The Role of the Epidermal Growth Factor Receptor Family in Prostate Cancer and their Potential as Therapeutic Targets

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Abstract

The Epidermal Growth Factor Receptor family has been associated with the development of advanced prostate cancer (Carrion-Salip *et al.*, 2012). The family consists of 4 tyrosine kinase receptors – EGFR, HER2, HER3 and HER4 (Burgess, 2008). Previous research has focussed on targeting the EGFR and HER2 receptors however, the role of HER3 in driving PI3K/Akt signalling and mediating drug resistance has shifted the attention towards this signalling partner. This project provides additional understanding regarding the functioning of this receptor family in prostate cancer and establishes the therapeutic potential of a novel pan-inhibitor. The expression levels of HER2 and HER3 were validated in patient biopsy samples and elevated cytoplasmic and nuclear expression was observed with disease progression. Patients differentially expressing low HER2 and strong HER3 levels correlated significantly with poor survival rates. The nuclear translocation of these receptors was further validated *in-vitro* upon ligand induced activation and their total and phosphorylated forms were found to be present in the nucleus and bound to chromatin. The differential expression of the receptors was also explored *in-vitro* and *in-vivo* by transient and stable over-expression and the importance of HER3 mediated signalling was established by the observation of increased MAP Kinase and PI3 Kinase cell signalling pathway activities. This over-activation translated to vital cellular processes in cancer progression, with significant increases in cell proliferation, migration and invasion. The role of these receptors in acquired drug resistance revealed elevated expression and activity of EGFR and HER3 receptors in Lapatinib resistant cells. AZD8931, an EGFR, HER2 and HER3 inhibitor, radically reduced the functioning of these receptors and their regulated cellular processes. The findings of this project opens new avenues for research and suggests the use of a pan-inhibitor as a novel and attractive therapeutic approach in the treatment of advanced prostate cancer.

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
ADAM	A disintegrin and metalloprotease
AR	Amphiregulin
AR	Androgen Receptor
ARE	Androgen Response Element
AWT	Androgen Withdrawal Therapy
BioCOSHH	Biological Control of Substances Hazardous to Health
BM	Basal Medium
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
BTC	Betacellulin
CAF	CBP-associated factor
CBP	CREB bind protein
COSHH	Control of Substances Hazardous to Health
COX	Cyclooxygenase
CREB	cAMP response element binding protein
CRPC	Castration- Resistant Prostate Cancer
DAB	Diaminobenzidine tetrahydrochloride
DBD	DNA-binding domain
DCC	Dextran-Coated Charcoal
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMSO	Dimethylsulphoxide
DMSO	Dimethylsulphoxide
DNA-PK	DNA-dependent protein kinase

DRE	Digital Rectal Examination
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EPG	Epigen
EPR	Epiregulin
ERK	Extracellular-signal regulated kinases
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FM	Full Medium
FOXO	Forkhead box O
FzM	Freezing Medium
GnRH	Gonadotropin Releasing Hormone
GPCR	G-protein coupled receptor
HB-EGF	Heparin-binding EGF-like growth factor
HDAC	Histone Deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Hinge region
HRG	Heregulin
HRP	Horseradish Peroxidase
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAF	Laminar Air Flow
MAPK	Mitogen-activated protein kinase
MAPKK	MAP Kinase Kinase
MAPKKK	MAP Kinase Kinase Kinase

MMP	Matrix Metalloproteinase
mTOR	Mechanistic Target of Rapamycin
mTORC	mTOR complex
NLS	Nuclear Localization Signal
Nrdp1	Neuregulin receptor degradation protein 1
NRG	Neuregulin
NTD	N-terminal domain
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PET	Polyethylene terephthalate
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PPE	Personal Protective Equipment
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin homolog
RTK	Receptor Tyrosine Kinase
SAPK	Stress activated protein kinase
SDM	Steroid Depleted Medium
SDS	Sodium dodecyl sulfate
SMRT	Silencing mediator for Retinoid and Thyroid hormone
SOP	Standard Operating Protocol
SRB	Sulforhodamine B
TAE	Tris-Acetic acid-EDTA
TBS	Tris Buffered Saline
TCA	Trichloroacetic Acid
TGF	Transforming Growth Factor
TKI	Tyrosine Kinase Inhibitor

TMA	Tissue Microarray
TRUS	Transrectal ultrasonography
TSC	Tuberous Sclerosis protein
TTBS	Tris Buffered Saline – Tween20
TURP	Transurethral resection of prostate
u-PA	Urokinase-type Plasminogen Activator
UV	Ultraviolet

Table of Contents

List of Figures

List of Tables

Chapter 1 Introduction

1.1 Introduction to cancer

Cancer is a disease that is caused by unregulated cell growth. There are many types of cancer that affect different parts of the body. The disease originates when normal cells evolve to a neoplastic state due to the acquisition of certain traits that enable them to become tumorigenic and eventually malignant. These traits have been described as the 'Hallmarks of Cancer' (Hanahan and Weinberg, 2011) (**Figure 1-1**).

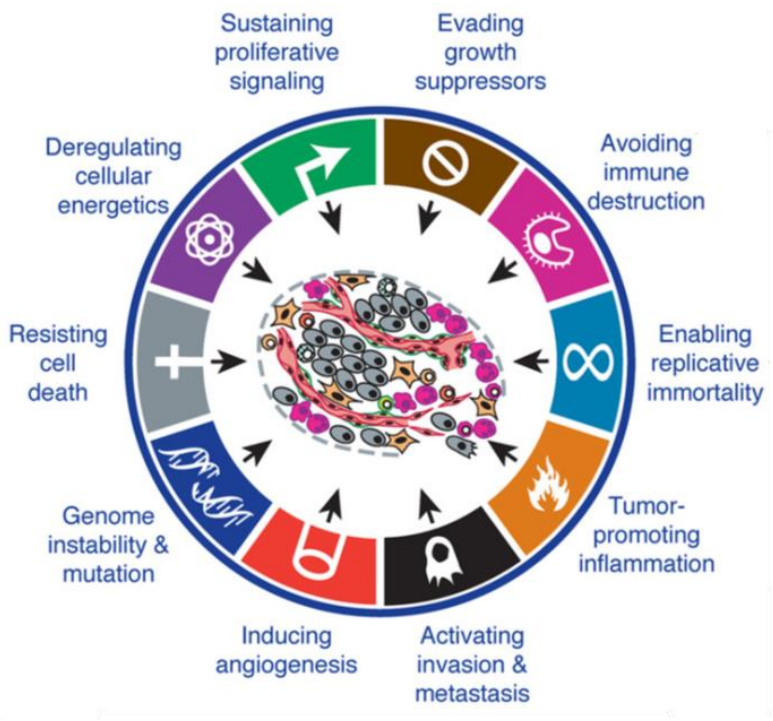

Figure 1-1: Hallmarks of Cancer

This diagram represents all the hallmarks of cancer discussed in the revised review by Hanahan and Weinberg (2011). The review explains how the cancer uses these hallmarks in the establishment and progression of the disease.

Research based upon these hallmarks, helps us understand the various mechanisms employed by the cancerous cell, developing and maintaining the disease. Key findings have helped in the development of many anti-cancer agents. However, many cancers continue to lack an adequate therapeutic regime. Hence, it is very important to review the disease and its various approaches for sustenance and survival, to help in identifying

and designing improved therapeutic targets.

This project focuses on the role of the epidermal growth factor receptor (EGFR) family members in prostate cancer and their potential role as therapeutic targets. These receptors have been largely researched for helping the cancer cells in attaining the 'sustaining proliferative signalling' hallmark of cancer and recent work indicates that they can also be contributing to attain additional hallmarks, thus helping the cancer to survive and progress. The following sections of this chapter give a brief understanding of the prostate gland and its associated cancer, the EGFR family receptors and their functions and previous research findings that helped develop the structure of this PhD project.

1.2 Prostate gland

1.2.1 Function of the prostate gland

The prostate gland is part of the male reproductive system where its main function is to produce a fluid that forms a part of the ejaculate. This fluid contains prostate specific antigen (PSA) produced by the prostate which helps in maintaining the liquid state of the semen thus, aiding the motility of the sperm (Lilja *et al.*, 1987; Vincent T. DeVita Jr., 2008). Testosterone, in the form of dihydrotestosterone (DHT), is required for the normal functioning of the prostate gland (Lamb and Neal, 2013).

1.2.2 Anatomy of the prostate gland

The prostate gland was first described in 1536 (Jathal *et al.*, 2011). It is an exocrine gland situated just below the bladder, behind the pubic bone and just in front of the rectum. The gland can be divided into three glandular zones (peripheral, central and transitional) and an anterior fibromuscular stroma (**Figure 1-2**).

Peripheral zone – This zone accounts for around 65% of the prostate's volume and is around the posterolateral peripheral parts of the gland. The prostatic acini present in this region have a role of discharging the prostatic secretions into the urethra at the time of ejaculation to help liquefy the seminal fluid. Prostatic carcinoma, chronic prostatitis and post-inflammatory atrophy are relatively more common in the peripheral zone. About 60-70% of prostatic cancers arise from the peripheral zone (Schulz *et al.*, 2003; Hedvig Hricak, 2009).

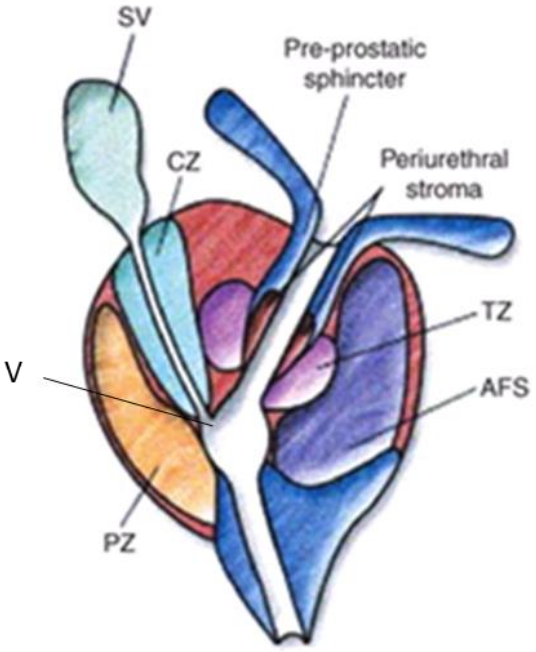

Figure 1-2: Normal prostate gland

The diagram describes the zones of the prostate gland. SV-seminal vesicle, CZ-central zone, V-verumontanum, PZ-peripheral zone, TZ-transitional zone, AFS-anterior fibromuscular stroma (Vincent T. DeVita Jr., 2008).

Central zone – The central zone makes up the majority of the prostatic base and accounts for around 25% of the prostate's volume. The zone is cone-shaped and surrounds the ejaculatory ducts. The ducts of this region join the urethra at the verumontanum. This zone comprises of epithelial cells that contain pepsinogen, a gastric proenzyme. Only around 5-10% of the cancers originate from the central zone (Reese *et al.*, 1988; Schulz *et al.*, 2003).

Transitional zone – This is the smallest zone of the prostate and accounts for only 5 - 10% of the prostate and is made up of two symmetrical lobules on either sides of the prostatic urethra. 10- 20% of prostatic carcinomas arise from this zone and has clinical relevance, as it grows with age and is mainly involved in the development of age-related benign prostatic hyperplasia (BPH) (Villers *et al.*, 1991; Hedvig Hricak, 2009; Amin M, 2010).

Anterior fibromuscular stroma – This region separates the transition zone from the central and the peripheral zones. This region is mainly composed of striated and smooth muscle. The distal and proximal regions of the stroma are important for voluntary and involuntary sphincter functions, respectively (Hedvig Hricak, 2009).

1.3 Pre-malignant prostatic diseases

1.3.1 Benign prostatic hyperplasia (BPH)

The normal prostate is made up of luminal, basal and neuroendocrine epithelial cells (**Figure 1-3**). The normal prostate epithelia predominantly consist of luminal cells whose main function is to produce prostatic secretory proteins. These cells possess the androgen receptor (AR) and are known to be androgen dependent (Liu *et al.*, 1997). Between the luminal cells and the basement membrane are another type of epithelial cells called the basal cells. These cells continuously line the human prostate and are known to express low levels of AR along with anti-apoptotic genes including Bcl2 (Abate-Shen and Shen, 2000). Another type of epithelial cells called neuroendocrine cells are distributed throughout the basal layer. These cells assist the growth of the luminal cells by providing paracrine signalling and are androgen-independent (Abrahamsson, 1999).

Benign prostatic hyperplasia is a condition wherein the prostate enlarges by the proliferation of epithelial cells and smooth muscles present in the transitional zone of the prostate (Lee *et al.*, 1997). It is a condition that has been observed in older men and has been diagnosed in 50% of men over 60 years of age. Previous findings also reveal that one or more symptoms associated with BPH are present in 75% of men over the age of 70 and ultimately 90% of men over the age of 85 reveal symptoms attributed to BPH (Platz *et al.*, 2002). The enlargement of the gland often obstructs the urethra and leads to lower urinary tract symptoms (LUTS) which include hesitancy, weak urine stream, sensation of incomplete emptying and also urgency, increased frequency of urination and nocturia (Priest *et al.*, 2012). These symptoms can have a significant impact on the quality of life considering the condition is rarely life threatening. For the condition to advance, the interaction between the epithelial cells and the surrounding stroma is crucial with the encouragement from the androgen receptor and its ligand dihydrotestosterone (DHT) (Cunha *et al.*, 1983).

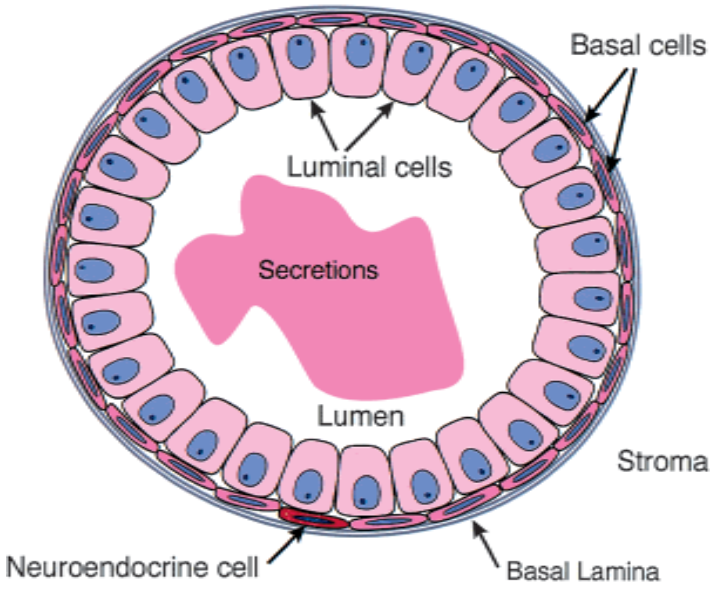

Figure 1-3: Schematic representation of a prostatic duct

The figure shows the prostatic duct comprising of the basal, luminal and neuroendocrine cells surrounded by stroma (Abate-Shen and Shen, 2000).

1.3.2 Prostatic intraepithelial neoplasia (PIN)

PIN was initially characterised by McNeal and Bostwick (1986). It is most commonly found in the peripheral zone and rarely in the transitional zone and the central zone which is similar to the incidences of prostate cancer (Montironi *et al.*, 2011). PIN can be characterized by certain changes in cytological features. It is identified by the presence of prostatic ducts that are lined by atypical cells. It is then categorised as low grade or high grade PIN depending on the degrees of nuclear enlargement, hyperchromasia and nucleolar prominence (Brawer, 2005). High grade PIN and prostate cancer have common markers and the disease is widely classed as a transitional stage between benign disease and invasive malignant carcinoma (**Figure 1-4**) (Sakr *et al.*, 1994).

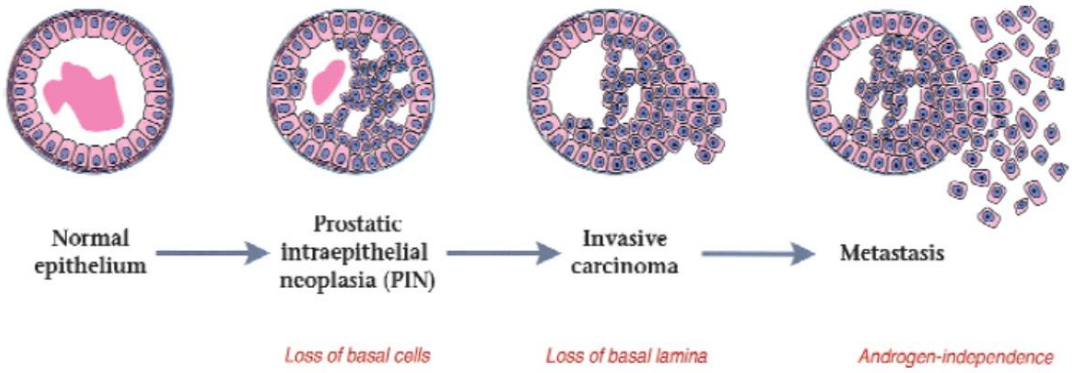

Figure 1-4: Schematic representation of the development and progression of prostate cancer

The diagram describes the cellular morphological changes that occur in the prostate during the progression from benign to malignant disease (Abate-Shen and Shen, 2000).

1.4 Prostate cancer

Prostate cancer is the second most frequently diagnosed cancer and the fifth leading cause of cancer death worldwide (Torre *et al.*, 2015). The first case of prostate cancer was established by histological examination in the mid-19th century (Adams, 1853) and research since then has led to a better understanding of the development of the disease and has subsequently resulted in the identification of multiple druggable targets.

1.4.1 Etiology

Many factors have been linked to the occurrence of prostate cancer. Some of these are mentioned below.

Age - Prostate cancer is most commonly diagnosed in older men. 65% of the diagnosed cases are from patients above the age of 65 (Jathal *et al.*, 2011). Similar trends have been observed in the UK (**Figure 1-5**). According to Cancer Research UK, an average of 36% of the total cases diagnosed between years 2009 and 2011 included men aged over 75 years and only 1% of the diagnosed cases include men aged under 50 years. Autopsy based studies have revealed that many men develop lesions at earlier ages (between 20 and 30) and suggests that prostate cancer can have a long initiation period (Gann, 2002).

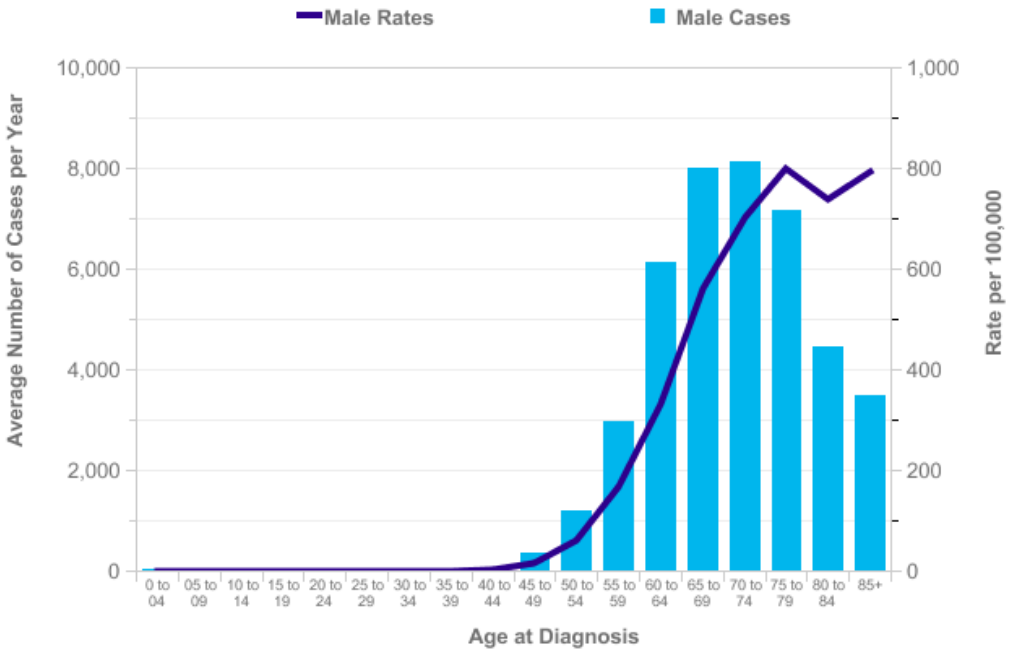

Figure 1-5: Age specific incidence rates in the UK (2009-2011) (CancerResearchUK, Prostate cancer - UK incidence)

The graph shows the average number of new cases diagnosed in the UK each along with the age specific incidence rates

Race – There have been observations made by comparing the incidence rates to race and ethnic background. It has been observed that African men are three – times more likely to be affected than Caucasian men while Asian men have a lower risk of developing the disease. However, migration to other places can affect the incidence rates in the ethnic groups. For example, immigration of Japanese men to the USA caused an increase in disease incidence within the race and this has been linked to the changes in various environmental factors like diet (Sonn *et al.*, 2005; Ben-Shlomo *et al.*, 2008).

Family history – The likelihood of developing the disease increases if there is a history of relatives diagnosed with prostate cancer. The risk is known to be greater if the relatives have been diagnosed at an early age (Jathal *et al.*, 2011).

The above mentioned factors have been established, but other risk factors are still under investigation. These include diet, oxidant damage, physical activity, endogenous hormones like insulin-like growth factor – 1 (IGF-1) and the presence of other medical conditions (Kolonel, 2001; Cimino *et al.*, 2014; Doolan *et al.*, 2014; Cao *et al.*, 2015;

Tsilidis *et al.*, 2015).

1.4.2 Diagnosis

The diagnosis of prostate cancer is routinely made by measuring the levels of PSA in the serum, digital rectal examination (DRE) and histological analysis of transrectal ultrasonography (TRUS)-guided biopsy samples. The aggressiveness of the disease is determined by assigning the appropriate Gleason score after histological analysis (Sciarra *et al.*, 2012).

Prostate specific Antigen (PSA)

PSA, also known as kallikrein-3, is a serine protease that belongs to the kallikrein family of proteases and is produced by the epithelial cells of the prostate tissue. As mentioned earlier, the main function of this protease is to liquefy the ejaculate to increase the motility of the sperm and to also dissolve the cervical mucus (Lilja *et al.*, 1987). Under normal conditions, the glandular ducts act as barriers in preventing the outflow of PSA into the serum. The increase in PSA serum levels has been linked to diseases of the prostate, including prostate cancer. The assessment of PSA in blood was first performed in 1980 (Kuriyama *et al.*, 1980) and the correlation with prostate cancer was established in 1987 (Stamey *et al.*, 1987). The screening for PSA for diagnosis of prostate cancer was subsequently approved by the U.S. Food and Drug Administration in 1994 and is currently the mostly widely used oncological biomarker (Ablin and Haythorn, 2009). However, there are limitations with respect to the specificity of PSA screening as elevated PSA levels can be detected in non-malignant conditions like BPH and prostatitis (Oesterling, 1991). The PSA cut-off levels for diagnosis are continually being revised. These revisions of the screening have led to the both under and over diagnosis and treatment of prostate cancer (Duskova and Vesely, 2015).

Histopathology

Despite the initial PSA screen and digital rectal examination, the presence of prostate cancer is confirmed only after histopathological examination of transrectal ultrasonography (TRUS)-guided biopsy samples. The samples are evaluated based on the Gleason grading system. The system was established by the combined effort of Dr. Donald. F. Gleason and the Veterans Administration Cooperative Urological Research Group (VACURG) (Gleason, 1966; Gleason and Mellinger, 1974). The analysis is performed by staining the biopsied tissue sections with haematoxylin and

eosin stains (a.k.a H&E staining) and observing the arrangement patterns of the cancerous cells. A Gleason score is then assigned by adding the 2 most commonly observed patterns. The Gleason patterning system has recently been revised and describes the different morphological changes commonly observed as seen in (**Figure 1-6**) (Epstein, 2011)

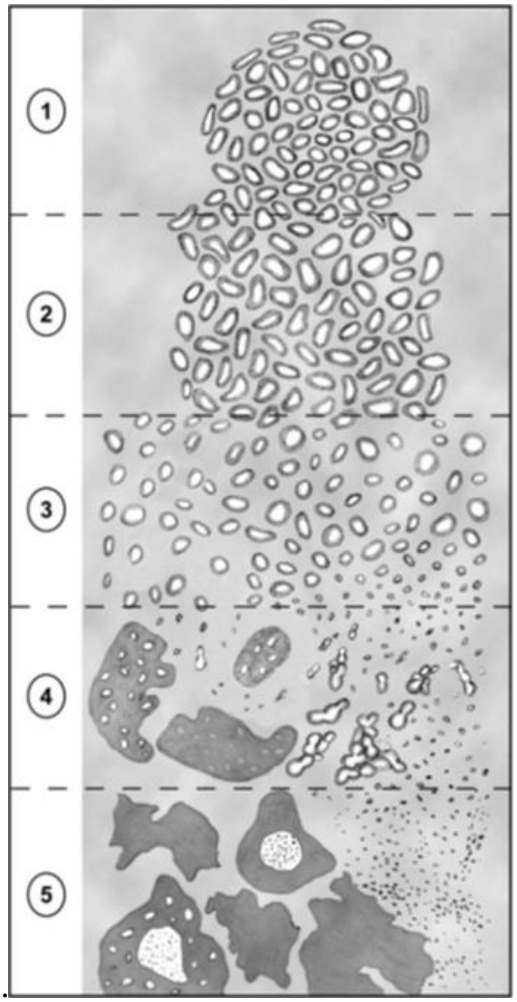

Figure 1-6: Schematic of different Gleason patterns

The above diagram describes the different Gleason patterns used in establishing a final Gleason score. Grade 1 comprised of well differentiated and closely packed glands and occurs quite rarely. Grade 2 can be identified with a slightly lesser well-defined mass and tumour-stromal boundary of the gland. The size and shape of the gland also vary. Grade 3 is the most common pattern observed in prostate carcinoma and consists of moderately differentiated glands of varied size and the most prominent feature is the presence of invasive stroma. Grade 4 is considered as high grade and poorly differentiated carcinoma and is characterised by the presence of ill-defined glands and chains of malignant epithelial cells. Grade 5 is the most undifferentiated pattern and can resemble other undifferentiated cancers of other organs. The highest Gleason sum scores are associated with metastatic disease and have poor prognosis (Humphrey, 2004; Epstein, 2011).

1.4.3 Treatment

Depending on the clinical stage of the cancer and whether the patients are considered to be at a low or high risk of cancer progression, different treatment regimens are followed. Organ confined disease is initially considered for conservative treatment with watchful waiting and active surveillance by using PSA and digital rectal examination (DRE) screening. This is a common choice in low risk and older patients, as the risk of dying from the disease is minimal and also to spare the patients from the morbidity caused by the treatment options (Xu *et al.*, 2012). However, localised prostate cancer can be treated surgically by performing radical prostatectomy which essentially involves the removal of the entire prostate along with the seminal vesicles (Trojan *et al.*, 2005). Other options include external beam radiotherapy and also brachytherapy which involves the permanent placement of radioactive seeds (iodine-125 or palladium-103) in the prostate (Yu *et al.*, 1999). When the disease is diagnosed as being locally advanced and metastatic, the therapy provided aims at preventing distance metastases and improving quality of life, respectively. These are achieved by androgen withdrawal therapy (AWT) and chemotherapy. The discovery of eliminating testosterone for prostate cancer treatment was described by Charles Huggins and Clarence Hodges and in recognition of their work were awarded a Nobel Prize in 1966 (Huggins and Hodges, 1941). Androgen-withdrawal causes a reduction in the androgen receptor (AR) signalling cascade (explained in section **1.5**), which is essential for prostate cancer maintenance and progression. AWT targets tumour growth that is androgen dependent and can be achieved by surgical and medical castration. Surgical castration involves complete removal of both the testes which is more commonly known as bilateral orchiectomy and can deplete the testosterone levels by >95% (Miyamoto *et al.*, 2004). This removal of the primary source of testosterone reduces the availability of androgens for tumour cell proliferation. Alternative methods, such as medical castration, target the hypothalamus – pituitary and testicular axis of androgen production by using oestrogens and gonadotropin releasing hormone (GnRH) analogues like Zoladex (Brogden and Faulds, 1995). Adrenal androgen production can also be inhibited by targeting CYP17 – a bi-functional enzyme present in the testis and adrenal glands and is involved in the biosynthesis of androgens from cholesterol (Ang *et al.*, 2009). Abiraterone, a recent drug developed by the Institute of Cancer Research in London, blocks the dual activity of CYP17 and results in the reduction of androgen synthesis (Goyal and Antonarakis, 2013). The enzyme 5α- reductase that converts testosterone to the potent form of

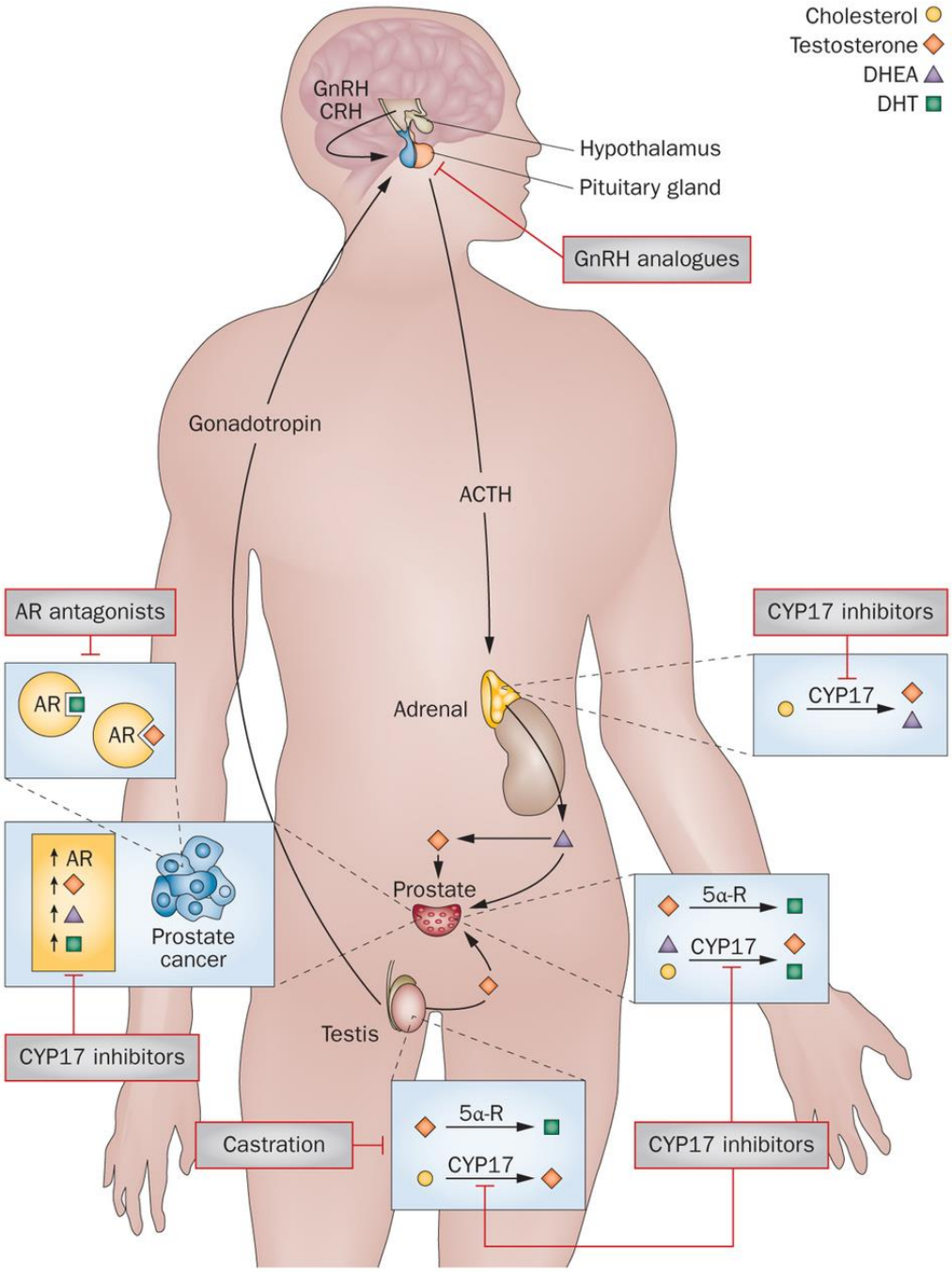

Figure 1-7: Surgical and medical castration in prostate cancer treatment

The above figure describes the various methods used in prostate cancer treatment for inhibiting androgen production and function. The hypothalamus and pituitary gland control the production of androgens in the testes and adrenal gland by the release of luteinising hormone and adrenocorticotropic hormone (ACTH) respectively. This hypothalamic–pituitary–gonadal axis can be controlled with the use of GnRH analogues. Surgical castration reduces 5α- reductase and CYP17 enzymes that play a role in gonadal androgen synthesis. However, adrenal androgens can still be produced by the production of dehydroepiandrosterone (DHEA) from cholesterol, which can then be converted to DHT in the prostate. This is inhibited with the use of CYP17 inhibitors like Abiraterone. Further reduction in the AR signalling can be achieved with the use of anti-androgenic compounds such as Enzalutamide and ARN-509 (Yin and Hu, 2014).

dihydrotestosterone (DHT) has also been used as a target to inhibit the activity of the androgen receptor (Titus *et al.,* 2014). Anti-androgens have also been used in the treatment of prostate cancer and function by competitive inhibition of androgen binding to the androgen receptor. First generation anti-androgens such as Casodex have been extensively used in prostate cancer treatment. However, they have been ultimately unsuccessful due to their poor binding affinity to the androgen receptor and to their agonistic properties (Masiello *et al.*, 2002). Second generation anti-androgens like Enzalutamide and ARN-509 have since been developed and have showed promise in clinical trial studies with the observation of increased anti-tumour activity (Scher *et al.*, 2010; Smith *et al.,* 2012). Treatment regimens can involve intermittent and maximal androgen blockade with the combined used of GnRH analogues along with antiandrogens (Perlmutter and Lepor, 2007).

Unfortunately, the majority of patients undergoing AWT eventually relapse with a more aggressive form of androgen-independent disease and is more commonly known as castration- resistant prostate cancer (CRPC) (Attard *et al.,* 2011). This advanced form of the disease most commonly metastasise to the bone and can lead to other ailments like spinal cord compression, fractures, anaemia and lastly death (Petrylak, 2014). Patients who present with this form of advanced metastatic disease are then treated by chemotherapeutic agents such as Docetaxel and Cabazitaxel in combination with the corticosteroid Prednisone as a final palliative treatment (Kelly *et al.*, 2012; Heidenreich *et al.*, 2013). The androgen receptor signalling axis remains overactive in CRPC and research in recent years has focussed on understanding the mechanisms sustaining this activity.

1.5 Androgen receptor

The androgen receptor is a nuclear transcription factor belonging to the steroid hormone receptor superfamily and uses androgens to carry out its biological functions. Its gene is located on chromosome X q (11-12) and comprises of 8 exons (Brown *et al.*, 1989) (**Figure 1-8**). These exons together encode the different structural components of the receptor and consists of the N-terminal domain (NTD), the DNA-binding domain (DBD), the 'hinge' region (HR) and the ligand-binding domain (LBD) (Jenster *et al.*, 1991) (**Figure 1-8**). The signalling of the androgen receptor initiates with the intracellular conversion of testosterone by 5α- reductase into a more potent form, 5α-

dihydrotestosterone (DHT) (**Figure 1-9**). The AR is located primarily in the cytoplasm and is kept inactive by the interaction of heat shock proteins (HSP90, HSP70, HSP56, and HSP27) (Zoubeidi *et al.*, 2007). The high affinity binding of DHT to the AR, displaces the HSPs and the conformational change allows the interaction of the N and C terminal domains of the AR and also to its co-activators (van de Wijngaart *et al.*, 2012). The NTD contains the activation function-1 (AF-1) and two transcription activation units (TAUs). TAU-1 encourages AR transcriptional activity upon activation,

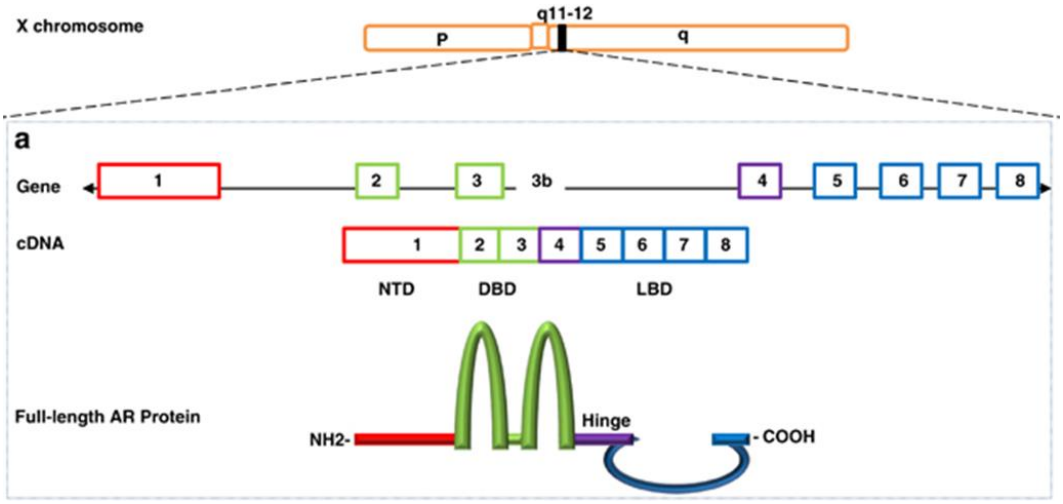

Figure 1-8: Structure of the androgen receptor

The gene of the AR comprises of 8 exons that make up its different domains – the N-terminal domain (NTD), the DNA-binding domain (DBD), the 'hinge' region (HR) and the ligand-binding domain (LBD). Ligand association allows conformational change of the receptor that then helps in recruiting co-activators to carry out its functional properties (Ferraldeschi *et al.*, 2015).

and TAU-5 provides constitutive activity to the AR in the absence of the ligand-binding domain (LBD) (Callewaert *et al.*, 2006). The NTD/LBD interaction stabilizes the AR protein by reducing the dissociation rate of the ligand and is crucial for its transcriptional activity (He *et al.*, 2000). The activation of the receptor is observed with its phosphorylation and dimerization to other activated forms of the AR (Nazareth and Weigel, 1996). The role of the nuclear localization signal (NLS) then takes over which is located between the DBD and the hinge regions and is responsible for nuclear transport of the receptor through the recruitment of the nuclear importin complex (Cutress *et al.*, 2008). The receptor then binds to specific androgen response elements (AREs) and recruits a variety of co-regulatory proteins and initiates the transcription of specific target genes such as PSA, leading to the regulation of important cellular responses such as survival and growth (Bennett *et al.*, 2010; Hsu *et al.*, 2011).

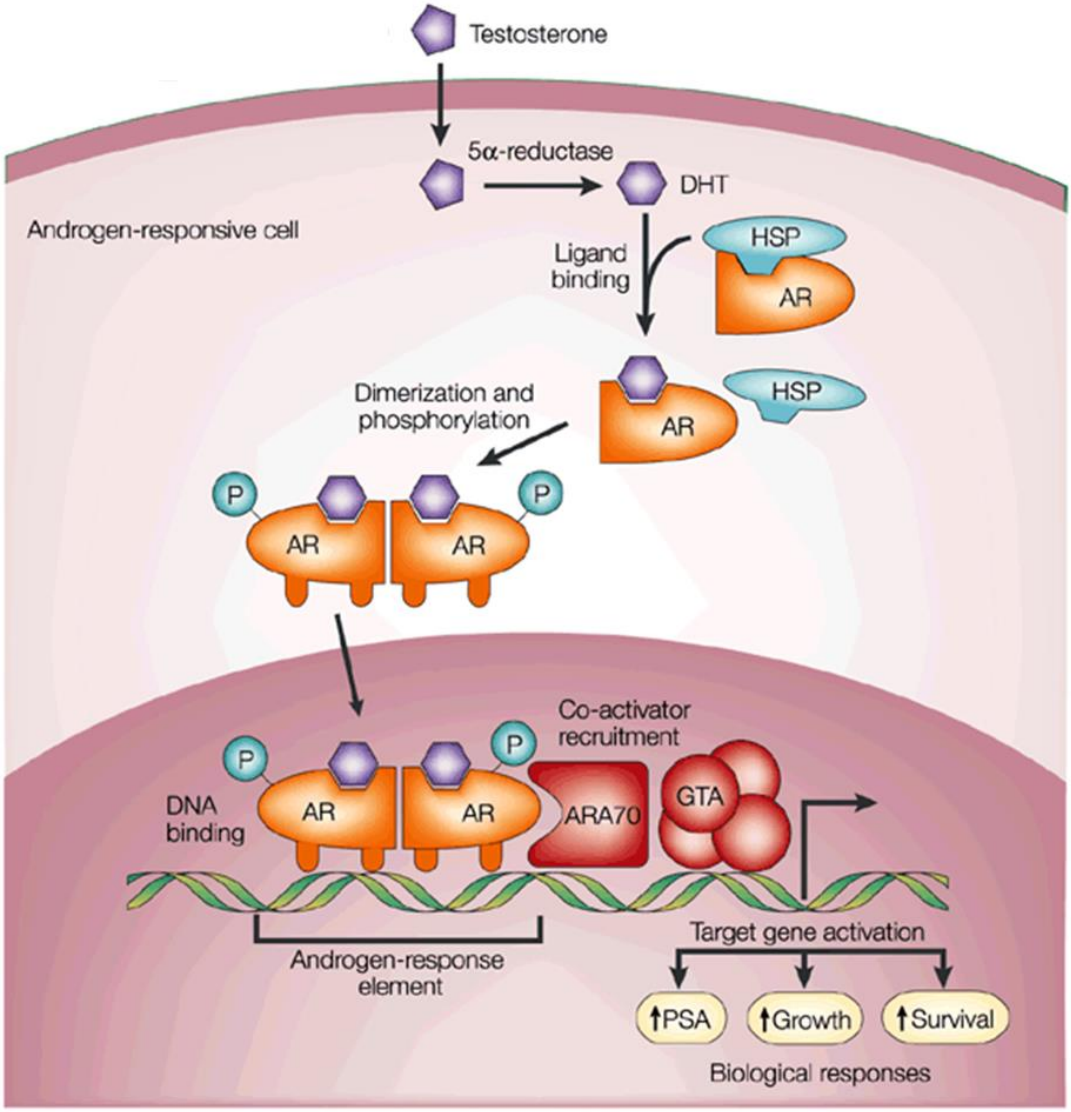

Figure 1-9: AR signalling cascade

Free testosterone gets converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Binding and strong association of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins and also results in receptor phosphorylation. The AR subsequently dimerizes and translocates to the nucleus where it binds to androgen-response elements in the promoter regions of specific target genes that are involved in biological responses such as growth and survival (Feldman and Feldman, 2001).

1.5.1 Androgen receptor signalling in castrate resistant prostate cancer

Despite androgen withdrawal therapy, PSA protein levels are detected in CRPC patients indicating that the AR signalling pathway is active. The sustained AR signalling mechanisms have been extensively researched in the recent years, a summary of which is explained in the below sections (**Figure 1-10**).

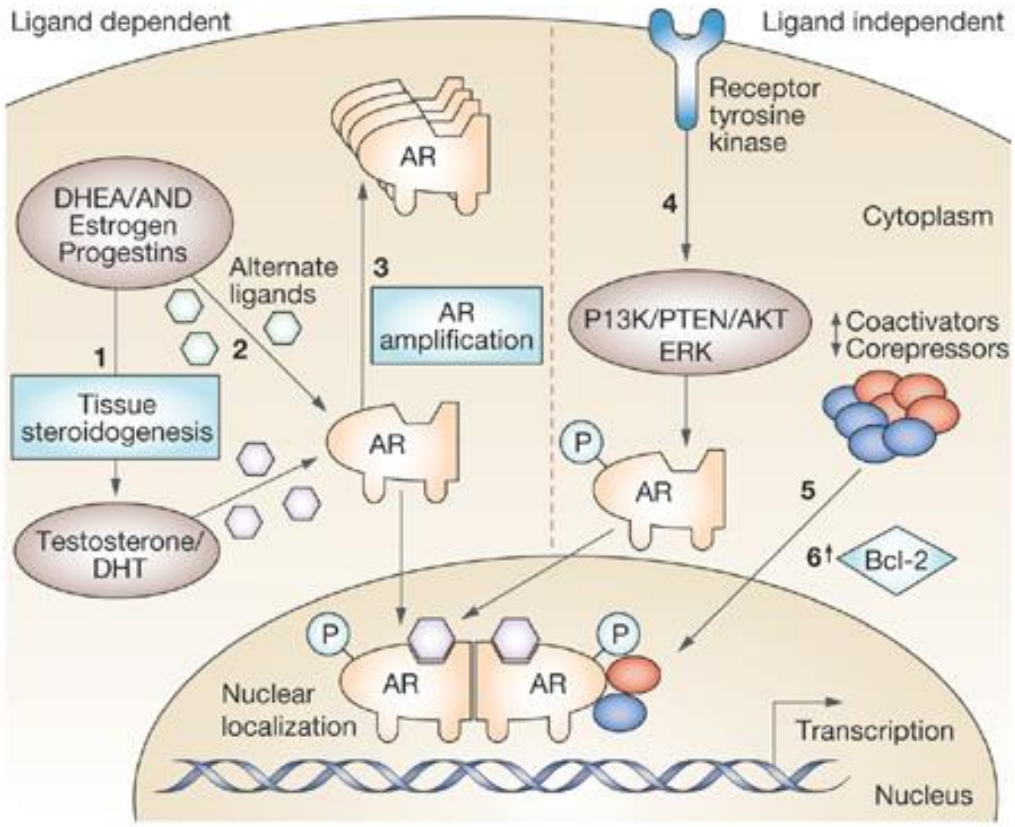

Figure 1-10: Mechanisms for AR activity in CRPC

The above figure represents the various mechanisms involved in the sustenance of the AR signalling in CRPC. (1) Despite castration, continual steroidogenesis adds to the production of testosterone and DHT assisting in AR activation (2) AR mutations can cause activation by alternative ligands and can also cause increased affinity to androgens. (3) The amplification of the receptor can lead to its abundance causing over activity. (4) Cross-talk with other signalling pathways can lead to the activation and nuclear translocation of the AR (5) Increased co-activators and decreased co-repressors can alter regulation of AR activity. (6) The AR independent bypass pathways functions through positively regulating of anti-apoptotic proteins, such as Bcl2 (Harris *et al.*, 2009).

Continual androgen production – Increased activity of enzyme 5α reductase can cause an enhanced rate of testosterone to DHT conversion, thus producing more ligand to activate the AR (Radmayr *et al.*, 2008). Substitute ligands like progesterone can persistently activate the AR to maintain its transcriptional function (Yuan and Balk, 2009). Additionally, the action of intratumoural steroidogenesis along with the conversion of circulating adrenal androgens provides continual and adequate androgen concentrations to activate the AR despite low testosterone caused by AWT (Locke *et al.*, 2008).

AR mutations – Mutations have been observed in the NTD, hinge and LBD of the receptor, at sites that are crucial for protein-receptor interaction, transactivation activity and ligand binding (Brooke and Bevan, 2009). The sensitivity to alternative ligands including other circulating steroid hormones such as oestrogen, progesterone along with AR antagonists can be caused by AR mutations. A well characterised mutation showing this effect is the missense mutation that causes a threonine to alanine amino acid change at position 877 (T877A) seen in the ligand binding domain of the AR. The mutation, originally characterised in the LNCaP cell line, can occur with the treatment of anti-androgen Flutamide and also steroid hormones like progesterone (Han *et al.*, 2005; Jennifer and Iain, 2005). Patients exhibiting resistance to the currently used second generation anti-androgen drugs like Enzalutamide and ARN-509, have been noted to attain AR mutations like T877A and other mutations like F876L (phenylalanine to leucine substitution), causing the drugs to have an agonistic effect and have also been linked to assist the cells in achieving drug resistance (Joseph *et al.*, 2013; Azad *et al.*, 2015).

AR over-expression and variant forms – AR over-expression can be caused by gene amplification or by increased transcriptional activity and protein stabilisation (Visakorpi *et al.*, 1995; Miyoshi *et al.*, 2000; Zhang *et al.*, 2009; Lin *et al.*, 2013). This can cause the AR to be hypersensitive to low levels of androgen and continue tumour proliferation (Waltering *et al.*, 2009). The over-expression of the AR has been reported to also cause a shift in the behaviour of the antiandrogen Casodex in an agonistic manner (Chen *et al.*, 2004).

The presence of AR variants has also been observed in advanced prostate cancer. The truncated version of the receptor contains the NTD and the DBD but not the LBD and is caused by structural rearrangements and/or alternative splicing of the AR gene (Hu *et*

al., 2009; Liu *et al.*, 2014). A variety of receptor variant forms have been discovered with different properties and have been targets for novel therapeutic agents (Lallous *et al.*, 2013). The AR-V7 variant is the most commonly detected AR variant in advanced prostate cancer and remains constitutively active (Hu *et al.*, 2012). Its activity has been reported to be enhanced by anti-androgenic drugs such as Enzalutamide and Abiraterone (Mostaghel *et al.*, 2011; Li *et al.*, 2013). Current research is focussing on understanding the diverse roles of these variant forms in advanced and drug resistant prostate cancer which can then further help in the formulation of more robust AR targeted therapies (Xu *et al.*, 2015).

Co-regulators and repressors – Altered expression and activity of co-regulators and co- repressors of the AR can also lead to the development of advanced prostate cancer (Chandrasekar *et al.*, 2015). These proteins form part of the transcriptional complex and can alter the activities of the AR by influencing its DNA binding, nuclear translocation, chromatin remodelling and is achieved by altering the phosphorylation, methylation, acetylation or ubiquitylation profiles (Lee and Chang, 2003; Wolf *et al.*, 2008). For example, co-activators that possess histone acetyltransferase (HAT) activity, such as cAMP response element binding protein (CREB)-binding protein (CBP)/p300 and p300/CBP-associated factor (p/CAF) can interact with AR and enable chromatin remodelling (Shen *et al.*, 2005). However, co-repressors like silencing mediator for retinoid and thyroid hormone receptors (SMRT) can disrupt the N and C terminal interaction of the AR and can do this in a ligand dependent or independent manner (Heinlein and Chang, 2002). By competing with co-activators such as SRC/p160, SMRT then recruits histone deacetylases (HDAC) that package the DNA into nucleosomes and thus inhibits the transcription machinery from binding to the promoter or enhancer regions and ultimately represses gene transcription (Liao *et al.*, 2003).

The above mentioned mechanisms involve the androgen receptor. The androgen independent ‘bypass’ mechanism also plays a role in the progression of prostate cancer. For example, the infiltration of B and T cells, caused by the inflammatory response of apoptotic prostate cancer cells, can cause an upregulation of Stat3 signalling which ultimately promotes androgen-independent growth (Ammirante *et al.*, 2010). Similarly, castration induced apoptosis can trigger the upregulation of anti-apoptotic genes such as Bcl2 (Setlur and Rubin, 2005).

1.5.2 Kinase signalling pathways regulating AR activity

The activity of the AR can also be controlled by crosstalk with other pro-survival and proliferative pathways and is discussed in the following sections.

The phosphatidylinositol 3-kinase (PI3K) pathway – This is one of the most commonly activated signalling pathway in cancer including prostate cancer (Courtney *et al.*, 2010; Chen *et al.*, 2014). The pathway controls many diverse cellular functions including cell proliferation, metabolism and differentiation (Engelman *et al.*, 2006). The activity of this pathway initiates by signals generated by growth factors like fibroblast growth factor (FGF) and insulin-like growth factor (IGF) and also G-protein coupled receptors (GPCRs) (Skolnik *et al.*, 1991; Stephens *et al.*, 1994). The generated signals activate the PI3 Kinases whose main function is to phosphorylate the downstream phosphatidylinositol and phosphoinositides at their 3' hydroxyl groups, which is achieved by the activities of their catalytic and regulatory subunits (Carpenter *et al.*, 1993). The oncogene RAS can also bind to the catalytic subunit p110 of PI3 Kinase integrating the PI3 Kinase and MAP Kinase signalling pathways (Goc *et al.*, 2011). The downstream serine/threonine kinase AKT is a target of the PI3 Kinase pathway and its phosphorylation at serine 473 increases its activity by 10-fold (Sarbassov *et al.*, 2005). Active AKT subsequently regulates the expression of genes like forkhead box O (FOXO), p27, BAD and eNOS that play a role in cell proliferation, metabolism and angiogenesis (Manning and Cantley, 2007). The downstream effector of AKT is mTOR, another serine/threonine kinase. The complexes involving mTOR –mTORC1 and mTORC2 regulate processes involving protein synthesis, cell proliferation and differentiation (Brown *et al.*, 1995).

The PI3K-AKT-mTOR pathway plays an important role in prostate tumourigenesis (Majumder *et al.*, 2003). The deregulation of the pathway has been linked to the reduced expression of PTEN tumour suppressor gene, a negative regulator of the PI3 Kinase pathway and has been observed in patients with a high Gleason score (McMenamin *et al.*, 1999). The importance of the mTORC1 and mTORC2 complexes in prostate cancer development has also been established by *in-vivo* knockout models (Guertin *et al.*, 2009; Nardella *et al.*, 2009).

The PI3 Kinase signalling pathway and the AR signalling axis collaborate with each other in the progression of castrate resistant prostate cancer. AKT can phosphorylate the AR at serine residues 217 and 791 leading to its activation and has been observed in

prostate cancer (Wen *et al.*, 2000). The inhibition of the AR signalling cascade by the various androgen withdrawal therapies causes a reduction in FKBP5 and subsequently, PHLPP- a phosphatase negatively regulating AKT. This causes an upregulation in PI3 Kinase signalling and supports prostate cancer progression (Carver *et al.*, 2011; Mulholland *et al.*, 2011). Since this pathway is commonly deregulated in many cancer types, there have been many inhibitors developed to act against its activity. There are a range of inhibitors that target different proteins in the pathway. The PI3K pan-inhibitors target the catalytic subunits of all its isoforms and are currently in Phase I/II clinical trials in combination with AR targeting drugs like Abiraterone and Enzalutamide (Edlind and Hsieh, 2014). AKT targeted inhibitors like AZD5363 have been shown to delay CRPC progression in combination with Casodex (Thomas *et al.*, 2013). The use of mTOR inhibitors (rapamycin and similar derivatives) has had limited success in prostate cancer clinical trials (Nakabayashi *et al.*, 2012; Templeton *et al.*, 2013). Currently, newer ATP site mTOR inhibitors like MLN0128 are in early stage clinical trials (Rathkoph, 2015). These inhibit both the mTORC complexes in an ATP dependent manner as opposed to allosteric mTORC1 specific inhibitors (rapalogues) that can cause an upregulation of AKT. The results from the clinical trials will determine the efficacy of targeting this pathway. There are also dual PI3K/mTORC1/2 inhibitors that target all the p110 isoforms of P13K along with the mTOR complexes and suggest to be more robust in inhibiting the signalling pathway. One such inhibitor, BEZ235 was shown to reduce tumour growth formation on its own. However, this inhibition led to an increase in HER3 levels and a reactivation of the AR signalling pathway was observed. The reduction in cell proliferation was more pronounced when the EGFR/HER2 signalling axis was inhibited along with the activity of the PI3 Kinase pathway (Carver *et al.*, 2011). The figure below is a summary of the pathway and the interventions used (**Figure 1-11**).

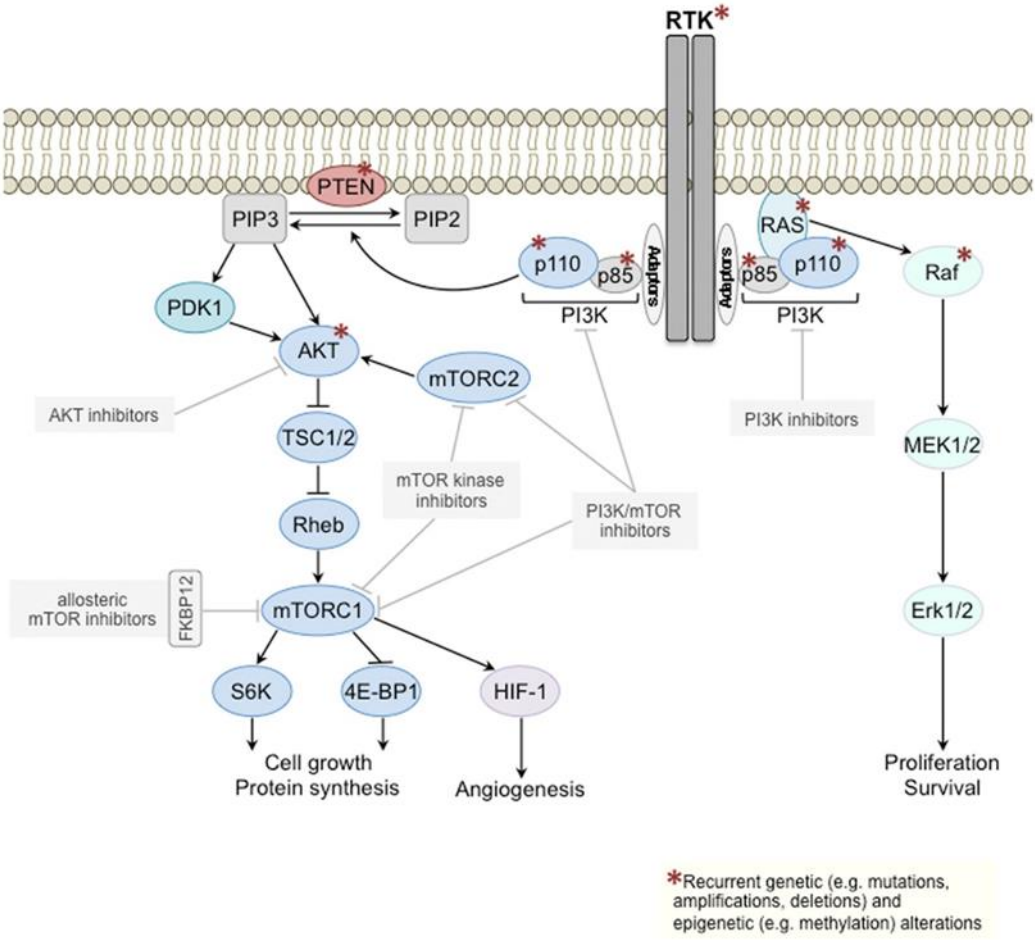

Figure 1-11: The PI3 Kinase pathway and its directed inhibitors

The PI3 kinase pathway can activate the AKT pathway and the MAP Kinase pathway. The indicated downstream components have been used as targets for therapy with the ultimate goal of inhibiting the signalling axis that contributes to overall cell survival. mTOR (mechanistic target of rapamycin); mTORC (mTOR complex); PI3K (phosphoinositide 3-kinase); PIP2 (phosphatidylinositol 4,5-bisphosphate); PIP3 (phosphatidylinositol (3,4,5)-triphosphate); PTEN (phosphatase and tensin homolog); RTK (receptor tyrosine kinase); TSC (tuberous sclerosis protein) (Downward and Weigelt, 2012).

The Mitogen-activated protein kinase (MAPK) pathways – The MAPKs are serine/threonine kinases that are involved in regulating a variety of cellular processes through the activation of different signalling cascades (McCubrey *et al.*, 2006). There are 4 different pathways regulated by the following MAP Kinases - the extracellular-signal regulated kinases (ERK1/2 or p42/44 MAP kinase), Big MAP kinase (BMK/ERK5), c-jun N-terminal kinase (JNK/stress activated protein kinase-1 (SAPK1)),and p38 MAPK (SAPK2/RK) (Koul *et al.*, 2013). Upstream MAP Kinase Kinases (MAPKK) that are in-turn activated by further upstream MAP Kinase Kinase Kinases (MAPKKK), regulate the activities of these key signalling proteins (Fang *et al.*, 2012). The signalling cascade is initiated by a variety of stimulants like growth factors, cytokines, death and stress receptors and also oxidative stress (Rodriguez-Berriguete *et al.*, 2012) (**Figure 1-12**).

The majority of ERK signalling is activated through the RAS-RAF signalling cascade (Junttila *et al.*, 2008) and has been associated with advanced prostate cancer and poor prognosis (Keller *et al.*, 2004). ERK signalling has been observed to upregulate interleukin-6 (IL-6), an inflammatory cytokine in CRPC (Nguyen *et al.*, 2014). ERK signalling also regulates NF-κB which then positively controls the expression of anti-apoptotic genes such as those belonging to the Bcl2 family (Aggarwal, 2000). Research on ERK5 has revealed its role in upregulating tumour growth, migration and invasion in advanced prostate cancer (McCracken *et al.*, 2008; Ramsay *et al.*, 2011).

The role of the JNK signalling in prostate cancer has been linked to inducing apoptosis due to androgen deprivation (Lorenzo and Saatcioglu, 2008). Proteins involved in cell adhesion, invasion, and migration such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA) were upregulated due to JNK signalling in prostate cancer cells and hence suggests to play a role in the disease progression (Hung *et al.*, 2009). p38 MAPK signalling can be activated by pro-inflammatory (IL-6) and anti-inflammatory (EGF) cytokines along with other stress signals (Whyte *et al.*, 2009). Similar to the other MAPK pathways, p38 has been seen to be over-expressed in prostate cancer leading to the upregulation of IL-6 and also regulates TGF-β-mediated activation of MMPs thus assisting prostate cancer growth and metastasis (Lin *et al.*, 2001; Shen *et al.*, 2010)

The use of MEK1 inhibitor PD184352 effectively reduced tumour growth by inhibiting ERK1/2 and ERK5 signalling activity (McCracken *et al.*, 2008). Recent studies have

shown that inhibiting both the PI3 Kinase and MAP kinase signalling with the use of dual PI3K/mTOR inhibitor GSK2126458 as well as AZD6244, a MEK inhibitor, inhibited prostate cancer tumour growth both *in-vitro* and *in-vivo* (Park *et al.*, 2015).

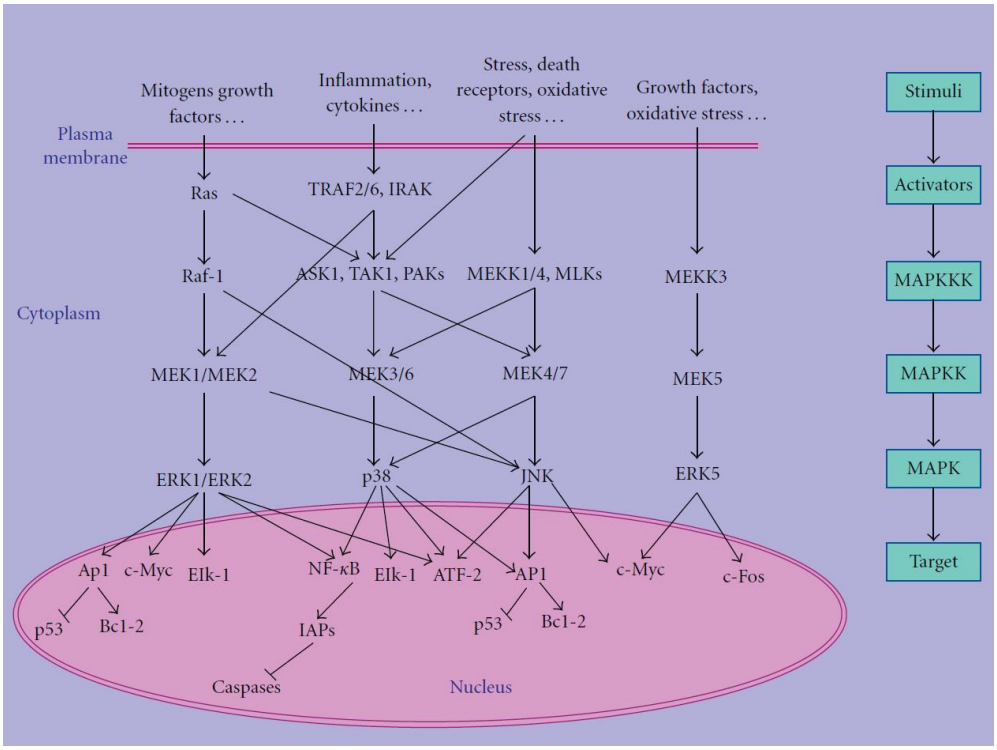

Figure 1-12: MAP Kinase signalling pathways

The diagram shows the 4 regulatory signalling pathways mediated through MAP kinases ERK1/2, p38, JNK and ERK5. The pathways are activated by various signals as indicated and regulate the expression of a range of proteins that are involved inhibiting apoptosis (inhibitory of apoptosis proteins -IAPs). The pathways also positively regulate the activities of transcription factors like c-myc and promotes cancer progression (Rodriguez-Berriguete *et al.*, 2012).

There are other signalling pathways that are altered in prostate cancer. Other growth factors like IGF along with its respective receptors have been reported to be over-expressed in prostate cancer (Nickerson *et al.*, 2001; Lin and Wang, 2010) and IGF inhibition was found to regress prostate cancer proliferation, migration and invasion (Kato *et al.*, 2015). FGF receptor expression was also found to be upregulated in prostate adenocarcinoma and the increased expression of its growth factor led to the increase in AR protein levels linking it to the AR-signalling axis (Acevedo *et al.*, 2007; Memarzadeh *et al.*, 2007). The expression of FGFR has also been linked to prostate cancer progression by increasing angiogenesis, cell migration and invasion (Feng *et al.*, 2012; Wan *et al.*, 2014). The various roles of multiple kinases are thus evident in advanced prostate cancer. The remaining sections of this chapter give a brief update on

the roles of the EGFR family in prostate cancer development and progression, leading to the aims and objectives of this project.

1.6 Epidermal growth factor receptor (EGFR) family

The EGFR family of receptors consists of four closely related type 1 transmembrane tyrosine kinase receptors; epidermal growth factor receptor (EGFR, also known as HER1 (Human Epidermal growth factor Receptor-1/ ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) and were assigned the gene symbol ErbB as they are homologous to Erythroblastic Leukemia Viral Oncogene (Roskoski, 2014). These receptors control activities such as cell division, differentiation, migration adhesion and apoptosis. Various studies have shown that these receptors have a vital role in tissue development and maintenance (Jathal *et al.*, 2011). The EGFR family are receptor tyrosine kinases (RTKs) that are known to activate both the MAPK and the PI3 Kinase pathway and hence play a major role in prostate cancer development and progression (Baselga and Swain, 2009). The properties of the members of the family are explained in the following sections.

1.6.1 Structure

The EGF receptors exist as inactive monomers but dimerize after being activated by mesenchymal ligands. The overall structure of the EGF receptors consists of an extracellular domain, a transmembrame domain, a tyrosine-kinase domain followed by a c-terminal domain (**Figure 1-13**) (Jathal *et al.*, 2011). The extracellular domain is further divided into four sub-domains. Subdomains I (amino acids 1–165) and III (amino acids 310–480) have leucine-rich repeats and assist ligand binding. Domains II (amino acids 165–310) and IV (amino acids 480–620) have multiple cysteine residues that are involved in the formation of disulphide bonds, and their interaction prevents subdomains I and III from binding to the ligand by disrupting the ligand-binding pocket. Ligand binding relieves these inhibitory interactions and encourages dimerization of one monomer and a second ligand bound monomer through the interaction of their domain II loops. The transmembrane domain consists of a mere 19–25 amino acid residues and is separated on either side by the extra and intracellular juxtamembrane segments. This is followed by the tyrosine-kinase domain and the c-terminal tail (amino acids 953–1186) (Lemmon *et al.*, 2014). The receptor dimer now activates the kinase domain and specific tyrosine residues within the cytoplasmic tail are phosphorylated.

These phosphorylated residues serve as docking sites for different proteins that subsequently activate intracellular signalling pathways (Di Lorenzo *et al.*, 2003; Sithanandam and Anderson, 2008; Jathal *et al.*, 2011).

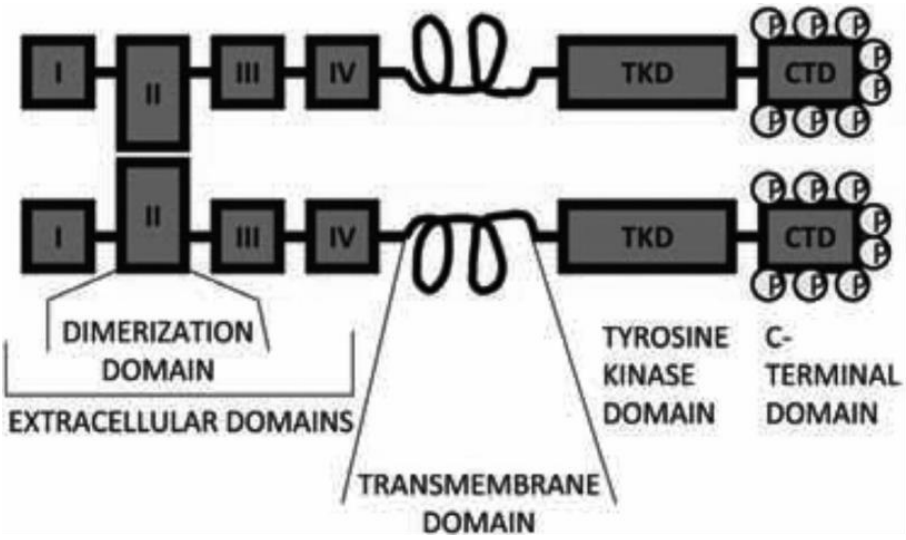

Figure 1-13: Structure of EGF receptors

All members of the EGF receptor family have a large extracellular ligand-binding region (with subdomains I-IV), a small intracellular transmembrane domain, a cytoplasmic tyrosine kinase domain and a C-terminal tail, which contains the phosphorylation sites for the effector signalling phosphotyrosine molecules. Subdomains I and III are involved in in ligand binding and are also known as called L1 and L2. Subdomains II and IV are also known as CR1 and CR2 and maintain the inactive conformation of the receptor. Ligand binding counteracts the inhibitory interactions and initiates receptor dimerization through domain II of two monomers. The stable receptor dimer then activates the kinase domain through phosphorylation of specific tyrosine residues in the cytoplasmic tail, which in-turn act as docking sites for a range of proteins resulting in the subsequent activation of intracellular signalling pathways (Jathal *et al.*, 2011).

1.6.2 Ligands and receptor isoforms

13 known ligands, including EGF, transforming growth factor alpha (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPG) and neuregulins 1 – 4 (NRG) can activate the EGF receptor family members (Han and Lo, 2012).

The functional activity of the receptors varies in spite of them having the same domain structures. The receptors can function through the formation of homodimers and heterodimers. EGFR, HER2 and HER4 possess active tyrosine kinase domains and their ligands for EGFR and HER4 are known. However, HER2 has no known ligand and HER3 lacks a functional tyrosine kinase domain and hence activation via

heterodimerization is essential for transmission of their signals (Sithanandam and Anderson, 2008; Baselga and Swain, 2009; Jathal *et al.*, 2011).

The receptors also express isoforms with EGFR having only one, and HER2 with two full length isoforms that only slightly differ due to their alternative mRNA splicing. HER3 also has 2 full length receptors with one of them missing the first 59 amino acids. HER4 however has 4 different isoforms (JMaCTa, JMaCTb, JMbCTa, and JMbCTb.) due to different versions of the extracellular juxtamembrane (JMa and JMb) and the carboxyterminal tail (CTa and CTb) and are also produced by alternative mRNA splicing. EGFR can be activated by seven growth factors (EGF, TGF-α, AR, EPG, BTC, HB-EGF and EPR) while HER3 is activated by Neuregulins 1 and 2. HER4 has also seven activating growth factors (Neuregulins 1- 4, BTC, HB-EGF and EPR) but HER2 has no ligands and lacks a functional ligand binding domain. A total of 614 possible combinations of receptors can occur when the 11 growth factors and the 28 possible dimers are considered. However, when the non-functional combinations of the HER2/HER2 and HER3/HER3 homdodimers are eliminated, a grand total of 611 active combinations are possible (Elenius *et al.*, 1997; Roskoski, 2014). The ligand precursors which are initially expressed as membrane proteins undergo protein ectodomain shedding which involves the proteins being cleaved and released by proteases belonging to the A disintegrin and metalloproteases family (ADAMs) (Blobel, 2005). The members ADAM 10 and ADAM17 are mainly involved in processing of ligands that activate the EGFR family (Scheller *et al.*, 2011). This protease family is also involved in processing other growth factors and their receptors and control a range of important cellular processes including neurogenesis and myogenesis (Rocks *et al.*, 2008).

The abundance and membrane distribution of the EGF receptors are tightly regulated by mechanisms such as Clathrin-dependent and independent endocytosis, ubiquitylation and degradation (Longva *et al.*, 2002). However, these mechanisms are altered in cancer cells causing receptor clustering in the plasma membrane and cause an increase in receptor dimerization and cell signalling (Casaletto and McClatchey, 2012). A recent study has reported that HER3 translocated to the nucleus via Clathrin-independent endocytosis and importin β1 complex (Reif *et al.*, 2015). There is also evidence suggesting that the signalling activity of these receptors is retained in the endosomes that can also help in transporting these receptors to the nucleus (Vieira *et al.*, 1996). The receptors also have nuclear localisation sequences (NLS) that help in the interaction with nuclear importin complexes involved in intracellular protein trafficking (Lo *et al.*,

2006).

1.6.3 Epidermal growth factor receptor (EGFR/HER1/ErbB1)

The EGF receptor was the first growth factor receptor to be identified in cancer cells (Carpenter *et al.*, 1975) after the discovery of its ligand EGF in 1962 (Cohen, 1962). EGFR gene is located on chromosome 7q12 and is 170 KD in size (Puri and Salgia, 2008; Ooi *et al.*, 2009). EGFR has been known for its role in epithelial cell development (Jathal *et al.*, 2011). Once activated by its ligand, it can initiate signalling pathways including Ras-Raf-MEK, PI3K-AKT-mTOR and JAK2-STAT (Han and Lo, 2012). The C-terminal domain contains five autophosphorylation sites (Purvis *et al.*, 2008). Among these sites, tyrosine (Tyr) 1068, Tyr1148, and Tyr1173 act as major sites and Tyr992 and Tyr1086 act as minor sites for phosphorylation (Chattopadhyay *et al.*, 1999). Depending on the site of phosphorylation, different downstream signalling cascades are initiated by EGFR. Phosphorylation at Tyr1068 leads to the recruitment of GAB-1 or Grb2, and subsequently activation of their downstream signalling pathways (Chattopadhyay *et al.*, 1999; Yarden, 2001).

EGFR and prostate cancer

As mentioned earlier, EGFR has been known to control signalling pathways that modulate growth, differentiation, adhesion, migration and survival of cancer cells and is known to be over-expressed in several types of cancer including prostate, breast, colorectal, lung, oesophageal, pancreatic and head and neck cancers (Yewale *et al.*, 2013). Progression of tumours in becoming invasive and metastatic has been associated with increased EGFR signalling. The activity of the receptor is increased in cancer due to activating mutations or over-expression. Mutations have been identified on exons 19, 20 and 21 in patients with prostate cancer and the presence of G735S, G796S and E804G mutations led to an increase in cell proliferation and invasion (Cai *et al.*, 2008). EGFR can contribute to cancer progression not only by activating signalling pathways, but can also translocate to the nucleus where it acts as a transcription factor for cell proliferative and survival genes including cyclin D1, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), aurora A and c-Myc. It has also been shown to have a role in DNA damage repair by interacting with DNA-dependent protein kinase (DNA-PK) (Brand *et al.*, 2011; Han and Lo, 2012). A truncated form of the receptor, EGFRvIII has been detected in advanced prostate cancer and its expression has been associated with poor survival outcome (Edwards *et al.*, 2006). This form lacks the

ligand binding domain (amino acids 6- 273) and remains constitutively active (Yewale *et al.*, 2013). In the normal prostate, EGFR levels are down regulated in an androgen dependent manner and this regulation is lost in advanced prostate cancer suggesting its role in driving androgen independent prostate cancer (Cai *et al.*, 2009; Traish and Morgentaler, 2009).

1.6.4 HER2 (Neu/ErbB2)

The HER2 receptor is encoded by the HER2 gene, a proto-oncogene located on chromosome 17q21. The protein has 1255 amino acids and is 185kD in size. The gene was initially identified in rat neuroectodermal tumours (hence also the name - Neu) (Schechter *et al.*, 1984). As mentioned earlier, HER2 lacks a functional ligand binding domain and relies on heterodimerization with another family member. However, the over-expression of HER2 has been reported to form functional homodimers (Ghosh *et al.*, 2011). HER2 has the strongest catalytic kinase activity and hence HER2 containing heterodimers have the strongest signalling activity. The HER2- HER3 heterodimer is the most potent in terms of strength of interaction, ligand induced tyrosine phosphorylation and downstream signalling (Sithanandam and Anderson, 2008; Baselga and Swain, 2009; Jathal *et al.*, 2011). The activity of HER2 is induced by the phosphorylation of 4 major sites Tyr1139, Tyr1196, Tyr1221/1222, and Tyr1248 (Olayioye *et al.*, 2000), however phosphorylation at Tyr1028 impairs the receptor's function (Dankort *et al.*, 1997). Other receptors such as insulin-like growth factor receptor I (IGF1) and estrogen receptor (ER) can also activate the receptor. HER2 majorly mediates the mitogen activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) pathway (Tai *et al.*, 2010; Gutierrez and Schiff, 2011).

HER2 and prostate cancer

HER2 amplification and over-expression can be tumorigenic and this has been reported in a number of human tumours, including in 18–25% of human breast cancers, ovarian cancers, gastric carcinoma, salivary gland tumours and prostate cancer (Baselga and Swain, 2009; Tai *et al.*, 2010) and is associated with poor prognosis (Edwards *et al.*, 2004). According to Signoretti *et al.* (2000), HER2 was found to be over-expressed in 25% of untreated primary tumours, 59% of tumours after hormone therapy and 78% of metastatic tumours. HER2 has also been associated with the progression to CRPC. Previous research has shown that HER2 can promote phosphorylation of AR and

activate AR signalling. This leads to stabilisation of AR protein levels and enhances its binding to the promoter regions of AR regulated genes (Wen *et al.*, 2000; Mellinghoff *et al.*, 2004). HER2 has also been associated as one of the factors responsible for promoting cell survival in an androgen-depleted environment. HER2 was negatively regulated by the AR in the presence of androgens and was thus inferred to play an important role in activating and re-establishing the AR pathway and encouraging prostate cancer cell survival following androgen ablation (Berger *et al.*, 2006). A soluble truncated form of the receptor consisting of only the extracellular domain has been located in the serum of prostate cancer patients over-expressing HER2 and correlates with aggressive disease (Domingo-Domenech *et al.*, 2008).

1.6.5 HER3 (ErbB3)

HER3 was identified in 1989. It is located on chromosome 12q13.2 and the protein is 180 kDa in size (Kraus *et al.*, 1989). HER3 mRNA has been detected in the early stages of development suggesting its role in cell growth and differentiation. In human adults, HER3 is detected in the brain, spinal cord, liver, prostate, kidney and lung tissues. Growth factors belonging to the Heregulin-1 (HRG-1) and HRG-2 groups are known to activate the receptor (Zimonjic *et al.*, 1995b; Seshacharyulu *et al.*, 2012). The C-terminal domain of HER3 is slightly longer than those of EGFR and HER2. HER3 was recently characterized to have very low catalytic activity but it is sufficient to phosphorylate HER2, its most favourable dimerization partner. Once activated, they phosphorylate each other's tyrosine sites thus creating docking sites for downstream signalling proteins (Shi *et al.*, 2010). The HER2-HER3 heterodimer mainly activates the MAPK and PI3K signalling pathways (**Figure 1-14**). Tyr1289 in HER3 serves as a binding site for the p85 regulatory subunit of PI3K and allows subsequent activation of the pathway. They also have a nuclear localization signal at its C-terminus (Sithanandam and Anderson, 2008). However, HER3 homodimers have been reported to have weak autophosphorylation activity (1/1000th the autophosphorylation activity of EGFR) despite being kinase-impaired (Shi *et al.*, 2010).

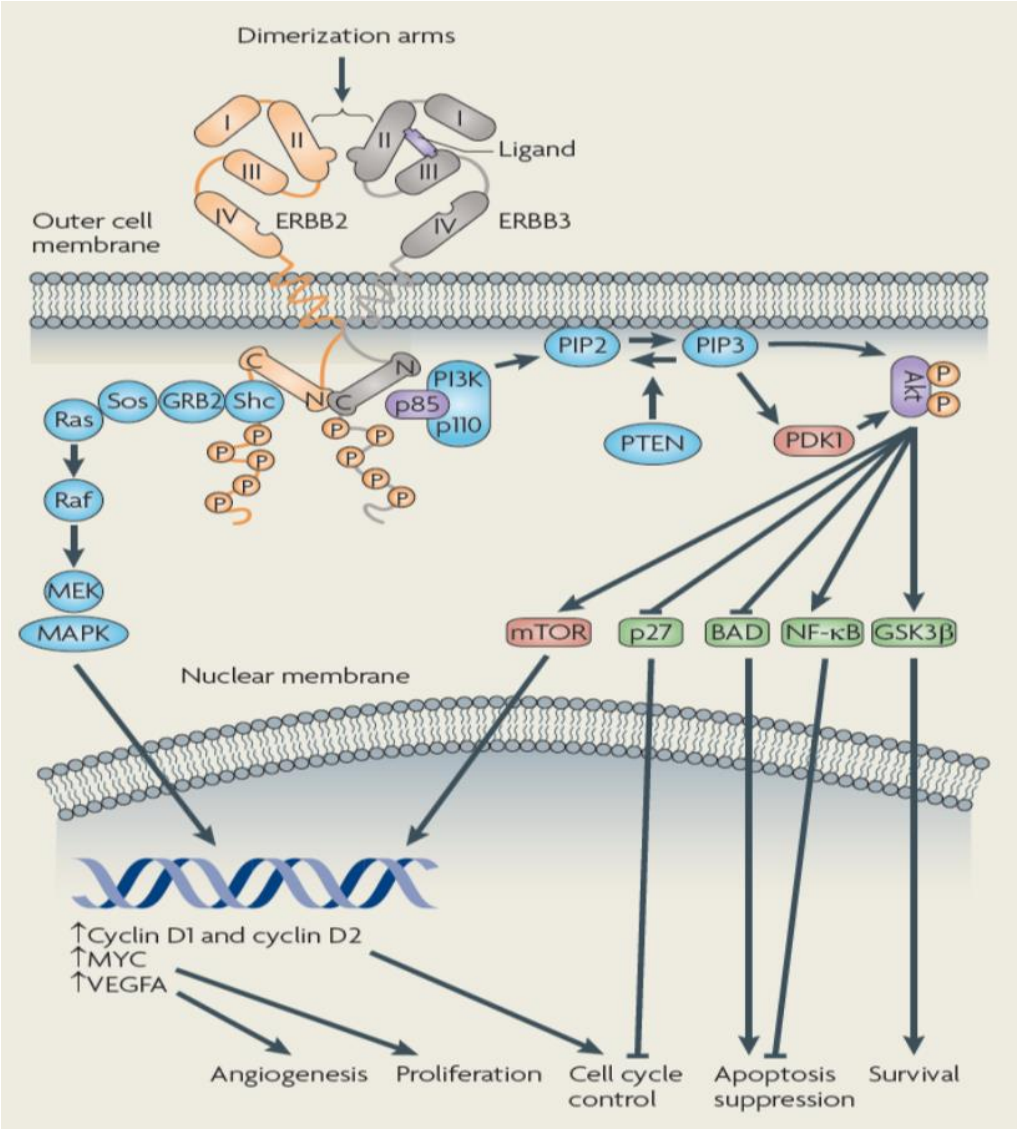

Figure 1-14: Downstream signalling of HER2 and HER3

Ligand binding initiates cell signalling by receptor dimer formation. Cross-phosphorylation of the dimer partners creates docking sites for the recruitment of downstream signalling partners to form signalling complexes. MAPK pathway and PI3K–Akt pathways are the two key signalling pathways activated by the EGFR family and promotes tumour cell proliferation and survival (GSK3β -glycogen synthase kinase 3β; NF-κB - nuclear factor-κB; PDK1 - pyruvate dehydrogenase kinase 1; PIP2 - phosphatidylinositol biphosphate; PIP3 -phosphatidylinositol triphosphate) (Baselga and Swain, 2009).

HER3 and prostate cancer

HER3 presence has been detected in prostate cancer and cancer cell lines. HER3 over-expression in tumours mainly results due to abnormal functioning of HER3 regulating mechanisms. Neuregulin receptor degradation protein 1 (Nrdp1) is an E3 ubiquitin ligase and was recently discovered to regulate HER3 degradation. Ubiquitination of HER3 occurs via the N-terminal RING finger domain of Nrdp1 and is also responsible for its turnover. Chen *et al.* (2010) reported that the AR functioning negatively regulated HER3 in an androgen dependent manner but not in CRPC and observed elevated levels of HER3 in castration resistance cells. Their research uncovered that in CRPC cells, AR was unable to regulate Nrdp1 transcription thus increasing HER3 levels, which in turn stabilised AR activity along with HER2. HER3 also regulates the AR through EBP1 (ErbB3 binding protein 1). Zhang *et al.* (2008) showed that EBP1 suppressed AR activity through physical interaction. EBP1 is initially bound to HER3 and upon HER3 stimulation, EBP1 gets phosphorylated and released. It then translocates to the nucleus and binds to AR promoter regions and prevents its activation. EBP1 over-expression down regulated expression of AR in LNCaPs, an androgen-dependent cell line. The presence of nuclear HER3 has also been linked to CRPC progression and elevated nuclear HER3 expression was found in lymph node metastases of the disease. The nuclear form of HER3 – p45 sHer3 – a secreted isoform of the receptor, has been found in bone metastases of the disease (Myers *et al.*, 1994; Chen *et al.*, 2007). HER3 over-expression was also been linked to a less favourable prognosis in prostate cancer (Sithanandam and Anderson, 2008; Jathal *et al.*, 2011). These findings reveal the diverse roles that HER3 holds in prostate cancer. Recently, HER3 mutations in the extracellular and kinase domains of the receptor have been identified in a variety of human malignancies, particularly in gastric and colon cancers. Interestingly, the G284R mutation in the extracellular domain is hypothesized to encourage a conformation suitable for its dimerization even in the absence of ligand (Jaiswal *et al.*, 2013). The presence of HER3 mutations in prostate cancer is not widely reported and should be investigated.

1.6.6 HER4 (ErbB4)

The HER4 gene is located on chromosome 2q (33.3–34) (Zimonjic *et al.*, 1995a). EGF family ligands (betacellulin, epiregulin, heparin-binding EGF-like ligand) and neuregulins can activate the receptor. HER4 can regulate diverse biological activities and cellular responses that mainly include activation of pro-apoptotic pathways, cell cycle arrest and forms complexes with transcription factors to modulate cell proliferation via activity of its intracellular domain. Unlike HER3, isoforms of HER4 have been observed due to alternative splicing of its mRNA. The intracellular C-terminal fragment of HER4 is known to translocate into the nucleus following gamma secretase-mediated cleavage (Koutras *et al.*, 2010; Seshacharyulu *et al.*, 2012).

HER4 and prostate cancer

HER4 expression is not seen in most prostate cancer cells and their expression appears to disrupt cancer cell growth (Baselga and Swain, 2009). HER4 expression is observed to be lost in most prostate cancer patients permitting HER3 to only dimerize with EGFR and HER2 (Jathal *et al.*, 2011). However, breast, oral cavity, esophagus, ovary, cervix and skin cancers have been detected with a truncated C-terminal HER4 in the cancer nuclei and have been linked to poor clinical prognosis (Seshacharyulu *et al.*, 2012).

1.7 EGFR family as therapeutic targets in prostate cancer

EGFR family members have been recognised as targets for therapy as they are over-expressed and deregulated in many types of cancer. They remain to be targets for the development of novel anticancer agents and to add to the range of existing small tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (Baselga and Swain, 2009) (**Figure 1-15**). In 1998, the first inhibitor of the EGFR family –Trastuzumab (Herceptin), was approved by the US Food and Drug Administration (FDA) (Goldenberg, 1999). This monoclonal antibody targets the extracellular region of HER2. Similarly Cetuximab, also a mAb, binds to the extracellular domain of EGFR and prevents ligand-receptor interaction and promotes receptor internalization (Martinelli *et al.*, 2009). The small molecule TKIs Gefitinib and Erlotinib reversibly inhibit EGFR and prevent autophosphorylation (Roskoski, 2014). Patients suffering with CRPC have been on clinical trials of Trastuzumab, Gefitinib and Erlotinib to test single-agent therapeutic efficacy. However, these trails reached only phase II as the results were unsatisfactory (Lara *et al.*, 2004; Canil *et al.*, 2005; Gravis *et al.*, 2008; Guerin *et al.*, 2008; Dhupkar *et al.*, 2010). Similar results were obtained with Pertuzumab, a second-generation anti-HER2 antibody (de Bono *et al.*, 2007). The dual kinase inhibitor Lapatinib (targeting both EGFR and HER2) instead had lesser side effects but provided no PSA responses (Sridhar *et al.*, 2010). The use of the more recent drug Afatinib (also targeting EGFR and HER2) has also shown limited success in clinical trials (Molife *et al.*, 2014). The poor success of EGFR and HER2 inhibitors has been associated with the over-expression of HER3. The role of HER3 in driving PI3K/AKT signalling and mediating drug resistance has shifted the attention on this signalling partner of EGFR and HER2 (Jathal *et al.*, 2011). Multi-EGFR family inhibitors have also been developed to inhibit more than one individual EGF receptor at one time. Previous research on *in-vitro* and *in-vivo* models has demonstrated greater anti-tumour activity by employing dual- or multi-EGFR family inhibitors (Hsieh and Moasser, 2007; Seshacharyulu *et al.*, 2012).

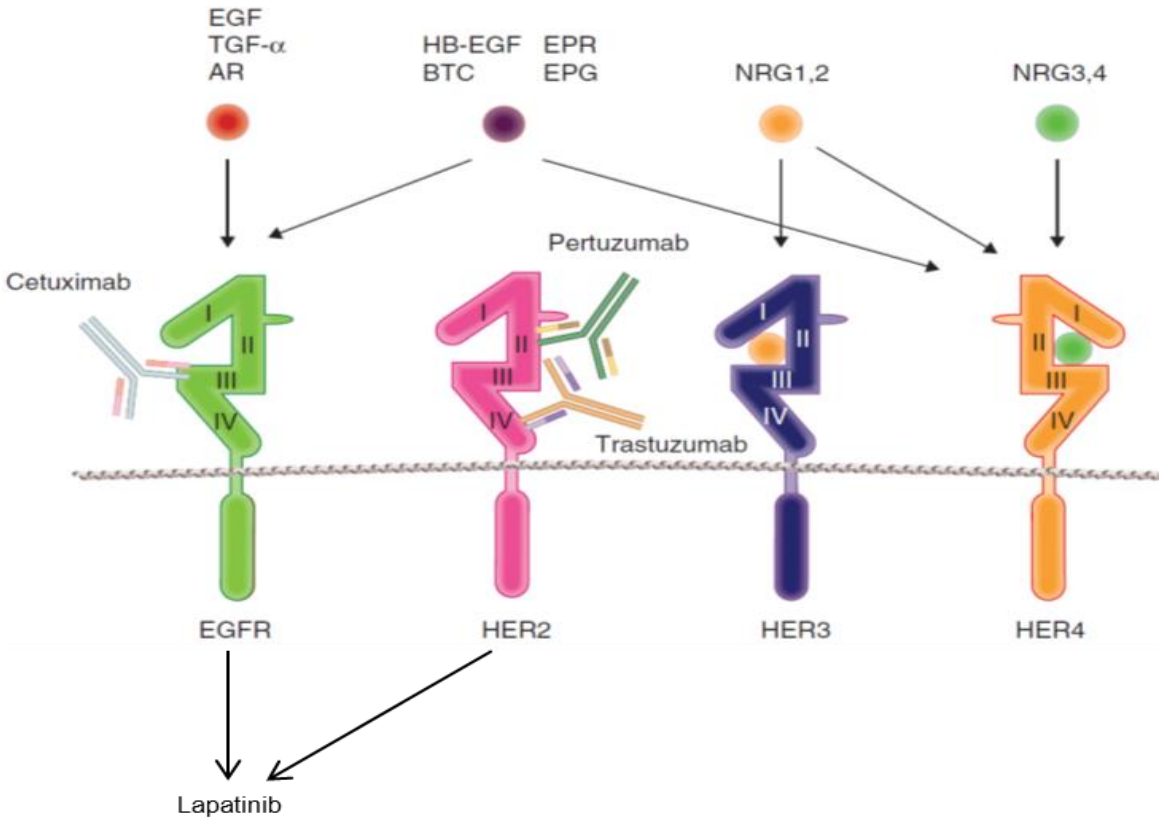

Figure 1-15: Inhibitors against EGFR family

The above figure describes a few of the different inhibitors that have used in the treatment for prostate cancer but have been largely unsuccessful as they target a single or dual member of the family (Huang *et al.*, 2009).

AstraZeneca's AZD8931 is a recently-documented pan-EGFR family inhibitor and targets EGFR, HER2 and HER3 signalling. Initial studies revealed greater effect of the inhibitor against the activity of the HER2-HER3 dimer and hence is expected to be of particular use in solid tumours that are managed through these receptors (Hickinson *et al.*, 2010). The characterisation of this drug was mainly performed on lung and breast cancer cell lines. The drug is currently in clinical trials for oesophageal cancer (CRUKD/12/007) and bowel cancer (CRUKD/12/015). Recent published results in breast cancer research suggest that the compound could be a novel strategy in the treatment of the disease (Mu *et al.*, 2014). However, there is no available literature on the use of this compound in prostate cancer.

It is evident from previous research that the EGFR family plays a significant role in many cancers like breast, ovarian, gastric and also the prostate. Many anticancer agents have been developed and tested against this receptor family. The outcomes of the clinical trials with the use of these agents have been different among the various cancer types. However, none of these agents have had a formidable outcome in prostate cancer. It is therefore essential to study the molecular mechanisms of the EGFR family in prostate cancer and try new therapeutic approaches that target the family.

1.8 Aims and objectives

The principle aims of this project are -

i. To characterise the expression levels of EGFR family receptors in prostate cancer tissues and cell lines

ii. To study their effects on cancer progression (by over-expression experiments)

iii. To study the efficacy of the pan inhibitor AZD8931 (AstraZeneca) in prostate cancer cell lines which specifically targets EGFR, HER2 and HER3.

The broader aim of this project is to identify patients who could benefit from pan-EGFR family inhibition and initiate a Phase I/II clinical trial with the compound alone or with combination of other treatments.

Chapter 2 Materials and Methods

2.1 General laboratory procedures

All laboratory procedures were carried out in accordance with the departmental safety policy as outlined in the 'Northern Institute for Cancer Research – Paul O'Gorman Building Safety Policy.' The Standard Operating Protocol (SOP) for the respective techniques was followed and Personal Protective Equipment (PPE) was worn at all times. The necessary COSHH (Control of Substances Hazardous to Health) and BioCOSHH (Biological Control of Substances Hazardous to Health) forms were filed before the commencement of the procedure.

2.2 Immunohistochemistry

2.2.1 Human tissue samples

Immunohistochemistry was carried out on archival formalin-fixed paraffin wax-embedded serial sections of clinical prostate samples that were cleared from the Newcastle upon Tyne ethics committee. A database of patients diagnosed with prostate cancer was generated from the Freeman Hospital, Newcastle upon Tyne database. Transurethral resection of prostate (TURP) samples from patients at the Freeman Hospital between 1993 and 2003 were collected. An anonymous database was maintained with information of all the samples (Demographic data, pathological data, hormone manipulation, and other treatment modalities). The database was securely stored according to good clinical laboratory practice guidelines

2.2.2 Processing and grading samples

Immediately after extraction, the tissue samples were fixed overnight in 10% formalin buffered to pH–7. They were then embedded in paraffin blocks after they were trimmed and processed in graded ethanol and xylene using the routine processing and embedding equipment (Miles Scientific VIP processor, Leica EG1160). Blocks selected for tissue microarray (TMA) construction were then cut at 5μm thickness using a standard

microtome onto slides (Superfrost Plus, Thermo Scientific) and stained with haematoxylin and eosin (H&E). The presence of the pathology of interest (various Gleason grades, PIN and BPH) was confirmed by Dr Mathers, uropathologist, RVI, Newcastle upon Tyne. The blocks were appropriately marked with different colours to represent different grades.

2.2.3 Construction of Tissue Microarray (TMA)

Tissue Microarrays (TMA) were constructed according to a pre-determined layout (X and Y co-ordinates for each sample) by using a MTA1 arrayer (Beecher instruments). Briefly, a recipient block was made out of paraffin wax. A 0.6mm wax core was taken from the recipient block at the appropriate location and the cavity created was then replaced by the appropriate tissue sample. The recipient block could be moved precisely on both the X and Y axes with the help of the micrometer. Subsequent cores were placed at 1mm intervals. Once the layout was completed in this manner, the block was incubated at 37ºC for 30 minutes to provide stability to the cores. The block was then cooled to room temperature and sectioning using a standard microtome and water bath. With a core depth of 3 mm, up to 600 sections of 5μm thickness could be cut from a single TMA. The slides were baked at 60ºC for 2 hours before storing them at room temperature.

2.2.4 Methodology

The samples were initially deparaffinised in xylene for 5 minutes (2 times) and rehydrated through a series of graded alcohols (100%, 100%, 70%, and 50%) with a final wash in distilled water. Antigen retrieval was achieved by incubating the slides in citrate buffer using a decloaker (**Table 2-1**). Endogenous peroxide activity was blocked by treating the samples with 3% hydrogen peroxide for 10 minutes. The sections were then washed under running tap water for 5 minutes and placed in TTBS. The area around the sections were marked with a hydrophobic PAP pen (Dako). Unspecific binding was blocked prior to antibody application by incubating the sections for 5 minutes using protein block (Dako). Primary antibody was incubated overnight at 4ºC. The next day, the slides were washed twice for 5 minutes in TTBS (Tris buffered saline – Tween20) and continued with the instructions from the detection system depending on the antibody species. After DAB (Diaminobenzidine tetrahydrochloride) incubation (5 mins) the slides were washed in tap water for 5 minutes, counter stained in Harris Haematoxylin for 15 seconds, immediately washed with tap water and treated with

Scotts tap water for 30 seconds. Finally, the tissue sections were dehydrated in a series of graded alcohols (50%, 70%, 100%, and 100%) followed by two 5 minute xylene washes, and mounted in DPX.

X-Cell-Plus HRP detection kit (Menapath)

After primary antibody incubation, the slides were incubated with either HRP (Horseradish Peroxidase) polymer (for rabbit Ab) for 30 minutes alone or universal probe for 30 minutes followed by a wash in TTBS for 5 minutes subsequently followed by incubation with HRP polymer as before (for mouse Ab). This was followed by a 10 minute wash in tap water and DAB incubation for 5 minutes after which the sections were counterstained, dehydrated and mounted as mentioned previously.

Scoring

Once stained, the TMA was then scored for nuclear and cytoplasmic staining. The expressions were scored by two independent observers (average values were finally considered), using a histoscore method, also known as H-score. The method considers the intensity of staining and the percentage of cells with the same intensity of staining across the sample. The score is calculated by using the formula:

$$\begin{aligned} Histoscore = {} & (1 \text{ x } \% \; cells \; with \; weak \; staining) \\ & + (2 \text{ x } \% \; cells \; with \; moderate \; staining) \\ & + (3 \text{ x } \% \; cells \; with \; strong \; staining) \end{aligned}$$

This method has been well established (Ahmad *et al.*, 2011) and is useful for scoring heterogeneous staining of samples. It provides a maximum score of 300 (100% cells with strong staining) and a minimum of 0 (100% cells with no staining) which makes the data quantifiable. Once the scores for cytoplasmic and nuclear HER2 and HER3 expression were established, the available patient clinic-pathological data was used to analyse the expression profiles in cancer patients.The expression was classed as being low (histoscore between 0-100), moderate (histoscore between 100-200) or strong (histoscore between 200-300) and the available survival data was analysed by plotting Kaplan-Meier Curves. This uses an appropriate nonparametric statistical test called the log-rank test. The test considers the null hypothesis that there is no difference between the populations in the probability of an event (here a death secondary to prostate cancer) at any time point (Bland and Altman, 2004).

Target	Ag retrieval	Primary Antibody	Washes	Detection kit
HER2	0.01 M sodium citrate buffer, pH 6.0 Decloaker (Menapath)	1:500 1% BSA in PBS as diluent Overnight incubation Santa Cruz Neu (3B5): Cat no - sc-33684 (Mouse monoclonal – raised against synthetic peptide corresponding to amino acids 1242-1255 of human HER2)	TTBS	X-Cell-Plus HRP Detection Kit (Menapath)
HER3		1:100 1% BSA in PBS as diluent Overnight incubation Santa Cruz ErBb3 (C-17): Cat no - sc-285 (Rabbit polyclonal – raised against a peptide mapping the c-terminus of human HER3)		

Table 2-1: IHC conditions

The antibodies used for IHC were first validated for its specificity by performing western blotting using appropriate control samples. A test TMA consisting of different tissue types (including benign and cancerous prostate tissue) was then used to optimise the correct antigen retrieval buffer and antibody concentration. A negative control (without primary antibody incubation) was included during the all the optimisation and final experiments.

Clinical details	TMA1	TMA4
Age (Mean;Range)	70 yrs (55-88)	72 yrs (55-89)
Total number of patients	n=76	n=81
Previous treaments	Naïve	Naive
Gleason sum score		
4-6	n=7	n=15
7	n=10	n=12
8-10	n=53	n=45
Gleason single score		
3	n=11	n=25
4	n=28	n=31
5	n=28	n=16
Organ confined disease	n=34	n=45
Locally invasive disease	n=27	n=28
Metastases	n=16	n=23
Subsequent metastates	n=16	n=18

Table 2-2: Clinical details of patients that were analysed for HER2 and HER3 expression.

2.3 Mammalian cell culture

2.3.1 Cell culture reagents

Full medium (FM)

RPMI 1640 medium (500 ml - Sigma Aldrich) containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (25 mM) and L-glutamine (2 mM) was supplemented with foetal calf serum (FCS) (56 ml=10% - Gibco). This standard medium was used to maintain prostate cancer cell lines that required androgens for their growth.

Steroid depleted medium (SDM)

Steroid depleted serum (56 ml=10% Gibco) was added to RPMI 1640 medium (500 ml -Gibco) containing HEPES buffer (25 mM) and L-glutamine (2 mM). This medium is required to culture cells in androgen depleted conditions. In principle, this is achieved by treating FCS using dextran-coated charcoal (DCC) (that depletes materials such as hormones and certain growth factors without having an effect on essential salts and amino acids. Any steroid depleted conditions in experiments were established using this medium as is referred to as SDM in this study.

Basal medium (BM)

RPMI 1640 medium (500 ml-Gibco) containing HEPES buffer (25 mM) and L-glutamine (2 mM) was supplemented with bovine serum albumin (BSA) at a final concentration of 250 µg/ml to produce a medium devoid of any serum. Experiments with
Heregulin treatments were carried out by starving the cells overnight (16 hours) in this media.

Freezing medium (FzM)

The composition of freezing medium for cells consists of 80% of the respective growth medium, 10% of dimethylsulphoxide (DMSO) (Sigma Aldrich) and 10% of serum

(serum in respective medium i.e. FM or SDM).

Phosphate buffered saline (PBS)

PBS containing sodium chloride - NaCl (137mM), potassium chloride - KCl (2.7mM), disodium hydrogen phosphate - Na_2HPO_4 .2 H_2O (10mM) and potassium dihydrogen phosphate (2mM) was set to pH–7.4 and autoclaved.

Trypsin/EDTA (Ethylenediaminetetraacetic acid)

10 X Trypsin stock (0.5g trypsin, 0.2g EDTA and 0.85g NaCl per litre (Sigma Aldrich)) was diluted to 1X working concentration using sterile PBS.

2.3.2 Cell lines and their characteristics

All cell lines were authenticated after purchase/donation and were only used at low numbered passage (i.e. frozen stocks were made soon after the cell lines were acquired. When a vial of cells were used, subsequent stocks were made and the cells in culture were passaged not more than 30 times).

LNCaP

The LNCaP prostate cancer cell line (Clone FGC- Fast Growing Clone; Passage 19) was purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). This cell line was derived from a 50- year old Caucasian male by using the needle aspiration biopsy sample of a lymph node metastasis showing metastatic prostate carcinoma. The cell line is well characterized confirming that they are androgen responsive and possess a mutated AR (Horoszewicz *et al.*, 1983).

LNCaP–AI

The LNCaP – Androgen Independent cell line was derived in-house by Prof. Craig Robson (Urology Research Group, NICR) from the LNCaP cell line. The cells were continuously passaged under androgen depleted conditions for a period of 9 months. The cell line was later characterized to be proliferative without the requirement of

androgens but retains androgen responsiveness. The parental LNCaP cell line with matched passage numbers was also maintained (McCracken *et al.*, 2008).

LNCaP-7B7

This cell line was a kind gift from Jan Trapman (Erasmus Medical Centre, Netherlands). The LNCaP-7B7 cell line was established by stably transfecting the LNCaP cell line with a pPSA luciferase reporter (Cleutjens *et al.*, 1996). This cell line can thus be utilised to investigate the effects on AR transcriptional activity using luciferase assays.

PC3

The PC3 cell line (Passage 27) was purchased from the European Collection of Animal Cell Cultures (EACC, Salisbury, UK). This cell line was derived from a metastatic bone lesion isolated from a 62-year old Caucasian male. The cell line is not hormone sensitive and does not express prostate specific antigen (PSA) or the androgen receptor protein. (Kaighn *et al.*, 1979).

2.4 Compounds

2.4.1 Heregulin-β1

Cells were treated with Heregulin (R&D Systems). The stock concentration was 1µg/ml in sterile PBS were stored at -80 ºC. Treatments were done by using a working concentration of 20ng/ml.

2.4.2 Dihydrotestosterone (DHT)

DHT (Sigma Aldrich) at 10mM stock concentration (dissolved in ethanol and stored at -80 ºC) was diluted 1:1000 in basal medium to get 10 µM stock. This was further diluted to the final required working concentrations.

2.4.3 AZD8931

The pan-HER inhibitor was provided by AstraZeneca. Stocks of 30mM of the compound were made in DMSO and stored at -80 ºC. Further dilutions were made in DMSO and stored at -20 ºC and repeated freeze thaw of the compound was avoided.

2.4.4 Lapatinib

Lapatinib was purchased from Selleck Chemicals and stocks of 30mM concentration were made in DMSO and stored at the recommended temperature of -20 ºC. Further dilutions were made in DMSO and stored at -20 ºC and repeated freeze thaw of the compound was avoided.

2.4.5 Enzalutamide

Enzalutamide was purchased from Selleck Chemicals and stocks of 30mM concentration were made in DMSO and stored at the recommended temperature of -80 ºC. Further dilutions were made in DMSO and stored at -20 ºC and repeated freeze thaw of the compound was avoided.

2.5 Generation of resistant cell lines

2.5.1 LNCaP-Lapatinib resistant cell line

LNCaP cells were continuously grown in the presence of Lapatinib using the GI_{50} concentration (1 µM) which was estimated using colony formation assay (**2.16.1**). After a few passages, the cells were treated with 3 µM of Lapatinib. A mixed populations of cells were grown that were resistant at this dose after which these cells were further subjected to a final concentration of 5 µM of the drug. The cells were maintained in the media for a minimal of 12 weeks prior to analysis. The cells hereafter were maintained in the presence of 5 µM Lapatinib.

2.6 Routine culturing of cell lines

All cell lines were routinely cultured using flasks and petri dishes (Corning) in a 37 ºC incubator (Sanyo) with 5% CO_2 humidified atmosphere. LNCaP–AI cells were maintained in SDM whereas all other cell lines were maintained in FM. All reagents were heated to 37 ºC prior to use. Handling of cell lines were done under sterile conditions in a Laminar Air Flow (LAF) hood. All reagents and equipment to be used inside the hood were made sterile by cleaning them with 70% alcohol (Fisher Scientific). Briefly, the media on the cells was removed, washed with PBS and incubated with Trypsin/EDTA (least amount required to cover surface area) at 37 ºC

until cellular detachment. Cells were then suspended at appropriate volumes in their respective growth medium to neutralize the effect of trypsin on the cells. The cells were pelleted at 400g for 5 minutes. The pelleted cells were re-suspended, counted if necessary and passaged (1:5 for LNCaP cell lines, 1:10 for other cell lines) onto fresh dishes or flasks.

Cells were subjected to routine mycoplasma testing in 1 month intervals using the Mycoalert mycoplasma detection kit (Cambrex, Berkshire, UK).

2.6.1 Cell counting

Cell concentration/ml was estimated with the use of an improved neubauer haemocytometer (VWR International). Briefly, 10 µl of re-suspended cells were applied to the haemocytometer and viewed under a light microscope using the 20x objective. The cell number present in the counting grid (25 large squares of the central 1 mm^2) was multiplied by 1 x 10^4 to get the cells/ml concentration of the cell suspension. Based on the number of cells required for the experiment, the volume of cells required was calculated with the use of this concentration.

2.6.2 Cryopreservation

Monolayer cultures were pelleted down and re-suspended in freezing medium. 1ml aliquots with cell concentrations between 2-5 x 10^6 cells /ml were made in cryovials (ThermoScientific) and labelled with the name of the cell line, passage number and date. The cells were frozen at a controlled rate to – 80 ºC and subsequently stored in liquid nitrogen. Frozen cells were re-cultured by defrosting the cells at 37 ºC and re-suspending the cells in 10ml of its respective fresh medium. The cells were then pelleted and cultured as mentioned in section **2.6**. Medium was replaced after 24 hours to remove dead cells.

2.7 Protein analysis

2.7.1 Sample preparation

Cell lysates were generated with the addition of RIPA buffer (150 mM NaCl pH 7.5; 50mM Tris-Hcl pH 7.5 (Fisher Scientific); 1% NP40 (BDH Chemicals); 1mM EDTA; 0.25% sodium deoxycholate (Sigma Aldrich). The buffer was supplemented with fresh

protease and phosphatase inhibitor tablets (Roche) following the recommended protocol. Briefly, cells were washed with chilled PBS and lysed in RIPA buffer for 30 minutes at 4 ºC after which the samples were sonicated for 5 minutes and cell debris was pelleted by centrifugation at 12,000g at 4 ºC for 10 minutes. The supernatant (whole cell lysate) was then used for further analysis. Protein concentration was estimated using BCA assay (Section **2.7.2**) and equal amounts of protein was added to appropriate amounts of 5x SDS sample buffer (250 mM Tris-HCl pH6.8; 10% SDS; 30% glycerol (Fisher Scientific), 5% β-mercaptoethanol, 0.02% bromophenol blue (Sigma Aldrich) and analysed using western blotting.

2.7.2 Protein quantification using BCA assay

Protein concentration of samples was estimated using the Pierce BCA protein assay kit. Briefly, a standard curve was made by serially diluting known concentrations of bovine serum albumin (BSA). 10 µl of all samples (standard curve and unknowns) were loaded in triplicate onto a 96 well plate and the working reagent was added using the sample to reagent ratio 1:20. The plate was placed on a shaker for 30 seconds and incubated at 37 ºC for 30 minutes. The absorbance was read at 570nm using the BioRad Model 680 microplate reader.

2.7.3 Western Blotting

SDS-PAGE (Sodium dodecyl sulfate - polyacrylamide gel electrophoresis)

Polyacrylamide gels were routinely prepared using the Bio-Rad Protean Mini-II series system. Clean glass gel casting plates of 0.75mm spacers were used. First the resolving gel was poured and overlaid with water. Once set, the water was poured off and the stacking gel was overlaid. To assist sample loading, a comb was placed in the stacking gel. The gels were placed in the running tanks containing 1x Tris-glycine running buffer. The respective samples were loaded along with a multicolour broad range protein ladder (Spectra Fermentas Life Sciences) to confirm protein transfer and to assess protein size. Gels were run at 180V. The proteins on the gel were then transferred onto a nitrocellulose membrane. (**Table 2-3**).

Electrophoretic transfer

Hybond C-Extra (Amersham Biosciences) and 2 pieces of Whatman filter paper (VWR) was first soaked in 1x transfer buffer. The gel was disassembled from the glass plates

and then placed on the membrane between the filter paper. Any air bubbles between these layers that might affect the transfer were removed by flattening the assembly. This was laid in a cassette between 2 sponges that aid the assembly to remain wet during the transfer. The cassette was placed in a Bio-Rad Protean II transfer cell system containing 1x transfer buffer and transfer was performed at 100V for 1 hour. (**Table 2-3**).

Immunoblotting

To prevent non-specific binding, the membrane was blocked in 5% (w/v) non-fat dried milk (Marvel) in 1x TBS for an hour at room temperature on an orbital shaker. The membrane was then washed with 1x TTBS for 5 minutes and then incubated with primary antibody solution for 1 hour at room temperature or at 4 ºC overnight with rotation. The antibodies were diluted in 5% (w/v) non-fat dried milk at the required concentration (**Table 2-4**, **Table 2-5** and **Table 2-6**) in 1x TTBS. Following incubation, the membrane was then washed twice with TTBS for 5 minutes to remove excess antibody and was further incubated for 1 hour at room temperature with the appropriate secondary antibody (HRP-tagged, Dako or Cell Signalling). Depending on the species, the secondary antibody was either rabbit-anti-mouse or swine-anti-rabbit. Finally, unbound antibody was removed by washing the membrane twice in TTBS for 10 minutes followed by a wash with TBS for 5 minutes.

Chemiluminescent detection

Luminol-based enhanced chemiluminescence (ECL) HRP substrate (Amersham Biosciences or ThermoScientific) was used to detect the protein of interest according to the manufacturer's instructions. The system uses the principle of oxidation of HRP which catalyses the luminol in the presence of peracid and phenolic enhancers, to emit light. The emitted light signal was captured on X-ray film (Super RX Fuji Medical X-ray Film^{TM}) in a high-speed cassette (Generic Research Instrumentation) and then developed and fixed using Mediphot 937 X-ray film processor (ACI-Colenta International, Cambridgeshire, UK).

Membrane stripping

The membrane was stripped of bound antibody by incubating in stripping buffer (**Table 2-3**) at 50 ºC incubator for 30 minutes with agitation. Membranes were then washed

with TTBS (twice for 10 minutes) before proceeding with immunoblotting. Membranes were re-probed no more than three times.

Technique	Reagent	Constituents
SDS-PAGE	6% stacking gel	6% acrylamide:bis-acrylamide (30% stock Sigma Aldrich) 125mM Tris-HCl, pH 6.8 (Fisher Scientific) 0.1% SDS (Fisher Scientific) 1% (w/v) ammonium persulfate (Sigma Aldrich) 0.2% TEMED (Sigma Aldrich)
	10% resolving gel	10% acrylamide:bis-acrylamide 375mM Tris-HCl, pH 8.8 0.1% SDS 1% (w/v) ammonium persulfate 0.2% TEMED
	5x Tris-Glycine Running buffer	125mM Tris-HCl 950mM Glycine (Fisher Scientific) 0.5% SDS
Western Blotting	10x Transfer buffer	250mM Tris-HCl, 1.5M Glycine 10% Methanol (Fisher Scientific)
	5x TBS (Tris buffered Saline)	100m M Tris-HCl 2.5M NaCl (Fisher Scientific) pH 7.5
	TTBS	0.1% Tween-20 (VWR) in 1x TBS
	Stripping buffer	62.5mM Tris-HCl, pH 6.7 2% SDS 0.78% β-mercaptoethanol

Table 2-3: Routinely used reagents in protein analysis

Antibody Target	Species	Company/Cat No	Ig Type	Concentration	Ab diluent and incubation time
EGFR	Rabbit	Cell Signalling (D38B1)/#4267	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
Phospho-EGFR	Rabbit	Cell Signalling Tyr 1068 (D7A5)/#3777	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
HER2	Rabbit	Cell Signalling (D8F12)/#4290	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
Phospho-HER2	Rabbit	Cell Signalling Tyr 1221/1222(6B12)/#2243	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
HER3	Rabbit	Cell Signalling (D22C5)/#12708	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
Phospho-HER3	Rabbit	Cell Signalling (Tyr1289) (21D3)/# 4791	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
AR	Rabbit	Santa Cruz N-20/ sc-816	Polyclonal	1:1000	5% Marvel Overnight at 4ºC

Table 2-4: List of Antibodies and their conditions used routinely in western blotting

Antibody Target	Species	Company/Cat No	Ig Type	Concentration	Ab diluent and incubation time
Phospho – AR (Ser 81)	Rabbit	Merck Millipore 04-078	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
PARP 1/2	Rabbit	Santa Cruz sc-7150	Polyclonal	1:1000	5% Marvel Overnight at 4ºC
α – tubulin	Mouse	Sigma Aldrich T 9026	Monoclonal	1:2000	5% Marvel Overnight at 4ºC
Histone H4	Rabbit	AbCam Ab10158	Polyclonal	1:1000	5% Marvel Overnight at 4ºC
AKT 1/2	Rabbit	Santa Cruz sc-1619	Polyclonal	1:1000	5% Marvel Overnight at 4ºC
Phospho- AKT 1/2/3 (Ser 473)	Rabbit	Santa Cruz sc-7985	Polyclonal	1:1000	5% Marvel Overnight at 4ºC
ERK 1/2	Mouse	Santa Cruz sc-135900	Monoclonal	1:500	5% Marvel Overnight at 4ºC

Table 2-5: List of Antibodies and their conditions used routinely in western blotting

Antibody Target	Species	Company/Cat No	Ig Type	Concentration	Ab diluent and incubation time
Phospho – ERK1/2	Mouse	Santa Cruz Sc-7383	Monoclonal	1:200	5% Marvel Overnight at 4ºC
ERK5	Rabbit	Cell Signalling #3372S	Polyclonal	1:1000	5% Marvel Overnight at 4ºC
Phospho – ERK5 Thr218/ Tyr220	Rabbit	Cell Signalling #3371S	Monoclonal	1:1000	5% Marvel Overnight at 4ºC
PSA	Rabbit	Dako A0562	Polyclonal	1:1000	1% Marvel 1hr at RT or Overnight at 4ºC

Table 2-6: List of Antibodies and their conditions used routinely in western blotting

2.8 siRNA transfections

siRNAs against the target of interest were obtained from Sigma Aldrich. The oligonucleotides were obtained in duplexes where the antisense strand sequence was generated by the company's software and is based on complementarity to the sense strand (**Table 2-7**). They were diluted in sterile diethylpyrocarbonate (DEPC) treated water to avoid contamination by RNase. Based on the stock concentration, the siRNA was diluted to a final working concentration of 25nM. Lipofectamine RNAiMax (Invitrogen) was used to facilitate the transfections. 3 µl of RNAiMax was used per microgram of siRNA. After determining the amount of siRNA required, a mastermix was made in basal medium and incubated for 30 minutes at room temperature. The cells were reverse transfected (adding siRNA first and then seeding cells slowly over it) in either full or steroid depleted medium. Efficiency of the siRNA was tested by monitoring the protein expression levels before and after transfection.

2.9 Plasmid transfections

Forward transfections were carried by estimating the amount of plasmid required for the experiment. 3 µl of the DNA transfection reagent -TransIT-LT1 Transfection Reagent (Mirus), was added per microgram of plasmid. A master mix was made in basal medium, incubated at room temperature for 30 minutes and then added drop wise onto previously seeded cells.

2.10 Immunofluorescence

Glass coverslips and forceps were sterilised by soaking in 100% ethanol for one hour. The coverslips were then allowed to dry by placing them against each well of a 6-well plate using forceps. Meanwhile, the cells required for the experiment were prepared. Once the coverslips were dry, the plate was slightly nudged to place the coverslips onto the well. The cells were seeded on top of the coverslips and left to attach. The next day, the cells were washed in PBS and either changed to full medium or starved in basal medium overnight. The next day, the cells were treated with Heregulin (20 ng/ml) for 2 hours. The medium was then removed and the cells were washed with PBS following which they were fixed with warm 4% para-formaldehyde (Alfa Aesar) for 10 minutes at

Target	Sequence of sense strand	
Scrambled	5´ UUCUCCGAACGUGUCACGU[dT][dT]	
HER3	HER3-1	5´ AAGAGGAUGUCAACGGUUA[dT][dT]
	HER3-2	5´ GAAGACUGCCAGACAUUGA[dT][dT]
	HER3-3	5´ GCAGUGGAUUCGAGAAGUG[dT][dT]
HER2	HER2-1	5´ GGACGAAUUCUGCACAAUG[dT][dT]
	HER2-2	5´ GACGAAUUCUGCACAAUGG[dT][dT]
	HER2-3	5´ CUACAACACAGACACGUUU[dT][dT]

Table 2-7: siRNA sequences

37 ºC followed by treatment with chilled methanol at -20 ºC for a further 10 minutes for permeabilization. Unspecific binding was prevented by blocking with serum-free protein block (Dako) for 5 minutes followed by primary antibody incubation. 50 µl of antibody (HER2 (sc-33684) 1:300 and HER3 (Cell Signalling #12708) 1:300) was placed on parafilm, kept in a hydrated chamber and the coverslips were inverted onto the antibody and incubated at room temperature for 1 hour. The coverslips were then washed in PBS for 5 minutes (2x) and similarly incubated with 50 µl of 1:500 Alexa Fluor (Invitrogen) secondary antibody for 1 hour at room temperature in the dark to prevent bleaching of the fluorescent probe. The species of the secondary antibody was either anti-mouse (488nm excitation) or anti-rabbit (633nm excitation) The coverslips were then washed in the dark with PBS for 5 minutes (3x) followed by one wash with distilled water for 5 minutes and then mounted onto glass slides using hard-set mountant with DAPI (Vectashield). The sides of the coverslips were sealed with clear nail varnish to prevent excess drying. The slides were then left to set in the dark at 4ºC. The slides were viewed with a confocal fluorescent microscope (Leica TCS SP2 UV laser scanning microscope) under x63 oil immersion using lasers of appropriate wavelengths. Optical sections of 1 µm increments in the Z-axis were taken by scanning across the XY-axis and a Z series was reconstructed from individual images taken throughout the depth of the section.

2.11 Cytoplasmic–nuclear–chromatin extractions

Cytoplasmic-nuclear extractions were carried out using the NE-PER nuclear and cytoplasmic extraction kit (ThermoScientific). $1x10^6$ cells were seeded on 90mm petri dishes. After 24 hours, the medium was changed on respective samples to basal medium after a wash with PBS. Depending on the experiment, the cells were treated with or without appropriate inhibitory compounds overnight. The next morning, the cells were treated with Heregulin (20ng/ml) for 30 and 120 minutes. The cells were then pelleted down in either full medium or steroid depleted medium and they were then used to extract cytoplasmic and nuclear fractions following the protocol provided. 80X protease and phosphatase inhibitors stocks were made and appropriate amounts to get a final concentration of 1X were added to the extraction buffers to keep the proteins and their modifications from degrading. The pellets left behind were taken as the chromatin fractions and were dissolved in 50 µl of SDS (sodium dodecyl sulphate) sample buffer (125 mM Tris-HCl pH 6.8; 2% SDS; 10% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) and were then subjected to a 5 minutes sonication programme using a Bioruptor (Diagenode) which incorporated cycles of 30 seconds on and 30 seconds off at the 'high' setting. The extracts were probed for α-tubulin (cytoplasm), PARP (nucleus) and histone H4 (chromatin) to ensure equal loading of the samples and purity of the extracts.

2.12 Plasmid vectors

pIRES-Hyg, pIRES-Hyg-HER2, pIRES-Hyg-HER3 plasmids (Antibiotic selection-Hygromycin) were a kind gift from Dr. Maurizio Scaltriti (Memorial Sloan-Kettering Cancer Center, New York) (Scaltriti *et al.*, 2007) and were used for transfections and as a template for the generation of lentiviral particles.

MMP9-Luciferase construct (Antibiotic selection - Ampicillin) was obtained as a kind gift from Dr. D. Boyd (MD Anderson Cancer Centre, USA). The constructs contain 5′ -flanking fragments upstream to the transcription initiation start site that is linked to a luciferase reporter (Gum *et al.*, 1996).

pFLAG-CMV-AR (Wild type; Antibiotic selection - Ampicillin) was a kind gift from Ralf Janknecht (Oklahoma University, Rochester, MN).(Gaughan *et al.*, 2011)

The pCMV (Antibiotic selection - Ampicillin) empty vector was used as a control for transfections and to ensure equal final amount of DNA used throughout the experiment carried out.(Gaughan *et al.*, 2011)

pCMV-β-gal (Antibiotic selection - Zeocin) was used as a control to normalise transfection efficiency.(Gaughan *et al.*, 2011)

2.13 Bacterial transformation and plasmid preparation

NEB 5α *E.coli* competent cells (New England Biolabs) were used for routine plasmid transformations. The cells were thawed on ice after recovering from storage at -80 ºC. 1 µl of plasmid (maximum concentration of 100 ng/µl) was then added to 25 µl of competent cells and mixed gently by pipetting followed by incubation on ice for 30 minutes. The cells were then subjected to a heat shock at 42 ºC for 45 seconds using a water bath and immediately placed on ice for 2 minutes. 1ml of pre-warmed Luria-Bertani (LB) media (10g/L tryptone, 5g/L yeast extract (Oxoid), 10g/L NaCl, pH 7.0) was then added to the cells and placed on a 37 ºC shaker for 1 hour at 200rpm. Cells were then spread out on LB agar plates (15g/L agar,10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, 20g/L pH 7.0) at different dilutions (10-100 µl and a concentrated sample after spinning the cells down on the highest speed for 1 minute) which contained the respective antibiotic for selection (Ampicillin 100 µg/ml, Zeocin 100 µg/ Kanamycin, 50 µg/ml). The plates were incubated at 37 ºC overnight.

The next day, the plasmid was prepared following instructions from the miniprep or maxiprep kit (Sigma Aldrich) for respective small scale and large scale preparations. Stocks of the culture were made by mixing equal volumes of bacterial culture and sterile glycerol in a cryovial and stored at -80 ºC.

2.14 Generation of stable over-expressing cell lines

2.14.1 Lentivirus production

The production of lentivirus was made by using the ViraPower lentiviral expression kit (Life Technologies) by following the manufacturer's guidelines.

Briefly, the full length gene of HER2 and HER3 was amplified from the pIRES-Hyg-HER2, pIRES-Hyg-HER3 plasmids respectively. The primers were designed so that the forward primer contained a 'CACC' overhang before the transcription initiation site to permit directional cloning into the pENTR/ D- TOPO vector and the reverse primer was stopped just before the stop codon to ensure that the insert was in-frame with the V5 epitope following recombination into the pLenti6/V5 DEST vector. (Note: The last amino acid of HER2 was changed to incorporate a methionine (ATG) instead of valine (GTG) and the second amino acid of HER3 was changed to glycine (GGG) instead of arginine (AGG). These changes were necessary to minimise the chance of the PCR product cloning in the opposite direction) (**Table 2-8**). The quality of the primers was checked using the online programme – PCR Primer Stats – Bioinformatics.Org. The PCR reaction was done by making a reaction mix as mentioned in **Table 2-9**. A negative control with water as template was used for each gene. The contents were mixed briefly and the PCR was carried out using the conditions mentioned in **Table 2-10** . The PCR product was run on an agarose gel to confirm the right product size.

Gene	Forward Primer	Reverse Primer
HER2	5' **CACC**ACCATGGAGCTGGCGG 3'	5'CA**T**TGGCACGTCCAGACC 3'
HER3	5' **CACC**GTCATG**G**GGGCGAA 3'	5'CGTTCTCTGGGCATTA 3'

Table 2-8: Primer sequences used for HER2 and HER3 amplification

Reagent	Volume
10x PFX reaction mix	5µl
Forward primer (10 µM)	1.5 µl
Reverse primer (10 µM)	1.5 µl
Template DNA (100ng)	1 µl
Accuprime Pfx DNA Polymerase	1 µl
Sterile water	40 µl

Table 2-9: PCR reaction mix

Cycle	Stage	Cycles	Temp	Time
Initial Denaturation	1	1	95 ºC	2 min
Denaturation	2	30	95 ºC	15 sec
Annealing			55 ºC	30 sec
Extension			68 ºC	4min 10sec (1min/kb insert)
Final extension	3	1	68 ºC	5 min
Storage	4		4 ºC	

Table 2-10: PCR conditions

Agarose gel electrophoresis

1% agarose gel was made in TAE buffer (40mM Tris-HCl, 20mM acetic acid and 1mM EDTA). The sample was mixed with equal amounts of DNA loading dye and once loaded alongside a ladder, they were run at 100V in TAE buffer till the dye front reached two-thirds the length of the gel. The gel was stained with SYBR® Safe DNA gel stain (Life Technologies) and imaged under UV (Ultraviolet) light using a Gel Documentation System (BioRad).

The manufacturer's guidelines were followed and the PCR product was ligated to pENTR/ D-TOPO. The plasmid was then transformed into competent cells as mentioned in (**2.13**). Resistant colonies were selected using kanamycin, validated by agarose gel electrophoresis and Sanger sequencing using M13 forward and reverse

primers with an additional 4 primers per gene making sure the correct sequence of the gene was incorporated in the right direction.

The HER2 and HER3 genes were transferred to the pLenti6/V5 DEST vector by allowing homologous recombination by combining the pENTR/ D-TOPO with the respective gene insert according to the manufacturer's guidelines. The recombined plasmid was then transformed into Stbl3 competent *E.coli* cells as mentioned in (**2.13**). Resistant colonies were selected using ampicillin, validated by restriction digestion, agarose gel electrophoresis and Sanger sequencing using CMV forward and V5 reverse primers with an additional 4 primers per gene making sure the correct sequence of the gene was incorporated in the right direction.

The viral particles were then produced in 293FT cells following the manufacturer's guidelines by transfecting the verified pLenti6 clone along with the ViraPower packaging mix which contained the pLP1, pLP2, pLP/VSVG plasmids in an optimized mixture to facilitate viral packaging. The control pLenti6 plasmid containing the LACZ gene was provided in the kit and was also used to produce viral particles which was further used to produce a LACZ control cell line. The protocol was followed after which the medium containing the virus was centrifuged at 500g for 15 minutes to pellet cells and debris. The supernatant was filtered using a Millex-HV 0.45µm PVDF filter. The viral particles were pelleted by ultracentrifugation at 20,000g for 4 hours at 4 ºC. The viral pellet was then re-suspended in 1ml of basal medium and 100 µl aliquots were stored at -80 ºC.

The virus was then titrated on PC3 cells by seeding out the cells in a 6-well plate at 30% confluency. The following day, the viral stock was serially diluted in range from 10^{-2} and 10^{-6} in a final volume of 1ml. After 24 hours, the medium was removed and replaced with fresh selection medium which contained Blasticidin (10µg/ml – predetermined concentration). The cells were cultured for 14 days replacing the selection medium every 3 days. The colonies formed were fixed with Carnoy's fixative and stained with 1% crystal violet. The excess stain was removed by washing with tap water and the plates were allowed to air dry and the colonies were counted. The viral titre was determined using the following formula.

$$Viral\ titre = mean\ colony\ number \times \frac{1}{dilution\ factor}$$

Using a multiplicity of infection (MOI) of 0.001 for HER2, HER3 and LACZ, PC3 cells were transduced with the respective virus and grown in selection medium to establish the stably over-expressing cell lines. Western blotting was performed to validate the over-expression of HER2 and HER3. The cell lines were continuously maintained in selection medium.

2.15 *In-Vivo* experiments

Athymic CD1 nude mice (male; 6 weeks old) were purchased from Harlan UK and maintained under aseptic conditions with the use of a laminar air-flow unit. Experiments were carried out under a Home Office License alongside the UKCCCR guidelines and were approved and reviewed by the Local Animal Welfare Committee. The mice were given tap water and fed with a pelleted diet (SDS Ltd, R & M No.3) *ad libatum*.

2.15.1 Xenografts

A total of 1.1 x 10^7 cells of PC3-HER2, -HER3 and -LACZ in full medium were subcutaneously injected into the flanks of nude mice in a final volume of 50 µl (10 mice per cell line). The mice were inspected thereafter for signs of tumour development. The tumour sizes were measured 3 times a week with callipers and recorded. When the tumour size reached 10 x 10 mm or > 15 mm in one dimension, the animals were culled and the tumours were excised into full medium containing antibiotics (Penicillin 100U/ml/Streptomycin 100µg/ml; Sigma Aldrich). The final tumour size was calculated by using the formula

$$\frac{length}{2} * (width)^2$$

The tumours were then divided into 3 parts under sterile conditions and the tumour was stored in - 10% formalin (Fisher Scientific) for subsequent histological analysis, liquid nitrogen for protein analysis and the final third of the sample was washed in PBS and cut up into small pieces with the use of sterile scissors and forceps. The pieces were then transferred into an Erlenmeyer flask containing 10 ml of filter sterilised full medium which contained antibiotics and collagenase type I (200IU/ml; 6 mg in 10 ml; Worthington Biochemical Corporation). The tissue was digested overnight at 37 ºC on an orbital shaker at low speed. The next morning, the mixture was pipetted repeatedly

with sequentially smaller pipette tips and finally taken though a needle and syringe until a broth-like mixture was produced. The cells were spun down at 400g for 5 minutes. The supernatant was removed and the pellet was washed twice with PBS. The pellet was then re-suspended in 10ml of full medium containing antibiotics and was centrifuged at 1000 rpm for 1 minute. The epithelial cell pellet was then re-suspended in full medium containing antibiotics and cultured back as cell lines. Blasticidin was included in the medium to avoid growth of any mouse cells.

2.16 Proliferation assays

2.16.1 Colony formation assay

1000-2000 cells/well were seeded onto a 6-well plate depending on the cell line and treated accordingly the next day depending on the experiment. The cells were allowed to form colonies over a period of 14 days after which the medium was removed and the cells were washed with PBS. The cells were fixed with Carnoy's fixative (3:1 Methanol: Acetic acid) for 1 minute and were allowed to dry. The colonies were then stained with 1% crystal violet and counted.

2.16.2 Sulforhodamine B (SRB) assay

This assay was performed on 96-well plates where 1000-2000 cells were seeded per well depending on the cell line in 90µl of normal growth medium using an Eppendorf repeater stream pipette. After 24 hours, the cells were treated with 10µl of 10x drug (final concentration 1x in total final volume of 100 µl) or DMSO or left untreated depending on the nature of the experiment. After 96 hours, the cells were fixed by adding 25µl of 50% ice cold trichloroacetic acid (TCA) for 1 hour at 4 ºC. The cells were washed with tap water, dried and 100µl of 0.4% SRB (w/v in 1% acetic acid) was added per well and left to stain the cells for 30 minutes at room temperature. The cells were then washed with 1% acetic acid to remove excess stain. After drying, the bound stain was dissolved by adding 100µl of 10mM Tris (pH 10.8) per well and placing on an orbital shaker for 30 minutes at room temperature. The absorbance was then measured at 570nm using the BioRad Model 680 microplate reader.

2.16.3 Cell counts

Cells were seeded at the required density in a 6-well plate and treated with appropriate

drugs depending on the experiment. After 96 hours, the cells were pelleted as mentioned in (**2.6**) and the pellet was re-suspended in 1ml of medium. The number of cells in each sample was counted and compared to the control sample.

2.17 Cell migration and invasion assays

The cell motility and metastatic potential of PC3 cells over-expressing either HER2 or HER3 were tested using the Boyden chambers. The inserts were made up of PET (polyethylene terephthalate) membrane with a membrane pore size of 8µm. The inserts were coated with matrigel (composed of extracellular matrix proteins - laminin, collagen IV, heparan sulfate proteo-glycan and entactin) for invasion (Corning – Cat No. 354480) whereas the uncoated inserts were used to measure migration (Corning – Cat No. 353097). The experiment was carried out by placing the inserts in a 24-well culture plate. The invasion inserts were pre-treated with basal media prior to the experiment according to manufacturer instructions. 600µl of basal media with Heregulin (20ng/ml) with or without drug/DMSO was added per well and the inserts were placed on top. Depending on the experiment, the cells were not treated, pre-treated with drug or control in basal media for 16 hours. The cells were then collected in SDM and re-suspended in basal media with or without drug/DMSO. $3x10^4$ cells were seeded in a final volume of 300µl in the upper chamber. The cells were allowed to migrate for 48 hours at 37 ºC and 5% CO_2. The cells that hadn't migrated or invaded were removed from the upper part of the chamber by wiping the inside of the insert with a cotton bud. The membrane was then fixed with methanol for 30 minutes at -20 ºC, stained with 1% crystal violet and the excess stain was removed with tap water. The inserts were then allowed to air dry and the membrane was carefully removed from the insert with the help of a scalpel. The membrane was then mounted on glass slides (bottom side upwards) with the help of DPX mountant. The number of cells was counted using a light microscope at 20x magnification. The average number of cells from 8 random views per membrane was considered. Each arm of the experiment was tested in triplicate and the entire experiment was carried out three times.

2.18 Luciferase assay

When LNCaP-7B7 cells were used, the cells were seeded out in quadruplets per experimental arm using a 24-well plate. The next day the cells were either starved in

SDM for 3 days. For the HER2-3 signalling inhibition, the cells were either treated with DMSO, AZD8931 or Enzalutamide (as inhibition control for AR activity). Once the experiment was completed, the cells were washed with PBS and lysed using 50 µl of 1x reporter lysis buffer (Promega) /well. 10 µl of lysate from each well was transferred onto an opaque flat-bottomed 96-well plate. The plate was then placed in the automated FLUOstar Omega (BMG Labtech) plate reader where 50 µl of Steady-Glo luciferase substrate (Promega) was injected per well, shaken for 5 seconds and the emitted luciferase counts per second was recorded. The data was then normalised to the protein concentration per well by performing a BCA assay on the samples. The average values were then used to interpret the results.

When PC3 cells were used, the cells were seeded out in quadruplets per experimental arm using a 24-well plate and were forward transfected (transfection after the cells have adhered) with the appropriate plasmids required for the experiment (HER2, HER3, MMP9, pCMV and β-gal). The experiment was carried out accordingly and once completed, the cells were lysed in 1x reporter lysis buffer and luciferase activity was recorded as explained above. The results were normalised to the results of a β-galactosidase assay which would reveal transfection efficiency. Briefly, 10 µl of lysate from each well was transferred onto a 96-well plate and equal amount of β-gal assay substrate (2mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG) and 100 mM sodium phosphate buffer, pH 7.3) was mixed and incubated at 37 ºC for 5 minutes. ONPG (substrate of β-galactosidase) is cleaved to galactose and o-nitrophenol to yield a pale yellow colour. The reaction was then stopped by adding 50 µl of 1M Na_2CO_3 and the absorbance was read at 415 nm using the BioRad Model 680 microplate reader. The average values were then used to interpret the results.

Chapter 3 Aberrant Expression of HER2 and HER3 in Human Prostate Cancer

3.1 Introduction

Early detection and diagnosis of prostate cancer have been very difficult due to the lack of clinical symptoms during disease development. Prostate cancer detection is traditionally achieved by performing digital rectal examination, PSA screening and histopathological analysis (Venderbos and Roobol, 2011). Due to the heterogeneity of the disease, the aggressiveness of the disease can vary from patient to patient. The use of biomarkers has helped with the detection and monitoring of the disease. Prostate cancer biomarkers can be classed into different categories; namely diagnostic, prognostic, predictive, and pharmacodynamic markers respectively. Diagnostic markers help to differentiate between benign and cancerous cell types and the advancement of the disease can be evaluated by prognostic markers. As the name suggests, predictive biomarkers assess the patient's response to the given treatment regiment. Pharmacodynamic biomarkers help to conclude an appropriate dosage of anticancer drug in the initial stages of therapy (Madu and Lu, 2010; Esfahani *et al.*, 2015).

The most common diagnostic, prognostic and predictive protein marker used in prostate cancer detection is prostate specific antigen (PSA). PSA screening has been a popular technique in the United States of America, as it is a non-invasive procedure and since its use, there has been a decrease in the number of metastatic patients at diagnosis (Cary and Cooperberg, 2013). Although being a prostate specific protein marker, PSA is not cancer specific. Elevated PSA levels have been detected in patients with benign prostatic hyperplasia leading to false positive tests. The rather poor specificity and sensitivity of PSA to cancer have led to needless biopsies and wrong/over diagnosis along with overtreatment of dormant disease (Nogueira *et al.*, 2010). Histopathological analysis helps to assess the aggressiveness of the cancer by assigning a Gleason grade to the cancer. The disease progression however differs between patients. The disease may be inactive or slow progressing, having no significant effect on mortality in some patients, while other patients may possess an extremely aggressive form of the disease leading to tumour metastasis which plays a substantial role in cancer death (Siddiqui *et al.*, 2004; Bickers and Aukim-Hastie, 2009).The limitations of the current biomarkers of

prostate cancer have created a sense of urgency and necessity for novel, reliable and specific molecular biomarkers that can enhance the detection and clinical monitoring of the disease. The current focus in biomarker research involves looking into deregulated signalling pathways in the cancer and identifying proteins that could be quantifiable. Such proteins and their regulated pathways can also serve as novel therapeutic targets along with their use of being diagnostic, prognostic and predictive markers of the disease. This kind of clinical screening will be additionally effective, as it will facilitate in providing the patients with appropriate and beneficial therapy, based on their individual molecular profiling.

As explained in the introductory chapter, the deregulation of the PI3 Kinase and MAP Kinase signalling pathways due to the over-expression of EGFR, HER2 and HER3 has been linked to the progression of aggressive prostate cancer. Previous research has linked the over-expression of EGFR and HER2 to poor prognostic disease. However, the use of EGFR and HER2 inhibitors in prostate cancer has not shown any promise and suggests that HER3 could be compensating for the inhibition of EGFR and HER2 (Chen *et al.*, 2011). As HER3 is a kinase dead receptor, it requires a dimerization partner to carry out its activity. The present study concentrates on the role of HER2-HER3 heterodimer in prostate cancer, as it the most potent heterodimer when receptor phosphorylation and downstream signalling activity is considered (Jathal *et al.*, 2011).

This chapter focusses on analysing the expression of HER2 and HER3 in clinical samples by performing immunohistochemistry on "in-house" constructed tissue microarrays (TMAs). These TMAs consist of patient biopsy samples collected over the years and serve as valuable tools to investigate protein expression during various stages of prostate cancer. The cohort also consists of matched paired biopsy samples that are taken from the same patients during different points after their initial diagnosis and helps in comparing the protein expression alterations, pre- and post-therapy. There is limited available literature on using matched clinical samples and profiling protein expression changes. The recorded clinic-pathological data has been used to correlate the changes in protein expression to disease progression and patient survival. The expression of downstream signalling proteins and other prognostic markers in the clinical samples were also analysed. The chapter concludes by also observing the protein expression in prostate cancer cell line models that are routinely used *in-vitro*.

3.2 HER2 and HER3 show elevated expression in advanced prostate cancer and their expression can be associated with poor prognostic disease - Analysis of TMA1

To evaluate the role of HER2 and HER3 in human prostate cancer, the expression levels of HER2 and HER3 in cancer samples was initially examined. This was done by performing immunohistochemistry on TMAs. The first TMA consisted of a collection of prostate cancer samples across a range of cancer stages taken from patients at the time of diagnosis. The results thus indicate the expression levels of the receptors prior to any anti-cancer treatment. BPH samples were also included in the TMA for comparison. **Figure 3-1** is a representative picture of the sample cores of the TMA after performing immunohistochemistry. Once stained, the TMA was then scored for nuclear and cytoplasmic staining using the histoscore method (**2.2.4**). Briefly, the method considers the intensity of staining and the percentage of cells with the same intensity of staining across the sample. The score is calculated by using the formula:

$$Histoscore = (1 \text{ x } \% \textit{ cells with weak staining}) + (2 \text{ x } \% \textit{ cells with moderate staining}) + (3 \text{ x } \% \textit{ cells with strong staining})$$

This method provides a maximum score of 300 (100% cells with strong staining) and a minimum of 0 (100% cells with no staining), which makes the data quantifiable. These scores were then used with the available patient clinic-pathological data to infer the results.

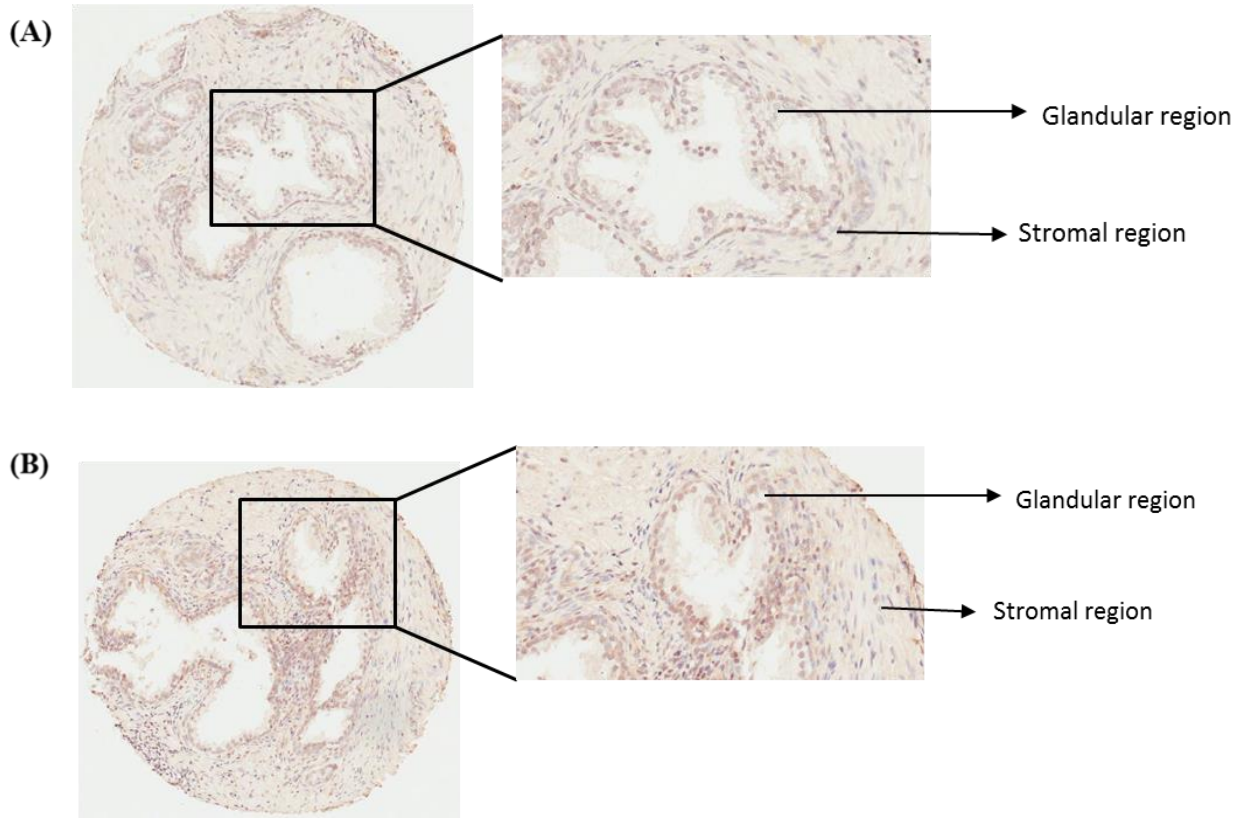

Figure 3-1: TMA cores showing cytoplasmic and nuclear staining for HER2 and HER3 by immunohistochemistry

Briefly, the samples were deparaffinised and rehydrated. Antigen retrieval was performed in 0.01M sodium citrate buffer pH-6.0 using a decloaker. Endogenous peroxide activity was blocked by treating the samples with hydrogen peroxide. The samples were then incubated with HER2 and HER3 antibodies at 1:300 dilutions overnight at 4ºC. (Concentration validated by performing IHC on test TMA). The Menapath kit was used to complete the procedure. A positive (prostate cancer) and negative control (secondary antibody only) was included in the experiment.The stained slides were then scanned using the Aperio Scanscope CS system (Spectra Healthcare). The images were stored in a secure database. The samples were then scored as having low expression (0-100), moderate (100-200) and strong (200-300).(A,B) Representative BPH samples stained for HER3 showing weak diffused cytoplasmic and moderate nuclear staining. The stromal regions as indicated in the picture was not included in the scoring process. Cancer samples (C,D,E and F) were similarly scored for low, moderate and strong cytoplasmic and nuclear expression.

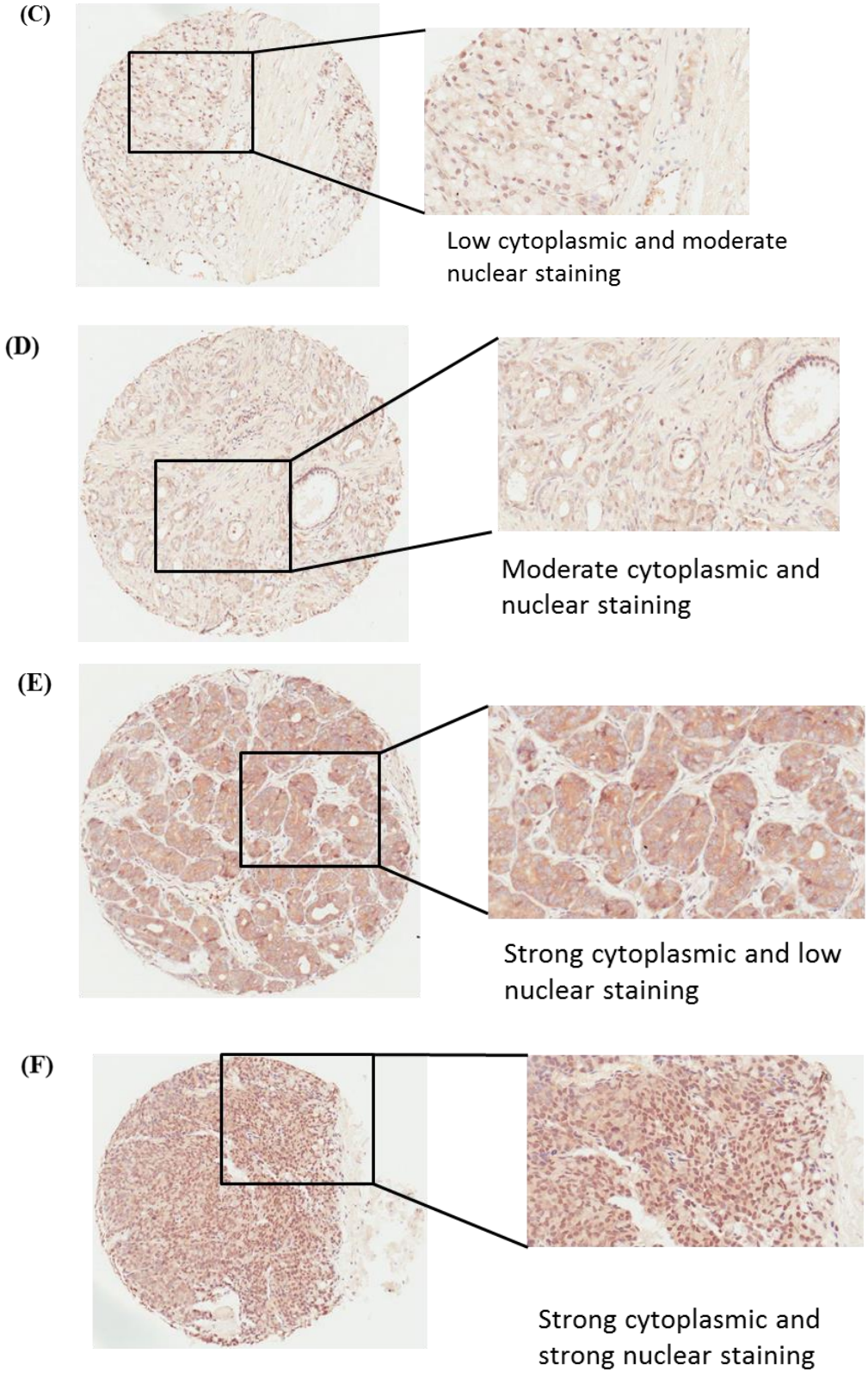

Figure 3-1: TMA cores showing cytoplasmic and nuclear staining for HER2 and HER3 by immunohistochemistry

Cancer samples (C,D,E and F) showing low, moderate and strong nuclear and diffused cytoplasmic expression as indicated.

3.2.1 Cytoplasmic and nuclear expression of HER2 and HER3 is significantly elavated in cancer samples when compared to benign samples

The differences in expression between benign hyperplasia and cancer samples was compared. It was observed that both cytoplasmic and nuclear expression of HER2 and HER3 was increased significantly in patients with cancer and not in those with benign prostatic hyperplasia. It should be noted that there was no nuclear HER2 expression in patients who presented with benign disease (**Figure 3-2**).

3.2.2 Cytoplasmic expression of HER3 is elavated with the grade of prostate cancer

Further comparisons were made across different Gleason grades to observe any differential expressions of the receptors in the cellular compartments. The Gleason single grades of the samples present on the TMA were considered for the comparison. An increase in cytoplasmic HER3 expression, but not nuclear, was revealed with increase in Gleason grade. No change in expression was observed across cytoplasmic and nuclear HER2 (**Figure 3-3**).

3.2.3 Cytoplasmic expression of HER2 and HER3 and nuclear expression of HER2 is stronger with cancer stage

The next comparison made was between the stages of the cancer, i.e. if the cancer was organ confined or locally invasive at diagnosis. **Figure 3-4** shows the observed increase in both HER2 and HER3 cytoplasmic expression when the cancer was known to be locally invasive. There was also an increase in HER2 nuclear expression when the disease was found to be locally invasive.

3.2.4 Cytoplasmic expression of HER2 is higher in patients who subsequently developed metastases after diagnosis

The samples from the patients were collected by transurethral resection of the prostate (TURP). These samples, when obtained, were analysed to see if they were metastatic or not. Analysis of the patient records revealed that many patients developed metastases post diagnosis. **Figure 3-5** and **Figure 3-6** show the comparison of receptor expression between the cellular compartments upon attaining a metastatic condition. It was observed that HER2 cytoplasmic expression was higher when patients developed subsequent metastases. However, no variation in receptor expression was observed in

the nuclear compartment with metastases at diagnosis or in patients who subsequently developed metastases.

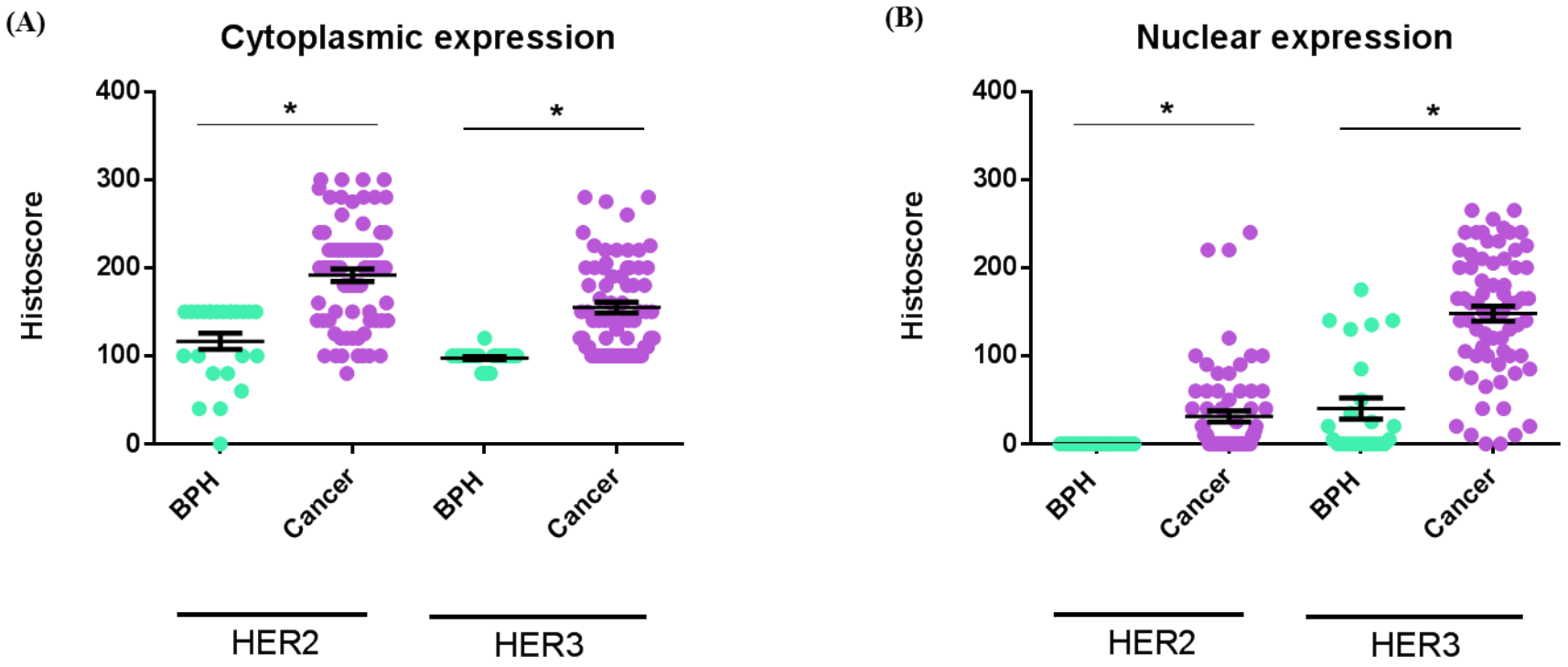

Figure 3-2: Cytoplasmic and nuclear expression of HER2 and HER3 is significantly higher in cancer patients when compared to those with BPH

This figure shows the significantly higher expression of both cytoplasmic (A) and nuclear (B) of HER2 and HER3 in cancer samples. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the BPH samples.

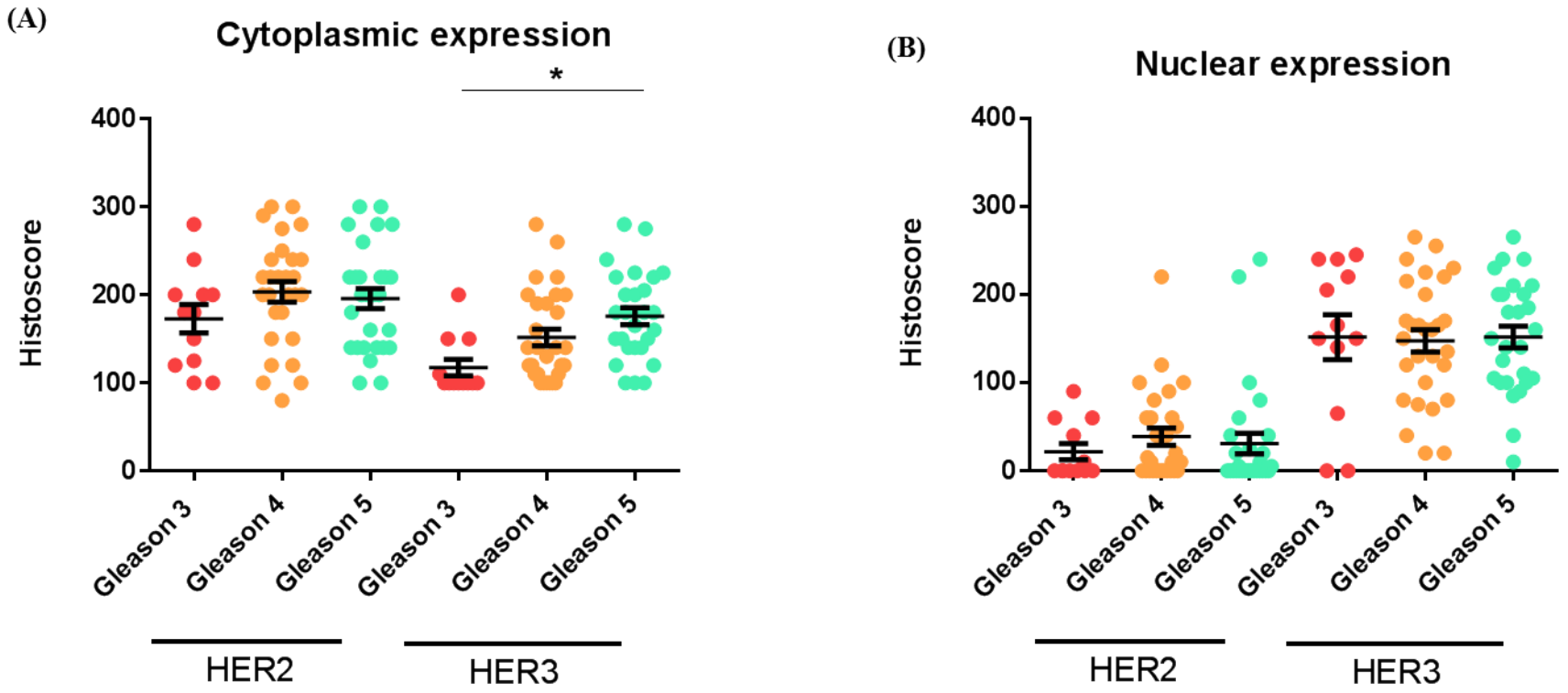

Figure 3-3: Cytoplasmic expression of HER3 is significantly higher with the grade of prostate cancer

This figure shows the significantly higher expression of cytoplasmic (A) HER3 with Gleason grade of prostate cancer. However, there was no significant change in HER2 cytoplasmic expression or nuclear expression of both HER2 and HER3 (B). One-way ANOVA was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to Gleason 3.

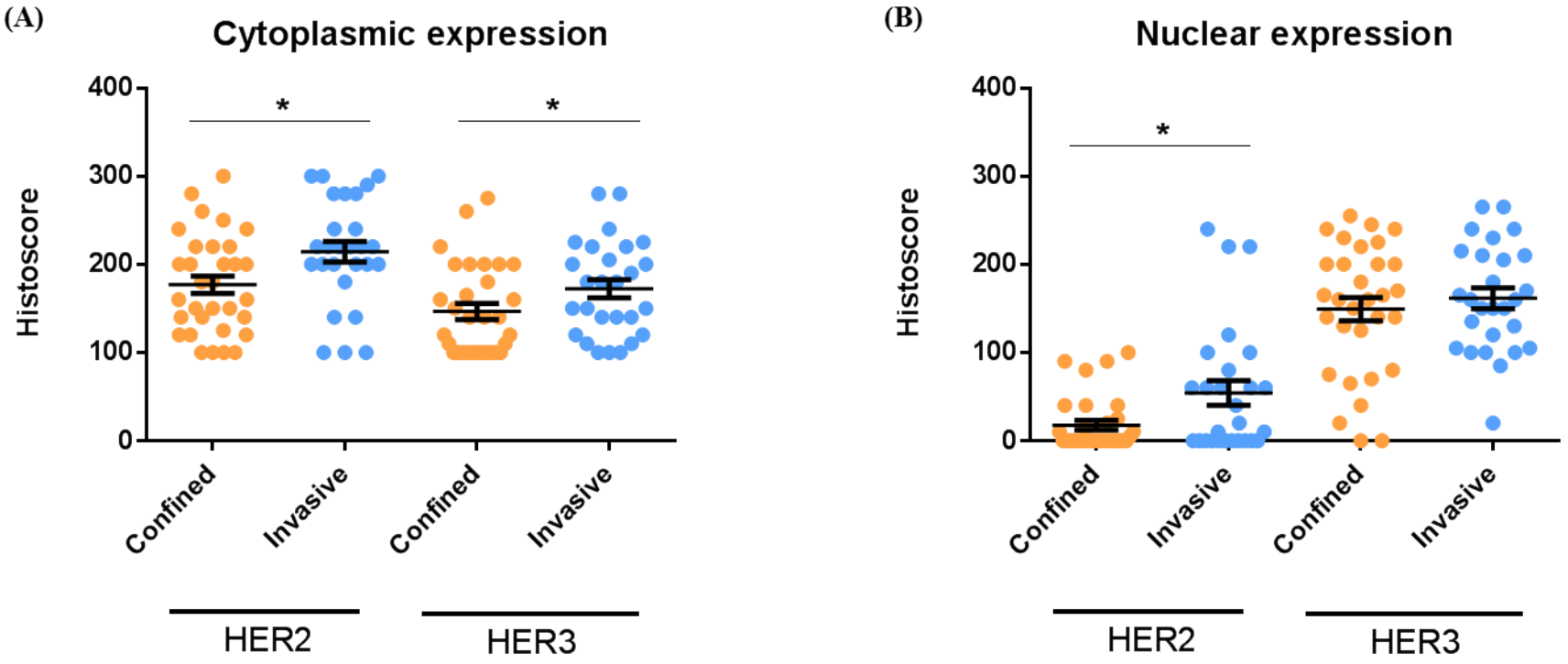

Figure 3-4: Cytoplasmic expression of HER2 and HER3 is higher with cancer stage

This figure shows the significantly higher expression of cytoplasmic (A) HER2 and HER3 with cancer stage. There were also a significant changes in HER2 nuclear expression (B). Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to organ confined disease.

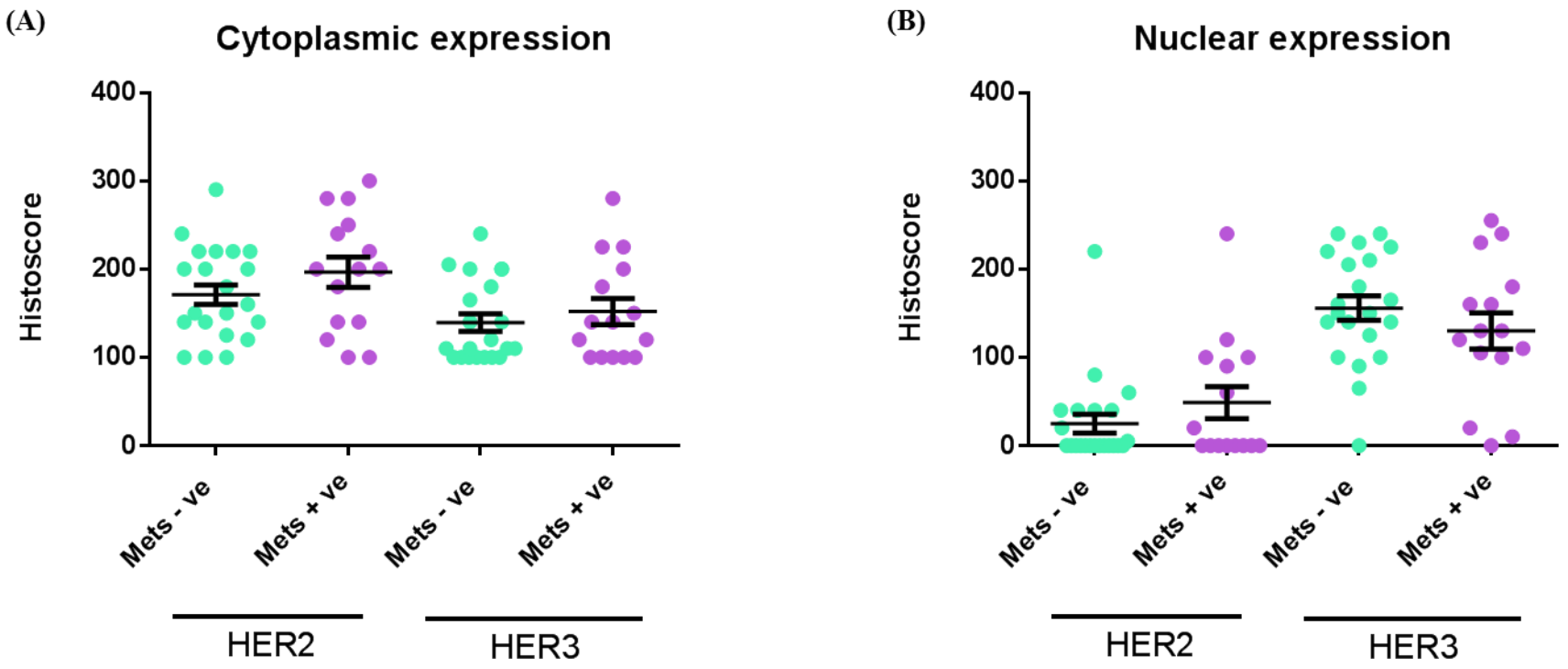

Figure 3-5: Comparison of cytoplasmic and nuclear expression of HER2 and HER3 upon metastases on diagnosis

This figure shows no significant increase of HER2 and HER3 in either of the cellular compartments when the cancer is metastatic at diagnosis. Student t-test was performed on the experiments to test for statistical significance.

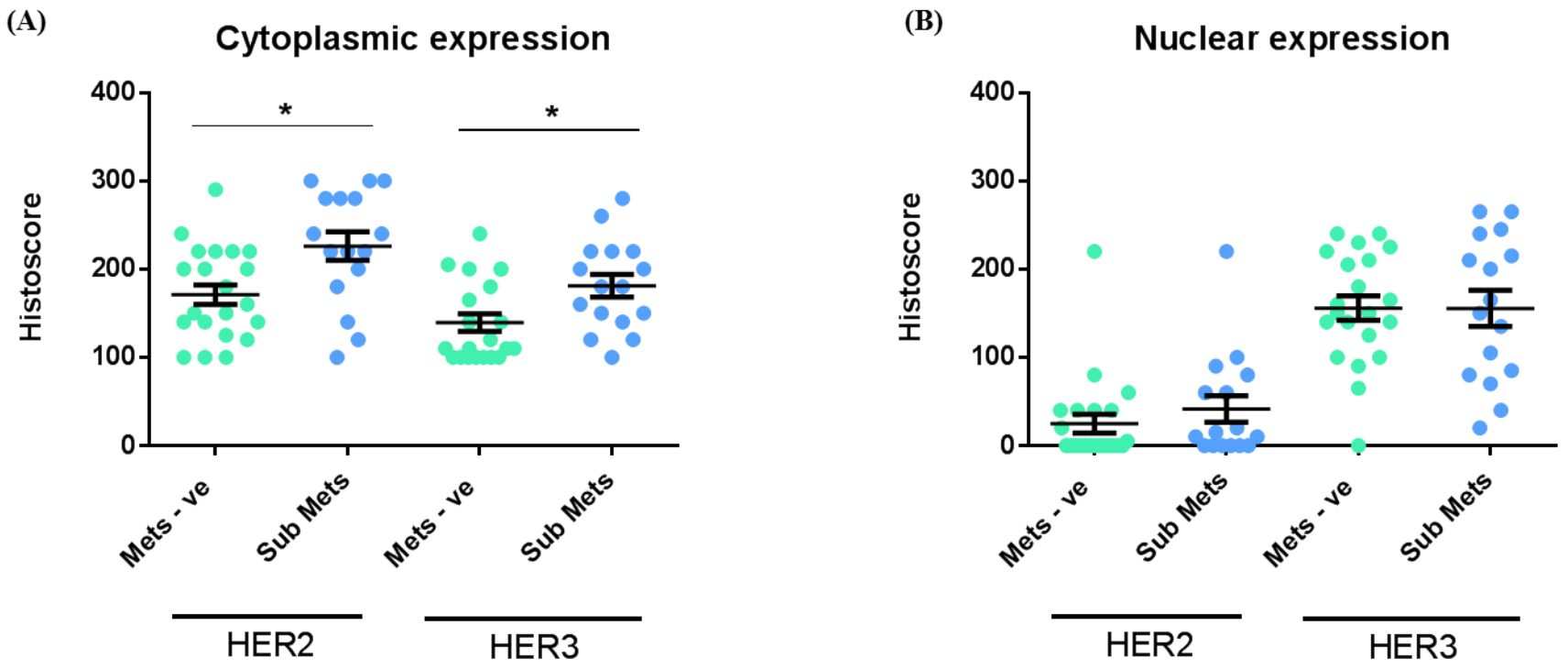

Figure 3-6: Cytoplasmic expression of HER2 is significantly higher in patients who subsequently develop metastases after diagnosis

This figure shows the significantly higher expression of cytoplasmic HER2 (A) in patients who subsequently develop metastasis after diagnosis. However, there was no significant change in HER3 cytoplasmic expression or nuclear expression of both HER2 and HER3 (B). Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to metastases –ve samples.

3.2.5 Strong HER2 and HER3 cytoplasmic expression correlates with poor patient survival

Further comparisons were made to examine the effect of receptor expression on patient survival. **Figure 3-7** and **Figure 3-8** show the correlation of cytoplasmic HER2 and HER3 expression on patient survival. The expression levels were categorised into 3 groups –low, moderate and strong expression. Kaplan–Meier curves were plotted using the available patient survival data against the different receptor expression groups. Comparisons were also made by combining the low and moderate expression groups and significant differences were observed.

3.2.6 Strong HER2 nuclear expression correlates with poor patient survival

Similar comparisons were made for nuclear expression of HER2 and HER3. Only HER2 nuclear expression demonstrated a significant correlation with patient survival (**Figure 3-9** and **Figure 3-10**).

3.3 Continued analysis with additional clinical samples – combined analysis of TMA1 and TMA4

It was noticed that certain groups in the analysis had very low patient numbers. A second TMA –"TMA4", was added to the analysis to increase the sample size. Similar comparisons were made and **Figure 3-11** shows that there is still a significant elevated expression of cytoplasmic and nuclear expression of HER2 and HER3 in cancer patients when compared to those with BPH. However, all the other comparisons showed no difference in cytoplasmic or nuclear expression of the receptors (**Figure 3-12** and **Figure 3-13**). It should be noted that there was an overall higher expression of nuclear HER3 when compared to HER2, even though there were no significant differences with cancer grade and presence of metastases.

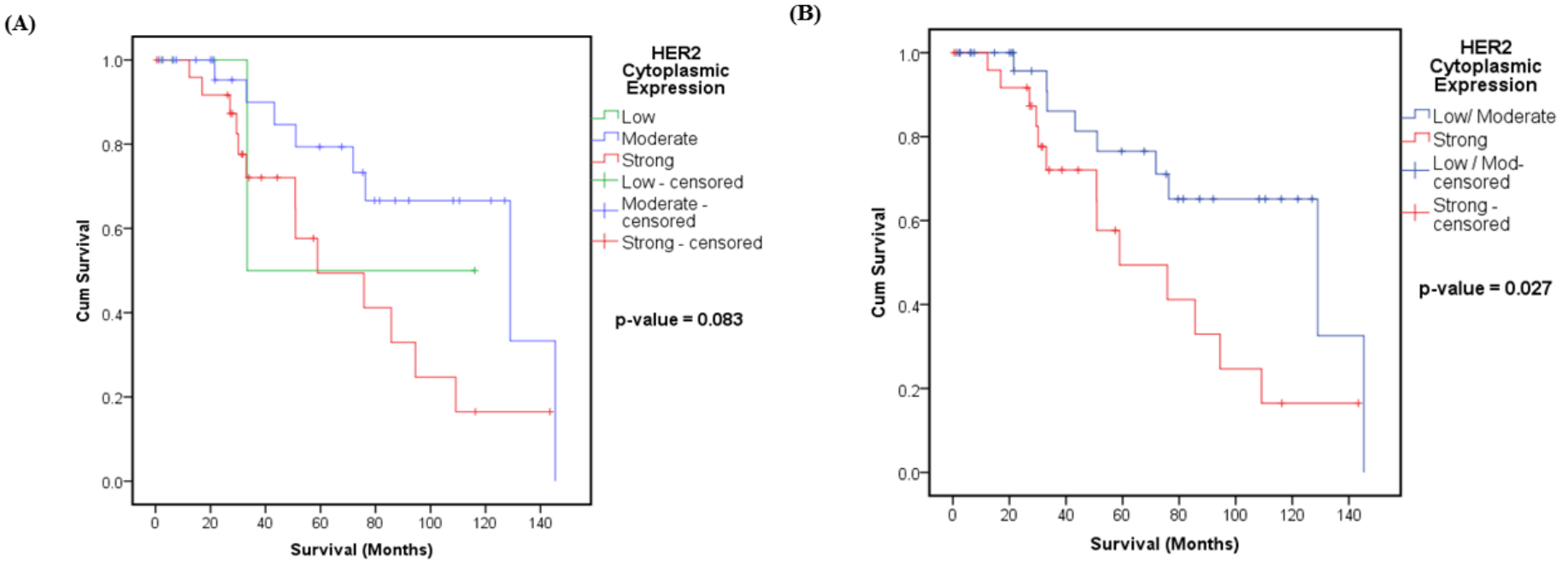

Figure 3-7: Kaplan–Meier curves showing an inverse correlation of HER2 cytoplasmic expression on patient survival

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER2 cytoplasmic expression on survival (A). Due to low patient number in the low expression group, a further comparison was made between combined low and moderate expression vs strong expression (B). This comparison shows a significant correlation of HER2 strong cytoplasmic expression on patient survival.

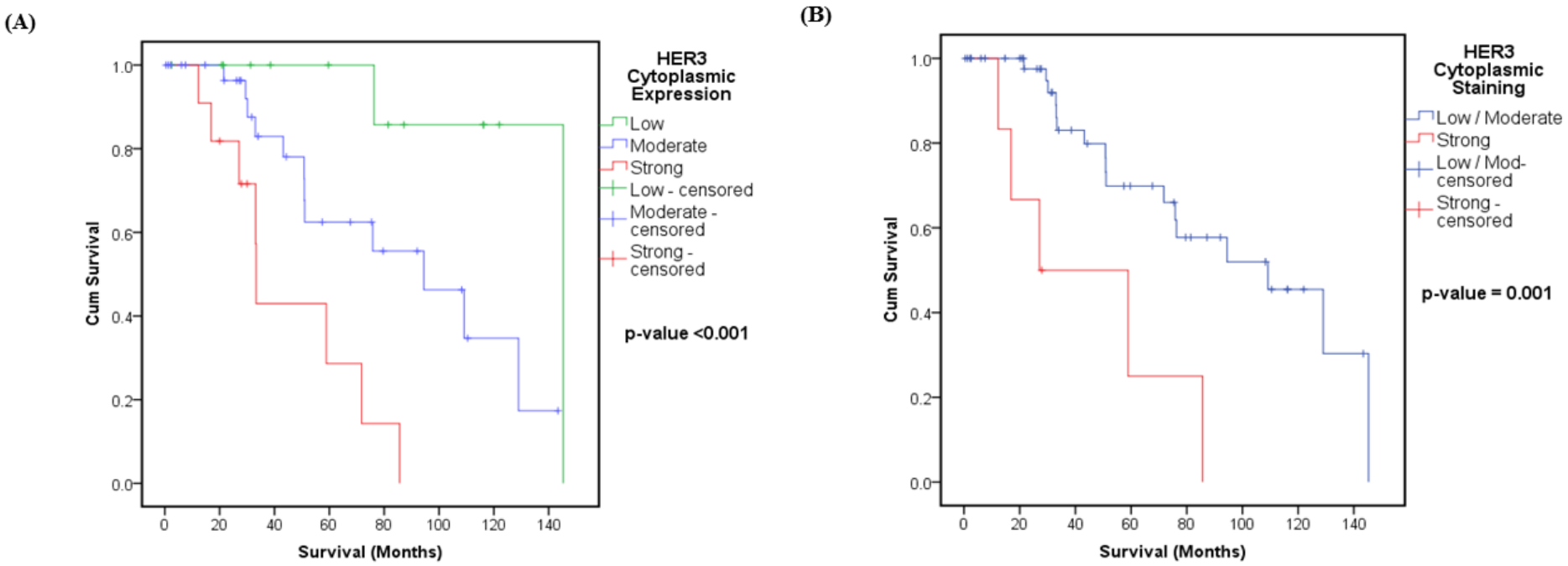

Figure 3-8: Kaplan–Meier curves showing an inverse correlation of HER3 cytoplasmic expression on patient survival

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER3 cytoplasmic expression on survival (A). A further comparison (similar to HER2 cytoplasmic expression) was made between combined low and moderate expression vs strong expression of HER3 (B). Both comparisons show a significant correlation of HER3 strong cytoplasmic expression on patient survival.

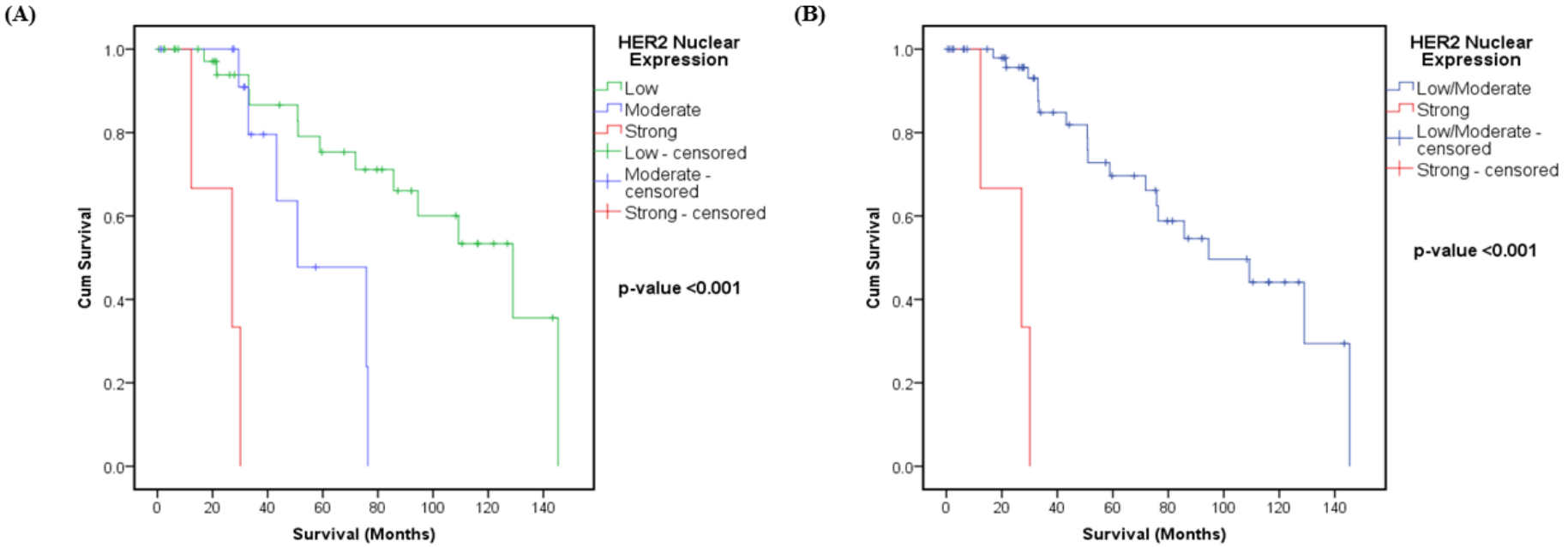

Figure 3-9: Kaplan–Meier curves showing an inverse correlation of HER2 nuclear expression on patient survival

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER2 nuclear expression on survival (A). A further comparison was made between combined low and moderate expression vs strong expression (B). Both comparisons show a significant correlation of HER2 strong nuclear expression on patient survival.

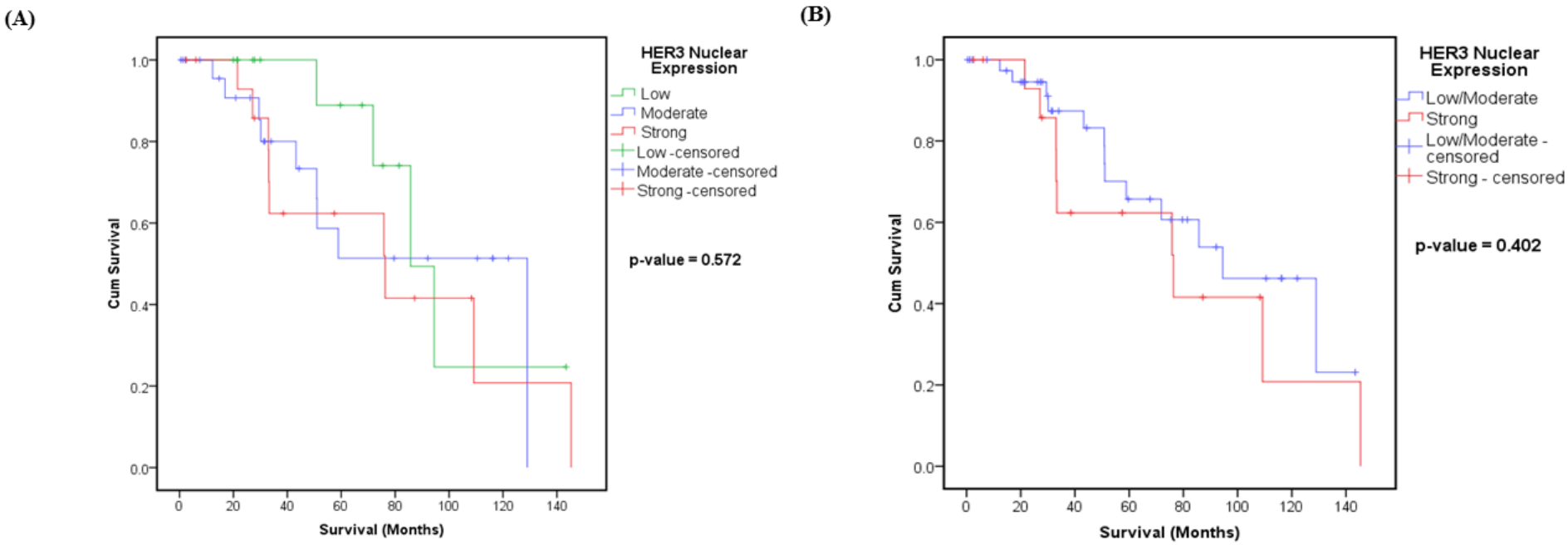

Figure 3-10: Kaplan–Meier curves showing no correlation of HER3 nuclear expression on patient survival

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER3 nuclear expression on survival (A). A further comparison was made between combined low and moderate expression vs strong expression (B). Both comparisons showed no significant correlation of HER3 nuclear expression on patient survival.

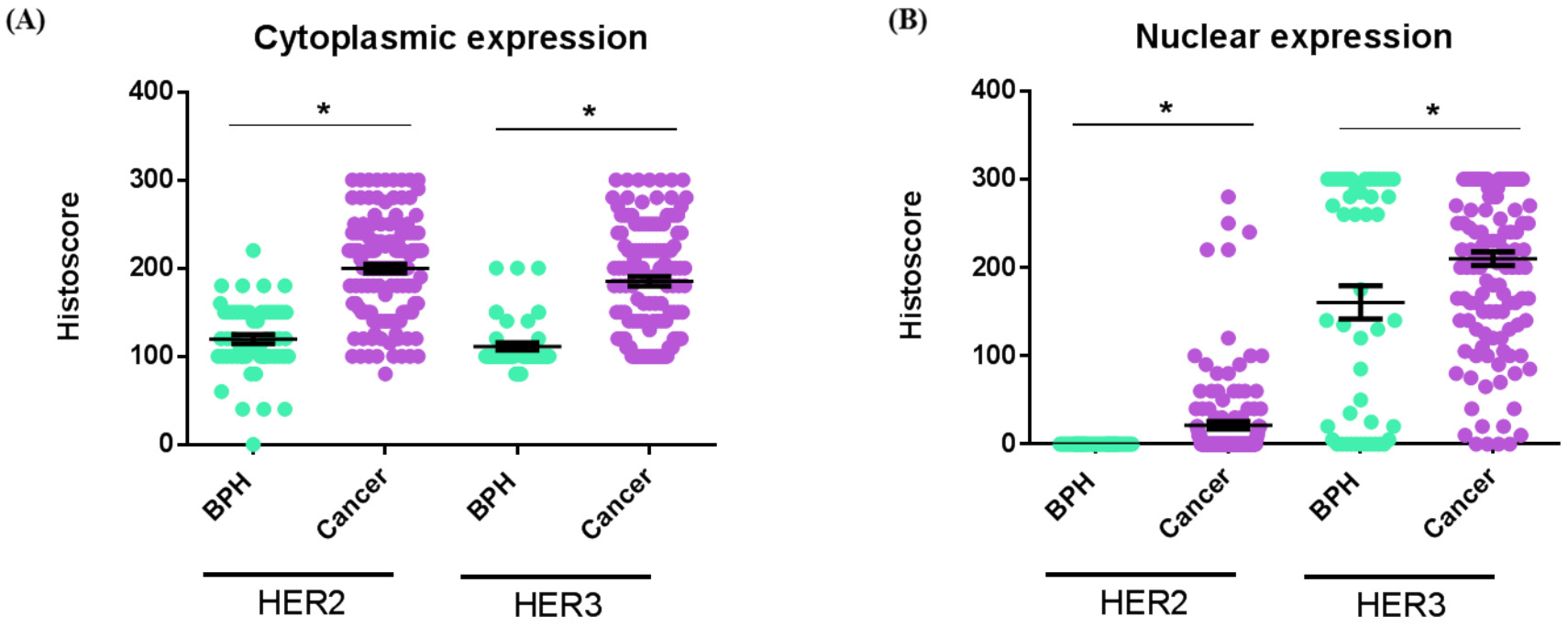

Figure 3-11: Increased sample size (TMA1 and TMA4 together) shows cytoplasmic and nuclear expression of HER2 and HER3 is significantly higher in cancer patients when compared to those with BPH

This figure shows the significantly higher expression in both cytoplasmic (A) and nuclear (B) of HER2 and HER3 in cancer samples. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the BPH samples.

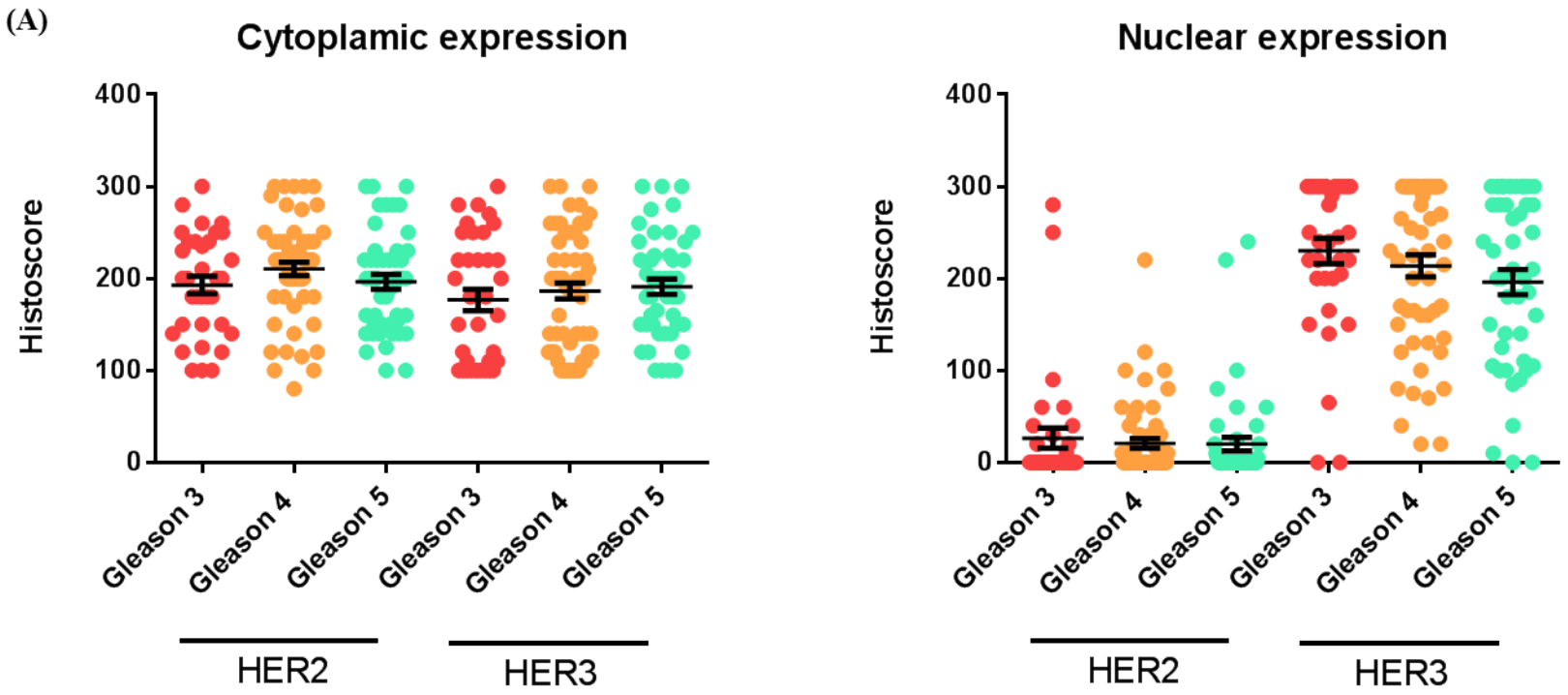

Figure 3-12: Increased sample size (TMA1 and TMA4 together) does not show any significant changes in HER2 and HER3 expression with cancer grade or stage

This figure shows the cytoplasmic and nuclear expression of HER2 and HER3 between cancer grades (A) or stage (B). One-way ANOVA was performed on the experiments to reveal if there was any statistical significance. It was observed that there were no significant changes of receptor expression within the groups

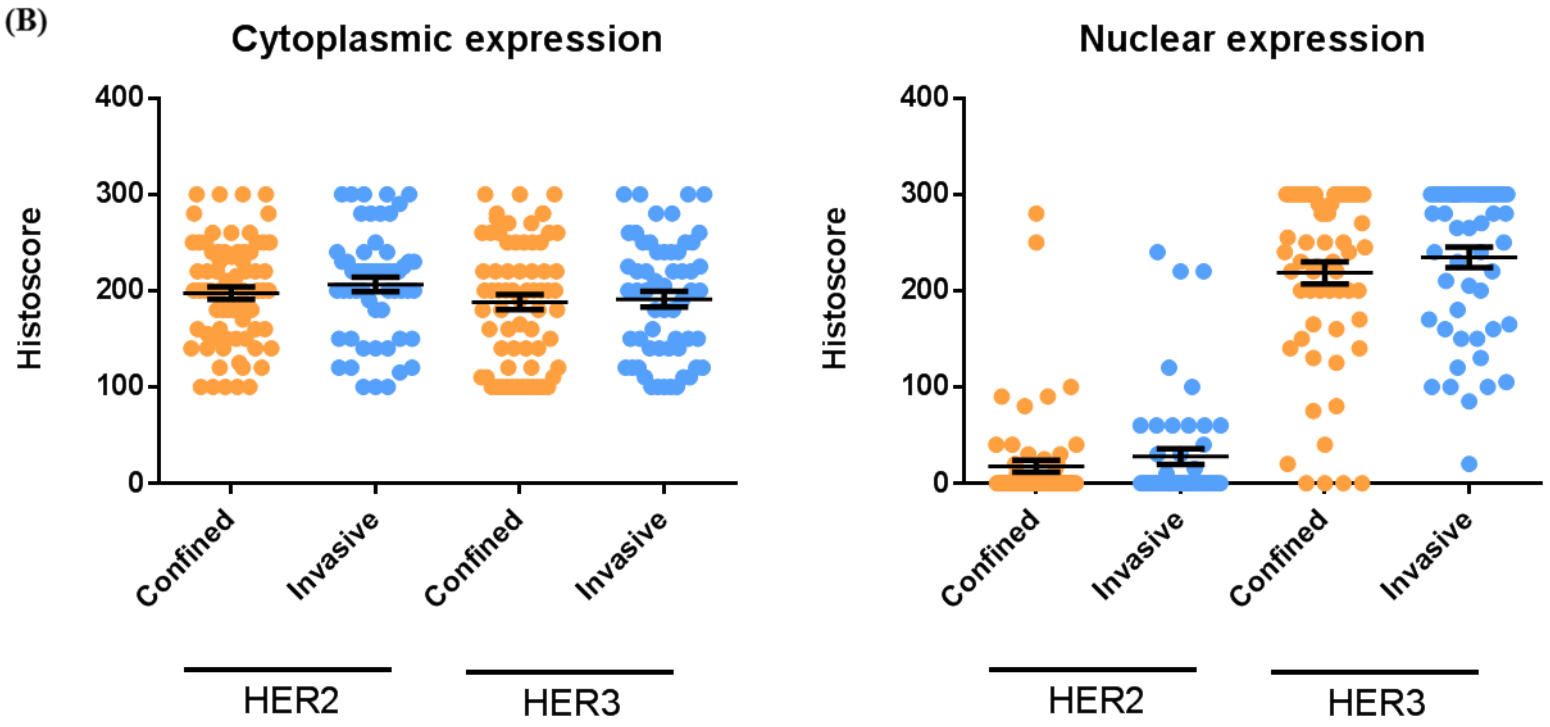

Figure 3-12: Increased sample size (TMA1 and TMA4 together) does not show any significant changes in HER2 and HER3 expression with cancer stage

This figure shows the cytoplasmic and nuclear expression of HER2 and HER3 between cancer stages (B). One-way ANOVA was performed on the experiments to reveal if there was any statistical significance. It was observed that there were no significant changes of receptor expression within the groups

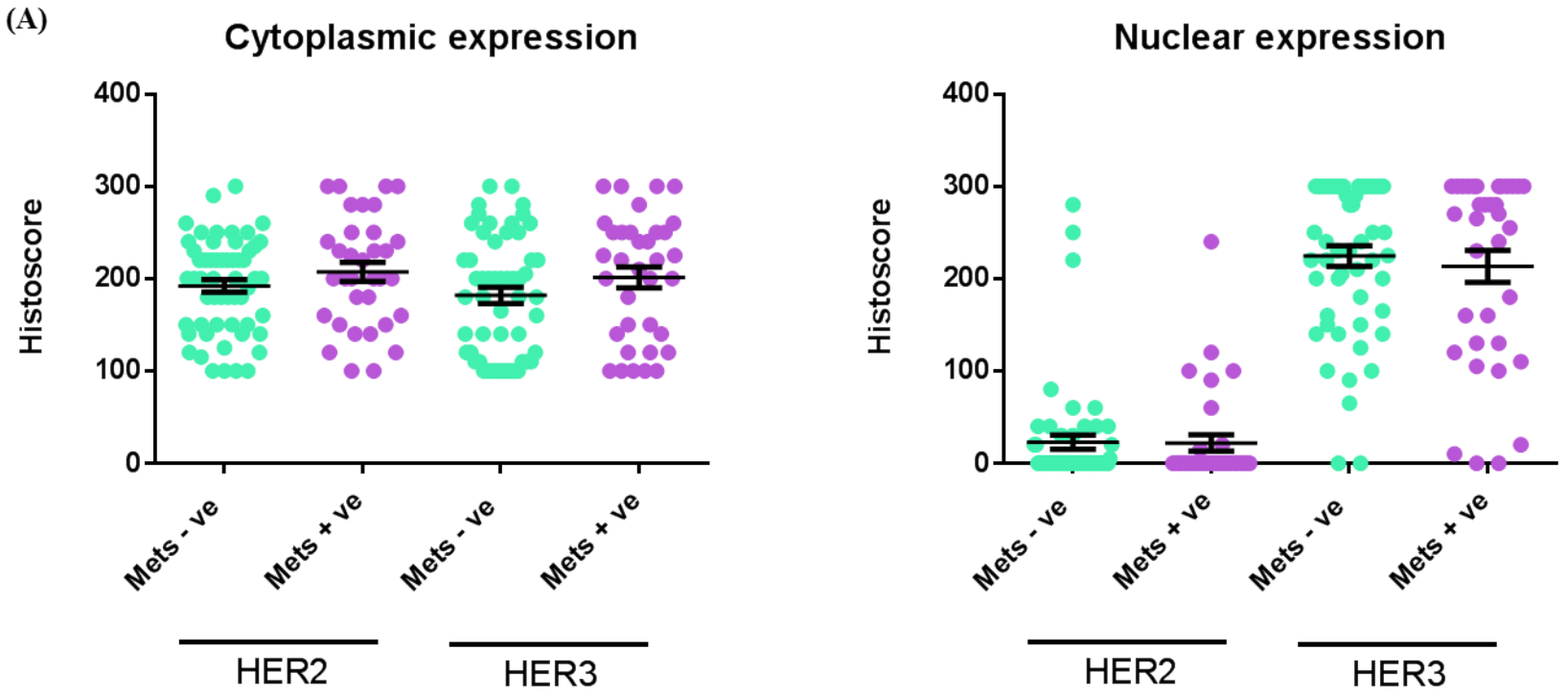

Figure 3-13: Increased sample size (TMA1 and TMA4 together) does not show any significant changes in HER2 and HER3 expression with metastases

This figure shows the cytoplasmic and nuclear expression of HER2 and HER3 between patients who presented with or without metastases at diagnosis (A) Student t-test was performed on the experiments to reveal if there was any statistical significance. It was observed that there were no significant changes of receptor expression within these groups.

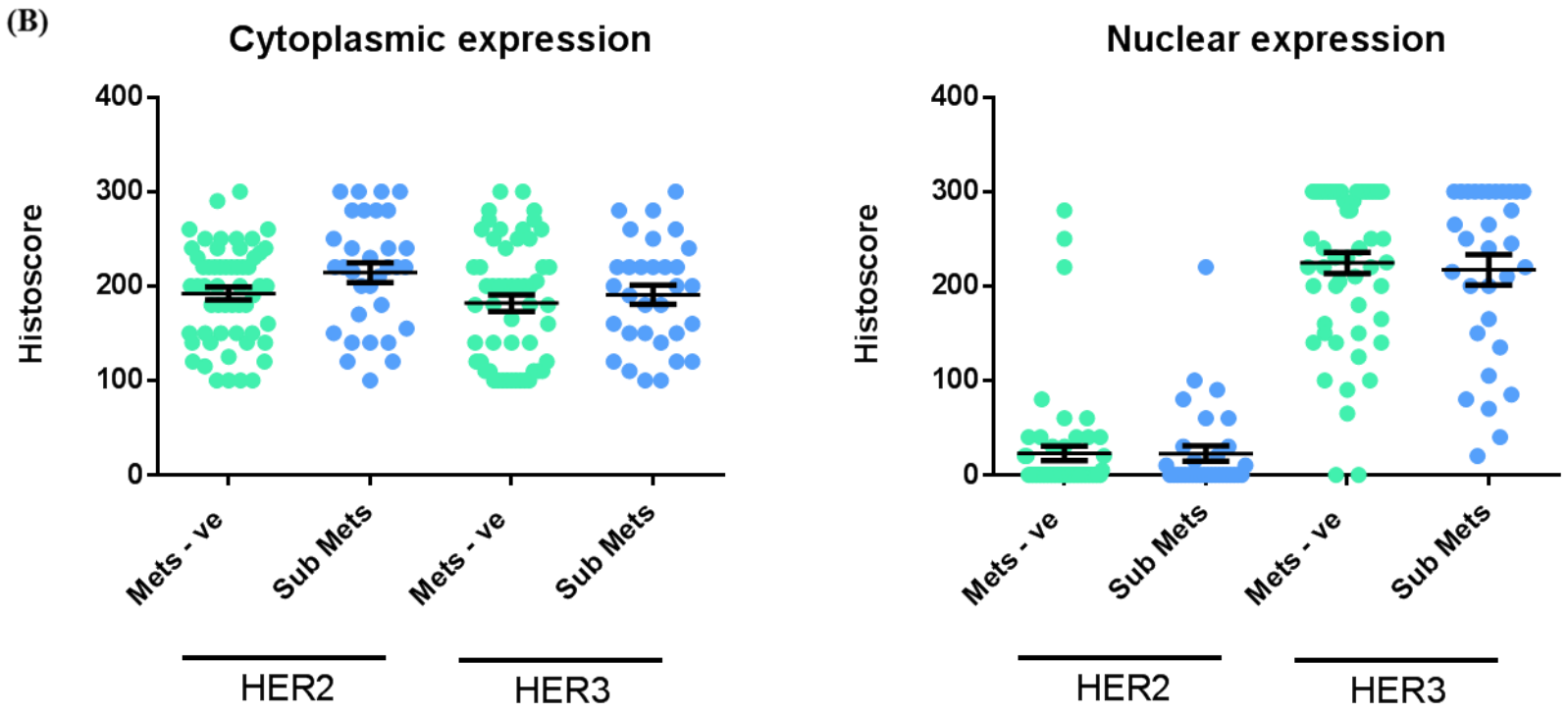

Figure 3-13: Increased sample size (TMA1 and TMA4 together) does not show any significant changes in HER2 and HER3 expression with metastases

This figure shows the cytoplasmic and nuclear expression of HER2 and HER3 between patients who presented without any metastases at diagnosis and those who developed subsequent metastases after diagnosis (B). Student t-test was performed on the experiments to reveal if there was any statistical significance. It was observed that there were no significant changes of receptor expression within these groups.

3.3.1 Patients presenting with low HER2 and strong HER3 cytoplasmic expression and those with low HER2 and strong HER3 nuclear expression have the poorest survival rates

The analysis of the combined TMA cohort was continued by further correlations with the patient survival data. When HER2 cytoplasmic expression was analysed, it was observed that there was no significant correlations between HER2 expression and patient survival (**Figure 3-14**). However, analysis of HER3 expression revealed a significant inverse correlation of HER3 cytoplasmic expression on patient survival (**Figure 3-15**).

The nuclear expression of HER2 and HER3 was similarly analysed. Patients had poorer survival rates with increased HER2 and HER3 nuclear expression (**Figure 3-16** and **Figure 3-17**). However, it should be noted that there were very few patient numbers with strong nuclear HER2 expression.

A final comparison was made to analyse the outcome of patient survival by analysing the co-expression of HER2 and HER3 (**Figure 3-18**).

It was observed that patients who had low/moderate HER2 along with strong HER3 cytoplasmic expression had the poorest survival rates when compared to the other groups. When the nuclear expression was considered, there were no patients who presented with both strong HER2 and HER3 expression and strong HER2 and low/moderate HER3 together. However, it was observed that patients who had low/ moderate HER2 along with strong HER3 nuclear expression had lower survival rates than patients who presented with low/ moderate expression of both HER2 and HER3.

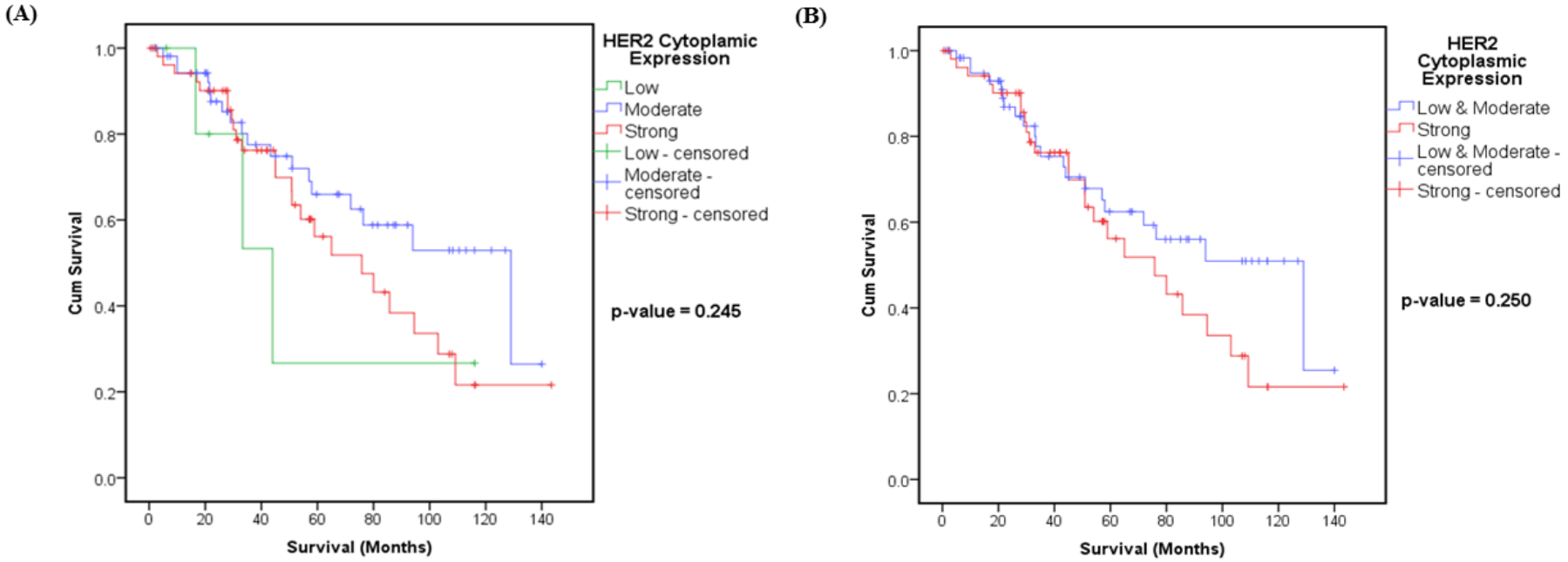

Figure 3-14: Kaplan–Meier curves showing the correlation of HER2 cytoplasmic expression on patient survival upon analysis of samples taken from 2 TMAs

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER2 cytoplasmic expression on survival (A). Due to low patient numbers in the low expression group, a further comparison was made between combined low and moderate expression vs strong expression (B). Both comparisons revealed no significant correlation of HER2 cytoplasmic expression on patient survival.

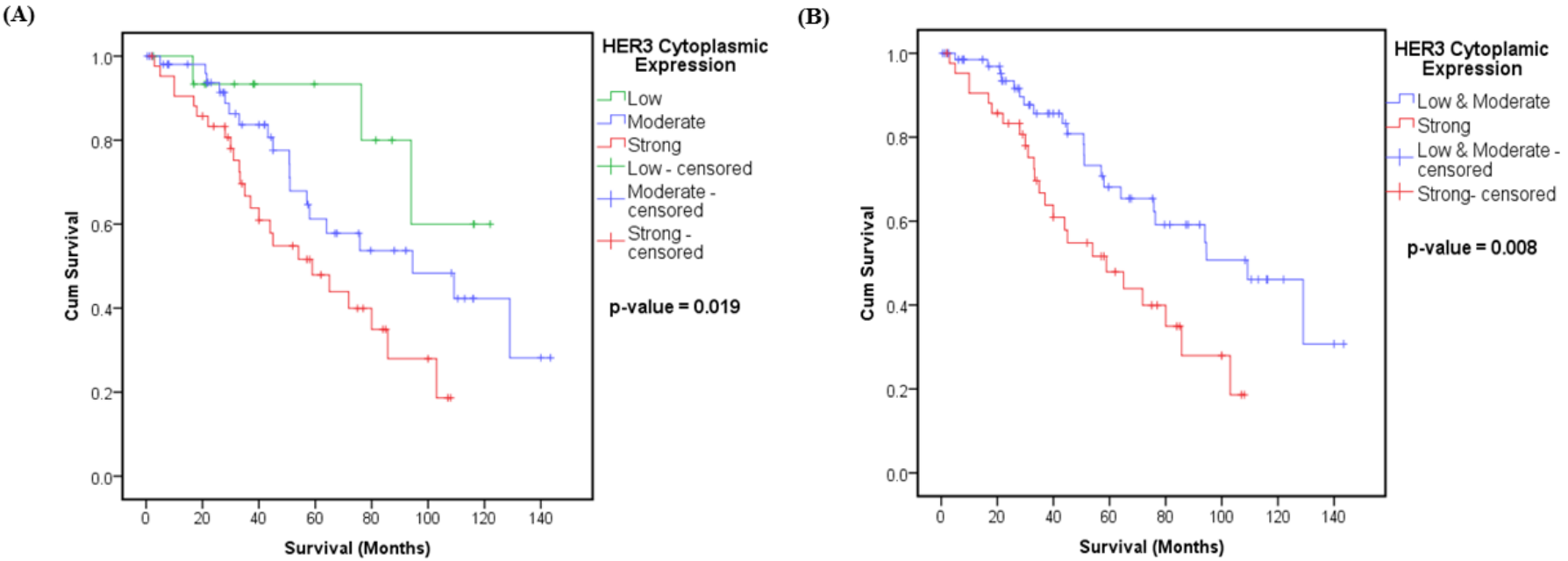

Figure 3-15: Kaplan–Meier curves showing an inverse correlation of HER3 cytoplasmic expression on patient survival upon analysis of samples taken from 2 TMAs

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER3 cytoplasmic expression on survival (A). Due to low patient numbers in the low expression group, a further comparison was made between combined low and moderate expression vs strong expression (B). Both comparisons revealed a significant correlation of HER3 cytoplasmic expression on patient survival.

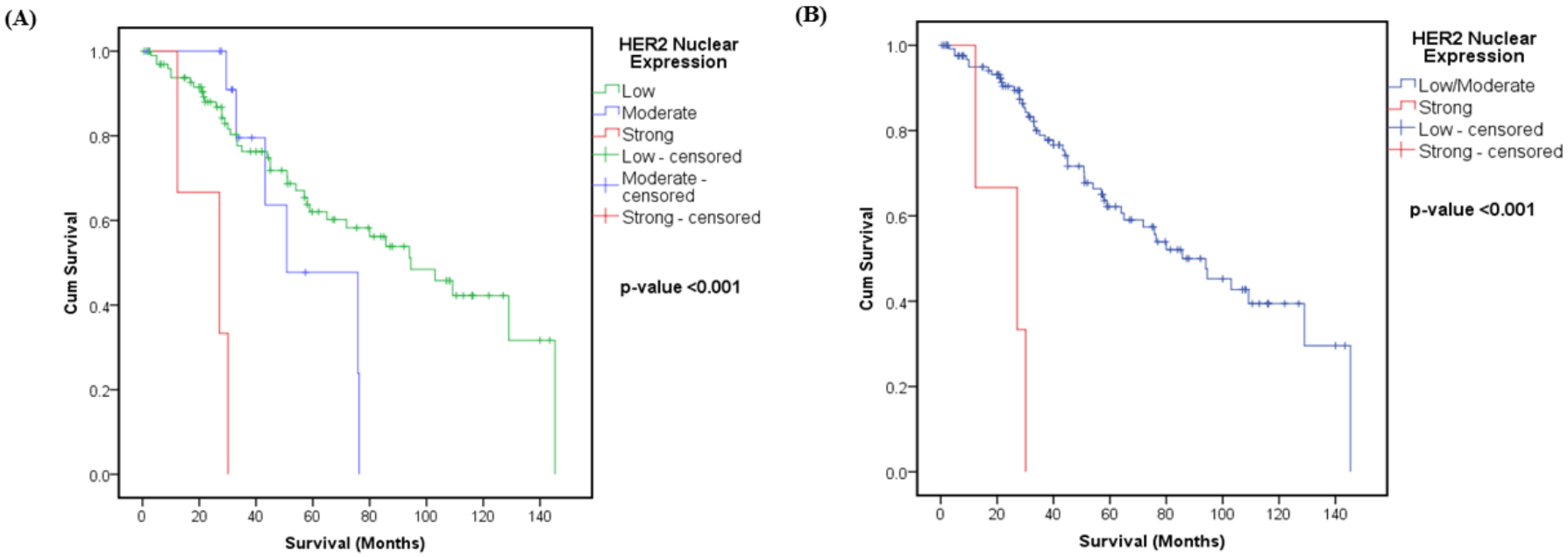

Figure 3-16: Kaplan–Meier curves showing an inverse correlation of HER2 nuclear expression on patient survival upon analysis of samples taken from 2 TMAs

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER2 nuclear expression on survival (A). A further comparison was made between combined low and moderate expression vs strong expression (B). Both comparisons revealed a significant correlation of HER2 nuclear expression on patient survival. There were very few patients who presented with strong nuclear HER2 expression.

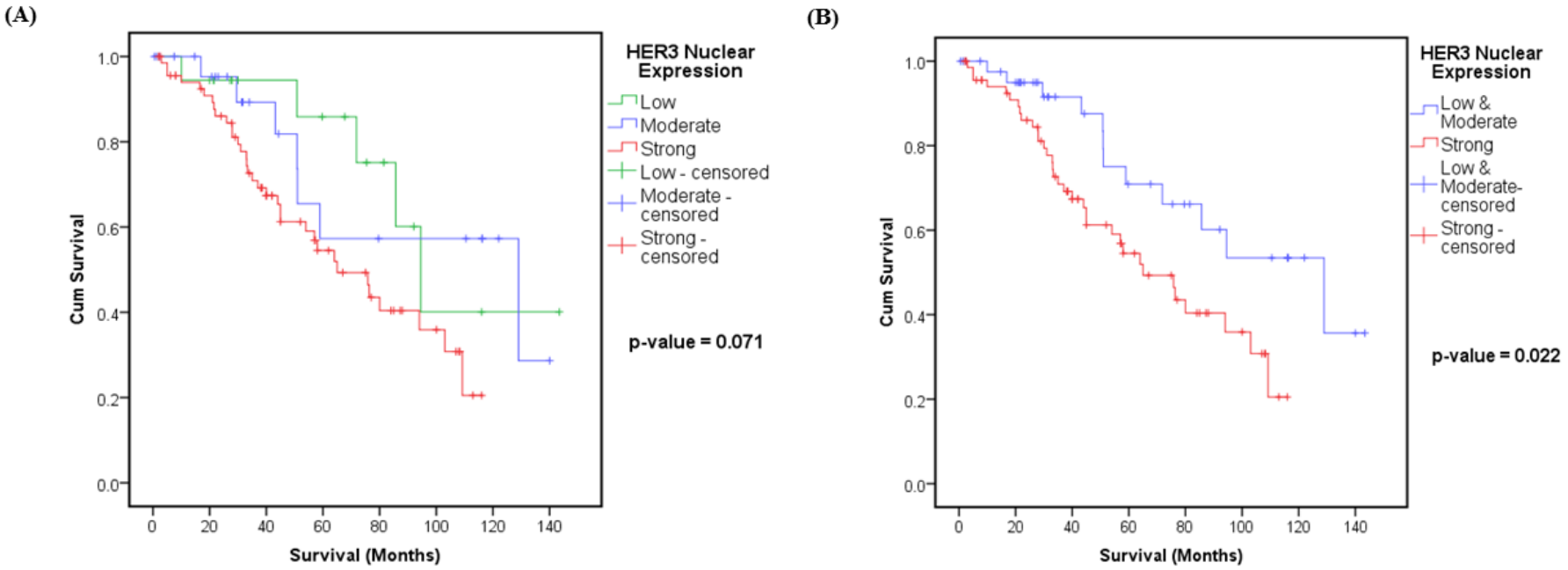

Figure 3-17: Kaplan–Meier curves showing an inverse correlation of HER3 nuclear expression on patient survival upon analysis of samples taken from 2 TMAs

The staining of the receptors was grouped into low, moderate and strong expression and the available patient survival data was used to estimate the correlation of HER3 nuclear expression on survival (A). A further comparison was made between combined low and moderate expression vs strong expression (B). The second comparisons revealed a significant correlation of strong HER3 nuclear expression on patient survival.

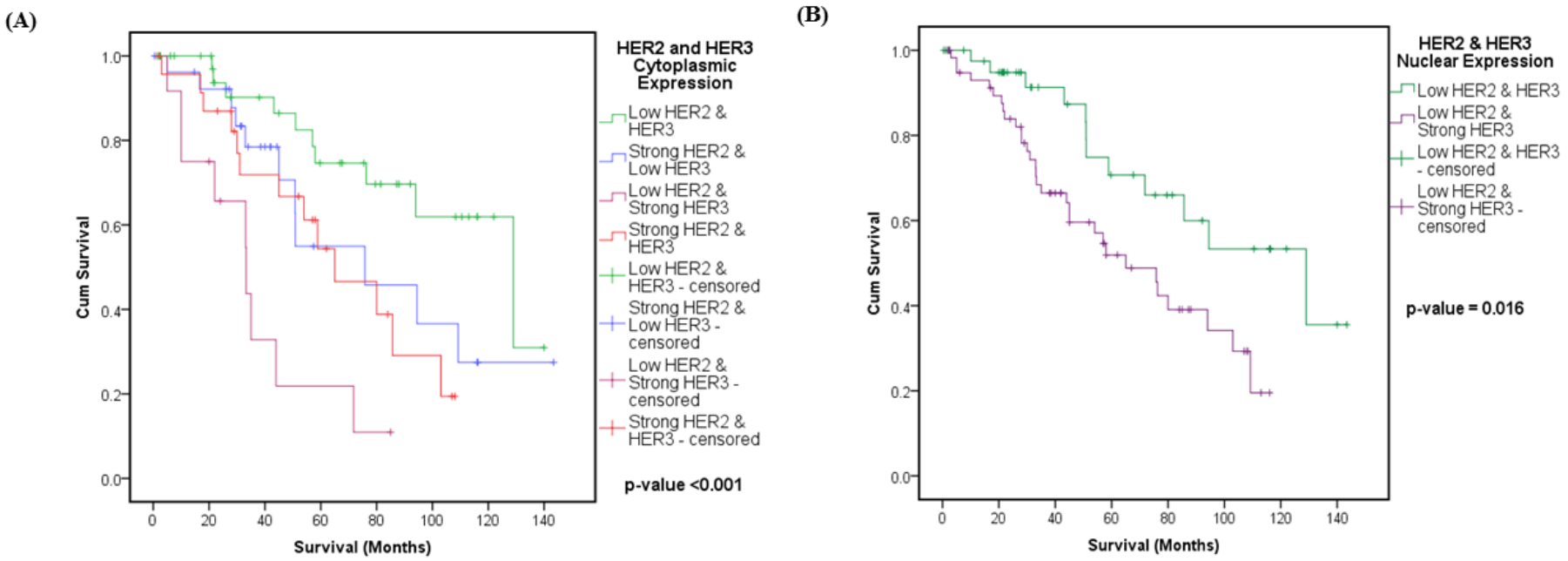

Figure 3-18: Kaplan–Meier curves showing an inverse correlation of HER2 and HER3 differential expression on patient survival upon analysis of samples taken from 2 TMAs

The staining of the receptors was grouped into 4 groups for combined cytoplasmic expression of HER2 and HER3 as indicated in the figure (A). The available patient survival data was used to estimate the correlation of their combined expression on patient survival. This analysis revealed that patients who expressed low /moderate levels of HER2 along with strong levels of HER3 had the worst outcome. For the combined nuclear expression, only 2 groups could be formed due to limited sample number (B). This analysis revealed that patients who had low HER2 along with strong HER3 nuclear expression showed a significant decrease in their survival rates.

3.3.2 Patients who developed castrate resistant prostate cancer presented with higher nuclear HER3 expression along with a loss of PTEN expression

A separate TMA – "TMA5", was considered and the expression of HER2, HER3, tumour suppressor gene – PTEN and pAKT - the downstream protein of HER2 and HER3 signalling pathway, were analysed. This TMA was of particular interest because it consisted of matched paired samples i.e. samples taken from the same patient before and after they received treatment. The pairs were divided into 3 groups – hormone naïve group which consisted of patients who had not received any treatment; hormone sensitive group which consisted of patients who had received androgen withdrawal therapy and were sensitive and continued to be sensitive to the treatment and the castrate resistant group, which consisted of patients who received treatment but had relapsed and presented with castrate resistant prostate cancer. There were no significant differences in receptor expression between the hormone naïve and hormone sensitive groups (**Figure 3-19**). However, the analysis revealed a significant increase in HER3 nuclear expression in patients who developed castrate resistant disease (**Figure 3-20**). The groups were also analysed for the loss of PTEN expression. It was observed that in the castrate resistant group 33.33% of samples had loss of PTEN which was a significant increase from the hormone sensitive group (6.25%) and hormone naïve group (4.35%) (**Figure 3-21**). However, no change in pAKT was observed between the three groups (**Figure 3-22**).

The analysis of the clinical samples proves that HER2 and HER3 expression plays a crucial role in advanced prostate cancer and inhibiting its signalling activity could help in cancer regression.

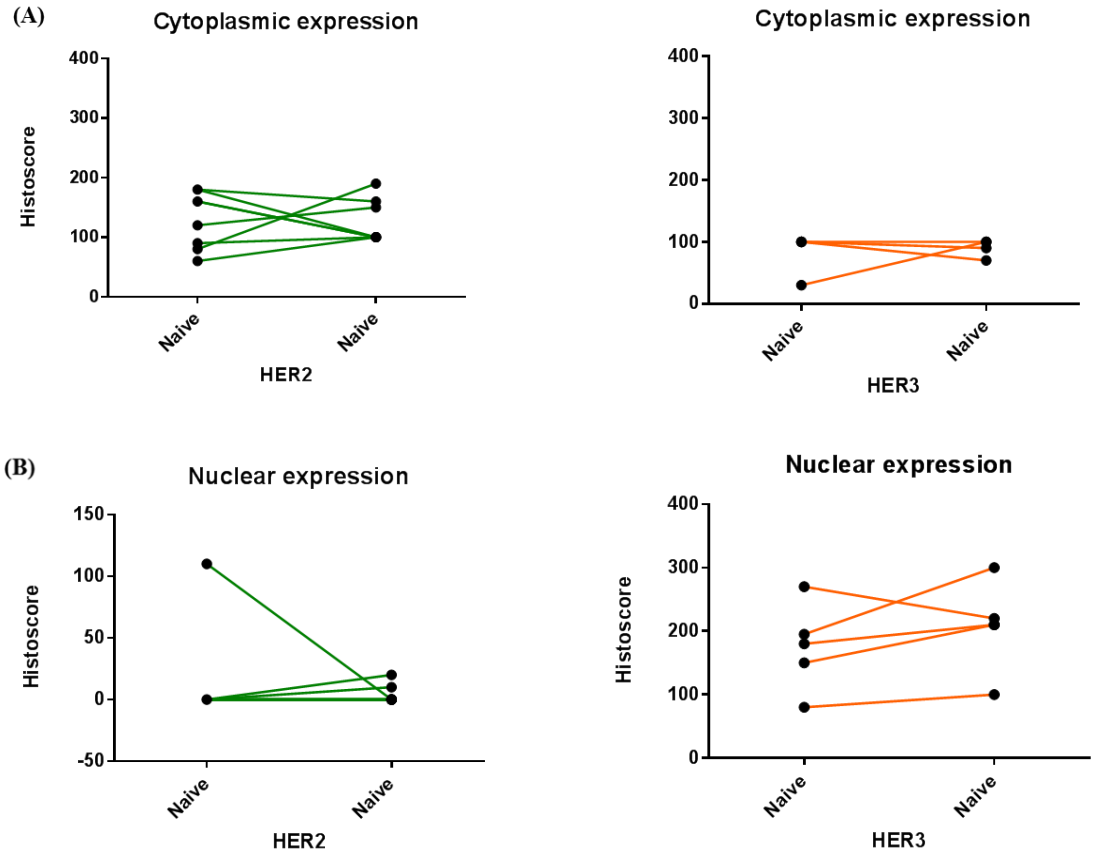

Figure 3-19: HER2 and HER3 expression does not change between matched patients who belonged to the treatment naïve group vs those who were sensitive to treatment

(A, B) There was no significant change in cytoplasmic and nuclear expression of HER2 and HER3 in matched samples from patients who were screened at an early and later stage. These patients had not received any treatment.

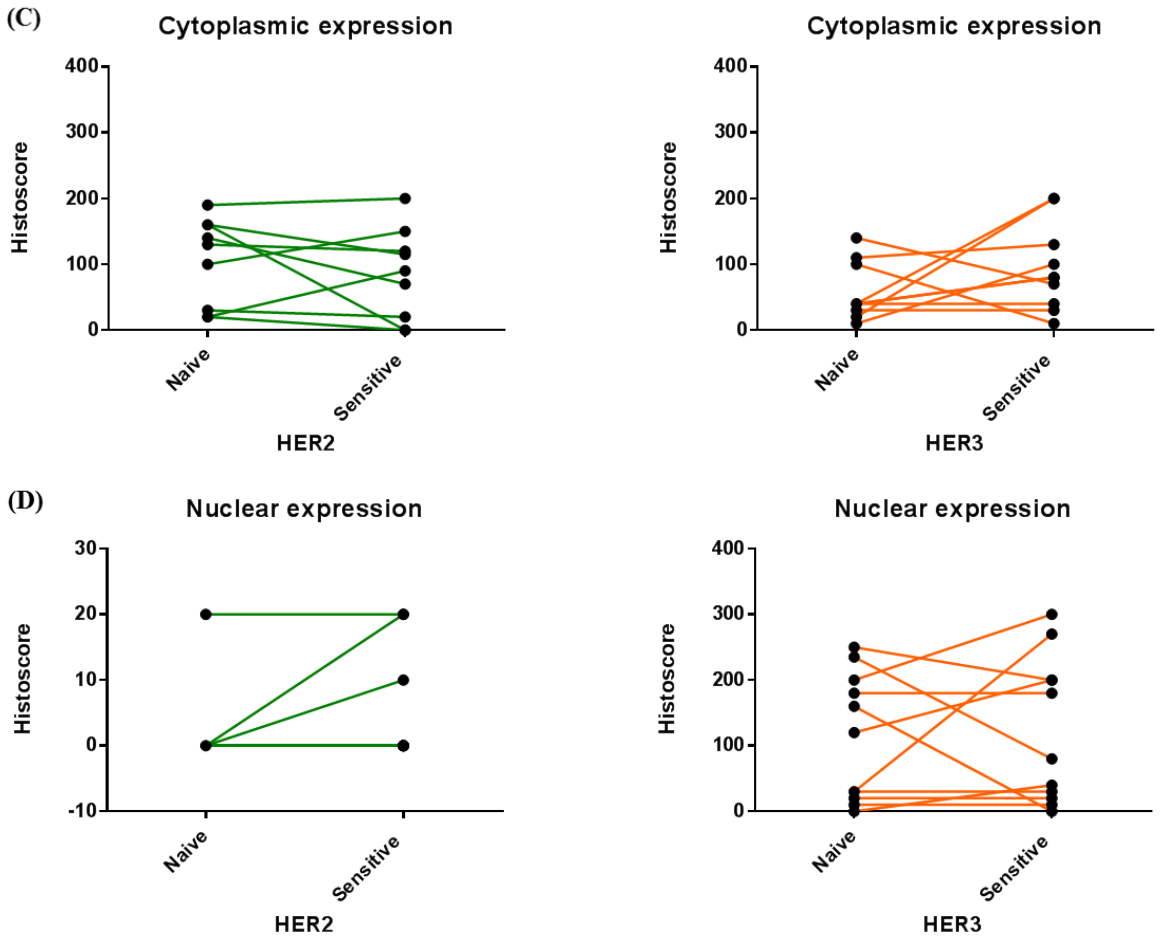

Figure 3-19: HER2 and HER3 expression does not change between matched patients who belonged to the treatment naïve group vs those who were sensitive to treatment (C, D) There was no significant change in the receptor expression in matched samples from patients who were screened before treatment and after they showed signs of being sensitive to the treatment.

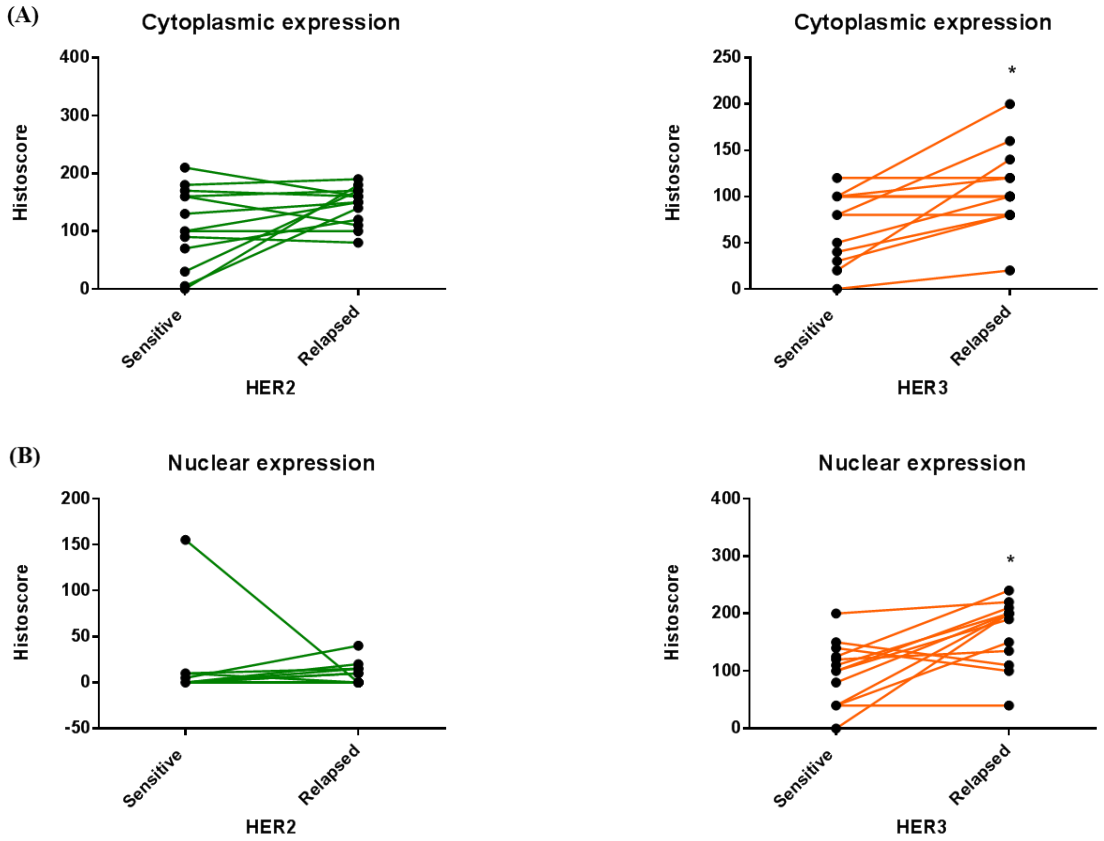

Figure 3-20: HER3 cytoplasmic and nuclear expression is significantly higher in matched patients who relapsed after being sensitive to therapy

(A) There was a significant change in cytoplasmic expression of HER3 in matched samples taken from patients who were screened after they received treatment and after they relapsed following treatment. (B) There was a significant change in nuclear HER3 expression in matched samples taken from patients who were screened after they were sensitive to treatment and after they relapsed following treatment. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the treatment sensitive group.

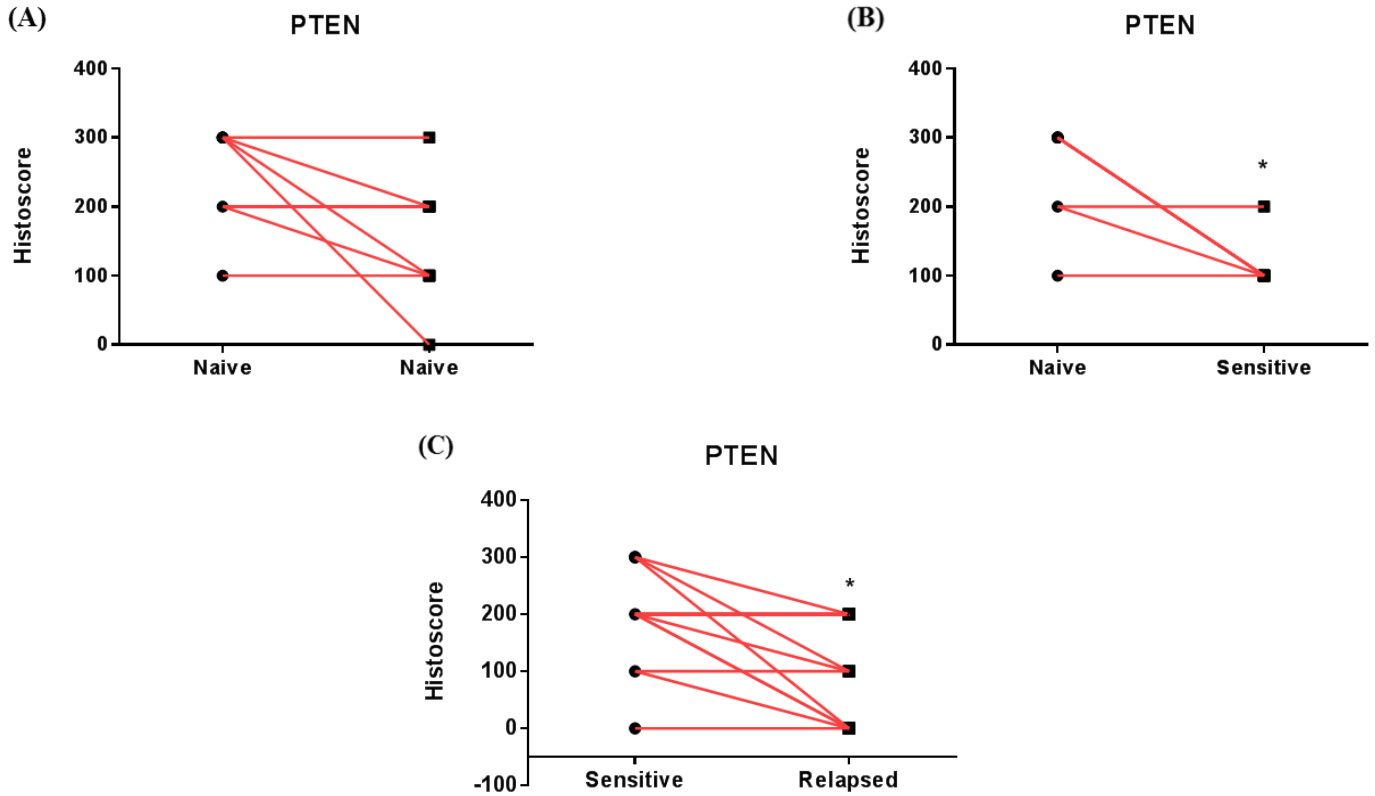

Figure 3-21: PTEN loss is observed in patients who become sensitive to treatment and in those who relapse after receiving treatment.

(A) There was no significant change in PTEN expression in matched samples from patients who were screened for prostate cancer at different stages. These patients had not received any treatment. However, a reduction in PTEN expression was observed in matched samples from patients who were screened before treatment and after they showed signs of being sensitive to the treatment (B) and in samples who eventually relapsed (C). Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the treatment naïve and sensitive group respectively. (Staining performed by Luke Martinson).

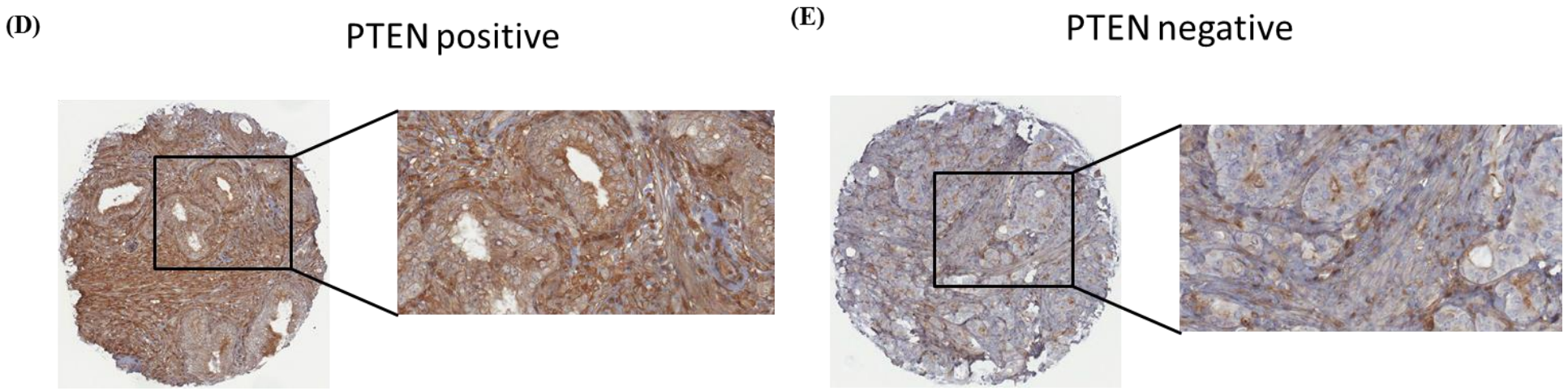

Figure 3-21: PTEN loss is observed in patients who become sensitive to treatment and in those who relapse after receiving treatment.

Representative patient samples showing strong expression of PTEN while they were sensitive to treatment (D) and reduced expression when they relapsed with aggressive disease (E) (Staining performed by Luke Martinson).

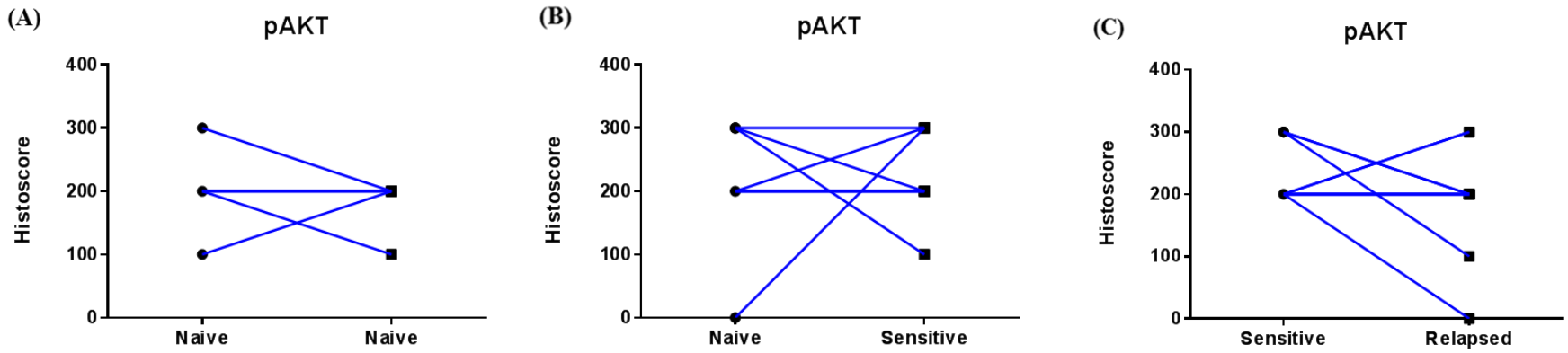

Figure 3-22: There is no change on pAKT levels in matched paired patient samples between the treatment naïve, treatment sensitive and relapsed groups.

pAKT levels were screened in matched paired samples between 3 groups of patient samples – naïve group consisted of patients who were screened at different time points after being diagnosed with prostate cancer (A); Naïve/sensitive group consisted of samples from patients who hadn't received any treatment and then who showed sensitivity to the treatment (B) and the sensitive / relapsed group which consisted of patients samples that were sensitive to treatment but subsequently relapsed with aggressive prostate cancer (C). There were no significant changes in pAKT levels in all the 3 groups. (Staining performed by Luke Martinson)..

3.4 Validating the expression of HER2 and HER3 in prostate cancer cell lines

After analysing the expression in clinical samples, the validation of receptor expression was continued in prostate cancer cell lines that were to be used in this project. The cell lines used were LNCaP and their derivative cell lines LNCaP–AI. The androgen – independent LNCaP–AI cell line was established by growing LNCaP cells in steroid depleted conditions and is used as a model of prostate cancer under androgen withdrawal conditions. Both the cell lines showed good levels of expression with and without Heregulin stimulation (**Figure 3-23**).

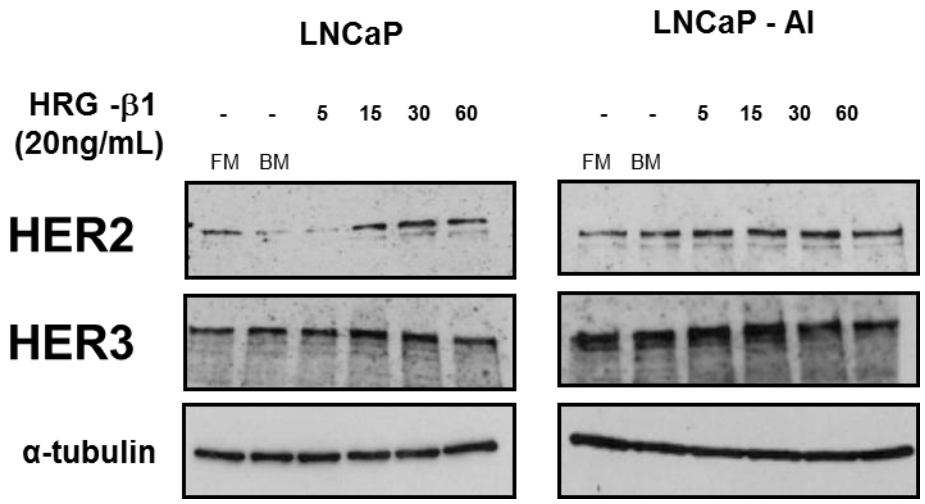

Figure 3-23: Expression of HER2 and HER3 in prostate cancer cell lines

The cells were seeded out in full medium (FM). The next day, the cells were washed with PBS and the media were changed on the control and treatment arms of the experiment to basal medium (BM) to starve the cells. After an overnight incubation, the cells were treated with Heregulin (HRG; 20 ng/ml) for 15 minutes after which the cells were lysed in RIPA buffer and the lysates were analysed for HER2 and HER3 expression by western blotting. The above result is a representation of 2 repeats.

3.5 Cellular localisation of HER2 and HER3

Having observed the nuclear localisation of these receptors in the clinical samples, the finding was further investigated *in-vitro* and the LNCaP cell line was used for validation. It was observed that when the cells were treated with Heregulin, both HER2 and HER3 translocated to the nucleus. This finding was confirmed by immunofluorescence (**Figure 3-24**) and western blotting (**Figure 3-25**).

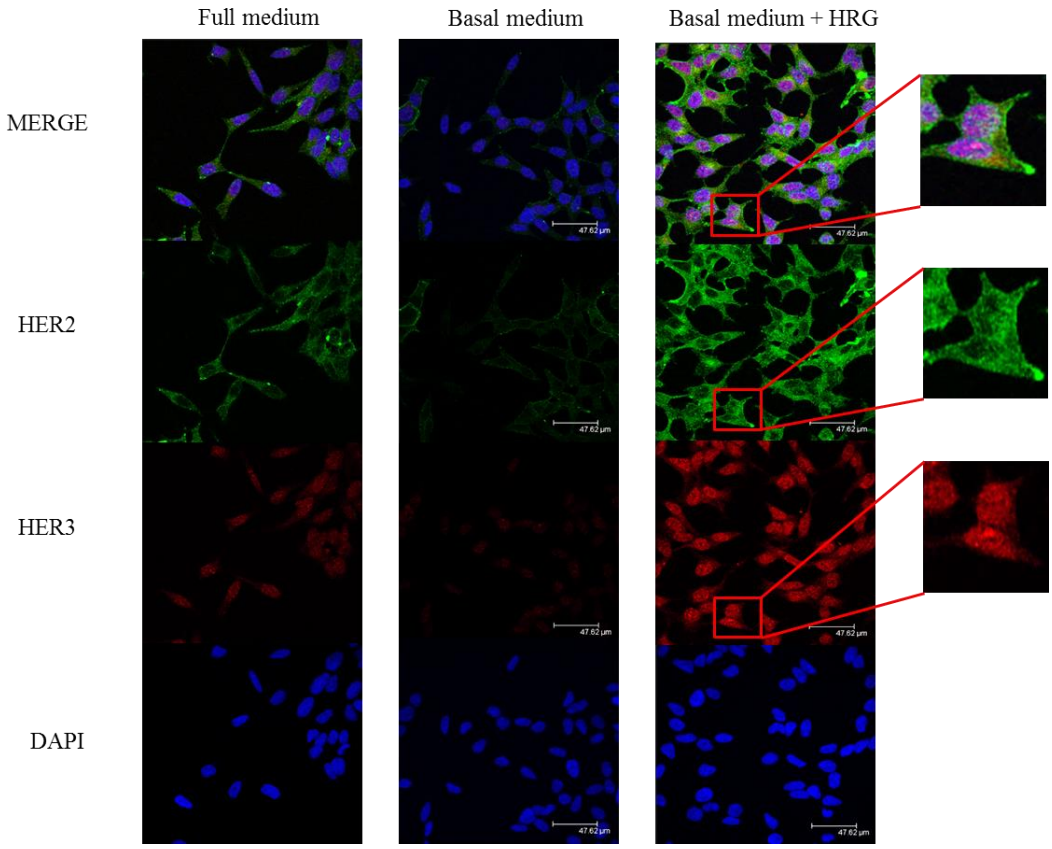

Figure 3-24: HER2 and HER3 translocate to the nucleus with Heregulin stimulation

LNCaP cells were seeded out in full medium on sterile coverslips. The next day, the relevant arms of the experiment were starved in basal medium overnight followed by treatment with Heregulin (HRG; 20 ng/ml) for 2 hours. The cells were then fixed onto the coverslips with 4% paraformaldehyde and permeabilized using chilled methanol. This was followed by blocking the cells with serum-free protein block, to avoid unspecific binding of primary antibody. The cells were incubated with the respective primary antibodies for 1 hour at room temperature. After the incubation, the cells were washed with PBS, followed by incubation with appropriate Alexa-Fluor secondary antibodies for another hour at room temperature. The cells were finally washed with PBS and mounted on the glass slides using Vectashield mountant containing DAPI to stain the nucleus. The images were observed under confocal microscope using Z-stack imaging taking of sections of 1 micron thickness. Appropriate secondary only negative controls were used to ensure specificity. The above result is a representation of 2 repeats

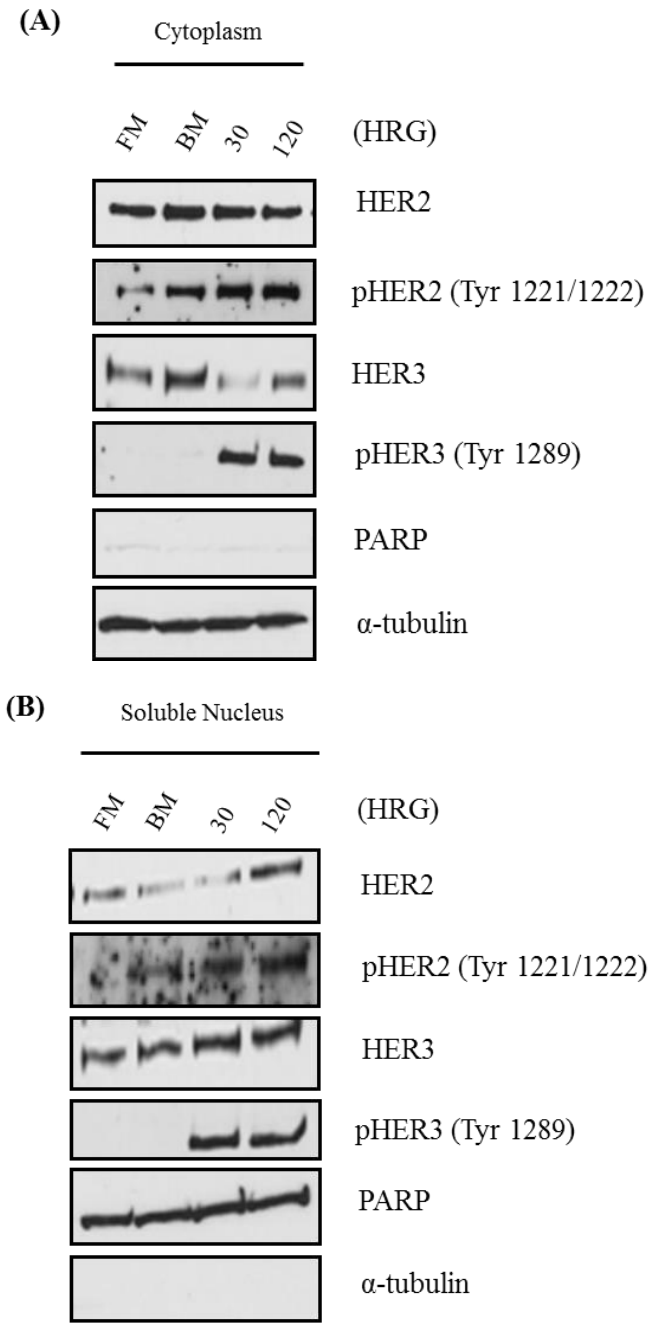

Figure 3-25: Total and phosphorylated forms of HER2 and HER3 translocate to the nucleus after Heregulin stimulation

LNCaP cells were seeded out in full medium. The following day, the medium was changed to basal medium on the control and the samples to be treated with Heregulin. After starving the cells overnight, the cells were treated with Heregulin (HRG; 20 ng/ml) for 30 minutes and 2 hours as indicated. The cells were then lysed and the cytoplasmic and nuclear compartments of the cells were then separated by following the protocol of the extraction kit. The lysates were then analysed for cytoplasmic (A) and nuclear (B) protein expression by western blotting. α-tubulin and PARP were used as controls for the cytoplasmic and nuclear compartments, respectively. The result revealed that Heregulin aided the total and active forms of the receptors to shuttle into the nucleus. The above result is a representation of 2 repeats

3.6 HER2 and HER3 translocate to the nucleus and is also present in the chromatin fraction upon Heregulin stimulation

The presence of these receptors in the nuclear compartment was further investigated. The remaining chromatin samples, extracted from the above experiment, were analysed by western blotting, using appropriate controls. Upon analysis it was observed that HER2 and HER3 were present in the chromatin fraction upon Heregulin stimulation (**Figure 3-26**).

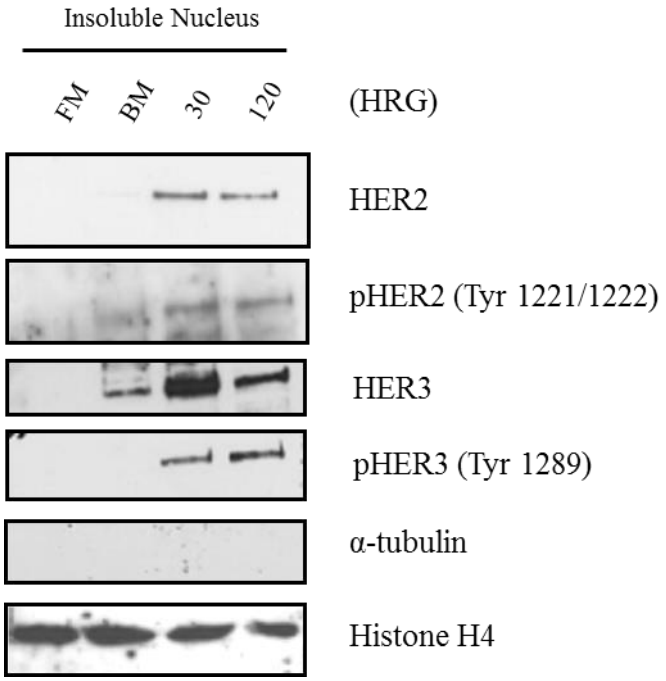

Figure 3-26: HER2 and HER3 shows association with chromatin upon Heregulin treatment

LNCaP cells were seeded out in full medium. The following day, the medium was changed to basal medium on the control and the samples to be treated with Heregulin. After starving the cells overnight the cells were treated with Heregulin (HRG; 20ng/ml) the following day for 30 minutes and 2 hours. The cells were then lysed and the cytoplasmic and nuclear compartments of the cells were then separated by following the protocol of the extraction kit. The insoluble pellet left behind was sonicated in laemmli buffer. The chromatin samples were then analysed for the presence of total HER2 and HER3 and their phosphorylated forms by western blotting. α-tubulin and histone H4 were used as controls. The result revealed that Heregulin aided the total and active forms of the receptors to be associated with the chromatin. The above result is a representation of 2 repeat

s

3.7 Discussion

There is an essential need for reliable and specific biomarkers that can differentiate between benign and aggressive prostate disease. The use of the historic biomarker, PSA, has resulted in over-dosing and treatment due to its lack of specificity, as it is present in both normal and diseased prostate cells (Crawford *et al.*, 2014). There has been a significant amount of research carried out to identify new, more specific biomarkers for prostate disease and its different stages. Due to the variability of disease between patients, it would be useful to screen patients for biomarkers before, during and after therapy. The patient's individual molecular profile can then be used to monitor and direct the patient towards more appropriate and beneficial therapies.

Prostate cancer progression has been linked to various mechanisms as explained earlier in the introductory chapter. The role of HER3 in prostate cancer is still poorly understood and the available data on clinical samples is not consistent and reveals some contradictions. For example, Koumakpayi *et al.* (2006) have observed higher nuclear HER3 in hormone-refractory disease, whereas Carlsson *et al.* (2013) have observed a downregulation in HER3 expression in prostate metastases. Since this project is focussed on the role of HER2 and HER3 expression and signalling in prostate cancer, an initial screen for these receptors was performed using clinical samples. Immunohistochemistry was performed using tissue microarrays that consisted of benign and prostate cancer samples. The recorded patient data was used to draw correlations between the protein expression and disease. The stained TMAs were scored for cytoplasmic and nuclear expression using the histoscore method, which made the data quantifiable.

Upon analysis, a significant increase in both cytoplasmic and nuclear expression of HER2 and HER3 was observed in cancer samples when compared to BPH samples (**Figure 3-11**). When the expression between different Gleason grades, cancer stages (**Figure 3-12**) and the presence of metastasis (**Figure 3-13**) was considered, it was observed that there was no significant differences between HER2 and HER3 expression. However, there remained a higher nuclear expression of HER3 in advanced and metastatic disease. The data of TMA1 alone revealed significant differences between the cytoplasmic HER2 and HER3 expression in all these groups (**Figure 3-3**, **Figure 3-4**, **Figure 3-5** and **Figure 3-6**). Upon close analysis of the data in these groups, it was

observed that the additional samples had stronger expression of receptor expression in groups that were previously seen to have low expression. Also, the additional TMA did not result in adding equal number of patient data to all the groups to be analysed. Hence it will be interesting to continue the expression analysis by adding the appropriate number of patient samples (based on a power calculations) in each data set to ensure equal sample distribution leading to a more accurate correlation of the data sets. The available patient records were used to correlate HER2 and HER3 cytoplasmic and nuclear expression with patient survival. When the independent cytoplasmic scores were considered, only strong HER3 expression significantly correlated with poor patient survival (**Figure 3-15**). Both HER2 and HER3 strong nuclear expression significantly correlated with survival outcome (**Figure 3-16**). However, it must be noted that there were very few patients who presented with high nuclear HER2.

The data from the matched paired patient samples suggests the importance of HER3 expression in advanced disease. In the treatment naïve group, the data suggests a trend to increasing nuclear HER3 expression as the disease progresses. A similar trend in increasing cytoplasmic and nuclear HER3 expression was observed in patients who showed sensitivity to treatment (**Figure 3-19**). Interestingly, there was a significant increase in both cytoplasmic and nuclear HER3 in patients who relapsed from the treatment (**Figure 3-20**). This result provides new insight to one of the many mechanisms adopted by advanced disease to survive and evolve. Another such mechanism, the reduction of the tumour suppressor gene PTEN, has been previously observed and described in prostate cancer (McMenamin *et al.*, 1999; Squire, 2009). The results herein are in agreement with these findings with the observation of a significant reduction in PTEN expression in patients who subsequently relapsed with a more aggressive disease after being sensitive to treatment. (**Figure 3-21**). PTEN loss has also been researched for its use as a predictive biomarker and could be useful and analysis of clinical samples has shown some direct correlation of PTEN loss and disease aggressiveness (Ahearn *et al.*, 2016). However there were no changes in the pAKT expression between the matched-paired samples used in this study (**Figure 3-22**). Upon analysis of the patient pairs it was seen that majority of the samples did not have a complete PTEN loss suggesting that the activation of this pathway could still be regulated and hence not resulting in a dramatic increase in pAKT. Saying this, the

expression of pAKT was still strong between all the groups suggesting that the pathway remains active in aggressive disease.

When co-expression of HER2 and HER3 was evaluated, patients who presented with low HER2 along with strong HER3 cytoplasmic expression had the worst prognosis. Similarly, patients who co-expressed low levels of nuclear HER2 along with strong nuclear HER3 showed poorer survival rates (**Figure 3-18**). There has been no previous published data analysing the co-expression of HER2 and HER3 expression in prostate cancer and correlating it to patient survival. The results obtained from this study using clinical material, suggests that these receptors, particularly HER3, could play an important role in advanced prostate cancer.

The observed data from the clinical samples encouraged the analysis of the expression of these receptors *in-vitro*. Two prostate cancer cell lines that serve as models to study the different stages on prostate cancer were used. LNCaP and their derivative LNCaP-AI typically represent androgen dependent and androgen-independent prostate cancer. Upon analysis using western blotting, it was observed that these receptors show substantial co-expression of HER2 and HER3 (**Figure 3-23**). An upregulation of receptor expression was also observed upon Heregulin treatment suggesting that the activation of the signalling pathways could be causing stabilisation of the receptors. It will be interesting to study the stabilisation mechanisms for these receptors in prostate cancer. The nuclear expression of HER2 and HER3 was also investigated *in-vitro* after observing their expression in clinical samples. LNCaP cells were stimulated with Heregulin and analysed for HER2 and HER3 expression using immunofluorescence. It was observed that both HER2 and HER3 are translocated to the nucleus when they are activated (**Figure 3-24**). This finding was validated by analysing the different protein expression, in the different cell compartments, by performing nuclear-cytoplasmic extractions. Both the total and active phosphorylated forms of HER2 and HER3 were present in the cytoplasm and nucleus upon activation by the addition of Heregulin (**Figure 3-25**). The study of nuclear HER2 and HER3 has been quite limited and very little data exists of their presence in prostate cancer cell lines. Koumakpayi *et al.* (2006) observed an increased presence of nuclear HER3 in AR-positive cell lines and suggested a link to AR regulation. There have been reports in other cancers suggesting that these receptors could be acting as transcription factors for key survival and proliferation genes (Xie and Hung, 1994; Wang *et al.*, 2004). To investigate these findings further in prostate cancer, chromatin extraction was performed. It was observed

that upon Heregulin treatment, there was increased association of these receptors to chromatin (**Figure 3-26**). The active phosphorylated forms of HER2 and HER3 were also found to be associated with chromatin. The presence of the active forms in the nucleus and them being bound to chromatin is a novel finding using a prostate cancer cell line.

The data presented in this chapter validates the expression of HER2 and HER3 in prostate cancer and also brings new insight to their role in the advancement and sustenance of the disease. The analysis of patient samples has suggested that the disease progression is reliant on HER2 and HER3 signalling and can be significantly correlated to poor prognosis. The presence of these receptors in the nucleus also provides an interesting avenue for further research. The investigation was continued by focussing on the functionality of these receptors in prostate cancer and these findings are discussed in the next chapter.

Chapter 4 The Association of HER2 and HER3 Over-expression and Aggressive Prostate Cancer

4.1 Introduction

The development of prostate cancer is commonly a slow process and can take several years to progress (Freedland *et al.*, 2005). The tumour initially originates in the prostate gland due to increased proliferation of prostate cells and can eventually invade surrounding organs like the seminal vesicles, bladder and urethra. As the cancer progresses, the proliferating cells make use of the blood and lymphatic system to reach distal sites in the body and causing the disease to affect other organs which include liver, lungs and bones (Lynch and Lynch, 1996; Jin *et al.*, 2011). Prostate cancer is initially treated by surgery or radiotherapy and more progressive disease is then treated by androgen –withdrawal therapy. However, the latter treatment is only temporarily effective as the disease progresses and attains a more aggressive nature, causing the cancer to recur. One of the causes that has been associated with cancer relapse is the disruption in cell signalling pathways within the cancer microenvironment (Culig *et al.*, 1994; Lonergan and Tindall, 2011). There have been many signalling pathways that have been associated with the advancement of prostate cancer including the MAP Kinase and the PI3 Kinase pathways (da Silva *et al.*, 2013).

MAP Kinase and PI3 Kinase pathways are known for their role in assisting tumour growth and metastasis in many types of cancer (Dhillon *et al.*, 2007). The MAP Kinases have been known to regulate various intracellular process including gene expression, cell differentiation and cell growth. The MAPK/ERK pathway is known to be activated by receptor tyrosine kinases such as EGFR, HER2 and HER3. This activation then leads to a signalling cascade causing the downstream protein ERK to become phosphorylated which in turn leads to the activation of a variety of transcription factors that control cell proliferation, differentiation and migration (Yarden, 2001). The PI3K/AKT pathway can also be activated by EGFR, HER2 and HER3 amongst other RTKs and cytokine receptors. The functioning pathway leads to the activation of AKT which then regulates other transcription factors similarly assisting in cell survival and proliferation (Manning and Cantley, 2007).

Signalling through the EGFR, HER2 and HER3 receptors and their roles in regulating the MAP Kinase and PI3 Kinase pathways has been an interesting subject for research and has led to a deeper understanding of their molecular signatures. These receptors can be activated by a variety of ligands as explained in section **1.6.2**. Upon ligand binding, the receptors change their conformation which assists in the formation of homodimers or heterodimers. The receptors then propagate the downstream signalling process by the transphosphorylation of the dimers. Amongst all the HER receptors, HER2 is the favoured partner for dimerization (Graus-Porta *et al.*, 1997). The association of HER2 and HER3 forms the most potent heterodimer combination as HER2 allows a slow rate of ligand disassociation and receptor internalisation and HER3 allows the most efficient cross phosphorylation of the receptors and creates docking sites for the recruitment of downstream signalling components. Signalling via these receptors is prolonged and causes enhanced cellular responses such as proliferation, migration and invasion (Zaczek *et al.*, 2005; Baselga and Swain, 2009).

As previously described, the androgen receptor plays a crucial role in prostate cancer development and progression. Various theories, including the outlaw pathway, suggest that signalling mediated via receptor tyrosine kinases have a significant effect on AR activity in prostate cancer. Previous research has associated the over-expression of EGFR and HER2 with poor prognostic disease in many cancers, including prostate cancer. However, therapies targeting these receptors haven't been successful. HER3 has since become an attractive area of interest and research in other malignancies has suggested a link to the receptor's over-expression and acquiring therapeutic resistance (Sergina *et al.*, 2007; Jiang *et al.*, 2012).

One of the crucial processes required for the malignant transformation of solid tumours includes tumour cell migration and invasion and, as mentioned above, is regulated by different signalling pathways. The degradation of the extracellular matrix is an essential step in achieving metastasis (Jin *et al.*, 2011). Proteinases such as matrix metalloproteinases (MMPs) help in the degradation of the surrounding extracellular matrix and assist tumour progression. Previous studies have revealed that deregulated signalling pathways have led to increased MMP expression (Overall and Lopez-Otin, 2002).

The data collected from the previous chapter has shown increased expression of both HER2 and HER3 in advanced prostate cancer. Particularly, the observed increased expression of HER3 in advanced disease has warranted further investigation. This chapter focusses on understanding the activity and the functional importance of the HER2-HER3 heterodimer in prostate cancer. For this study, the expression levels of both these receptors have been manipulated by creating relevant cell line models and they have been assessed and validated by using a range of functional assays that are used in monitoring key cellular processes in metastatic disease

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4.2 Transient over-expression of HER2 and HER3 increases downstream signalling of PI3 Kinase and MAP Kinase pathways

The analysis of prostate cancer tissue samples and cell lines shows that HER2 and HER3 are over-expressed in this disease. To investigate their role in the disease, HER2 and HER3 stably over-expressing cell lines were established from PC3 cells, an AR negative prostate cancer cell line, expressing low levels of these proteins. It also has low activation of MAP Kinase and PI3 Kinase pathways hence serves as a good model to study the activation of these pathways when HER2 and HER3 are overexpressed. The empty vector lane shows relatively low levels of HER2 and HER3 expression which are increased by transfection of HER2 and HER3 plasmids (Error! Reference source not ound.).

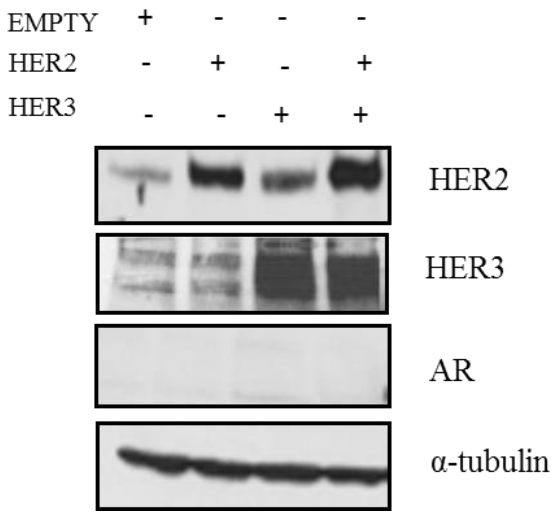


Figure 4-1: Validation of expression of HER2 and HER3 in PC3 cells

PC3 cells were forward transfected either with empty vector, pIRES-HER2, pIRES-HER3 or pIRES-HER2 and pIRES-HER3 plasmids together in full medium for 72 hours. Empty vector was used as a control and to ensure equal plasmid concentration (2 µg) was added to each experimental arm. The cells were then lysed in RIPA buffer and analysed for HER2 and HER3 expression levels The above figure is a representation of 2 repeats.

The downstream effects of HER2 and HER3 over-expression were further investigated upon Heregulin stimulation. It was observed that both the MAP Kinase and PI3 Kinase pathways showed increased signalling activity (**Figure 4-2**).

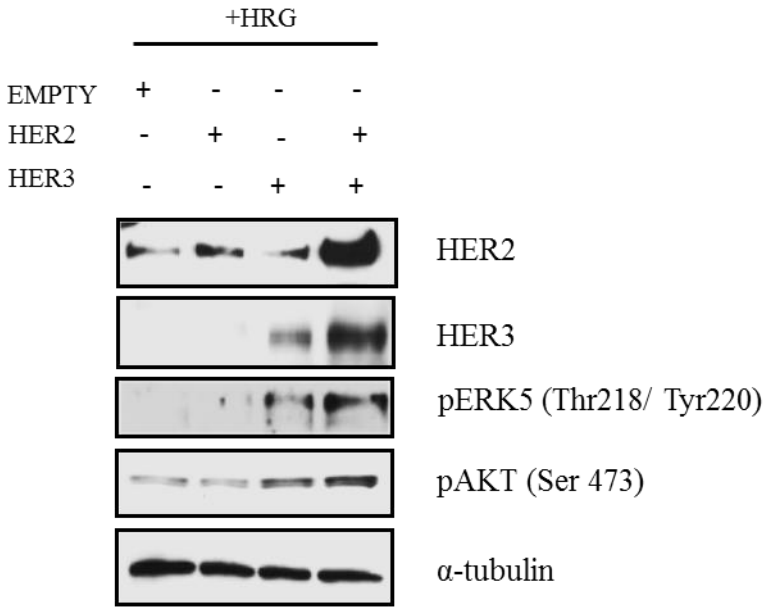


Figure 4-2: HER2 and HER3 over-expression causes increased signalling of MAP Kinase and PI3 Kinase pathways

PC3 cells were forward transfected either with empty vector, pIRES-HER2, pIRES-HER3 or pIRES-HER2 and pIRES-HER3 plasmids together in full medium. Empty vector was used as a control and to ensure equal plasmid concentration (2 µg) was added to each experimental arm. The following day the cells were starved overnight in basal medium followed by 15 minutes of Heregulin (HRG; 20 ng/ml) treatment. The cells were then lysed in RIPA buffer and analysed for HER2 and HER3 expression levels along with pERK5 and pAKT levels to observe the activity of MAP Kinase and PI3 Kinase pathways, respectively. Increased expression of both pERK5 and pAKT indicated increased signalling of their pathways caused by the over-expression of HER2 and HER3 The above figure is a representation of 2 repeats.

4.3 Generation of HER2 and HER3 over-expressing cell lines

4.3.1 Lentivirus production

Lentiviral particles containing HER2 and HER3 were produced as mentioned in **2.14.1**. Briefly, HER2 and HER3 were amplified from pIRES plasmids using a forward primer that contains at 5' – CACC overhang sequence to promote directional cloning into the pENTR/D-TOPO vector and it was ensured that the sequence did not contain the stop codon to retain the sequence in-frame with the C-terminal V5 tag following recombination into pLenti6/V5-DEST vector. HER2 and HER3 amplification was verified by agarose gel electrophoresis and sequencing (**Figure 4-3 (A)**). Primers were designed to verify the gene sequence by referring to The National Center for Biotechnology Information (NCBI) database. HER2 (NCBI Reference Sequence -

M11730.1) amplicon was confirmed to be 3869 bp and HER3 (NCBI Reference Sequence - NM_001982.3) as 4097 bp in size.

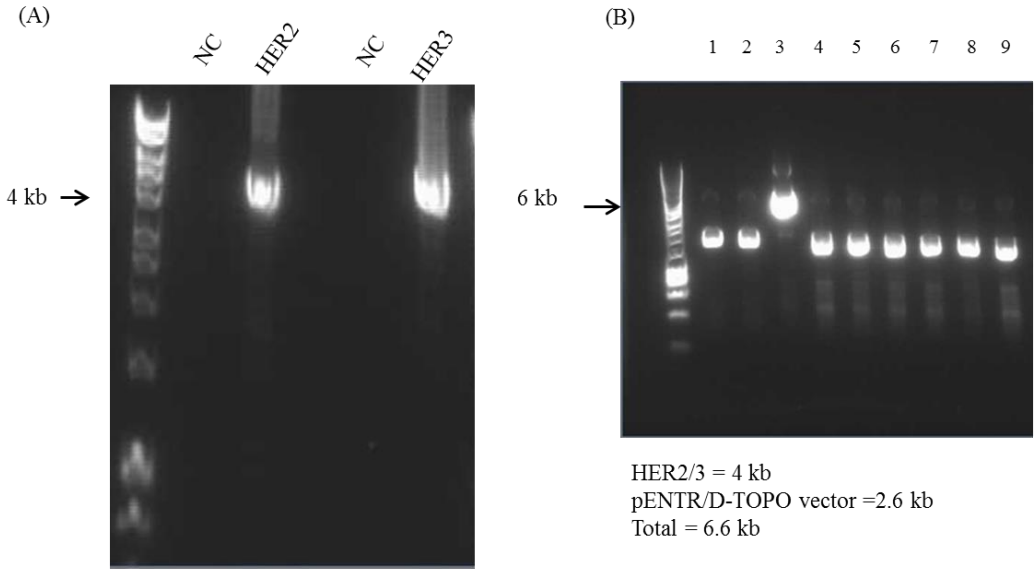


Figure 4-3: Confirmation of PCR and cloning by agarose gel electrophoresis

(A) PCR amplicons of HER2 and HER3 along with their respective negative controls were run and visualised on a 1% agarose gel (B) The HER2 and HER3 amplicons were cloned into the pENTR/D-TOPO vector. The transformed clones (lanes1-9) were run on an agarose gel and the positive clones (Lane 3) were selected and used for further analysis.

After cloning into the pENTR/D-TOPO vector, positive clones were identified by agarose gel electrophoresis (**Figure 4-3 (B)**) and were then mini-prepped and the directional cloning and gene sequence was confirmed by Sanger gene sequencing by using primer sequences that covered the entire gene. A confirmed clone was then incubated with the pLenti6/V5-DEST vector to clone the gene from the pENTR/D-TOPO vector by homologous recombination. Clones were transformed, selected by antibiotic selection, mini-prepped and confirmed by agarose gel electrophoresis, restriction digestion (**Figure 4-4**) and Sanger sequencing. The correct clone was then used to generate viral particles.

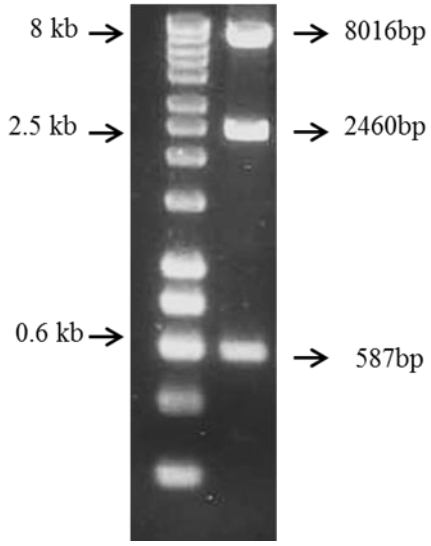


Figure 4-4: Confirmation of directional cloning by restriction digestion.

The sequences of the gene and the vector were analysed for restriction sites using the online tool restriction mapper. 1µg of pLenti6/V5-DEST with the insert was then digested using the restriction enzyme *XhoI* according to the guidelines. Successful digestion would yield 3 fragments of sizes 8016bp, 2460bp and 587bp if the insert was integrated in the correct direction as seen above.

4.4 Profiling established cell lines

The titre for the virus was established and PC3-LACZ, -HER2 and -HER3 lines were generated as mentioned in (**2.14**). Briefly, the respective viruses were transduced to PC3 cells with the same MOI and the transduced cells were selected by Blasticidin antibiotic selection.

4.4.1 Validating HER2 and HER3 expression in the established stable cell lines

The V5 antibody was first validated to detect the over-expressed proteins. Whole cell lysates were analysed by western blotting using the V5 antibody. However, the antibody presented with a non-specific band in the non-transduced sample at approximately the same size of the HER2 and HER3 proteins as seen in **Figure 4-6**. For this reason, only total HER2 and HER3 antibodies have been used to investigate the protein expression in the over-expressing cell lines. Over-expression of HER2 and HER3 was confirmed in the cell lines (**Figure 4-7**) after which the cells were analysed to check for any functional differences.

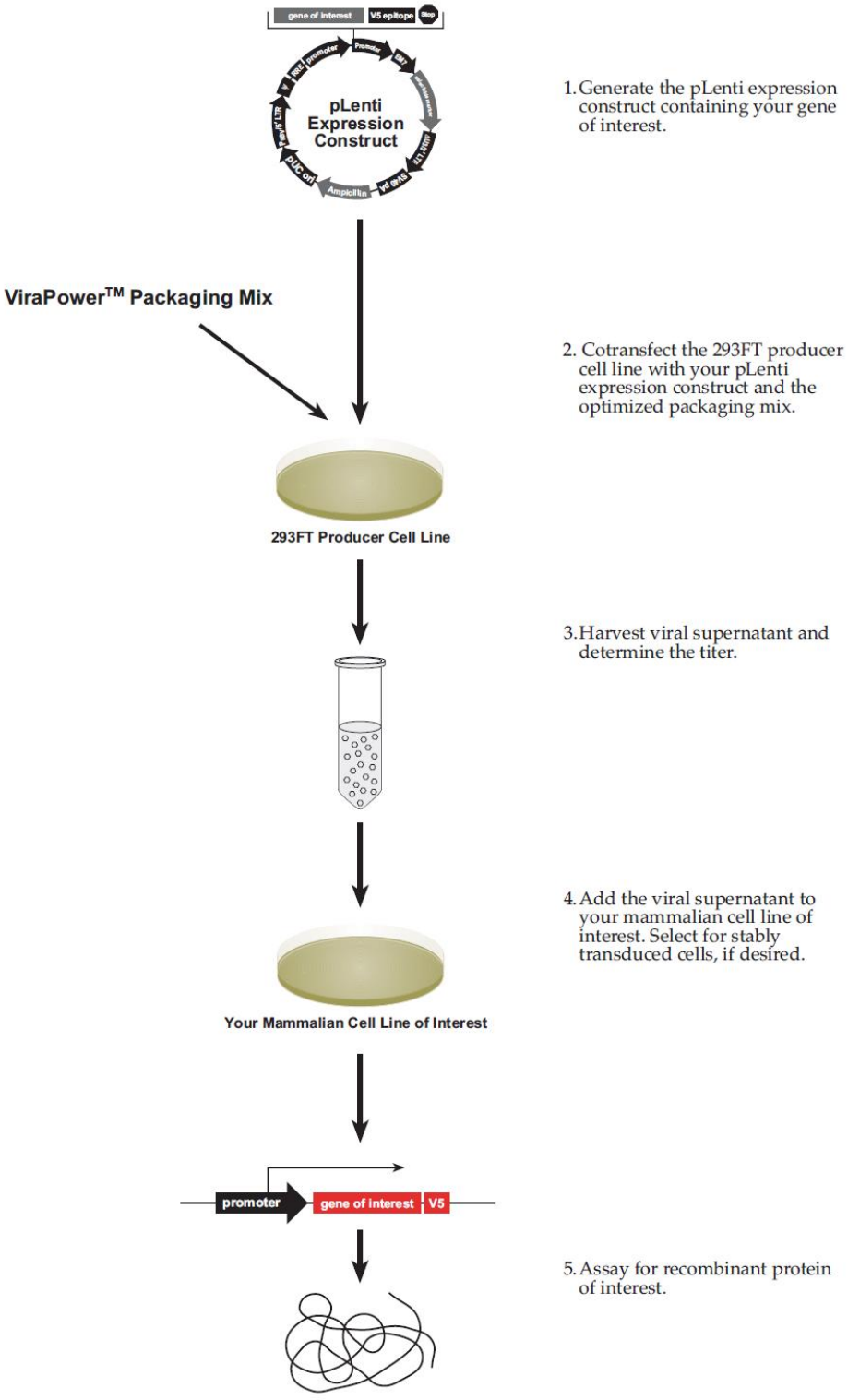


Figure 4-5: Schematic of lentiviral production using the pLenti expression system (Invitrogen)

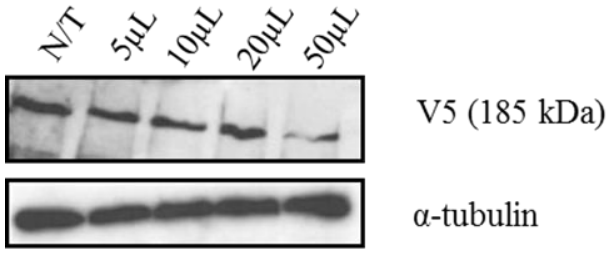


Figure 4-6: Western blot using the V5 antibody.

PC3 cells were transduced with increasing amounts of HER3 lentivirus. The next day the medium was changed and the cells were allowed for grow for another 24 hours after which the cells were lysed in RIPA buffer and analysed by western blotting. Unfortunately, this antibody could not be used in subsequent experiments as the non-transduced sample presented with a non-specific band at approximately the same size of HER2 and HER3.

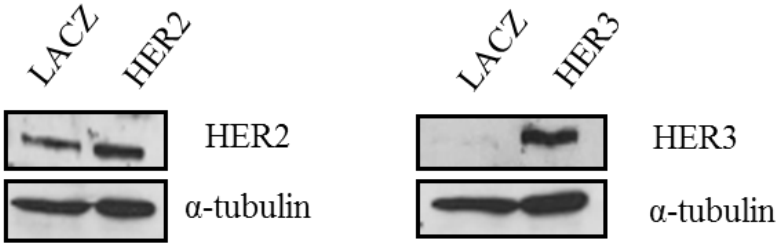


Figure 4-7: Western blot analysis confirming over-expression of HER2 and HER3.

Over-expressing cell lines were seeded out in 6-well plates. The next day, the medium was changed with selection medium and the cells were allowed to grow for 24 hours after which they were lysed in RIPA buffer and the whole cell lysates were analysed for total HER2 and HER3 expression The above figure is a representation of 2 repeats.

4.4.2 HER2 and HER3 over-expression leads to increased PI3 Kinase and MAP Kinase signalling

Potential functional changes due to the increased expression of HER2 and HER3 were analysed by observing the changes in their signalling pathways. The cell lines were treated with Heregulin and the downstream proteins of the PI3 Kinase and MAP Kinase pathways were analysed. An increase in pAKT and pERK5 levels was observed in the over-expressing cell lines (**Figure 4-8**) suggesting that these cell lines maintain higher signalling activity that are controlled by HER2 and HER3.

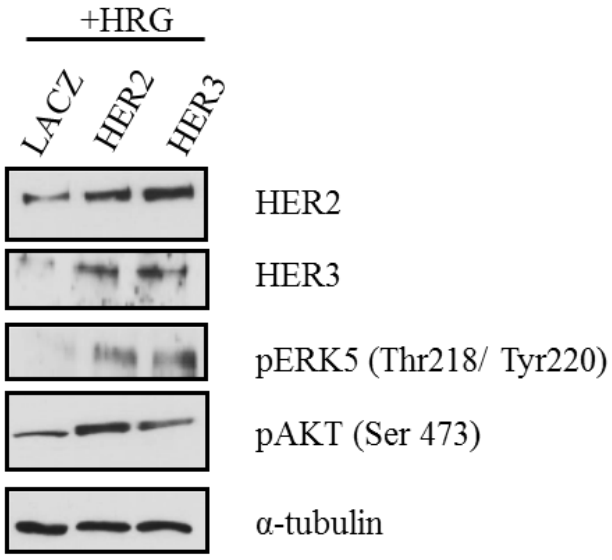


Figure 4-8: HER2 and HER3 over-expression leads to increased PI3 Kinase and MAP Kinase signalling.

PC3-LACZ, -HER2 and -HER3 over-expressing lines were seeded out in full medium. The following day, the cells were starved overnight in basal medium and the next day they were treated with Heregulin (HRG; 20ng/ml) for 15 minutes. The cells were then lysed in RIPA buffer and the lysates were analysed for protein expression using western blotting. The figure shows the over-activation of both HER2 and HER3 along with that of pERK5 and pAKT. The above figure is a representation of 2 repeats.

4.4.3 HER2 and HER3 over-expression leads to increased cell growth

Any changes in cell growth due to expression of HER2 and HER3 were analysed by performing SRB assays. The PC3-LACZ line was used as a control. The analysis revealed increased cell growth in HER2 and HER3 over-expressing cell lines (**Figure 4-9**). This result was further validated by performing cell count changes over 96 hours of proliferation (**Figure 4-10**).

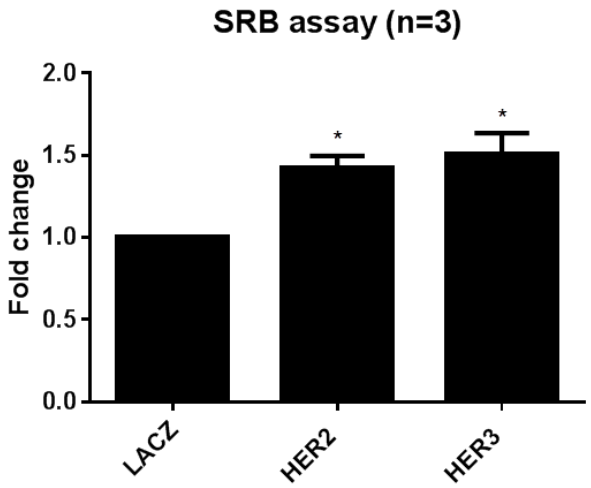


Figure 4-9: HER2 and HER3 over-expression causes increased cell growth

Briefly, the different cell lines were seeded out in a 96-well plates. The cells were allowed to grow for 96 hours in full medium after which the cells were fixed with chilled 50% trichoroacetic acid (TCA) for 1 hour at 4 ºC. The cells were then washed with tap water, dried and stained with 0.4% SRB in 1% acetic acid for 30 minutes at room temperature. Excess staining was prevented by rinsing the cells in 1% acetic acid. The retained dye on the cells was then dissolved in 10mM Tris (pH 10.8) and the absorbance was recorded at 570nm. The experiment was repeated 3 times and the data was normalised to PC3-LACZ. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05 when compared to the control LACZ line.

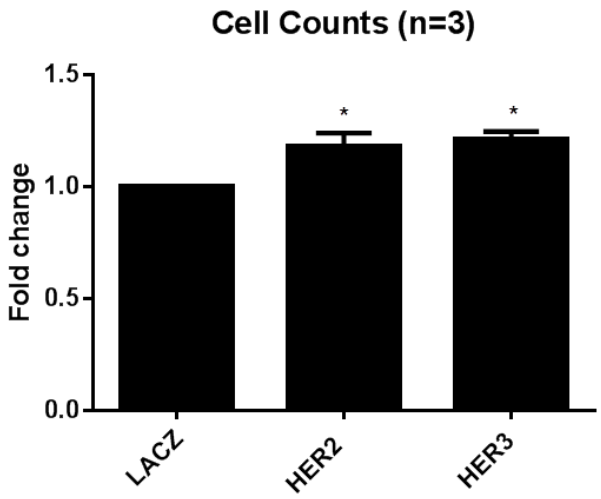


Figure 4-10: HER2 and HER3 over-expression causes increased cell proliferation

Briefly, 100,000 cells of each cell line were seeded in full medium on a 6-well plate. The following day, the medium was changed to selection medium and the cells were left to grow for 96 hours. The cells from each cell line were then counted using a haemocytometer. Each cell line was counted 3 times and the experiment was performed 3 times. The changes in proliferation were calculated using GraphPad Prism. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the control LACZ line.

4.4.4 HER2 and HER3 over-expression leads to an increase in cell migratory potential

Since HER2 and HER3 are over-expressed in advanced prostate cancer, it was hypothesised that these proteins could have an impact on cell migration. To test this hypothesis, the association between the differential expression of HER2 and HER3 and matrix metallopeptidase 9 (MMP-9) was investigated.

Matrix metallopeptidase 9 (MMP-9)

MMP-9 belongs to the family of proteases called matrix metalloproteinase (MMP). These proteins are involves in breaking down the extracellular matrix. Their activity has been linked with cancer, immunological and cardiovascular diseases. In cancer, they initiate tumour migration, invasion and angiogenesis by degrading the extracellular matrix and collagen IV in the basement membrane (Groblewska *et al.*, 2012).

4.4.5 HER2 and HER3 over-expression increases MMP-9 transcription

The activity of MMP-9 was assessed by a luciferase reporter assay. This was performed by using a construct that had the luciferase gene linked downstream to the MMP-9 promoter. PC3 cells were used for this experiment and they were transfected with either empty vector as control or HER2 and HER3 plasmids. MMP-9 transcription was found to be increased in the presence of HER2, HER3 and both receptors (**Figure 4-11**).

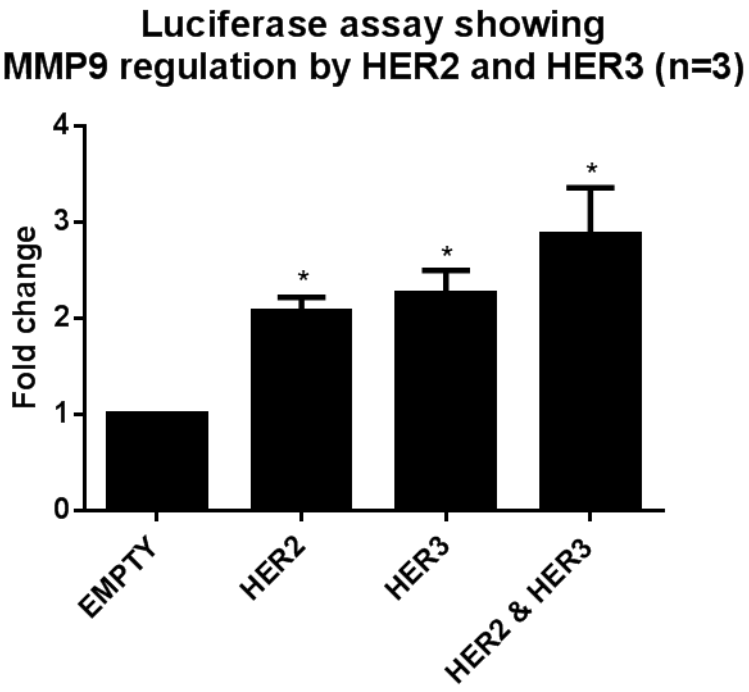


Figure 4-11: HER2 and HER3 increases MMP-9 promoter activity

PC3 cells were seeded out in quadruplets per experimental arm using a 24-well plate and were forward transfected with 100 ng of MMP9, 50 ng of βgal and 300 ng of HER2 and HER3. pCMV empty vector was used for the control and to ensure equal amounts of plasmid were added to all the experimental arms. The cells were then starved in basal medium overnight and treated with Heregulin (20 ng/ml) for 24 hours to activate HER2 and HER3 receptors. The cells were then lysed and luciferase substrate was added. The emitted luciferase counts were measured using a plate reader. A β-Gal assay was performed on the same samples and the data obtained was used to normalise for luciferase activity. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the empty vector control. MMP-9 promoter activity was increased in the presence of HER2, HER3 and both receptors.

This was further investigated by performing migration assays using Boydon chambers (**2.17**). PET (polyethylene terephthalate) membrane inserts with a pore size of 8 µm were used. The cells were seeded at the top of the chamber and allowed to migrate towards the chemoattractant which was placed in the bottom compartment of the chamber. The result revealed that the over-expressing lines did possess higher migratory potential compared to the control LACZ control cell line (**Figure 4-12**).

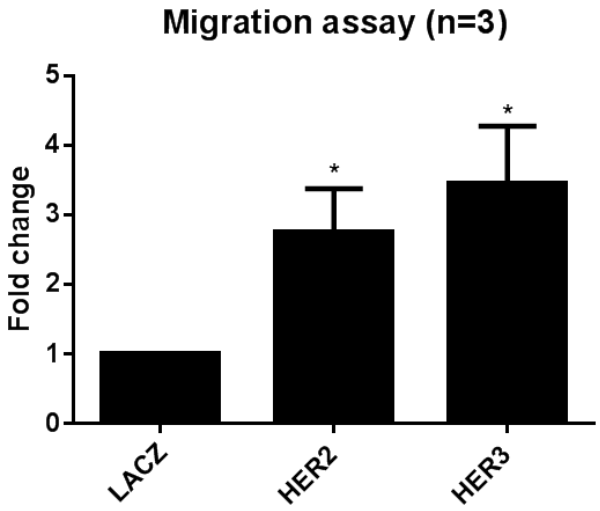


Figure 4-12: HER2 and HER3 over-expression causes an increase in cell migration

The over-expressing stable cell lines were used for performing migration assays using Boydon chambers. PET membrane inserts with a pore size of 8 µm was used. 600 µl of basal medium with Heregulin (20 ng/ml) was added per well and the inserts were placed on top. $3x10^4$ cells were seeded in a final volume of 300 µl in the upper chamber. The cells were allowed to migrate for 48 hours. The cells that hadn't migrated were removed using a cotton bud and the cells that had migrated were fixed on the membrane using methanol, stained with crystal violet, mounted on a slide and counted. Each arm of the experiment and the entire experiment itself was performed in triplicate and normalised to the control LACZ line. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05.

4.4.6 HER2 and HER3 over-expression leads to an increase in cell invasion potential

Potential changes to the cell's invasion capability due to the increased activity of HER2 and HER3 were also investigated. The experiment was similarly conducted, except that the Boyden chambers were coated with matrigel containing extracellular matrix proteins to mimic *in-vivo* conditions (**2.17**). As expected, the experiment proved that HER2 and HER3 over-expression can correlate to an increase in cell invasion potential, when compared to the LACZ control line (**Figure 4-13**).

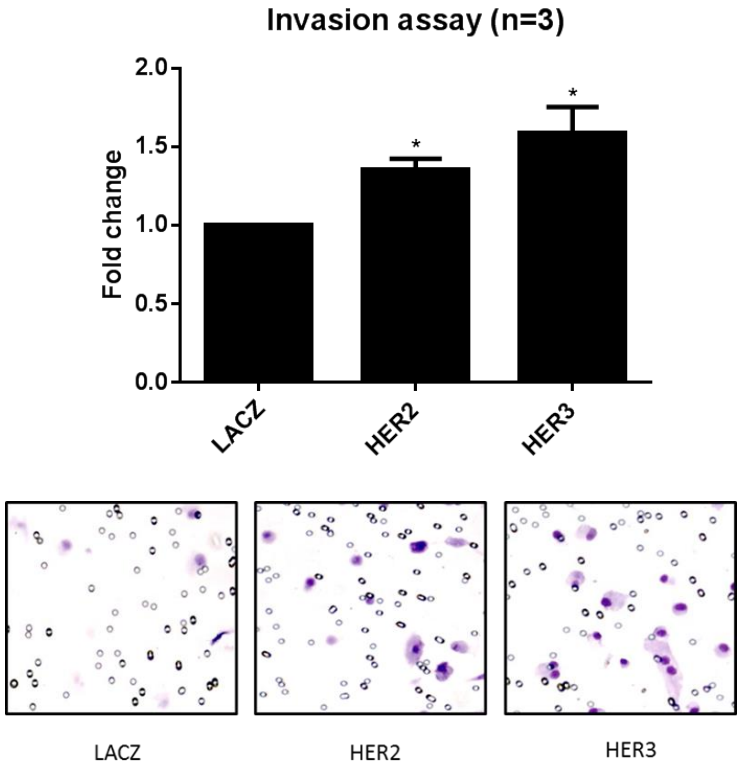


Figure 4-13: HER2 and HER3 over-expression increases cell invasion potential

The over-expressing stable cell lines were used for performing invasion assays using Boydon chambers. PET membrane inserts with a pore size of 8 µm was used. The chambers were coated with matrigel. 600 µl of basal medium with Heregulin (20 ng/ml) was added per well and the inserts were placed on top. $3x10^4$ cells were seeded in a final volume of 300 µl in the upper chamber. The cells were allowed to migrate for 48 hours the cells that hadn't invaded were removed using a cotton bud and the cells that had invaded were fixed on the membrane using methanol, stained with crystal violet, mounted on a slide and counted. Each arm of the experiment and the entire experiment itself was performed in triplicate and normalised to the control LACZ line. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05.

4.5 Validating HER2 and HER3 functionality *in-vivo*

After observing the increase in proliferation, migration and invasion the next step involved validating the findings of the differentially functional cell lines *in-vivo*. Athymic CD1 nude mice were injected with either PC3-LACZ, -HER2 or -HER3 over-expressing cell lines, used for the formation of xenografts (**2.15.1**). The *in-vivo* experiment revealed no differences in tumour size between the cell lines (**Figure 4-14**).

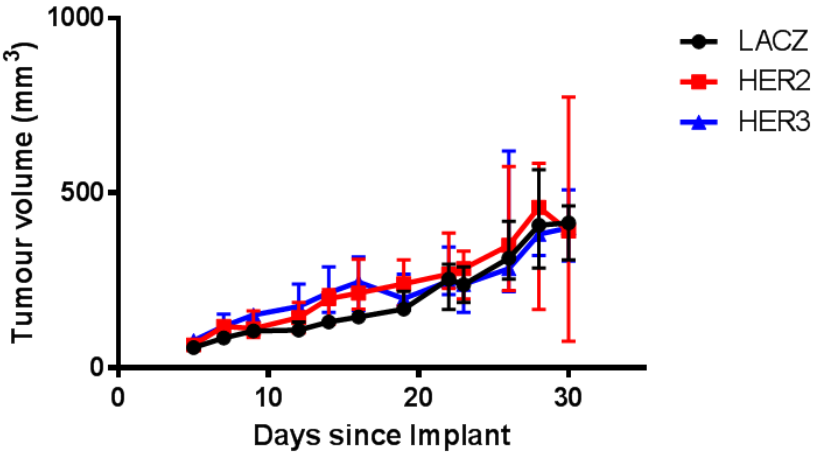


Figure 4-14: *in-vivo* experiment revealing growth rates of xenografts established from different cell lines

10 mice were used per experimental arm. A total of 1.1 x 10^7 cells of PC3-HER2, -HER3 and -LACZ cell lines in full medium were subcutaneously injected into the flanks of the athymic CD1 nude mice in a final volume of 50 µl (10 mice per cell line). The mice were inspected thereafter for signs of tumour development. The tumour sizes were measured 3 times a week with callipers and recorded. The study was stopped as soon as the tumours reached a size of 10 x 10 mm or > 15 mm in one dimension. The tumour volume was calculated by using the following formula $\frac{length}{2} * (width)^2$

The resected tumours were used for further analysis. A mixed population of tumours was used to establish cell lines (**2.15.1**). Blasticidin was incorporated in the media to prevent the culture of mouse cells. The tumours were also fixed appropriately and were used to validate protein expression through immunohistochemistry and western blotting. The cell lines established from the xenografts were used to confirm the over-expression of the receptors. The expression was compared in two different conditions. The first condition was comparing the expression of HER2 and HER3 in their normal growth medium i.e. full medium (FM). The other condition involved starving the cells in basal medium overnight followed by Heregulin stimulation. Interestingly, differential

expression was only observed when the cells were treated with Heregulin (**Figure 4-15**).

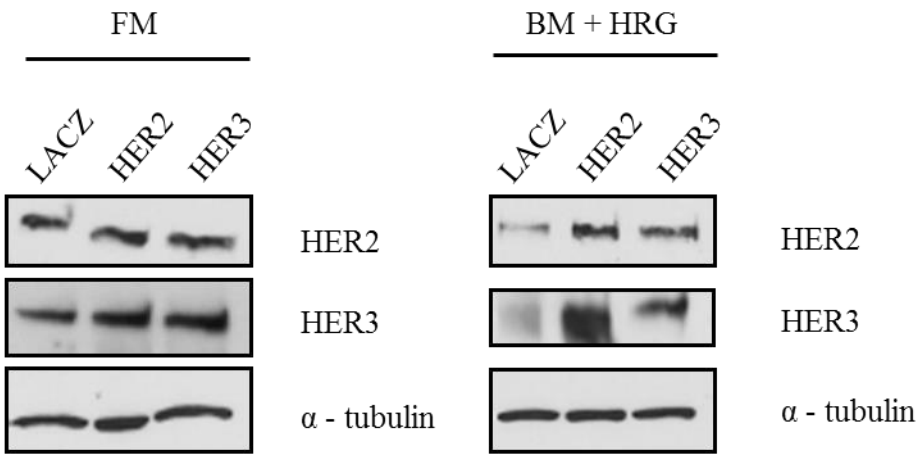
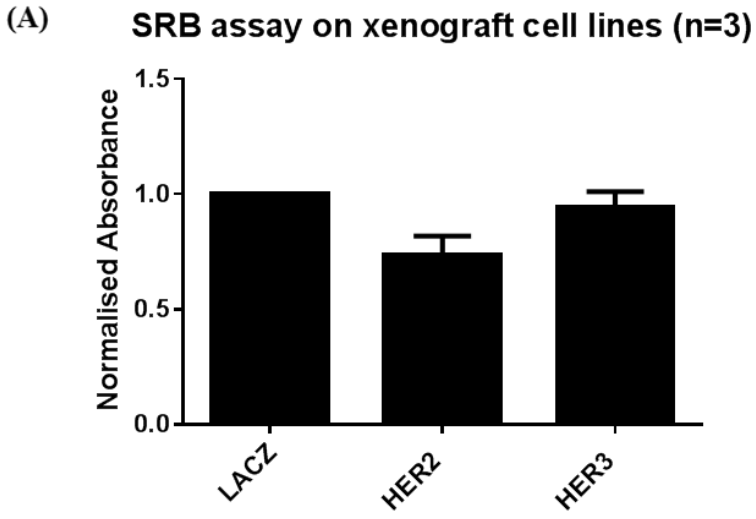


Figure 4-15: Differential expression of HER2 and HER3 under +/- Heregulin stimulated conditions in cell lines established from xenografts

The cells were seeded down in full medium and either left alone or starved in basal medium overnight and treated with Heregulin (HRG; 20ng/ml) for 15 minutes. The cells were then lysed in RIPA buffer and analysed for HER2 and HER3 expression by western blotting. The above result is a representation of 2 repeats

The proliferation assays were also performed as described before, on the xenograft cell lines to observe if there was any variability before and after implantation. From the results obtained (**Figure 4-16**) it was noted that the cells behaved differently post implantation as there was no increased proliferation in the HER2 and HER3 over-expressing cell lines as seen before.



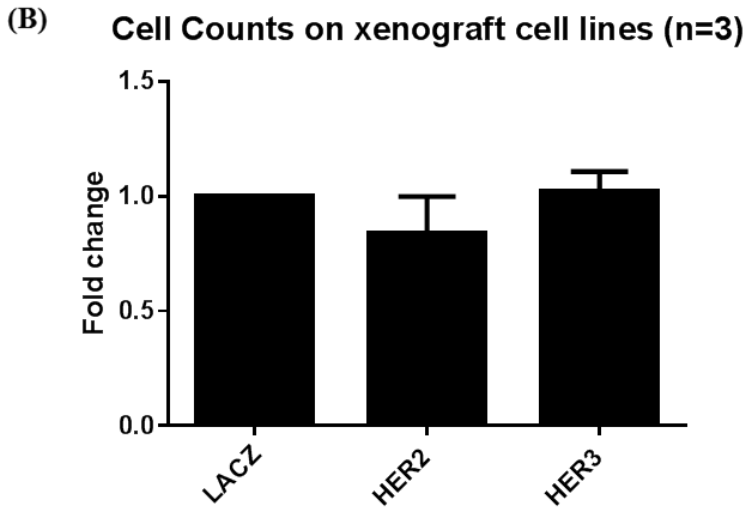


Figure 4-16: Proliferation assays revealing no significant change in proliferation in the PC3-LACZ, -HER2 and -HER3 over-expressing cell lines which were established from xenografts

(A) The xenograft cell lines were seeded on 96-well plates in selection medium. The cells were allowed to grow for 96 hours after which the cells were fixed with chilled 50% TCA for 1 hour at 4 ºC. The cells were then washed with tap water, dried and stained with 0.4% SRB in 1% acetic acid for 30 minutes at room temperature. The cells were then rinsed in 1% acetic acid to remove excess dye. The dye on the cells were dissolved in 10mM Tris (pH 10.8) and the absorbance was recorded at 570nm. The experiment was repeated 3 times and the student t-test performed on the data. There were no significant differences compared to the control LACZ line.

(B) 100,000 cells of each cell line were seeded in full medium on a 6-well plate. The following day, the medium was changed to selection medium and the cells were left to grow for 96 hours. The cells from each cell line were then counted using a haemocytometer. Each cell line was counted 3 times and the experiment was performed 3 times. The changes in proliferation were calculated using GraphPad Prism. Student t-test was performed on the experiments. There were no significant differences compared to the control LACZ line.

The initial profiling experiments were performed in normal growth conditions (**Figure 4-9**, **Figure 4-10**). However, the cells from the xenografts showed no over-expression in normal growth conditions (**Figure 4-16**). These differences were further investigated in resected tumours. It was observed that the tumours had varied sizes and hence it was chosen to analyse the smallest and the largest tumours obtained from the different cell line xenografts.

IHC analysis revealed that the larger tumours had stronger expression of HER2 and HER3 (**Figure 4-17** and **Figure 4-18**). There was also strong nuclear staining of HER3

in the larger tumours. This finding led to an investigation of potential differences in the downstream targets of HER2 and HER3 signalling. The same tumour samples were stained for pAKT as an indicator of active PI3-Kinase signalling. The result indicated that the pathway was more active in the larger tumours which was correlated to the high expression of HER2 and HER3 (**Figure 4-19**).

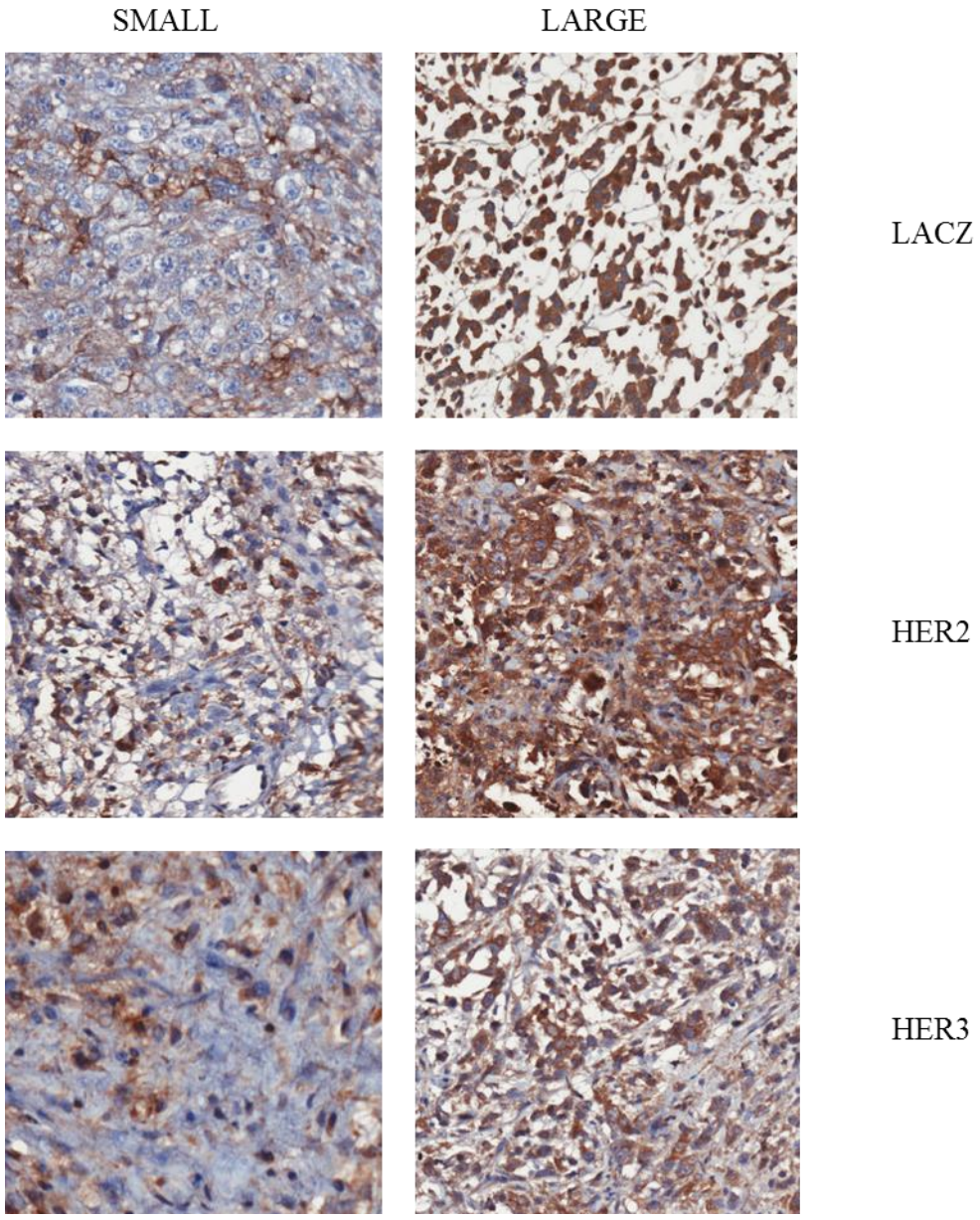


Figure 4-17: Expression of HER2 in xenografts of different sizes

Immediately after extraction, the xenografts were fixed overnight in 10% formalin buffered to pH–7. They were then embedded in paraffin blocks after they were trimmed and processed in graded ethanol and xylene using the routine processing and embedding equipment. Sections of 5µm thickness were cut using a standard microtome and placed onto slides. IHC was performed for HER2 on the sections as mentioned in (**2.2.4**).

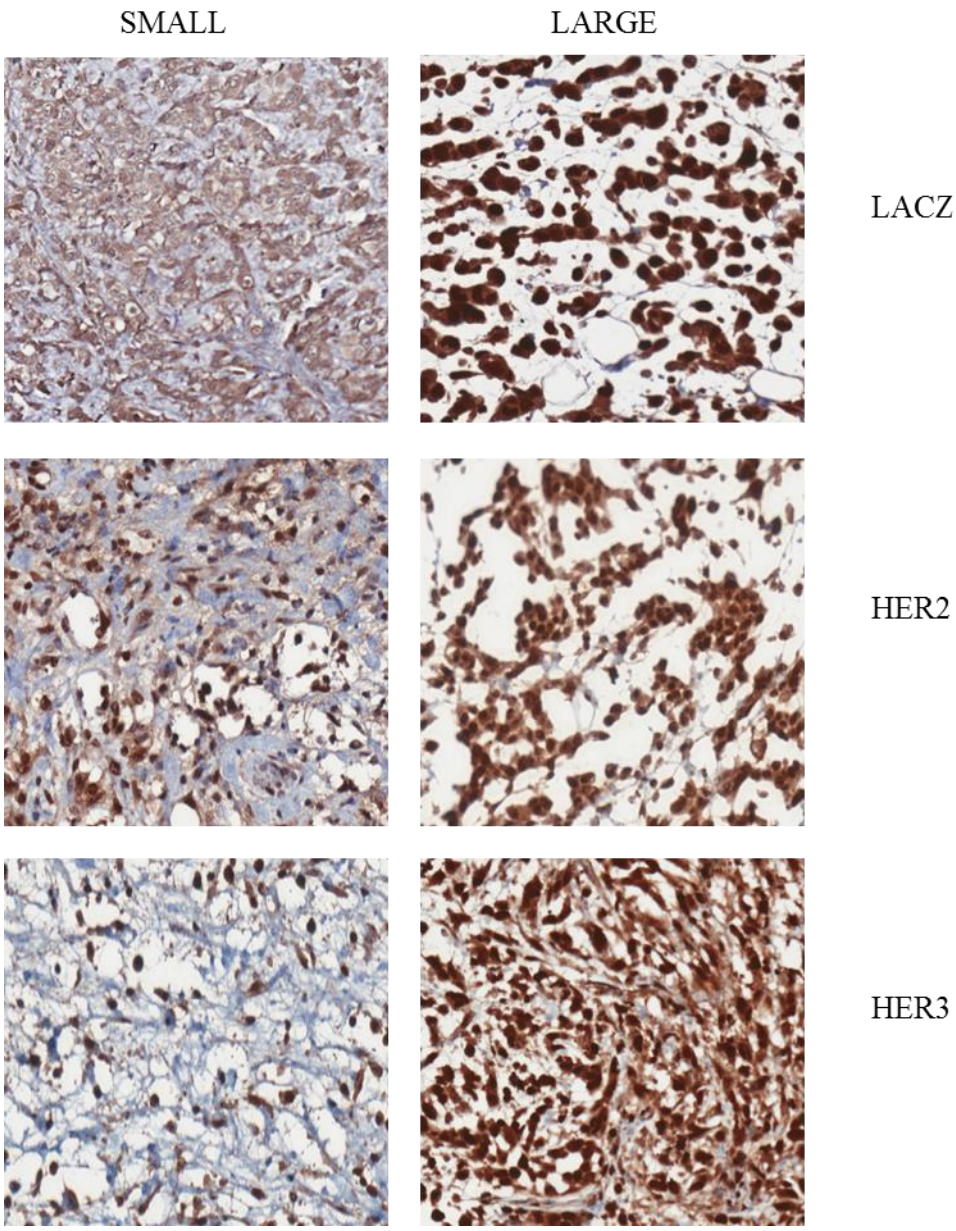


Figure 4-18: Expression of HER3 in xenografts of different sizes

Immediately after extraction, the xenografts were fixed overnight in 10% formalin buffered to pH–7. They were then embedded in paraffin blocks after they were trimmed and processed in graded ethanol and xylene using the routine processing and embedding equipment. Sections of 5μm thickness were cut using a standard microtome and placed onto slides. IHC was performed for HER3 on the sections as mentioned in (**2.2.4**).

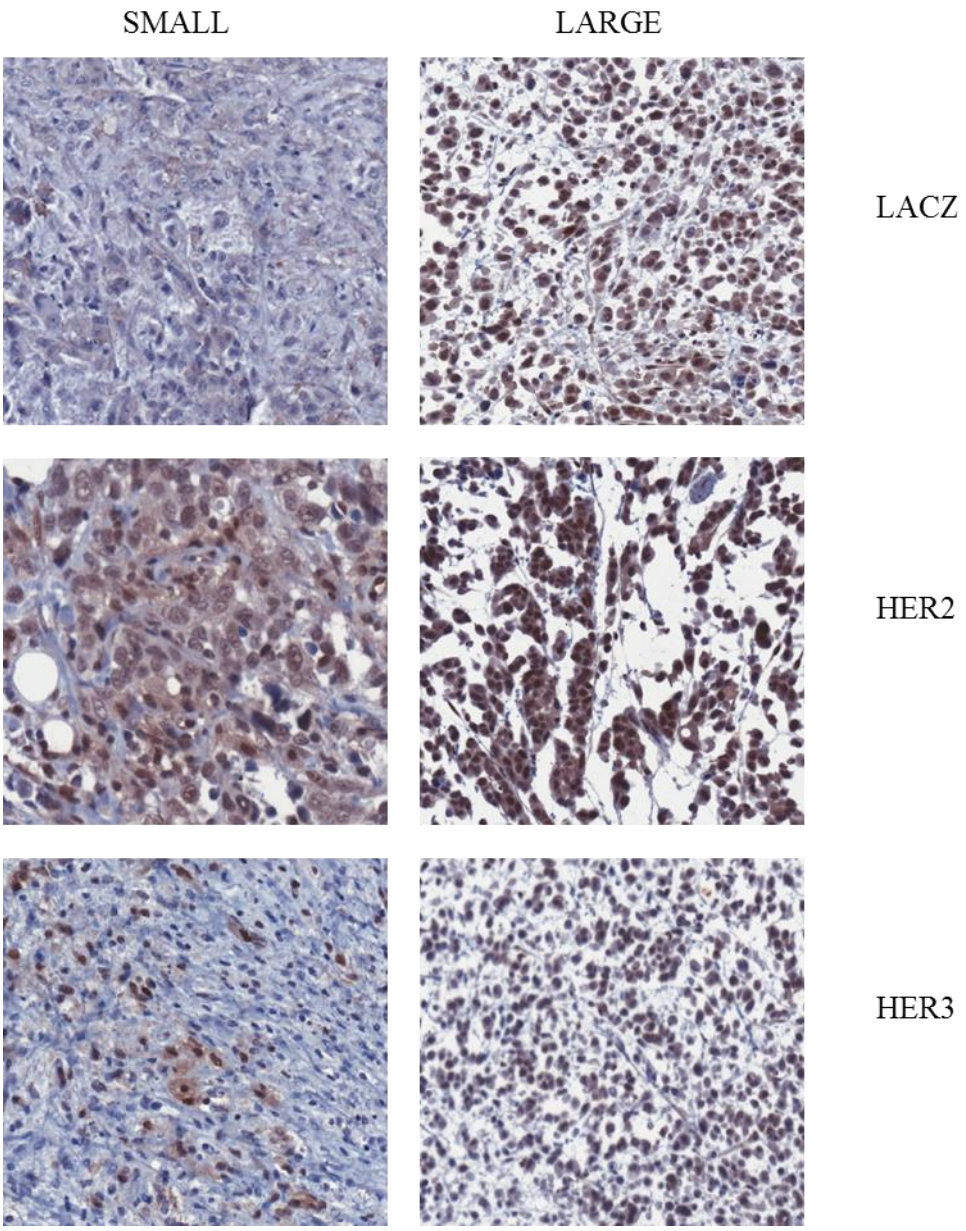


Figure 4-19: Expression of pAKT in xenografts of different sizes

Immediately after extraction, the xenografts were fixed overnight in 10% formalin buffered to pH–7. They were then embedded in paraffin blocks after they were trimmed and processed in graded ethanol and xylene using the routine processing and embedding equipment. Sections of 5µm thickness were cut using a standard microtome and placed onto slides. IHC was performed for pAKT on the sections as mentioned in (**2.2.4**).

4.6 HER2 and HER3 over-expression increases AR activity and expression

4.6.1 AR activity increases through HER2-HER3 mediated signalling

The LNCaP-7B7 cell line was used to test the effect of HER2 and HER3 signalling on AR activity. This cell line was derived from the LNCaP cell line and stably over-expresses the luciferase gene downstream the PSA promoter (**2.3.2**) which can be used to measure promoter's activity. The FM and the SDM treated samples were compared to ensure that the cells were starved by observing the decrease in the PSA promoter's activity (**Figure 4-20 (A)**). The same experiment was conducted using the LNCaP-AI 7B7 cell line. This cell line was established from the LNCaP-AI cell line that grows in steroid depleted media. A luciferase gene downstream to the PSA promoter was similarly incorporated in this cell line and was used as a model to investigate the effect of HER2 and HER3 signalling on AR activity in androgen independent disease. The results revealed increased activity of PSA promoter with Heregulin treatment in both androgen dependent (**Figure 4-20 (B)**) and independent cell lines (**Figure 4-20 (C)**).

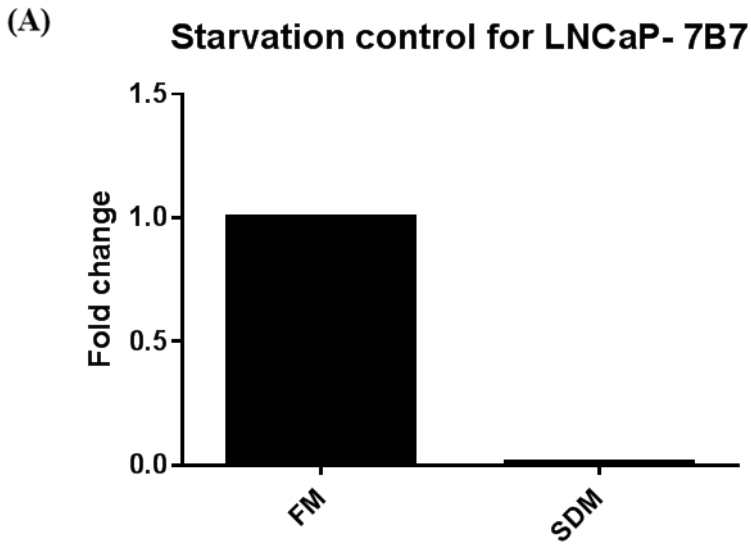


Figure 4-20: HER2-HER3 mediated signalling increases AR promoter activity

(A) LNCaP-7B7 cells were seeded out in 24-well plates and starved in SDM for 3 days as a control for AR activity. After starvation, the cells were lysed in reporter lysis buffer and luciferase substrate was added to measure the luciferase activity. The luciferase counts per second were measured using the plate reader. The data was normalised to the protein concentration per well by performing a BCA assay.

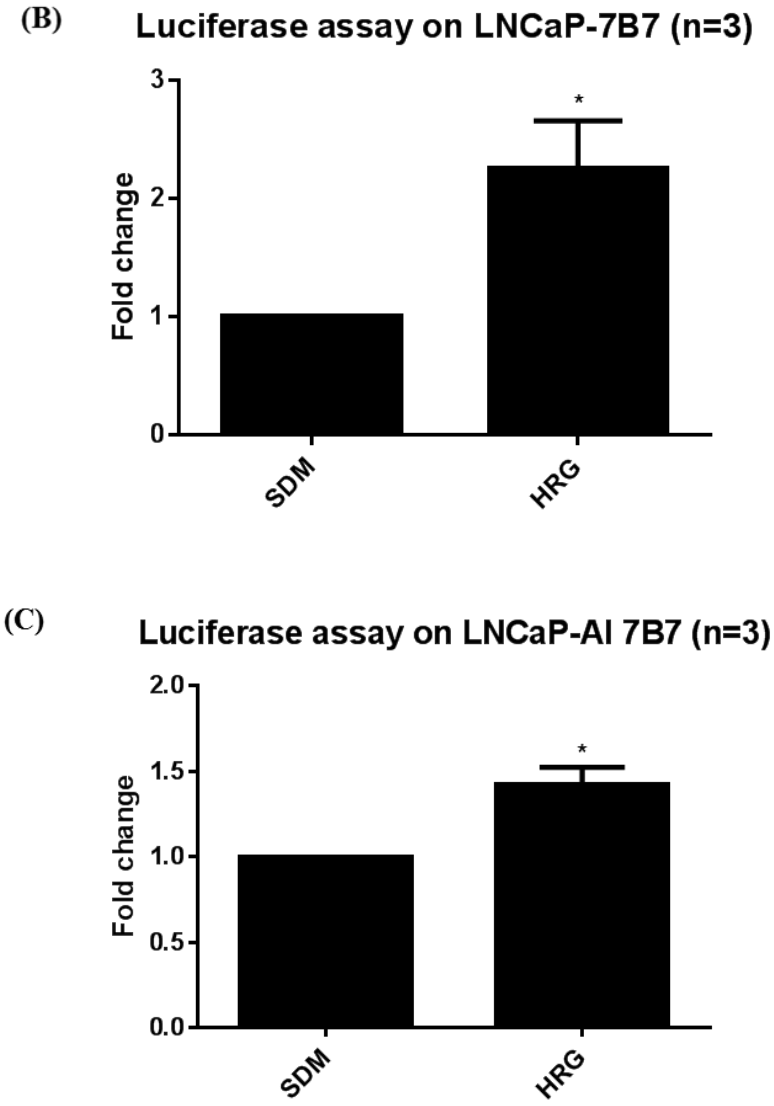


Figure 4-20: HER2-HER3 mediated signalling increases AR promoter activity

(B) LNCaP-7B7 cells were starved as described above, followed by Heregulin (HRG; 20ng/ml) treatment for 16 hours. The cells were then lysed and analysed as explained.

(C) For LNCaP-AI 7B7 cells, the cells were seeded out in their normal growth medium (SDM) and treated with Heregulin (HRG; 20ng/ml) for 16 hours the next day. The cells were similarly lysed and analysed.

Student t-test was performed on all the experiments and * indicates statistical significance with p-value <0.05.

To prove that this activity was indeed through the AR, the cells were also treated with the drug Enzalutamide, an AR antagonist. Minimal activity of the PSA promoter was observed when the cells were treated with Enzalutamide (**Figure 4-21**).

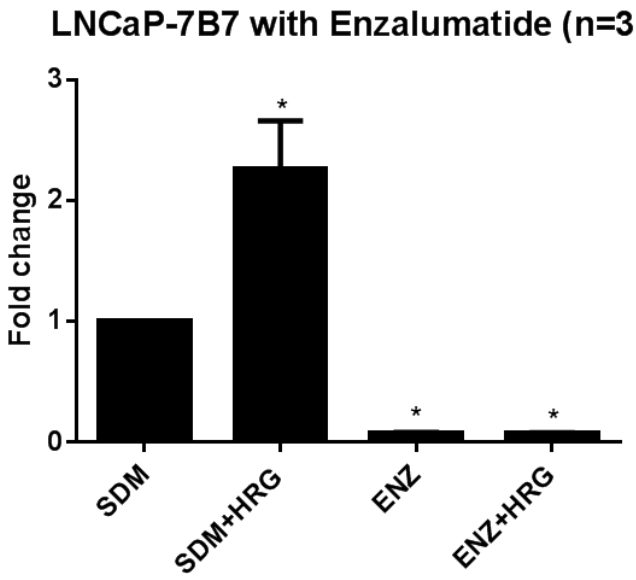


Figure 4-21: The effect of Heregulin on the PSA promoter acts through the androgen receptor.

The cells were seeded out in 24-well plates, starved in SDM for 3 days. 16 hours prior to Heregulin treatment, the cells were treated with either Enzalutamide (ENZ) (10 µM) or DMSO as control. The cells were then treated with Heregulin (HRG; 20ng/ml) for an additional 16 hours. The cells were then lysed in reporter lysis buffer and luciferase substrate was added to measure the luciferase activity. The luciferase counts per second were measured using the plate reader. The data was normalised to the protein concentration per well by performing a BCA assay (**2.7.2**). Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05.

4.6.2 AR expression increases with HER2 and HER3 mediated signalling

An investigation of the effect of HER2 and HER3 on androgen receptor was continued by transiently over-expressing HER2 and HER3 in the AR-negative PC3 cells. These cells were then transfected with equal amounts of the androgen receptor and the changes in protein expression under androgen and Heregulin stimulation were observed (**Figure 4-22(A)**). AR expression increased in the presence of DHT and Heregulin. This finding was further verified by transfecting the AR in the PC3-HER2 and -HER3 over-expressing cell lines, using the PC3-LACZ cell line as a control (**Figure 4-22 (B)**).

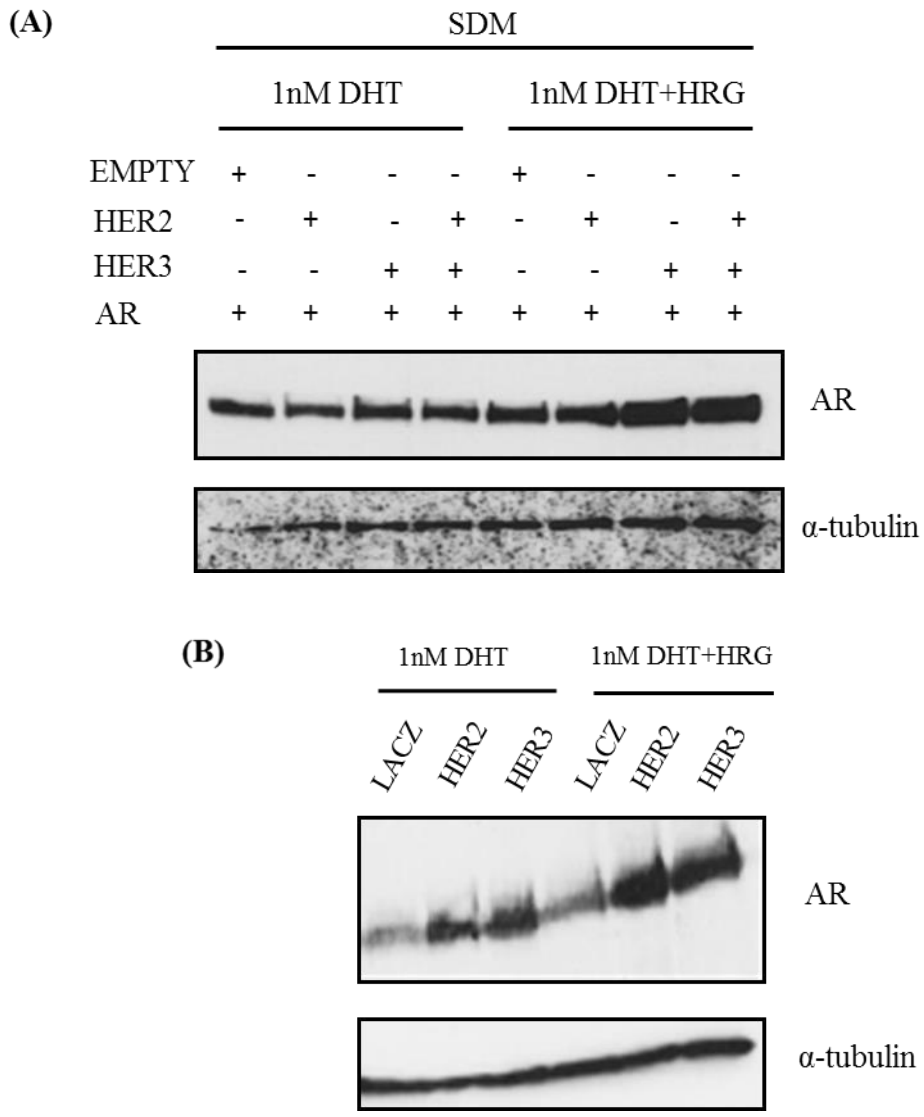


Figure 4-22: AR expression increases in the presence of Heregulin and low levels on androgens

(A) PC3 cells were seeded down in full medium and transfected with equal amounts of AR, empty vector control, HER2 and HER3. The final amount of plasmid going into each arm was consistent and was balanced using the empty vector. The cells were then starved in SDM for 3 days after which they were treated with either 1nM DHT alone or with both DHT and Heregulin (HRG; 20ng/ml) for 24 hours. The cells were then lysed in RIPA buffer and AR protein expression was analysed using western blotting. (B) In this experiment equal amount of androgen receptor was transfected between the different over-expressing cell lines and the difference in its expression was observed under similar conditions as mentioned above. The above figure is a representation of 2 repeats.

4.7 Discussion

There have been many signalling pathways implicated in the malignant transformation of prostate cancer. The findings of the previous chapter, i.e. the observed over-expression of HER2 and HER3 in patient samples, suggests that the activation of these receptors could be leading to sustained signalling of their regulated pathways, thus ultimately leading to favourable conditions for tumour growth and survival. The analysis of the clinical samples also revealed that differential expression of HER2 and HER3 could be associated with the varied survival outcomes of the patients. Hence, it was deemed relevant to establish an appropriate model to study the individual effects of these receptors. For this purpose, the PC3 cell line was chosen as it expresses low levels of HER2 and HER3. It is also a cell line that can be easily transfected and this was verified with the use of HER2 and HER3 plasmids as seen in **Error! Reference source ot found.**. This experiment validated that the expression levels of HER2 and HER3 could be individually manipulated in PC3 cells. The downstream effects of this increased expression were also investigated and it was noted that upon Heregulin stimulation both the MAP Kinase (pERK5) and the PI3 Kinase (pAKT) pathways were activated as seen in **Figure 4-2**. The same samples were also analysed for pERK1/2 but there was no expression detected even in the HER2 and HER3 overexpressed cells leading to evaluating the activation of the pathway by looking at pERK5 instead. It would have been useful to have a pERK1/2 positive control to establish any problems with the antibody used. This experiment also revealed that the cells expressing high amounts of HER3 led to the strongest activation of both the above mentioned signalling pathways.

However, transient over-expression of proteins isn't stable for long periods of time and the number of gene copies incorporated per cell can differ. Hence it was opted to establish stable over-expressing cell lines by transducing PC3 cells with lentivirus containing HER2, HER3 or the control LACZ gene. This method allowed for the stable integration of the gene into the cells and also for the selection of only the over-expressing cells by Blasticidin antibiotic selection. Since the virus was titred and the multiplicity of infection (MOI) was <1, it can be assumed that the cells that over-express these proteins have incorporated only a single copy of the gene making them a more robust model for investigation. The virus was made and titred as explained in **2.14.1**. **Figure 4-3** and **Figure 4-4** show that the genes were successfully incorporated

into the plasmid which was then packaged to produce the respective viral particles. The established cell lines were maintained in Blasticidin selection medium and it was observed that the cells did not present with any morphological changes. The V5 tag could not be used to detect the over-expressing proteins, as an unspecific band was detected even in non-transduced cells (**Figure 4-6**). So, the over-expression of the receptors was validated by using the total HER2 and HER3 antibodies, as seen in **Figure 4-7**. The downstream changes were also analysed and it was observed that both the MAP Kinase and PI3 Kinase pathways were similarly upregulated between the over-expressing cell lines (**Figure 4-8**). This result validated the outcomes of the transient over-expression experiment but it however did not reveal any differential abilities between HER2 and HER3. This could be due to the fact that the basal levels of the partner receptors are also upregulated in the cell lines allowing for similar levels of downstream activity to be established.

The cell lines were further used to validate any functional differences. The first set of experiments was used to study the effect of HER2 and HER3 over-expression on cell proliferation. SRB assays and cell counts were performed and it was observed that both the HER2 and HER3 over-expressing cell lines showed significant differences in the rates of cell proliferation (**Figure 4-9**, **Figure 4-10**). Since the over-expression of these receptors has been observed in advanced disease, the potential effects on cell migration and invasion were also investigated. Matrix metalloproteinases are a family of proteins that have been known to play a key role in normal physiological processes, but also in diseases such as cancer. MMPs in cancer mainly facilitate tumour migration and invasion by breaking down the extracellular matrix and hence enabling cells to establish new colonies of growth. These proteins have been investigated and have been known to be over-expressed and activated in many types of cancers (Egeblad and Werb, 2002). In this study, the transcriptional regulation of MMP-9 was investigated by transiently over-expressing HER2 and HER3 in PC3 cells. A luciferase reporter construct that consisted of the MMP-9 promoter was also transfected to analyse its transcriptional activation upon activation of HER2 and HER3 mediated signalling. The result showed that the presence of both HER2 and HER3 led to the highest transcriptional activation of MMP-9. It was also observed that both the receptors could individually upregulate MMP-9 transcriptional activity (**Figure 4-11**). Migration and invasion assays with the use of Boyden chambers were further performed to validate this finding. For this experiment, the stable over-expressing cell lines were used and receptor activation was ensured with

the treatment of Heregulin. It was observed that both the HER2 and HER3 over-expressing cell lines showed increased cell migratory and invasion potential when compared to the control LACZ cell line (**Figure 4-12** and **Figure 4-13**). Interestingly, the HER3 cell line seemed to possess a stronger potential when compared to the HER2 over-expressing cell line. The profiling of the cell lines however showed that the downstream signalling pathways were not different between the lines. This suggests a potential role of nuclear HER3 acting as a transcription factor in the upregulation of MMP-9 and should be investigated further.

These results suggested that the established cell line models could be further used to investigate the role of HER2 and HER3 in prostate cancer progression. An *in-vivo* study was considered to study the rate of xenograft formation with the use of the stable over-expressing cell lines. Analysing the results in this manner gives would provide additional information about the cells behaviour as they will be growing in a more physiologically relevant environment, i.e, by interacting with the surrounding tissues that could aid their survival by providing them with the necessary proliferative signals. These environments are currently being studied *in-vitro* with the use of 3D cultures and ex-vivo cultures. For this study, athymic CD1 nude mice were used and 10 mice were used per experimental arm. The cells were injected into the flank of the mice and the xenograft formation was regularly monitored as mentioned in (**2.15.1**). The mice were sacrificed when the xenograft reached a size of 10 x 10 mm or > 15 mm in one dimension. From **Figure 4-14** it was observed that there were no significant changes in the xenograft formation between the over-expressing cell lines and the control LACZ cell line. The resected xenografts were used for further analysis. Although the calliper measurements of the xenografts indicated they had reached their maximum permissible size , it was observed that this measurement could not be correlated with the resected tumours. This suggested that the implantation in the mice was variable leading to irregular xenograft formation. The tumours once resected were found to be quite cystic and hence difficult to measure. The resected xenografts were collected under sterile conditions and were used for further profiling. The cell lines established from the xenografts were grown in Blasticidin selection medium and hence ensured that they were not contaminated with mouse cells. Protein lysates from these cell lines were collected after culturing the cells in their normal growth conditions and when they were treated with Heregulin. Interestingly, it was observed that HER2 and HER3 expression was significantly increased only with Heregulin treatment (**Figure 4-15**). This result

suggests that there was improper Heregulin stimulation *in-vivo* thus leading to inadequate expression and activity of HER2 and HER3. The proliferation of these cell lines was also investigated by performing SRB assays and cell counts which revealed no significant changes (**Figure 4-16**). The xenografts were also analysed for protein expression by immunohistochemistry. Since the sizes of the resected tumours were different, comparisons of protein expression were made between a small and large xenograft taken from each cell line. A downstream mark for pAKT was also included in the analysis. **Figure 4-17**, **Figure 4-18** and **Figure 4-19** show that the larger sized tumours had more expression of HER2, HER3 and pAKT. This finding indirectly suggests that HER2 and HER3 expression and its ability to activate the downstream signalling can be linked to tumour formation and growth. Staining these samples with Ki67 would be indicative if the cells possess increased proliferative capabilities between the LACZ, HER2, HER3 cell lines. It would also be useful to analyse these samples by performing some H and E staining to observe the pathology of the tumours. As the tumour samples were not validated by H and E it is quite difficult to draw and conclusions from the IHC analysis. Lysing the frozen tissue material and analysing it for protein expression would also give additional information about the protein expression in the xenografts. Since these cell lines showed a pronounced effect of cell migration and invasion, it would also be useful to perform the *in-vivo* experiments by intravenously implanting the cells and performing a lung colony formation assay. It would also be useful to carry out a smaller pilot study using these alternative assays to ensure success rates and then carry out a larger study to observe any significant changes.

The next set of experiments performed was to interpret the effects of HER2 and HER3 on the androgen receptor (AR). The AR is known to be the driver of prostate cancer and its over-expression and activity have been extensively researched. The development of the aggressive form of androgen-independent prostate cancer as discussed in Feldman and Feldman (2001) has been established as a benchmark review, and since has led to further investigations for the greater understanding of the disease. In the review, the 'outlaw pathway' is one of the mechanisms suggested for attaining androgen independence wherein the AR can be activated by growth factors such as insulin-like-growth factor -1 (IGF-1) and epidermal growth factor (EGF) and also by receptor tyrosine kinases like HER2. In this study, the effect of HER2-HER3 mediated signalling on the AR was initially investigated by using a luciferase reporter assay. The

LNCaP-7B7 cell line was used which stably expresses a PSA-luciferase construct. Since PSA is an AR regulated gene, the use of this cell line serves as an appropriate model to study AR activity. The functioning of the cell line was validated by starving the cells in steroid depleted medium and as expected, it was observed that the transcriptional activity of PSA was reduced (**Figure 4-20 (A)**). These cells were then stimulated with Heregulin under appropriate conditions and it was observed that activated HER2 and HER3 caused an increase in PSA transcriptional activity (**Figure 4-20 (B)**). A similar result was obtained when the LNCaP-AI 7B7 cell line was used. This cell line grows in androgen deprived conditions and hence serves as a model for androgen-independent prostate cancer (**Figure 4-20 (C)**). To prove that these changes are occurring through the activity of the androgen receptor, the cells were treated with the anti-androgen drug Enzalutamide and treated with Heregulin. Upon drug treatment, it was observed that PSA transcriptional activity was inhibited thus proving that the influence of HER2 and HER3 signalling on androgen receptor's activity (**Figure 4-21**).

After validating the role of HER2 and HER3 mediated signalling on AR's activity, the investigation was continued to observe the effect of differential expression of HER2 and HER3 on AR protein expression. Since the differential expression on the receptors was observed in advanced prostate cancer, the experiment was designed to mimic those conditions. In advanced disease, patients are usually treated by androgen ablation which ultimately leads to the low levels of androgens to be present in circulation. For the experiment, PC3 cells were used and transfected with equal amounts of AR along with either HER2 or HER3 or both the receptors. An empty vector control was also used. From **Figure 4-22 (A)** it was observed that AR expression was increased in the presence of low levels of androgens and Heregulin, particularly in the presence of HER3 and hence strongly suggests that HER2-HER3 mediated signalling plays a crucial role in maintaining the AR levels and its activity in advanced prostate cancer. To validate this finding, the experiment was repeated in the stable over-expressing cell lines. The result similarly revealed the enhanced expression of the AR upon HER2-HER3 activation (**Figure 4-22 (B)**.

The findings of this chapter provide a better understanding of these receptors in prostate cancer. The analysis of the differential expression of these receptors along with their downstream effects – particularly the migration, invasion and AR expression studies, have suggested that HER3 over-expression could be leading to enhanced and prolonged signalling activity that is more efficient in sustaining the disease. The study of the

expression and activity of HER2 and HER3 in clinical samples and *in-vitro* models provided sufficient data to continue this study by investigating their potential as drug targets

Chapter 5 Demonstrating the Therapeutic Potential of HER2-HER3 Heterodimer Inhibition

5.1 Introduction

Signalling pathways within the cell are highly regulated to maintain the equilibrium of important processes including cell proliferation, differentiation and cell death. Many of these vital processes are maintained by receptor tyrosine kinases (RTKs). Receptor tyrosine kinases and their properties have been extensively studied and their role in cell signalling activation and regulation has been well established. These receptors can transform into oncogenic proteins and trigger tumourigenesis when they acquire mutations or become structurally altered (Gschwind *et al.*, 2004). This phenomenon has been observed in many solid tumours including cancers of the breast, prostate, colon and lung. Many types of inhibitors have been clinically developed and examined since the identification of RTKs as therapeutic targets and these inhibitors are known as tyrosine kinase inhibitors (TKIs). Numerous small molecule inhibitors (SMIs) and monoclonal antibodies (mAbs) have been developed in recent years and have transformed the therapeutic options for malignant diseases. Focussing on the EGFR family, many such inhibitors have been developed with the intention to inhibit their activity and cause tumour regression. Since these proteins have been shown to be oncogenic across a variety of tissues, the targeted therapies have been used in clinical trials in different malignancies but with varied outcomes. For example, Trastuzumab, a monoclonal antibody against HER2, has been more successful in the treatment of HER2 positive breast cancer when compared to HER2 positive prostate cancer (Jathal *et al.*, 2011). The next approach taken in the TKI development involved designing drugs that targeted two members of the protein family to overcome the disadvantages of single-agent targeted therapy. This resulted in the use of TKIs such as Lapatinib – which is a small-molecule inhibitor against the EGFR and HER2 receptors. However, phase II clinical trials using Lapatinib in early stage and metastatic prostate cancer were unsuccessful (Sridhar *et al.*, 2010). The dual receptor inhibition has been reasoned to be unsuccessful due to the ability of the cancer cell to sustain active signalling by the upregulation of alternative receptors. This upregulation can lead to more active heterodimer or homodimer formation and safeguard the continual signalling of the pro-survival pathways. This mechanism is also linked to enabling the cancer to become drug

resistant; a major setback commonly faced in targeted therapy (Sergina *et al.*, 2007). Re-sensitising cells to existing cancer therapy is a strategy currently being investigated for prostate cancer treatment and has been addressed with the use of pan-inhibitors. Pan-inhibitors such as Canertinib and Dacomitinib, which target EGFR, HER2 and HER4 are currently being investigated in the treatment of melanoma and non-small cell lung cancers (Djerf Severinsson *et al.*, 2011; Enting and Spicer, 2012). However, these drugs aren't used in prostate cancer treatment as HER4 hasn't been known to have a role in prostate cancer development and has in fact not been detected in prostate cancer tissues and cell lines (Grasso *et al.*, 1997). A recently developed pan-inhibitor from AstraZeneca – AZD8931, is designed to target the kinase activity of EGFR, HER2 and HER3 (Hickinson *et al.*, 2010). Its role is currently being investigated in breast cancer and other solid tumours (Tjulandin *et al.*, 2014). However, there is no data available regarding the use of this inhibitor in prostate cancer.

The clinical and *in-vitro* analyses of HER2-HER3 expression and their mediated signalling in this study have revealed supporting information and justifies its potential as therapeutic targets. This chapter focusses on profiling the use of the novel pan-inhibitor-AZD8931 in prostate cancer along with investigating the role of HER3 in acquiring drug resistance.

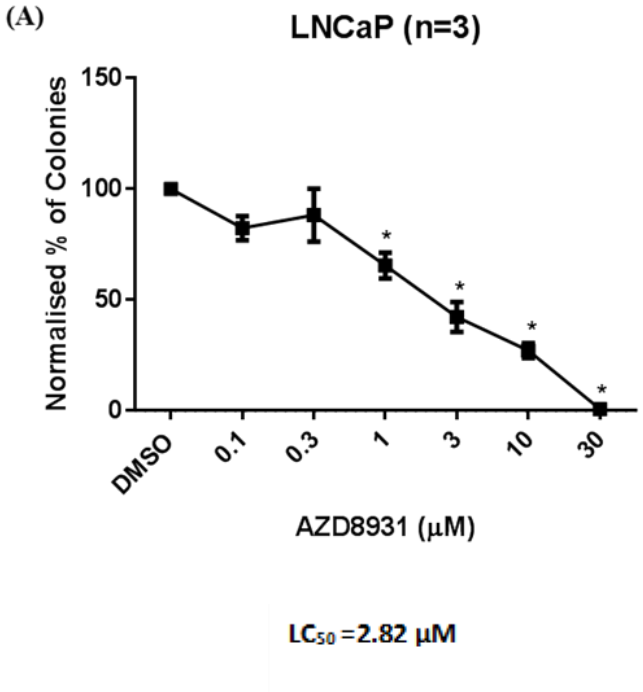
5.2 AZD8931-pan EGFR family inhibitor

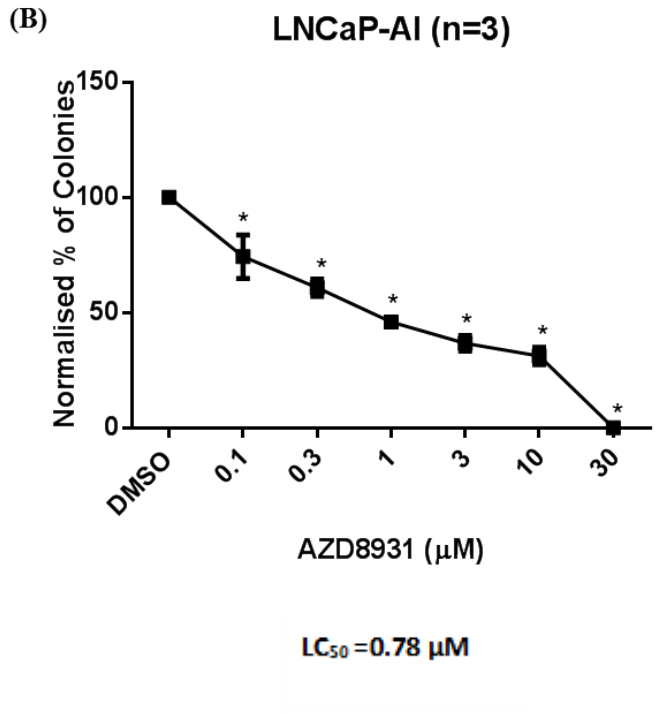
Use of AZD8931

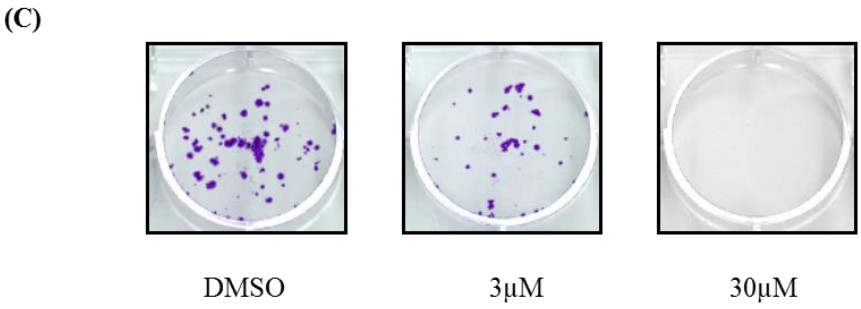
The inhibitor was obtained from AstraZeneca and was reconstituted and stored according to the guidelines. Briefly, a 30mM stock was made in DMSO which was stored at -80 ºC and was used to make further stocks of lower concentrations. Working aliquots were stored at -20 ºC and frequent freeze-thaw cycles were avoided to ensure the stability of the compound was maintained.

5.3 AZD8931 reduces and colony forming ability in prostate cancer cell lines

LNCaP and LNCaP–AI cell lines were used to estimate the LC_{50} values of AZD8931 by performing colony forming assays. The assay analyses the ability of cells to form colonies in the presence of drug. The reduction in the number of colonies formed over a period of time would indicate the reduction in cell survival. This method was chosen for its sensitivity as it can estimate a more accurate LC_{50} of the drug as opposed to SRB assays that estimates cell growth The analysis revealed that AZD8931 reduced cell proliferation in both the androgen dependent and independent cell lines (**Figure 5-1**). The respective LC_{50} concentration was used for all the subsequent experiments.







Representative pictures showing colonies growing under none, moderate or high concentrations of AZD8931

Figure 5-1: Colony forming assay revealing AZD8931 reduces cell survival in prostate cancer cell lines

LNCaP and AI cell lines (A and B respectively) were seeded in 6-well plates at low densities (2000 cells) and treated with respective drug doses the next day. The cells were allowed to grow for 14 days in full medium to form colonies after which they were fixed using Carnoy's fixative and stained with crystal violet. The colonies were counted (C) and the reduction in proliferation and the LC_{50} values were calculated using GraphPad Prism. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the control DMSO.

5.4 AZD8931 reduces HER2 and HER3 translocation to the nucleus and reduces their activity in the cellular and nuclear compartments

In the previous chapter, it was shown that Heregulin activates HER2 and HER3 and their active forms along with the respective full length receptors were detected in the cytoplasm and nuclear cell compartments. After estimating the LC_{50} values, the action of AZD8931 on the receptor nuclear translocation was investigated. From **Figure 5-2** it was observed that with AZD8931 treatment, LNCaP cells retained the total receptors in the cytoplasm and their presence in the nucleus was reduced suggesting that AZD8931 can inhibit the nuclear translocation of HER2 and HER3. It was also observed that AZD8931 reduced the presence of the active forms in the nucleus.

5.5 AZD8931 reduces HER2 and HER3 association with chromatin

After observing the reduction of HER2 and HER3 nuclear translocation upon AZD8931 treatment, the investigation of the compound was continued by analysing its ability to inhibit chromatin association of these receptors. In **Chapter 3**, it was shown that Heregulin treatment enhances chromatin association of HER2 and HER3 (**Figure 3-26)**. The insoluble chromatin pellets collected after nuclear and cytoplasmic extractions were dissolved in 1X SDS sample buffer and sonicated to reduce the viscosity due to their high chromatin content. Histone H4 was used as a positive control for the chromatin fractions and α-tubulin as a negative control. **Figure 5-3** shows that AZD8931 reduces the association of the total and active forms of HER2 and HER3 to chromatin.

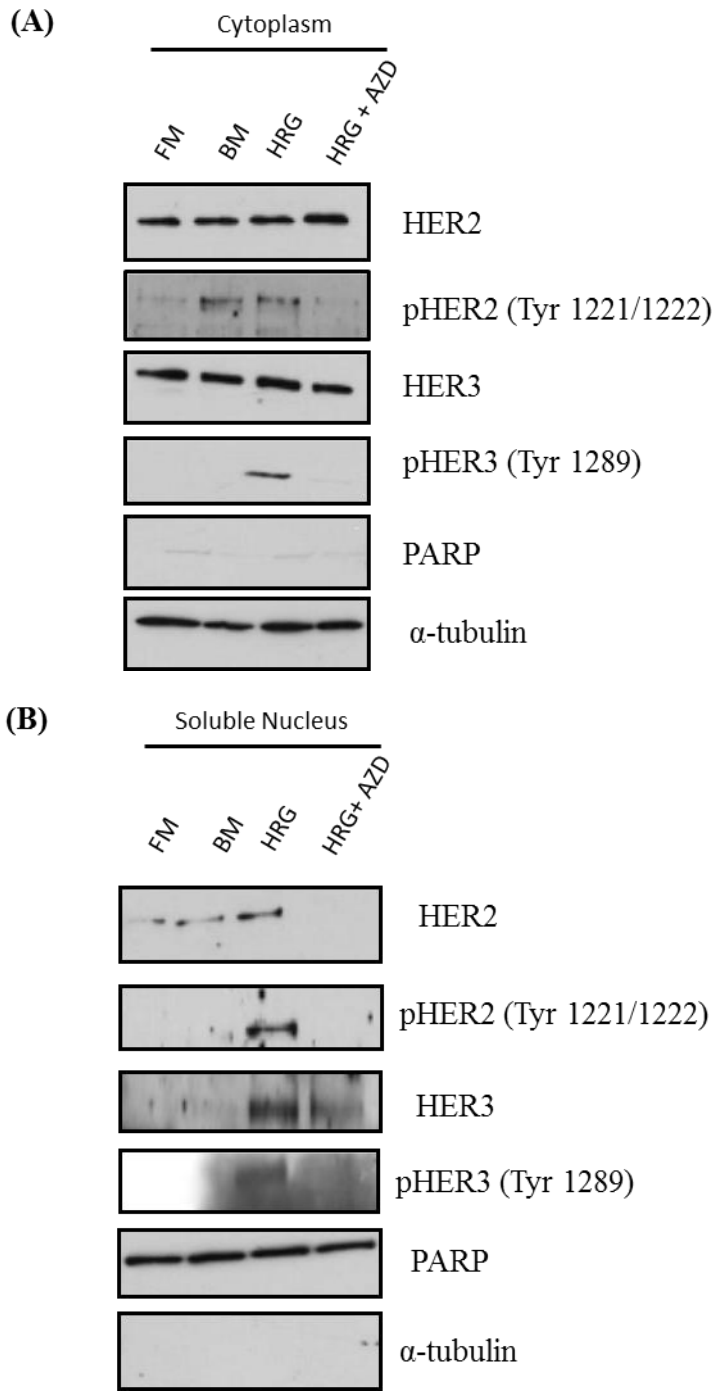


Figure 5-2: AZD8931 reduces nuclear translocation of total and phosphorylated forms of HER2 and HER3

LNCaP cells were seeded out in full medium. The following day, the medium was changed to full medium or basal medium and the indicated sample was pre-treated with 3 µM AZD8931 (LC_{50}). The cells were then treated with Heregulin (HRG; 20ng/ml) the following day for 2 hours after which the cells were lysed and the cytoplasmic and nuclear compartments of the cells were separated (by following the extraction kit protocol). The lysates were then analysed for cytoplasmic (A) and nuclear (B) protein expression by western blotting. α-tubulin and PARP were used as controls for the cytoplasmic and nuclear compartments respectively. The result revealed that AZD8931 reduced the translocation of the total and active forms of HER2 and HER3 into the nucleus.

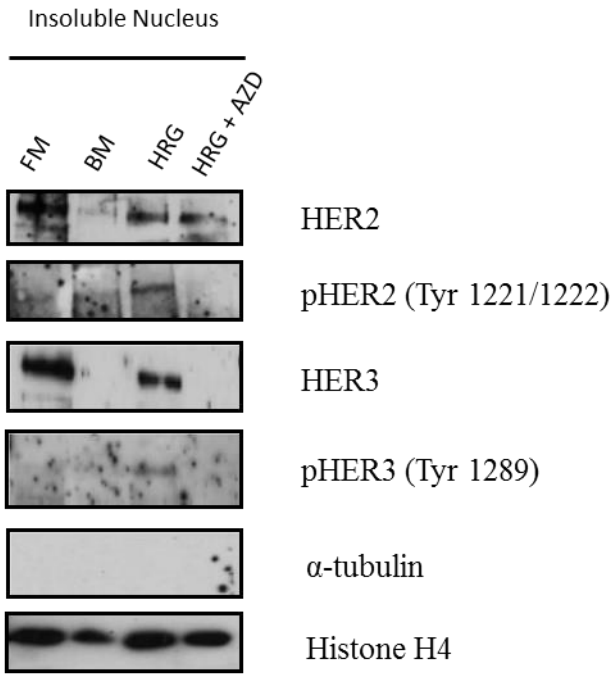


Figure 5-3: AZD8931 reduces total and phosphorylated forms of HER2 and HER3 from associating to chromatin

LNCaP cells were seeded out in full medium. The following day, the medium was changed to full medium or basal medium and the indicated sample was pre-treated with 3 µM AZD8931 (LC_{50}). The cells were then treated with Heregulin (HRG; 20ng/ml) the following day for 2 hours after which the cells were lysed and the cytoplasmic and nuclear compartments of the cells then separated by following the protocol of the extraction kit. The insoluble chromatin fraction was solubilised by sonicating the pellet in 1X SDS sample buffer. Histone H4 and α-tubulin were used as controls to ensure the purity of the chromatin samples. The result revealed that AZD8931 reduced the association of total and active forms of HER2 and HER3 to chromatin in the presence of Heregulin.

5.6 AZD8931 reduces activity of EGFR family and their signalling pathways

LNCaP cells were used to study the effects of Heregulin and AZD8931 on EGFR, HER2, HER3 and their downstream signalling pathways. The cells were stimulated with Heregulin with or without the presence of AZD8931. **Figure 5-4** shows that the drug reduced the activity of EGFR, HER2 and HER3 along with downstream marks of the PI3 Kinase and MAP Kinase pathways. A reduction in phosphorylation of AR was also observed in the presence of AZD8931.

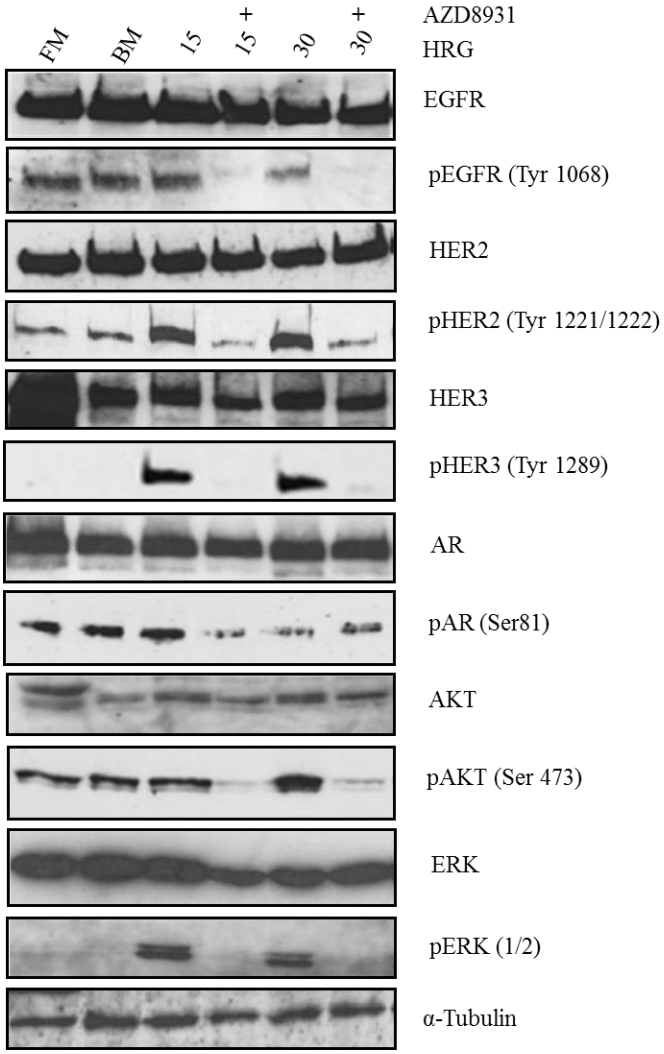


Figure 5-4: AZD8931 reduces the activity of EGFR family along with their downstream targets including AR in LNCaP cells

The cells were seeded down in full medium, starved in basal medium and treated with or without drug (using LNCaP LC_{50} – 3 µM) overnight. Equal amounts of DMSO were added to the untreated samples to ensure that any variation observed was due to the drug alone. The following day, the cells were treated with Heregulin (HRG; 20ng/ml) for 15 minutes after which the cells were lysed in RIPA buffer and analysed by western blotting.

5.7 AZD8931 reduces AR activity

In the previous chapter it was seen that HER2 and HER3 increased the activity of androgen receptor (**Figure 4-20**) in the LNCaP-7B7 cell line. The cell line contains a chromosomally integrated PSA-luciferase promoter, thus enabling the analysis of AR activity on the transcription of its downstream targets like PSA. The same experiment was repeated along with AZD8931. DMSO was added as a control to the untreated samples. **Figure 5-5 (A)** shows that the activity of AR is reduced by the effect of AZD8931. This was also seen in the LNCaP-AI 7B7 cell line which grows in androgen independent conditions (**Figure 5-5 (B)**). To validate that HER2 and HER3 inhibition mediated the reduction in PSA promoter activity, the experiment was repeated by inhibiting HER2 and HER3 by using respective siRNAs for protein knockdown. The siRNAs were first validated by western blotting (**Figure 5-6 (A)**) and were then used on the LNCaP–7B7 cell line. **Figure 5-6 (B)** shows that the reduction of HER2 and HER3 levels results in the reduction of AR activity.

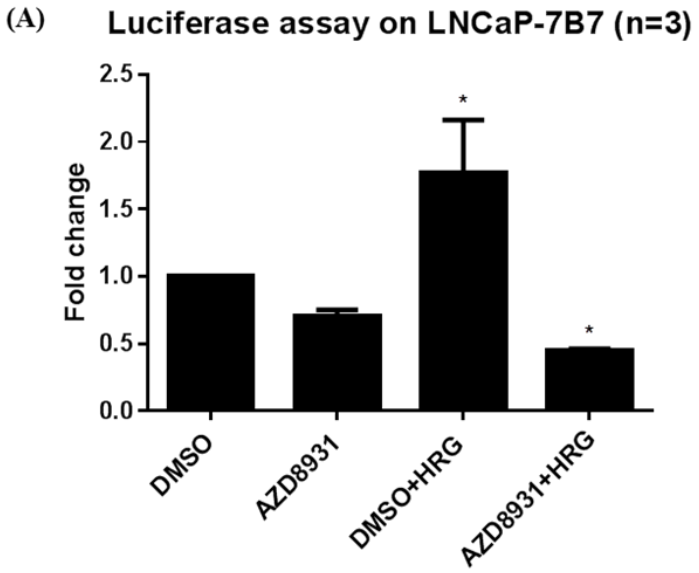


Figure 5-5: AZD8931 reduces AR promoter activity

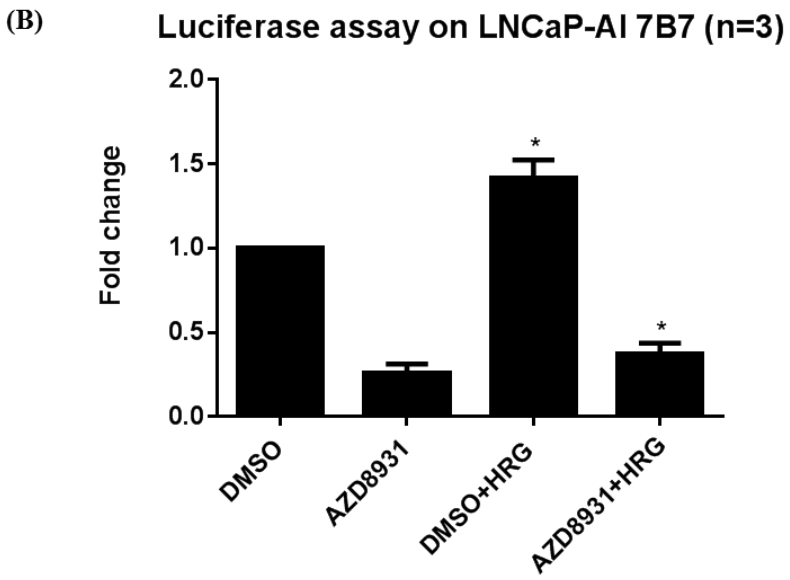


Figure 5-5: AZD8931 reduces AR promoter activity

LNCaP-7B7 cells were seeded out in quadruplets per experimental arm, using a 24-well plate. The cells were then starved in SDM for 3 days. 16 hours prior to the starvation period, the cells were treated with either DMSO or AZD8931 (3 µM AZD8931-LC_{50}) after which the cells were treated with Heregulin (HRG; 20ng/ml) for another 16 hours. When the LNCaP-AI 7B7 cell line was used, the cells were seeded out in their normal growth medium (SDM) and the cells were pre-treated with AZD8931 the next day followed by 16h of Heregulin stimulation. The cells were then lysed in reporter lysis buffer and luciferase substrate was added to measure the luciferase activity. The luciferase counts per second were measured and the data was normalised to the protein concentration per well by performing a BCA assay. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05. The data showed that AZD8931 reduces Heregulin stimulated AR activity in both LNCaP–7B7 (A) and LNCaP-AI 7B7 cell lines (B).

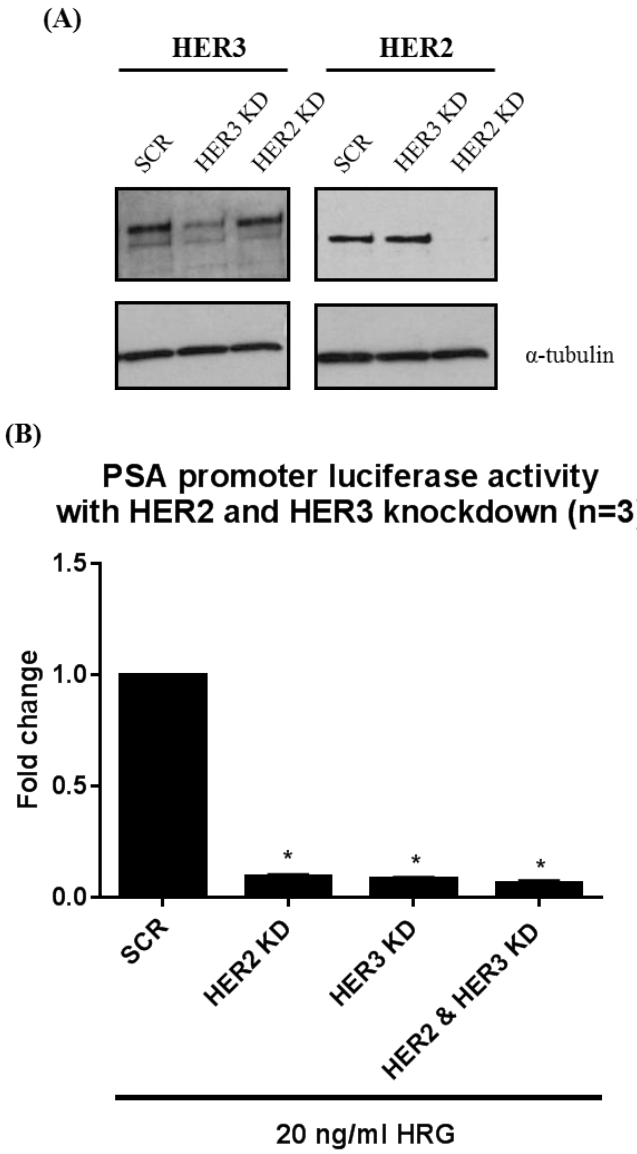


Figure 5-6: Knockdown of HER2 and HER3 using siRNA shows similar effect to that of AZD8931

(A) HER2 and HER3 siRNAs were validated using western blotting. LNCaP cells were seeded out in full medium in a 6-well plate. The next day, the cells were transfected with respective siRNAs so that the final concentration on the cells was 25nM. A scrambled siRNA sequence (SCR) was used as a control. The knockdown was performed for 96 hours after which the cells were lysed in RIPA buffer and protein expression was analysed using western blotting. The figure shows that the siRNAs are specific and reduce their targeting protein levels.

(B) The validated siRNAs were used on the LNCaP-7B7 cell line which was previously used to measure PSA promoter activity. HER2 and HER3 knockdown was performed similarly as mentioned above except the transfections were done in SDM for 96 hours after which the cells were treated with Heregulin (HRG; 20ng/ml) for 16 hours. The luciferase activity was then measured and the data was analysed as previously described. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05. The figure shows that the reduction of HER2 and HER3 protein levels reduces Heregulin stimulated AR activity.

5.8 AZD8931 reduces cell migratory and invasion potential

The effects of AZD8931 on cell migratory and invasion potential were also investigated. In the previous chapter it was seen that HER2 and HER3 increased cell migration (**Figure 4-11** and **Figure 4-12**) and invasion (**Figure 4-13**). The same experiment was repeated along with the pre-treatment with AZD8931 prior to Heregulin stimulation. DMSO was added as a control to the untreated samples. **Figure 5-7** revealed that AZD8931 reduced the activity of MMP9. This was further validated by performing migration and invasion assays using the HER2 and HER3 over-expressing cell lines. AZD8931 reduced both the cell migratory and invasion potential of these cells (**Figure 5-8**).

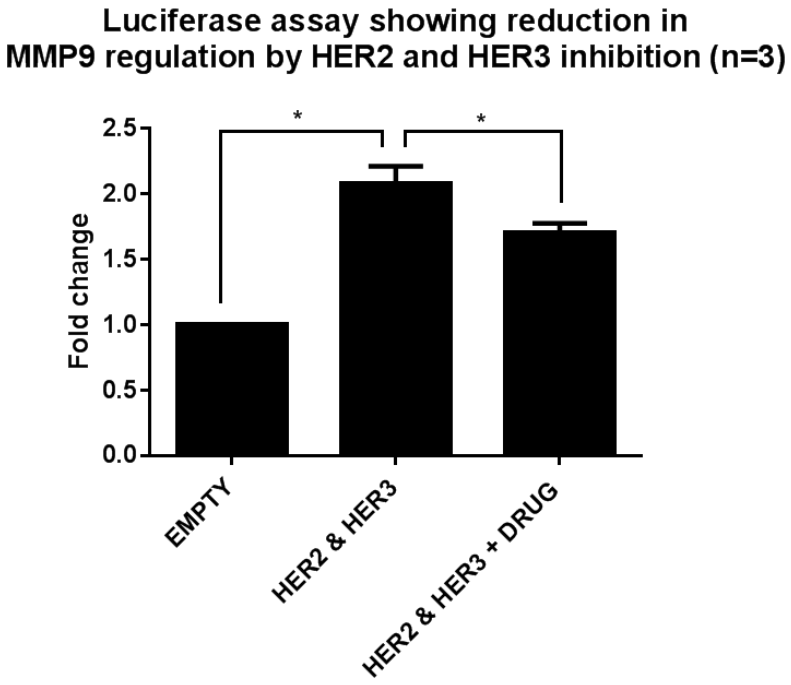


Figure 5-7: AZD8931 reduces HER2 and HER3 induced MMP9 activity

MMP-9 expression is reduced in the presence of AZD8931. PC3 cells were seeded out in quadruplets per experimental arm using a 24-well plate and were forward transfected with 100ng of MMP9, 50ng of βgal and 300ng of HER2 and HER3. pCMV empty vector was used for the control and to ensure equal amounts of plasmid were added to all the experimental arms. The cells were allowed to transfect for 24 hours after which the cells were starved in basal medium with or without treatment of 3 µM AZD8931 (LC_{50}) for 16 hours. DMSO was added as a control to the untreated samples. The cells were then treated with Heregulin (HRG; 20ng/ml) for 16 hours after which the cells were lysed in 1x reporter lysis buffer and the luciferase activity was recorded. The data was normalised to the results of a β-galactosidase assay that revealed transfection efficiency. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05.

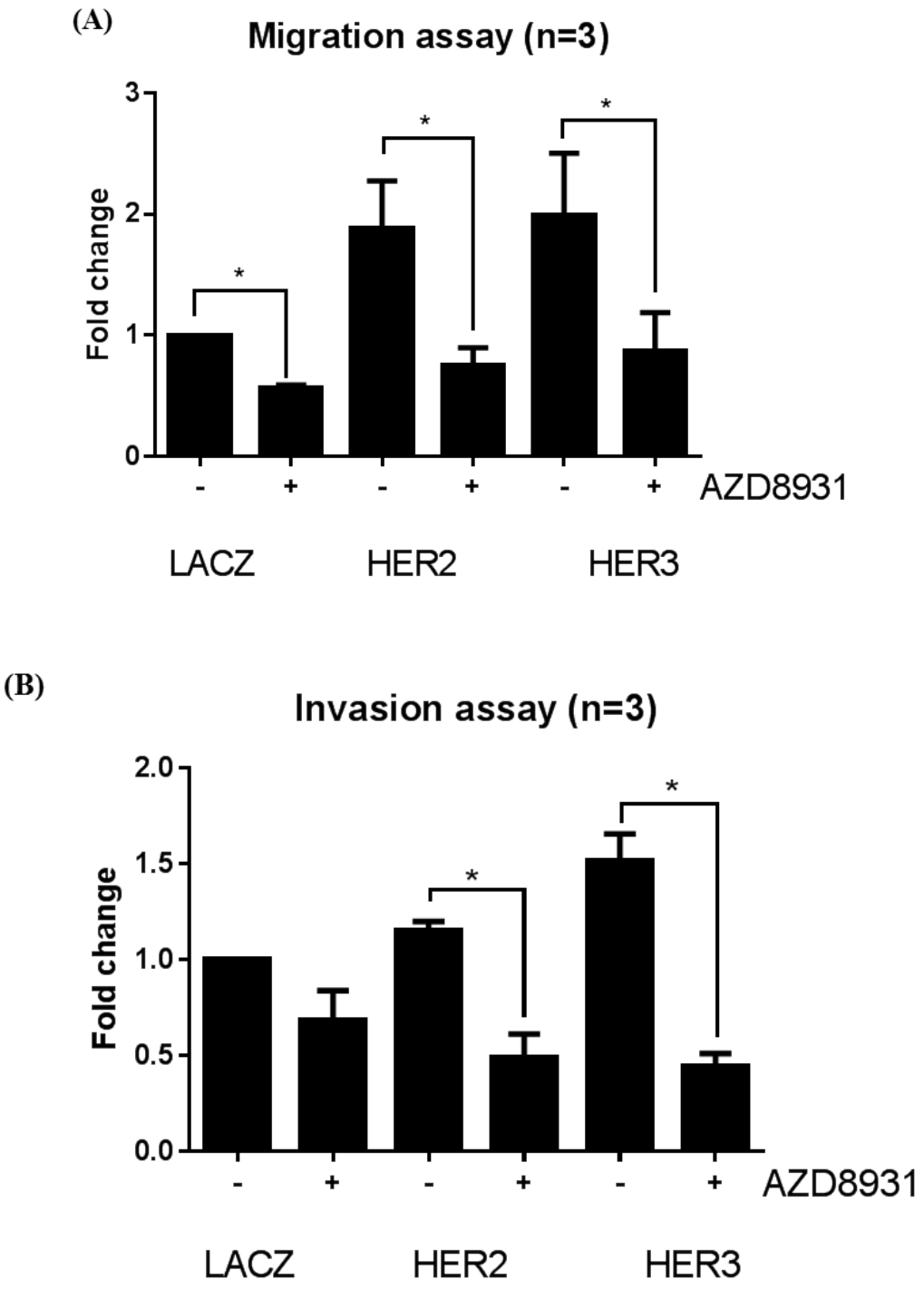


Figure 5-8: AZD8931 reduces cell migratory and invasion potential

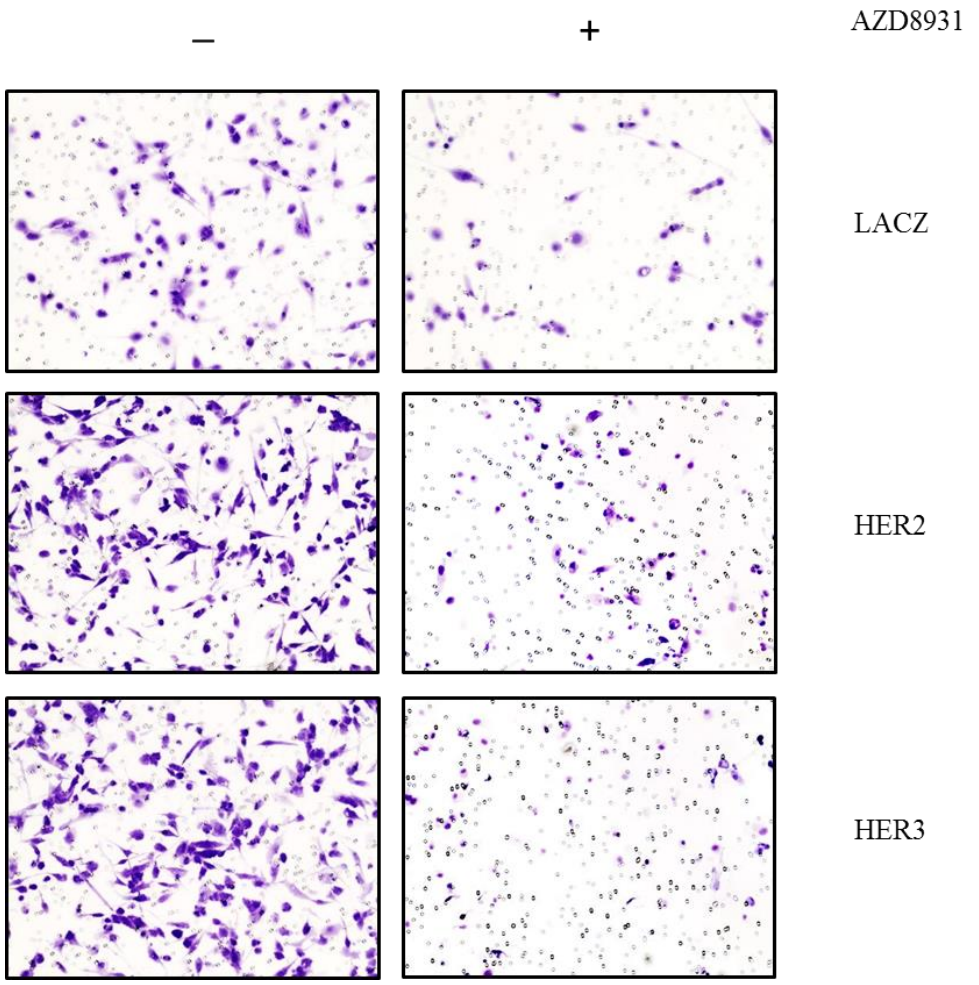


Figure 5-8: AZD8931 reduces cell migratory and invasion potential

The over-expressing stable cell lines were used for performing migration and invasion assays using Boydon chambers (A and B). PET membrane inserts with a pore size of 8µm was used. The invasion chambers were coated with matrigel. 600µl of basal medium with Heregulin (20ng/ml) and 3 µM AZD8931 (LC_{50}). /DMSO was added per well and the inserts were placed on top. The cells were pre-treated with drug or DMSO in basal medium for 16 hours. The cells were then collected in SDM and re-suspended in basal medium with drug/DMSO. $3x10^4$ cells were seeded in a final volume of 300µl in the upper chamber. The cells were allowed to migrate/invade for 48 hours The cells that hadn't migrated/invaded were removed using a cotton bud and the cells that had migrated were fixed on the membrane using methanol, stained with crystal violet, mounted on a slide and counted. Each arm of the experiment and the entire experiment itself was done in triplicate and normalised to the control LACZ line. Student t-test was performed on the experiment and * indicates statistical significance with p-value <0.05. Figure C is a representation of the stained migration/invasion inserts containing the cells of the 3 respective cell lines – before and after AZD8931 treatment.

5.9 Comparative experiments using Lapatinib

Lapatinib – another TKI that targets only 2 members of the EGFR family – EGFR and HER2 was used to compare the sensitivity of AZD8931. The preliminary experiment performed was to compare the effect on proliferation in prostate cancer cell lines. Colony forming assays were similarly performed using the LNCaP and the LNCaP-AI cell lines and it was observed that the cell lines were more sensitive to Lapatinib than AZD8931 (**Figure 5-9**).

However, the use of Lapatinib has been unsuccessful in prostate cancer clinical trials and suggests that the cancer may have attained other mechanisms in overcoming inhibition. This was investigated through the establishment of Lapatinib resistant cell lines.

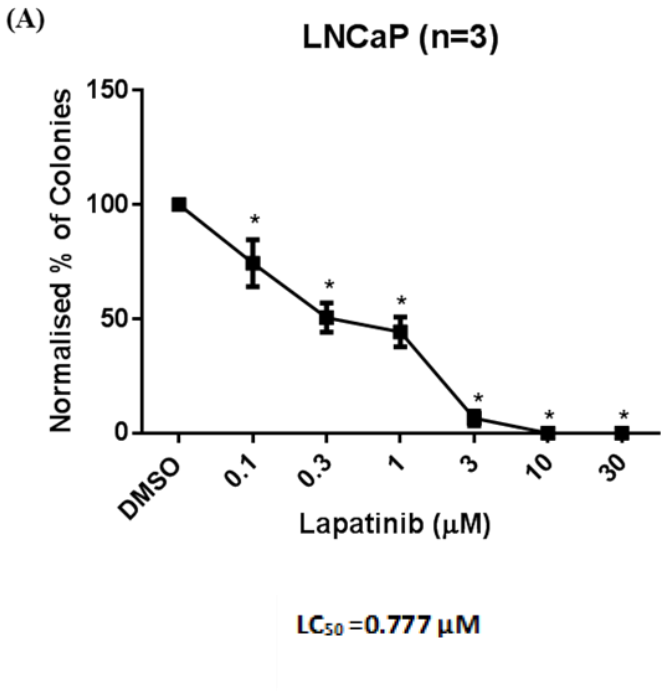


Figure 5-9: Lapatinib reduces cell proliferation in prostate cancer cell lines

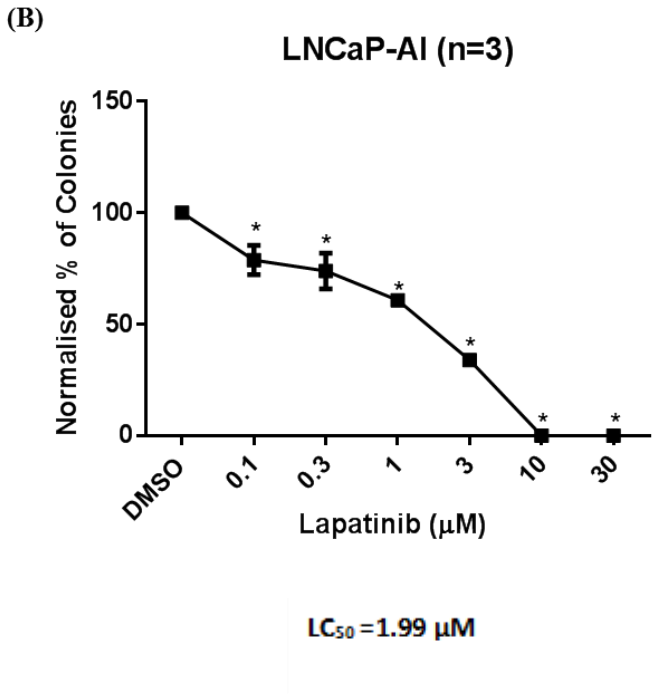


Figure 5-9: Lapatinib reduces cell proliferation in prostate cancer cell lines

LNCaP and LNCaP-AI (A and B, respectively) were seeded in 6-well plates at low densities (2000 cells) and treated with indicated doses of Lapatinib the next day. The cells were allowed to grow for 14 days to form colonies after which they were fixed using Carnoy's fixative and stained with crystal violet. The colonies were counted and the reduction in proliferation and the LC_{50} values were calculated using GraphPad Prism. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the control DMSO.

5.9.1 Establishing Lapatinib resistant cell line

Lapatinib sensitive cell lines inhibit the activity of HER3 by inhibiting the activity of its preferred partner HER2. However, previous research in different cancer cell lines has shown that Heregulin induces Lapatinib resistance by increasing HER3 expression (Sato *et al.*, 2013). However, there is very little literature that explains the mechanisms of Lapatinib resistance in prostate cancer. Therefore a Lapatinib resistant cell line was established using the LNCaP cell line. Briefly, the cells were grown in increasing doses of Lapatinib over a period of 3 months. The maximum concentration of drug that could be used was 5 μM as higher doses had very poor solubility.

5.9.2 The presence of lapatinib in lapatinib resistant cells does not affect their survival

Cell survival assays were first performed to test the resistance. Colony forming assays

were performed on both - Lapatinib sensitive and resistant LNCaP cell lines. **Figure 5-10** shows that there was no reduction in survival in the Lapatinib resistant lines in the presence of Lapatinib.

Cell proliferation was then evaluated by performing SRB assays. This showed that the Lapatinib resistant lines had reduced proliferation in the presence of drug (**Figure 5-11 (A)**). However, the overall proliferation was still higher compared to the Lapatinib sensitive lines. An additional experiment was performed with the use of live cell imaging for the analysis of cell proliferation using the Incucyte incubator. This is an incubator in which cells can be grown and the cell confluence can be regularly monitored by taking live images, which can then be analysed from time zero to observe any changes in proliferation. The result showed that the resistant lines grew slower in the presence of Lapatinib (**Figure 5-11 (B)**).

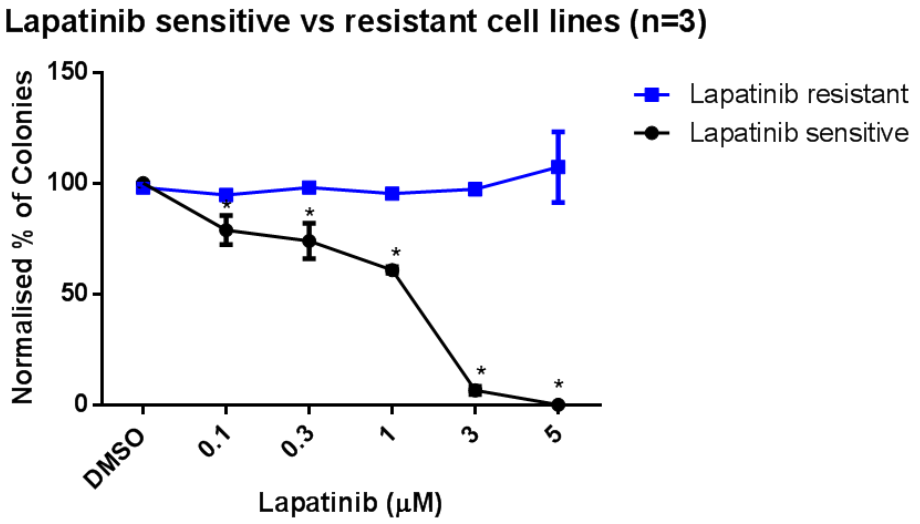


Figure 5-10: Colony forming assay showing Lapatinib resistant lines growing in the presence of drug

LNCaP–Lapatinib resistant and sensitive lines were seeded in 6-well plates at low densities (2000 cells) and treated with indicated doses of Lapatinib the next day. The cells were allowed to grow for 14 days to form colonies after which they were fixed using Carnoy's fixative and stained with crystal violet. The colonies were counted and the reduction in proliferation and the LC_{50} values were calculated using GraphPad Prism. Student t-test was performed on the experiments and * indicates statistical significance with p-value <0.05 when compared to the control DMSO.

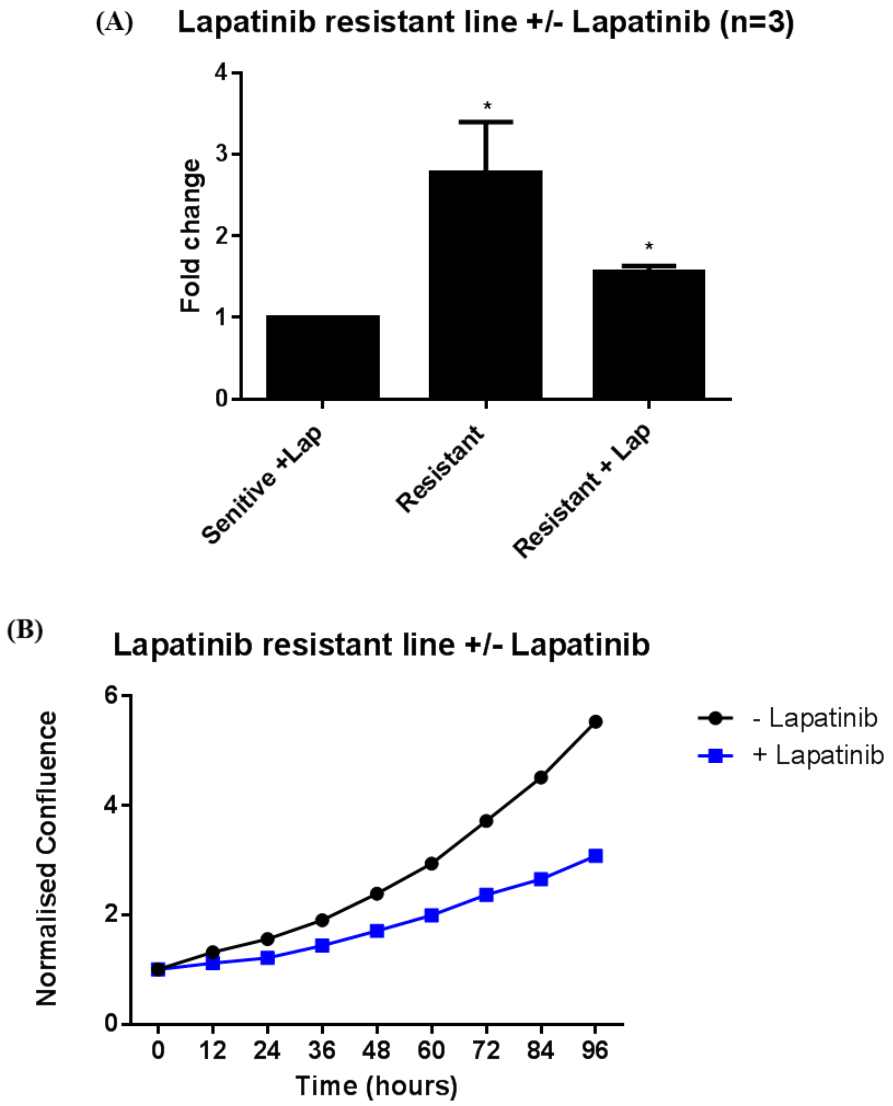


Figure 5-11: Lapatinib resistant cell line has reduced proliferation in the presence of Lapatinib

(A) LNCaP and Lapatinib resistant cell lines were seeded on 96-well plates and the next day were treated with or without 5 µM Lapatinib. The cells were allowed to grow for 96 hours after which the cells were fixed with chilled 50% TCA for 1 hour at 4 ºC. The cells were then washed with tap water, dried and stained with 0.4% SRB in 1% acetic acid for 30 minutes at room temperature. The cells were then rinsed in 1% acetic acid to remove excess dye. The dye on the cells was dissolved in 10mM Tris (pH 10.8) and the absorbance was recorded at 570nm. The experiment was repeated 3 times and student t-test was performed on the experiment. * indicates statistical significance with p-value <0.05 when compared to the control – LNCaP Lapatinib sensitive line.

(B) Lapatinib resistant lines were seeded out in 12-well plates and treated with or without Lapatinib the next day. The cells were placed in the Incucyte incubator for 96 hours. The captured images were analysed using the Incucyte software. The software analysed the changes in well confluence from time zero till 96 hours. The fold change in cell confluence was then plotted on a graph to observe any changes. The result revealed that the cells are not dependant on Lapatinib for their growth.

5.9.3 EGFR, HER2 and HER3 over-expression and activity leads to Lapatinib resistance which can be abrogated by AZD8931

The cell line was further investigated to establish the mechanism of reaching Lapatinib resistance. The expression of EGFR, HER2 and HER3 was compared between the parental LNCaP cell line and the Lapatinib resistant cell line in their normal growth conditions. **Figure 5-12 (A)** shows that the Lapatinib resistant cell line has a profound increase in EGFR and HER3 protein expression. There did not appear to be a substantial increase in HER2 protein expression in the Lapatinib resistant line.

However, when the active form of the receptor was considered, HER2 was still active in the resistant line upon Heregulin stimulation. The increased over-expression of EGFR and HER3 would suggest the maintenance of the pHER2 levels in the resistant line. The active forms of EGFR and HER3 were also observed in the resistant line when they were treated with Heregulin. The activity of these receptors was reduced in the parental LNCaP cell line when treated with Lapatinib (**Figure 5-12 (B)**). The observations of the resistant cell line still being responsive to Heregulin and the increased activity of EGFR, HER2 and HER3 suggest that the PI3Kinase and MAP Kinase signalling pathways are maintained, eventually aiding cell survival and creating drug resistance. This sustained activation of the receptors was abrogated with the treatment of AZD8931.

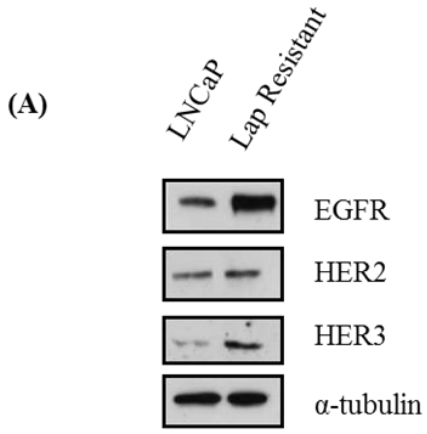


Figure 5-12: Lapatinib resistant lines remain Heregulin responsive and AZD8931 reduces this response.

(A) LNCaP and Lapatinib resistant cell lines were grown in FM for 24 hours. The Lapatinib resistant lines were grown in 5 μM Lapatinib. After 24 hours, the cells were lysed in RIPA buffer and the whole cell lysates were analysed for protein expression using western blotting. The figure shows the Lapatinib resistant cell line have an increase in EGFR, HER2 (slight increase) and HER3 expression when compared to the parental LNCaP cell line.

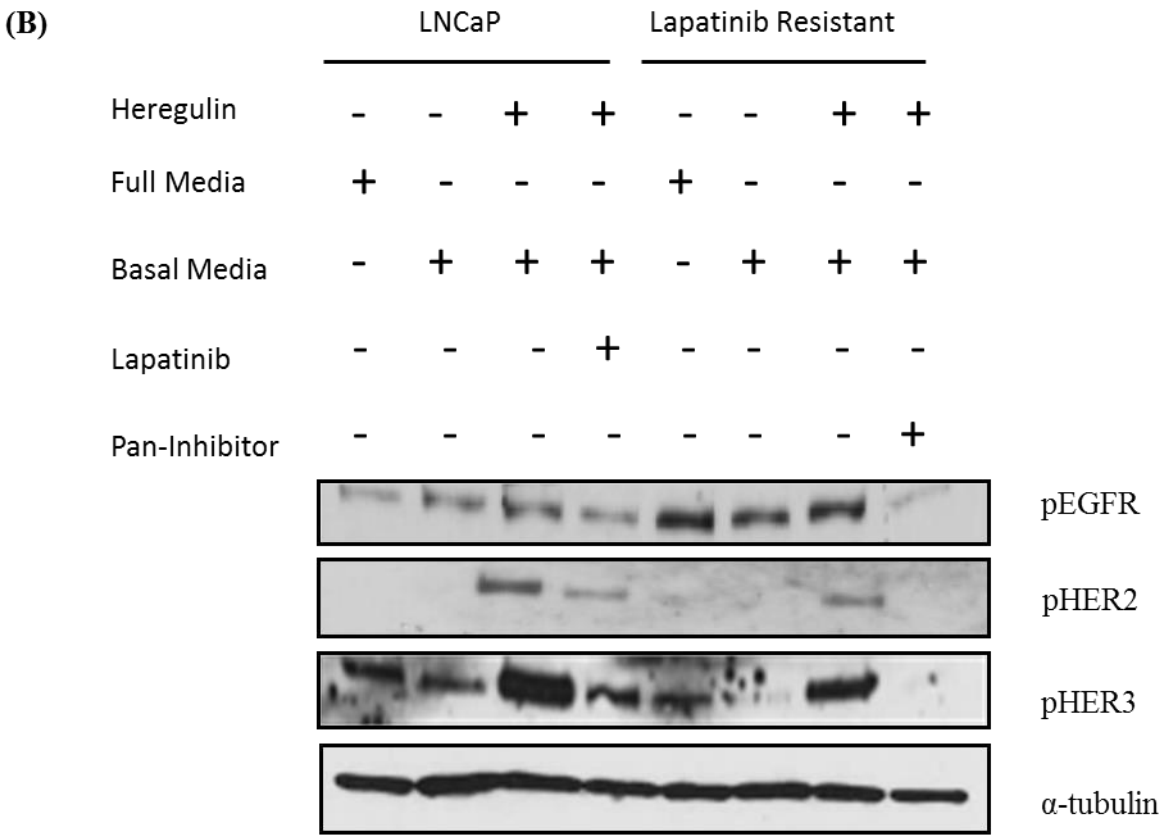


Figure 5-12: Lapatinib resistant lines remain Heregulin responsive and AZD8931 reduces this response.

(A) LNCaP and Lapatinib resistant cell lines were seeded out in FM. The next day, fresh medium containing Lapatinib (5 µM) was added to the resistant cell line and the parental cell line was supplemented with fresh medium alone. After 24 hours, the cells were lysed in RIPA buffer and the whole cell lysates were analysed for protein expression using western blotting. The figure shows the Lapatinib resistant cell line have an increase in EGFR, HER2 (slight increase) and HER3 expression when compared to the parental LNCaP cell line.

(B) Lapatinib resistant cells were seeded down in full medium and starved in basal medium overnight and treated with or without drug (Lapatinib – 1 µM, AZD8931 – 3 µM) overnight. Same amount of DMSO was added to the untreated samples to ensure that any variation seen was due to the drug alone. The following day, the cells were treated with Heregulin (HRG; 20ng/ml) for 15 minutes after which the cells were lysed in RIPA buffer and analysed by western blotting. The figure shows that the Lapatinib resistant cell line is still responsive to Heregulin and shows active forms of EGFR, HER2 and HER3. This effect can be abrogated with the use of AZD8931.

5.9.4 AZD8931 is more effective in abrogating HER2-HER3 mediated signalling when compared to Lapatinib.

The previous experiments conducted in this study have suggested that the differential expression of HER2 and HER3 and their mediated signalling could be creating advantageous conditions for the progression of prostate cancer. An experiment was conducted to establish the effect of AZD8931 in cells exhibiting these conditions whilst comparing the action of Lapatinib. It was observed that the presence of HER2 and HER3 together activated both the PI3 Kinase and the MAP Kinase pathways and this was confirmed by the increased presence of activated AKT and ERK5 respectively (**Figure 5-13**). This enhanced activity was reduced by the addition of both Lapatinib and AZD8931. However, it was observed that the AZD8931 exhibited a more pronounced inhibition of the activated pathways.

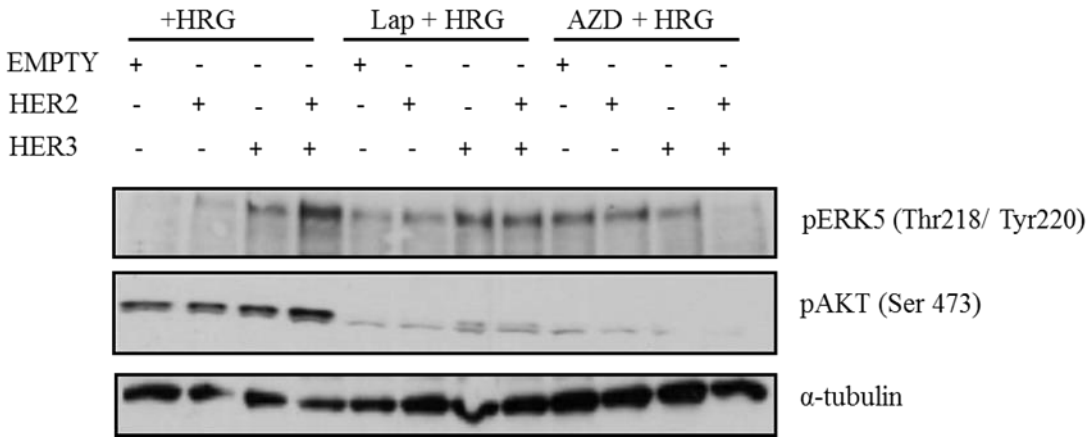


Figure 5-13: AZD8931 is more effective in inhibiting MAP Kinase and PI3 Kinase signalling in cells differentialy expressing HER2 and HER3.

PC3 cells were forward transfected either with empty vector, pIRES-HER2, pIRES-HER3 or pIRES-HER2 and pIRES-HER3 together in full medium. Empty vector was used as a control and to make sure equal amounts of plasmid (2µg) were added to each experimental arm. The following day the cells were starved overnight in basal medium along with the indicated drug treatment (1 µM Lapatinib and 3 µM AZD8931) and this was followed the next day by 15 minutes of Heregulin (HRG; 20ng/ml) treatment to ensure receptor activation. The cells were then lysed in RIPA buffer and analysed for HER2 and HER3 expression levels along with pERK5 and pAKT levels to observe the activity of MAP Kinase and PI3 Kinase pathways, respectively. Increased expression of both pERK5 and pAKT indicated increased signalling of their pathways caused by the over-expression of HER2 and HER3. The addition of both Lapatinib and AZD8931 reduced the activities of the signalling pathways with AZD8931 showing the more prominent effect.

5.10 Discussion

Current research in the field of prostate cancer treatment is focussing on the potential benefits of patient stratification, based on their molecular profiling. This would provide useful information and reveal the survival mechanisms the cancer has utilised which could then be targeted using appropriate drugs. One of the mechanisms the cancer employs for survival is the over-expression of receptor tyrosine kinases that have a role in regulating signalling pathways that are important for cell survival and proliferation. This study so far has profiled a few mechanisms that can lead to the progression of prostate cancer with the over-expression of HER2 and HER3 and suggests the potential for these receptors to be therapeutic targets. The novel inhibitor AZD8931 has been used in this study and observes the effects of pan-inhibition of EGFR, HER2 and HER3 in prostate cancer.

Since this drug hasn't been used in prostate cancer before, an initial proliferation assay was performed to estimate the LC_{50} drug concentration using different prostate cancer cell lines that represent androgen dependent and androgen independent prostate cancer. The LNCaP-AI cell line was the most sensitive cell line upon AZD8931 treatment and validates that EGFR, HER2 and HER3 play a crucial role in maintaining cell survival in an androgen-independent environment (**Figure 5-1**).

The treatment of AZD8931 also influenced the expression and activity of HER2 and HER3 in the different compartments of the cell. The nuclear expression of HER2 and HER3 was observed in clinical samples and their translocation into the nucleus upon activation and being recruited to chromatin has been demonstrated in **Chapter 3** (**Figure 3-18**, **Figure 3-24**, **Figure 3-25** and **Figure 3-26**). The use of AZD8931 resulted in a dramatic inhibition of the expression and activity of HER2 and HER3 in the nuclei of LNCaP cells (**Figure 5-2 (B)**). However, when the cytoplasmic compartment was considered, the total and active forms of HER2 were not reduced, suggesting that they are retained in the cytoplasm (**Figure 5-2 (A)**). The chromatin fraction was also investigated and revealed a reduction in the active and total bound receptors with AZD8931 treatment (**Figure 5-3**). Since the presence of these forms of the receptor is remarkably reduced in the starved cells and the AZD8931 treated cells, it suggests that phosphorylation of these receptor plays a key role in their recruitment to chromatin. The role of these receptors as transcription factors is still not fully understood in prostate cancer. The reduced ability of HER2 and HER3 to bind to

chromatin upon AZD8931 would suggest that their role in prostate cancer sustenance could also be inhibited and needs to be investigated further.

The next set of experiments focussed on the effect of AZD8931 on HER2 and HER3 mediated signalling. The experiments performed in the previous chapters revealed that the activation of the PI3 Kinase and MAP Kinase pathways depended on the differential expression of HER2 and HER3. However, these experiments were performed in the AR negative PC3 cell line. The activation of the signalling pathways and the inhibitory effects of AZD8931 was tested in the AR positive LNCaP cell line. The activation of the receptors using Heregulin showed an increase in the phosphorylation of EGFR, HER2 and HER3 along with the downstream proteins including AKT and ERK. However, this activation was abrogated with the addition of AZD8931 (**Figure 5-4**). It was also observed that AR was phosphorylated at Serine 81, a site known for positively regulated the activity of the receptor (Coffey and Robson, 2012). This observation also validated the luciferase reporter data in **Chapter 4** (**Figure 4-20**) which showed the increase in the transcription of PSA, which is an AR regulated gene. The addition of AZD8931 also reduced the phosphorylation of the AR and the reduction in its activity was further validated by using the LNCaP-7B7 and LNCaP–AI 7B7 cell lines (**Figure 5-4** and **Figure 5-5**), where the transcriptional activity of PSA was also supressed. The experiment was also repeated using siRNA targeting HER2 and HER3. The specificity of the siRNA was verified by the addition of the respective siRNAs on LNCaP cells, for 96 hours, and the expression of HER2 and HER3 was compared using western blotting. The siRNAs proved to be specific as it did not knockdown its respective partner receptor. It was observed that the PSA transcription was reduced with the inhibition of either partner of the HER2 and HER3 heterodimer, thus revealing the importance of their signalling in regulating the androgen receptor (**Figure 5-6**).

The inhibition of the HER2-HER3 signalling axis also exposed various functional consequences. The treatment with AZD8931 reduced the ability of the cells to migrate and invade as seen in **Figure 5-7** and **Figure 5-8** and indicates the benefit of using a pan-inhibitor against the EGFR family in advanced prostate cancer.

The use of other tyrosine kinase inhibitors such as Lapatinib has largely been unsuccessful in prostate cancer treatment (Sridhar *et al.*, 2010; Whang *et al.*, 2013). This study investigated Lapatinib resistance and the resistant cell lines were first validated. **Figure 5-11** showed that the cells has reduced proliferation in the presence of

drug but overall had higher proliferation when compared to the Lapatinib sensitive LNCaP cells. The experiment would be more informative if comparisons were made using LNCaP sensitive cells with and without the presence of drug. An over-expression of EGFR and HER3 receptors was also observed in the LNCaP-Lapatinib resistant cell line suggesting their role in overcoming EGFR and HER2 inhibition (**Figure 5-12**). The cells also retained their ability to be responsive to Heregulin treatment. Increased activity of EGFR suggests that the EGFR-HER3 heterodimers and their mediated signalling could be assisting in cell survival even in the presence of drug. The addition of AZD8931 inhibited the activities of EGFR, HER2 and HER3 in the Lapatinib resistant cell line and provided initial evidence of restoring drug sensitivity (**Figure 5-12**). AZD8931 also seemed to inhibit the downstream phosphorylation of MAP Kinase and PI3 Kinase signalling pathways more efficiently when compared to Lapatinib (**Figure 5-13**). This experiment used the drug concentration (LC_{50}) that was estimated in LNCaP cell lines. These concentrations were used as they were quite low and showed fewer signs of cell toxicity but were still in excess of the IC_{50} concentrations (**Table 5-1**). A comparison of the data by using a drug concentration after estimating the LC_{50} values in PC3 cells for both AZD8931 and Lapatinib would be more appropriate. Quantification of the respective western blots can also reveal the extent of downregulation of the phosphorylation of EGFR, HER2 and HER3.

Compound Name	**EGFR (IC_{50})**	**HER2 (IC_{50})**	**HER3 (IC_{50})**
AZD8931	4 nmol/L	3 nmol/L	4 nmol/L
Lapatinib	33 nmol/L	9 nmol/L	13 nmol/L

Table 5-1: IC_{50} values of EGFR, HER2 and HER3 with AZD8931 and Lapatinib

AZD8931 has been shown to be more potent in inhibiting the phosphorylation of EGFR, HER2 and HER3 (Hickinson *et al.*, 2010)

Compound Name	Maximum tolerated dose (MTD), mg	C_{max} (Peak serum concentration), µg/mL
AZD8931	240	1.46
Lapatinib	1600	2.13

Table 5-2: Pharmacokinetics of AZD8931 and Lapatinib

The available Phase I clinical data was used to compare the pharmacokinetic parameters of AZD8931 and Lapatinib (Burris *et al.*, 2005; Tjulandin *et al.*, 2014)

In summary, the over-expression of the EGFR, HER2 and particularly HER3 can lead to enhanced cell signalling events that are important for cell survival, proliferation, migration and invasion. **Table 5-1** and **Table 5-2** show the available pharmacokinetics data for Lapatinib and AZD8931. AZD8931 is more sensitive in inhibiting the activities of EGFR, HER2 and HER3. The phase I studies using AZD8931 were done in a range of solid tumours but did not include patients who had prostate cancer. It would be interesting to correlate the maximum tolerated dose (MTD) to the dose used this study. It will be useful to perform a clinical trial using AZD8931 in prostate cancer patients and see if they respond well at the MTD. The data collected in this project validates the importance of these receptors in prostate cancer and indicates that HER3 mediated signalling to be crucial in driving these functions especially in advanced and drug resistant disease. The inhibition of HER3 along with its dimerization partners HER2 and EGFR signifies the strong potential of the use of a pan-inhibitor for the treatment of advanced prostate cancer.

Chapter 6 General Discussion

Prostate cancer is the most commonly diagnosed cancer in men in Australia, Europe and the USA (Jemal *et al.*, 2011). The UK holds the 15th place in Europe for mortalities caused by prostate cancer, the highest being Lithuania (CancerResearchUK). Despite the improvement in our knowledge of prostate cancer, management of the disease remains a challenge. The normal functioning of the prostate and the majority of its malignancies are regulated by androgens. Of those cases unsuitable for curative treatments, an initial favourable response is observed in 80% with the use of androgen withdrawal therapy alone, however a more aggressive form of the disease inevitably develops and, whereas previously termed "androgen-independent" disease, is now known as castration-resistant prostate cancer (CRPC) (Kirby *et al.*, 2011). Patients who present with metastatic CRPC have a poor prognosis and it has been estimated that they have a mean survival of 16-18 months (Tilki and Evans, 2014). Castrate resistant prostate cancer remains a therapeutic challenge and recent research in the past decade has focussed on targeting the androgen receptor and its signalling with the development of drugs such as Abiraterone acetate and Enzalutamide. The metastatic disease is also treated by chemotherapeutic agents such as Docetaxel and Cabazitaxel. However, the use of the above mentioned therapies has generated many clinical questions. Firstly, the unavailability of a reliable biomarker for AR-targeted therapies is challenging for clinicians in deciding treatment plans that are beneficial to patients and the optimal sequence of administering these therapies remains unknown. Additionally, regardless of effective blockade of androgen receptor biosynthesis and signalling, the cancer continues to progress (Tran *et al.*, 2009; de Bono *et al.*, 2011; Fizazi *et al.*, 2012) and suggests that the cancer employs alternative mechanisms for the development and maintenance of castrate resistant disease.

One such mechanism is the up-regulation of growth and survival pathways by the over-expression of receptor tyrosine kinases (RTKs). RTKs and their activity are highly controlled in normal cells. However, this regulation is lost in transformed cells and RTKs present with abnormal activity. This phenomenon has been observed in many human cancers including breast, gastrointestinal and non-small-cell lung cancers (Gschwind *et al.*, 2004). This study has focussed on the tyrosine kinases belonging to the EGFR family. The EGFR family consists of 4 member receptors – EGFR, HER2,

HER3 and HER4. Their structure typically constitutes of a ligand binding region, a transmembrane region and a cytoplasmic tyrosine kinase domain. The activation of these receptors is controlled by the expression of their ligands. These ligands all belong to the EGF- related peptide growth factor family and are produced as transmembrane precursors that are then cleaved by proteolysis forming the soluble growth factors. Ligand binding facilitates signalling by the formation of specific homodimers and heterodimers that subsequently activate the cytoplasmic tyrosine kinase domains of the receptor by cross-phosphorylation of the tyrosine residues in the tail zone of each receptor (Hynes and MacDonald, 2009). These phosphorylation events enable the recruitment of specific downstream signalling molecules which can initiate the signalling cascade. However, HER2 and HER3 differ in their properties and possess a non-functional ligand binding domain and tyrosine kinase domain, respectively. They can hence be activated only after heterodimerization with other activated receptors. However, the HER2-HER3 heterodimer is the most effective combination for the downstream activation of signalling pathways. Once active, these receptors are involved in various cellular processes including cell proliferation, differentiation and migration (Graus-Porta *et al.*, 1997; Olayioye *et al.*, 2000; Hynes and Lane, 2005).

The starting point of this study was to validate the expression of HER2 and HER3 using prostate cancer patient material. An increase in HER2 and HER3 expression was observed by performing immunohistochemistry on formalin fixed prostate cancer samples. The samples were scored for the cytoplasmic and nuclear expression of HER2 and HER3. A total of 195 patient samples spread between two microarrays was analysed. The analysis of the samples revealed that both HER2 and HER3 cytoplasmic and nuclear expression increased in prostate cancer samples when compared to benign disease (**Figure 3-11**). However, upon analysis of samples from different Gleason grades and stages of cancer, it was observed that there were no significant variations in HER2 and HER3 expression. Interestingly, an overall high expression of nuclear HER3 was observed (**Figure 3-12** and **Figure 3-13**). A significant correlation of increased protein expression with Gleason grade and different stages of cancer was observed when the samples of the first TMA was considered and suggests that further analysis with more patient material might be beneficial (**Figure 3-3** and **Figure 3-4**). The insufficient sample size could also be due loss of samples from the TMA. The construction of a TMA involves selecting the appropriate tissues for analysis and embedding representative cores to the recipient paraffin wax block according to a pre-

designed layout. However, due to the variations of availability of the required tissues, the final depth of the embedded samples tends to be unequal in the TMA leading ultimately to a loss of the limited samples. Further loss of samples also occurs as the samples are processed through the stages of antigen retrieval and various wash steps involved in the protocol for immunohistochemistry. The limited and inconsistent availability of patient follow-up data led to varied sample numbers in the groups compared. Hence further analysis of the groups with increased sample size would be beneficial for a conclusive result. It would be useful to collect appropriate number of patient samples in each group to ensure equal distribution of samples for a more robust comparison and to observe any statistical significance. That being said, the preliminary results obtained in this study agree with other research findings. Previous reports have shown an increase of HER2 and HER3 expression in prostate cancer samples across all Gleason grades (Ahmad *et al.*, 2011). Nuclear HER3 has also been detected in advanced prostate cancer samples and has been linked to poor prognosis (Koumakpayi *et al.*, 2006). This study also revealed that the differential expression of HER2 and HER3 could be related to patient survival. Patients who presented with low levels of HER2 along with high levels of HER3, both in the cytoplasm and nucleus died significantly quicker hence suggesting an important role of HER3 expression in disease advancement (**Figure 3-18**). The role of HER3 was further strengthened with the analysis of matched-paired patient samples. A total of 69 matched patient samples were analysed for the changes in expression of HER2 and HER3 as they progressed with the disease. The samples were spread across three groups – the hormone naïve group did not receive any androgen–deprivation therapy and biopsy samples from these patients were collected at an initial stage and at a later stage of their disease. The hormone sensitive group consisted of samples taken from patients before they received androgen–deprivation therapy and at a later stage when they were still sensitive to the treatment. The final relapsed group consisted of samples that were taken from patients who were sensitive to treatment and eventually relapsed with aggressive disease. Upon analysis, an upregulation of HER3 cytoplasmic and nuclear expression was observed in the relapsed patients (**Figure 3-20**) enforcing the hypothesis of this study that HER3 could be playing a crucial role in the progression and sustenance of metastatic castrate resistant prostate cancer. A reduction in the tumour suppressor gene PTEN was also observed in the treatment sensitive and relapsed samples although no changes was observed in the downstream pAKT levels. (**Figure 3-21** and **Figure 3-22**). On closer observation of the data there did not seem to be a complete loss of PTEN suggesting the

remaining regulatory role of the tumour suppressor gene. This could be resulting in prevention of increased activation of AKT however still sustaining its activity. Loss of PTEN expression for tumour development and progression is observed in many types of cancers including prostate cancer (Squire, 2009). This mechanism ensures sustained cell signalling by enabling the activation of AKT. Also, the presence of six consensus phosphotyrosine sites at the C-terminal region of HER3 help in the binding of the SH2 domain of PI3 K thus implicating its critical role in the activation of the PI3 Kinase-Akt pathway (Jiang *et al.*, 2012). This suggests that the PI3 Kinase pathway is constitutively active in advanced prostate cancer and the upregulation of HER3 expression is playing a role in this activation.

The presence of nuclear HER2 and HER3 expression in patient biopsy samples led to the investigation of their expression and cellular translocation *in-vitro* using the LNCaP cell line. Upon activation, both HER2 and HER3 translocated to the nucleus and this was confirmed by western blotting and immunofluorescence (**Figure 3-24** and **Figure 3-25**). In the nucleus it was also observed that these receptors bind to chromatin upon activation (**Figure 3-26**). The nuclear expression of EGFR family members has been previously reported. The presence of EGFR in the nucleus has been widely studied and it has been implied to function as a transcription factor acting on chromatin remodelling as well as DNA repair (Nowsheen and Yang, 2012; Dittmann *et al.*, 2013). In prostate cancer, nuclear EGFR activity has been shown to promote bone metastasis by upregulating the transcription factor TWIST1 and downregulating the expression of the miRNA, miR-1 (Chang *et al.*, 2015). Studies in breast cancer have revealed that nuclear HER2 activates the transcription of COX2 gene (Wang *et al.*, 2004). Studies reporting the presence of nuclear HER3 are limited and also indicate roles of regulating gene transcription. Research performed on various cancer types reveals that HER3 localisation and its outcome seems to be dependent on the cancer type and stage. For instance, non-small cell lung cancer (NSCLC) mainly exhibits cytoplasmic HER3 expression. However, the nuclear expression of HER3 is associated with metastatic disease and overall poor survival (Jiang *et al.*, 2012). Interestingly, patients with uveal melanoma show increased survival rates with the presence of nuclear HER3 expression (Trocme *et al.*, 2012). In prostate cancer, the presence of nuclear HER3 has been linked to advanced disease and poor outcome (Koumakpayi *et al.*, 2006). Even though research

in other cancers suggest transcriptional regulatory roles of HER2 and HER3 in the nucleus, these findings need to be further investigated and validated in prostate cancer.

The significance on the outcome on patient survival due to the differential expression of HER2 and HER3 in the clinical samples led to further *in-vitro* investigation. The study was done in PC3 cells that allowed the study of the differential expression of HER2 and HER3 and served as an appropriate model. It was observed that the downstream activation of both the PI3 Kinase and MAP Kinase pathways was substantially increased with HER3 over-expression and this effect was seen both by transient and stable over-expression of HER2 and HER3 (**Figure 4-2** and **Figure 4-8**). The transient over-expression of HER2 and HER3 together in cells should have been confirmed by using plasmids that had different antibiotic selection or with plasmids that incorporated different fluorescent tags. This would enable the selection of cells overexpressing both HER2 and HER3 and would confirm the downstream effects. The study also showed that this differential over-expression and increased signalling activity led to the increase in cell growth, migration and invasion (**Figure 4-9**, **Figure 4-10**, **Figure 4-11**, **Figure 4-12** and **Figure 4-13**). It should be noted that the cell growth assays is a measure of total protein content after 72 hours and does not reflect any discrepancies caused during the setting up of the experiment (time zero). It would hence be informative to repeat these experiments with an additional control for data normalisation. EGFR expression and signalling through the AKT pathway have been previously reported to positively regulate MMP9 activity in non-small cell lung cancer (Pei *et al.*, 2014). This study shows the significance of HER3 mediated signalling and its ability to increase the cell migratory and invasion potential and coincides with the expression profiles of the analysed patient material. Unfortunately, an attempt to study the downstream effects of this differential expression and activity through the observation of the growth of *in-vivo* xenografts was unsuccessful. Although the measurements of the final tumour sizes indicated that there was no change in growth rate, the resected tumours were of different sizes and suggested improper implantation of the PC3-LACZ, -HER2 and -HER3 over-expressing cell lines. The analysis of the resected tumour cells revealed no change in cell proliferation amongst the different cell lines (**Figure 4-16**). However, the expression of HER2 and HER3 increased with Heregulin stimulation, though not when they were grown in full media *in-vitro,* suggesting that there was improper Heregulin stimulation to the cells in the *in-vivo* setting (**Figure 4-15**). The analysis of the resected tumours also revealed that the tumours of larger sizes had stronger expression of HER2,

HER3 and downstream regulatory protein pAKT indirectly suggesting that the expression and activation of HER2 and HER3 are necessary for tumour growth (**Figure 4-19**). It will beneficial to perform additional *in-vivo* experiments to study the increased migration and invasion potential of the over expressing cell lines. Experiments such a lung colony forming assay would be a more appropriate model to study these effects. It would also be beneficial to perform a preliminary study to ensure success rates and then power the study accordingly.

The differential expression observed in the patient samples along with the observations from the *in-vitro* studies suggest an important role of HER2-HER3 signalling in prostate cancer progression. The androgen receptor has been established as the key regulatory protein in driving prostate cancer and its deregulated expression and activity is the main event in establishing and maintaining advanced castrate resistant prostate cancer (Kahn *et al.*, 2014). As previously described (**1.5.1**) there are many theories describing the mechanisms of deregulation of AR expression and activity. The effect of the cross talk between AR and other signalling pathways is of particular interest. This effect was investigated by observing changes in the expression of prostate specific antigen, which is an AR target gene. Luciferase reporter assays confirmed the upregulation of PSA promoter activity upon activation of HER2-HER3 signalling in both androgen dependent and androgen independent cell lines (**Figure 4-20**). The involvement of the androgen receptor activity was proven by the reduction in PSA promoter activity upon treatment with AR antagonist Enzalutamide in the presence of HER2-3 activity (**Figure 4-21**). The differential expression and activity of HER2 and HER3 also increased AR protein expression in the presence of low levels of androgens (**Figure 4-22**). Previous research in prostate cancer has investigated the role of the EGFR family signalling in prostate cancer. Traish and Morgentaler (2009) demonstrated that EGFR levels are regulated in an androgen dependent manner in prostate cancer. This over-expression of EGFR has been associated with stabilisation of the sodium/glucose co-transporter 1 (SGLT1) helping with cell survival by meeting the energy demand (Weihua *et al.*, 2008). EGFR has also been reported to be involved in chromatin remodelling and DNA repair (Dittmann *et al.*, 2013) and proves to be a novel avenue for further research in prostate cancer. Research by Mellinghoff *et al.* (2004) resulted in an important article on prostate cancer signalling, adding to the limited knowledge on the impact of kinase signalling on the androgen receptor in prostate cancer. Their work showed that HER2 mediated signalling enhanced the ability of the AR to bind to its respective androgen

responsive elements. Hsu *et al.* (2011) further showed that increased HER2 expression led to the stability of androgen receptor in an androgen independent environment. The inhibition of the PI3 Kinase pathway led to an increase in AR stability and transcriptional activity by the upregulation of HER2-HER3 mediated signalling and was reported by Carver *et al.* (2011). The EGFR-HER2-HER3 signalling can contribute to the positive regulation of the AR through multiple pathways. EGF mediated signalling has been reported to phosphorylate the AR at Tyr534 through the activity of Src kinase (Liu *et al.*, 2010). Nuclear EGFR can also be implicated in the regulation of the AR as it enhances the expression of Aurora A kinase, which in turn has been shown to activate the AR by phosphorylation at sites Tyr282 and Tyr293 (Hung *et al.*, 2008; Shu *et al.*, 2010). HER2-HER3 mediated signalling also can also enhance AR activity through the activity of Ack1 (activated cdc42-associated kinase). Ack1 once activated can phosphorylate the AR at Tyr267 and enable its function (Liu *et al.*, 2010). The activity of the MAP Kinase pathway by EGFR-HER2-HER3 signalling can also activate S6 ribosomal kinase that has been previously implicated with AR regulation and prostate cancer growth (Whitworth *et al.*, 2012). These findings all suggest the important roles played by these receptors in the progression of androgen independent prostate cancer. However, the inhibition of these receptors has been unsuccessful in prostate cancer treatment as the therapeutic agents used were designed only against one or two members of the EGFR family (Morris *et al.*, 2002; Lara *et al.*, 2004; Ziada *et al.*, 2004; Canil *et al.*, 2005; Guerin *et al.*, 2008).

The above findings led to the investigation of the use of AZD8931, a pan-EGFR family inhibitor from AstraZeneca. The compound has been tested predominantly in breast, lung and head and neck cancers and is currently in phase II clinical trials for solid tumours (Hickinson *et al.*, 2010; Barlaam *et al.*, 2013; Tjulandin *et al.*, 2014). However, prostate cancer has not been included in these studies and the data presented in this project are the novel and preliminary findings of the outcomes of AZD8931 in an *in-vitro* setting. The LC_{50} concentrations for the compound were first established by performing colony forming assays. The androgen dependent LNCaP cell line and the androgen independent LNCaP-AI cell line were used for comparison. Interestingly, the androgen-independent cell line was more sensitive to the compound with a LC_{50} concentration of 0.78 µM when compared to the androgen dependent cell line, which had a concentration of 2.82 µM (**Figure 5-1**) and adds to the theory of the EGFR family playing a key role in androgen independent disease. After observing this effect on cell

survival, the investigation was continued by observing the nuclear translocation of HER2 and HER3. Treatment with AZD8931 demonstrated retention of the total HER2 and HER3 in the cytoplasm (**Figure 5-2 (A)**) and this was confirmed by observing the reduced expression of both the total and activated forms of HER2 and HER3 in the nucleus (**Figure 5-2 (B)**). This was then further analysed and a reduced presence of these receptors and their active forms bound to chromatin was observed (**Figure 5-3**). The role of the nuclear expression of these receptors needs further investigation, however this result suggests that the inhibitory role of the compound can act against any positive regulatory functions of these receptors. The compound was then tested against the activity of the downstream signalling cascades in the LNCaP cell line. The treatment with AZD8931 reduced the activation of EGFR, HER2 and HER3 in the presence of the activating ligand, Heregulin. There were no observed reductions in the total levels of these receptors. The reduction of the receptors' activities led to the reduction in both the MAP Kinase and PI3 Kinase signalling pathways and this was confirmed by the reduction in the pERK and pAKT levels, respectively. Similar to the EGF receptors, no reduction was observed in the total levels of ERK and AKT. The effect on the androgen receptor was also established by the presence of lower active site - serine 81 phosphorylation on the androgen receptor upon AZD8931 treatment (**Figure 5-4**). The reduction in AR activity was further validated by luciferase reporter assays with the use of the LNCaP-7B7 and LNCaP-AI-7B7 cell lines that stably express the PSA-luciferase promoter. The initially shown positive effect of HER2-HER3 mediated signalling on AR activity was lowered with the addition of AZD8931 (**Figure 5-5**). This effect of AZD8931 was also verified with the use of siRNA (**Figure 5-6**). The reduction in the activity of downstream signalling cascades along with that of the AR led to the exploration of these outcomes on cell migration and invasion potential. It was observed that the expression of matrix metalloproteinase MMP9 was reduced with the treatment of AZD8931 (**Figure 5-7**). The migration and invasion assays performed with the use of the compound also proved to effectively reduce these cellular processes (**Figure 5-8**). The final part of this study included the study of tyrosine kinase inhibitor resistance in prostate cancer focussing on the EGFR family. As previously mentioned, the use of the dual kinase inhibitor, Lapatinib, has been largely unsuccessful in prostate cancer treatment (Sridhar *et al.*, 2010; Whang *et al.*, 2013). The current studies suggest that the signalling activities of EGFR and HER2 were still being carried out with the partnering of HER3. This was addressed by comparing the effect of Lapatinib between LNCaP and LNCaP-AI cell line proliferation. It was observed that the androgen independent cell

line had a higher LC_{50} concentration (**Figure 5-9**).This could be contribute to the lack of success of Lapatinib is CRPC. Previous profiling of this cell line showed an overall higher expression of HER3 (**Figure 3-23**) and higher sensitivity to AZD8931 (**Figure 5-1**), hence further validating the role of HER3 mediated signalling in androgen independent disease. The added role of this signalling in drug resistance was tested using the LNCaP-Lapatinib resistant cell line. The cell line developed acquired resistance to Lapatinib upon gradual dose escalation and once established was continuously maintained in normal growth medium with a final Lapatinib concentration of 5µM. The cell line was then profiled for its resistance and it was shown through colony forming assays that the cells were able to grow in different concentrations of Lapatinib (from 0-5µM), unlike the sensitive parental cell line (**Figure 5-10**). The resistance cell line was however had a reduced rate of cell proliferation when grown in the presence of the drug. However, this rate of proliferation was still significantly higher when compared to the LNCaP sensitive cells thus proving its resistance (**Figure 5-11**). When the protein expression was analysed, an increased expression of EGFR and HER3 was observed. The resistant cells were also still responsive to Heregulin, demonstrating a clear increase in pEGFR levels. The addition of AZD8931 efficiently reduced the effect of active EGFR, HER2 and HER3 receptors in the Lapatinib resistant cell line (**Figure 5-12**). The final experiment in this study was to compare the effects of both Lapatinib and AZD891 in HER2 and HER3 differentially expressing cells. PC3 cells were transiently over-expressed with either HER2, HER3 or both receptors together as previously described and showed increased MAPK and PI3 Kinase pathway activation with HER3 over-expression. The addition of Lapatinib reduced the PI3 Kinase pathway activation but the MAP Kinase pathway remained active with HER3 over-expression. However, AZD8931 demonstrated better activity in reducing both of these downstream signalling pathways, even in the presence of high HER3 levels (**Figure 5-13**). This suggests that pan-inhibiton of the receptors could be more effective in patients that show differential expression of HER2 and HER3. However, since these receptors are expressed and play a role in a variety of tissues in the body, the implications of pan-inhibition should be considered. However, the phase I trial using AZD8931 in other solid tumours showed that the compound was well tolerated at a maxium dose of 240 mg and had minor side effects like diarrhea and acne (Tjulandin *et al.*, 2014). A trial including prostate cancer patients having differential strong expression of HER2 and

HER3 could be more informative. It would also be interesting to study AZD8931 resistance and then subsequently study the effect of appropriate combination therapies.

The results from this study sheds more light towards the understanding of the role of HER2-HER3 mediated signalling in advanced prostate cancer. The validation of the presence of the these receptors in the nucleus and their being associated to chromatin, along with its inhibition with the use of AZD8931, stimulates many questions regarding the roles played by these receptors in prostate cancer progression and drug resistance. Investigation of transcription regulatory roles of these receptors by performing ChIP-sequencing would be a good starting point for further studies. Research in breast cancer has revealed that upon androgen stimulation, AR and FOXA1, a transcriptional regulator also over-expressed in prostate cancer, binds to HER3 gene regulatory regions upon androgen stimulation (Ni et al., 2011). On similar lines, the regulatory roles of AR on HER3 expression is reported to be lost in castrate resistant prostate cancer by the loss of its regulatory effect on Nrdp1, an E3 ubiquitin ligase that is known to target HER3 for degradation (Chen et al., 2010). Further investigation of these receptors' transcriptional regulation, intracellular translocation and recycling mechanisms may unveil more deregulated mechanisms in advanced prostate cancer and could serve as new targets for therapeutic intervention. The differential expression of these receptors has revealed the importance of HER3 in advanced prostate cancer. The clinical data, along with the *in-vitro* analysis show increased activity of both the MAP Kinase and PI3 Kinase pathways. The role of HER3 in predominantly activating the PI3 Kinase pathway is well established. Patients samples analysed in this study suggests that this pathway is still active in aggressive disease due to the loss of PTEN expression, along with the upregulation of HER3 cytoplasmic and nuclear expression. Since the PI3 Kinase pathway is critical for cell survival, it's over activity has been linked to drug resistance. However, the inhibition of this pathway in prostate cancer has had limited success due to the upregulation of HER2-3 mediated signalling and dual pathway inhibition has been suggested for better anti-tumour activity (Carver *et al.*, 2011). The combination of AKT inhibition along with the inhibition of EGFR-HER2-HER3 mediated signalling has proven effective in reducing breast cancer tumour growth (Crafter *et al.*, 2015) and suggests its use in prostate cancer treatment could be beneficial. Signalling through the EGFR family has also been linked to tumour cells acquiring drug resistance, in particular as the result of the recently described, critical role of HER3 mediated signalling (Dey *et al.*, 2015). For example, a recent study in

prostate cancer has shown that modulating the amounts of EBP1 – HER3 binding protein, affected Lapatinib sensitivity (Awasthi *et al.*, 2015). The work in this project on Lapatinib resistance further validates the role of HER3 in prostate cancer and the study is currently being continued by performing a wider screen for deregulated mechanisms with the use of a reverse phase protein array (RPPA). Another recently published article this September on breast cancer research showed that the targeting of nuclear HER2 inhibited the cells from acquiring and also overcoming Trastuzumab resistance, suggesting a similar mechanism could be useful in prostate cancer treatment (Cordo Russo *et al.*, 2015). Interest also lies in linking the HER2-HER3 mediated signalling and AR as the driver of therapeutic resistance in prostate cancer. The combined use of the anti-androgen drug Enzalutamide along with Lapatinib has revealed enhanced anti-tumour activity in castrate resistant prostate cancer by inhibiting the activity of YB-1, a transcription factor regulated by AKT, and this factor in turn positively regulates HER2 expression (Shiota *et al.*, 2015). YB-1 has been reported to be over-expressed in prostate cancer and has been linked to resistant and advanced disease progression (Shiota *et al.*, 2011). Interestingly, studies in non-small cell lung cancer have shown that the inhibition of YB-1 led to a depletion in HER3 levels (Kashihara *et al.*, 2009), suggesting a potential novel regulatory mechanism in prostate cancer which requires further exploration. Current research in our group has also confirmed an increase in HER2 and HER3 levels in an Enzalutamide resistant cell line (established in house) and these changes in expression are being investigated by gene microarray. **Figure 6-1** shows the summary of the findings of this project. The data collected from this project, along with the recent published findings, suggest that the simultaneous inhibition of EGFR, HER2 and HER3, perhaps in combination with androgen receptor blockade or more recently identified targets such as YB-1, could lead to an enhanced, prolonged delay to drug resistant / castrate resistant disease, and ultimately improve mortality rates in advanced prostate cancer.

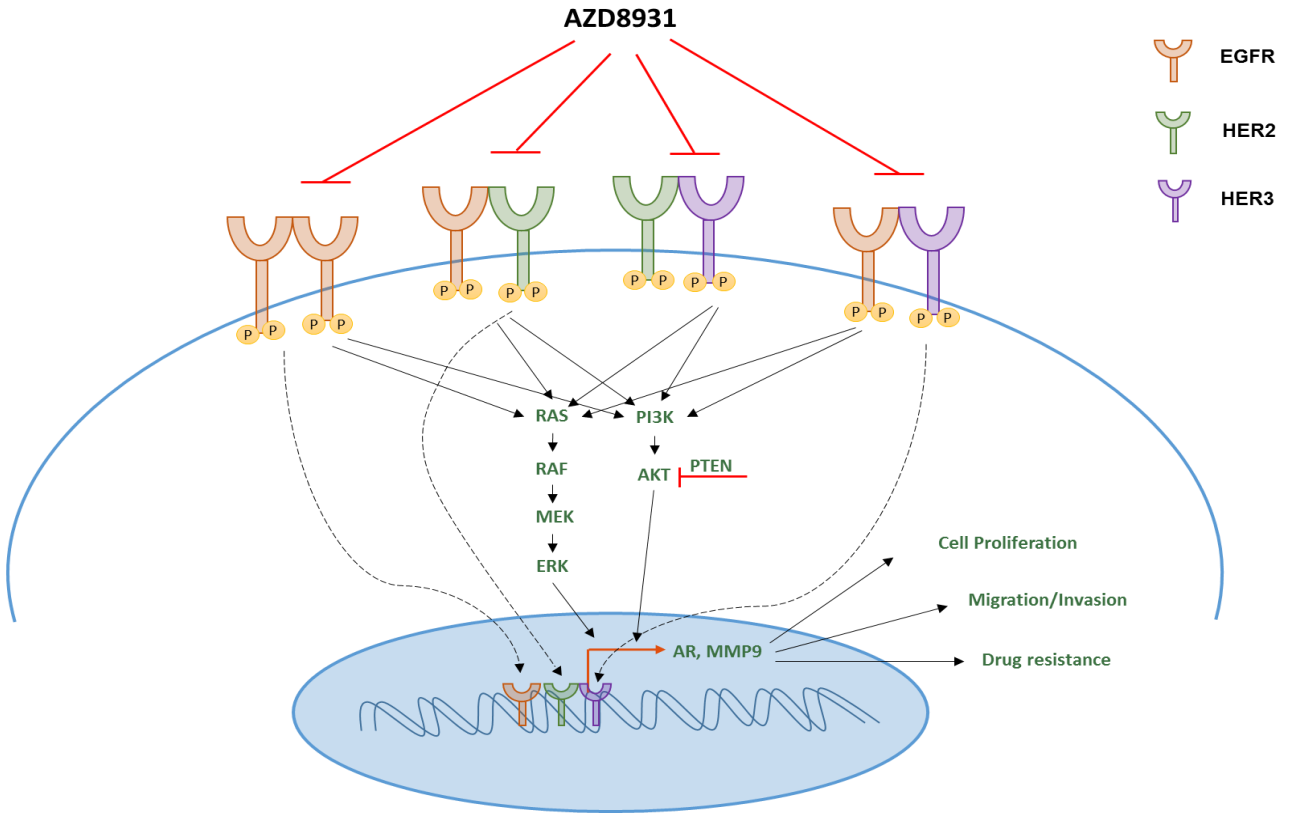


Figure 6-1: Diagram summarising the findings of this PhD project

The activity of EGFR/HER2/HER3 receptors activate the downstream MAP Kinase and PI3 Kinase signalling pathways that regulate a diverse range of cellular processes like migration and invasion that are beneficial for tumour growth. The receptors also translocate to the nucleus and can regulate the transcription of genes that can support tumour growth and can also help in attaining drug resistance. Targeting EGFR, HER2 and HER3 suggests a novel strategic approach in the treatment of advanced prostate cancer, whose growth is dependent on these receptors.

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