RETROSPECTIVE EVALUATION OF THE CLINICAL SIGNIFICANCE OF MULTIDRUG RESISTANCE PROTEINS IN POOR RESPONSE TO TREATMENT OF PC

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ABSTRACT

PC remains one of the most significant public health issues contributing to mortality in Sub-Sahara Africa, with South Africa being one of the most affected. Increased resistance to chemotherapy in PC patients is fast becoming a major concern and has led to increase investigation of its mechanisms. The present study investigated the expression of two genes, ABCB1 and CYP1B1, implicated in drug resistance in cancer cells. We also applied bioinformatics to unravel the molecular pathways and cascades linked to drug resistance that may be triggered following upregulation of these genes. Samples were collected from archived PC patient specimens obtained through pre-treatment biopsies. cDNAs synthesized from RNAs isolated from the samples, were subjected to qPCR analysis. qPCR analysis revealed a low expression of ABCB1, with concomitant high expression of CYP1B1 in PC cells. Gene enrichment and network analysis revealed ABCB1 to be associated with ABC transporters and LncRNA-mediated mechanisms of therapeutic resistance WP3672, while CYP1B1 is associated with ovarian steroidogenesis, tryptophan metabolism, steroid hormone biosynthesis, benzo(a)pyrene metabolism WP696, sulindac metabolic pathway WP2542 and estrogen receptor pathway WP2881. Both ABCB1 and CYP1B1 were associated with microRNAs in cancer and nuclear Receptors Meta-Pathway WP2882. STRING analysis further predicted a protein-protein interaction of ABCB1 and CYP1B1 with Glutathione S-transferase Pi; Catechol O-methyltransferase; UDP-glucuronosyltransferase 1-6; Leucine rich Transmembrane and O-methyltransferase (LRTOMT); and Epoxide hydrolase 1, and a score of 0.973, 0.971, 0.966, 0.966 and 0.966, respectively. Furthermore, molecular docking analysis of the regimen drug, docetaxel and CYP1B1 revealed a potent molecular interaction, with a binding energy of -20.37 Kcal/mol. These results indicate the susceptibility of the studied cancer patients to drug resistance via increased expression of ABCB1 and CYP1B1 in tumour samples of the poor responders' category, and their associated molecular pathways. This is further depicted by the potent molecular interaction of CYP1B1 with docetaxel.

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DECLARATION OF INDEPENDENCE

I, Lemohang Gumenku, hereby declare that the dissertation entitled: **Retrospective Evaluation of the Clinical Significance of Multidrug Resistance Proteins in Poor Response to Treatment of PC,** submitted by me for Masters in Biomedical Technology at Central University of Technology, is my own independent work and has not previously been submitted by me at another University or Faculty for admission to a degree or diploma or any other qualification.

__13/12/2023___

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SUPERVISOR'S DECLARATION

I, Prof. M.P. Sekhoacha, the supervisor of the dissertation entitled: **Retrospective Evaluation of the Clinical Significance of Multidrug Resistance Proteins in Poor Response to Treatment of PC**, hereby certify that the work in this project was done by Lemohang Gumenku at the Health Science Department, Central University of Technology, and the Department of Pharmacology, University of the Free State.

I hereby approve submission of this dissertation and affirm that this has not been submitted previously to this or any other situation for admission to a degree or any qualification.

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Signature Date

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LIST OF ABBREVIATIONS

ADT	**Androgen Deprivation Therapy**
AI	**Artificial Intelligence**
AJCC	**American Joint Committee on Cancer**
AR	**Androgen Receptor**
AR-VT	**Androgen Receptor Variant**
CRPC	**Castration-Resistant PC**
CYP	**Cytochrome**
DCE	**Dynamic Contrast-Enhanced**
DNA	**Deoxyribonucleic Acid**
DRE	**Digital Rectal Examination**
FDA	**Food and Drug Administration**
FFPE	**Formalin Fixed Paraffin Embedded**
G	**Gleason**
HGPIN	**High Grade Prostatic Intraepithelial Neoplasia**
HSREC	**Health Science Research Ethics Committee**
IACR	**International Agency for Cancer Research**
ISUP	**International Society of Urological Pathology**
LH	**Luteinizing Hormone**
LHRH	**Luteinizing Hormone Releasing Hormone**
mCRPC	**Metastatic Castration-Resistant PC**
MDR-1	**Multidrug Resistance 1/P-glycoprotein**
mL	**Milliliter**
ML	**Machine Learning**
mpMRI	**Multiparametric Magnetic Resonance Imaging**

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MRI	**Magnetic Resonance Imaging**
MRP	**Multidrug Resistance Proteins**
NHLS	**National Health Laboratory Service**
NHREC	**National Health Research Ethics Committee**
PC	**Prostate Cancer**
PIN	**Prostatic Intraepithelial Neoplasia**
PIRADS	**Prostate Imaging-Reporting and Data System**
PSA	**Prostate-Specific Antigen**
RNA	**Ribonucleic Acid**
RT–PCR	**Reverse Transcription Polymerase Chain Reaction**
TNM	**Tumor Lymph Node Invasion Metastasis**
TPUS-GB	**Trans Perineal Ultrasound-Guided Biopsy**
TRUS	**Transrectal Ultrasound**
TRUS-GB	**Transrectal Ultrasound-Guided Biopsy**
WHO	**World Health Organization**

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CHAPTER ONE: STUDY OVERVIEW

1.1 Introduction

With a startling daily diagnosis rate of over 4,300 cases and roughly five Prostate Cancer (PC)-related fatalities each day, PC poses a significant health challenge in South Africa (Foundation, 2016). The urgency of the situation is highlighted by the high incidence and deficiencies of the available treatments. A number of distressing clinical symptoms, such as dysuria, oligospermia, hemospermia, and urinary complications, are associated with PC, including its aggressive progression and frequent metastasis (Xie, 2019). Chemotherapy is the main treatment option for managing metastatic PC, but alarmingly high treatment failure rates are now a reality due to the emergence of drug resistance. The prediction that PC's epidemiological burden will increase unless novel strategies to combat drug resistance and metastasis are investigated is of the utmost importance (Hee Choi, 2014).

Individual responses to treatment vary widely, a phenomenon that is attributed to the interaction of several genes that control drug response and toxicity. Evidence suggests that genetic markers involved in drug transport and metabolism have potential as more accurate predictors of therapeutic success. Particularly through polymorphisms in the genes responsible for drug resistance and metabolism, genetic diversity plays a crucial role in determining how differently each patient responds to the same treatment regimen (Abidi, 2013). South African researchers have not yet fully delved into this therapeutic frontier, despite international research beginning to harness the potential of genetic biomarkers for predicting drug responses in cancer treatment.

By examining the relationship between drug responses and potential genetic polymorphisms connected to drug transport and metabolism, this research project sets out on a mission to close this gap. Its ultimate goal is to create a comprehensive pharmacogenetic database that can predict chemo-resistance specific to patients, providing crucial information about possible treatment outcomes. The ABC transport proteins, particularly Multidrug resistance 1 (MDR-1), which are crucial for drug delivery, and the Cytochrome (CYP) 450 metabolic enzymes, such as CYP1B1 and CYP3A4, which have implications for chemotherapy metabolism, clinical chemo-resistance, and the overall outcome of chemotherapy, are the study's main focus areas.

In light of this pressing healthcare concern, it is imperative to explore new avenues for enhancing PC treatment, particularly in the advanced castration-resistant PC (CRPC) setting. While first-line chemotherapy with docetaxel has become standard, questions persist regarding the efficacy of prednisone alongside docetaxel (Petrylak, 2004).

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Moreover, the emergence of resistance to docetaxel highlights the need for predictive biomarkers to guide treatment decisions, an area where current clinical practice is deficient. This study seeks to address these critical issues, offering a novel approach to addressing molecular strategies to combat drug resistance in PC patients.

1.2 Problem Statement

PC is a significant public health concern in South Africa (particularly the CRPC), with a high rate of mortality. The mortality rate has been linked to development of drug resistance, leading to inefficacy of chemotherapy and treatment failure. Epidemiological projections indicate that the burden of PC is expected to increase, making it imperative to explore new avenues to combat drug resistance and metastasis. This study addresses the pressing issue of PC treatment resistance and seeks to uncover molecular strategy of drug resistance in PC patients.

1.3 Rationale for the Study

1. *High Incidence and Mortality*: PC is a serious health issue in South Africa, where there are over 4,200 new cases diagnosed every day and a substantial amount of deaths. High mortality rates are caused by the limited specificity and efficacy of current treatment options.

2. *Chemotherapy challenges*: Despite being an essential treatment option for metastatic prostate tumors, chemotherapy is frequently unsuccessful due to drug resistance. To improve patient outcomes, drug resistance strategies must be developed.

3. *Genetic Variation in Drug Response*: Genetic factors that affect drug transport and metabolism cause individual responses to chemotherapy to vary. More individualized treatment strategies and increased treatment effectiveness may be made possible by understanding the genetic markers linked to drug resistance.

4. *Pharmacogenomics' Potential*: By customizing therapies to patient profiles, pharmacogenomics, the study of how genetics affect drug response, has the potential to completely transform the way cancer is treated. Despite its enormous potential, South Africa has not conducted much research on the use of pharmacogenomics in the treatment of .

1.4 Aim of the study

The aim of the research is to profile proteomic and genetic variants of identified biological markers that influence chemo-resistance in PC treatment, from stored specimen (formalin fixed paraffin embedded (FFPE) tissues) of PC patients who have received chemotherapy treatment.

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1.5 Study objectives

The specific objectives are:

1. To collect archived PC patient specimens obtained through pre-treatment biopsies, distinguishing them into three categories: poor responders, good responders, and responders with significant toxicity after receiving chemotherapy.
2. To apply gene expression analysis to investigate the association between patients' response to treatment and genetic variations of selected biomarkers in the patients.
3. To evaluate the expression of target proteins (MDR-1 and CYP1B1) in obtained specimens and correlate the expression levels to patients' response to treatment.
4. To apply bioinformatics in correlating expressed genes with pathways linked to chemo-resistance and treatment outcome in PC patients and develop recommendations.

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CHAPTER TWO: LITERATURE REVIEW

2.1 Functional Anatomy of the Prostate Gland

The prostate gland is the male reproductive system's largest accessory exocrine organ (Figure 2.1). It stores and secretes seminal plasma, a moderately alkaline fluid that accounts for 50-75% of the human sperm volume (3mL). This fluid is necessary for the spermatozoa's longevity as well as the liquefaction of the sperm. In healthy individuals, the prostate gland is about the size of a walnut/chestnut and weighs between 7 and 16g (Bhavsar, 2014). The prostate gland is found in the sub-peritoneal compartment between the pelvic diaphragm and the peritoneal cavity, posterior to the lower portion of the symphysis pubis, anterior to the rectum, and interior to the urinary bladder. The prostate gland, sometimes known as the "walnut gland," is conical in shape and surrounds the proximal urethra as it exits the bladder (Centers for Disease Control and Prevention, 2020).

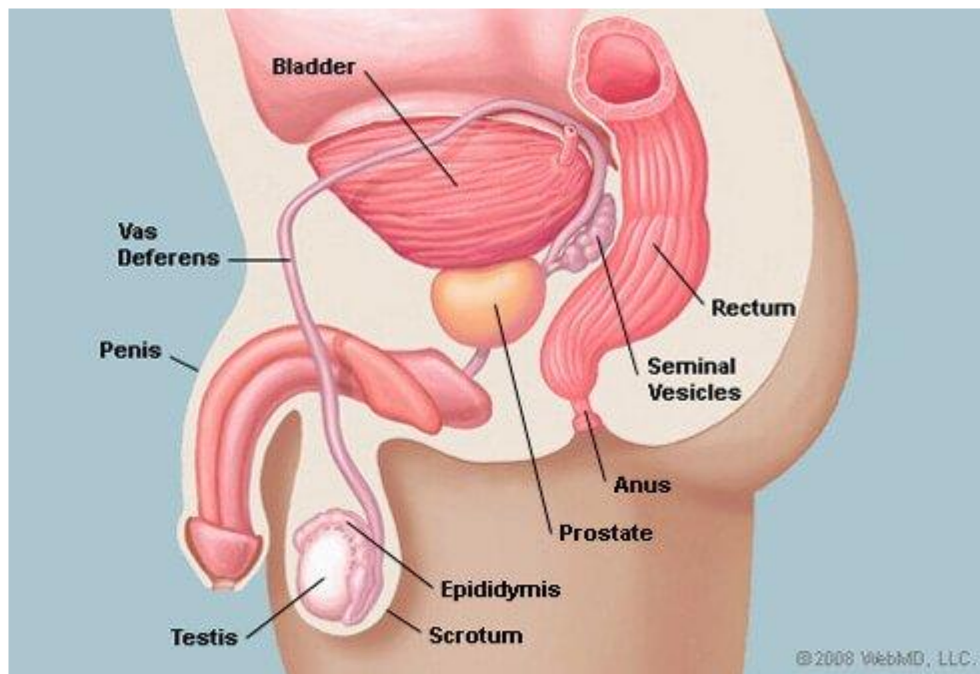

Figure 2.1 Sagittal View of the male pelvis with the prostate gland's position. Adapted without permission (Sharma, 2017).

The human prostate gland has three distinct glandular zones: peripheral zone, which is mainly composed of the posterior part of the gland surrounding the distal urethra; central zone surrounding the ejaculatory ducts, and transitional zone surrounding the proximal urethra and continues to enlarge throughout life (Figure 2.2). Virtually 70-80% of PC occurs in the peripheral zone and approximately 10-20% in the transitional zone. Only

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about 2.5% of PC occurs in the central zone, and inclined to be more aggressive (Leskela, 2007).

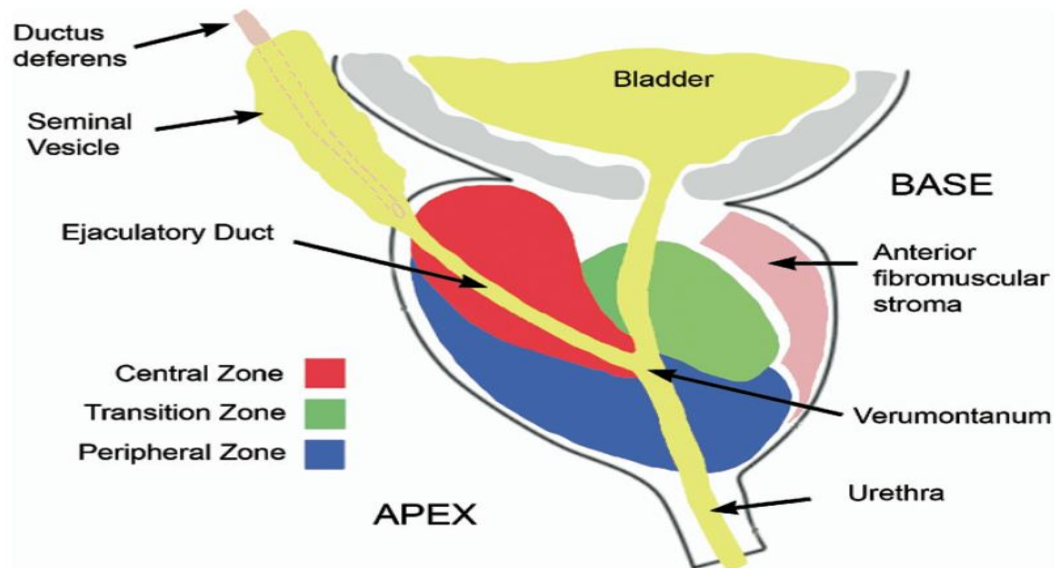

Figure 2.2 Sagittal view of prostate gland. Adapted without permission (Cohen, 2008).

2.2 Basic Concepts in Cancer

Cancer is a leading cause of death worldwide. In 2020, the World Health Organization (WHO) and the International Agency for Cancer Research (IACR) reported 19.3 million new diagnoses of cancer, and 10 million cancer deaths (Sung, 2021). Since genetic mutation is the primary cause of cancer development, all malignancies are genetic in nature. A tumor can arise from the accumulation of just somatic mutations, from the germline inheritance of one or more mutations followed by the acquisition of additional somatic mutations (Oláh, 2005).

Chemical carcinogens like beryllium, arsenic, benzene, asbestos, cadmium, chromium, nickel, ethylene oxide, radon, and vinyl chloride have been implicated in the pathogenesis and progression of cancer (Adeola, 2016). As shown in Figure 2.3, carcinogenesis is grouped into four stages. Cellular origins of tumors and how they are driven by cellular components, are identified in each of the stages. Alteration of a cell's genetic makeup is the first step of the initiation procedure. The injured cells release chemicals that are carcinogenic, which mutate the nucleic acids of adjacent cells, making them malignant. In order to provide the affected cells with a unique environment to develop into malignant and immortalized cells, promotion during the expansion stage leads the affected cells to

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accumulate as well as a change in the fundamental circumstances of the subsequent round of target cells. Tumor promoters might not be able to start the malignant stage because cancer cells already have self-promoter activity (Jones, 2002; Weston, 2003).

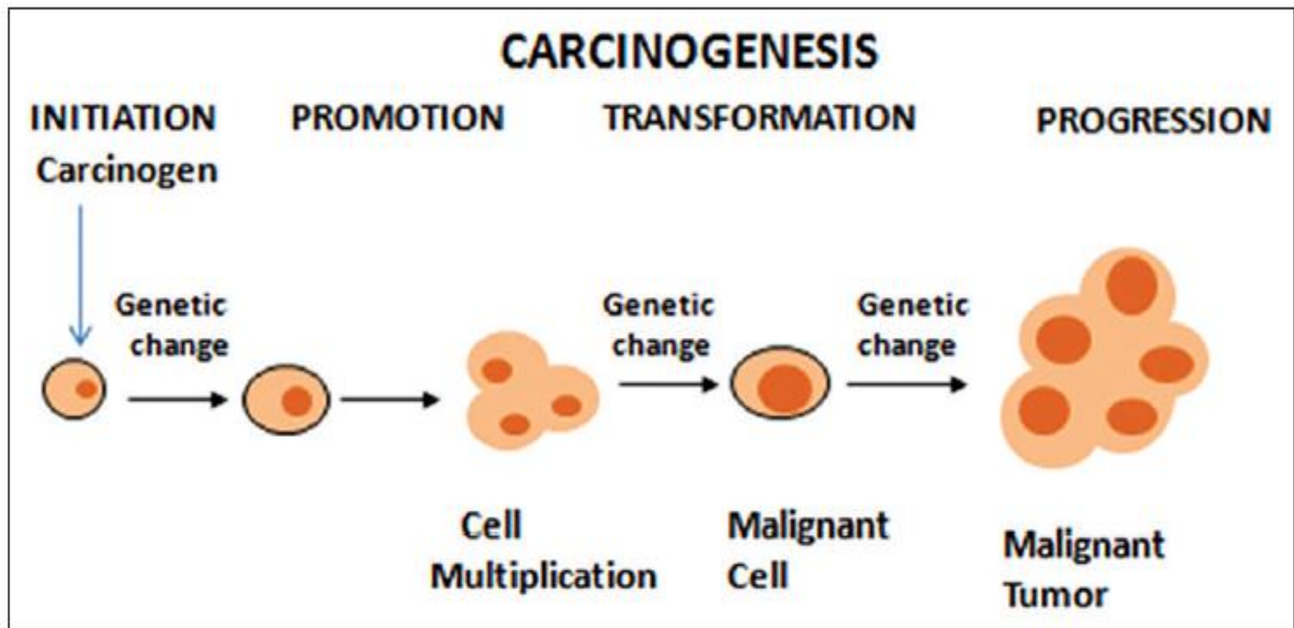

Figure 2.3 Different stages of carcinogenesis. Adapted without permission (Kaur, 2014).

2.3 Prostate Cancer

PC is a heterogeneous adenocarcinoma that originally develops from the epithelial cells in the peripheral zone of the prostate gland, which is in the male reproductive system (Semenas, 2012). PC poses a threat to male health starting at the age of 40 years. It clinically manifests with dysuria, oligospermia, hemospermia, frequent micturition, and involuntary urination (Xie, 2019). PC is characterized by a rapid rate of growth and metastasis (Figure 2.4). The various genetic and enzymatic changes that occur at each stage of this growth cycle provide the tumor cells an advantage over their normal counterparts in terms of survival and may also confer treatment resistance (Herberts, 2022). The transformation of healthy epithelial prostate cells into malignant epithelial prostate cells involves a wide range of genetic alterations in molecular pathways that control normal cell proliferation and differentiation, including androgen receptor (AR) signaling (Ho, 2008).

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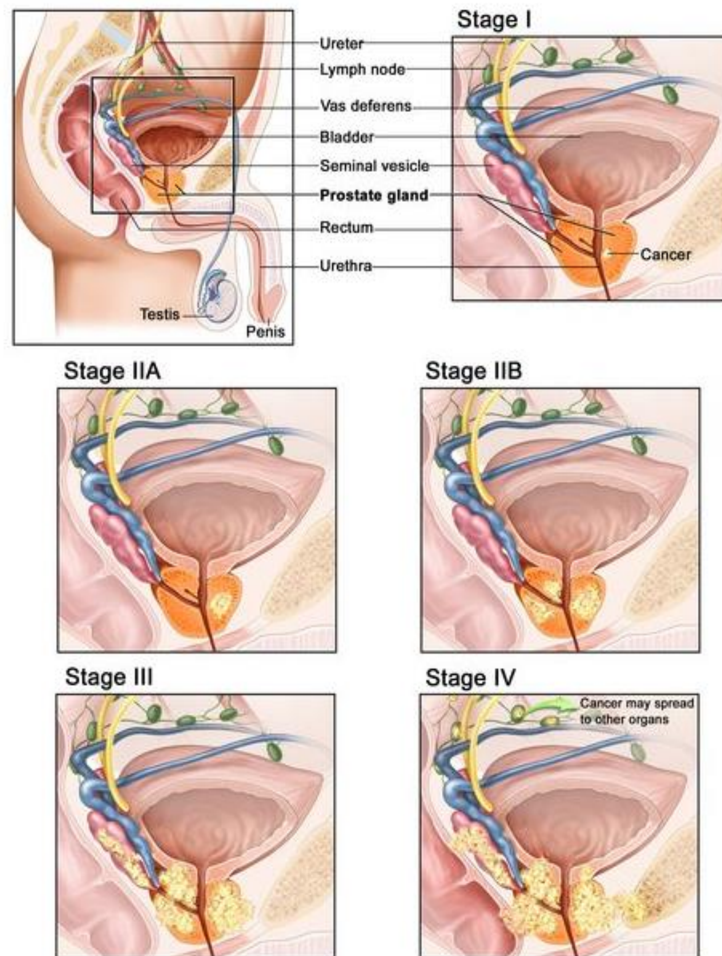

Figure 2.4 Staging of PC. Adapted without permission (Board, 2021).

2.3.1 Prostate Cancer Epidemiology

PC has a significant global impact, ranking among the top five cancers in terms of both incidence and mortality (Sung, 2021). Insights into the involvement of individual risk factors and population screening habits in the epidemiology of PC can be gained by looking at patterns in PC incidence and mortality across the global population over time. In 2020, GLOBOCAN/ IARC reported 19.3 million PC cases and 10 million PC deaths to have occurred worldwide. In terms of mortality, PC was estimated as the leading cause of cancer deaths in 46 countries located in sub-Saharan Africa and Latin America, with few exceptions (Sung, et al., 2021). In South Africa, around 4300 men are diagnosed with PC each day, and roughly five men die from the disease (Foundation, 2016). Despite extensive study into the prevention, identification, and treatment of PC, the success of cancer pharmacotherapy is hampered by multidrug resistance (Hee Choi, 2014).. Hence, the need for more research to identify new cancer drug targets, understand factors that influence response to treatment, and suggest appropriate regimes for individual patients

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based on their genetic and proteomic profile of specific biomarkers. It is hypothesized that this approach could lead to reduced disease-related mortality rate.

2.3.2 Etiology and Risk Factor

Several ways that individual biology and lifestyle factors affect the chance of developing PC have been identified by epidemiologic studies of the disease (Pernar, 2018). Well-established PC risks include advanced age, ethnicity, genetic variables, and family history (Table 2.1). Other factors linked to PC are diet (such as increased consumption of saturated animal fat and red meat, lower intake of fruits, vegetables, vitamins, and coffee), obesity, physical inactivity, inflammation, hyperglycemia, infections, and environmental exposure to chemicals or ionizing radiation (Rawla, 2019).

PC is the most often diagnosed malignancy in elderly men, and it is uncommon in men under the age of 40. Similar to other epithelial malignancies, PC incidence increases faster after age 55. This pattern may be observed in both developing and developed countries, as well as with the incidence of PC worldwide (Kimura, 2018). Several studies have suggested that genetic predisposition may play a role in inherited genetic background especially when it has been linked to elevated risks of PC in several studies, accounting for roughly 5% of disease risks (Ferris-i-Tortajada, 2011). When high-penetrance genetic "risk" alleles are inherited, the risk is multiplied by several orders of magnitude, as opposed to more prevalent low-penetrance sites that only increase the risk slightly. Major susceptibility loci for PC have been discovered on genes in seven separate loci, according to gene linkage studies. African males are more likely to have chromosomal 8q24 polymorphisms, which have been linked to an increased risk of PC (Freedman, 2006; Okobia, 2011). Some studies have found that Africans have a high rate of polymorphisms in tumor-suppressing genes such EphB2 (Robbins, 2011) and cell-apoptosis-regulating genes like BCL2 (Hatcher, 2009). African men have also been reported for a more aggressive type of the disease, which has been linked to genetic and physiological differences (Wu, 2012).

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Table 2.1 The following is a summary of the evidence regarding main risk factors for total prostate cancer (Rawla, 2019).

Risk factor	Strength of evidence
Increased risk	
Older age	Strong
African descent	Strong
Family history	Strong
Genetic risk loci	Strong
Taller height	Probable

2.3.3 Morpho-pathology of Prostate Cancer

The morphological changes in the prostate are the earliest indication of a malignant transformation that could occur in a healthy prostate (Figure 2.6). PC, according to literature, begins with changes in the structure and size of the prostate gland known as prostatic intraepithelial neoplasia "PIN" (Heidenreich, 2011). With the loss of the basal cell layer and basement membrane, PIN lesions advance to invasive adenocarcinoma (luminal phenotype), resulting in varied tumor grades, ranging from indolent to more aggressive types of PC, as well as metastasis and castration resistance (Rybak, 2015). PIN is a pre-neoplastic lesion in humans that is characterized by luminal epithelial hyperplasia, larger nuclei and nucleoli, and a reduction in basal cells while preserving an intact basement membrane (Shen, 2010). It can be categorized into four architectural types: tufting, micropapillary, cribriform, and flat. The clinical consequences of these various patterns appear to be indistinguishable. PIN is a continuum between low-grade and high-grade forms, with high-grade PIN (HGPIN) thought to be an early invasive carcinoma precursor (Abate-Shen, 2000).

HGPIN has clinical significance since it identifies patients who are at risk of developing cancer. Clinicians are more likely to detect HGPIN and overlook concomitant cancer with the growing use of prolonged biopsy methods. Androgen deprivation therapy reduces the incidence and severity of PIN and may help with chemoprevention. According to preliminary research, selective estrogen receptor modulators may also prevent HGPIN from progressing to PC (Chen, 2022).

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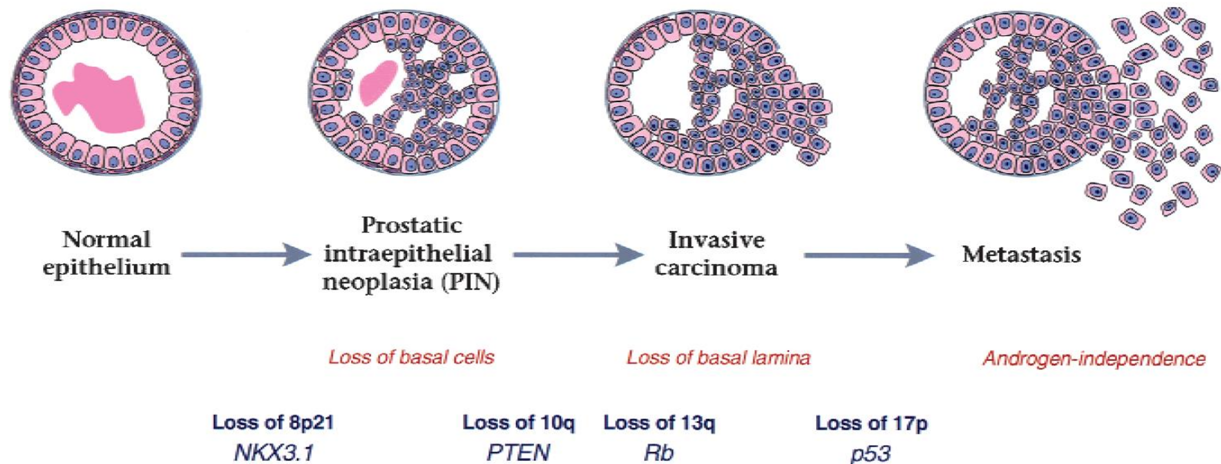

Figure 2.5 Prostate cancer progression pathway in humans. Loss of particular chromosomal regions and potential tumour suppressor genes are linked to progression stages. Adapted without permission (Abate-Shen, 2000).

2.3.4 Grading and Morphologic Classification of Prostate Cancer

Clinicians may understand the severity or aggressiveness of an illness by grading and classifying it, which also allows for international uniformity of nomenclature. The classification further assists in determining the best treatment choice for an illness. The standard approach to histologic grading of prostate adenocarcinoma continues to be Gleason grading. The International Society of Urological Pathology (ISUP) depicts common development patterns of prostatic cancer, including 2015 modified Gleason grading schematic diagram (Humphrey, 2017).

2.3.4.1 Gleason's Grading and Scoring System

According to histology, PC tissue typically has a mix of benign glands; preneoplastic (PIN) foci, and neoplastic foci of different severity. Gleason established a grading system to account for this heterogeneity, which is now the most widely used by pathologists as an excellent prognostic indicator (Gleason, 1992). Gleason grades are used to describe PC development patterns (Figure 2.7) and are associated to disease severity. Gleason scores range from Gleason 1 (G1) to Gleason 5 (G5), with G1 indicating tissue with the highest degree of similarity to normal tissue and the best prognosis, and G5 indicating poorly differentiated tissue with the worst prognosis (Li, 2018).

A total score is calculated by adding the scores from the most representative pathological samples. Scores of 2–10 are conceivable, however scores of less than 6 are uncommon. The Gleason score is then used to categorize prostate tumors as low-grade (<6), intermediate-grade (7), or high-grade (8–10). Gleason scores are more closely

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associated with grade groups than with these categories. Then, based on the grade group, Prostate-Specific Antigen (PSA), and clinical stage (Tumor Node Metastasis staging), risk stratification is done (Barsouk, 2020).

The newly proposed grades have been referred to as a "new grading system," despite the fact that it might alternatively be considered a "novel grouping," of a substantially modified original Gleason grading system, due to considerable modifications in criteria and reporting compared to Gleason's original grading system. Table 2.2 lists the histologic definitions of the five grade groups in the new grading system.

Table 2.2 Histologic definition of new grading system

Table 2.2 Histologic definition of new grading system
Grade group 1 (Gleason score 3 + 3 = 6): Only individual discrete well-formed glands
Grade group 2 (Gleason score 3 + 4 = 7): Predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands
Grade group 3 (Gleason score 4 + 3 = 7): Predominantly poorly formed/ fused/cribriform glands with lesser component of well-formed glands
Grade group 4 (Gleason score 8) - Only poorly formed/fused/cribriform glands or - Predominantly well-formed glands and lesser component lacking glands - Predominantly lacking glands and lesser component of well-formed glands
Grade group 5 (Gleason scores 9–10): Lack of gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands
For cases with >95% poorly formed/fused/cribriform glands or lack of glands on a core or at radical prostatectomy, the component of <5% well-formed glands is not factored into the grade. Poorly formed/fused/cribriform glands can be a more minor component.

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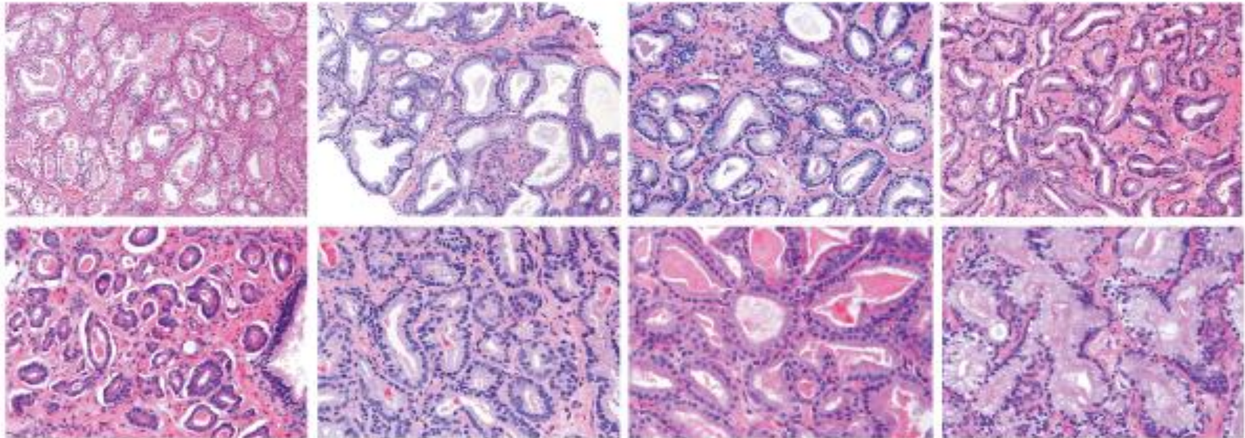
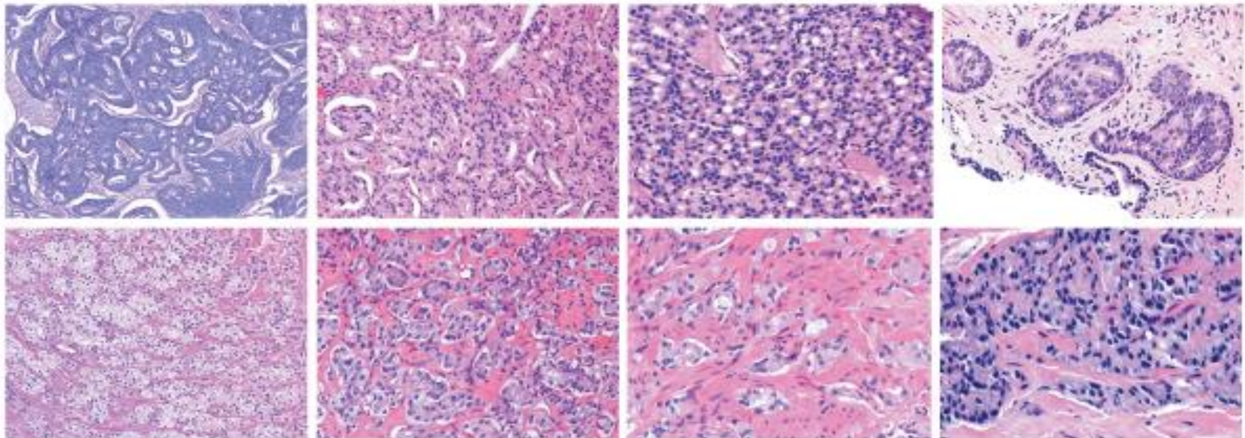
Discrete Well-formed Glands (Gleason Patterns 1-3)



Cribriform/Poorly-formed/Fused Glands (Gleason Pattern 4)



Sheets/Cords/Single Cells/Solid Nests/Necrosis (Gleason Pattern 5)

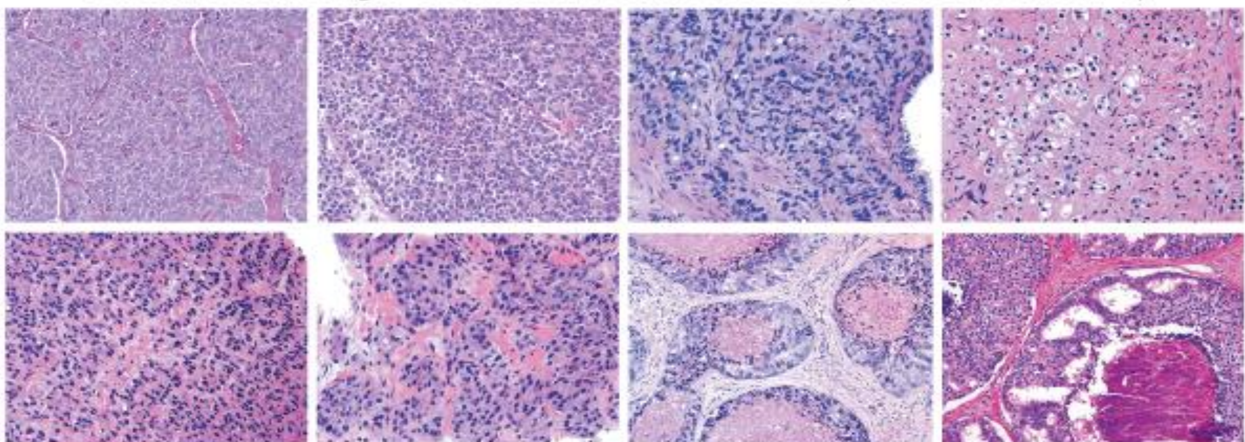


Figure 2.6 The severity of prostate cancer is graded. According to the ISUP, the Gleason grading scale was updated in 2015. Pattern 1 (at the top) through 5 (at the bottom) are graded . Adapted without permission (Epstein, 2016).

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2.3.4.2 World Health Organization Histologic Classification

The World Health Organization (WHO) histologically classified prostate tumors in 2016, focusing on a new entity, new acinar adenocarcinoma variations, and novel immunohistochemical stains for diagnosis, grading, risk stratification, and molecular genetics of acinar adenocarcinoma of the prostate (Figure 2.8 - Appendix).

2.3.5 Clinical Staging of Prostate Cancer

Accurate clinical staging is essential in assessing a patient's disease risk and guiding treatment decisions. Traditionally, the initial clinical evaluation relied on findings from the digital rectal examination (DRE) and histological confirmation of PC (Mark, 2018). However, the landscape of clinical staging has evolved significantly with recent advancements in MRI technology, which offer enhanced diagnostic precision. In both initial biopsy protocols and repeat biopsies for patients with previously negative results, Magnetic Resonance Imaging (MRI) now plays a pivotal role in identifying abnormal regions suitable for core biopsy (Borley, 2009).

The T (tumor extent), N (lymph node invasion), and M (presence or absence of metastases) staging system for PC was first introduced in 1992, when the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer adopted a unified TNM staging system for PC. The staging of PC in the 2010 AJCC (Table 2.2) differs from that of the 2002 version in various ways. Extraprostatic extension and microscopic bladder neck invasion were also added to the T3a category, Gleason score was recognized as the recommended grading system, and preoperative PSA were introduced into stage grouping as prognostic markers (Cheng, 2012).

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Table 2.3 AJCC stage grouping. Adapted without permission (2010 edition).

Stage	T	N	M	PSA (ng/ml)	Gleason score
I	T1a-c	N0	M0	<10	≤ 6
	T2a	N0	M0	<10	≤ 6
	T1-2a	N0	M0	X	X
IIA	T1a-c	N0	M0	<20	7
	T1a-c	N0	M0	≥10 and <20	≤6
	T2a	N0	M0	<20	7
	T2b	N0	M0	<20	≤7
	T2b	N0	M0	X	X
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1-2	N0	M0	≥20	Any Gleason
	T1-2	N0	M0	Any PSA	≥8
III	T3a-b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

2.3.5.1 Primary Tumor

PC does not develop in the same way all across the prostate. Although prostate tumors are frequently multifocal, the peripheral zone accounts for 80 - 85 %, the transition zone for 10 - 15% , and the central zone for 5 - 10% of cases. The peripheral zone, which is placed posteriorly and abuts the rectum, is receptive to DRE and biopsy under Transrectal ultrasound (TRUS) guidance. The anteromedial prostate is in the transition zone and it is not commonly sampled with a systematic TRUS-guided biopsy. The central zone, which includes part of the prostate's base and the area around the ejaculatory ducts, is rarely the source of cancer, but it is frequently invaded by the spread of larger tumors from the periphery zone (Mark, 2018). The following symbols are used in the intepretation of primary tumors:

TX Primary tumour cannot be assessed;
TO No evidence of primary tumour;
T1 Clinically inapparent tumour, neither palpable nor visible by imaging;
T1a Tumour (non-palpable) as incidental histological finding at transurethral resection of prostate in 5% tissue resected;
T1b Tumour (non-palpable) as incidental histological finding at transurethral resection of prostate in > 5% of tissue resected;

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T1c Tumour (non-palpable) identified by needle biopsy (for elevated serum PSA): includes bilateral nonpalpable tumour on needle biopsy;
T2 Tumour confined within prostate (including prostatic apex, prostate capsule) that is either palpable or visible on imaging or (with p-prefix) demonstrated in radical prostatectomy specimen;
T2a Tumour involving one-half of one lobe or less;
T2b Tumour involving more than one-half of one lobe but not both lobes;
T2c Tumour involving both lobes;
T3 Tumour extends through prostatic capsule;
T3a Extra-capsular extension (ECE);
T3b Invasion of seminal vesicle(s);
T4 Tumour fixed or invades adjacent structures: bladder neck, external sphincter, rectum, levator muscles and pelvic wall (Borley, 2009).

2.3.5.2 Regional lymph nodes

The regional lymph nodes below the bifurcation of the common iliac arteries are referred to as pelvic lymph nodes (Mark, 2018). The following symbols are used in the intepretation of regional lymph nodes:

NX Regional lymph nodes cannot be assessed;
N0 No regional lymph node metastases;
N1 Regional lymph node metastases within true pelvis, below common iliac artery bifurcation, either unilateral or bilateral (Borley, 2009).

2.3.5.3 Metastases

Outside of the actual pelvis, there are distant lymph nodes. The aorta, common iliac, deep inguinal, superficial inguinal (ie, femoral), supraclavicular, cervical, scalene, and retroperitoneal lymph nodes are among the distal lymph nodes. PC metastasis most commonly occurs in the bones, and these lesions are frequently osteoblastic rather than osteolytic. Lung and liver metastases are frequently discovered late in the disease's progression (Mark, 2018). The following symbols are used in the intepretation of metastases:

MX Distant metastases cannot be assessed;
M0 No distant metastases;
M1a Non-regional lymph node metastasis;
M1b Metastasis to bone(s);
M1c Other site(s) of metastasis (Borley, 2009).

M - Metastasis; N - node; T - tumor; X - unknown.

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2.3.6 Screening and Diagnosis of Prostate Cancer

Serum prostate specific antigen (PSA) testing improved PC survival rates by allowing for early discovery of the illness, which allowed for resection and local treatment before it spread (Mohler J. A., 2016). Routine PSA testing of low-risk men, on the other hand, has been found to have drawbacks. In one study, 15% of males obtained a false positive result after ten years of testing, prompting them to undertake needless and invasive diagnostic procedures such as biopsy (Kilpeläinen, 2011). According to studies, 20–50 percent of men diagnosed with PC as a result of PSA testing may have been "over-diagnosed," meaning that their disease would have remained asymptomatic throughout their lives, making the treatment they received unnecessary (Fenton, 2018).

However, recently developed machine learning (ML) and artificial intelligence (AI) techniques have led to new PC classifications. The paradigm of PC screening, diagnosis, and treatment has recently changed to a more individualized approach due to the availability of novel molecular markers and the development of advanced imaging techniques like prostate-specific membrane antigen positron emission tomography scans and multiparametric magnetic resonance imaging (mpMRI). The most recent recommendations say that any male at risk for developing PC should first undergo an MRI of the prostate before having a prostate biopsy (Cornford & Mottet, 2021). Additional tissue cores are obtained from suspected MRI lesions to improve prostate sampling if the MRI is positive. Furthermore, MRI has been shown to provide better staging accuracy than DRE, allowing for a more conservative dissection in patients (Falagario, 2020). While Gleason grading and histological analysis of PC are based on glandular architecture and phenotypic appearance, new techniques for high-throughput sequencing of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) extracted from cancer cells have helped to characterize PC at a genotypic level (Maggi, 2021).

Transrectal ultrasound-guided biopsy (TRUS-GB) and trans perineal ultrasound-guided biopsy (TPUS-GB) are the two standard procedures for prostate biopsy (Lopes, 2015). TRUS-GB remains the gold standard despite the increased risks of sequelae (rectal bleeding, fever, sepsis, hematuria, and acute urine retention) (Xiang, 2019). TRUS is excellent for guiding the biopsy needle and determining prostate gland volume, but it lacks sensitivity and specificity for detecting and staging PC. When the initial TRUS biopsy result is negative, but the suspicion of PC persists, clinical guidelines now recommend that an mpMRI be performed (Mottet N. B., 2017). mpMRI approaches have improved imaging sensitivity for PC, transforming the disease's diagnostic and treatment prospects (Rapisarda, 2020).

A biopsy is routinely performed after a positive PSA test. Biopsies for PC have a 1% likelihood of requiring hospitalization as a result of the procedure's complications. To guide the biopsy and estimate prostate size for diagnosis, trans-rectal ultrasound and/or MRI are frequently employed. The cancer is assessed using the Gleason Score if the

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biopsy is positive (Barsouk, 2020). Other criteria such as DRE, clinical history, or treatment decision should not be included for determining the PI-RADSTM v2 Assessment Category. Although biopsy should be explored for Prostate Imaging-Reporting and Data System (PIRADS) 4 or 5, but not for PIRADS 1 or 2, PI-RADSTM v2 does not offer management recommendations because they must evaluate criteria other than the MRI findings, such as laboratory/clinical history, local preferences, expertise, and standards of care (Weinreb, 2016). Assessment of lesions in the central zone and anterior fibromuscular stroma, evaluation of the transition zone, modification of criteria for DWI scores 2 and 3, and clarification of the distinction between positive and negative enhancement on Dynamic contrast-enhanced magnetic resonance imaging (DCE) MRI are among the changes to the interpretation criteria of mpMRI data incorporated into PI-RADS v2.1 (Turkbey, 2019).

2.3.7 Treatment and Management of Prostate Cancer

Advances in PC diagnosis and treatment have enhanced clinicians' capacity to classify patients by risk and propose therapy based on cancer prognosis and patient preference (Litwin, 2017). Surveillance, prostatectomy, and radiotherapy are the standard treatments for stage I–III patients. Androgen ablation by surgical or pharmacological castration can bring about lasting remission in all stage IV and high-risk stage III patients (Table 2.4). In this case, first-generation anti-androgens like flutamide and bicalutamide can aid. However, in the stage IV situation, castration resistance, which is characterized by genomic mutations in the androgen receptor, invariably occurs, and the prognosis is poor (Trewartha, 2013).

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Table 2.4 Common Prostate Cancer Treatment Options and Potential Adverse Effects. Adapted without permission (Shah, 2018).

Treatment Option	Disease Progression	Potential Adverse Effects
Active surveillance	Localized	Illness uncertainty
Radical prostatectomy	Localized	Erectile dysfunction Urinary incontinence
External beam radiation	Localized and advanced disease	Urinary urgency & frequency, dysuria, diarrhea and proctitis Erectile dysfunction Urinary incontinence
Brachytherapy	Localized	Urinary urgency & frequency, dysuria, diarrhea and proctitis Erectile dysfunction Urinary incontinence
Cryotherapy	Localized	Erectile dysfunction Urinary incontinence and retention Rectal pain and fistula
Hormone therapy	Advanced	Fatigue Hot flashes, and flare effect Hyperlipidemia Insulin resistance Cardiovascular disease Anemia Osteoporosis Erectile dysfunction Cognitive deficits
Chemotherapy	Advanced	Myelosuppression Hypersensitivity reaction Gastrointestinal upset Peripheral neuropathy

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2.3.7.1 Active surveillance

Active surveillance is a structured program that employs monitoring and expected intervention as the main technique in the management of PC (Choo, 2002). For patients who have low-risk cancers or those who have a short life expectancy, active surveillance has been recognized as the best option. The criteria for active surveillance have recommendations that are usually based on the factors: disease characteristics, health conditions, life expectancy, side effects, and patient preference (van den Bergh, 2009). Assessing the PSA level, clinical progression, or histologic progression are used as PC trigger points (Lawrentschuk, 2011).

The advantages of active surveillance are the preservation of erectile function, decreased costs of treatment; avoidance of needless treatment of inactive cancers; and sustaining of life quality and normal activities. Its disadvantages include the likeliness of cancer metastasis before treatment, missed opportunity for a remedy, need for a complex therapy with side effects for larger and aggressive cancers, reduced chances of potency preservation mostly after surgery, and chances of increased anxiety by patients, and frequent medical checks (Costello, 2020).

2.3.7.2 Radical Prostatectomy

Radical prostatectomy is the procedure of medically removing the prostate gland by open and/or laparoscopic surgery (Mellman, 2011). The procedure requires making small incisions on the abdomen or via the perineum. The benefits of radical prostatectomy are few complications, quick recovery, and reduced blood loss (Mouraviev, 2006).

Salvage radical prostatectomy is usually recommended to patients with local recurrence in the absence of metastases after undergoing external-beam radiation therapy, brachytherapy, or cryotherapy. This may however lead to increased morbidity. Patients with younger than the age of 70 with organ-confined PC, with a life expectancy higher than 10-year who have little to no comorbidities, are best suited for radical prostatectomy. However, there are a few complications associated with its use. These complications include incontinence and erectile dysfunction arising from surgical damage to the urinary sphincter and erectile nerves (Mouraviev, 2006).

2.3.7.3 Cryotherapy

This method involves the use of surgical insertion of cryoprobes into the prostate under ultrasound guidance. It involves the freezing of the prostate gland to a temperature of -100 to -200 degrees Celsius for about 10 minutes (Baskar, 2012). However, there are reports of complications associated with the use of this method, these include urinary incontinence and urinary retention, erectile dysfunction, fistula, and rectal pain (Shah, 2018).

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2.3.7.4 Radiation

Radiation therapy is regarded as one of the most effective therapies that kills PC cells using high radiation beams. Radiations are sent to cancerous cells through various techniques such as brachytherapy (the use of seeds placed in the body) and external beam (where the energy is projected through the skin) to the cancerous sites. Radiation therapy aims at specifically transferring high-energy rays or particle doses directly to the prostate without affecting the normal tissues. These doses are based on the level of PC. This treatment is considered an acceptable therapy for patients who are not suitable for surgical procedures (Institute, 2012). This method of treatment does not only kill or delay cancer growth, but it can also harm healthy cells nearby. Damage to healthy cells has unfavorable consequences and the treatment may be unaffordable to most patients (Mohler J. K., 2006). Various techniques of radiation therapy are discussed below.

2.3.7.4.1 Brachytherapy

This includes the direct placement of radioactive sources into the prostate gland with the aid of seeds, injections, or wires under the guidance of transrectal ultrasound. This often involves 2 techniques vis-à-vis the low-dose and high-dose rates. The low dose rate refers to the permanent implantation of seeds in the prostate tissue, which loses radioactivity gradually (Potosky, 2000). While the latter refers to the supply of a dose of radiation to the prostate tissues with great risk of leakage to other surrounding organs. The advantage associated with brachytherapy is that it can be completed within a day or less. There is a minimal risk of incontinence in patients without a previous transurethral resection of the prostate. Erectile function is not also affected. Its disadvantages are usually a requirement for general anesthesia, acute urinary retention risks, and persistent irritative voiding symptoms (da Silva, 2015).

2.3.7.4.2 External beam radiation therapy

External beam radiation therapy is a commonly used treatment technique that involves emitting strong x-ray beams specifically targeting the prostate tissues. It radiates higher prostate radiation doses, with less emission to the surrounding tissues. Radiation therapy is considered a very effective intermediate- and high-risk PC treatment when used together with androgen deprivation therapy (ADT) (Trewartha, 2013). It is a suitable therapy for attenuating metastasizing cancer cells. This technique is more advantageous than surgical therapy, it can treat early stages of cancer, it is associated with lesser risks such as bleeding, myocardial infarction, pulmonary embolus, urinary incontinence, and erectile dysfunction. It can also relieve symptoms such as bone and joint pain. Side effects of radiation include urinary urgency and frequency, erectile dysfunction, dysuria, diarrhea, and proctitis (Shah, 2018).

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2.3.7.5 Radium-223 Therapy

The radium-223 dichloride (Xofigo) technique makes use of a substance used for therapy in patients with metastatic PC that is resistant to hormone therapy. Its ability to mimic calcium makes radium-223 dichloride be selectively absorbed by the cancer cells in bone tissue. This technique has been reported to have a great impact on the survival and recovery of metastatic PC patients leading to delayed onset of bone fracture and pain (Seidenfeld, 2000).

2.3.7.6 Hormonal therapy

Hormonal therapy, known as the androgen deprivation therapy (ADT), is a technique applied in the treatment of advanced and/or metastasized PC. Its therapeutic mechanism is based on the blockage of testosterone production and its actions on PC cells (Foundation, 2016). Thus, reducing male hormonal levels which prevent the action of androgen on the androgen receptors (Heidenreich A. B., 2014). This is often achieved using bilateral orchiectomy or medical castration via administration of luteinizing hormone releasing hormone (LHRH) analogues or antagonists (Seidenfeld, 2000). LHRH analogue initially increases the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by stimulating hypophysis receptors. Thus, enabling the drug to downregulate the hypophysis receptors with concomitant reduction of LH and FSH levels, leading to suppressed testosterone production (Rosario, 2016). Leuprolide, goserelin, triptorelin, and histrelin are among the common LHRH agonists.

The antagonists however bring about their action by blocking the hypophysis receptors, thereby triggering the immediate inhibition of testosterone synthesis (Albertsen, 2009). ADT has been associated with acute and long-term side effects. These include fatigue, hot flashes, flare effect, hyperlipidemia, insulin resistance, cardiovascular disease, anemia, osteoporosis, and sexual dysfunction (Braga-Basaria, 2006; Tzortzis, 2017).

2.3.7.7. Immunotherapy

Immunotherapy or biological therapy is based on stimulating or suppressing the immune system. The treatment uses vaccines designed to work with the patient's immune system to fight cancer cells (Molina, 2011). Sipuleucel-T (Provenge) is among such vaccines and it is designed for advanced and metastatic PC cells that have developed resistance to hormone therapy. It is developed from the immune cells by collecting the white blood cells and activating with prostatic acid phosphatase (Crawford, 2015). This is then associated with a protein that can trigger the immune system before infusing into the blood (Mohan, 2011). Sipuleucel-T (Provenge; Dendreon) is an autologous dendritic cell-based immunotherapy used in treating asymptomatic patients. It is intravenously administered in 3 doses over one month. Its lesser side effects make it more favorable compared to

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other chemotherapies. Its side effects include fever, nausea, chills, and muscle aches (Dunn, 2011).

2.3.7.8. Chemotherapy

Chemotherapy is the type of therapy that uses anti-cancer drugs to kill or inhibit the growth of cancerous cells (table 2.5). There has been tremendous progress in the treatment of PC following decades of understanding genetics, diagnosis, and treatment. The most common chemotherapy drug for PC is docetaxel. Since its approval, there have been other Food and Drug Administration (FDA)-approved agents namely enzalutamide, abiraterone, and cabazitaxel which have shown an increased survival rate in clinical trials (Green, 2012).

Metastatic castration-resistant PC (mCRPC) represents the most aggressive and prevalent stage in the progression of PC. This stage is characterized by its diverse drivers of disease progression and complex mechanisms leading to drug resistance (Boudadi, 2016; Labecque et al., 2019). While there is a wide array of treatment options available for mCRPC (Table 2.5), it remains an incurable condition. It is becoming increasingly evident that this disease continually evolves, developing various mechanisms of resistance with each successive therapy (Drake, 2013). An understanding of how metastatic PC cells proliferate and interact within the surrounding tumor microenvironment can help identify critical pathways responsible for driving the disease's progression.

Table 2.5 FDA-Approved anticancer treatments for mCRPC. Adapted without permission (Stein, 2014).

Treatment	Mechanism	Indication	Combination agent	Date of initial FDA approval for mCRPC
Docetaxel (Taxoterel)	Taxane chemotherapy (microtubule inhibitor)	mCRPC	Oral prednisone	May 2004
Cabazitaxel (Jevtana)	Taxane chemotherapy (microtubule inhibitor)	mCRPC	Oral prednisone	June 2010
Abiraterone acetate (Zytiga)	CYP17 (androgen synthesis) inhibitor	mCRPC	Oral prednisone	April 2011
Enzalutamide (Xtandi)	Androgen receptor inhibitor	mCRPC	-	September 2012

FDA, Food and Drug Administration.

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2.3.7.8.1 Abiraterone

Abiraterone is a second-generation therapy targeted at adrenal and tumor androgen production. It is associated with the irreversible inhibitions of the hydroxylase and lyase activities of CYP17A, AR pathways, and 3β-hydroxysteroid dehydrogenase activity and is used to treat PC that has metastasized to other parts of the body (Molina, 2011). Abiraterone has also been proven to be a potent inhibitor of other microsomal drug-metabolizing enzymes including CYP1A2 and CYP2D6 (Zhu, 2013). Clinical data of abiraterone has indicated remarkable results, there are however reports of variable responses and concomitant increasing PSA levels. Abiraterone is correlated with high CYP17A upstream mineralocorticoids, with concomitant side effects including edema, hypertension, fatigue, and hypokalemia (Abidi, 2013).

2.3.7.8.2 Docetaxel

This is regarded as the first-line standard therapy for PC cells that are castration-resistant. It is an anti-microtubule agent which attaches to β-tubulin to inhibit microtubule depolymerization, thereby suppressing mitotic cell division and initiating apoptosis (Benoist, 2016). CYP3A is a major requirement for the activation of Docetaxel. The development of Docetaxel resistance has been associated with a relapse. Docetaxel resistance has been attributed to increased upregulation of the multidrug resistance (MDR) 1 gene that encodes P-glycoprotein (Cookson, 2013).

2.3.7.8.3 Cabazitaxel

This is a novel antineoplastic semi-synthetic derived from the needles of various species of yew trees (Taxus). It is usually sold under the name Jevtana, Cabazitaxel is a second-generation therapy aimed at suppressing docetaxel resistance (Stein, 2014). It has a low affinity for P-glycoprotein owing to its additional methyl groups. It is metabolized in the hepatic tissues by CYP3A4/5 and CYP2C8 (10% -20%). Hypotension, bronchospasm, renal failure, neurotoxicity fatigue, alopecia, and generalized rash/erythema are among the common side effect associated with its use. There have also been reports of diarrheal deaths related to cabazitaxel therapy resulting in electrolyte imbalances and dehydration (Cookson, 2013).

2.3.7.8.4 Enzalutamide

Enzalutamide is a second-generation Androgen Receptor (AR) inhibitor that was recognized as one of the chemotherapeutic drugs for PC in 2012. This drug focuses on the androgen pathway and has functions such as) competitively inhibiting the binding of androgen to the androgen receptor; (2) inhibiting nuclear translocation and recruitment of cofactors; and (3) inhibits the association of the activated androgen receptor with (Drake, 2013). Enzalutamide targets androgens such as testosterone and dihydrotestosterone. Its therapeutic mechanism includes:

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1. Competitive inhibition of androgen binding to the androgen receptor
2. Inhibition of nuclear translocation and co-factors recruitment
3. Inhibition of the binding of DNA with activated androgen receptor

The side effects of enzalutamide include fatigue, asthenia, diarrhea, and vomiting (Watson, 2015).

2.3.8 Treatment challenges of Prostate Cancer

Despite the various treatment options (Table 2.4), mCRPC is yet an incurable disease. Over time, the disease continues to develop resistance to different conventional treatment options (Semenas, 2012). This has led to continuous research on understanding the growth, metastasis, tumorigenesis, tumor microenvironment, and tumor environmental interactions that promote the disease progression.

2.3.8.1 Drug resistance

Castration resistance is a well-documented phenomenon in advanced PC, signifying a critical juncture in the progression of the disease. This resistance mechanism enables the persistence of androgen signaling within tumors, primarily achieved through the amplification of androgen receptor synthesis and the disruption of co-expressors and coactivators associated with the androgen receptor (Giacinti, 2018). Recent research has shed light on the significance of this resistance mechanism, underscoring its impact on the clinical landscape.

One of the notable consequences of this castration resistance is the reduced efficacy of treatments like enzalutamide and abiraterone acetate, which are pivotal in managing metastatic PC. Such resistance has been attributed to the overexpression of the active androgen receptor variant (AR-VT) in patients (Obligacion, 2006). These recent findings emphasize the clinical challenges posed by the emergence of resistance mechanisms, demanding innovative approaches to therapy. It's worth noting that the role of androgens in PC development is pivotal, which is why many treatment strategies are centered around androgen hormone inhibition. This approach has proven beneficial, especially for patients who have developed resistance to anticancer drugs (Pelekanou, 2016). Cutting-edge research continues to explore ways to enhance the efficacy of androgen-targeted therapies in light of evolving resistance mechanisms, thus offering hope for better patient outcomes.

Recent studies have also unveiled the complex interplay of mutations in contributing to drug resistance in cancer cells (Boulos, 2020). These mutations enable cancer cells to bypass the targeted pathways, further complicating treatment strategies. Notably, alterations in intrinsic pathways such as the androgen receptor signaling pathways, the MAPK/ErK pathway, the endothelin A receptor (EAR), and the Akt/PI3K pathways, along

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with exacerbated androgen receptor expression, have been implicated in contributing to ADT resistance (de Jonge-Peeters, 2007). Understanding these intricate molecular processes is essential for devising more effective therapies and improving patient outcomes in the face of evolving resistance mechanisms in PC.

2.3.8.2 ABC Transporters

Transporter proteins play a pivotal role in drug resistance by acting as efflux pumps located on the plasma membrane, thereby contributing significantly to the phenomenon of multidrug resistance. These transporters are crucial in regulating the influx and efflux of drugs and xenobiotics in and out of cells, impacting the efficacy of therapeutic interventions (Deeley, 2006). In the context of PC, several transporters, including Multidrug Resistance Proteins (MRPs) and P-glycoprotein (MDR-1), have been identified as key players in the development of drug resistance (Hwang, 2012). The upregulated expression of these transporters has been closely associated with the enhanced efflux of drugs, thereby culminating in multidrug resistance (Oyama, 2004). The intricate involvement of these transporters in drug resistance has garnered significant attention in recent research, underscoring their clinical importance.

Among these transporters, MRP2 has been reported as one of the most potent contributors to resistance against natural product agents. In contrast, MRP3 exhibits lower resistance, particularly to agents like etoposide. Furthermore, MRP4 and MRP5 are known to be responsible for mediating resistance to nucleoside analogs and the transportation of cyclic nucleotides, adding complexity to the resistance mechanisms (Kinoshita, 2004). Notably, MRP4's role extends beyond nucleoside analog resistance. It has also been implicated in resistance to a range of chemotherapeutic agents, including camptothecins, cyclophosphamide, topotecan, methotrexate, and nucleoside analogs (Tokizane, 2005). These findings highlight the multifaceted nature of drug resistance in PC and underscore the critical role of transporter proteins in mediating resistance to a broad spectrum of therapeutic agents.

This growing body of research provides valuable insights into the molecular mechanisms underpinning multidrug resistance in PC. A deeper understanding of these transporters and their specific roles in resistance will undoubtedly pave the way for the development of more effective treatment strategies and personalized approaches to combat drug resistance. The dynamic landscape of transporter-mediated resistance continues to be a focal point in ongoing research, offering hope for improved outcomes in PC management.

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2.3.8.3 Cytochrome P450

Cytochrome P450 (CYP450) enzymes constitute a versatile and extensive superfamily of heme-containing monooxygenases responsible for the metabolism of a broad array of xenobiotics and endogenous substrates (Oyama, 2004). These enzymes, particularly those involved in phase I drug metabolism, have garnered significant attention in the context of PC. In the human prostate, several CYP450 enzymes, including CYP1A2, CYP1B1, CYP2C19, CYP2D6, CYP3A5, and CYP4B1, have been identified in both normal and tumorous tissue, underscoring their role in drug metabolism (Obligacion, 2006). Among these enzymes, CYP4B1 has been associated with the activation of arylamines through N-hydroxylation, a process linked to an increased risk of bladder tumors and PC (Kinoshita Y, 2004). Furthermore, CYP1B1, a member of the CYP450 superfamily, has gained recognition as an important tumor biomarker. Its overexpression has been documented in drug-resistant PC, with one mechanism involving the 2-hydroxylation of flutamide (Rochat, 2001).

CYP17A1, a crucial enzyme responsible for the sequential hydroxylase and lyase steps in androgen biosynthesis, has become a significant target for PC treatment (Devore, 2012). Notably, alterations in key genes involved in androgen biosynthesis have been identified in CRPC. A study comparing CRPC with primary tumors revealed a substantial increase in the relative expression of numerous transcripts, including CYP17A1 (Green, 2012). These findings underscore the pivotal role of intratumoral steroidogenesis in facilitating CRPC survival within a castrate environment.

Testosterone is a hormone essential for the growth and function of luminal prostate cells, which are frequently the origin of PC. Interestingly, CYP3A enzymes have been found to hydroxylate testosterone and dehydroepiandrosterone into less active metabolites. This has led to the investigation of associations between CYP3A polymorphisms and PC, suggesting a potential link between these genetic variations and the development of PC (Honrado, 2007). These studies collectively highlight the intricate interplay between CYP450 enzymes and PC, shedding light on their multifaceted roles in drug metabolism, resistance, and androgen biosynthesis. The findings offer potential avenues for developing more targeted therapeutic approaches in the battle against PC and emphasize the importance of personalized medicine to improve patient outcomes.

2.3.8.4 Mutations in androgen receptor

The development of androgen resistance, a hallmark of PC progression, arises from a disruption in androgen sensitivity. Androgen receptor (AR) signaling, a central regulatory pathway in the prostate gland, exerts a profound influence on its development, activity, and homeostasis. AR functions by modulating gene transcription through its binding to androgen response elements on specific genes, as well as facilitating the nuclear

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translocation of the androgen receptor. Recent research has illuminated the critical role of gene alterations within the AR signaling pathway in the context of PC (Zhou, 2015).

A substantial proportion of AR mutations result in single-amino-acid substitutions, with the majority occurring within the androgen-binding domain of the AR protein. Notably, the T877A mutation, found in approximately 30% of metastatic castration-resistant PC (CRPC) patients, stands out as the most prevalent mutation. This mutation, among others, has significant implications for the development of AR resistance to therapies targeting the androgen receptor (Aurilio, 2020). Further mutations, such as H874Y and W435L, result in an augmented AR binding to co-regulators, ultimately leading to heightened AR transcriptional activity. These mutations have been associated with the emergence of resistance to AR-targeted therapies, underscoring the complexity and adaptability of PC in the face of treatment (Tikhomirova, 2005). Understanding the intricate genetic changes within the AR signaling pathway is paramount in unraveling the mechanisms behind resistance and disease progression in PC. It not only informs therapeutic strategies but also paves the way for the development of more effective, personalized treatments that consider the unique genetic profile of each patient. These recent studies shed light on the evolving landscape of AR mutations and their implications, offering hope for improved therapeutic approaches and better patient outcomes in the battle against PC.

2.3.8.5 Drug Metabolism

Recent studies have revealed that drug-metabolizing enzymes, particularly cytochrome P450 (CYP450) enzymes, are intricately involved in the metabolism of various crucial anticancer agents (McFadyen, 2004). However, the metabolism of these drugs is subject to significant inter-individual variations, making it challenging to achieve consistent therapeutic benefits from chemotherapy across patients. Notably, there can be several-fold differences in the clearance of anticancer drugs among individuals, highlighting the need for a more personalized approach to cancer treatment (Oyama, 2004; Rooseboom, 2004). Inter-individual differences in P450-mediated drug actions are predominantly attributed to genetic factors, particularly polymorphisms. Polymorphisms can arise from gene duplication or amplification, resulting in either defective or increased enzyme activity (Johansson, 1993; Aklillu, 1996). This genetic diversity can significantly impact an individual's response to anticancer treatments.

Polymorphic xenobiotic metabolizing CYP450 enzymes are typically categorized into two classes. Class I includes enzymes like CYP1A1, CYP1A2, CYP2E1, and CYP3A4, which are relatively well-conserved, and exhibit limited functional polymorphisms. They primarily participate in the metabolism of pre-carcinogens and drugs. In contrast, Class II comprises highly polymorphic enzymes such as CYP2B6, CYP2C9, CYP2C19, CYP2D6,

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and CYP2A6, which play a significant role in drug metabolism and can exhibit extensive genetic variability (Oscarson, 2001).

While recent advances in utilizing polymorphic P450 enzymes as potential drug targets in cancer therapy hold promise, they represent a novel and potentially effective alternative for future cancer treatment. However, the impact of these polymorphisms on the outcomes of anticancer drug treatments remains a subject of ongoing research. A deeper understanding of the relationship between genetic variations in P450 enzymes and treatment responses is essential for the development of more tailored and effective cancer therapies. These findings underscore the importance of personalized medicine in optimizing cancer treatment strategies and improving patient outcomes. elucidated.

2.3.9 Pharmacodynamics and Clinical efficacy: Docetaxel and Prednisone

Advanced CRPC is a complex issue that necessitates a multidisciplinary treatment. The priority remains the preservation of quality of life and supporting care (Saad, 2010). Since the publication of two pivotal phase III trials (TAX-327 study and the SWOG 99-16 study) that demonstrated a significant survival benefit in mCRPC in 2004, first-line chemotherapy with docetaxel and prednisone has been a standard treatment in mCRPC (Petrylak, 2004). According to the TAX327 data, docetaxel has a well-known adverse effect profile (Table 2.6) that includes neutropenia (32% Grade) and non-hematological toxicities such as alopecia (65%), nail alterations (30%), sensory neuropathy (30%), and change in taste (dysgeusia; 18%) (Omlin, 2015). Despite the fact that docetaxel is routinely given in combination with prednisone as a result of the TAX327 research, there is no convincing biological evidence for glucocorticoids and taxanes working together. Thus, it is unclear if prednisone adds to the efficacy of docetaxel or is only a byproduct of the drug's licensing procedure (Ndibe, 2015).

Prednisone is a corticosteroid routinely utilized in the treatment of advanced PC. Corticosteroids are sometimes administered to relieve pain from bone metastases (Haywood, 2015), to manage cancer-related fatigue (Yennurajalingam, 2013), or to minimize the toxicity of chemotherapy (Dorff, 2013). Corticosteroids have also been linked to positive antitumor responses, in addition to their palliative uses (Lorente, 2014). However, prednisone is not always co-administered with docetaxel in modern clinical practice, for a variety of reasons (Lafeuille, 2013). For instance, some oncologists are concerned about the long-term effects of prednisone, which include glucose intolerance, osteopenia, fluid retention, and peptic ulcers, among other things (Auchus, 2014). Furthermore, there is a theoretical risk of prednisone activating the AR, which could lead to PC growth (Chang, 2001).

Docetaxel is the first semisynthetic taxane to show a survival benefit in patients with mCRPC, as well as pain relief and enhanced quality of life (Oudard, 2015). It binds to β-tubulin and prevents disassembly of the microtubule network which can be detrimental

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for the cells since it leads to the stabilization of the mitotic spindle during the G2-M phase of the cell cycle leading to cell death by mitotic catastrophy. Moreover, it also acts by inhibiting anti-apoptotic Bcl2 family members, lowering the apoptotic threshold and allows for stressed cells to undergo apoptosis (Kavallaris, 2010). Docetaxel's clinical use has resulted in small increases in survival, owing to the development of resistance (Kharaziha, 2015).

There are currently no clinical biomarkers that can predict whether a CRPC patient will respond to this treatment or develop resistance to it. In previous studies, the following guidelines were used as outcome measures; tumor response was based on the Prostate-Specific Antigen Working Group criteria (good response was regarded as a 50% decrease in PSA) and standard Response Evaluation Criteria in Solid Tumors for measurable disease (Bubley, 1999; Therasse, 2000), adverse events were assessed according to the National Cancer Institue Common Toxicity Criteria, version 3, clinical outcome (categories being symptom relief (performance status and/or pain relief and/or reduced analgesic consumption), and progression-free interval (Ning, 2010).

Table 2.6 Common toxicities associated with the use of docetaxel. Adapted without permission (Omlin, 2015).

Category	Toxicities
Dermatological	Alopecia, peripheral edema, erythema, maculopapular rash
Allergic	Anaphylactic reactions, hypersensitivity reactions
Hepatic	Increased liver enzymes
Gastrointestinal	Nausea, anorexia, vomiting, diarrhea
Pulmonary	Bronchospasms, pleural effusions
Hematological	Neutropenia, anemia, thrombocytopenia, leukopenia
Neurological	Peripheral neuropathy, rare toxicity
General	Lethargy, epiphora, headache

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CHAPTER THREE: METHODOLODGY

3.1 Archived Biopsy Samples

This study utilized archived biopsy samples (formalin fixed paraffin embedded tissues) obtained from a well-established clinical repository, Universitas hospital, to investigate critical aspects of PC. These biopsy specimens, collected as part of routine, medical practice prior to chemotherapy treatment, provide a valuable resource for gaining insights into the disease's characteristics and progression. Based on the sensitivity & treatment outcome of the patients with biopsy, three groups were created: good responders, poor responders and those that produce excessive toxicity.

3.2 Ethics clearance

Ethics clearance was applied for from the Health Science Research Ethics Committee (HSREC) of the University of the Free State, which is registered with the National Health Research Council (NHREC). Once obtained, permission was applied for from the Provincial Health Research Committee and National Health Laboratory Service (NHLS), which are gate keepers providing sight over public health facilities. Then a search in the database of Free State Provincial Department of Health was conducted to obtain records of PC statistics and identifying points of treatment for patients.

Finally, permission for sample collection was applied for from Universitas hospital. It must be noted that for anonymized biological specimen, consent from patients from which the specimen was collected was not necessary (according to National Ethics Guidelines: Department of Health Ethics in Health Research Guidelines, Processes, Structures and Procedures, 2015). The patients' specimens were kept anonymous. As a result, there was no reflection of the patients' clinical information, allowing the specimens to be de-identified. The specimens were used purely for genetic and proteomic analysis, with the results connected to the de-identified specimens' treatment outcomes.

3.3 Sample collection

Archived formalin fixed paraffin embedded (FFPE) PC tissue specimen was obtained retrospectively from Universitas Academic hospital, Department of Anatomical Pathology Tissue Archive. The specimen being requested were from 2016 to 2022 records.The tissues were taken in sterile nuclease free-tubes and thereafter transferred to the respective laboratories. Aspects considered during collection include variables such as age (Patients' age ranged between 40 and 80 years (median 60 years)), treatment administered (Docetaxel and Prednisone), and clinical complications encountered such as excessive toxicity. The clinical and pathological variables included stage of the cancer, tumor type and grade (Table 3.1).

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In this pilot study, 15 patient specimens (x2 = 30: each sample with normal and tumor) were used as test sample to lay a foundation of causes of chemo resistance and effects on treatment outcome. Histopathology evaluation was performed to assess the percentage of tumor and the Gleason score in each tissue samples. From each tissue block, 2-mm biopsy punch samples were collected from both the tumor and normal tissue compartments.

3.4 Study Design

RETROSPECTIVE *IN VITRO* STUDY

In this pilot study, the study samples consist of 15 (x2: tumour & normal) archived specimen (5 in each of the 3 categories): responders who experienced excessive toxicity, poor and good responders, where the results of these three categories were

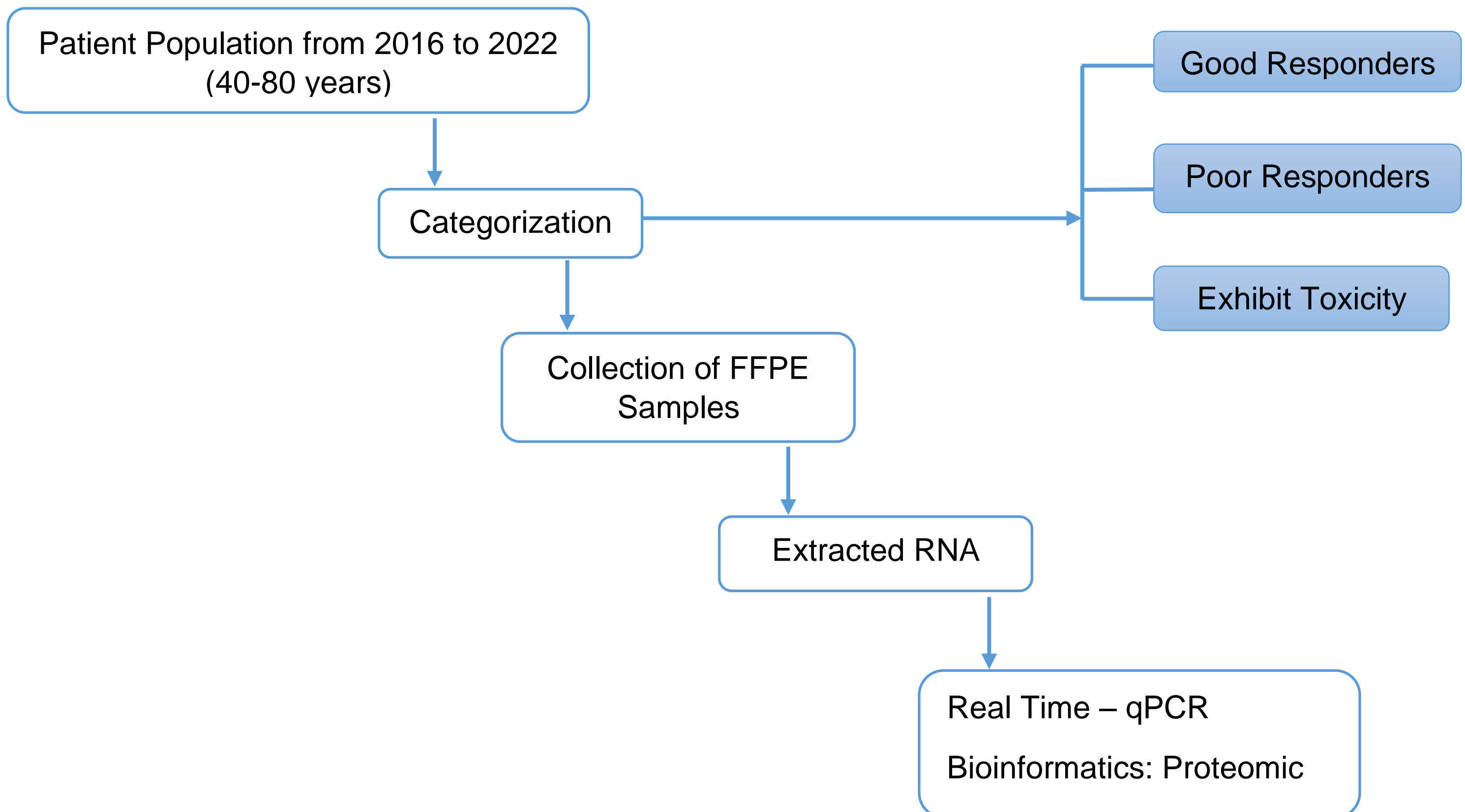

Figure 3.1 Flow chart of the study

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3.4.1 Research Team

1. Patient Selection: for the archived Specimen

NB - The clinical oncologist (**who is not part of the project team**) had access to patients' details including their clinical data, which was used to identify the tissue samples that fit into the three categories of samples required. Once the tissue samples were identified, both the Anatomical pathologist and the study team did not know the identity of the tissue sample owner because the tissue was named according to the category it belonged to (anonymized). Only the Oncologist had an identity code which she kept confidential.

2. Performing Microtomy

- Mr. L Gumenku [Principal investigator]
- Dr L Muller [Anatomical Pathologist]

3. Lab work: RNA Extraction, RT-qPCR & Bioinformatics

- The remaining study activities were conducted under the guidance and supervision of the two designated supervisors.

Table 3.1 Clinical characteristics of PC patients

Patient Category	Clinical Stage	Symptomatic improvement	Gleason Score (Gs)	Gleason grades (Gg)	Serum PSA level (ng/ml)	Number of Cycles of Docetaxel (Months)	Progressive Disease
Good responders group							
HU0046753 4	IV	Yes	7 (4+3)	3	>5000	2-10	No
ABFB7080N 0F	IV	Yes	7 (4+3)	3	22,3	2-10	No
JA0065649 5	IV	Yes	7 (4+3)	3	7000	2-10	No
JA0068259 2	IV	Yes	8 (4+4)	4	300	2-10	No
ABPD3201N OF	IV	Yes	7 (3+4)	2	>2000	2-10	No
JE01904355			7 (4+3)	3	4513		

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Poor responders group							
HU00548286	IV	No	8 (4+4)	4	Any PSA	>2000	Yes
HU00482964	IV	No	9 (4+5)	4	209	2-10	Yes
HU00347562	IV	No	7 (4+3)	3	75	2-10	Yes
HU00642960	IV	No	7 (4+3)	3	103	2-10	Yes
10	IV	No	Any Gs	Any Gg	Any PSA	2-10	Yes
Excessive toxicity group							
JE0106237	IV	No	9 (4+5)	-	11,000	2-10	Yes
FPE4068545	IV	No	9 (4+5)	Any Gg	Any PSA	2-10	Yes
13	IV	No	Any Gs	Any Gg	Any PSA	2-10	Yes
14	IV	No	Any Gs	Any Gg	Any PSA	2-10	Yes
15	IV	No	Any Gs	Any Gg	Any PSA	2-10	Yes

3.5 Extraction of RNA and Proteins from FFPE Tissue

3.5.1 Performing Microtomy

The rotary microtome was used to cut the FFPE tissue sections according to the method of Sy et al., 2019. Before cutting, the paraffin blocks were grouped into complete cases. Excess wax was removed from the sides of the plastic cassettes by scraping or melting. The paraffin blocks were then be placed onto the cold plate so that they can cool for trimming. The knife blade was carefully inserted into its holder on the microtome, while ensuring that the blade is positioned correctly. The knife tilt was ensured that is correct (clearance angle is usually between 3 and 8°, but this is dependent on the type of blade used). The lever was tightened and the knife guard was placed in the up position to cover the edge of the blade. The hand wheel brake was made sure that is on before inserting paraffin blocks into the chuck holder. The clamp of the paraffin block holder was released by pulling the vertical lever forward and snapping the cassette into the cutting position. The paraffin block was oriented so that any epithelial surface is upper most, thus ensuring that the knife edge cuts the epithelium last. The brake (lever/button) was released on the hand wheel. Using the coarse advance wheel, the block was brought close, up to the knife blade.

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When the block was close to the knife blade, the trimming button was then used to advance cut into the paraffin block until a full face of the tissue is exposed. Trimming is usually done at a thickness ranging from 10 to 30 µm. The last few trimming sections were cut at the designated final thickness in order to polish the block face. Once trimmed, the paraffin block was placed facing down on the ice block and leaving it there until it was thoroughly chilled. The trimming blade was replaced with a new blade. Once the block was chilled, sections were cut at designated thickness, ensuring the block is oriented in the same way as it was for the trimming process. The hand wheel was turned at a steady rhythm. The speed is determined by the consistency of the tissue. Soft tissues were cut optimally at a slower speed, whereas firmer tissues, such as skin or fibrous tissue required the hand wheel to be turned at a faster speed. The paraffin ribbon was picked up carefully by using a small paintbrush to remove the end closest to the blade with an upward motion. The paraffin ribbon was then layed down onto the water bath. The water bath was heated to a few degrees below the melting temperature of the paraffin. A clean paint brush was used to smooth out the paraffin sections. The individual paraffin section or ribbon of consecutive sections was poured in the 2 ml nuclease free-tube prelabeled to match the case number. Sections with wrinkles or bubbles were not choosen.

3.5.2 RNA Extraction

As per the manufacture's protocol, 400 µl of Deparaffinization Solution was added to the samples in the nuclease free tubes and Incubated at 55°C for 1 minute, then vortex briefly. Deparaffinization Solution was removed from the sample and the following mixture was added: DNase/RNase-Free Water (95 µl), 2X Digestion Buffer (95 µl) and Proteinase K (10 µl). The samples were first incubated at 55°C for 1 hour and then incubated at 94°C for 20 minutes to de-crosslink the samples. 600 µl DNA/RNA Lysis Buffer was added to the tissue and mixed thoroughly, then centrifuged at maximum speed for 1 minute to remove insoluble debris. The supernatant was transferred into a Zymo-Spin™ IICR Column[1] with the collection tube and centrifuged, then saved the flow-through. 1 volume of ethanol (95-100%) was added to the flow-through (1:1) and mixed well. The mixture was then transferred into a new Zymo-Spin™ IICR Column[1] in a Collection Tube and centrifuged. The flow-through was discarded. 400 µl of DNA/RNA Wash Buffer was added to the column, centrifuged and discarded the flow-through. For each sample to be treated, 80 µl of DNase I Reaction Mix (5 µl of DNase I and DNA digestion buffer - per sample) was prepared in a nuclease-free tube and mixed by gentle inversion then added directly into column matrix and incubated at room temperature (20-30°C) for 15 minutes. 400 µl of DNA/RNA Prep Buffer was added into the column and centrifuged. The flow-through was discarded. 700 µl of DNA/RNA Wash Buffer was added to the column and centrifuged. The flow-through was discarded. 400 µl of DNA/RNA Wash Buffer was added and centrifuged the column for 1 minute to ensure complete removal of the wash buffer. Then the column was transferred carefully into a nuclease-free tube. 50 µl of

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DNase/RNase-Free Water was added directly to the column matrix and centrifuged. The eluted RNA was used stored in the fridge at -80^{0}.

3.6 Background and samples submitted

The goal of this project was to analyze the expression of the genes: ABCB1 and CYP1B1 in RNA samples extracted from FFPE tissues. The specific objective for the CPGR were as follows:

1. Quality control analysis of the FFPE RNA samples.
2. Design qPCR primers for CYP1B1 using Primer-BLAST. The primers for ABCB1 and the reference genes (GAPDH, HPRT, HSPCB) were already available for the project.
3. Out-source the synthesis of the qPCR primers to Integrated DNA Technologies (Whitehead Scientific).
4. Synthesize first-strand cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher).
5. Optimize and validate qPCR assays.
6. Gene Expression Analysis of the 2 target genes and 3 reference genes in the RNA samples using technical triplicates of each sample.

3.7 qPCR primers

The qPCR primers used for the project (Table 3.2) were either designed using Primer-BLAST, selected from literature, or sourced from PrimerBank. Primer-BLAST primer design parameters are shown in Table 3.3.

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Table 3.2 Primer nucleotide sequences and product lengths for the target and reference genes amplification by qPCR

Gene Symbol	Primer Name	Primer Sequence (5' ------- 3')	Amplicon Size	Reference
ABCB1	3-abcb1Fp1	GGA CTG TAA CTG ACT GCC TTG C	124 bp	This study
	3-abcb1Rp1	GGC AGT TTG GAC AAG ATG ACT CC		
CYP1B1	CYP1B1pp3F	GCTGCAGTGGCTGCTCCT	81 bp	(Finnström et al. 2001)
	CYP1B1pp3R	CCCACGACCTGATCCAATTCT		
GAPDH	3-GAPDHFp	AGT CCC TGC CAC ACT CAG	123 bp	(Nolan et al. 2006)
	3-GAPDHRp	TAC TTT ATT GAT GGT ACA TGA CAA GG		
HPRT1	HPRT1F	GAC CAG TCA ACA GGG GAC AT	132 bp	(Liu et al. 2015)
	HPRT1R	CCT GAC CAA GGA AAG CAA AG		
HSPCB	HSPCBF	TCT GGG TAT CGG AAA GCA AGC C	80 bp	(Jacob et al. 2013)
	HSPCBR	GTG CAC TTC CTC AGG CAT CTT G		

Table 3.3 Primer-BLAST primer design selection criteria

Primer Design Criteria	Selected Parameters
Primer Location	3'- end
PCR product size (bp)	Min = 80 Max = 200
Primer T_m (°C)	Min = 60 Opt = 63 Max = 65
Max. T_m difference [°C] between the primers	1
Primer size (nucleotides)	Min = 18 Opt = 21 Max = 24
Primer GC content (%)	Min = 40 Max = 60
GC clamp	1
Max Poly-X	3
Max GC in primer 3' end	3
SNP handling	Primer binding site may not contain known SNP
Intron inclusion	Primer separated by at least one intron on the corresponding genomic DNA
Intron length range	Min = 200

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3.8 qPCR Assay Optimization and Validation

3.8.1 Standard Curves – calculation of PCR efficiency to measure assay performance

The PCR efficiency (linearity of amplification) of the qPCR assays was assessed by generating a standard curve using 2-fold and 10-fold dilution series of the universal cDNA template. The Cq values obtained at each dilution were then plotted against the log of the cDNA dilution and the amplification efficiency € calculated from the slope of the standard curve using the equation $E = (10^{-1/slope})-1$, where E is PCR efficiency. The ideal efficiency is 100% (slope = -3.3), but an efficiency in the range of 90 – 110% (slope between -3.5 and -3.2) is acceptable according to the MIQE guidelines (Bustin et al. 2009). The qPCR primers used in the project showed qPCR efficiencies within the acceptable range and with high linearity ($R^2 \geq 0.99$) except for ABCC10 (E = 117.96%) and CYP3A4 (E = 111.85%).

3.8.2 Assay Specificity

Specificity of each assay during the optimizations was verified using non-reverse transcribed RNA (NRT), no template control (NTCs) and assay specificity was validated by melt curve analysis. No amplification was seen with NRT and NTC samples. All qPCR assays showed amplification of single amplicons as shown by single Tm peaks on melt curves (Figure 3.1).

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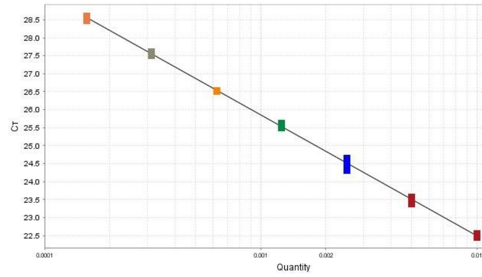
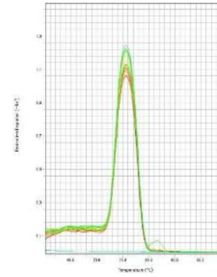
A. ABCB1 Standard Curve



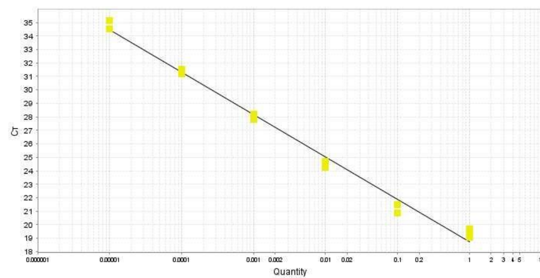
R^2 = 0.998, Slope = -3.36, Efficiency = 98.4%

B. ABCB1 Melt Curve



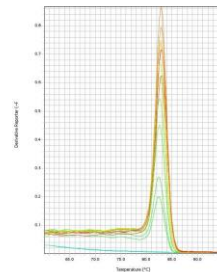
T_m (amplicon) = 75.6°C

C. CYP1B1 Standard Curve



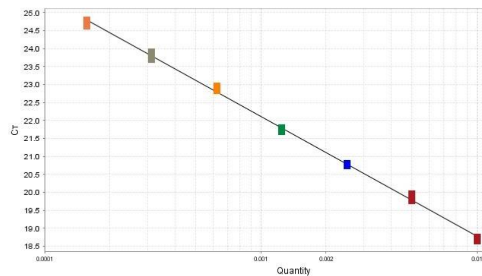
R^2 = 0.99, Slope = -3.141, Efficiency = 108.13%

D. CYP1B 1Melt Curve



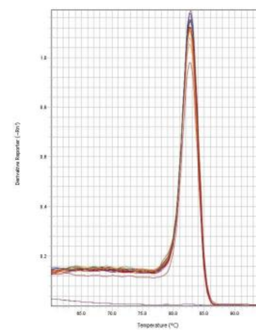
T_m (amplicon) = 83°C

E. GAPDH Standard Curve



R^2 = 0.998, Slope = -3.33, Efficiency = 99.7%

F. GAPDH Melt Curve



T_m (amplicon) = 82.7°C

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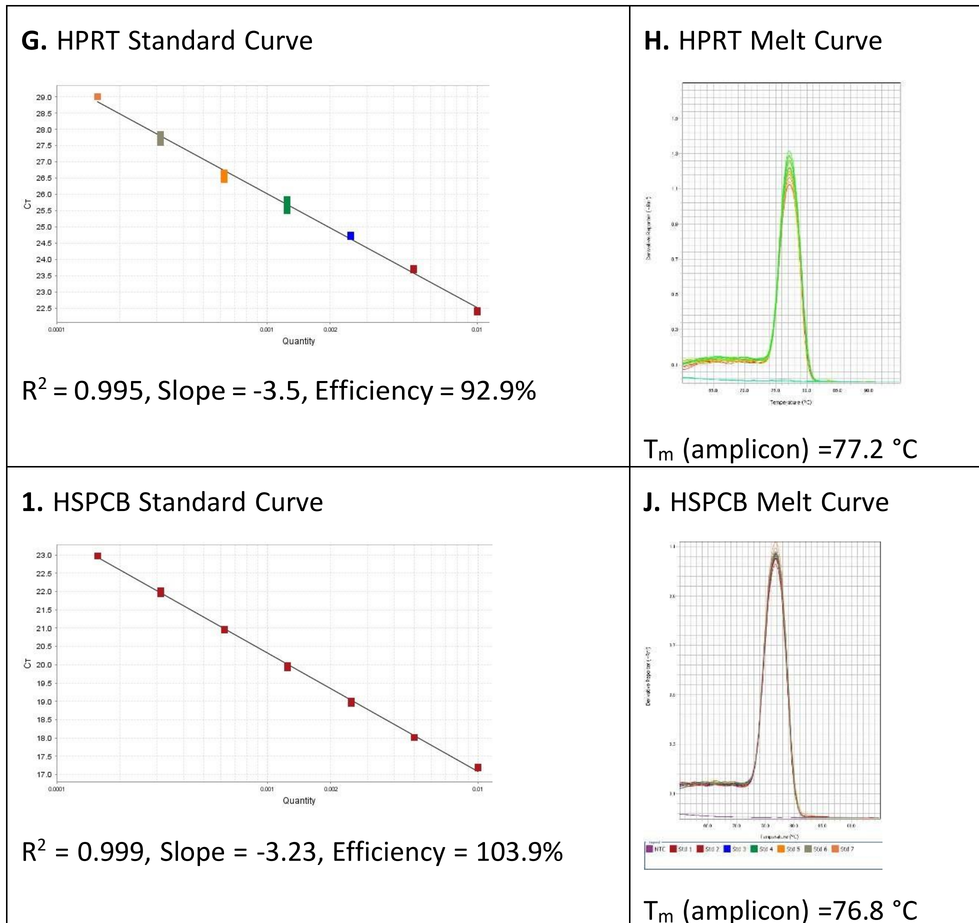

Figure 3.2 Determination of qPCR efficiencies of target and reference genes. Standard curves (on the left) using 2-fold dilution series of the XpressRef Universal cDNA for ABCC1 (A) and 10-fold dilution series for the rest of the amplicons. CT (Cq) cycle s versus log cDNA concentration input were plotted to calculate the slope. The corresponding qPCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. Melt curve plots (on the right) of qPCR assays show a single Tm peak for a specific amplicon in each assay. Tm of each amplicon of each assay are shown

3.9 Gene Expression Analysis

3.9.1 Validation of the preamplification uniformity

- To determine whether amplicons are amplified uniformly and without bias.
- To assess uniformity of preamplification, ΔΔCq is measured by comparing expression levels of target gene(s) in control cDNA sample before and after preamplification (Table 3.4)
- GAPDH was used as a uniformity reference gene
- ΔΔCq = 0 ± 1.5 indicates preamplification uniformity (Table 3.5)

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Table 3.4 Mean Cq values of target gene ABCB1 and reference genes GAPDH, HPRT and HSPCB in non-amplified and preamplified cDNA

Sample Name	Cq^{abcb1}	Cq^{abcc1}	Cq^{GAPDH}	Cq^{HPRT}	Cq^{HSPCB}
CTRL_PreAmp	20.85	20.95	14.05	22.10	14.77
CTRL_NoPreAmp	30.88	30.93	23.35	30.58	ND

CTRL_PreAmp, XpressRef Universal cDNA that was preamplified

CTRL_NoPreAmp, non-amplified XpressRef Universal cDNA

Table 3.5 Determination of preamplification uniformity of ABCB1

Target	$\Delta C_{q\ (NoPreAmp)}$	$\Delta C_{q\ (preAmp)}$	$\Delta\Delta Cq$
ABCB1	7.53	6.80	-0.73
ABCC1	7.53	6.90	-0.63

ΔCq (NoPreAmp) = Cq (target) - Cq (GAPDH)

ΔCq (preAmp) = Cq (target) - Cq (GAPDH)

$\Delta\Delta$Cq = ΔCq (preAmp) - ΔCq (NoPreAmp)

3.9.2 qPCR data

Expression values (Cq) of each target and reference gene were collected and sorted according to biological groups (Normal vs Tumour). These are shown in Table 3.6.

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Table 3.6 Mean Cq values (of 3 replicates) for each target and reference gene using preamplified cDNA samples as templates for qPCR

Sample Name	Biological Group	Cq^{ABCB1}	Cq^{CYP1B1}	Cq^{GAPDH}	Cq^{HPRT}	Cq^{HSPCB}
4N	Normal	28.3	24.12	21.4	33.8	22.4
5N-1	Normal	30.1	23.86	17.6	UND	19.8
5N-2	Normal	32.8	28.92	27.2	28.2	24.0
7N	Normal	27.8	22.93	17.8	27.7	19.7
13N	Normal	26.1	20.38	15.6	UND	17.3
15N	Normal	30.1	22.01	18.1	28.2	18.3
16N	Normal	27.1	21.56	16.7	27.3	18.8
17N	Normal	30.2	25.04	24.2	27.8	22.8
18N	Normal	UND	30.42	29.1	25.4	26.5
20N	Normal	29.4	24.80	20.7	24.8	21.0
21N	Normal	28.9	24.43	22.1	26.3	22.4
4T	Tumour	UND	28.18	25.9	26.8	27.2
5T-1	Tumour	31.6	23.56	17.4	26.3	19.6
5T-2	Tumour	29.8	23.63	18.7	26.1	20.0
7T	Tumour	27.0	22.08	18.5	UND	20.0
13T	Tumour	25.9	20.93	15.9	29.7	17.4
15T	Tumour	28.3	21.37	19.0	UND	18.9
16T	Tumour	27.9	21.73	16.9	32.6	18.8
17T	Tumour	UND	24.06	20.3	29.9	20.9
18T	Tumour	29.7	24.76	22.2	28.4	23.2
20T	Tumour	28.9	20.59	19.6	31.6	20.1
21T	Tumour	29.2	24.28	20.4	30.2	21.4
CTRL	Control Sample	20.9	20.83	14.1	22.1	14.8

UND, Cq value undetermined. Control Sample, XpressRef Universal Total RNA (QIAGEN) used as a positive control for gene expression.

Reference gene selection - Evaluation of the stability of the reference genes

HSPCB was ranked the most stable of the 3 genes by all the RefFinder algorithms, followed by GAPDH (Table 3.7). Both HSPCB and GAPDH were recommended for normalization of the Cq values of the target genes during relative quantitative data analysis.

Table 3.7 Ranking and stability values of the 3 reference genes

Algorithm	HSPCB	GAPDH	HPRT
ΔCT	1	2	3
BestKeeper	1	2	3
NormFinder	1	2	3
GeNorm	1	1	3
Overal Rank	**1**	**2**	**3**

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The rank order (1-3) for each gene is shown for each algorithm (in parenthesis). The overall rank order of the reference genes is shown.

3.10 Experimental procedures

3.10.1 Primer design for qPCR

Primers for the target genes, ABCB1 was designed using the NCBI Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the parameters shown in Table 3.2 to result in 80 -200 bp amplicons. The primers for CYP1B, GAPDH, HPRT1, and HSPCB were sourced from literature and validated.

3.10.2 Primer Preparation

The qPCR primers were sourced from IDT and were resuspended in TE buffer (10mM Tris, pH 8.0, 1mM EDTA) buffer to a 100µM stock concentration, as per manufacturer instructions.

3.10.3 cDNA synthesis

cDNA was synthesized from 100 ng FFPE total RNA using the Maxima H Minus cDNA Synthesis MasterMix with dsDNase Kit, according to the manufacturer's protocol (Thermo Fisher Scientific Pub. No. MAN0016393, Rev.B.0). A 1 µg of a commercial XpressRef Universal Total RNA (QIAGEN) was also reverse transcribed and used for validation of the qPCR assays and as a positive control. NRT negative control reactions were performed alongside to assess for genomic DNA contamination of the RNA sample. An NTC reaction was also included to assess for reagent contamination and primer dimers.

3.10.4 cDNA preamplification

For FFPE cDNA pre-amplification, the TaqMan PreAmp Master Mix (Thermo Fisher) was used according to the manufacturer's instructions. All qPCR primers were pooled by combining 1 µl of each 100 µM primer stock to make a final volume of 200 µl in TE buffer to give a final concentration of 500 nM each primer. The preamplification reaction was performed in a final volume of 5 µ l containing 2.5 µ l TaqMan PreAmp Master Mix, 0.5 µl pooled primer mix and 2 µl cDNA, and amplified using the cycling conditions: one cycle at 95°C for 10 min, 14 cycles at 95°C for 15 s and 60°C for 4 min. At the end of the cycling program the reactions were diluted 1:20 in TE Buffer. Pre-amplified DNAs were stored at −20°C until used for qPCR assays.

3.11 qPCR validation

3.11.1 qPCR reaction efficiency

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PCR efficiency was determined using standard curves generated with 2- and 10- fold dilutions of the XpressRef Universal Total cDNA for each target gene. Real-time qPCR amplifications were performed in triplicate in 10 µl volume containing 5 µl 2x PowerUp SYBR Green Master Mix (Thermo Fisher), 0.5 µl forward and reverse primer (500 nM final concentration), 2 µl nuclease- free water and 2 µl cDNA. The reactions were performed on the QuantStudio 12K Flex PCR System (Applied Biosystems) using the following cycling conditions: 2 min UDG activation at 50°C, 2 min polymerase activation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 57°C followed by a dissociation curve analysis. Standard curves were generated by plotting the CT values against the log of each serial dilution.

3.11.2 qPCR Assays

qPCR was performed in a total reaction volume of 10ul consisting of 2 µl diluted preamplified cDNA as template, 5µl of 2x PowerUp SYBR Green I Mastermix (Thermo Fisher), and both the forward and reverse primers to a final concentration of 500 nM. NTC reactions were included in all assays as negative controls. Each reaction was run in triplicate. Reactions were performed on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using the following cycling parameters: 50°C for 2 min; initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 15 secs and 57°C for 1 min. A melt curve analysis was performed on all reactions at the end of the PCR run using default parameters. Amplification data were analyzed with Life Technologies QuantStudio 12K Flex Software v1.2.4, applying user-defined thresholds to obtain Cq-values. Outliers in technical replicate reactions, reactions that showed no amplification and reactions that showed multiple Tm peaks during melt curve analysis were removed from analysis. Data was finally exported into Excel spreadsheets for further analysis.

3.11.3 Reference gene stability

The stability of the reference genes was evaluated using the web-based tool RefFinder (https://blooge.cn/RefFinder/) which runs four well-established algorithms (GeNorm, BestKeeper, NormFinder and comparative delta-CT) to simultaneously compare and rank the three candidate reference genes (from most to least stable).

3.12 Proteomics study

3.12.1 Signalling pathway enrichment of ABCB1 and CYP1B1 expression

To identify the signalling pathways and network associated with the expressions of ABCB1 and CYP1B1, both genes were subjected to gene set enrichment using the Enrichr online server (https://maayanlab.cloud/Enrichr/) (Kuleshov et al. 2016; Xie et al. 2021). The signaling pathways were mapped based on their p-value ranking using the KEGG pathway, WikiPathway and PFOCR_Pathways.

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3.12.2 Protein–Protein Interaction Network Analysis of CYP1B1 and ABCB1

ABCB1 and CYP1B1 were subjected to protein–protein interaction network analysis to predict functional proteins that may contribute to their interactions and network, using the STRING version 12 online server (https://string-db.org/).

3.13 Molecular Docking Analysis

Following the patients treatment regimen with docetaxel, we went further to investigate the molecular interaction between drug and the studied genes, ABCB1 and CYP1B1, *in silico*.

The Molecular Operating Environment (MOE 2015.10) software was employed the *in silico* study. The Protein Data Bank website (PDB) (https://www.rcsb.org/) was used to obtain 3D models of CYP1A1 (PDB ID 3PM0) (Raju et al. 2022). For ABCB1, the amino acids sequences of the protein (P08183) were obtained from the Universal Protein Resource (UniProt) (https://www.uniprot.org/) and modelled using the Swiss Model online tools (https://swissmodel.expasy.org/). The active ligands were created using 3D protonation, partial charge calculation, and energy reduction using Force Field MMFF94x. Proteins were created without adding repeat chains, and water. MOE Quick Prep was used to fix structural defects, perform 3D protonation, and compute partial charge. MOE was utilized to generate the best binding pocket under specific conditions, using a triangle matcher as the placement method and London dG as the major scoring function. An extra refinement step was performed utilizing the rigid receptor technique with the GBVI/WSA dG score function to retain poses with the protein's largest hydrophobic, ionic, and hydrogen-bond interactions. Then, the compound-enzyme complex was visually analyzed using BIOVIA Discovery studio visualizer.

3.14 Statistic analysis

Data from all experiments was captured electronically by the researcher in Microsoft Excel. Any further analysis was done by the statistician using SAS Version 9.2. Descriptive statistics namely frequencies and percentages were calculated for the categorical data. Means and standard deviations or medians and percentiles were calculated for numerical data. A significance level of 0.05 was used.

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CHAPTER FOUR: RESULTS

4.1 Relative Gene Expression of ABCB1

The results of ABCB1 expression levels in the FFPE samples are shown in Figure 4.1. In addition, Figure 4.2 further compares this samples based on how the patients responded to docetaxel treatment (Good and Poor Responders). ABCB1 codes for the MDR ABC transporter which is involved in transporting of docetaxel across the cell membrane. High expression of this protein means high transport of the drug out of the cell, affording the drug less time to exert the desired cytotoxic effect, which could lead to a poor response to treatment with docetaxel. As shown in Figure 4.1, the expression of ABCB1 was lower in prostate tumours compared to the normal prostate tissues where it was highly expressed. However, when compared between good and poor responders, ABCB1 was significantly expressed in both responders as shown in Figure 4.2.

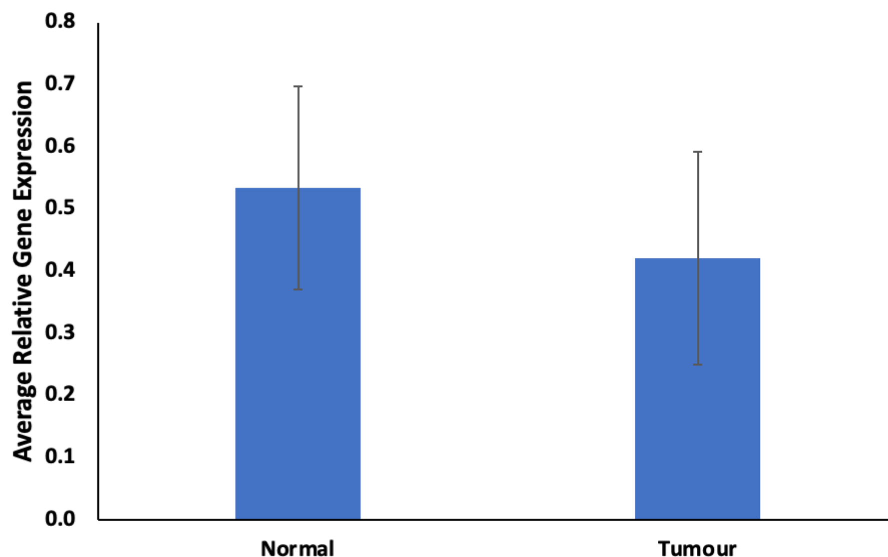

Figure 4.1 Expression of ABCB1 in prostate tumour. Values = mean ± SE; n = 3

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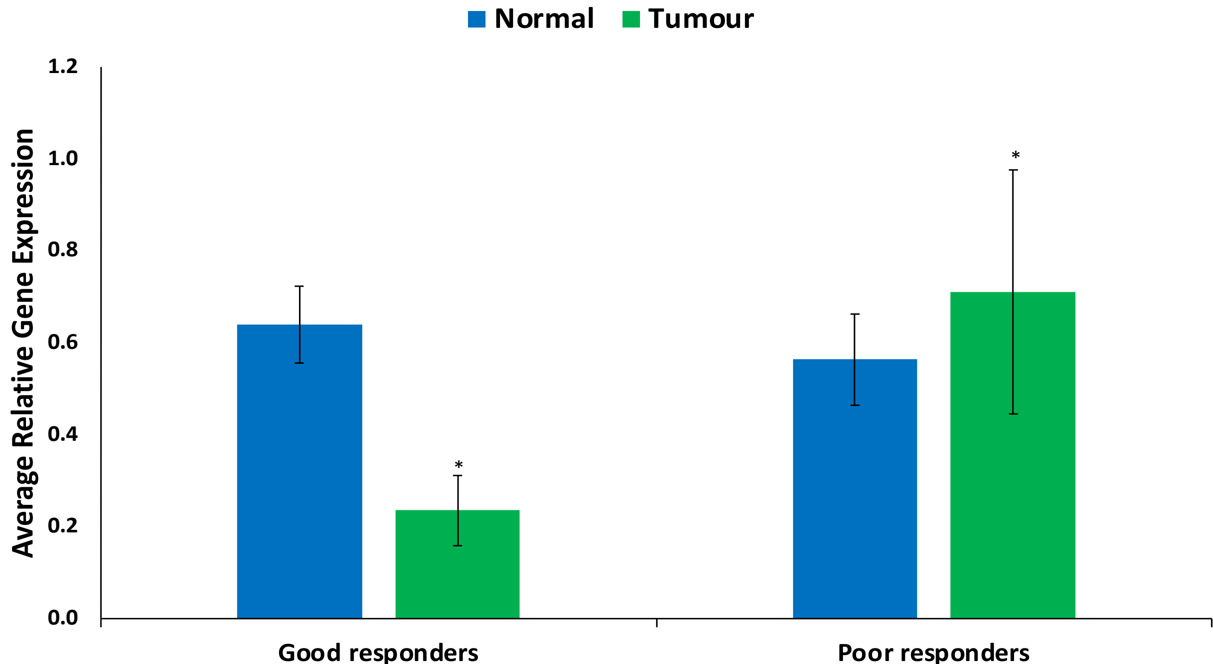

Figure 4.2 Expression of ABCB1 in prostate tumour of good and poor responders. Values = mean ± SE; n = 3. *Statistically significant (p<0.05) in comparison to normal prostate tissues (normal)

4.2 Relative Gene Expression of CYP1B1

The CYP1B1 expression results are well presented in Figure 4.3 and Figure 4.4. The CYP1B1 gene codes for an enzyme that belongs to the CYP1 family, which is a subfamily of the cytochrome P450 superfamily. This enzyme is essential to the metabolism of docetaxel. Certain types of cancer, including PC, have been linked to elevated levels of CYP1B1. As shown in Figure 4.3 CYP1B1 was significantly ($p<0.05$) expressed in prostrate tumours compared to normal prostate tissues. In Figure 4.4, CYP1B1 was also significantly ($p<0.05$) expressed in prostrate tumours of poor responders compared to good responders and normal cells. There seems to be high expression of CYP1B1 in the Good responders category than in the Poor responders. Within the Good responders, Normal tissue expressed higher level of CYP1B1 than the tumour. However, the opposite seems to happen with the Poor responders where CYP1B1 expression significantly increased in the tumour.

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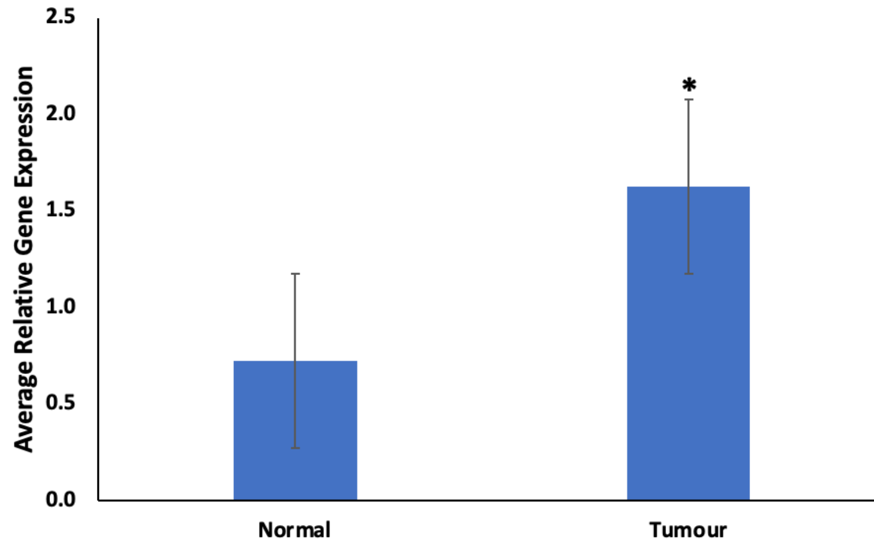

*Figure 4.3 Expression of CYP1B1 in prostate tumours. Values = mean ± SE; n = 3. *Statistically significant (p<0.05) in comparison to normal prostate tissues (normal)*

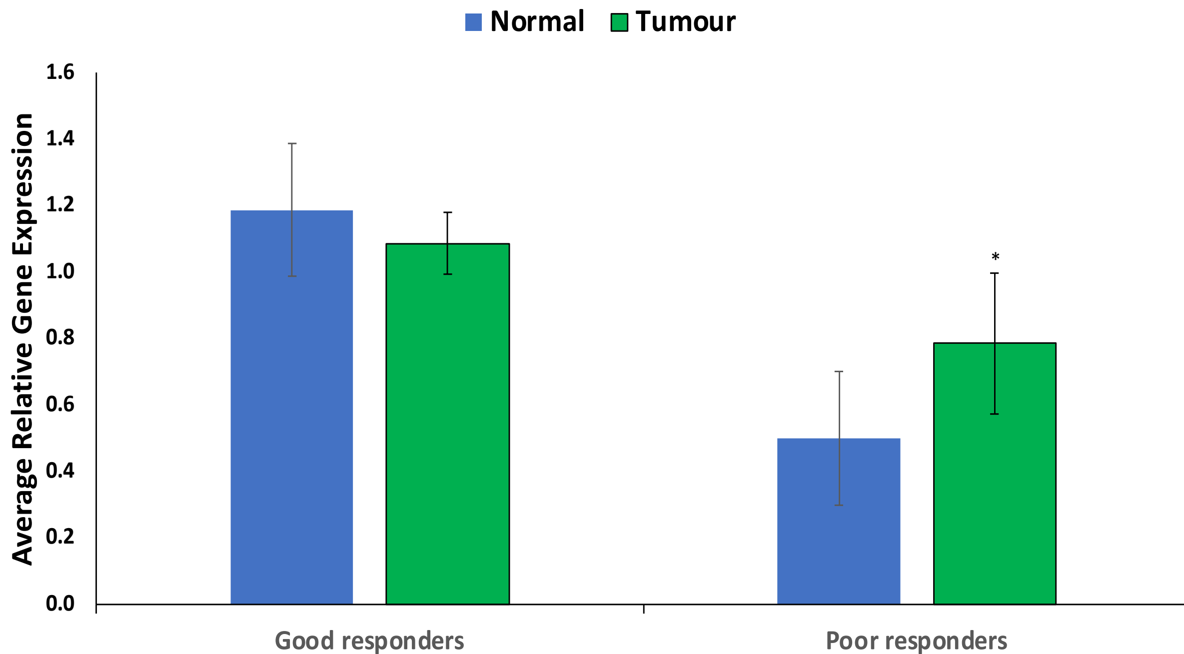

*Figure 4.4 Expression of CYP1B1 in prostate tumours of good and poor responders. Values = mean ± SE; n = 3. *Statistically significant (p<0.05) in comparison to normal prostate tissues (normal)*

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4.3 Signalling Pathway Enrichment of CYP1B1 and ABCB1 Expression

Signalling pathway enrichment analysis is a computational method used in bioinformatics and systems biology to identify and interpret patterns in high-throughput biological data, such as gene expression data or proteomic data. The goal is to determine whether specific signalling pathways are overrepresented or enriched in a list of genes compared to what would be expected by chance. Enrichment of ABCB1 and CYP1B1 revealed a total of 15 GO molecular functions and pathways, with p values < 0.05 as shown in Table 4.1.

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Table 4.1 Molecular function and network analysis following gene enrichment

Molecular function and pathways	Library	p-value	q-value	z-score	Combined score
MicroRNAs in cancer	KEGG_2021_Human	0.0002395	0.002155	39380	328300
Nuclear Receptors Meta-Pathway WP2882	WikiPathway_2021_Human	0.0002536	0.004599	39362	325900
Sulindac Metabolic Pathway WP2542	WikiPathway_2021_Human	0.0004999	0.004599	4999	37990
PMC5346035 Nihms844046F1	PFOCR_Pathways	0.0004999	0.002168	4999	37990
PMC4844852 Nihms-744712-F0001	PFOCR_Pathways	0.0004999	0.002168	4999	37990
PMC7235470 RMV-30-e2109-g001	PFOCR_Pathways	0.0004999	0.002168	4999	37990
PMC5953846 Nihms963973F8	PFOCR_Pathways	0.0004999	0.002168	4999	37990
PMC5896366 Dmd.118.080663F2	PFOCR_Pathways	0.0004999	0.002168	4999	37990
LncRNA-mediated mechanisms of therapeutic resistance WP3672	WikiPathway_2021_Human	0.0005999	0.004599	3999	29660
Benzo(a)pyrene metabolism WP696	WikiPathway_2021_Human	0.0008998	0.004827	2499	17520
Estrogen Receptor Pathway WP2881	WikiPathway_2021_Human	0.0013	0.004827	1666	11070
Tryptophan metabolism	KEGG_2021_Human	0.004196	0.01096	486.8	2664
ABC transporters	KEGG_2021_Human	0.004495	0.01096	453.5	2451
Ovarian steroidogenesis	KEGG_2021_Human	0.005094	0.01096	399	2106
Steroid hormone biosynthesis	KEGG_2021_Human	0.006091	0.01096	332.3	1695

As shown in Figure 4.5, the network map revealed ABCB1 to be associated with ABC transporters and LncRNA-mediated mechanisms of therapeutic resistance WP3672, while CYP1B1 is associated with ovarian steroidogenesis, tryptophan metabolism, steroid hormone biosynthesis, benzo(a)pyrene metabolism WP696, sulindac metabolic pathway

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WP2542 and estrogen receptor pathway WP2881. Both ABCB1 and CYP1B1 were associated with microRNAs in cancer and nuclear Receptors Meta-Pathway WP2882.

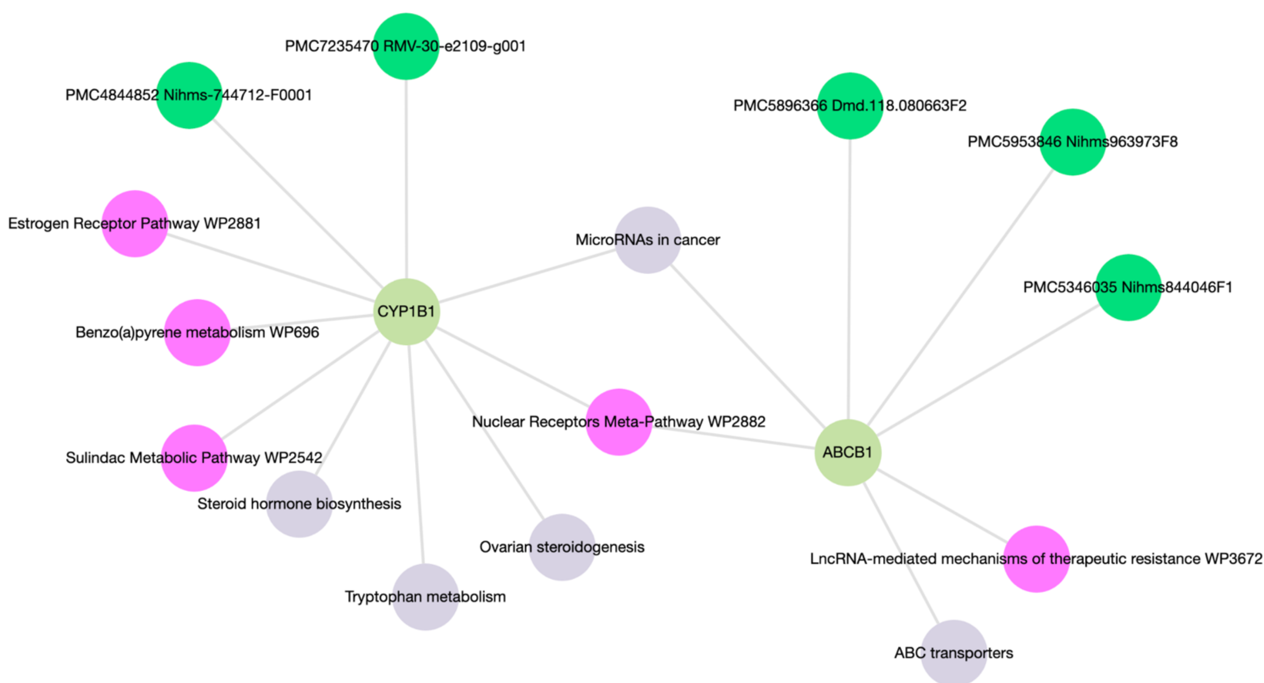

Figure 4.5 Network map of molecular function and pathways following gene enrichment

4.4 Protein-Protein Interaction Network Analysis of CYP1B1 and ABCB1

Protein-Protein Interaction Network Analysis is a computational approach used in bioinformatics and systems biology to study the interactions between proteins within a cell or an organism. Proteins seldom act in isolation; rather, they form intricate networks by physically interacting with each other to carry out various cellular functions. As shown in Figure 4.6, STRING analysis predicted a protein-protein interaction of ABCB1 and CYP1B1 with Glutathione S-transferase Pi (GSTP1); Catechol O-methyltransferase (COMT); UDP-glucuronosyltransferase 1-6 (UGT1A6); Leucine rich Transmembrane and O-methyltransferase (LRTOMT); and Epoxide hydrolase 1 (EPHX1), and a score of 0.973, 0.971, 0.966, 0.966 and 0.966, respectively (Table 4.2).

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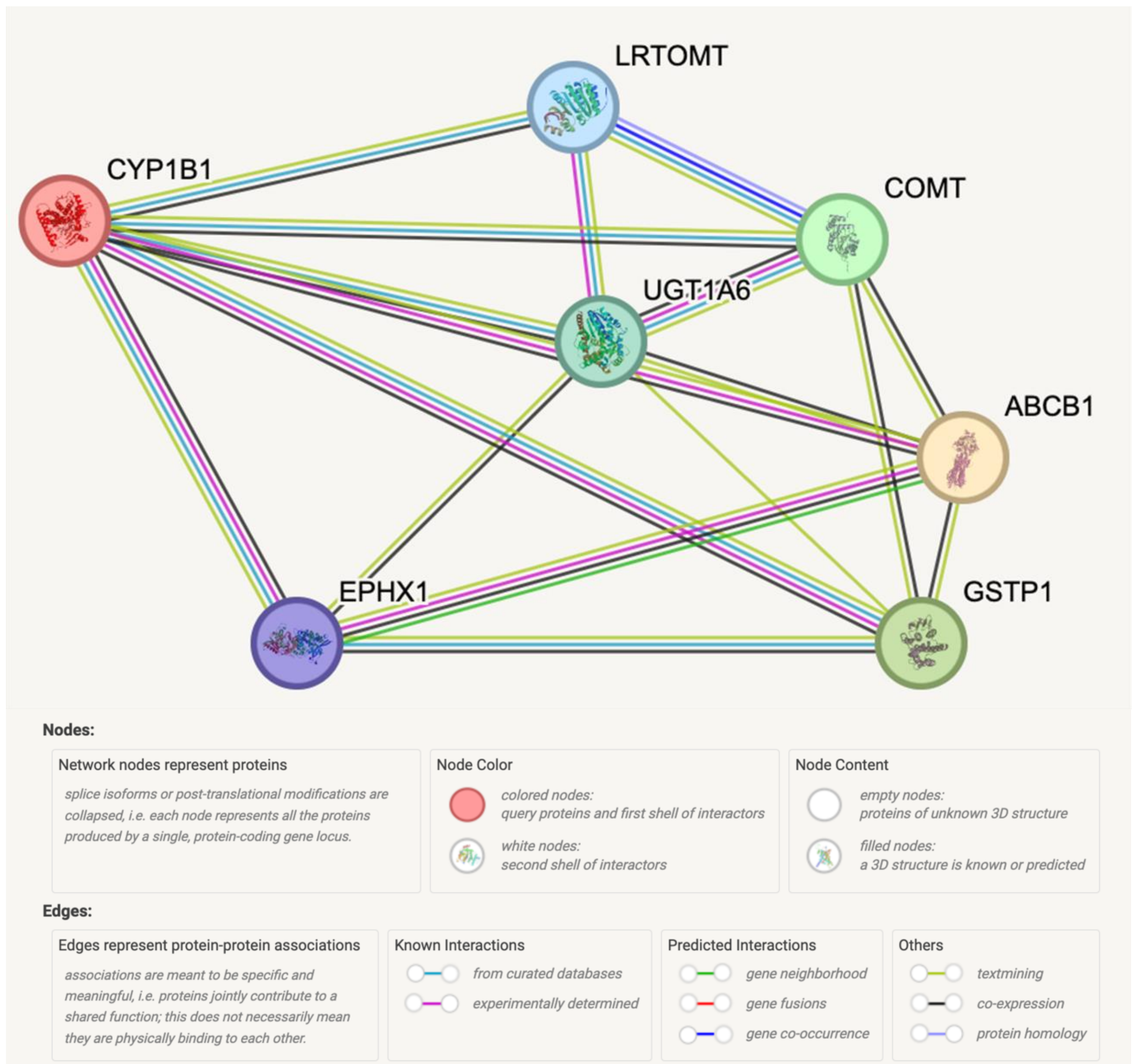

Figure 4.6 Protein–protein interaction network of CYP1B1 and ABCB1 genes with predicted functional proteins visualized by STRING

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Table 4.2 Predicted functional proteins and score

Protein	Score
GSTP1	0.973
COMT	0.971
UGT1A6	0.966
LRTOMT	0.966
EPHX1	0.966

GSTP1: Glutathione S-transferase Pi; **COMT**: Catechol O-methyltransferase; **UGT1A6**: UDP-glucuronosyltransferase 1-6; **LRTOMT**: Leucine rich Transmembrane and O-methyltransferase; and **EPHX1**: Epoxide hydrolase 1.

4.5 Molecular Docking Analysis of ABCB1 and CYP1B1

A computational method called molecular docking analysis is used in structural biology and drug discovery to forecast and examine molecular interactions, usually between a target macromolecule (like a protein) and a small ligand (like a drug candidate). Molecular docking aims to forecast a ligand's preferred conformation, orientation, and binding affinity during its interaction with a target receptor. Molecular docking analysis of docetaxel with ABCB1 and CYP1B1 revealed potent molecular interactions between the drug and the genes as portrayed by the energy scores (Table 4.3).

Table 4.3 Free binding energy interaction of docetaxel with ABCB1 and CYP1B1

Drug	Genes	Protein	Bond	Score of binding energy Kcal/mol
Docetaxel	ABCB1	P08183	H-donor H-donor H-donor H-acceptor	-15.25
	CYP1B1	3PM0	H-acceptor H-acceptor Pi-H	-20.37

The interactive bonds were identified as hydrogen bonds (H-acceptor and Pi-H) as shown in Table 4.3 and Figures 4.7A and 4.8B.

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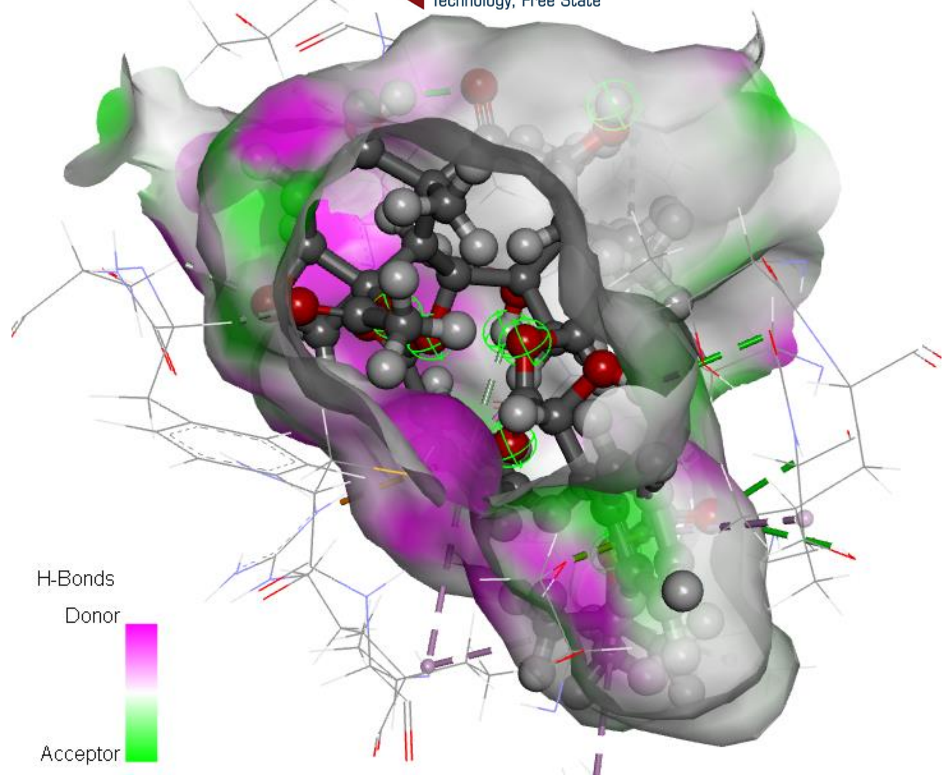
(A)



(B)

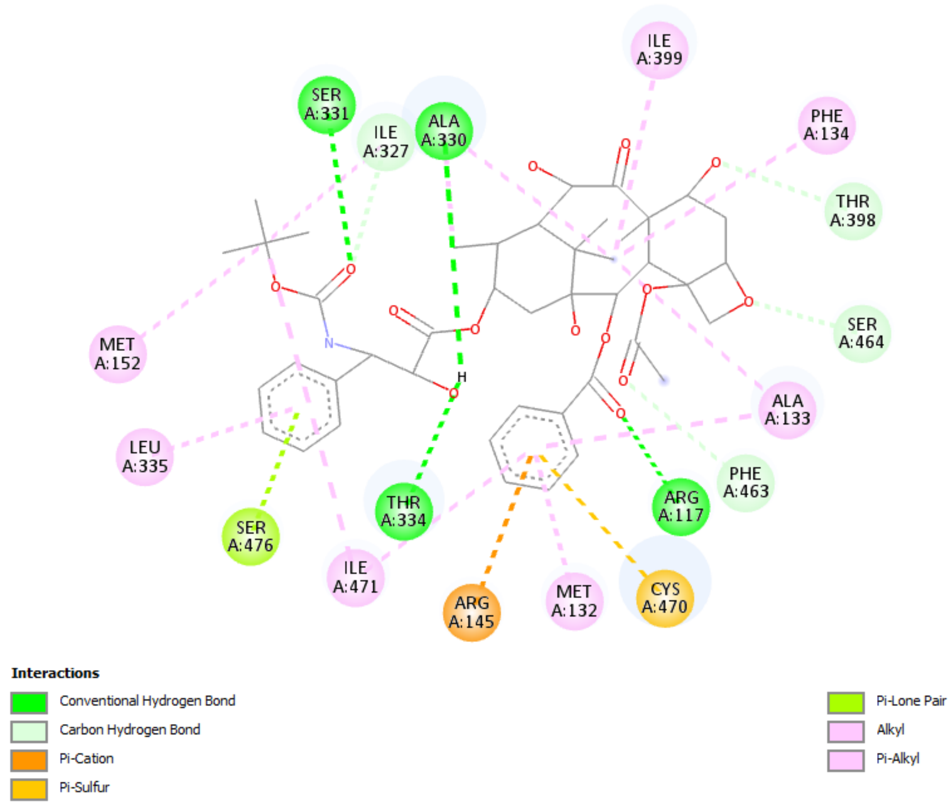


Figure 4.7 (A) 3D and (B) 2D representations of the molecular interaction of Docetaxel with CYP1B1

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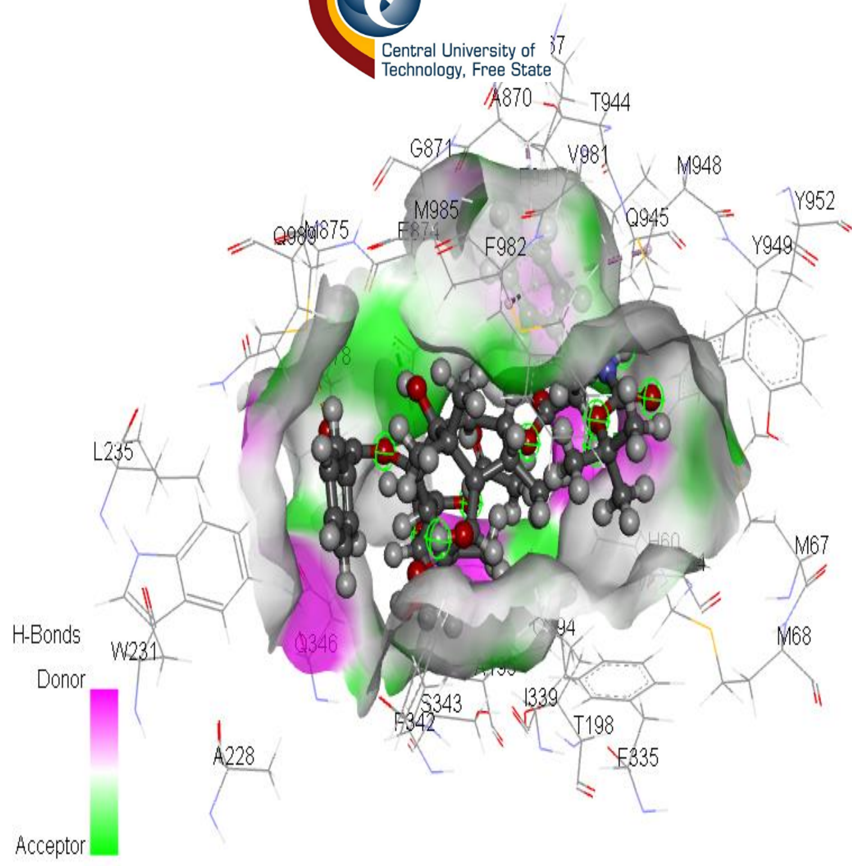
(A)



(B)

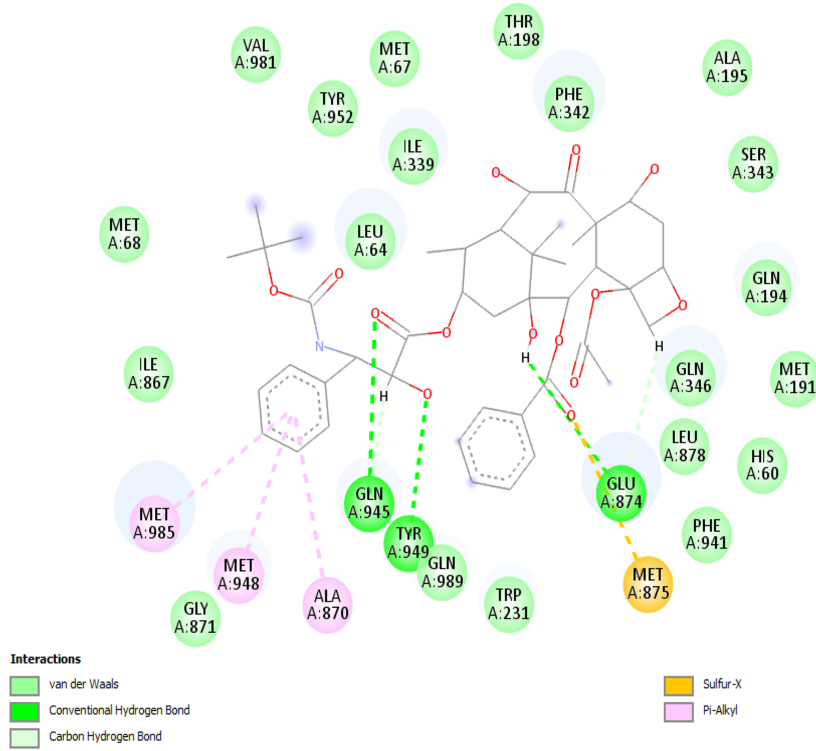


Figure 4.8 (A) 3D and (B) 2D representations of the molecular interaction of Docetaxel with ABCB1

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CHAPTER FIVE: DISCUSSION

The increased cases of PC particularly in sub-Saharan Africa, is a cause of major concern owing to the region's poor economy and health infrastructure. Despite limited availability of medication, the increasing rate of drug resistance in PC patients continue to contribute to the morbidity and mortality of the disease. Thus, further portraying a double-edged sword. Upregulations of proteins involved in drug metabolism has been implicated in drug-resistance in PC. The present study investigated the expression of proteins reported for drug-resistance vis-à-vis ABCB1 and CYP1B1 in PC tumours and normal prostate tissues in categories of patients according to their responses to treatment with docetaxel; good responders (chemo sensitive) and poor responders (chemo resistant).

ABCB1 plays a major role in drug resistance as its overexpression has been implicated in several multidrug-resistant cancers. Its upregulation has been reported in cancer cells treated with chemotherapeutic agents such as paclitaxel, doxorubicin, and cisplatin *in vitro* (Skinner et al. 2023; Wu et al. 2021). This upregulation triggers a series of molecular and biochemical processes that further exacerbates chemotherapeutic resistance. In the present study, the low expression of ABCB1 in prostate tumours (Figure 4.1) may indicate low chemotherapeutic resistant via the ABCB1 pathway. The low expression further indicates suppression of the identified GO and biochemical pathways following gene enrichment of ABCB1 (Figure 4.3 and Table 4.1). The low expression further translates to the low activation of the ABC Transporter pathways which play a crucial role in drug transport, with combined score of 0.5 Normal tissues and 0.4 Tumour tissues as depicted in Table 4.1. However, the high expression of ABCB1 in the poor responders (Figure 4.2), portrays chemo-resistance in the patients. This corroborates with identified pathways following enrichment and network analysis of ABCB1 (Table 4.1 and Figure 4.5). ABCB1 is a member of the ABC transporters, which translocate substrates across membranes (Rees et al. 2009). Although studies of ABC transporters in PC are limited, their roles in tumorigenesis and chemo-resistance have been reported (Demidenko et al. 2015; Muriithi et al. 2020). LncRNAs have been well reported for their role in drug resistance in cancer. It induces drug resistance by several mechanisms including remodeling of chromatin, ceRNAs, chromatin interactions, and natural antisense transcripts (NATs), thereby modulating apoptosis ((Ding et al. 2021) Ye et al. 2022). The low expression in the good responders indicates suppressed drug resistance, and therefore insinuates deactivation of ABC transporters and LncRNA-mediated mechanisms of therapeutic resistance.

CYP1B1 belongs to polypeptide 1 and family 1 of cytochrome P450 enzymes, and it is expressed in almost all tissues in the body where they are involved in several metabolisms including drug metabolism, redox and energy homeostasis (Falero-Perez et

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al. 2018; Shah et al. 2019). Its overexpression has been reported in several malignant tumours, and has been implicated in chemotherapeutic resistance in these tumours (Chen et al. 2023; McFadyen et al. 2001; Zhu et al. 2015). Zhu et al. (2015) reported a correlation between CYP1B1 expression and increased resistance of epithelial ovarian cancer cells to paclitaxel. This is further corroborated by Chen et al. (2023) who linked CYP1B1 expression with anti-PD-1 resistance in colorectal cancer. In the present study, the increased expression of CYP1B1 in prostate tumours (Figure 4.3), including in the poor responders (Figure 4.4) therefore portrays a high potential of drug resistance among the patients. This correlates with several studies on increased CYP1B1 expression and drug resistance in PC (Beuten et al. 2008; Lin et al. 2022; Zhang et al. 2013).

The identified pathways following enrichment and network analysis of CYP1B1 (Table 4.1 and Figure 4.5), have been implicated in the pathophysiology of PC. CYP1B1 has been shown to activate these pathways leading to drug resistance and progression of PC (Angel and DiGiovanni 2018). In PC, malignancy is driven by steroid metabolism via unconventional pathways where CYP1B1 catalyzes the transformation of estradiol to 4-Hydroxy-17β-estradiol (4-OHE2) (Lin et al. 2022). These pathways subvert pharmacologic blockades and boost flux by employing secondary enzymes such that have been reported to circumvent canonical pathways and disrupt treatment plans (Sharifi et al. 2012). Abnormal tryptophan metabolism has been implicated in the pathophysiology of PC, and has been linked with chemotherapy resistance via the aryl hydrocarbon receptor (AhR) (Li et al. 2021; Li et al. 2022). An increased mRNA expression of CYP1B1 is a marker of AhR (Chuang et al. 2022).

The role of the predicted functional proteins (enzymes) following STRING analysis of CYP1B1 and ABCB1 (Figure 4.6 and Table 4.2) in drug resistance in cancers have been reported. GSTs have been linked to chemotherapy resistance via direct detoxification of the drug (Townsend and Tew 2003), and GSTP1 has been suggested as a marker of drug resistance (Nakagawa et al. 1990). UDP-glucuronosyltransferase enzymes including UGT1A6 have been well reported for their metabolic inactivation of drug therapies in tumours (Allain et al. 2020; De Almagro et al. 2011). Overexpression of EPHX1 has been reported in castration-resistant PC (Ju et al. 2021).

To further understand the potential molecular interaction between the studied genes, and the regimen drug, docetaxel, we carried out a molecular docking analysis. The predicted free energies of -15.25 and -20.37 Kcal/mol (Table 4.3) suggests potent molecular interactions of the drug with ABCB1 and CYP1B1, respectively. This interaction can be attributed to the identified bonds which binds the drug to the active site of the genes as shown in Figures 4.7A - 4.8B. Previous studies have demonstrated increased survival rate in PC patients treated with docetaxel (de Morrée et al. 2017; Puente et al. 2017; Vale et al. 2023). However, the therapeutic efficacy has been reported to be compromised in drug-resistant PC cells (Corcoran et al. 2012; Hashemi et al. 2023; Sekino and Teishima

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2020). Furthermore, high expression of CYP1B1 has been implicated in the resistance of PC cells to docetaxel (Lin et al. 2022; McFadyen et al. 2001; Pastina et al. 2010). Multidrug resistance has also been reported in Docetaxel therapy in PC cells with high expression of ABCB1 (Linke et al. 2022; Seo et al. 2020). Thus, the potent molecular interaction of docetaxel with ABCB1 and CYP1B1 (Table 4.5 and Figures 4.7 and 4.8) may insinuate potential resistance of the cancer cells to docetaxel via increased expression of ABCB1 and CYP1B1. This correlates previous reports on increased expression of ABCB1 and CYP1B1 and cell proliferation following treatment with docetaxel (Martinez et al. 2008; Seo et al. 2020).

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CHAPTER SIX: CONCLUSION

Taken together, these results indicate the susceptibility of the studied cancer patients drug resistance via increased expression of ABCB1 and CYP1B1 in tumour samples of the poor responders' category, and their associated molecular pathways. This is further depicted by the potent molecular interaction of both ABCB1 and CYP1B1 with the regimen drug, docetaxel. Although the study was initially planned to compare the three categories of patients; good responders, poor responders and those who exhibited excessive toxicity, the study was limited to only comparing the two groups of specimen (good and poor responders) due to the shortage of patients who exhibited excessive toxicity in the South African government database. The study was also limited to expression studies without investigation of the identified pathways and predicted enzymes activities (obtained from proteomics studies). We therefore propose further investigation of these pathways and enzyme activities in both pre-treated and post-treated patients with PC. This will give a clear understanding on the interaction of the drug and the expressed proteins in order to cognize how genetic factors affect drug transport and metabolism cause individual responses to chemotherapy vary, as well as give a clearer picture of the potential molecular mechanism of the drug effect in patients. This pilot study served as an initial exploration, focusing on delivering a proof of concept and establishing a methodology. However, it is imperative to underscore that a more extensive study is indispensable for comprehensive insights. Subsequent investigations must employ freshly obtained patient biopsies, as opposed to archived specimens, to ensure an ample supply of RNA and proteins for precise expression level analyses.

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APPENDIX

Epithelial tumours	
Glandular neoplasms	
Acinar adenocarcinoma	8140/3
Atrophic	
Pseudohyperplastic	
Microcystic	
Foamy gland	
Mucinous (colloid)	8480/3
Signet ring–like cell	8490/3
Pleomorphic giant cell	
Sarcomatoid	8572/3
Prostatic intraepithelial neoplasia, high-grade	8148/2
Intraductal carcinoma	8500/2
Ductal adenocarcinoma	8500/3
Cribriform	8201/3
Papillary	8260/3
Solid	8230/3
Urothelial carcinoma	8120/3
Squamous neoplasms	
Adenosquamous carcinoma	8560/3
Squamous cell carcinoma	8070/3
Basal cell carcinoma	8147/3
Neuroendocrine tumours	
Adenocarcinoma with neuroendocrine differentiation	8574/3
Well-differentiated neuroendocrine tumour	8240/3
Small cell neuroendocrine carcinoma	8041/3
Large cell neuroendocrine carcinoma	8013/3
Mesenchymal tumours	
Stromal tumour of uncertain malignant potential	8935/1
Stromal sarcoma	8935/3
Leiomyosarcoma	8890/3
Rhabdomyosarcoma	8900/3
Leiomyoma	8890/0
Angiosarcoma	9120/3
Synovial sarcoma	9040/3
Inflammatory myofibroblastic tumour	8825/1
Osteosarcoma	9180/3
Undifferentiated pleomorphic sarcoma	8802/3
Solitary fibrous tumour	8815/1
Solitary fibrous tumour, malignant	8815/3
Haemangioma	9120/0
Granular cell tumour	9580/0
Haematolymphoid tumours	
Diffuse large B-cell lymphoma	9680/3
Chronic lymphocytic leukaemia / small lymphocytic lymphoma	9823/3
Follicular lymphoma	9690/3
Mantle cell lymphoma	9673/3
Acute myeloid leukaemia	9861/3
B lymphoblastic leukaemia/lymphoma	9811/3
Miscellaneous tumours	
Cystadenoma	8440/0
Nephroblastoma	8960/3
Rhabdoid tumour	8963/3
Germ cell tumours	
Clear cell adenocarcinoma	8310/3
Melanoma	8720/3
Paraganglioma	8693/1
Neuroblastoma	9500/3
Metastatic tumours	

Tumours of the seminal vesicles	
Epithelial tumours	
Adenocarcinoma	8140/3
Squamous cell carcinoma	8070/3
Mixed epithelial and stromal tumours	
Cystadenoma	8440/0
Mesenchymal tumours	
Leiomyoma	8890/0
Schwannoma	9560/0
Mammary-type myofibroblastoma	8825/0
Gastrointestinal stromal tumour, NOS	8936/1
Leiomyosarcoma	8890/3
Angiosarcoma	9120/3
Liposarcoma	8850/3
Solitary fibrous tumour	8815/1
Haemangiopericytoma	9150/1
Miscellaneous tumours	
Choriocarcinoma	9100/3
Seminoma	9061/3
Well-differentiated neuroendocrine tumour / carcinoid tumour	8240/3
Lymphomas	
Ewing sarcoma	9364/3
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) {917A}. Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification {756A}, taking into account changes in our understanding of these lesions.

Appendix 1. Prostate tumours' histological classification by the World Health Organization in 2016 Adapted without permission (Humphrey, 2016).

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