the environment, continue to overstress the environment due to being in an overwhelmed state [74]. These reactive compounds eventually build up and cause mutagenic damages and genome-destabilizing DNA lesions [75]. Some damages include either arrested or promoted transcription, induced point mutations, replication errors, or DNA repair inhibition [76]. G to T transversion, a type of point mutation, was seen in Ras [77] and p53 genes [78] in several cancers which brings about the potential of ROS and RNS activating or inactivating proto-oncogenes and tumor suppressor genes, respectively. Other alterations that can contribute to tumorigenesis, can occur at an epigenetic level. These damages occur via aberrant DNA methylation and histone modifications [79].

b. Inflammatory Tumor–Microenvironment

The activation of TLRs, on the surface of innate immune cells, gives way to several intracellular events such as the activation of NF-κB signaling pathways. This, in turn, produces higher amounts of proinflammatory cytokines, chemokines, and NO [80]. Furthermore, these damages alter the growth and migration of epithelial cells, which produces more cytokines and chemokines [80]. TAMs release interleukins and prostaglandins which act to suppress anti-tumor responses. Additionally, they release angiogenic factors, such as endothelin-2 [81] and VEGF [82], to increase tumorigenic vascularization. Furthermore, tumor cell migration and proliferation are stimulated via the release of epidermal growth factors. Other functions of TAMS include the degradation of the basement membrane of cells and remodeling stromal matrices. This further promotes the invasion of tumor cells and metastasis [83]. Proinflammatory transcription

factors, expressed in tumor cells, further induce the production of cytokines, chemokines, and enzymes that contribute to building an inflammatory microenvironment [84]. Signaling, using autocrine and paracrine methods within the tumor microenvironment, may be altered and lead to inflammation. This promotes a carcinogenic environment that promotes cell survival, proliferation, and metastasis [84].

c. Inflammasomes

Inflammasomes are another component found within an inflamed environment. They are protein complexes assembled by innate immune cells and are characterized by having pattern recognition receptors [85]. These proteins play a role in promoting an inflammatory response, by activating interleukins, via the regulation of caspase-1 [85]. These interleukins, such as IL-18, induce the production of IFN-γ in NK cells and T-cells which promotes anti-pathogen responses via the production of reactive oxygen and nitrogen species [86].

d. Cytokines and Chemokines

Cytokines and chemokines form a complex network of interactions within the inflammatory environment. This network, consisting of complex signaling between stromal, immune, and tumor cells, promotes tumorigenic behavior such as cell survival and replication [87]. Cytokines are compounds that regulate the behavior of immune cells such as activation and differentiation; they could be peptides, proteins, or glycoproteins [87]. Examples include interleukins (IL); those associated with prostatic diseases include IL-1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, and IL-23. Other important cytokines include TNF-α and TGFβ [88]. Certain interleukins, such as IL-6,

IL-8, have been well-studied and documented to play an important role in prostate carcinogenesis [87, 89].

IL-6 is released by a variety of cells including inflammatory cells, PCa cells, and stromal cells [90]. Some of its roles include acute phase inflammation mediation, B-cell and T-cell differentiation and activation regulation, and cell growth support. IL-6 is shown to be secreted by PCa cells upon exposure to a macrophage-filled environment and undergoing an inflammatory response [90]. Tumor-infiltration, via myeloid and lymphoid cells, aids in the progression of PCa development. Tumor phenotypes identified the majority of these infiltrates to be CD4+ cells. T-helper 17 cells differentiation, derived from CD4+ lymphocytes, are regulated via IL-6 [91].Myeloid-derived suppressor cells, which play a role in suppressing T-cells, have been found to play a role in PCa development and are also regulated via IL-6 [92]. Moreover, IL-6 also induces an epithelial–mesenchymal transition (EMT) in the morphology of benign prostate epithelial cells to acquire an invasive phenotype. Certain pathways are seen to be activated in IL-6 induced PCa cell growth [92].

Interleukin-8 (IL-8) is another cytokine that plays a role in the development of PCa. Like IL-6, it is secreted by several cells and factors such as NF-κB, TNF-α, IL-1β, chemotherapy agents, hormones, and steroids. IL-8's functions are mediated by CXCR1 and CXCR2, two surface receptors. The functions include the induction of several signaling pathways which could contribute to tumorigenesis [93]. IL-8 promotes angiogenesis, via a paracrine manner, and oncogenic signaling and invasion, via an autocrine fashion [94]. Androgen-sensitive cell lines have lower levels of IL-8 compared to androgen-non-responsive, metastatic, or advanced stages of cell lines [93]. Introducing IL-8 to androgen-responsive cell lines resulted in the downregulation of

androgen-receptor, proliferation with less androgen-dependence, higher levels of tumor cell survival factors such as AKT, SRC, and NF-κB, increased VEGF production, higher resistance to antiandrogens, increased motility and invasion, and increased production of matrix metalloproteinases [93, 94]. Additionally, IL-8 was shown to recruit myeloid-derived suppressor cells within the microenvironment. This was demonstrated in a clinical study that revealed high circulating levels of these cells, along with IL-8 and IL-6, and increased with later stages of the disease [94].

e. Inflammation-induced epithelial to mesenchymal transition

Growing evidence indicates that by triggering a developmental mechanism known as epithelial to mesenchymal transition (EMT), the tumor microenvironment transmits inflammatory signals that increase the metastatic potential of cancer [95]. The transition from an epithelial to mesenchymal phenotype occurs at the epithelial lining via the disintegration of cell-to-cell or cell-to-ECM adherence [96, 97]. One key mechanism is the breakdown of E-cadherins, an important component of the adherence junctions in epithelial linings. This is often correlated with the initiation of EMT that will eventually subside intercellular communications [95]. Upon EMT, important mesenchymal markers are heightened. These include N-cadherin, vimentin, zinc proteins, E-cadherin transcriptional repressors, among others. These markers eventually give the mesenchymal phenotype with enhanced cell motility [98].

As stated earlier, immune cells, like DCs, TAMs, NK cells, regulatory T cells, neutrophils, B cells and MDSCs, constitute a significant proportion of the microenvironment of the tumor and function as mediators of inflammation-induced EMT [96]. Cells must have the potential to undergo this process regardless of their oncogenic content in order for EMT to occur and have adequate signals that facilitate

the induction of EMT [95]. In addition, studies have shown that when induced with TGF-β1, epithelial cells will undergo different degrees of EMT, resulting in the activation of SMAD transcription factors that then stimulate EMT proteins [99, 100]. Furthermore, oncogenic pathways such as Ras, Notch, and Hedgehog are shown to be involved in stemness by TGF-β induction of EMT [101]. TAMs, MDSCs, and Tregs generate TGFβ1 within the tumor microenvironment, worsening cancer to a more destructive and invasive state [102-104]. Reciprocally, for immune cell recruitment and polarization, tumor cells generate TGF-β1; these cells come together to form a tumor-permissive microenvironment that induces EMT [24, 96].

An important cytokine that plays a role in inducing systemic inflammation is Tumor necrosis factor (TNF). During early tumorigenesis, TNF is involved in recruiting inflammatory cytokines, epithelial adhesion and growth factors to the wounded tissue [82]. TNF also plays a role in angiogenesis, an important hallmark of cancer, by inducing angiogenic enzymes and factors [105].Some players include fibroblast growth factors, VEGF, and thymidine phosphorylase. One important factor, induced by TNF, is nuclear factor-κB (NF-κB), a transcription factor that supports many genes involved in carcinogenesis, i.e. STAT3 [106]. The NF-κB pathway becomes disrupted, in PCA, which triggers the progression to an androgen-independent stage. This further progresses to a castration-resistant condition that becomes fatal. The activation of the NF-κB is seen as an important event for surveillance of PCA progression [107]. This is due to the observation of consecutive NF-κB activation in prostate tumors with metastatic potential, not in benign prostate tissues [108].

## F. Effectiveness of NSAIDs and Statins on Prostate Cancer

### *1. NSAIDs*

Non-steroidal anti-inflammatory drugs (NSAIDs) are a family of drugs which have the ability to suppress pain and fever through inflammation inhibition. Cyclooxygenase enzyme (COX-1 and COX-2) activity on their corresponding receptors can be reversibly inhibited through NSAIDs. Cyclooxygenase, also named prostaglandin-endoperoxide synthase (PTGS), is an enzyme family responsible for prostanoids formation. NSAIDs are also found to reduce incidence or even prevent progression of PCa COX-2 pathway suppression [109]. Additionally, they can induce cancer cell apoptosis and protect or repair DNA from damage. COX-1 is found to be expressed in many tissues while COX-2 expresses specificity in prostate inflammatory cells in PCa. Aspirin is seen to irreversibly inhibit, non-specifically, both COX-1 and COX-2 [110].

In a clinical study, the use of aspirin or NSAIDs was found to be associated with a decreased risk of PCa. Another study looked at the effects of aspirin in radical prostatectomy or radiation therapy treated localized prostate cancer. Results showed that aspirin-users, especially in high-risk patients, had a lower disease-specific mortality compared to non-users. When looking at the association between the pre-diagnosis use of aspirin in low doses and mortality in newly diagnosed PCa patients, there was no protective association; however, a small reduced risk of disease-specific mortality was still observed. Higher aspirin dosage had stronger associations. A significant association with lower disease-specific mortality was observed in high-risk PCa patients in a cohort study assessing post-diagnosis aspirin usage. Furthermore, a reduced risk of distant metastasis was observed with the use of pre- or post-diagnostic NSAIDs [109]. A Swedish cohort study assessed a large sample of aspirin/non-aspirin NSAIDs users and found that

there was a decreased risk of prostate cancerogenesis in long time use of non-aspirin NSAIDs users. Aspirin's protective factor was found to be enhanced upon the simultaneous use of statins, seen in 63% of aspirin users. The statin's enhancing effect was seen upon removal from the analysis and resulting in a decrease effect of aspirin and non-aspirin NSAIDs [110].

Piroxicam

Piroxicam (PXM) is an NSAID drug belonging to the oxicam family of drugs belonging to the oxicam group of NSAIDs [111]. PXM has shown anti-tumor properties in cell lines such as human malignant mesothelioma and human oral squamous cell carcinoma cells, via apoptotic activity [112]. Furthermore, a study tested the effects of PXM on breast cancer cell lines, showing effectiveness as a potential therapeutic drug. The drug acted on a time and dose-dependent behavior. Cell viability assays showed that PXM induced apoptosis on the MCF-7 cell line. The exposure caused an accumulation of ROS with an upregulation of Akt phosphorylation, making it essential for apoptotic activity [112].With MDA-MB-231 cells, there was downregulated Akt phosphorylation with insignificant influence on ROS levels. The effect of the drug on other breast cancer cell lines showed that metastatic potential could be an important factor [112]. In one study, PXM was tested with antibiotherapy to assess treatments for prostate abnormalities with high PSA levels, however, it was not shown to be highly effective as a stand-alone treatment [113]. There was little literature on the effects of PXM on PCa. This leaves room for further exploration of PXM on PCa, and its progressive stages, to assess its full role as a potential therapeutic NSAID drug.

### *2. Statins*

Statins are drugs that act on the active site of the catalyzing enzyme ,3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, through inhibition [114]. This enzyme aids in the conversion of HMG-CoA into mevalonate within the cholesterol biosynthesis pathway [115]. These drugs are commonly prescribed as part of hypercholesterolemia and cardiovascular disease treatment. Clinical studies suggest that statins may play a role in the prevention of prostate carcinogenesis by lowering cholesterol levels and reducing raft-dependent signaling via lipid raft disruption which in turn reduces PCa cells proliferation and survival [116]. Additionally, isoprenoid synthesis inhibition, plasma membrane adhesion prevention, and small GTPase proteins activation, which are all players in cellular proliferation, differentiation, apoptosis, and migration, are observed. Moreover, there is a decrease in pro-inflammatory cytokines being secreted [116].

There are two generally proposed mechanisms to statins in prostate cancer [117]. The first mechanism is an indirect effect via its cholesterol-reducing properties which in turn increases chances to inhibit prostate carcinogenesis [118]. There are reports that androgens could be synthesized from cholesterol in late-stage PCa cells, hence making them independent of testicular androgens [118]. Androgens and their receptors (AR) play a significant role in castration-resistant prostate cancer (CRPC) progression shown by elevated levels of cholesterol-induced steroid precursor of androgens, pregnenolone, testosterone, and dihydrotestosterone; this in turn activates androgen receptors [119, 120]. In CRPC, cholesterol and its regulation are altered within the cell, which could indicate that regulated cholesterol could be a precursor for androgen, synthesized de novo within the tumor, in the absence of testicular androgens. The second mechanism is via a direct

effect. Studies have shown the apoptotic and antiproliferative properties of statins in PCa via several mechanisms. First of these is the inhibition of mevalonate production which in turn reduces cholesterol production and isoprenoid synthesis. Second, Thr-160 phosphorylation of cdk2, and thus its activity, is inhibited. This in turn enhances P21 needed for apoptosis. Third, fanesyl pyrophosphate and geranyl pyrophosphate can be inhibited to suppress proliferation and migration of cancer cells as they are precursors of Ras and Rho translation to the cell membrane [117].

The effects of statin still remain under debate [109]. Almost all meta-analyses that looked into statin-use randomized clinical trials have concluded an insignificant effect of statin on reducing the risk of prostate cancer risk; one meta-analysis found that statin-using patients showed a significant reduction in advanced prostate cancer incidence. These trials contained several limitations, which may impact the analysis and conclusion of statin use. Overall, these studies are inconclusive to the use of statin in overall prostate cancer risk. And so, denying statin as a protective factor should be avoided. This inconclusive state is also seen in a 3.5-year longitudinal study when neither a positive nor a negative correlation between the use of statin and PCa risk was deduced [116]. A retrospective cohort study, however, found a statistically significant reduction in prostate cancer mortality after examining the usage of statins post PCa diagnosis with a higher risk reduction in statin-using patients' pre-diagnosis and during treatment. Another retrospective cohort study also assessed PSA levels and found that non-statin users had a significantly higher risk of PCa with PSA levels approximately 8% lower in statin-users when compared to nonusers [116]. Observational studies further demonstrate disparities in the inconclusive results due to several factors such as health-care access, lifestyle, dietary factors, comorbidities, and others [116].

The potential protective effects of statins in PCa were demonstrated in preclinical studies using cell culture. PC-3 cells were treated with lipophilic and hydrophilic statins to examine its effects on bone marrow stroma invasion. Clonogenic assays showed that the number of colonies were significantly reduced in the stroma coculture, with the use of lipophilic statins, with a cellular morphological change of via inhibiting lamellipodia formation. This, however, was not seen with hydrophilic statins, due to the potential need for active transport across the cell membrane. This was suggestive of the idea that preventative measures in PCa were not demonstrated through cholesterol synthesis reduction [116]. In vitro studies showed the protective effects of statins in PCa through cell apoptosis induction, arresting the G1 cell cycle phase, autophagy, and the androgen receptors degradation. A study found that a lipophilic statin drug, simvastatin, accumulates into cells and induces necrosis in PC-3 cells at 10 μM but demonstrating toxicity to human cells [116]. In vivo studies revealed that certain pathways that regulate angiogenesis, tumor invasions, and apoptosis, are affected by statins. One pathway could be via the inhibition of Akt phosphorylation and prenylation of oncogenic proteins. Another mechanism is through dysregulating the mevalonate pathway that could limit the oncogenic proteins production [116].

Atorvastatin

A synthetic statin, used in investigating its role on PCa cells, is Atorvastatin. The 3-hydroxy-3-methylglutaryl CoA reductase inhibitor has been clinically used for hypercholerolemia. The drug has been found to be involved in inducing apoptosis in PCa. The reductase inhibitor interferes with the synthesis of geranylgeranyl-pyrophosphate and farnesyl-pyrophosphate among others. Geranylgeranyl-pyrophosphate plays an important

role in the function of Rho proteins while farnesyl-pyrophosphate for Ras. These molecules play a vital role in signaling cell proliferation and survival. Thus, studies assume that atorvastatin could possibly interfere with Ras/Rho activity, thus inhibiting growth and inducing cancer cell apoptosis [121]. While this is implied, it has been demonstrated that Atorvastatin reduces oxidative stress and inflammation through possible metabolites [122].

## G. Aims of the Study

Upon examining NSAIDs and Statins, it is shown that they comprise a therapeutic potential in treating PCa. Using PXM and Ato can be an example of how previously approved drugs can be repurposed and used in treatments other than their initial traditional use, especially being considered in different medical fields, mainly cancer.

Henceforth, the overall aim of this thesis project is to investigate the effect of Piroxicam (PXM) and Atorvastatin (Ato), alone or in combination, on murine PCa cell lines, PLum-AD (androgen-dependent) and PLum-AI (androgen-independent) cells, in 2D and 3D cultures.

# CHAPTER II

# MATERIALS AND METHODS

## H. Cell Culture:

### *1. PLum-AD and PLum-AI: Murine Prostate Cancer Cell Lines*

Our laboratory has previously generated a novel murine *in vitro* system which recapitulates PCa progression from a primary androgen-dependent state, namely PLum-AD cells, to an advanced androgen-independent state, namely PLum-AI cells [123, 124]. Molecular, functional and pathophysiological characterization of both murine cell models has been formerly observed validating that PLum-AD cells, indeed, represent primary PCa while PLum-AI cells display an aggressive nature and thus, representing the advanced stages of the disease [124].It is important to note that both cells carry the same genetic background (*Pten-/-TP53-/-*) [123, 124].

### *2. Cell Growth*

PLum-AD and PLum-AI cell lines were cultured and maintained in Advanced DMEM/F12 medium (Gibco) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich), 5µg/mL Plasmocin Prophylactic (InvivoGen), 1% Glutamine (Sigma-Aldrich), 1% Gentamicin (Gibco), 1% HEPES (Sigma-Aldrich), and 1% Epidermal Growth Factor (EGF). Cells were incubated at 37°C in a humidified incubator containing 5% $CO_2$. Typically, the media over the cells was replenished every 2-3 days. When cells reached 70 to 80% confluency, they were washed with phosphate buffered saline (PBS) then they were trypsinized with trypsin- ethylene-diamineteraacetic acid (EDTA) for five minutes at 37˚C. The action of Trypsin was then inhibited by a ratio of 1:1 complete media with

10% FBS. Cells were then centrifuged for 5 mins at 900 rpm to obtain a pellet. The pellet was then resuspended in media and transferred into new 25 $cm^2$ tissue culture flasks for maintenance and to be expanded before an experiment. For starting any experiment, cell number was calculated using a hemocytometer according to the following formula: cells per 1ml = average number of cells x dilution factor x volume of suspension x $10^4$. Cells were counted using 0.4% trypan blue dye exclusion assay.

### *3. Preparation of PXM and Ato*

Piroxicam (PXM) was supplied as a powder from Sigma-Aldrich and was reconstituted in dimethylsulfoxide (DMSO) at a final concentration of 75 mmol /l solution.

Atorvastatin (Ato) was supplied as a powder from Biorbyt and was dissolved in DMSO at a final concentration of 20 mmol /l. The two drugs were aliquoted and stored at -20˚C.

## I. MTT Cell Proliferation Assay:

The anti-proliferative effect of PXM and Ato *in vitro* was investigated using the MTT ([3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay. PLum-AD and PLum-AI were plated in triplicates in 100 µl complete media in 96-well culture plates, at a density of $2x10^3$ cells per well. Cells were incubated overnight, then treated with DMSO or various indicated concentrations of PXM alone, Ato alone, or the combination of both drugs diluted in complete media for 24, 48 and 72 hours. For each time point, the media containing treatment was removed, fresh complete media was added, and 10µL of 5mg/mL MTT reagent (dissolved in 1X PBS) was added to each well

and incubated at 37°C for 3 hours. In this step, metabolically active/viable cells had the ability to convert the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals due to the high levels of NADH and NADPH, which is a measure of mitochondrial metabolic activity. After which the reagent was removed and 100µL of solubilizing solution (Isopropanol) was added to solubilize the formed crystals. The plate was covered by foil and incubated for 1 hour at room temperature. Finally, the reduced MTT optical density was measured at a wavelength of 595nm using an ELISA reader (Multiskan EX). The percentage of cell proliferation is expressed as percentage growth relative to control wells and treated wells at indicated concentrations. The data are derived from the mean of triplicate wells of three independent experiments.

**J. Cell Viability / Trypan Blue Exclusion Assay:**

The effect of PXM and Ato *in vitro* on the viability of cells was assessed using trypan blue dye exclusion method. The technique is based on the disruption of the cell membrane that distinguishes non-viable from viable cells. Trypan blue is an azo dye that is cell impermeable and therefore only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins, thereby rendering the cell a blue color and thus viable cells can be visualized under microscope as white cells compared to the blue-colored non-viable cells. In this experiment, cells were seeded in duplicates in 300µl media in 24-well plates, at a density of $20\times10^3$ cells per well. Cells were then treated with DMSO or various indicated concentrations of PXM or Ato for 24, 48, and 72 hours. At each time point, cells were washed with PBS, detached by trypsin, collected, and resuspended in 1000 µl media. 50µl of cell suspension was then mixed with 50µl of trypan blue and cells were counted on the four corner chambers of a

hemocytometer by the previously mentioned formula. Cell viability is expressed as percentage growth relative to control and treated wells. The data is derived from the mean of duplicates wells of three independent experiments.

**K. Wound Healing Assay:**

The ability of PLum-AD and PLum-AI to migrate was assessed using the scratch/wound healing assay. For this assay, $15\times10^4$ cells were seeded in duplicates in a 24-well plate and were incubated until 80 to 90% confluence. Cells were then treated with 10 mg/mL of Mitomycin C (Sigma-Aldrich) for 30 to 45 mins to block cellular proliferation and ensure that the effect of wound closure is due to cell migration and not to cell proliferation. Next, a uniform scratch was made down the center of the well using a 200 µl micropipette tip. Detached cells were removed by washing with PBS and the remaining attached cells were cultured in complete media, with or without treatment. Microscopic photos were subsequently taken at 0, 3, 6, 9 and 15 hours. The distance traveled by the cells into the wounded area was computed from the closure of the wounds. Quantification of the distance of the wound closure was assessed over time and results are expressed as the percentage of the wound closure upon treatment compared with the control condition. The data is derived from the mean of duplicates wells of three independent experiments.

**L. Sphere-Formation Assay:**

This assay was performed according to a protocol optimized in our laboratory [123, 125]. $2x10^3$ cells/well of 24-well plates were suspended in cold growth factor-reduced Matrigel ™ /serum- free Advanced DMEM medium (1:1) in a total volume of

10 μL. PLum-AD and PLum-AI cells were seeded uniformly in a circular manner around the bottom rim of the well and allowed to solidify in the incubator at 37°C for 1 hour. Subsequently, 100 μL of serum-free media, with or without treatment, was added gently in the middle of each well. Spheres were replenished with warm media every 2 days and were counted after 6 to 7 days. The count was assessed using sphere count formula (% of control): Sphere Count (in %) = (number of spheres counted ÷ number of control spheres) × 100. Results are represented as a percentage of the sphere count of the treated spheres compared with the untreated ones. The average diameter of spheres was calculated (average of at least 30 spheres per condition). Zeiss Axiovert microscope was used for the acquisition of bright-field images.

**M. Statistical Analysis:**

Statistical analysis was performed using GraphPad Prism 6 analysis software. The significance of the data was analyzed using one-way and two-way analysis of variance (ANOVA). P values of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were considered significant.

# CHAPTER III

# RESULTS

## A. Piroxicam and Atorvastatin reduce cell proliferation of PLum-AD and PLum-AI cell lines *in vitro* using MTT assay

First, our objective was to assess the effect of PXM and Ato, alone or in combination, on the proliferation of the two murine PCa cell lines, using the MTT assay (**Figure 3**, **Figure 4**, and **Figure 5**). The anti-proliferative effect of PXM and Ato, alone or in combination, on PLum-AD and PLum-AI, which represent primary and advanced murine PCa cells, respectively, was reflected by a potent reduction in the proliferation rate, detected at different concentrations, in a dose- and time-dependent manner.

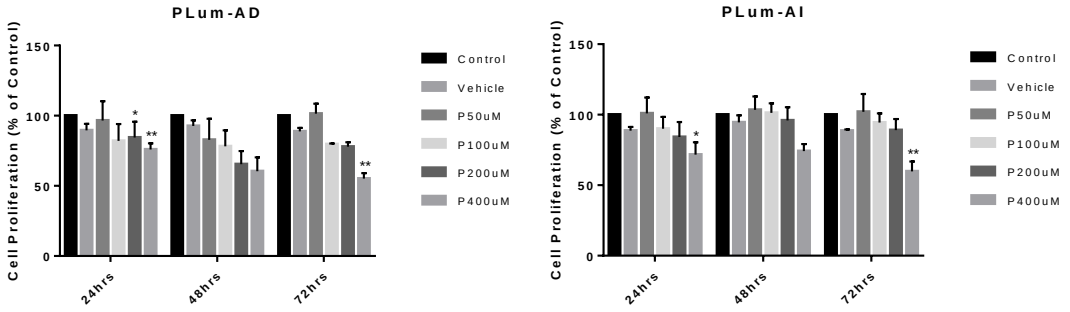


**Figure 3. Piroxicam reduces cell proliferation of both PLum-AD and PLum-AI in a dose-dependent manner.** After incubation of PLum-AD and PLum-AI cells for 24, 48, and 72 h with or without treatment (PXM), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

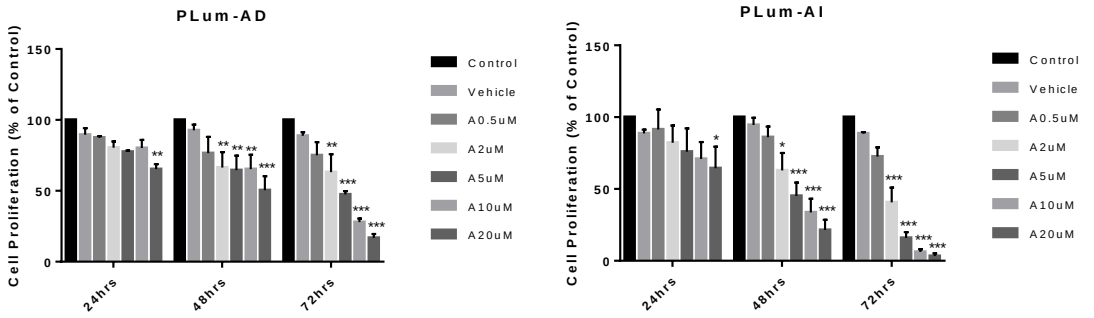


**Figure 4. Atorvastatin reduces cell proliferation of both PLum-AD and PLum-AI in a time and dose-dependent manner.** After incubation of PLum-AD and PLum-AI cells for 24, 48 and 72 hours with or without treatment (Ato), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

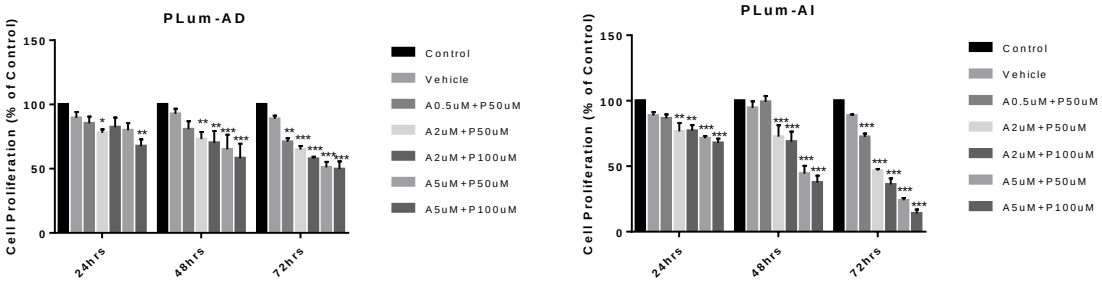


**Figure 5. The combination of Atorvastatin and Piroxicam concentrations didn't show an enhanced effect on cell proliferation of both PLum-AD and PLum-AI in a dose and dose-and-time-dependent manner, respectively.** After incubation of PLum-AD and PLum-AI cells for 24, 48 and 72 hours with or without treatment (Ato+PXM), cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

### B. Piroxicam and Atorvastatin inhibit PCa viability *in vitro* using Trypan Blue Exclusion assay:

As treatment with PXM and Ato showed anti-proliferative activities on both murine PCa cell lines, we further examined their effect on the cells' viability. Using the trypan blue exclusion method, treatment with PXM and Ato, alone and in combination, reduced the viability of the PLum-AD and PLum-AI cells, in a time- and dose-dependent manner. These results are consistent with the previous ones obtained with the MTT assay **(Figure 6, Figure 7,** and **Figure 8).**

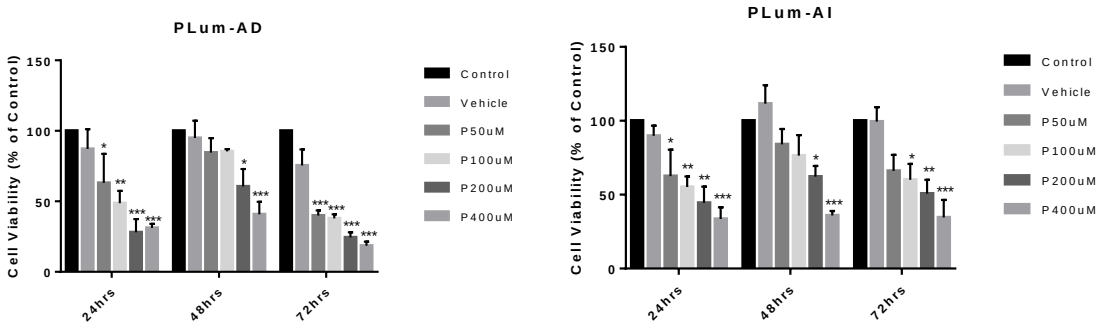


**Figure 6. Piroxicam reduces cell viability of both PLum-AD and PLum-AI in a dose-dependent manner.** After incubation of PLum-AD and PLum-AI cells for 24, 48, and 72 hours, with or without treatment, cell viability was determined using Trypan Blue assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

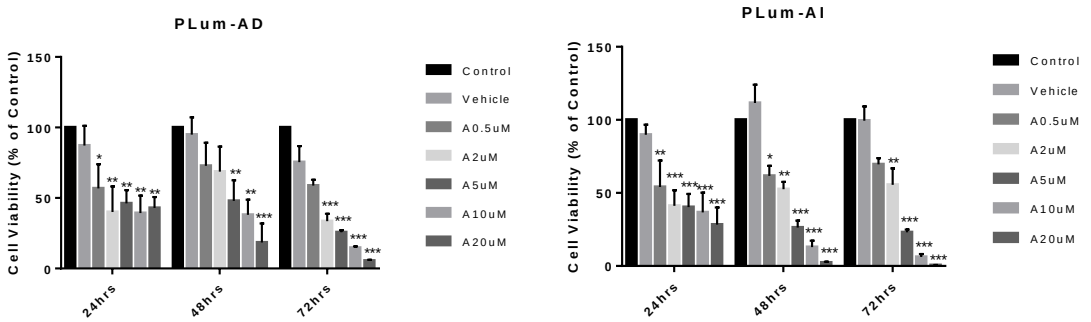


**Figure 7. Atorvastatin reduces cell viability of both PLum-AD and PLum-AI in a time and dose-dependent manner.** After incubation of PLum-AD and PLum-AI cells for 24, 48, and 72 hours, with or without treatment, cell viability was determined using Trypan Blue assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

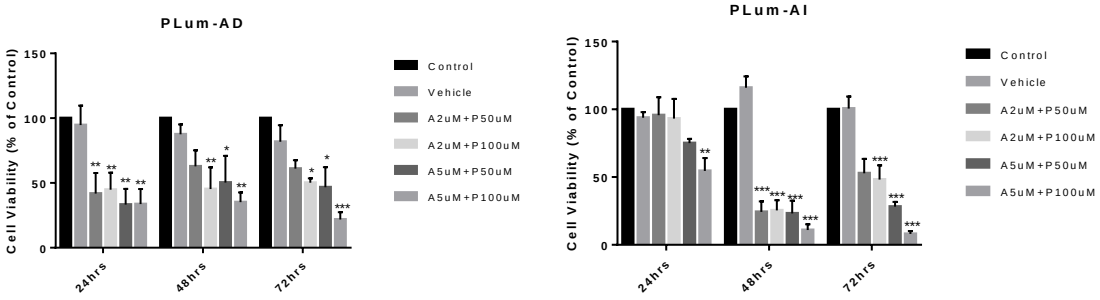


**Figure 8. The combination of Atorvastatin and Piroxicam didn't show an enhanced effect on cell viability of both PLum-AD and PLum-AI in a dose and time-and-dose-dependent manner, respectively.** After incubation of PLum-AD and PLum-AI cells for 24, 48, and 72 hours, with or without treatment, cell viability was determined using Trypan Blue assay. Results are expressed as a percentage of the treated group compared to its control at every time point. Data represent an average of three independent experiments and are reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

### C. Piroxicam and Atorvastatin attenuate PCa cell migration *in vitro* using wound healing assay:

Next, we investigated the effect of PXM and Ato drugs on the migratory ability of both murine PCa cell lines using a scratch/wound healing assay. This assay confirms that primary localized PCa can turn aggressive and develop the potential to metastasize to distant body organs mainly bones, brain, lymph nodes, liver, and thorax [126]. Also, it allows us to observe a mimicked version of the migration patterns of invasive tumor cells *in vivo*, and to further promote our understanding of cell-to-cell interactions.

In this experiment, PLum-AD and PLum-AI cells were either left untreated (control) or treated with indicated concentrations of PXM and Ato, alone or in combination. Cellular proliferation was inhibited with mitomycin C prior to wound formation. Representative bright-field images were taken at three different time points.

The results obtained showed that, while the wound almost completely healed in the control condition, 15 h treatment with PXM and Ato, alone or in combination, significantly suppressed the wound closure/migration ability of both cell lines compared to the control **(Figure 9, Figure 10, Figure 11)**. The results obtained reveal that PXM and Ato display an inhibitory effect on PCa cell migration, and potentially invasion.

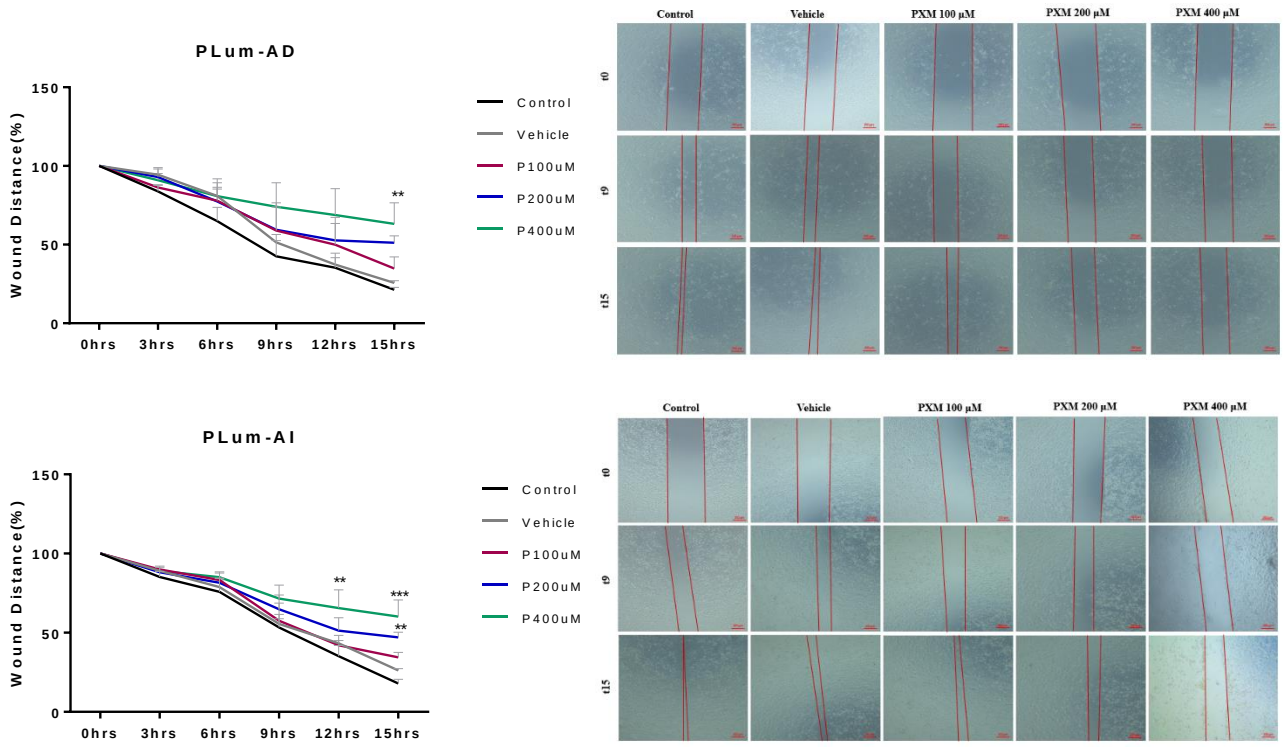


**Figure 9. Piroxicam reduces cell migration of PLum-AD and PLum-AI in a time-dependent manner.** After the cells reached 80 to 90% confluency, a scratch was made in a 24-well plate using a 200 µL tip. Representative bright-field images were taken at 5x magnification every three hours, with or without treatment, showing the effect of PXM on cell migration. The distance of wound closure (wound distance in %) in both cell lines was assessed over time, analyzed by the Carl Zeiss Zen 2012 image software, and represented by the following graphs. The data represents an average of three independent experiments and was reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

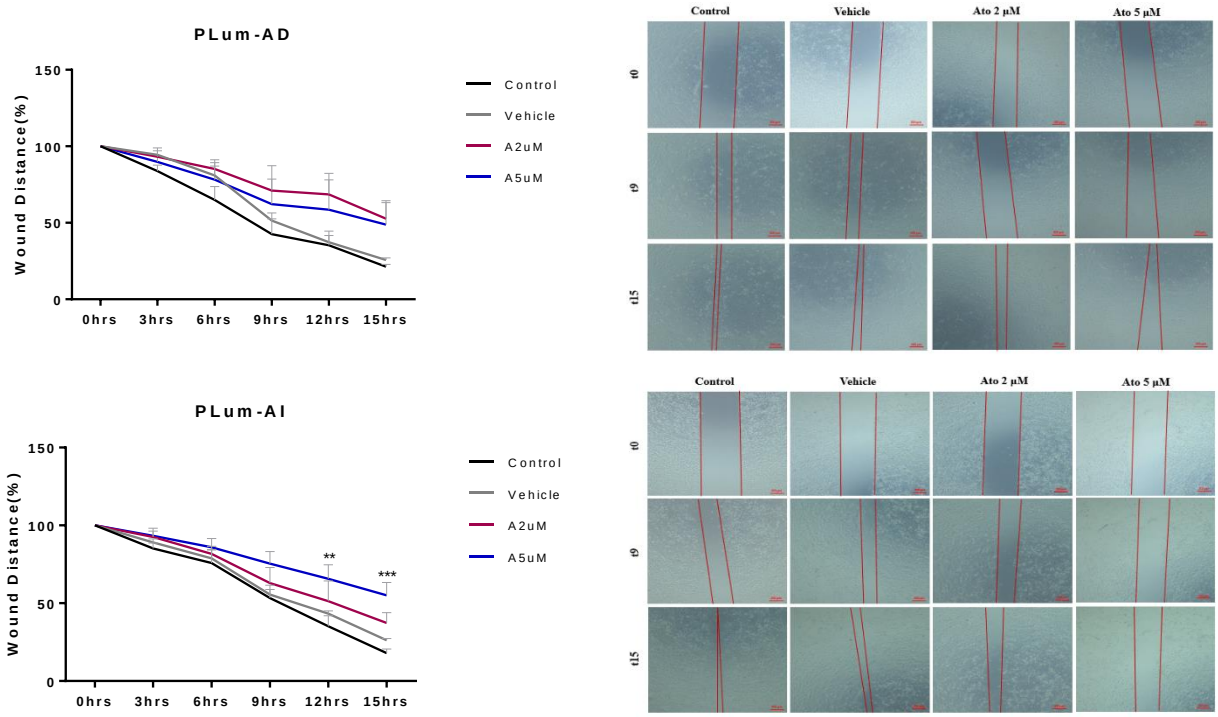


**Figure 10. Atorvastatin reduces cell migration of PLum-AD and PLum-AI in a time-dependent manner.** After the cells reached 80 to 90% confluency, a scratch was made in a 24-well plate using a 200 µL tip. Representative bright-field images were taken at 5x magnification every three hours, with or without treatment, showing the effect of Ato on cell migration. The distance of wound closure (wound distance in %) in both cell lines was assessed over time, analyzed by the Carl Zeiss Zen 2012 image software, and represented by the following graphs. The data represents an average of three independent experiments and was reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

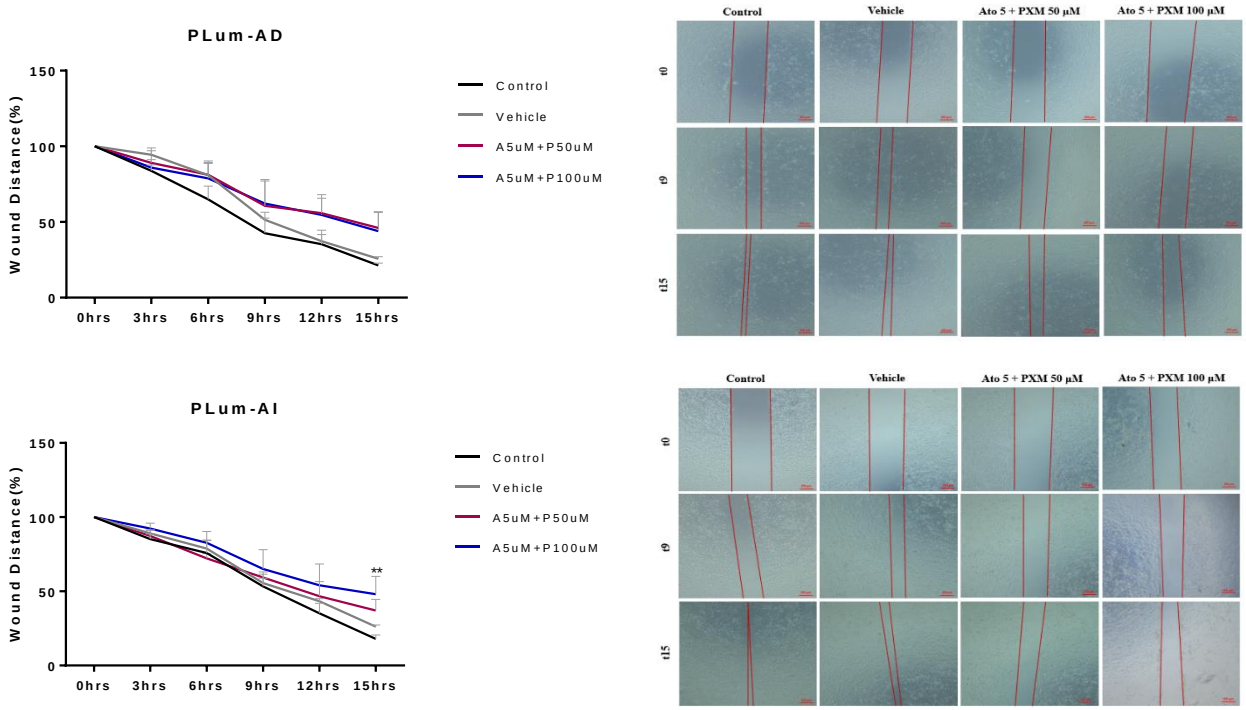


**Figure 11. The Combination of Atorvastatin and Piroxicam reduces cell migration of PLum-AD and PLum-AI in a dose and time-dependent manner.** After the cells reached 80 to 90% confluency, a scratch was made in a 24-well plate using a 200 µL tip. Representative bright-field images were taken at 5x magnification every three hours, with or without treatment, showing the effect of the combination of Ato and PXM on cell migration. The distance of wound closure (wound distance in %) in both cell lines was assessed over time, analyzed by the Carl Zeiss Zen 2012 image software, and represented by the following graphs. The data represents an average of three independent experiments and was reported as mean ± SEM ($p<0.01$, Two-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

### *D.* Piroxicam and Atorvastatin both decrease the stem/progenitor cell properties of PLum-AD and PLum-AI *in vitro*

The ability to grow as non-adherent spheroids in sphere medium has been widely used to assess the self-renewal capability of CSCs and is one of the characteristics of prostate CSCs. To examine the effects of PXM and Ato on the CSCs population of PLum-AD and PLum-AI, the sphere-forming assay was performed.

Matrigel™ was used to embed single-cell suspensions of PLum-AD and PLum-AI which were maintained for 6 to 7 days. The stem/progenitor cell-like properties of both cell lines were evaluated upon exposing the cell lines to treatment with PXM and Ato, alone or in combination. An inverted light microscope was used to visualize, count, and measure the size of the generated prostate spheres, in the different treatment conditions compared to the control **(Figure 12A, and Figure 13A)**.

Our results revealed that the number and the size of the cultured prostatospheres both had a significant reduction in both cell lines upon treatment with PXM and Ato **(Figure 12 and Figure 13)**. This suggests that treatment with PXM and Ato potentially targets the stem/progenitor-like population.

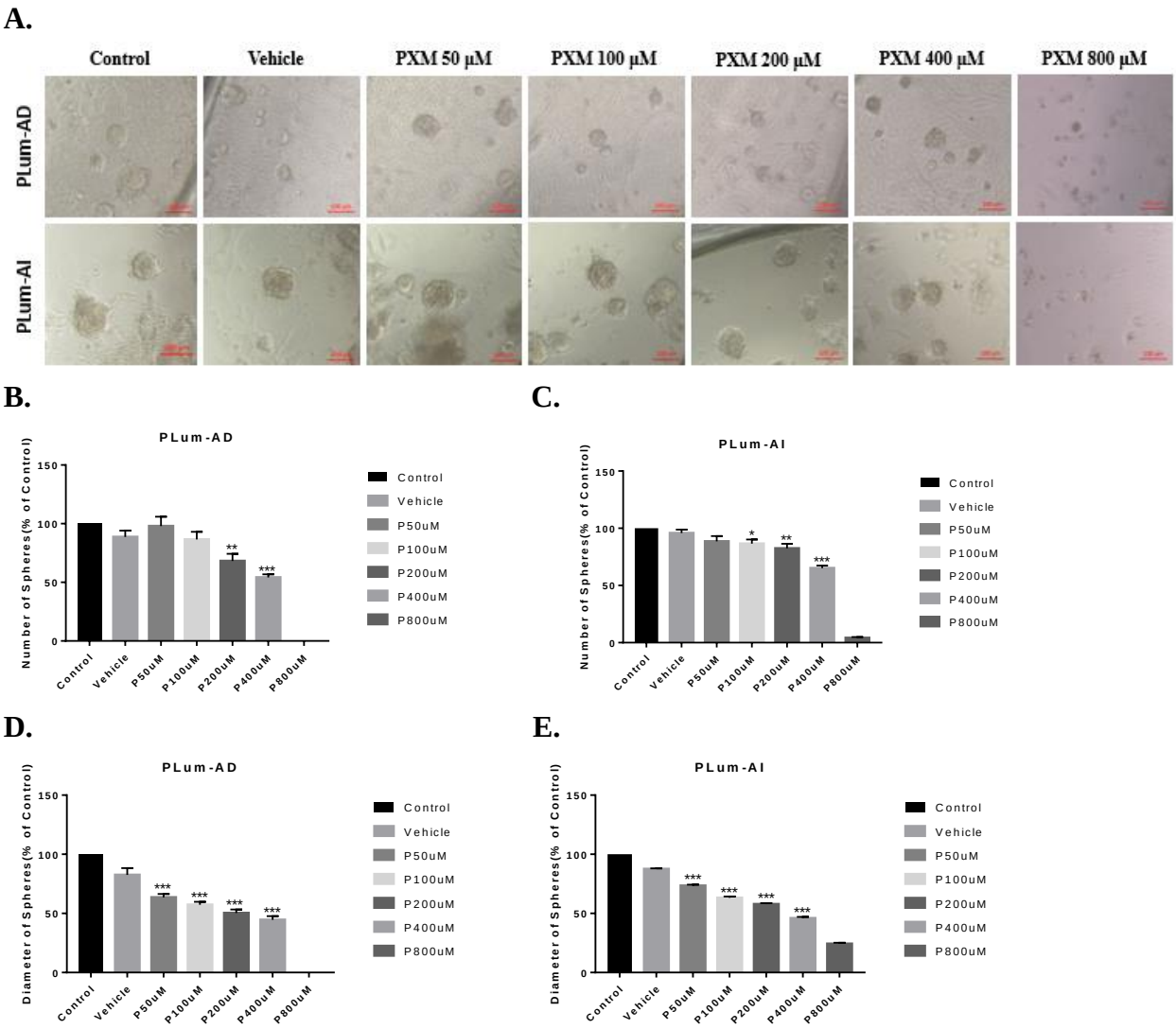


**Figure 12. Piroxicam augments the sphere count and the diameter of both PLum-AD and PLum-AI spheres. (A)** Representative bright-field images of PLum-AD and PLum-AI spheres with the different conditions embedded in Matrigel$^{TM}$ at day 7 after plating. Scale bar = 100 µm. Images of cultured spheres for all conditions were visualized and taken by Axiovert inverted microscope and analyzed by Carl Zeiss Zen 2012 image software. Spheres formed in each well were counted at days 6 to 7 for both cell lines. Incubation of the spheres with PXM significantly decreased the number of spheres in both cell lines **(B, C)**. When measuring the diameter of the spheres formed, a significant effect of PXM was observed in PLum-AD and PLum-AI cells, compared to their controls **(D, E)**. Data represent an average diameter (µm) of 30 to 40 measured spheres in each condition of three independent experiments, except for PXM 800 µM which was represented from only two independent experiments. Data are reported as mean ± SEM ($p<0.01$, One-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

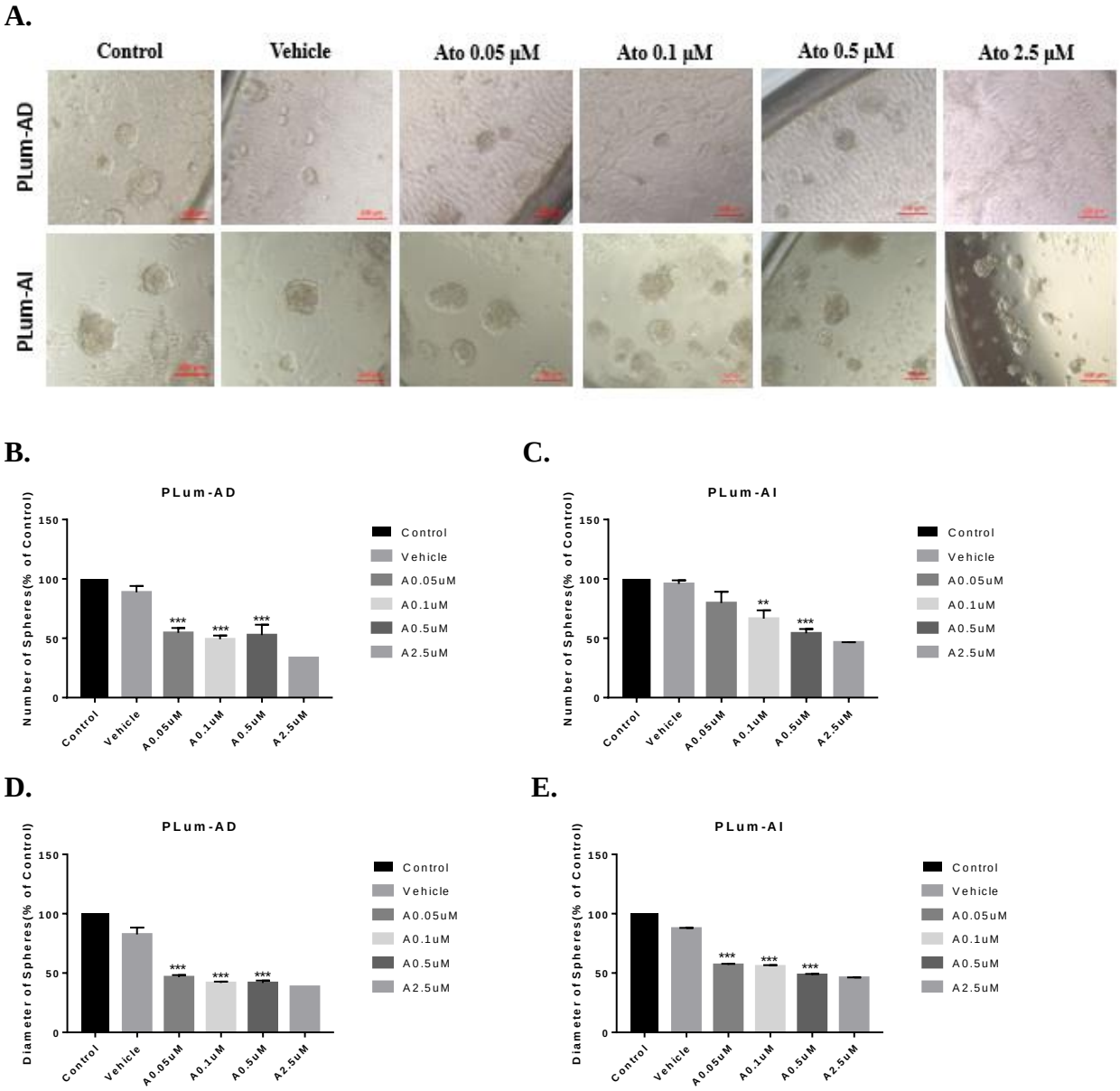


**Figure 13. Atorvastatin augments the sphere count and the diameter of both PLum-AD and PLum-AI spheres. (A)** Representative bright-field images of PLum-AD and PLum-AI spheres with the different conditions embedded in Matrigel™ at day 7 after plating. Scale bar = 100 µm. Images of cultured spheres for all conditions were visualized and taken by Axiovert inverted microscope and analyzed by Carl Zeiss Zen 2012 image software. Spheres formed in each well were counted at days 6 to 7 for both cell lines. Incubation of the spheres with Ato significantly decreased the number of spheres in both cell lines **(B, C)**. When measuring the diameter of the spheres formed, a significant effect of Ato was observed in PLum-AD and PLum-AI cells, compared to their controls **(D, E)**. Data represent an average diameter (µm) of 30 to 40 measured spheres in each condition of three independent experiments, except for Ato 2.5 µM which was represented from only two independent experiments. Data are reported as mean ± SEM ($p<0.01$, One-way ANOVA; * $P<0.05$; ** $P<0.01$; ***$P<0.001$).

# CHAPTER IV

# DISCUSSION

With the lack of full understanding of what makes PCa develop and progress, the observation of how certain drugs can interfere in a disease's development offers insight in these ambiguous mechanisms. These drugs are then repurposed in the aim of targeting cancer and halting its progression. Their cytotoxic effects are investigated and utilized for potentially offering a treatment to cancer. Current and recent studies are looking into how drugs can be repurposed to tackle the complex tumor microenvironment that is key to the tumorigenesis and progression of PCa.

NSAIDs were shown to reduce overall and advanced prostate cancer risk in extensive epidemiological studies and meta-analysis [127, 128]. Their effects also reduced prostate cancer specific mortality rates in high-risk patients [129, 130]. In regards to statins, the cholesterol-lowering drugs have demonstrated reduced aggressiveness and prostate-cancer specific mortality [131-134].

The cells' proliferation, viability, migration, and stemness are known to play an important role in prostate cancer development and progression. So the aim of our study was to look into the antineoplastic effects of PXM and Ato at different concentrations, on PLum-AD and PLum-AI cell lines. We started by analyzing the anti-proliferative effects of those drugs in reducing cellular proliferation which is problematic in the administration of treatment. MTT and trypan blue assays demonstrated the significant reduction of proliferation and viability, respectively, when high concentrations of PXM and Ato were used within 72 hours of incubation. This work exhibited the sensitivity of PLum-AD and PLum-AI cells in a time- and concentration-dependent manner. An MTT assay showed that different concentrations of PXM and Ato treatment greatly inhibited the proliferative

ability of our PCa cell lines. Cell viability assay also showed a significant reduction in cell viability upon administration of the PXM and Ato drugs. Our results are in concordance with other studies. He et al. showed that the combination of aspirin with atorvastatin significantly reduced the growth and induced apoptosis of prostate cancer cells [135]. Our results also showed consistency with previously established results of these drugs tested on other cancer cell lines [136-138] such as canine mammary tumor cell line CMT-U27 [137]. Rai et al. showed that PXM induced apoptotic cell death of the human breast cancer cells MCF-7 but not of the more aggressive breast cancer cells MDA-MB-231 [112].

Next, we continued by analyzing the anti-migration effects of our drugs. Migration is an important step in cancer progression. The ability of cancer cells to metastasize to other organs is the most endangering feature of malignancy. Cancer cells migrate, invade the ECM, enter the blood circulation, fixate to a new site, and finally egress to form distant foci [139]. Therefore, we tested the effect of PXM and Ato on migration using wound healing assay. Our results showed that both drugs significantly decreased the ability of metastatic prostate cancer cells to migrate. Treated cells failed to migrate and close up the scratch whereas the control wound of both PLum-AD and PLum-AI sealed within around 15 hrs. This experiment suggested that PXM and Ato have high inhibitory properties of metastasis. Our drugs showed this potential in harmony with other literature which also showed the inhibitory effect of migration in different cancer cell types. A study by Wynne et al. showed that PCa cell migration was inhibited by NSAID through Nag-1 induction via the p38 MAPK-p75NTR pathway [140]. Moreover, a meta-analysis by Zhao et al. investigated that the NSAID exposure, pre- or post-diagnosis, reduced the risk of distant metastasis. This reduction was significant in prostate and breast

cancer [141]. In addition, Ato has indicated a significant influence on melanoma and breast cancer cells by reducing their metastasis to the lung and bone [142-144]. Interestingly, this was in agreement with a study that showed the ability of lipophilic statins to remarkably reduce prostate cancer migration and colony formation in the bone marrow stroma [145].

The presence of a small subset of stem-like cancer cells, or CSCs, have played a role in causing drug resistance and therapy failure. These cells have capabilities of differentiation, self-renewal, and tumor initiation, making them an important target for identification to provide insight of PCa development. This calls for the need of developing a treatment that targets both PCa cells and CSCs. The ability to target these cells via these drugs has been suggested. Hence, a 3D sphere-formation assay was done to study the in vitro growth of prostate epithelial stem/progenitor cells. Tumor sphere-formation capacity was suppressed in PLum-AD and PLum-AI cells when treated with PXM and Ato; and this was demonstrated via the inhibition of number of tumor spheres formed and reduced sizes. This demonstrates the potential of both drugs to play a role in suppressing the tumor and preventing recurrence. However, an alternation in results was interestingly detected in this 3D assay, as the drugs had a higher effect of reduction on PLum-AD than PLum-AI spheres. This wasn't the reported case in the previous 2D experiments where the drugs were more effective on PLum-AI cells. This furthermore highlights the importance of this 3D sphere-formation assay, mimicking the physiological environment, rendering it more relevant and predictive than 2D cultures [146]. In spheres, the cell morphology mimics its natural form since cell-cell and cell-ECM interactions closely resemble what occurs naturally *in vivo*, yielding valuable results. This provides us with

more accurate data about cell-cell interactions, proliferation, viability, migration, stemness, and hence better drug discovery [147, 148].

*Future Directions and Conclusion:*

Therefore, future research on fully understanding and targeting of CSC populations is a must. In vivo survival and efficacy studies are also suggested when analyzing the effects of PXM and Ato in PCa animal models. Last but not least, future prospects should look into the precise molecular mechanisms which could help in developing strategies that target specific processes that could inhibit PCa progression.

In conclusion, we investigated the therapeutic potential of PXM and Ato in inhibiting cell proliferation, viability, migration, and stemness of PLum-AD and PLum-AI prostate cancer cell lines. We validated this on a cellular level, using 2D and 3D cultured cells. These preliminary data are encouraging and beg for further investigation. Our findings highlighted the importance of drug repurposing and its influence on potentially improving cancer treatment.

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