The Role of EGFR, HER 2-4, Heregulin and Downstream Signalling in Prostate Adenocarcinoma

Samuel John Sydney Grimsley

MSc: Cancer Sciences and Molecular Pathology

University of Glasgow

Faculty of Medicine

February 2008

ABSTRACT

Background

Currently evidence regarding influence of the HER tyrosine kinase family - EGFR, HER2, HER3 and HER4 - during disease progression in prostate adenocarcinoma is conflicting – both poor prognosis and no influence on outcome are reported. A small cohort pilot study of paired hormone sensitive (HSPC) and refractory (HRPC) specimens demonstrated HER2/HER4 as positively prognostic in HSPC. Heregulin (HRG), a principle HER family ligand, has previously been noted to have a differential effect on HSPC (decreased proliferation) and HRPC (increased proliferation) cell lines. This study determines influence of HER family and HRG in a larger HSPC cohort and whether influence mechanisms involve proliferation or apoptosis.

Patients and Methods

Immunohistochemical staining for HRG, KI67 (proliferation), TUNEL (apoptosis) was performed on pilot study specimens. Further IHC for EGFR, HER2, HER3, HER4, HRG, KI67 and TUNEL was performed on HSPC tissue microarrays. Correlations between target protein expression and the outcomes time to biochemical relapse and overall survival were determined.

Result

High expression of HER/HRG was correlated with improved prognosis particularly in androgen deprivation treated subcohort (e.g. high EGFR and longer time to relapse p=0.02, high HER2 and delayed relapse p=0.002, high HRG and delayed relapse-p=0.004). High expression of multiple markers increased association significance

(e.g. high HER1-4 and delayed relapse p=0.001). No correlations between HER and proliferation or apoptosis were seen.

Conclusion

The HER family and HRG are positively prognostic in prostate adenocarcinoma. This has implications for the use of HER family as outcome predictors to guide management.

CONTENTS

ACKNOWLEDGMENTS

I dedicate this work to my wife Athena Reid-Grimsley without whose patience and support it would not have been remotely possible to complete the project.

I would also like to acknowledge the following people who have assisted in completing this project;-

Dr Joanne Edwards - Project supervisor, Laboratory Support, assistance with scoring

Mr. Mark Underwood - Clinical Support	Pamela McCall - Assistance with scoring
Suba Shini - Assistance with scoring	Mustapha Hilmy – Assistance with scoring
Wilson Angerson - Statistical Assistance	Stuart McCracken - Provision of TMAs/patient data

SPECIFIC ATTRIBUTION

The scientific background was researched and presented in the background section by the candidate as was the method section.

The Heregulin antibody was optimised for the lab by the candidate who performed all HRG IHC staining of slides and TMAs and was primary scorer. Co-scoring was performed by S Shini and J Edwards. Western Blot was performed by the candidate.

KI-67 Antibody and TUNEL Assay were optimised, and both slides/TMAs stained and scored by the candidate with co-scoring by M Hilmy (KI-67) and J Edwards (TUNEL).

As mentioned in the text the staining/scoring of slides for HER1-4 and EGFRvIII were performed in a previous pilot study. Additionally TMA staining for HER1-4 was performed by J Edwards and scored by J Edwards/P McCall

All Statistical analysis was performed by the candidate.

The Discussion and Conclusion were written by the candidate.

ABBREVIATIONS

ACTH	Adrenocortocotrophic Hormone
ADT	Androgen Deprivation Therapy
AP	Activated Protein
APS	Ammonium Persulphate
AR	Androgen Receptor
ARA70	Also known as NCOA4 – Nuclear receptor coactivator 4
ARE	Androgen Response Elements
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CaP	Prostate Cancer
CDK	Cyclin-dependent Protein Kinases
DAB	3,3'-diaminobenzidine
DBD	DNA Binding Domain
DES	Diethylsilboestrol
DHT	Dihydrotestosterone
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor Variant III
EGFR-WT	Epidermal Growth Factor Receptor Wild Type
ELISA	Enzyme-Linked ImmunoSorbent Assay
FGF	Fibroblast Growth Factor
FISH	Fluorescence In-Situ Hybridisation
GnRH	Gonadotrophin Releasing Hormone

GPCR	G-protein Coupled Receptor
HER	Human Epidermal Growth Factor Receptor
HNSCC	Head and neck squamous cell carcinoma
HRG	Heregulin
HRP	Horseradish Peroxidase
HRPC	Hormone Refractory Prostate Cancer
HSPC	Hormone Sensitive Prostate Cancer
ICCC	Inter-Class Correlation Coefficient
IgG	Immunoglobulin G
IHC	Immunohistochemistry/Immunohistochemical
IL	Interleukin
kD	kilo Daltons
LBD	Ligand Binding Domain
LH	Luteinising Hormone
LHRH	Luteinising Hormone Releasing Hormone
LREC	Local Research Ethics Committee
LSAB	Labelled streptavadin-biotin
mAB	Monoclonal Antibody
MAB	Maximal Androgen Blockade
MAP	Mitogen Activate Protein
MAPK	Mitogen Activate Protein Kinase
MREC	Multicentre Research Ethics Committee
MMP	Matrix Metalloproteinase
NLS	Nuclear Localisation Signal
Nrdp1	Also known as RNF41 – Ring Finger Protein 41

NRG	Neuregulin
NSCLC	Non-small cell lung carcinoma
OS	Overall Survival
PI3K	Phosphoinositide 3 Kinase
PIN	Prostatic Intraepithelial Neoplasia
PLC-γ	Phospholipase C
PKC	Protein Kinase C
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Holologue
PVDF	Polyvinylidene Difluoride
SDS	Sodium Dodocyl Sulphate
STAT	Signal Transducer and Activator of Transcription
TACE	Tumour necrosis alpha converting enzyme
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
TGF-α	Transforming Growth Factor α
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TMA	Tissue Microarray
TRUS	Trans-rectal Ultrasound
TTBS	Tris Buffered Saline – Tween
TTDFR	Time to Death from Biochemical Relapse
TTR	Time to Biochemical Relapse
TURP	Trans-urethral Resection of Prostate

LIST OF FIGURES/ILLUSTRATIONS

Chapter 4

LIST OF TABLES

CHAPTER 1: BACKGROUND

1.1 PROSTATE ADENOCARCINOMA

Prostate Carcinoma (CaP) is a significant and growing health issue in the UK and the rest of the developed world and the focus of many areas of research. Adenocarcinoma comprises over 95% of CaP (Tanagho et al. 2004) arising primarily in the peripheral zone (85%) or transitional zone (15%) of the prostate.

In the UK incidence of CaP has risen significantly to become the most commonly diagnosed cancer in males. There were 31 900 CaP diagnoses in the UK in 2003 (Cancer Research UK: UK Prostate Cancer Statistics) representing 23% of new male cancers and 12% cancers overall. Incidence varied between UK regions with 110/100 000 in England, 140/100 000 in Wales 95/100 000 in Scotland and 86/100 000 in Northern Ireland (Cancer Research UK: UK Prostate Cancer Statistics). There has been a rise in UK incidence from ~34/100 000 in 1975 to 109/100 000 in 2003. This increase may be due to a combination of implementation of widespread Prostate Specific Antigen (PSA) testing and the generally aging demographic of the Western world. Age remains the principle risk factor for development of CaP rising from extremely few cases diagnosed below the age of 50 increasing to 120/100 000 at 55-59, 450/100 000 at 75-79 to just under 900/100 00 at over 85 (Selley et al 1997).

Worldwide the USA has the highest incidence in CaP with over 120/100 000 males compared to 54/100 000 males in the UK in 2002 (Ferlay et al 2002). Post mortem analysis indicates a 40% lifetime risk of CaP development (Tanagho et al. 2004).

UK CaP mortality has also increased over the past 30 years however not to the same extent as incidence. In 2005 there were 10 000 CaP deaths (34/100 000) again varying between the regions England 34/100 000, Wales 36/100 000, Scotland 31/100 000, Northern Ireland 26/100 000 (Cancer Research UK: UK Prostate Cancer Statistics) This represents 13% male cancer deaths, the 2nd most frequent cause of cancer death behind lung. As with incidence, mortality rises sharply with age from virtually no patients below the age of 50 to 800/100 000 at over 85 years old. UK age standardised mortality was 25/100 000 in 2005 compared to 20/100 000 in 1975 however the rise took place in the 1980s and now appears to have been a slight fall since the early 1990s (Majeed et al. 2000). Similar falls have been seen in the USA (Tarone et al. 2000) and Europe (Levi et al. 2000), this has been attributed to increased screening, treatment improvement (Hankey et al. 1999) and changes in cancer death attribution or coding (Feuer et al. 1999, Swerdlow et al. 2001) but a definite explanation has yet to emerge.

1.1.1 HORMONAL TREATMENT OF PROSTATE ADENOCARCINOMA

Treatment options for CaP are determined by stage as defined by the TNM staging system. For local organ-confined CaP (T1-2) there are several potentially curative options including radical prostatectomy, radical radiotherapy and brachytherapy. In general the mainstay of locally advanced (T3-4) or nodal/distant metastatic disease is endocrine therapy alone or combined with radiotherapy (at this time prostatectomy also an option in low grade T3a disease). While clinical trials are ongoing there are few chemotherapeutic agents e.g. Docetaxel (Tannock et al. 2004), that have been shown to be effective in prostate cancer, certainly none that are more effective with a better side effect profile than endocrine therapy in newly diagnosed CaP. In patients

with low grade, low volume disease a strategy of watchful waiting – delaying treatment until biochemical progression occurs – can also be employed

1.1.1.1 HORMONAL REGULATION

Endocrine treatment is based on the interaction of CaP cells with the androgen production axis (Fig. 1). Production of Testosterone, the principle androgen in men, is coordinated centrally via the hypothalamic-pituitary-gonadal axis and feedback loop. The axis is initiated by release of gonadotrophin-releasing hormone (GnRH, also termed Luteinising Hormone Releasing Hormone) by the hypothalamus. GnRH is released in pulsatile, circadian and seasonal manner as well as variation at key developmental times of life such as puberty. GnRH acts on the anterior pituitary gland stimulating production and release of luteinising hormone (LH) into the circulation. In a parallel system corticotrophin-releasing hormone released from the hypothalamus stimulates adrenocorticotropic hormone (ACTH) secretion by the pituitary. LH acts on Leydig cells in the testes stimulating production of testicular testosterone which makes up 95% of circulating testosterone. Testosterone is also produced in the adrenal gland in response to ACTH, in both testis and adrenals it is produced via the gamma-5 metabolic pathway of steroid hormone synthesis.

Circulating testosterone is mainly bound to sex hormone binding globulin with only a small fraction free. Testosterone enters prostatic cells by passive diffusion and is converted to the more potent dihydrotestosterone (DHT) by 5-α-reductase or to oestradiol by 5-α-aromatase. DHT has multiple cellular functions but also acts as a negative feedback agent acting on the hypothalamus to reduce GnRH production completing a negative feedback loop. Oestradiol also forms a negative feedback loop

acting at the pituitary to reduce LH secretion. Endocrine therapies for CaP act via interference with the androgen production axis (see below)

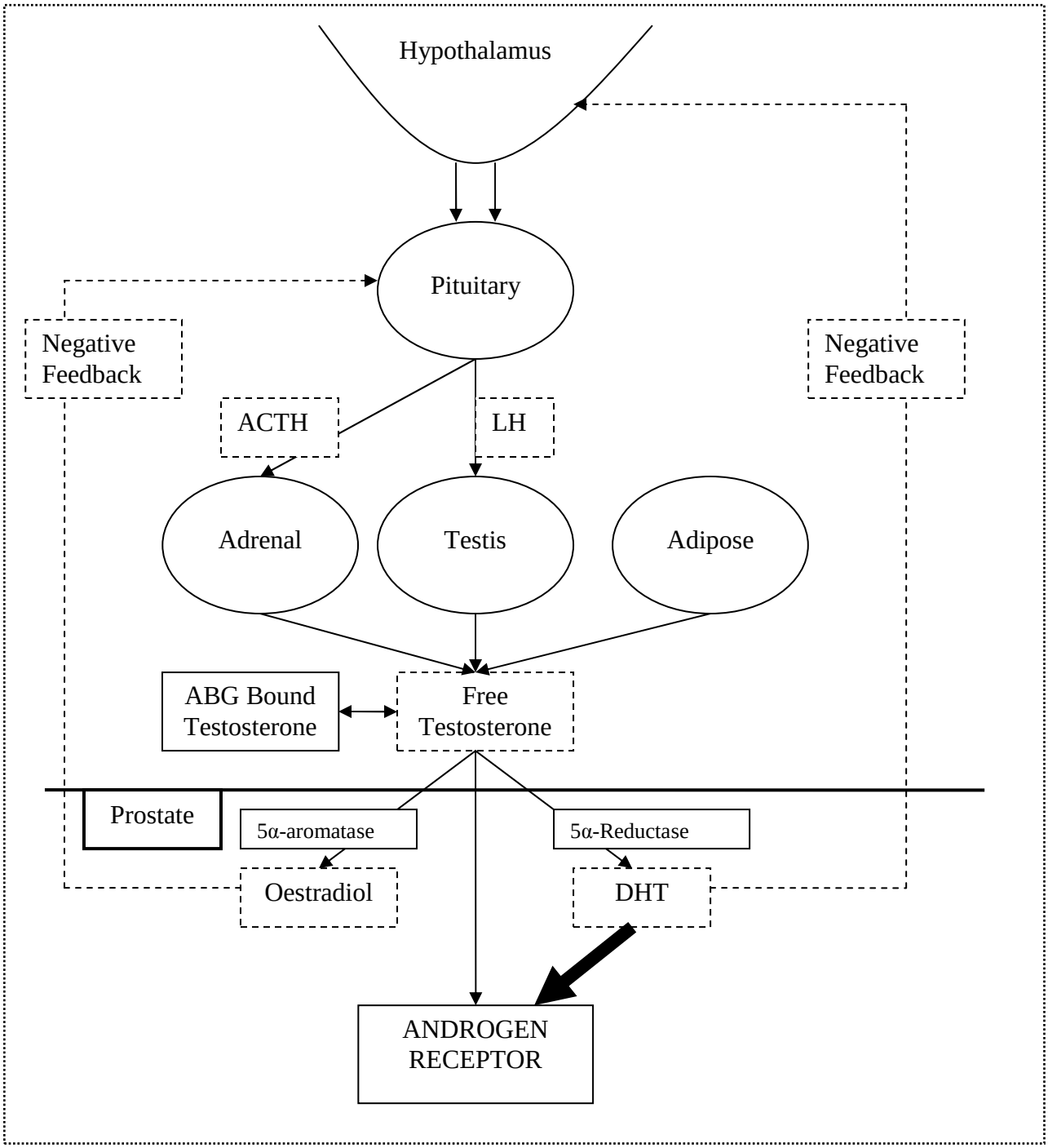


Figure 1.1: Regulation of Testosterone Production

1.1.1.2 THE ANDROGEN RECEPTOR

Prostatic testosterone and DHT both act by binding to the androgen receptor (AR) (Fig. 1.2); DHT has a 10 times greater affinity than testosterone (Suzuki et al. 2003). The AR is a steroid hormone receptor encoded by a gene on the long arm of the X chromosome and comprises 4 main functional domains (Culig et al. 2002);

- Amino-terminal regulatory domain – contains the activation function region AF-1 which allows binding of multiple co-factors and protein kinase pathway members and is important in transactivation/transcription regulation. (Rochette-Egly 2003)
- DNA-binding domain (DBD) – includes 2 zinc finger motifs that bind to androgen response elements (ARE) nucleotide sequences in the promoter regions contained within target androgen regulated genes (Lee and Chang 2003)
- Dimerisation/Hinge domain – responsible for dimerisation and translocation to the nucleus following ligand binding. Contains nuclear localisation signal (NLS) region that facilitates this. Phosphorylation of this region may cause inactivation of the AR. (Rochette-Egly 2003)
- Ligand-binding domain – contains the site for DHT/testosterone binding causing AR activation and the activation function AF-2 region which also interacts with cofactors influencing transcription (Rochette-Egly 2003)

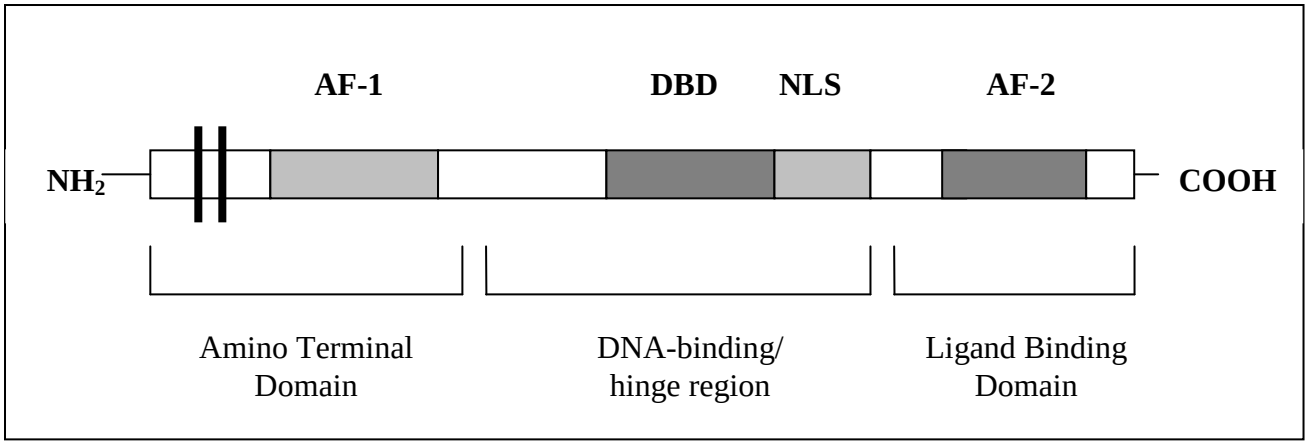


Figure 1.2: Structure of the Androgen Receptor. Bands in the amino terminal region represent polymorphic trinucleotide repeats e.g.CAG

Pre-activation AR is bound to heat shock proteins which prevent degradation. Ligand binding induces a conformational change and dissociation from these heat shock proteins. This is followed by phosphorylation of AR stabilising the ligand-receptor complex which then dimerises (Lee and Chang 2003) and translocates to the nucleus where the DNA-binding region binds to AREs initiating androgen directed transcription. After binding transcription is further regulated by co-factors which bind to AR aiding formation of a stable pre-initiation complex facilitating transcription. Androgen directed transcription results in an increase in cell proliferation and decrease in apoptosis stimulating prostatic tissue growth amongst other effects.

1.1.1.3 METHODS OF HORMONAL TREATMENT OF PROSTATE ADENOCARCINOMA

Hormonal treatment of CaP also termed Androgen Deprivation Therapy (ADT) is based on the principle that, in a similar fashion to breast cancer cells expressing oestrogen/progesterone receptors, CaP cells express AR making CaP androgen dependent. Androgen suppression as a treatment for CaP has been noted since 1941 when Huggins et al. demonstrated reduction in CaP related symptoms following surgical castration or diethylsilboestrol (DES) therapy after which survival benefits of

endocrine treatment in metastatic CaP were shown (Nesbit et al. 1950). The oestrogen DES acts on the pituitary (Fig 1.3) to reduce pituitary LH production in response to hypothalamic GnRH as oestrogens act naturally as negative feedback agents in the androgen production axis. With reduced LH production stimulation of testicular testosterone production falls (Blackard et al. 1970). As mentioned above endocrine therapy is utilised where curative radical therapy is inappropriate either due to the nature of the cancer – local or distant metastasis, or the patient – elderly and/or significant comorbidities. Additionally hormone therapy is used as neoadjuvant treatment with radiotherapy in T3 disease and as second line therapy in patients who suffer recurrence after prostatectomy/radiotherapy.

Since its inception methods of hormonal therapy have developed with neither orchidectomy nor DES remaining first line. The sites of action of ADT agents are shown in Fig.1.3 Common agents include

- Androgen Antagonists (also known as antiandrogens) – interfere with activation of AR androgen-androgen receptor complex formation directly in tumour cells by competitive binding to AR or inactivating androgens before binding. Target androgens whether the source is testicular or adrenal. Examples include 2-hydroxyflutamide, the metabolite of the agent flutamide, bicalutamide and cyproterone acetate. Antiandrogens can be used as single agents (Wellington et al. 2006) but are more commonly used in combination with other medications or orchidectomy. While antagonistic to CaP in most cases antiandrogens have stimulated cell proliferation in certain CaP cell lines and conversion from antagonist to agonist activity may be one mechanism behind hormone escape (see below).

- Gonadotrophin Releasing Hormone Agonists – target the hypothalamic-pituitary-gonadal hormone axis to reduce circulating androgen concentrations. These act at the pituitary by first stimulating LHRH receptors but ultimately causing receptor downregulation and thereby blocking hypothalamic-pituitary signaling and gonadotrophin secretion (Schally et al. 1992). GnRH agonists such as Goserelin therefore block the production of testicular but not adrenal testosterone. The agonistic nature of theses agents entails a brief flare-up with increased testosterone risking a transient worsening of disease hence a short course of antiandrogen is often used to cover the first dose of GnRH inhibitor. Side effect profile includes impotence, loss of libido, hot flashes, gynaecomastia and osteoporosis; it does not include the increased risk of cardiovascular complications of DES and is considerably better than that of cytotoxic chemotherapeutic agents.
- Maximal Androgen Blockade – Antiandrogen with GnRH analogue or orchidectomy

Rate of response to hormonal therapy as assessed by symptom relief, reduction in primary tumour size or fall in acid phosphatase/PSA varies 40-80% between studies (Murphy et al. 1980, Wein 2007). Endocrine treatment is not considered curative although at least one study has claimed this (Johansson et al. 1981)). A complete response with disappearance of all detectable disease is seen in only 5-10% (Murphy et al 1980). Progression despite hormonal treatment, also known as hormone escape or progression from hormone sensitive CaP (HSPC) to hormone refractory/resistant CaP (HSPC) occurs almost universally (Feldman et al. 2001) with time to progression

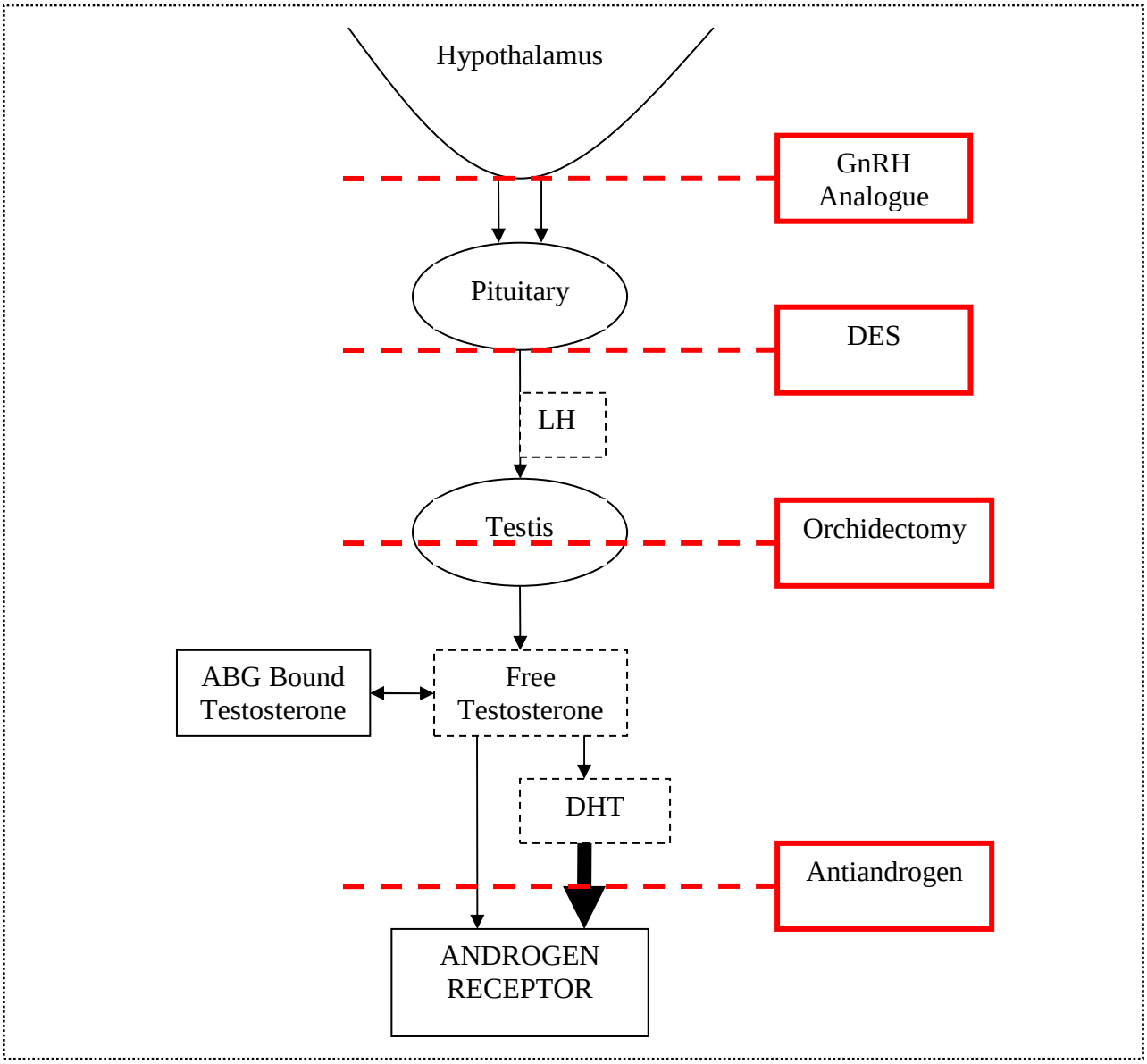


Figure 1.3: Sites of action for hormonal therapy for CaP

18 – 36 months varying between studies (Trachtenberg et al 2002, Wein 2007). Hormone escape occurs in 2 stages, a transition stage with tumour cells still requiring androgen to proliferate but not requiring androgen to survive then an outgrowth phase with androgen required neither for survival nor growth (Craft & Chhor). Progression can be defined as either physically – by increase in size of lesion/s by 25% or appearance of new lesion (Wein 2007) or biochemically – by rise in acid phosphatase

or, much more frequently, PSA from a nadir achieved after commencement of hormone therapy.

Prognosis in CaP is much reduced following hormone escape with mean time to death from escape 9-12 months (Henry et al. 1999). Much research in prostate cancer has focussed on factors contributing to hormone escape and potential treatments for HRPC. Current available strategies involve further hormonal manipulation e.g. removal of antiandrogen (20-30% demonstrate secondary PSA response)/conversion to maximal androgen blockade and cytotoxic chemotherapy e.g. Docetaxel.

1.1.2 MOLECULAR BIOLOGY OF PROSTATE ADENOCARCINOMA; AN OVERVIEW

Many genes and gene products have been investigated for their influence on CaP development, in particular oncogenes, the androgen receptor, growth factors, growth factor receptors and their transcription pathways.

9% CaP can be linked to one of a number of genes which increase likelihood of CaP development inherited in an autosomal dominant fashion. Such inheritance is seen more frequently in tumours presenting at a younger age (Carter et al. 1992). Deletion of Tumour Suppressor Genes at several chromosomal loci (Isaacs et al. 1995) including 8p, 10q, 13q (Retinoblastoma-Bookstein et al 1990), 16q (Breast Cancer Antioestrogen Receptor 1 (Fromont et al. 2007), 17p and 18q have been linked to localised CaP. In particular deletion at 8p22 is found in 70% localized CaP (Macoska et al. 1994) and E-cadherin at 16q deleted in 60% of metastatic CaP (Umbas et al. 1994). As in other cancers p53 mutation is associated with 50% high grade metastatic

CaP (Navone et al. 1993). A Tumour Suppressor Gene mapped to 10q23 which codes for protein PTEN (Li et al. 1997), a tyrosine phosphatase, which appears to be mutated in a large number of cancer cell lines with a greater proportion CaP cell lines (100%) versus other cancer types (31% glioblastoma, 6% breast Ca etc). Mutation of PTEN results in loss of expression therefore the PI3K/AKT pathway is upregulated (Davies et al. 1999).

Given the importance of androgenic stimulation in normal prostatic development as well as CaP (Feldman et al. 2001) and the pivotal role of androgen therapy in CaP attention relating to hormone escape has focussed on dysfunction of the androgen receptor (AR) in development of CaP and subsequent development of hormone escape. Evidence for AR involvement of AR in HRPC includes high AR expression in recurrent CaP (Van der Kwast et al. 1991)) and inhibition of proliferation of hormone refractory cell lines by in vitro inhibition of AR.

Several mutations have been identified affecting the ligand binding region of AR which allow a number of alternative ligands even antiandrogens such as hydroxyflutamide (Taplin et al. 1999) to stimulate transcriptional activity and tumour growth. Such mutations have been shown to develop in response to antiandrogen therapy itself and form the theoretical basis for the secondary PSA response when discontinuing antiandrogen therapy following hormone escape. Additional mutations have been noted in the transactivation amino-terminal domain allowing AR stimulated transcriptional activity without ligand binding (Wallen et al. 1999). These include a CAG polymorphism site where a shorter CAG repeat length is associated with increased CaP risk (Irvine et al. 1995). More than 60 AR mutations have been

described in CaP (Debes et al. 2002) however such mutations are only found in 10-20% CaP in vivo (Suzuki et al 2003, Taplin 2003).

The rate of AR gene amplification has been shown to be significantly higher following development of hormone escape (Brown et al. 2002, Edwards et al. 2003). Approximately 20-30% of HRPC samples have AR amplification compared to 0-5% in HSPC (Edwards et al. 2003) and this study also showed an association between AR amplification and reduced survival. While most tumours with AR amplification also demonstrated increased AR protein expression increased protein expression did not influence survival although this may reflect more accurate detection of gene amplification by FISH than corresponding protein expression by IHC. A xenograft-model study demonstrated increased AR mRNA expression related to hormone escape independent of gene amplification (Chen CD et al. 2000) although this does not represent the normal mechanism of AR upregulation in prostate cancer. These studies indicate that AR gene amplification and consequent increased AR protein expression in CAP cells allow a response to the reduced androgen levels in patients on GnRH analogues/antiandrogens thereby allowing progression in spite of hormonal therapy.

Increased phosphorylation of AR via a number of MAP kinase/AKT kinases also increased sensitivity to low androgen levels (Rochette-Egly 2003) and increased MAP kinase correlates with CaP grade and stage (Uzgare et al. 2003) and is found in increased levels in HRPC (Bakin et al. 2003). AR phosphorylation is one of a number of mechanisms whereby AKT may influence CaP development, higher AKT activity is found in CaP than benign prostate disease (Liao et al 2003) and it is higher in HRPC cell lines (Ghosh et al. 2002). Activation of Akt has been significantly

associated with decreased survival in hormone refractory tumours (Edwards et al. 2006) and strongly correlates with phosphorylation of the AR at serine 210 in the same patient cohort (McCall et al 2008).

Additionally the roles of several cofactors which bind to AR and alter its transcriptional activity have been investigated in relation to CaP and hormone escape. Examples include CBP (cAMP response element binding protein) which is overexpressed in HRPC and has been shown to allow hydroxyflutamide to activate AR in vitro (Gelfanov et al 2001) and ARA70, also upregulated in HRPC, has been shown to allow bicalutamide to act as an AR ligand (Yeh et al. 1996). C-Jun a cofactor which binds to the intracellular AR domain allows ligand independent signal transduction (Rochette-Egly 2003). Other cofactors e.g. STAT3 bind with AR and effect translocation of AR to the nucleus (Lee SO et al. 2003). The STAT3 pathway has been shown to be activated by interleukin-6 accompanied by conversion from HSPC to HRPC (Tam et al 2007), in fact IL-6 has been suggested as being linked to prostate cancer morbidity for over 10 years (Twillie et al. 1995).

Increased expression of a number of growth factors (EGF, TGF-α, FGF etc.) have been linked to the earliest stages of CaP including changes in stromal-epithelial cell interactions and angiogenesis necessary for tumour development and invasion. Increased vascular endothelial growth factor stimulated by other growth factors in tumour epithelial cells plays in particular has been linked to angiogenesis and subsequent invasion (Foster et al. 2002, Bhowmick et al 2004, Chung et al. 2005). TGF-α has been reported as having a tumour suppressor function (Kambhampati et al. 2005).

Several intracellular signal transduction pathways have been implicated in development of HRPC via bypassing AR. In addition to acting on the AR the MAP kinase pathway members are upregulated in HRPC and hormone escape has been shown occur by transfection with Raf via the MAP kinase pathway (Bakin et al. 2003,) independent of AR. C-Jun combines with c-Fos forming transcription factor Activated Protein 1 (AP1) which can activate AR targets without AR involvement (Edwards, Krishna et al. 2004). Components of the hedgehog signalling cascade are upregulated in CaP cell lines compared to normal prostate tissue and may stimulate growth in CaP (Karhadkar et al 2004). Several ligands and members of the Wnt/β-catenin signalling pathway are overexpressed in HSPC and HRPC cell lines (Chen G et al. 2004). Additionally several neuropeptides acting via G-protein-coupled receptors influence multiple tumorigenic cascades to upregulate CaP proliferation and metastasis (Mimeault et al. 2003).

1.2 HER FAMILY

1.2.1 HER1-4

The HER (Human Epidermal Growth Factor Receptor) family of transmembrane glycoprotein receptor molecules consists of 4 members; Epidermal Growth Factor Receptor (EGFR also termed HER1 and ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) and their variants. EGFR was first noted to be connected with oncogenesis in the 1980s during study of the avian erythroblastosis tumour virus which encodes an aberrant form of human EGFR (Burden et al 1997). HER2 was then identified due to homology to but distinction from EGFR (Schechter et al. 1984) and its amplification in mammary carcinoma (King et al. 1985). The gene loci for the HER family are EGFR 7p12, HER2 17q11, HER3 12q13, HER4 2q34. Classically the EGFR family members are usually considered as transmembrane receptors and as such are located in the cell membrane unless internalised to the cytoplasm as part of signalling cascade (Carraway et al. 2001). One specific pathway involves cleavage of HER4 by tumor necrosis factor α converting enzyme and presenilin-dependent γ secretase allowing the cytoplasmic portion to be internalised and accumulate in mitochondria stimulating apoptosis (Vidal et al. 2005). However, more recent research has revealed that HER3 may also be involved with signaling pathways within the cell nucleus (Koumakpayi et al. 2006) associated with oncogenesis.

HER family members have a common structure (Fig 1.4) with 3 domains;-

- Extracellular domain – the ligand binding domain (LBD) containing 2 cysteine rich regions and is glycosylated. The ligand specificity of this region varies between family members
- Transmembrane domain – single helix of 23 hydrophobic amino acids which anchors the receptor to the cell membrane

- Intracellular domain – has tyrosine kinase (TK) activity, responds to ligand binding with initiation of signal transduction

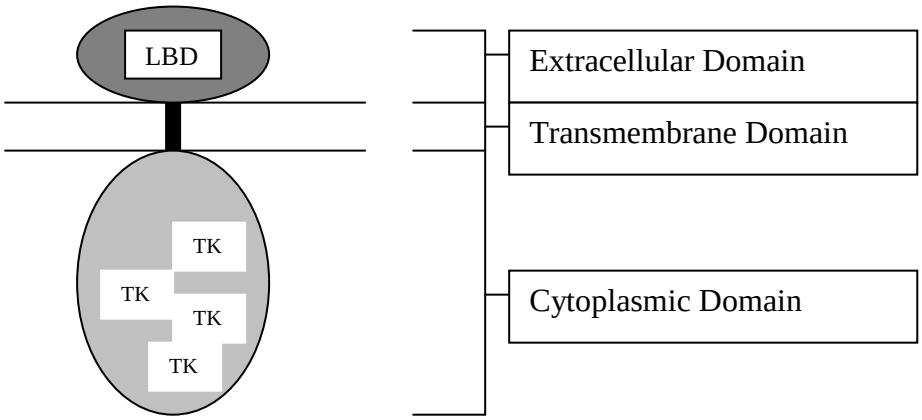
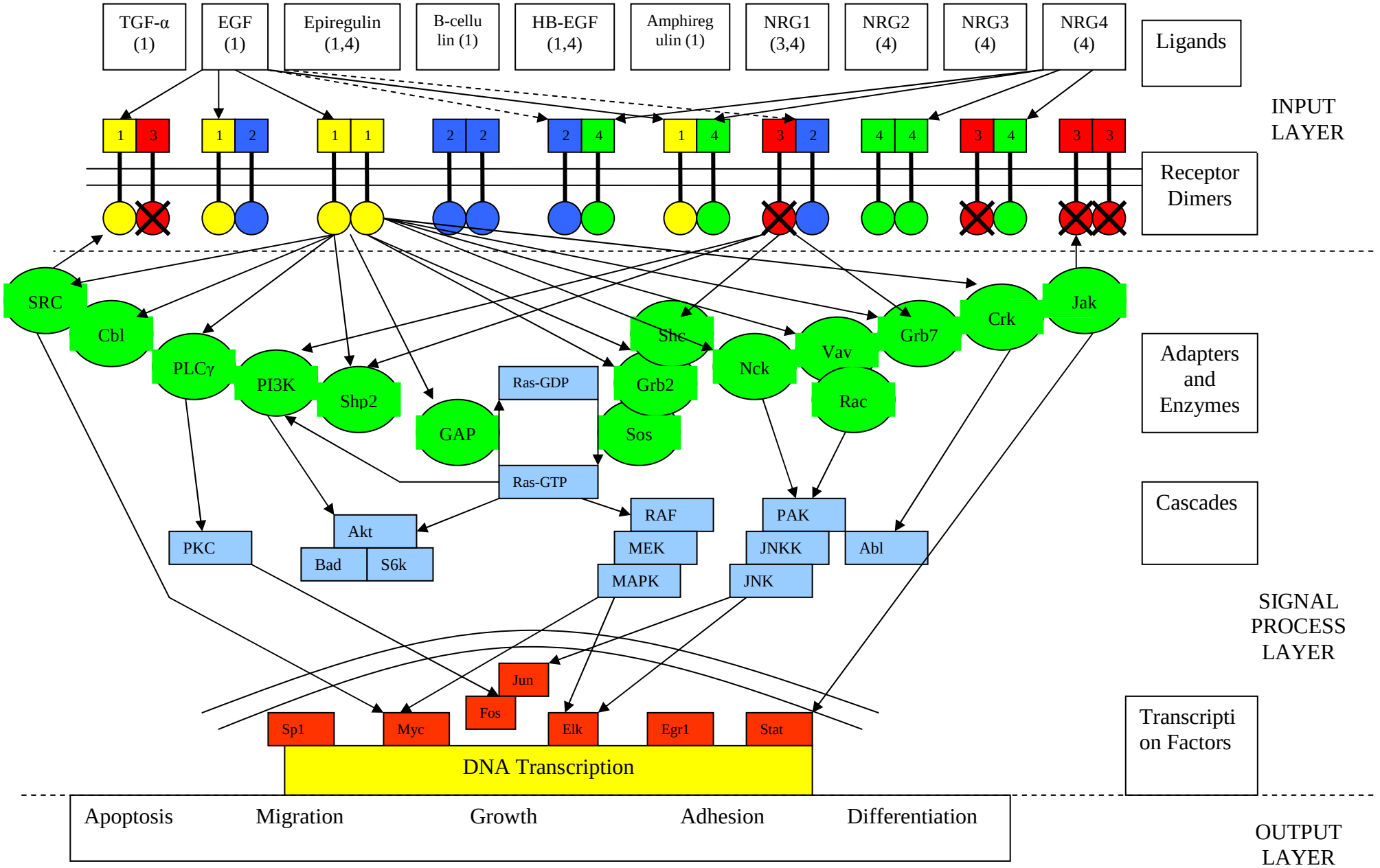


Figure 1.4: Generic structure of HER family member.

Total molecular weight of the EGFR molecule is 170 kD 40 kD of which is carbohydrate moieties (Ennis et al. 1991). HER2 has a weight of 185 kD. While there is a great deal of homology between family members only EGFR and HER4 have all domains fully functioning – HER3 has impaired tyrosine kinase activity largely relying on heterodimerisation (Guy et al. 1994) (see below) to function, HER2 has no known specific ligand (Klapper et al. 1999).

The mechanisms by which the HER family translate extracellular ligand signalling into cell activity is complex and has been termed a layered signalling network (Fig 1.5) (Yarden et al. 2001). The first or input layer consists of the HER family molecules themselves and their ligands. All HER ligands belong to the Epidermal Growth Factor (EGF) family containing a 60 amino acid EGF-like domain and 3 disulphide-bonded intramolecular loops. Ligands binding to EGFR include EGF,

Figure 1.5: Layered signalling network of HER family



heparin binding EGF-like growth factor, epiregulin, TGFα, amphiregulin and betacellulin (Warren et al. 2006). HER3 and HER4 ligands are termed neuregulins (NRG) a large family made up of splice variants of 4 NRG genes (Falls et al. 2003). Heregulins (HRG) are one subtype of NRGs. Some ligands have narrow specificity e.g. EGF, NRG4 while others can bind to 2 receptor types e.g. epiregulin (Jones et al. 1999).

Ligand binding initially stimulates dimerisation of HER family members, both hetero- and homodimers are formed with HER2 being the most common co-receptor in heterodimers (Mass et al. 2004, Graus et al. 1997) and HER2-HER3 the strongest dimer combination (Slikowski et al. 1994). Several factors are thought to make HER2 the preferred choice in heterodimers. HER2 increases the ligand binding affinity of its heterodimer partner (Slikowski 1994) and the specific crystal structure of HER2 is dimer favourable (Garret et al. 2003). HER2 heterodimers have greater activity than others due to decreased ligand dissociation rate (Karunagaran et al. 1996), defective ubiquitin degradation (Lenferink et al. 1998) (see below) and multiple pathway activation by the HER2-HER3 heterodimer (Ben-Levey et al. 1994).

Specific inter-receptor binding and dimer makeup are mediated by ligands but also by relative availability of HER family molecules for example while HER2 has no direct ligand, overexpression increases HER2 participation in heterodimer formation. Indeed this can change cellular ligand specificity as ligands better at recruiting HER2 as a co-receptor will be favoured (Yarden 2001). HER2 does not typically form homodimers but can do in the case of mutation or overexpression (Brennan et al. 2000, Penuel et al. 2002). The precise mechanism of dimerisation has not been fully defined as each

of the 3 domains has been shown to be involved and none fully responsible for dimer linkage (Warren et al. 2006).

The signal-processing layer is the next layer in the signal network and consists of an array of enzymes, proteins and secondary messengers involved in multiple inter-related transduction cascades. Which messengers are involved depends on the initial ligand, the structure and effector sites of HER family dimer and the types and availability of phosphotyrosine-binding proteins associated with the tails of HER molecules following dimerisation. Signals resulting from different dimers have been shown to be different (Wilkinson et al. 2002). The initial phosphorylation is of tyrosine residues within the intracellular domain of the HER dimer itself these sites then allow protein substrate binding and further phosphorylation. The first substrates are adaptors such as Src, PI3K, jak, Ras etc. which then activate an array of signal transduction cascades. Different HER molecules activate different adapters e.g. EGFR activates c-Cbl, Grb2 amongst others whereas HER3 does not but instead activates Shc and Grb7 (Yarden 2001).

The Ras activated MAP kinase pathway is a target of all HER family members and PI3K/AKT pathway components couple directly with HER3/HER4 and indirectly with EGFR/HER2 with consequently differing levels of activation (Soltoff et al. 1996). Other cascades recruited by HER family members including Protein Kinase C and stress activated protein kinase translate into nuclear transcription pathways involving fos, jun, myc and zinc finger containing transcription factors (Yarden 2001).

The final output layer refers to the cellular outcomes generate by the signalling cascade principally cell growth, division, migration, adhesion, differentiation and apoptosis. In addition to original ligand and HER receptor involved cell context determines outcome (Yarden 2001). Heterodimers particularly those including HER2 are known to be more mitogenic (Reese et al 1995).

HER family tyrosine kinase molecule levels are regulated via 2 main mechanisms, endocytosis of receptors with subsequent degradation and alteration of receptors with modulator proteins (Sweeney et al. 2004). The mechanism by which EGFR is endocytosed and degraded has been the subject of much study. The initial step is ligand stimulated localisation of membranous EGFR to clathrin coated pits via EGFR interacting with clathrin by way of AP2 adapters mediated by epsin (Warren 2006) following which the pits are internalised. Recent studies have shown the ubiquitin ligase cbl to play a vital role in this process (Shtiegman et al. 2003); stimulation by EGF causes phosphorylation of EGFR's intracellular domain tyrosine residues including a binding site to which cbl is recruited subsequently being tyrosine phosphorylated activating its ubiquitin ligase activity (Levkowitz et al. 1999). Cbl then stimulates attachment of monoubiquitin moieties which are thought to induce internalisation via endocytosis (Mosesson et al. 2003) although other studies suggest endocytosis can occur without ubiquitylation. Once internalised endosomal EGFR will undergo lysosomal degradation if already ubiqutinylated otherwise it is returned to the surface (Duan et al. 2003). Thereby ligands that stimulate EGFR activity also reduce EGFR levels. HER2-4 do not undergo ligand mediated endocytosis to the same extent as cbl does not attach to them as efficiently (Levkowitz et al. 1996). Degradation related mechanisms of controlling HER2-4 levels are less well defined however E3 ubiquitin ligases may be involved (Diamonti et al. 2003, Qiu et al.

2002);- Nrdp1 binds to HER3 and HER4 whether ligand stimulated or not marking receptors for endocytosis and degradation. Overexpression of both cbl and Nrdp1 downregulate HER expression at the cell membrane. Mechanisms controlling E3 ligase levels are poorly defined. HER4 is internalised and functions as a cytoplasmic protein for longer than EGFR. It is regulated by 2 separate pathways cleaved by a PKC dependent proteolytic pathway and by the PKC independent tumour necrosis alpha converting enzyme (TACE) (Rio et al. 2000)

Negative modulator proteins interact with receptors to alter their response to ligand binding negatively regulating receptor activity. Examples include splice variants of HER family extracellular domains herstatin, a HER2 splice variant, which binds to and inhibits EGFR and HER2 (Azios et al. 2001, Doherty et al. 1999). A similar variant exists for HER3; other negative modulators include potato carboxypeptidase inhibitor (Blanco-Aparicio et al. 1998).

The layered signalling network of the HER family interacts with other signalling networks. Interaction from a variety of signals including hormones and cytokines is generally mediated by protein kinases which phosphorylate HER family members thereby altering activity in response to their usual ligands (Carpenter et al. 1999). One example is G-protein coupled receptors some of whose ligands (e.g. lysophosphatidic acid, carbachol, thrombin) require transactivation of the HER network. GPCRs act via matrix metalloproteinases or Pyk2/Src cascade increasing tyrosine phosphorylation of EGFR/HER2 increasing downstream signalling and magnifying the mitogenic effects of the original ligand. Another example is the growth hormone activation of tyrosine kinase jak2 which phosphorylates EGFR indirectly activating the MAP Kinase

pathway. Other factors that interact with the HER network include Il-6 and PKC (Yarden 2001)

1.2.2 EGFR VARIANT III

There have been several EGFR variant mutations defined some of which have been associated with tumours (Moscatello et al. 1995). It has been postulated by at least one author that expression of mutated forms of EGFR explains contradictory findings regarding EGFR levels in benign and malignant prostate tissue i.e. while some studies indicate no significant difference in expression in malignancy others show decreasing expression with malignant transformation (Olapade-Olaopa et al. 2000). Of all the mutations of wild type EGFR (EGFR-WT) the most common is termed EGFR variant III (EGFRvIII). This variant has a mutation in the external domain which involves loss of the first 2 extracellular subdomains but preserves the intracellular signalling segments (Wong et al. 1990). No ligand binds to the altered extracellular potion but the instead the receptor is constitutively active activating the signal cascade in the absence of ligand binding.

EGFRvIII has been detected in different tumour types including CaP (Olapade-Olaopa 2000) but has not been detected to the same extent in benign tissue and has been postulated as having a role in tumourigenesis.

1.2.3 HEREGULIN

Heregulins (HRG), also termed neu differentiation factors or glial growth factor, are a family of growth factors encoded on chromosome locus 8p12 (Holmes et al. 1992) which contain an EGF-like sequence, an immunoglobulin homology unit and a

cytoplasmic domain with different family members made up of alternative splice variants. Principle HRG variants are α1, α2, α3, β1, β2 and β3 (Lyne et al. 1997) with variation in the 3rd cysteine loop of the EGF-like domain determining α or β isoform status (Holmes et al. 1992).

HRG family members, both α and β isoforms, principally act as ligands to HER3 and HER4 acting via the HER family signalling cascade to effect cell processes including growth, proliferation and differentiation (Leung et al. 1997) with different variants having varying potency and different effects on the same cell line (Sartor et al. 2001). HRG was initially thought to be a ligand for HER2 but has transpired to activate HER2 via its heterodimerisation with HER3 or HER4. HRG activity has been shown to be concentration dependent; in breast cell lines AU565 and MDA-MB-453 low HRG levels are mitogenic but at high levels HRG initiates differentiation and cell growth inhibition (Sartor et al 2001). Response to HRG has also been shown to be dependent on receptor (e.g. HER2) density and cell types.

1.3 HER FAMILY IN CANCER

Expression of HER family members has been extensively investigated in a number of cancer types with the best known being HER2 in breast Ca as this has given rise to both a prognostic test (HercepTest™, DAKO) and an immunotherapeutic treatment trastuzamab (Herceptin, Roche). Cancer pathogenesis related to the HER can be due to hyperactivity at various levels of the signalling network

- Ligand overproduction
- Receptor upregulation
- Constitutive receptor activity

Oncogenic viruses including HBV and avian erythroblastosis virus act by inappropriate manipulation of the signalling network. HER network hyperactivity effects oncogenesis by mechanisms including enhanced proliferation of tumour cells – clonal expansion, increased cell growth and decreased apoptosis (Yarden 2001).

1.3.1 EGFR

Overexpression and mutations/variants of EGFR has been noted in many tumour types. EGFR overexpression leading to oncogenesis is more often accompanied by concomitant ligand expression than overexpression alone being responsible (Yarden 2001). EGFR overexpression through gene amplification has been noted in 40% gliomas with overexpression associated high grade and poor outcome (Wikstrand et al. 1998). In breast cancer EGFR, HER2 and HER3 expression is associated with increased cellular proliferation while HER 4 is non- or anti proliferative (Tovey et al. 2004). HER 1-3 positive tumours have a significantly poorer prognosis than HER negative and HER 4 positive tumours (Witton et al. 2003). Again EGFR overexpression is associated with reduced disease free survival and overall survival

(Tsutsui et al. 2002). Other tumours in which EGFR overexpression has been demonstrated include head and neck squamous cell carcinoma (HNSCC) (Magne et al 2001), non-small cell lung carcinoma (NSCLC), gastric, colorectal, pancreatic ovarian, oesophageal, renal and bladder cancers (Saloman et al. 1995, Bellezza et al. 2006) and is considered a negatively prognostic factor in general (Ennis et al. 1991, Nicholson et al. 2001). Tumours which can secrete EGF and other ligands coupled with EGFR overexpression can set up an autocrine loop allowing tumour proliferation without extraneous stimulation providing a possible mechanism for hormone escape (Normanno et al. 1995, Sato et al. 1999). EGFRvIII has been linked to tumours of lung, ovary and breast (Moscatello 1995)

1.3.2 HER2

HER2 is overexpressed in several cancer types most notably ductal breast CA where 15-30% show significant gene amplification (Slamon et al. 1987). Overexpression is correlated with several negative tumour factors – increased size, high grade, greater proliferation, aneuploidy, lymph node spread and reduction/absence of hormone receptors (Ross et al. 1998, Paik et al. 2000) however expression is greater in earlier breast CA than advanced. HER2 amplification is associated with malignant transformation (Hudziak et al 1987. Zhou et al. 2003) significantly poorer prognosis (Ross 1998), higher risk of recurrence and disease related death with node negative (Press et al. 1997) and node positive (Slamon 1987, Tandon et al. 1989) invasive disease and has also been associated with increased risk of resistance to antioestrogen therapy (Borg et al. 1994) through cross-talk with the oestrogen receptor complex (Schiff et al. 2003) if present. HER2 has been shown to modulate response to chemotherapy with increased resistance to some (Zhang et al. 2003) regimens and

increased sensitivity to others e.g. anthracycline based agents (Pritchard et al. 2006). HER2 oncogene is also amplified in ovarian CA (Edwards and Mukherjee 2004).

HER2 positive tumours can now be identified with the immunohistochemical (IHC) Herceptest or fluorescence in-situ hybridisation (FISH) and treated with trastuzamab, a monoclonal antibody to HER2 which downregulates HER2 expression altering downstream signalling increasing apoptosis and blocking growth stimulation and VEGF production (Ferretti et al. 2007) with a consequent significantly increased time to disease progression (Slamon 2001). Trastuzamab use is dependent on the result of testing for HER2 with its use being indicated by strong immunohistochemical staining, contraindicated in weak or absent staining and intermediately stained specimens determined by additional Fluorescence in-situ hybridisation testing.

1.3.3 HER3/HER4

HER3 has been found in several cancer types including breast, gastric, colonic and endometrial adenocarcinomas (Poller et al. 1992) but gene amplification and overexpression are rare (Yarden 2001). Co-expression of HER2 with HER3 or EGFR predicts outcome in oral squamous cell carcinoma (Xia et al. 1999).

Conversely to other HER family members HER4 expression which includes nuclear expression, is lower in breast CA than in benign tissue and is associated with lower grade. As mentioned above HER4 is non- or antiproliferative and positively prognostic in breast CA particularly when compared to HER1-3 positive tumours (Tovey 2004). In a study of childhood medulloblastoma involving IHC and Western blotting for all 4 main family members HER4 co-expressed with HER2 in the HER2-

HER4 heterodimer was shown to prognostic value in terms of 25 year survival. An association between HER2-4 and transcription factor AP-1 expression was also noted (Gilbertson et al. 1997).

1.3.4 ANTITUMOUR THERAPY

Given the role of the HER family in several tumour types many agents that target HER family members have been investigated as antitumour therapy. The best known is Herceptin (trastuzamab), licensed in the UK for HER2 positive breast tumours to prevent/postpone recurrence. Several antitumour agent types have been developed (De Bono et al. 2002) including

- Monoclonal antibodies to HER family members
- Small molecule tyrosine kinase inhibitors
- Conjugates of specific antibodies with cytotoxic agents or radionuclides
- Gene therapy
- Antisense Oligonucleotides

Monoclonal antibodies (mAb) bind with high affinity to a specified target competing with normal ligands. They prevent ligand binding and induce receptor endocytosis and degradation with consequent reduction in downstream signal transduction resulting in delayed tumour growth and spread (Aguilar et al. 1999, Chen X, et al. 2000). An example of anti-EGFR antibody is Cetuximab (Erbitux, Merck) which has a binding affinity to the extracellular domain of EGFR 10 times that of EGF itself. Cetuximab binding blocks EGFR signalling cascades causing cell growth arrest in G1 and has been shown to inhibit growth, stimulate apoptosis, reduce volume and enhance the action of cytotoxins and radiotherapy in EGFR positive tumour

xenografts (Baccus et al. 1993, Daly et al. 1997). Cetuximab has been used, either alone or in combination with in trials involving multiple tumour types including non-small cell lung CA, HNSCC, colorectal and pancreatic CA (De Bono et al. 2002). Trastuzamab is a mAb with high affinity for the external domain of HER2 used now in HER2 positive breast CA. Other antibodies to HER2 include 2C4 used in trials against ovarian and lung CA. Antibodies to HER3 and HER4 have not been explored to the same extent.

Tyrosine kinase inhibitors (TKI) bind to the intracellular domain of HER family members preventing activation of the next level of the signalling cascade. These inhibitors usually target a specific HER family member but can block others through inhibition following heterodimerisation (Peles et al. 1992). TKIs have a lesser specificity than mAbs and greater concentrations are required however they can be oral agents (mAbs must be intravenous and are more likely to fail to recognise receptor mutations and variants (Bellezza 2006). Inhibitors can be reversible or irreversible. There are several TKIs targeting EGFR; these include Iressa (Gefitinib, AstraZeneca) which competitively binds to ATP binding at the tyrosine kinase site of EGFR decreasing CDK2 kinase activity inducing G_0/G_1 arrest and, at higher doses, apoptosis (De Bono 2002) and antiangiogenesis (Bellezza et al. 2006). Gefitinib has been used in advanced non-small cell lung carcinoma and in trials concerning breast, HNSCC, colorectal, uterine (De Bono 2002) and glioma cancers (Mass 2004). Tarceva (OSI-744, OSI Pharmaceuticals) also targeting EGFR has been used in studies concerning advanced SCCHN, pancreatic and ovarian CA. Both Gefitinib and Tarceva affect HER2 and HER3 signalling via heterodimers with EGFR (De Bono 2002). Tyrosine kinase inhibitors that target HER2 exist and include GW572016

(colorectal and breast CA). CI-1033 (lung and breast CA) targets all HER family members (Mass 2004). Adenovirus type 5 early region 1 (EA1) gene product has been studied as gene therapy designed to target HER2. This has been shown to inhibit HER2 transcription acting as a tumour suppressor in HER2 overexpressing ovarian cancer cells in mice prolonging survival (Hung et al. 2000). Antisense oligonucleotides directed against EGFR used in CaP cell lines in nude mice have been shown to inhibit tumour growth and angiogenesis causing tumour necrosis (Rubenstein et al. 1996).

1.4 HER FAMILY IN PROSTATE ADENOCARCINOMA

HER family members have been found to be expressed in prostate tissue benign, hyperplastic, precancerous (prostatic intraepithelial neoplasia, PIN) and cancerous (Nasu et al. 2006, Rana et al. 2006) but the relationship between levels of and changes in expression in relation to malignant transformation, severity of disease, hormone escape and overall prognosis remain controversial with conflicting evidence produced by different studies.

1.4.1 EGFR

EGFR is expressed in prostatic epithelial cells with a greater levels in basal than luminal epithelial cells (Maygarden et al. 1994) with EGF also detectable in high levels in prostatic tissue and secretions (Elson et al. 1984). EGFR is detectable in up to 100% benign tissue (Rana 2006), 29-88% benign hyperplasia (Di Lorenzo et al. 2004) and in 17 - 100% (Mellon et al. 1992, Di Lorenzo et al. 2002) of prostate adenocarcinomas with this high variability possibly due to definitions of high expression varying between studies, heterogeneity in CaP particularly metastasis and HRPC specimens or differences in IHC technique (Hernes et al. 2004). EGFR has been shown to essential for androgen induced proliferation. EGFR expression has not been found to related to Gleason score in most studies in which this has been assessed (Moul et al. 1996, Hernes 2004) although this has been shown in at least one (Di Lorenzo 2002). Amount/Intensity of staining in benign tissue and PIN has been reported as greater than in CaP (Mellon et al. 1992) in some studies while others indicates the converse – significantly greater EGFR expression in CaP and high grade PIN than in benign tissue and low grade PIN. EGFR overexpression in metastatic CaP has been seen in several studies (Kumar et al. 1996, Kim et al 1999, Di Lorenzo

2002). Kumar et al. showed EGFR messenger RNA expression as greater in malignant compared to benign prostatic tissue.

As in other cancer types EGFR acts via an array of signal transduction pathways in CaP oncogenesis including P13K/Akt, MAP Kinase and PKC. Inhibition of these signalling cascades in HSPC and HRPC cell lines inhibits growth blocking G1 to S phase transition by upregulating $p27^{KiPI}$ protein which in turn inhibits cyclin-dependent protein kinases (CDKs) controlling the cell cycle (Mimeault 2003). The EGFR activated PI3k cascade stimulates growth and angiogenic factors while MAPK/PLC-γ cascades increase cell motility (Graff et al. 2002, Ghosh et al. 2002). Another postulated mechanism behind HER carcinogenesis is overexpression of the EGFR-HER2 heterodimer compared to other HER dimers which has been noted in several cancer types. EGFR-HER2 has a greater affinity for EGF and lesser degradation compared EGFR homodimers (Xia 1999). EGF-EGFR acting via PI3K/Akt within membrane microdomains known as caveolae and rafts also effects an antiapoptotic signal maintaining CaP cells in the absence of androgen signalling: inhibiting PI3K causes apoptosis in these cells although this effect is lessened by activating EGFR with EGF suggesting alternative antiapoptotic pathways exist (Lin et al. 1999). In CaP EGFR also acts via reducing cellular ceramide levels to effect antiapoptotis.

In CaP EGFR and its downstream messengers interact with other signal transduction pathway. In AR positive cell lines endocytosis of EGFR is reduced altering downstream signalling via adaptor proteins possibly accounting for reduced invasiveness of AR positive cell lines (Bonaccorsi & Marchiani 2004). Several

neuropeptides interact with EGFR pathways via G-protein coupled receptors (Shah GV et al. 1994) with the MAP Kinase pathway being the point of convergence.

Previous studies have shown EGFR influence on hormone escape and CaP prognosis. Shah RB et al compared EGFR levels in tissue microarrays (TMA) of HSPC and HRPC tumours with HRPC status associated with increased expression although it did not achieve statistical significance on multivariate analysis. Zellweger et al. (2005) similarly showed greater expression 16% versus 1% in hormone refractory and metastatic CaP compared to localized disease. Several other studies concur with this (Glynne-Jones et al 1996, Maddy et al. 1989, Myers et al. 1997)); Di Lorenzo (2002) demonstrated increased EGFR expression in HRPC than in neoadjuvant hormone treated CaP and greater expression in both than in hormone naïve tissue. In this study increased EGFR expression was associated with advanced stage, high Gleason score, decreased time to hormone escape and relapse with results more pronounced if EGFR and HER2 overexpression are combined. Additional studies further demonstrated poor prognosis with increased EGFR expression (Fowler et al. 1998, De Miguel 1999). In Bartlett et al. (2005) a cohort of matched HSPC and HRPC tumour specimens with EGFR gene copies assessed by FISH and EGFR protein expression assessed though IHC, no EGFR gene amplification was shown however increased EGFR copy number in HRPC was observed and was associated with reduced overall survival. This increase in copy number was not associated with gene amplification or change in protein expression. Increased EGFR protein expression was observed in some patients (~25% showed significant increase in EGFR and/or HER2 expression) but was not associated with reduced overall survival (OS) however patients demonstrating a rise in EGFR expression following hormone escape did show a

significantly reduced time to death following relapse (TTDFR) (p=0.0004). This indicates that while increased EGFR expression may be involved in hormone escape and CaP progression this does not occur via gene amplification as is the case with HER2 association with breast CA. Hernes (2004) showed a similar significant rise in EGFR expression in the HRPC specimen of paired HSPC and HRPC samples. No association between EGFR expression and prognosis was found in this study. EGFR and EGFR ligand expression is greater in AR negative androgen independent CaP cell lines than in AR positive again linking EGFR expression with more aggressive disease (Vincentini et al. 2003). Alternatively Moul (1996) agrees with Hernes in that no correlation between EGFR and outcome is shown.

Several mechanisms have been suggested whereby EGFR could effect hormone escape. There is a high degree of overlap between androgen and EGFR activated changes in gene expression (Oosterhoff et al. 2005) and EGFR is one of a number of factors that have been shown to activate AR in the absence of androgenic stimulation (Mimeault 2003) indicating the EGFR pathway as a bypass allowing cell proliferation during androgen suppression. Further evidence is demonstrated in prostate cancer xenografts in mice where castration has been shown to increase concentrations of EGF related growth factors (Torring et al. 2005). As in other tumour types EGF and TGF-α are secreted by some HRPC cell lines forming an autocrine loop (Fong et al. 1992, De Miguel 1999, Kim 1999) independent of androgen influence. Autocrine growth stimulation may involve upregulation of transmembrane EGF-like ligand cleavage and activation by matrix metalloproteinases (MMPs) (Marinissen et al 2001). Such loops are not seen in benign prostate and HSPC cell lines (MacDonald et al. 1992). The MAP kinase pathway has also been implicated in the autocrine loop as

it is activated by EGFR and stimulates pro-EGF release at the cell surface (Lin et al. 1999).

Antitumour agents targeting EGFR have been utilised against CaP in trials. Iressa (gefitinib) has been used in CaP cell lines with both AR positive cell lines and AR negative sensitive to antiproliferative/apoptotic sensitive to it (Vincentini 2003). CaP cell line proliferation and metastasis are inhibited in both in vitro assays and in vivo – nude mice (Angelucci et al. 2006). Gefitinib has been shown to inhibit invasion and proliferation on HRPC cell lines via suppression of the PI3K/Akt pathway (Bonaccorsi & Marchiano 2004). Studies in HRPC cell lines have demonstrated gefitinib as having a greater antitumour effect than Herceptin (Formento et al. 2005). Additionally when gefitinib is used in conjunction with bicalutamide both agents potentiate each others antiproliferative action (Sirotnak et al. 2002). Unfortunately a phase II trial of gefitinib in 40 HRPC patients showed no improvement in PSA or objective disease progression and high levels of side effects, principally diarrhoea, fatigue and rash, were noted (Canil et al. 2005) with similar lack of effect seen in another trial (Rosenthal et al. 2003). It should be noted that these trials did not involve populations selected for EGFR expression and studies with gefitinib in Breast Ca have demonstrated a much greater response in EGFR selected populations (Polychronis et al. 2005). Antitumour agents that targeting both EGFR and HER2 have also been explored, despite Formento et al (2004) showing no benefit in targeting both (using combination trastuzamab and gefitinib) compared to targeting one alone. GW572016 (lapatinib, Tykerb, GlaxoSmithKline) a tyrosine kinase inhibitor that targets both has been associated with tumour apoptosis and regression of metastases (Spector 2005)

1.4.2 HER2

Evidence for HER2 expression in the development of CaP is contradictory (Yeh 1999). Level of HER2 expression in primary CaP varies widely between studies with 0 -100% (Zhau 1992, Shi et al. 2001, Savainan et al. 2002) immunohistochemically positive compared to 0-100% (Lyne 1997, Hernes 2004) in benign prostate tissue. Different studies have shown both greater expression of HER2 in CaP than benign prostate tissue (Okegawa et al. 2006, Hernes 2004) and no significant difference (Mellon et al. 1992). In general HER2 expression in CaP is lower than other tumour types both in positivity rate and intensity (Edwards 2003). HER2 expression is primarily cytoplasmic or membranous (Ware 1990) with greater expression in basal than luminal cells (Lyne 1997). Lara et al. (2002) found HER2 overexpression to be infrequent with IHC, FISH and ELISA. HER2 expression is correlated with Gleason score and stage in some studies (Ross et al. 1998, Sadasivan et al 1993, Shi 2001) but not in others (Mellon 1992, Lara 2002, Hernes 2004). Other evidence for HER2 involvement in CaP includes high HER2 expression in PIN indicating a role in early tumourigenesis (Kuhn et al. 1993, Ware 1991) and Osman et al. (2000) demonstrating raised serum HER2 in patients with metastatic CaP. Evidence to the contrary includes the absence of HER2 overexpression/gene amplification in several commonly used CaP cell lines and xenografts (Ullen et al. 2005).

The relationship between HER2 and CaP hormone escape and prognosis is likewise unclear with conflicting evidence. HER2 expression in HRPC is extremely varied from 0 to 85% (Signoretti et al. 2000, Shi 2001, Savainan 2002). Again high variation has been put down to varying IHC antibodies and techniques, scoring criteria and/or tissue heterogeneity. Several authors have demonstrated greater HER2 expression in

HRPC than HSPC in non-paired samples (Shi 2002, Xie et al. 1995, Signoretti 2001, Di Lorenzo 2002), in Shi et al 9% HER expression in untreated CaP compared with 50% in CaP treated with hormonal therapy and 85% in established HRPC, a similar progression was shown by Signoretti. Serum levels of HER2 were higher in metastatic HRPC than in metastatic HSPC patients (Osman 2000). Comparing paired HSPC and HRPC samples, Bartlett et al. (2005) described only low level (6.5%) HER2 gene amplification in contrast to the 25-30% seen in breast CA. Previous studies in CaP have reported an amplification rate of up to 41% (Reese et al. 1995, Ross et al. 1997) however these studies did not include a chromosome 17 probe as used in Bartlett et al. allowing no distinction between amplification and increased copy number. In other studies amplification has been linked to hormone escape (Craft & Shostak 1999). Bartlett et al. concluded that gene amplification was rare in CaP and not clinically significant and several other previous studies concur (Fournier et al. 1995, Mark et al. 1999, Savainan 2002). As with EGFR, increased gene copy number (again not associated with gene amplification or change in gene expression) following hormone escape was associated with decreased overall survival but the significance of this is unclear. Increased copy number is usually due to unstable genome and random duplication and is not thought to represent upregulation of gene expression in the same manner as gene amplification. It was not considered of clinical importance in Bartlett et al. There was no change in average HER2 expression following hormone escape in the full population however increased HER2 expression in the HRPC escape for individual paired samples was associated with significantly lower TTDFR ($p=0.0037$) indicating a possible role of HER2 overexpression in hormone escape. In this study if patients with increased EGFR and HER2 following hormone escape were combined the significance value for reduced TTDFR was even lower ($p=0.0003$).

Hernes et al. (2004) actually demonstrated a fall in HER2 expression in paired samples which did not achieve statistical significance rather than a rise however high HER2 expression in the HRPC specimen was again significantly associated with decreased TTDFR. Lara et al. (2002) amongst others (Savainan 2002, Calvo et al. 2003) demonstrated no link between HER2 overexpression and hormone escape. Xenograft studies in mice have indicated greater HER2 expression in androgen independent compared to androgen dependent human CaP cells (Agus et al. 1999). Poor prognosis has been linked to tissue HER2 expression in other studies (Zhau 1992, Sadasivan et al. 1993, Okegawa 2006) – in Di Lorenzo (2004) et al and Morote et al. (1999) the outcome demonstrated was disease specific death, in Okegawa (2006). risk of biochemical recurrence Serum HER2 is also correlated with increased risk of disease specific death (Osman 2000). 2 studies have shown HER2 expression as an independent predictor of worse prognosis on multivariate analysis (Veltri et al. 1994, Morote 1999) Other studies have shown no relationship of HER2 with prognosis on multivariate or univariate analysis (Ware 1991, Mellon, 1992, Ross 1997). HER2 expression in these studies was assessed primarily using either IHC or FISH.

As noted above androgen signalling pathways and their interaction with/bypass by other pathways are key to hormone escape therefore HER2-AR pathway interaction provides a mechanism of HER2 influence over the same phenomenon. Cross talk between HER2 and AR pathways shown by Mellinghoff et al. (2004) who used a dual EGFR-HER2 inhibitor PKI-166 to demonstrate the inhibitory effect of EGFR/HER2 acting via reduced AR transcription. It was further shown that the HER2-HER3

heterodimer rather than EGFR modulated AR function in the absence of androgens by stabilising AR levels and optimising binding of AR to androgen regulated genes. A role of the HER2-HER3 heterodimer in hormone escape was therefore suggested.

In Yeh (1999) AR positive cell lines HER2 has been shown to induce AR transactivation with transfection of HER2 gene increasing PSA secretion and proliferation rate. This study also indicates HER2 transactivates AR via the MAP Kinase pathway and this action is not blocked by antiandrogens indicating a possible pathway to hormone escape. HER2 and HER3 expression, stimulated by HRG, have been shown to increase AR transactivation and tumour proliferation in a recurrent CaP cell line in the absence of androgen (Gregory et al. 2005). As well as direct cross-talk between HER2 and AR interaction via downstream mediators such as IL-6 (Qiu et al. 1998)

Spontaneous HER2 homodimerisation in the presence of extreme overexpression of HER2 has also been shown to induce androgen independent AR transactivation. Wen et al. (2000) suggested HER2 induced hormone independence could be mediated via the PI3K/Akt signalling pathway while Pfeil et al. (2004) demonstrated androgen ablation therapy causing overexpression and resistance to inhibition of the PI3K/Akt pathway itself. Craft et al. (1999) induced HER2 gene expression in a HSPC cell line demonstrating the consequent development of a hormone independent state. Overexpression of HER2 has been shown to be induced by low androgen environments in vitro and in vivo (Berger et al. 2006).

An array of anti HER2 agents have been used in CaP trials. The HER2 mAb trastuzamab when used with HSPC and HRPC cell lines in xenografts models. HRPC tumours had no response but in HSPC tumours significant growth inhibition was observed (Agus 1999) and androgen dependence was proposed as required of

trastuzamab response. Li et al. (2004) correlated trastuzamab response only with HER2 levels. If trastuzamab was combined with the chemotherapeutic Paclitaxil (Taxil, Bristol-Myers Squibb) in animal models growth inhibition was seen in both androgen dependent and independent tumours to a greater extent than either individual agent (Agus 1999). This has not translated into a successful clinical trial; one trial with profiling correlation of HER-2 expression, androgen dependence and trastuzamab effect and Paclitaxil used after clinical failure (Morris et al. 2002) showed trastuzamab as ineffective with all patients progressing and the combination uncertain.

An alternative HER2 mAb 2C4 (Pertuzumab, Genentech) has been shown to inhibit growth of HSPC and HRPC cell lines and xenografts based on the same cell line (Menodoza et al. 2002) A phase I trial involving multiple types of solid metastatic malignancies at a terminal stage indicated some successful inhibition of HER2 heterodimerisation and stabilisation of disease. The TKI Lapatinib with action against EGFR and HER2 has been shown to significantly inhibit HER2/HER3 proproliferative action on recurrent CaP cell lines. When this cell line was denied growth factors lapatinib continued to have an inhibitory effect suggesting the existence of a HER2 autocrine loop (Gregory 2005).

In summary while reported expression of HER2 in relation to HSPC, HRPC and prognosis is conflicted there is still evidence including HER2-AR interactions, greater signalling effects of the HER2-HER3 dimer than EGFR dimers and response to some targeted therapies that HER2 has a key and potentially targetable role in CaP progression.

1.4.3 HER3

While studied to a lesser extent than EGFR and HER2, HER3 has been consistently found to be expressed in both benign and malignant prostatic tissue (Myers et al. 1994, Prigent et al. 1992). Koumakpayi et al. (2006) demonstrated >90% cytoplasmic HER3 expression in all types prostate cancer with no significant difference to benign tissue noted on IHC. However nuclear expression was significantly higher in CaP than benign prostate tissue with HRPC cell line expression being significantly higher than that of HSPC tissue. In this study higher HER3 nuclear expression was correlated with higher Gleason score. Another study showed HER3 expression in CaP but not benign prostate tissue however there was no relationship between HER3 expression and Gleason score (Leung 1997). Western blotting showed greater nuclear HER3 expression in HSPC cell lines as than HRPC contradicting IHC results. Comparing HER3 in paired HSPC and HRPC samples Hernes (2004). demonstrated a greater expression in HRPC samples than HSPC 21% vs. 15% which did not achieve significance. One important role of HER3 was suggested by Lee H et al (2001) who showed a naturally secreted form of HER3 (p85-sErbB3) as an important negative regulator of HRG action in stimulation of cell membrane HER 2-4 acting via competitive binding to HRG. As noted above in Gregory et al. (2005) HER2 and HER3 expression, stimulated by HRG, was shown to increase AR transactivation and tumour proliferation in a recurrent CaP cell line in the absence of androgen. At least one study has linked HER3 to poor prognosis, Leung et al (1997) demonstrated expression of HER3 and its ligand HRG-α expression in CaP but not benign tissue and indicated HER3 expression linked to poor response to androgen therapy and decreased survival. Koumakpayi et al (2006) demonstrated low nuclear HER3 as a predictor of biochemical recurrence in CaP.

1.4.4 HER4

In contrast to the other 3 family members, early studies indicated that HER4 was not expressed in CaP at al (Grasso et al 1997) however more recently this has been contradicted (Hernes 2004, Lyne 1997). Lyne et al. demonstrated high HER4 expression 95-100% in benign prostatic basal and luminal cells, 67% in PIN and 23% in CaP (prostatectomy). As in HER1-3 Hernes et al compared HER4 expression in paired HSPC and HRPC specimens; in this study HER4 expression rose slightly following hormone escape from 24% to 29% positivity but this did not achieve significance. In addition expression of HER4 in the HRPC sample was associated with improved 2 year survival to a degree nearly achieving statistical significance ($p=0.054$). This is in contrast to prognosis HER1-3 in this study as expression of these was associated with worse prognosis (although of these statistical significance occurred only with HER2) but in agreement with prognostic data for HER family in breast CA. In vitro, a constitutively active HER4 mutant inhibits formation of colonies in DU-145 and PC-3 CaP cell lines (Williams et al. 2003) .

1.4.5 EGFR VARIANT III

Several studies have linked EGFRvIII expression and/or constitutive EGFR expression with development of CaP and androgen resistance (Myers 1997, Sherwood et al.1998, Schwartz et al. 1999 Olapade 2000, Di Lorenzo 2002). EGFRvIII has been detected only in tumour cells and not in benign prostate tissue (Olapade 2000). It has been speculated that failure to recognise EGFR variants, in particular EGFRvIII, is one of the reasons behind the disparate results relating to EGFR-WT expression in HSPC and HRPC development. Olapade-Olaopa et al. stained benign and malignant prostate tissue for EGFR-WT and EGFRvIII. EGFRvIII was primarily found in the

perinuclear cytoplasm rather than the membrane where EGFR-WT was most commonly located. While there was a progressive decrease in EGFR-WT expression with increased malignancy (i.e. most in benign tissue, medium in PIN/CaP, least in metastatic CaP), EGFRvIII showed a counterpointing progressive increase – none in benign tissue, most in metastatic and poorly differentiated disease. There was significantly greater EGFRvIII expression in HRPC compared to HSPC samples in this study although there was no similar or opposite difference in EGFR-WT expression. In terms of prognosis EGFRvIII expression was found to be significantly associated with serum PSA and time to disease progression but not on overall survival. This article postulated that as CaP progresses it expresses the constitutively active EGFRvIII in preference to EGFR-WT and that this increase in ligand independent mitogenesis may occur due to loss of usual ligand input e.g. reduced androgens/androgen receptors with EGFRvIII overexpression effecting malignant progression and hormone escape. EGFRvIII has been found to be associated with anti KI67 mAb, a marker of cell proliferation, also indicating an association with increased CaP activity (Olapade-Olaopa et al 2001).

1.4.6 HEREGULIN

Heregulin expression in benign and malignant prostate tissue was assessed by Lyne (1997). In this study high expression was found in benign basal cells and stroma (100%) with intermediate expression in benign luminal cells (58%) and low expression in PIN (5%) and prostatectomy derived CaP (10%). In CaP cell lines some HRG mRNA was detectable on Southern Blotting but no HRG protein expression was detectable. In vitro HRG-β treatment of androgen sensitive LNCaP prostate cancer cells caused inhibition of cell proliferation whereas no such effect was seen in AR

negative cell lines. In a separate study HRG treatment of androgen resistant CWR-R1 prostate cancer cell lines increased proliferation (Gregory 2005). Whether this differing activity in androgen independent tissue or the concentration dependent mitogenesis/ antiproliferation activity of HRG described above are involved in the mechanisms by which the HER family affects cancer progression has not been clearly defined. Lyne et al suggested that lack of HRG expression in CaP, coupled with previous reports that the chromosomal locus 8p12-21 which includes the HRG gene is often lost in PIN/CaP (Emmett-Buck et al. 1995), indicates HRG acts as a tumour suppressor and its loss is an early stage in prostate oncogenesis. The differential effects on AR positive and negative tumour lines were also suggested as a driving force behind inability to prevent tumour progression at low androgen levels.

1.5 PILOT STUDY: HER FAMILY IN PROSTATE ADENOCARCINOMA

It was speculated that conflicting published results relating to the HER family and prostate cancer may be due to a piecemeal approach dealing with one or two family members at a time. Very few published studies examine all 4 members of the family ± ligands – exceptions are (Hernes 2004) and Grasso (1997). A pilot study has been conducted at this centre involving all 4 main members of the family and EGFR variant III to give a clearer picture of the overall role of HER proteins

Immunohistochemical analysis was use to investigate protein expression of HER 1-4 and EGFRvIII in matched hormone sensitive and resistant prostate tumours samples from 74 patients. These patients all had had a tissue diagnosis of locally advanced or metastatic CaP, underwent chemical or surgical (orchidectomy) androgen ablation but subsequently suffered hormone escape as determined by biochemical relapse (see method section) and had a further pathological specimen taken. Specimens were prepared as slides, stained with commercial antibodies to EGFR, HER2, HER3, HER4 and EGFRvIII and scored by 2 independent observers utilising a weighted histoscore technique (see method section). Scoring results were used to divide specimens into low score (< 3^{rd} quartile) and high score (> 3^{rd} quartile) and were analysed to determine effect of histoscore on endpoints time to hormone escape and mortality.

This pilot study demonstrated that HER3, HER4, EGFRvIII were expressed at significantly higher levels than EGFR/ HER2. Unlike previous studies EGFR overexpression was not associated with survival following hormone escape, neither was high or low EGFR expression associated with differing time to relapse. High

HER2 expression in hormone sensitive tumours was associated with increased time to biochemical relapse (p=0.0001). This translated into longer overall survival (p=0.0021).

As previously noted, high HER2 expression in HRPC samples was associated with significantly reduced time to death following biochemical relapse (p=0.039). Additionally, a significant rise in HER2 expression in between the first and second matched samples was associated with significantly reduced survival after biochemical relapse (p=0.012). Differing HER3 had no significant effect on measured endpoints

HER4 overexpression in hormone sensitive tumours was associated with longer time to biochemical relapse (p=0.042). EGFRvIII was associated with shorter time to biochemical relapse (p=0.037).

Multivariate analysis involving all 5 family members, Gleason Score and metastasis at diagnosis demonstrated HER 2 was an independent positive predictive marker of time to relapse in hormone sensitive tumours (p=0.014). Multivariate analysis did not demonstrate any of the 5 family members as significantly influencing overall survival.

1.6 SUMMARY, AIMS AND OBJECTIVES

Published literature concerning the role of the HER family is contradictory in many aspects. While there are several indications that the HER family and HRG provide opportunities for diagnosis, prognosis and therapy a clear candidate has yet to emerge. The pilot study was motivated by the aim to define the roles of the HER family in prostate cancer development and progression by examining all HER family members and utilising multivariate analysis to clarify the picture. Several interesting results have been thrown up by the pilot study most notably a positive prognostic role for HER2 in contradiction to much existing literature. Several avenues invite further exploration.

- Expansion of study numbers
- HER family ligands have not been explored – given the concentration and androgen level differentiation effects noted above HRG is of greatest interest
- There has been little use of markers of proliferation and apoptosis to define whether tumourigenesis effects mediated by HER family/HRG occur primarily through one or the other process.

The research questions for this study have been formulated with these in mind with the overall objective of determining the role of HER family in CaP development and progression.

1.6.1 RESEARCH QUESTIONS

1. Are expression of HER 1-4 and EGFRvIII correlated with response to therapy/ time to relapse/time to death in prostate cancer?
2. Is Heregulin (HRG) involved in mechanisms by which HER family proteins affect cancer progression?
3. Do HER family proteins effect oncogenesis via cell proliferation or reduced cell death?
4. Are trends seen in the pilot study borne out with a larger patient base?

CHAPTER 2: PATIENTS AND METHODS

2.1 ETHICAL APPROVAL AND FUNDING

Ethical approval for the pilot study (Edwards et al. 2006) was obtained from Multicentre Research Ethics Committee (MREC) for Scotland reference MREC/01/0/36. Ethical approval for this study was obtained from the Glasgow Royal Infirmary Local Research Ethics Committee (LREC) with application title 'What is the Role of EGFR, HER 2-4, EGFR variant III, Heregulin and Downstream Signalling in development/progression of Prostate Adenocarcinoma?' reference 05/S0704/89.

Funding was kindly provided by the Aileen Lynn Bequest Fund committee based at the Royal College of Physicians and Surgeons of Glasgow.

2.2 PATIENT COHORTS

2.2.1 COHORT 1: PILOT STUDY COHORT WITH PAIRED HORMONE SENSITIVE AND HORMONE RESISTANT SPECIMENS

This is the patient cohort utilised in the pilot study (Edwards 2006) and includes paired single slides of matched androgen sensitive and androgen insensitive samples. This cohort was collected from hospitals within the West of Scotland Deanery with specimens originally taken between 1984 and 2004. For inclusion within the study inclusion and exclusion criteria were;-

1. A pathological diagnosis of prostate adenocarcinoma with a pathological specimen taken at this stage; either a biopsy (usually obtained via trans-rectal ultrasound (TRUS) sampling or shavings obtained from a trans-urethral resection of prostate (TURP) procedure. In all cases this sample was obtained before any hormonal therapy was commenced making it a hormone naïve/sensitive sample (HSPC).
2. The patient commenced on hormonal therapy either an antiandrogen such as bicalutamide (Casodex, AstraZeneca), a gonadotrophin releasing hormone (GnRH) agonist such as goserelin (Zoladex, AstraZeneca) or maximal androgen blockade (MAB); a combination of antiandrogen and GnRH agonist. Alternatively the patient could have undergone bilateral orchidectomy.
3. A significant tumour response to the hormonal therapy as determined by a >50% fall in PSA following commencement confirming the hormone sensitivity of the primary tumour.
4. Subsequent tumour hormone escape as determined by two consecutive rises in PSA of >10% with the initial PSA value above 0.5 despite continued

hormonal therapy. The tumour is thereafter classified as hormone resistant/ refractory (HRPC)

5. A further pathological sample of the tumour obtained subsequent to hormone escape. Usually this is a sample obtained by TURP performed to alleviate symptoms of bladder outlet obstruction secondary to the tumour and constitutes the HRPC sample.

In the pilot study 74 sets of paired samples were utilised. Subsequently to the pilot study 7 patients were added to the cohort however due to the gradual nature of additions to the cohort coupled with depletion of slides through use in other studies staining for each target could not be carried out in all slide pairs. In this study a cohort of 81 paired samples were stained for at least one target.

Within this cohort of 81 patients mean age was 70.0 years (SD ± 8.0, range 41.1 – 98.0). At diagnosis at least 56 (69.1%) patients had locally advanced disease (T3-4). Gleason scores in HSPC specimens were low (1-4) in 2 (2.5%) patients, intermediate (5-7) in 35 (43.2%), high in 8-10 in 43 (53.8%) and not recorded in the remainder. 18 (22.2%) patients had known metastatic disease at diagnosis, 54 (66.7%) had no metastases with the metastatic status of the remainder unknown. By definition all of the patients in this cohort had undergone hormone escape; mean time to relapse was 36.5 ± 31.3 months. At last known follow up 69 (85.2%) patients were deceased, 8 (9.9%) patients were alive with the status of the remainder lost to follow up. Mean time to death/last follow up was 61.0 months (SD ± 43.1).

Table 2.1: Summary Statistics for All Cohort 1 Patients

N	81
Age	70 ± 8 years Range 41.1 – 98.0 years
Gleason Score ▪ Low ▪ Medium ▪ High	 2 35 43
T Stage at Diagnosis ▪ T1-T2/unknown ▪ T3-T4	 25 56
Metastasis at Diagnosis ▪ Yes ▪ No ▪ Unknown	 21 35 25
Biochemical Relapse ▪ Yes ▪ No ▪ Unknown	 81 0 0
Time to Relapse	36.5 ± 31.3 months
Final Status ▪ Alive ▪ Deceased ▪ Unknown	 69 8 4
Follow up	61.0 ± 43.1 months

2.2.2 COHORT 2: EXPANDED COHORT WITH HORMONE SENSITIVE SPECIMENS

Due to the fact that in the pilot the majority of significant results were found in the hormone sensitive samples the decision was made to expand the hormone sensitive samples cohort. The criterion for this cohort was to have had a pathologically confirmed diagnosis of prostate adenocarcinoma with a specimen taken before commencement of any hormonal therapy. This specimen can be obtained from a biopsy, TURP or radical prostatectomy sample if this was the primary treatment.

Cohort 2 was made up of the HSPC samples from cohort 1 supplemented by specimens obtained from Newcastle prepared as tissue microarrays i.e. with multiple tumour cores per slide. Where possible there was more than one tumour core per patient on the TMAs to compensate for possible tissue heterogeneity. 4 different TMAs were utilised including CaP specimens from a total of 276 patients.

- TMA1 consisted of samples from 76 patients all obtained from TRUS biopsy or TURP. There were 2-3 CaP samples per patient. A range of treatment methods were utilised with only a proportion of patients receiving hormonal therapy
- TMA2 consisted of samples from patients all obtained from TRUS biopsy or TURP. There were 2-3 CaP samples per patient. A range of treatment methods were utilised with only a proportion of patients receiving hormonal therapy.
- TMA3 consisted of samples from patients all obtained from radical prostatectomy. There were 4 CaP samples per patient. To our knowledge none of these patients underwent hormonal therapy.

- TMA4 consisted of samples obtained from patients obtained from TURP or TRUS biopsy. There was 1 sample per patient, all patients received hormone therapy.

Together the patients from cohort 1 and those from the TMAs gave a total of 357 patients. Within this second cohort of 357 the median age was 70.7 years (range 39.0 – 103.4 years) mean age was 70.4 years (SD ± 9.2). At diagnosis 65 (18.2%) of tumours were Stage T1, 53 (14.8%) T2, 86 (24.1%) T3, 30 (8.4%) T4 with the remainder of unknown/unrecorded stage. Gleason scores in HSPC specimens were 1-4 in 4 (1.1%), 5-7 in 206 (57.7%), 8-10 in 94 (26.3%) and not recorded in the remainder. 74 patients (20.7%) had known metastatic disease at diagnosis, 187 (52.4%) had no metastases with the metastatic status of the remainder unknown.

Patients within the cohort underwent a variety of treatment modalities. 227 patients (63.3%) including all those from the pilot study cohort underwent hormone therapy – antiandrogens, GnRH analogues, maximal androgen blockade or bilateral orchidectomy. At least 45 (12.6%) underwent radical retropubic prostatectomy. Over the recorded course of their disease 194 patients (54.3%) suffered biochemical relapse as defined above, 64 (17.9%) had no relapse and the relapse status was not recorded in 99 patients (27.7%). Mean time to relapse was 35.1 months (SD ± 32.3). At last known follow up 213 patients (59.7%) patients were deceased, 110 patients (31.9%) were alive with the status of the remainder unclear. Mean time to death/last follow up was 69.8 months (SD ± 54.0).

Table 2.2: Summary Statistics for All Cohort Patients with Hormone Treated Subgroup

	HORMONE TREATED PATIENTS	ALL PATIENTS
N	227	357
Age	71.4 ± 8.6 (41.1 – 103.4)	70.4 ± 9.2 (39.0 – 103.4)
Gleason Score ▪ Low ▪ Medium ▪ High	 4 116 69	 4 206 94
T Stage at Diagnosis ▪ T1 ▪ T2 ▪ T3 ▪ T4	 34 26 31 25	 65 54 86 30
Metastasis at Diagnosis ▪ Yes ▪ No ▪ Unknown	 57 110 60	 74 187 96
Biochemical Relapse ▪ Yes ▪ No ▪ Unknown	 182 36 12	 194 64 99
Time to Relapse	34.1 ± 32.1 months	35.1 ± 32.3 months
Final Status ▪ Alive ▪ Deceased ▪ Unknown	 43 167 17	 110 213 34
Follow up	73.2 ± 58.6 months	69.8 ± 54.0 months

2.3 IMMUNOHISTOCHEMISTRY

The main principle of immunohistochemistry (IHC) is the use of specific antibodies to the antigen to be detected to stain tissues prepared as slides. Once the primary antibody has bound to the antigen of interest, a secondary antibody is utilised which binds to the primary and amplifies the staining. This secondary antibody is labelled with an enzyme such as 3,3'-diaminobenzidine (DAB) or visible marker (fluorochrome) which gives a measurable visual representation of the level of binding of the primary antibody thus allowing the level of the target antigen to be assessed.

2.3.1 GENERAL STEPS IN IMMUNOHISTOCHEMISTRY

In this study 6 different specific protein marker antigens were targeted

- EGFR
- HER2
- HER3
- HER4
- EGFRVIII
- HRG

Additionally, as mentioned in the aims and objectives, the patient cohorts were also stained for markers of cell proliferation and apoptosis to assess their relationship with the main targets of this study.

- KI67-MIB antibody – a marker of cell proliferation
- ApopTag® TUNEL Assay (Chemicon International)– a marker of cell apoptosis

The IHC staining process of these markers varies somewhat in detail but follows the same basic steps in each case. The scoring process for assessing staining levels varies

somewhat between the main study proteins and the proliferation/apoptosis markers and will be addressed later in this chapter

Tissue Preparation

Tissue specimens had previously been prepared as formalin-fixed, wax-embedded 3-4μm thick sections with size dependent on the source of the sample for single slides and multiple 5mm diameter sections on TMAs. Specimens were mounted on 3-aminopropylethoxysilane coated slides. As the sections on TMAs are small and prone to damage/destruction during the IHC process TMAs are heated at 80°C for 5 minutes before other tissue preparation as this renders TMA samples less prone to damage. This process is not required for larger single slide sections

Before IHC staining tissue sections are dewaxed in 2×2-5 minute xylene baths then rehydrated in a series of alcohol baths;- 2×5 minutes 100% alcohol, 1×3 minutes 90% alcohol then 1×3 minutes 70% alcohol.

Antigen Retrieval

This process serves to counter any loss of immunoreactivity that occurs in tissues due to formalin-fixation and wax-embedding. During formalin fixation methylene bridges can form within tissue sections masking the relevant antigenic sites causing reduced or absent antibody-antigen interaction. Antigen retrieval breaks methylene bridges to expose antigenic sites (Fig 2.1a) and is achieved by incubating rehydrated tissue sections in a citrate or TRIS buffer at high temperature and/or pressure. The principle method used in this study was 1mM citrate buffer (1:100 dilution Epitope Retrieval Buffer, DAKO) in a pressure cooker heated in a microwave for 5 minutes followed by

a 20 minute cool down period. Other methods used in this study include heated at pressure in Tris EDTA Buffer (10mM Trizma Base, 0.25 mM EDTA), incubated in 0.1% trypsin in 0.1% calcium chloride at 37°C water bath, HercepTest™ (DAKO) epitope retrieval solution in water bath at 95-99°C and incubation with Protein Kinase K 20μg/ml at 25°C. The precise methods of antigen retrieval used for each marker are listed in tables 2.3 and 2.4 below.

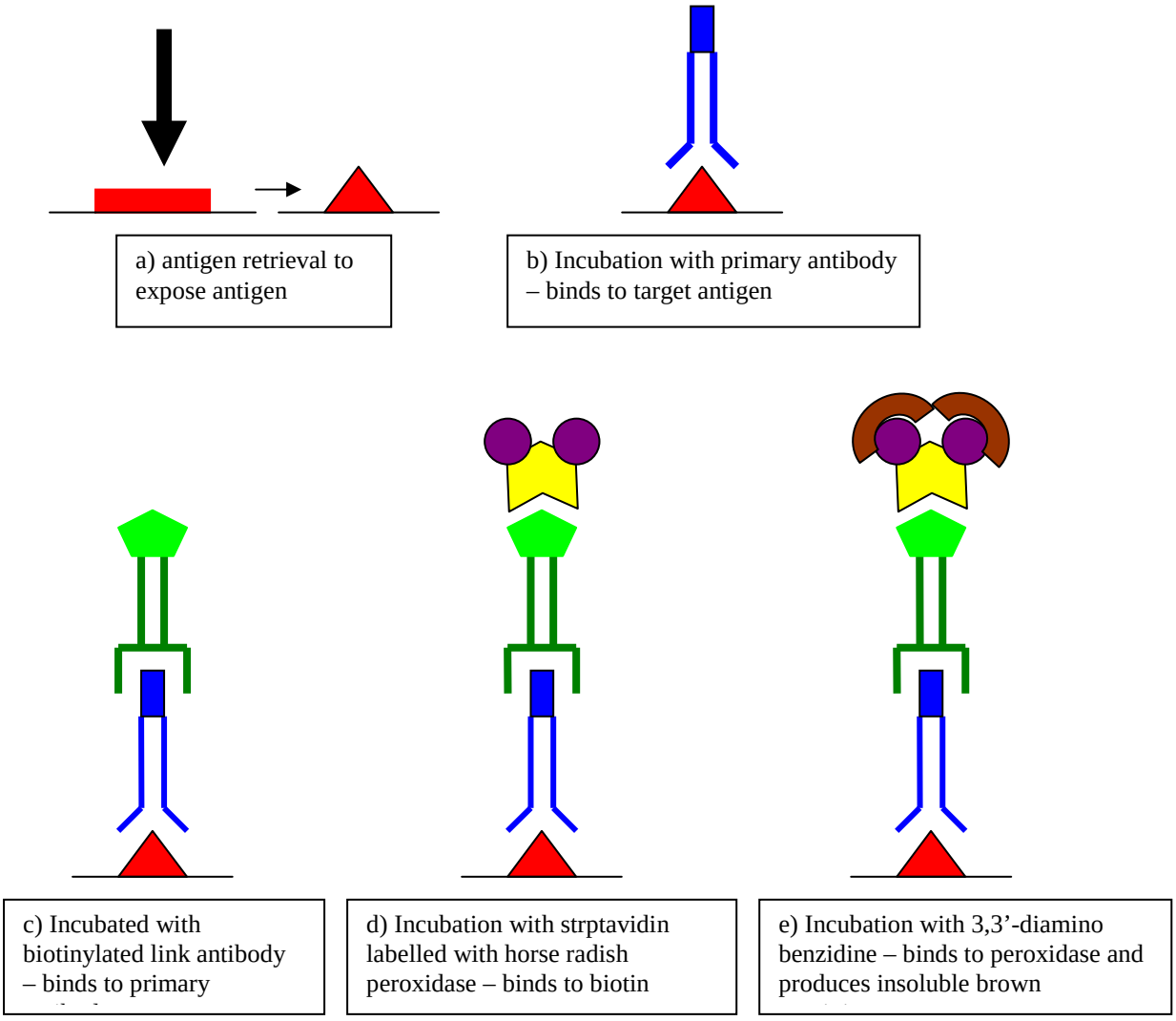
Blocking of Background Staining

Endogenous peroxidase within prepared tissue sections can react with diaminobenzidine (see below) causing non-specific background staining during the IHC process interfering with assessment of the presence of the target antigen. Blocking of this process can be achieved by incubating with 0.3% Hydrogen Peroxide (H_2O_2) for 10-20 mins followed by wash in distilled water. Additionally unintended hydrophobic bonds can form between immunoglobins and prepared tissue resulting in non-specific binding of both primary and secondary antibodies and further background staining. This can be counteracted by incubation with 1.5% horse serum (Vector) in Tris Buffered Saline (TBS) for 10 minutes after which the blocking serum is poured off but not washed. The relative position of blocking processes and antigen retrieval varies between different IHC protocols

Incubation with Primary Antibody

At this stage the prepared, blocked tissue sections were incubated with the primary antibody which binds to the target antigen (Fig 2.1b). The specific concentration and duration and temperature of incubation for each antibody was determined by optimisation of the antibody i.e. trial runs with a range of concentrations/durations

Figure 2.1: Schematic Representation of Immunohistochemical Staining using the example of the streptavidin-biotin method which relies on the high affinity of avidin and biotin to each other.



temperatures in samples known to stain positively with the aim of finding the conditions providing the best result – strongest specific staining with the least possible background and not overstained so a comparison between low and high expression is possible. The antibodies to EGFR, EGFRvIII, HER2, HER3 and HER4 were optimised during the pilot study and the regimens determined in the pilot study were

used. The antibodies to HRG and KI67 were optimised in this study (see below). The TUNEL apoptotic assay is provided with specific instruction and is pre-optimised. The specific incubation concentrations and conditions used for each antibody in this study are listed in tables 2.3 and 2.4.

Quality Control

Each IHC run performed included both a positive and negative control. The positive control used was a tissue section known to stain positively with the specific antibody – in most cases a specific tissue section shown to be strongly positive in the pilot study. The same positive controls were used in different runs of the same antibody although the section used varied between different antibodies. Positive controls go through every single step of a run with the aim of confirming the success or failure of staining; specific positive controls used are listed in table 2.5. Negative controls were all isotype matched prostatic tissue sections which went through every step of the run except incubation with the primary antibody with the aim of checking specificity of staining i.e. demonstration of staining in the negative control indicates background staining rendering a run unreliable.

Incubation with Secondary Antibody

Following incubation with the primary antibody the tissue sections were washed with TBS buffer for 2×5 minutes then incubated with a secondary antibody (Fig 2.1c, 2.1d). One secondary antibody method utilised in this study is the labelled streptavadin-biotin (LSAB plus-DAKO) visualisation which involves a 2 step process, first the specimen is incubated for 15 minutes at 25°C with biotin-attached antibody directed at the primary antibody washed in TBS then incubated with avidin affixed to a peroxidase for 15 minutes at 25°C and washed again. Avidin has a high affinity for

biotin and the result of this dual incubation is peroxidase bound via avidin, biotin and secondary antibody to the primary. Another secondary antibody used in this study was Envision™ (DAKO) which consists of a dextran large molecule backbone with enzyme molecules including horseradish peroxidase attached as well as antibodies which bind to the primary. It is the attached peroxidase which is vital to the next stage. Incubation is carried out at room temperature in a humidified chamber usually for 30 minutes. The envision system is noted to have high sensitivity and low background staining.

Visualisation

Following a further wash in 2×5 minutes TBS the tissue sections were incubated with 3,3'-diaminobenzidine (DAB) in the form of chromagen DAB substrate (DAKO) in specific DAB diluent (DAKO) usually in ratio 1:50. An alternative preparation is 4 drops DAB stock solution, buffer, 2 drops H_2O_2, 2 drops buffer in 5mls distilled water (Vector). When a substrate chromagen binds to peroxidase (Fig 2.1e) a colour reaction occurs producing a brown colour product with the amount of brown staining corresponding to the level of peroxidase present therefore the level of secondary and primary antibody hence the level of expression of the target antigen in the tissue section. Incubation with DAB is carried out at room temperature for 5-10 minutes followed by washing in water for 10 minutes.

Counterstaining

Following chromagen staining tissue sections were counterstained to provide contrast to the brown colour allowing assessment of the level of positive staining. Slides are immersed in haematoxylin for 30-45 seconds, washed then submerged in Scots Tap

Water Substitute for 45 seconds and washed again. Haematoxylin stains tissue not already chromagen stained vivid red and the Scots Tap converts this red to a blue colour particularly in contrast to chromagen brown.

Dehydration and Mounting

The final steps in the IHC process prepare the tissue section for viewing under light microscope and scoring. The slides are dehydrated in a series of alcohol baths;- 1×1 minute 70% alcohol, 1×1 minute 90%, 2×1 minute 100% then 2×1 minute xylene. Dehydrated slides are then mounted onto cover slips using DPX mountant (Dibutyl Phtalate and xylene) as an adhesive.

2.3.2 OPTIMISATION OF HEREGULIN

As it had neither been used in the pilot study nor as part of any study at this institution the antibody for HRG (HRG Clone V10081 (Biomedia)) was optimised for use in this study. Identical slides prepared and incubated at varying concentrations of primary antibody/durations.

- 1:100 for 10 mins at 25°C humidified
- 1:100 for 30 mins at 25°C humidified
- 1:200 for 10 mins at 25°C humidified
- 1:200 for 30 mins at 25°C humidified

The other steps used in HRG staining protocol used are listed in table 3. Incubation for 30 mins at either concentration rendered slides too heavily stained to allow differential scoring of expression. Incubation for 10 minutes gave an appropriate level of staining with 1:100 stronger than 1:200. Incubation with concentration 1:100 for 10 minutes was chosen as the optimal method and used thereafter for all HRG staining.

2.3.3 SPECIFIC STEPS IN IMMUNOHISTOCHEMISTRY

Table 2.3: Specific Antigen Staining Protocols

Antigen	Tissue Preparation	Blocking	Antigen Retrieval	Primary Antibody	Secondary Antibody	Visualisation	Counterstain	Dehydration
EGFR	xylene 2×4 mins, alcohol 100% 2×4 mins, alcohol 90% 1×2 mins, alcohol 70% 1×2 mins	Pre-retrieval: 0.3% H_2O_2 for 20 mins Post-retrieval: 1.5% horse serum	Incubated in 0.1% trypsin (Sigma) in 0.1% calcium chloride at 37°C water bath for 10 mins	EGFR clone 31G7 (Zymed) slides incubated 1:50 antibody: antibody diluent (DAKO) 25°C for 1 hour	LSAB Plus	DAB (Vector)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.
HER2	xylene 2×4 mins, alcohol 100% 2×4 mins, alcohol 90% 1×2 mins, alcohol 70% 1×2 mins		HercepTest™ (DAKO) epitope retrieval solution in water bath at 95-99°C	HercepTest 7.5g/ml rabbit anti-human HER2 polyclonal antibody for 30 mins at 25°C humidified chamber		DAB (Vector)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.
HER3	xylene 2×4 mins, alcohol 100% 2×4 mins, alcohol 90% 1×2 mins, alcohol 70% 1×2 mins	0.3% H_2O_2 for 20 mins. avidin/biotin blocking kit, 2.5% horse serum for 20 mins	none required	HER3 clone H3.105.5 (MS-303-PABX, Neomarkers) Slides incubated in 1:20 antibody:antibody diluent for 2 hours at 25°C humidified	ImmPRESS Anti-mouse Ig (peroxidase) kit (Vector)	DAB (Vector)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.

HER4	xylene 2×4 mins, alcohol 100% 2×4 mins, alcohol 90% 1×2 mins, alcohol 70% 1×2 mins	0.3% H_2O_2 for 20 mins avidin/biotin blocking kit, serum free blocking solution (DAKO) for 10 minutes	none required	HER4 clone HFR1 (ME-637-PO, Neomarkers) Slides incubated in 1:50 antibody:antibody diluent for 2 hours at 25°C	LSAB Plus	DAB (Vector)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.
EGFRvIII	xylene 2×4 mins, alcohol 100% 2×4 mins, alcohol 90% 1×2 mins, alcohol 70% 1×2 mins	Pre-retrieval: 0.3% H_2O_2 for 20 mins Post-retrieval: 5% horse serum for 1 hour	Tris EDTA Buffer (10mM Trizma Base, 0.25 mMEDTA) heated at pressure for 5mins	EGFRvIII clone ZMD.82 (Zymed) Slides incubated in 1:50 antibody:antibody diluent at 25°C humidified	LSAB Plus	DAB (Vector)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene..
HRG	2×2 mins xylene, 2×2 100% alcohol, 1×2 mins 90% alcohol, 1×2mins 70% alcohol	Post-retrieval: 1% H_2O_2 for 10 mins	Citrate Buffer heated at pressure for 5 mins, 20 mins cooling	HRG Clone V10081 (Biomedia) Slides incubated in 1:100 antibody:antibody diluent for 10 minutes at 25°C	Envision (DAKO) for 30 mins	Chromagen DAB 1:50 (DAKO)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.

2.3.4 KI67 ASSAY

KI67 is a nuclear protein principally associated with cellular proliferation. It is expressed in all active phases of the cell cycle (G1, S, G2 and M) but is not expressed in the resting G0 phase. Precise location of KI67 varies with cell cycle phase. In G1 the perinucleolar region, in later phases throughout the nucleus, being mainly localized to the nuclear matrix., in mitosis, it is present on all chromosomes. KI67 is thought to be involved in regulating the cell cycle and cell division. IHC is carried out in a similar fashion to other antigens however staining is only in the nucleus.

The antibody used for KI67 staining was KI67 MIB Clone M7240 (DAKO). For optimisation identical slides were prepared and incubated with the antibody at varying concentrations

- 1:50 for 1 hour at 25°C humidified
- 1:100 for 1 hour at 25°C humidified
- 1:150 for 1 hour at 25°C humidified

Steps used in KI67 staining are listed in tale 4. The 1:150 concentration was found to have the optimal staining level

2.3.5 TUNEL ASSAY

As apoptosis occurs many fundamental cellular changes occur including nuclear condensation, segmentation, fragmentation and formation of apoptotic bodies. The TUNEL assay functions by enzymatically labelling the free 3’-OH termini that are produced during apoptotic fragmentation of DNA typically located in the nucleus and apoptotic bodies. These 3’-OH ends are not seen in significant numbers in normal/proliferative nuclei and this type of DNA cleavage does not typically occur in cell necrosis. The TUNEL assay can detect apoptosis at a relatively early stage where

chromatin condensation has occurred and DNA strand breaks are few but still significantly greater than in normal/proliferative cells. As part of the TUNEL assay prepared, blocked tissue sections are incubated in terminal deoxynucleotidyl transferase (TdT) and reaction buffer; nucleotides in the buffer (which may be labelled with dioxigenin or unlabelled) are enzymatically added by the TdT to the 3'-OH terminals. Labelled and unlabelled nucleotides form an oligomer in a random sequence but in a ratio promoting binding by anti-dioxigenin antibodies. Secondary labelling is incubation with an anti-dioxigenin antibody conjugated with peroxidase and further addition of DAB (3,3'diaminobenzidine) produces a visible stain as in other IHC processes.

In lieu of optimisation instructions from the ApopTag TUNEL Assay kit were followed specifically. Specific steps are listed in table 4.

Table 2.4: Specific Staining Protocols for KI67 and TUNEL Assay

Antigen	Tissue Preparation	Antigen Retrieval	Blocking	Primary Antibody	Secondary Antibody	Visualisation	Counterstain	Dehydration
KI67	2×2 mins xylene, 2×2 100% alcohol, 1×2 mins 90% alcohol, 1×2mins 70% alcohol	Citrate Buffer heated at pressure for 5 mins, 20 mins cooling	Post Retrieval: 1% H_2O_2 for 10 mins	KI67 MIB Clone M7240 (DAKO) Slides incubated in 1:150 antibody:antibody diluent for 1 hour at 25°C humidified	Envision (DAKO) for 30 mins	Chromagen DAB 1:50 (DAKO)	Haematoxylin and Scots Tap	1×1 min 70% alcohol, 1×1 min 90% alcohol, 2×1 min 100% alcohol then 2×1 min xylene.
TUNEL Assay	3×5mins xylene, 2×5mins 100% alcohol, 1×3mins 90% alcohol, 1×70% alcohol	Incubation with Protein Kinase K 20µg/ml for 10 mins at 25°C	Post-Retrieval 3% H_2O_2 for 5 mins, ApopTag Equilibration Buffer	ApopTag TdT enzyme 3:7 reaction buffer Slide incubated for 1 hour at 37°C humidified	ApopTag Anti-Dioxigenin Peroxide Antibody incubated for 30 mins at 25°C humidified	ApopTag DAB Slides incubated at in 1:20 DAB substrate:diluent for 3-6 mins at 25°humidified	Methylgreen (0.5g methylgreen in sodium acetate (1.36g in 100ml dH_2O pH adjusted to 4)) for 10 minutes	2×20 dips dH_2O, 1×30 seconds dH_2O, 2×20 dips N-Butanol, 1×30 mins, 3×2 mins xylene

Table 2.5: Antibodies used in IHC

TARGET	ANTIBODY	SOURCE	CONCEN-TRATION	POSITIVE CONTROL
EGFR	clone 31G7	Zymed	1:50	Specific CaP tissue section from pilot
HER2	Anti-human HER2 polyclonal antibody Rabbit HercepTest	DakoCyto-mation	7.5g/ml	Specific CaP tissue section from pilot
HER3	H3.105.5 MS-303-PABX	Neomarkers	1:20	Specific CaP tissue section from pilot
HER4	clone HFR1 MS-637-PO	Neomarkers	1:50	Specific CaP tissue section from pilot
EGFRVIII	clone ZMD.82	Zymed	1:50	Specific CaP tissue section from pilot
HRG	Clone V10081	Biomedia	1:100	CaP (Specific Sample)
PROLIFERATION	KI67 MIB Clone M7240	DakoCyto-mation	1:150	Prostate Tissue (specific sample)
APOPTOSIS	TDT Enzyme* Anti-Dioxigenin (sheep polyclonal) TUNEL Assay ApopTag	Chemicon	3:7 in buffer As in TUNEL kit	Rodent Mammary Gland

* enzymatic addition of labelled nucleotides takes the place of the primary antibody

2.4 IMMUNOHISTOCHEMISTRY SCORING

The principle IHC scoring is assessment of the level of specific protein expression by determining the relative levels of the visualisation factor in different samples. Accuracy of scoring is ensured by use of 2 independent scorers with agreement in results between the 2 scorers correlated. Within this study 2 methods of histoscore; weighted histoscore for target antibodies primarily staining cytoplasm and cell membrane and nuclear counting for the cell proliferation and apoptotic markers that primarily stain the nucleus.

2.4.1 SCORING – WEIGHTED HISTOSCORE METHOD

For each of the target proteins EGFR, HER2, HER3, HER4, EGFRvIII and HRG tissue levels were assessed using the weighted histoscore method previously demonstrated in multiple studies including McCarty et al (1986), Witton et al. (2004) Edwards et al. (2005) and Kirkegarrd et al (2006). On viewing the full section the prostate tumour cells are identified and the level of staining of the cytoplasm, cell membrane and nucleus are separately assessed with the intensity of staining categorised as negative (0), weak (1), moderate (2), and strong (3) and the percentage of tumour cells within each intensity category estimated. The weighted histoscore was calculated using the formula

Histoscore = (0 × negative tumour cells) + (1 × % weakly stained tumour cells) + (2 × % moderately stained tumour cells) + (3 × % strongly stained tumour cells)

This formula gives a weighted histoscore value (called the HSCORE by McCarty) between minimum 0 and maximum 300. Separate values are calculated for cytoplasm, cell membrane and nucleus for each target protein.

Accuracy of score was determined by use of 2 independent observers for each target protein with the results of the 2 observers correlated. In single slide tissue sections all slides are analysed by both observers and the results for each slide compared. Results were considered discordant if scores differed by more than 50 and these individual cases re-evaluated by both observers. The inter-class correlation coefficient (ICCC) was used to assess variation in expression scoring between the 2 observers for all markers. This reliability measure assesses differences between the observers in each case comparing it to the overall variation between all scorings. ICCC as a determinant of validity was explored in Kirkegaard et al who stated that an ICCC > 0.7 was a minimum requirement for acceptable variation therefore within this current study agreement between 2 observers was considered satisfactory for a specific marker +if ICCC > 0.7 was achieved. Final scores used were the mean of the 2 observer scores. TMAs were scored in full by a single observer with a 2nd independent observer scoring a minimum of 10% of TMA specimens and the results of these double scored specimens compared. If ICCC of the double scored TMA specimens > 0.7 without adjustment this was taken as confirmation of accuracy of the 1st scorer. If ICCC < 0.7 at least a further 10% were double scored with ICCC calculated again with the process repeated until either ICCC > 0.7 or all TMA specimens were double scored. It should be noted that no further specimens for any marker required further double scoring after the first calculation of ICCC. The final histoscores used were the mean of the 2 scorers for double scored specimens and that of the 1st scorer in all others. ICCCs for all markers are listed in the results section below.

2.4.2 SCORING – NUCLEAR COUNT METHOD

Cell proliferation marker KI67 and apoptotic marker TUNEL assay stain only the nuclei of proliferating/apoptotic cells respectively with levels of these processes assessed by calculating the relative numbers of stained and unstained tumour cell nuclei. In single slide specimens 15 separate fields at 20×magnification which included tumour were assessed using a 10 line grid overlying the field. All tumour cell nuclei lying on a grid line were assessed as either positive or negative and counted with the aim of counting 100 nuclei per field therefore 1500 nuclei per specimen. Where fewer than 100 tumour nuclei were present on grid lines all tumour nuclei in the field were counted up to a maximum of 100 – all tumour cell nuclei present if there were fewer than 100. Where there were persistently fewer than 100 tumour nuclei per field up to 20 fields were viewed. Where fewer than 15 separate 20× magnification fields could be found with tumour within a tissue section the maximum of separate 20× magnification fields that could be found with tumour in were used. Therefore ~1500 tumour cell nuclei or all those present in up to 20 fields of the sample were counted. In TMAs all tumour cell nuclei within a sample were counted.

Marker expression was calculated as the percentage of all cells counted that were positive. This method has been demonstrated previously in Hilmy et al.

Positive Nuclear Score = 100 × (Number of Positive Tumour Nuclei/Total Number Tumour Nuclei)

All specimens were assessed by a single scorer with a 2nd independent scorer double scoring at least 10% of samples including TMAs. Positive nuclear scores of the 2 scorers

were compared and the ICCCs calculated for the double scored specimens. If ICCC > 0.7 without adjustment for the samples assessed this was taken as a confirmation of accuracy of the 1st scorer. If ICCC < 0.7 at least a further 10% samples were double scored and the ICCC calculated until ICCC > 0.7 or until all were double scored. It should be noted that, due to this method being a count and less subjective than the weighted histoscore method, no further double scoring was required after the initial ICCC calculation indeed ICCCs calculated by this method were all > 0.9. The final positive nuclear scores used were the mean of the 2 scorers for double scored specimens and that of the 1st scorer in all others. ICCCs for all markers are listed in the results section below

2.5 WESTERN BLOTTING

Western blotting is a technique which allows detection, identification and quantification of specific proteins within a sample using electrophoresis to divide denatured proteins into a spectrum by molecular weights within a gel followed by transfer of the resulting proteins to a PVDF membrane. The membrane is then incubated with a primary antibody to recognise a specific protein from the spectrum and a secondary antibody to the primary which allows visualisation of the protein via chemiluminescence/chemifluorescence/x-ray film. Use of a known primary antibody allows detection of a specific protein within a mixed sample (e.g. one derived from lysed tissue sections) with confirmation of molecular weight thereby identity via comparison of a protein's position following electrophoresis relative to proteins of known molecular weight in a protein ladder. Intensity of signalling following visualisation processes indicates quantity of the target protein within the original sample.

A further indication for use of Western Blotting is confirmation of specificity of the primary antibody. If an antibody is truly specific to one protein it will attach only to that protein within the spectrum produced by gel electrophoresis with the resultant completion of the Western Blotting process resulting in a single band. Conversely an antibody with poor specificity will result in multiple bands. Within this study Western Blotting is only used for this last indication. At this centre the specificity of EGFR, HER3, HER4 and EGFRvIII had been confirmed in previous studies including the pilot and HER2 specificity is assured by use of the commercial HercepTest; however the HRG antibody

(Clone V10081, Biomedia) had never been used here and a Western Blot utilising this antibody was performed.

2.5.1 WESTERN BLOTTING OF HEREGULIN ANTIBODY

Western Blotting was carried out using the Bio-Rad Mini-Protean 3 Electrophoresis System. The initial step was preparation of 10% resolving gel – polymerisation of the acrylamide and bis-acrylamide catalysed by TEMED and APS form the gel. Protein migration during electrophoresis is determined by size of gel pores which are in turn governed by the amount of acrylamide-bis in the gel mixture – increased acrylamide/increased gel percentage decreases pore size thereby making gel suitable for separating smaller proteins.

Table 2.6: Constituents of 10% Resolving Gel

REAGENTS	10% RESOLVING GEL
40% Acrylamide/Bis-Acrylamide (Sigma)	12.49ml
0.5M EDTA	330µl
2M Tris, pH 8.9	8.35ml
10% SDS	500µl
dH20	28.33ml
10% APS	300µl
TEMED	30µ

A mould was assembled from 2 spacer plates fixed into a casting frame and gel poured into the mould between spacer plates and isopropanol poured on top of the gel which is allowed to set over 30 mins. The isopropanol serves to remove air bubbles from and flatten the top of the setting gel. Once the gel was set the isopropanol was poured off and blotted and 4.5% stacking gel prepared and poured onto the resolving gel filled to the level of the top of the spacer plates. A gel comb, which creates the wells into which the

denatured protein samples would later be placed, was positioned in the stacking gel which is then allowed to set over 30 minutes.

Table 2.7: Constituents of 4.5% Stacking Gel

REAGENTS	4.5% STACKING GEL
40% Acrylamide/Bis-Acrylamide (Sigma)	5.63ml
0.5M EDTA	400µl
2M Tris, pH 8.9	6.35ml
10% SDS	500µl
dH20	37.22ml
10% APS	30µl
TEMED	10µ

After the stacking gel was set the comb was removed and the gel rinsed in 1× running buffer

Table 2.8: Buffer Constituents used in Western Blotting

BUFFERS IN WESTERN BLOTTING	REAGENTS
10× Running Buffer	200mM Tris, 2M Glycine, 1% SDS (diluted to 1× in dH_2O)
2× Sample Buffer	1ml 0.5M Tris/HCl pH 6.8, 08ml Glycerol, 1.6ml 10% SDS, 0.4ml 2-MerCaptoethanol, 0.2ml 0.05% Bromophenol Blue, 4ml dH_2O
10× Transfer Buffer	248mM Tris, 1.3M Glycine, 20% Methanol (diluted to 1× in dH_2O)
Gel Loading Buffer	5% 2-merCaptoethanol
10× TBS	0.1M Tris/HCl, 1.5M NaCl, pH 7.4 (diluted to 1× in dH2O
0.001% TTBS	1ml Tween 20 in 1l 1×TBS

Meanwhile protein sample – cell lysate of prostate cancer cells of the LNCaP cell line – was prepared via protein denaturation. 2 volumes protein were added to 2× sample buffer in an Eppendorf tube which was boiled at 100°C for 2 minutes then spun down to separate solid remnants. A molecular weight marker (Biotinylated Protein Ladder – cell

signalling Technology) was also boiled for 2 minutes with gel loading buffer (1µl marker in 9µl buffer) in a separate tube.

The sample buffer, specifically the SDS transfers a negative charge to the denatured proteins. It is this charge that allows the movement required for electrophoresis as they will migrate towards the anode if placed in an electric field. Proteins will migrate through the acrylamide gel at a rate determined by molecular weight with lower rates travelling more quickly thus proteins are separated.

The set gel was placed between glass plates in an electrode assembly in a mini buffer tank surrounded by 1× running buffer. The prepared protein sample and markers were loaded into the wells in the stacking gel with a fine tip pipette avoiding overspill from the wells. Once loaded the gel was run with a charge of 40mA for 1 hour. The denatured proteins migrate from the stacking to the running gel.

The next step is transfer of separated proteins onto a polyvinylidene difluoride (PVDF) membrane so that it can be labelled. In this study the Mini-Trans Blot Cell (Bio-Rad) system was used. A PVDF membrane cut to be slightly larger than the running gel was first pre-treated in 100% methanol for 1 minute then soaked in 1× transfer buffer (table 2.8) with fibre pads and 3M Whatman paper cut to the same size as the membrane. The gel was removed from the electrode assembly, the stacking portion removed and the running gel placed in transfer buffer for 15 minutes. A ‘transfer sandwich’ was then created with the running gel lying against the PVDF membrane with both packed

between Whatman paper (3 sheets either side), fibre pads and gel cassettes, air bubbles are carefully removed by rolling with a glass rod. The sandwich was placed in an electrode assembly in a mini tank filled with transfer buffer itself placed in a Bio-Ice cooling unit with a magnetic stirrer to maintain even buffer temperature. The sandwich was incubated overnight (~18 hours) with the electrode assembly set at 10V which causes the charged proteins to transfer from the gel to the membrane maintaining the dispersion pattern established by electrophoresis.

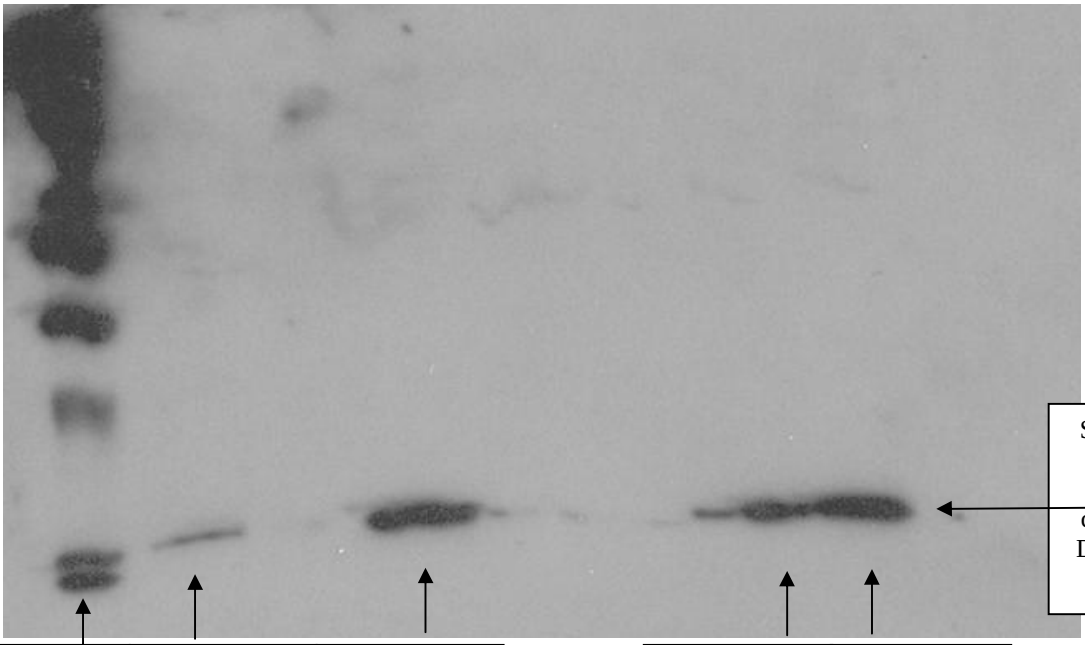
The next stage in the Western Blotting process is blotting of the membrane to prevent the primary antibody binding non-specifically to it. The sandwich was disassembled and the membrane incubated in 5% Marvel (non-fat dry milk) blocking solution in TBS-Tween (TTBS) (table) for 1 hour at 25°C on an orbital shaker which causes continuous stirring. The impregnated, blocked PVDF membrane was incubated with the primary antibody HRG antibody (Biomedia) 1:100 in 5% Marvel/TTBS at 4°C overnight on an orbital shaker.

Following primary antibody incubation the PVDF membrane was washed in TTBS for 3×10 mins then incubated with secondary antibody. In this study the secondary antibody used was 1:10 000 anti-mouse IgG (Cell Signalling Technology) linked to horseradish peroxidase (HRP) which recognises the HRG antibody. Additionally HRP-linked antibiotin antibody (Cell Signalling Technology) 1:1000 was used to detect the biotinylated marker ladder. The PVDF membrane was incubated with these secondary antibodies for 1 hour at 25°C on an orbital shaker.

The final step in Western Blotting is visualisation. In this study the ECL plus (Amersham) chemiluminescent method was utilised; – horse radish peroxidase oxidises luminal into an excited state which then emits light during its decay (chemiluminescence). In ECL plus the chemiluminescent agent substrate is Lumigen PS-3 Acridan oxidised by HRP to form acridinium ester intermediates which react with the peroxidase to produce light emissions at 430nm which can be detected by radiographic film. Following incubation with the secondary antibody the membrane was again washed in TTBS for 3×10 mins TTBS. The ECL plus components were heated to room temperature then mixed in amount 3mls solution A: 75μL solution B (40:1). In semi-darkness to prevent interference with chemiluminescence and premature non-specific exposure of the radiographic film the membrane was placed protein side up on a sheet of saran wrap, the ECL solution pipetted onto it and the membrane incubated for 5 minutes at 25°C. After this incubation the ECL reagents were poured off, the membrane blotted and wrapped in another piece of saran. The membrane was placed in full darkness with 4 autoradiographic films in succession for different durations; 30 seconds, 1, 5 and 15 minutes.

The radiographic films were subsequently developed and all showed the marker ladder and a single band at 7000Daltons corresponding with cellular Heregulin (Figure 2.2). This confirms that the HRG antibody used in this study has the appropriate specificity to be used in this study's IHC.

Figure 2.2: Western Blot of HRG antibody (Clone V10081, Biomedia)



Protein samples of LNCaP Prostate Cancer Cell Lysate protein mixed with SDS sample buffer (to confer a charge to the lysate constituent proteins) are loaded onto a resolving gel block with a separate molecular weight marker ladder and electrophoresed across it to create a dispersion pattern of its constituents delineated by decreasing molecular weight. The proteins are transferred to a PVDF membrane while maintaining the dispersion pattern. Following blotting to prevent non-specific binding labelled with the PVDF membrane is incubated with 1:100 HRG antibody at 4°C overnight. The primary antibody is washed off then the specific proteins labelled by antibody are discerned by incubation with secondary antibody anti-mouse IgG which binds to the primary and is also linked to a horseradish peroxidase moiety. The HRP labelled PVDF membrane is placed in a chemoluminescent agent (Lumigen PS-3 Acridan) and heated which produces light emissions which can be captured on radiographic film (pictured). Thus only the specific proteins within the lysate constituent dispersal pattern labeled by the primary antibody will ultimately produce a light emission and register on film. The HRG antibody produces a single band on Western Blot corresponding to Heregulin confirming its specificity as an antibody.

2.6 STATISTICS

As in the pilot study the purpose of the statistics was to determine whether any significant association existed between expression of the target markers and the outcome measures;-

Time to biochemical relapse (TTR) - time elapsed between tissue diagnosis of CaP and the occurrence of biochemical relapse as defined above and in the pilot study

Time to death from relapse (TTDFR) - time elapsed from biochemical relapse as defined above to patient death from any cause. This outcome measure was used only for cohort 1

Overall survival (OS) - time elapsed from tissue diagnosis of CaP to death from any cause

Initially Kaplan-Meier regression analysis was performed comparing both above median (High) and above upper quartile (Very High) marker expression with the outcome measures. As usual a p-value < 0.05 was taken as indicating a significant association. For those markers that demonstrated a significant association a univariate COX regression analysis was also performed to confirm significance and give a value for the hazard ratio (increased risk factor) with confidence interval. Multivariate COX regression was performed for those markers demonstrating significance to determine if this was independent of Gleason score and metastasis at present. A further multivariate analysis was performed including all markers maintaining significance through the first multivariate COX to compare the significance of these markers.

In Cohort 1 when comparing HSPC and HRPC mean expression Wilcoxen analysis was used to determine any significant rise or fall after hormone escape as this is linked data (before and after) with a non-parametric distribution. Dividing the cohort into those

whose individual marker expression had risen or fallen, Kaplan-Meier analysis was again used to determine if a rise or fall in expression of a marker had any association with any of the outcome measures.

Correlation analysis was performed on expression of all markers in the HSPC cohort to discern any correlations between expression of pairs of markers initially.

All statistics were performed using the SPSS program.

CHAPTER 3: RESULTS – COHORT 1

This chapter records the results obtained from cohort 1 which was used in the pilot study and consists of paired HSPC and HRPC specimens taken from the same patient at diagnosis (HSPC) then following established hormone escape (HSPC). The manner in which these patients were identified and samples obtained is described in the method section. Results gathered from the larger cohort 2 consisting of HSPC samples are described in chapter 4.

3.1 PATIENTS

In the pilot study 74 sets of paired samples were utilised. Subsequently 7 patients were added to the cohort however due to the gradual nature of additions to the cohort coupled with depletion of slides through use in other studies staining for each target could not be carried out in all slide pairs. In this study a cohort of 81 paired samples were stained for at least one target.

3.1.1 PATIENT DATA FOR COHORT 1

Table 3.1: Patient Data for Cohort 1

N	81
Age	70 ± 8 years Range 41.1 – 98.0 years
Gleason Score ▪ Low (2-4) ▪ Medium (5-7) ▪ High (8-10)	 2 35 43
T Stage at Diagnosis ▪ T1-T2/unknown ▪ T3-T4	 25 56
Metastasis at Diagnosis ▪ Yes ▪ No ▪ Unknown	 20 35 26
Biochemical Relapse ▪ Yes ▪ No ▪ Unknown	 81 0 0
Time to Relapse	36.5 ± 31.3 months
Final Status ▪ Alive ▪ Deceased ▪ Unknown	 8 69 4
Follow up	61.0 ± 43.1 months

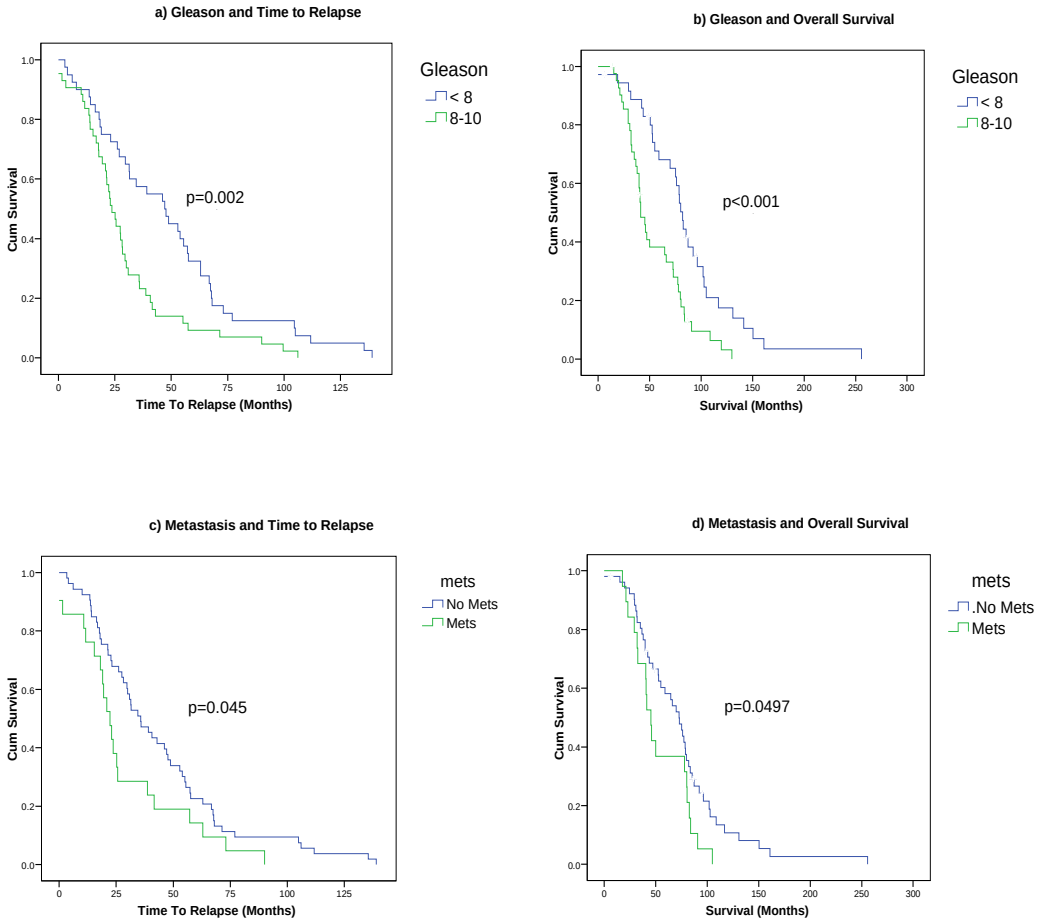
Table 3.2: Summary of Patient Tissue Samples Within Cohort 1 Stained for each Marker in this Study and the Pilot Given Sample Attrition

MARKER	PATIENTS	HSPC SAMPLES	HRPC SAMPLES	PAIRED SAMPLES
EGFR	74	74	74	74
HER2	52	52	52	52
HER3	53	50	52	49
HER4	59	53	54	48
EGFRvIII	69	63	69	63
HRG	69	53	61	45
KI-67	71	61	69	59
TUNEL ASSAY	62	57	50	45

3.1.2 CORRELATION OF GLEASON SCORE AND METASTASIS WITH STUDY OUTCOMES IN COHORT 1

In this cohort High Gleason score (8-10) was associated with reduced time to biochemical relapse ($P<0.001$) and overall survival ($P=0.002$). Metastasis at presentation was associated with reduced time to relapse ($P=0.045$) and survival ($P=0.0497$). These values are in accordance with known prostate cancer natural history and thus help validate the database.

Figure 3.1: Correlation of Gleason Score and Metastasis at Diagnosis with Time To Relapse and Overall Survival in Cohort 1



a) Kaplan-Meier plot correlating Gleason Score and Time To Relapse. b) Kaplan-Meier plot correlating Gleason Score and Overall Survival. c) Kaplan-Meier plot correlating Metastasis at Diagnosis and Time To Relapse. d) Kaplan-Meier plot correlating Metastasis at Diagnosis and Overall Survival

3.2 IMMUNOHISTOCHEMICAL EXPRESSION AND INTER-OBSERVER CORRELATION

As detailed in the method section scoring accuracy was confirmed by double scoring conducted by 2 independent observers. For the semi-qualitative weighted histoscore method (i.e. HRG in this study) all full tissue sections were double scored. For the more quantitative and objective nuclear staining count at least 10% of samples were double scored for each marker with the full scoring set for the first observer accepted if the inter class correlation coefficient (ICCC) was > 0.7. The figure of ICCC > 0.7 was chosen in reference to Kirkegaard et al (2006) which stated after multipaper review of IHC scoring that an ICCC > 0.7 was a minimum requirement for acceptable variation

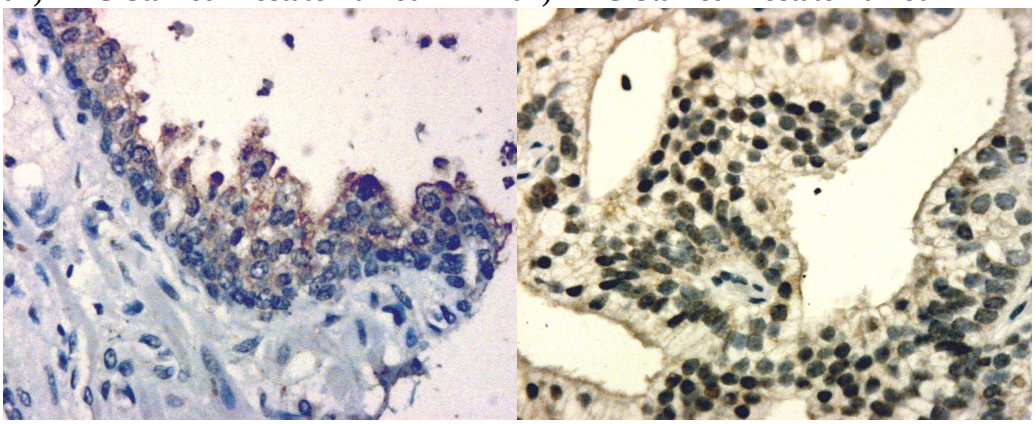
3.2.1 HEREGULIN

3.2.1.1 DESCRIPTION OF STAIN PROFILE

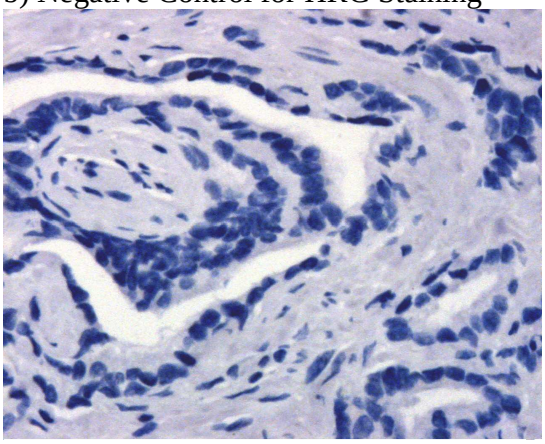
Within cohort 1 HRG expression was noted in the cytoplasm, cell membrane and nucleus with cytoplasmic expression being most frequent;- 99% of tumours had cytoplasmic staining seen by at least one observer. Nuclear (54% of tumours) and membranous (46% of tumours) expression were seen with lesser frequency. HRG expression was observed in at least one cellular location was seen in 99% of tumours analysed.

Figure 3.2: Heregulin Immunohistochemistry

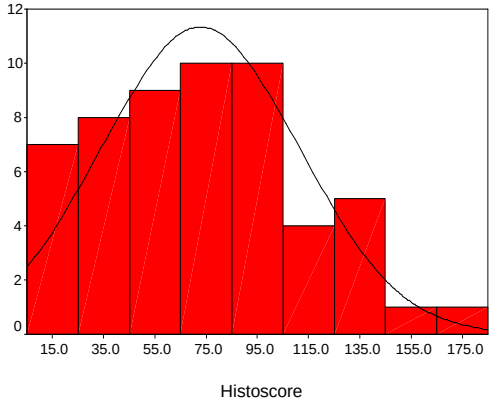
a1) HRG Stained Prostate Tumour a2) HRG Stained Prostate Tumour



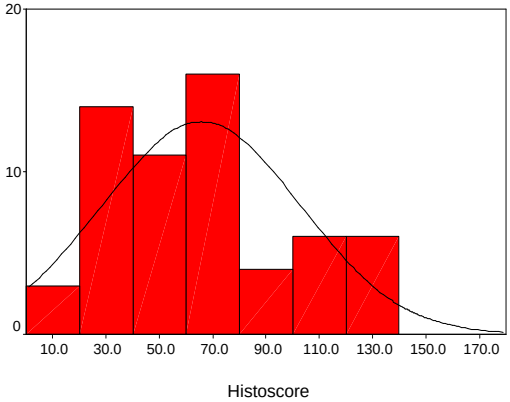
b) Negative Control for HRG Staining

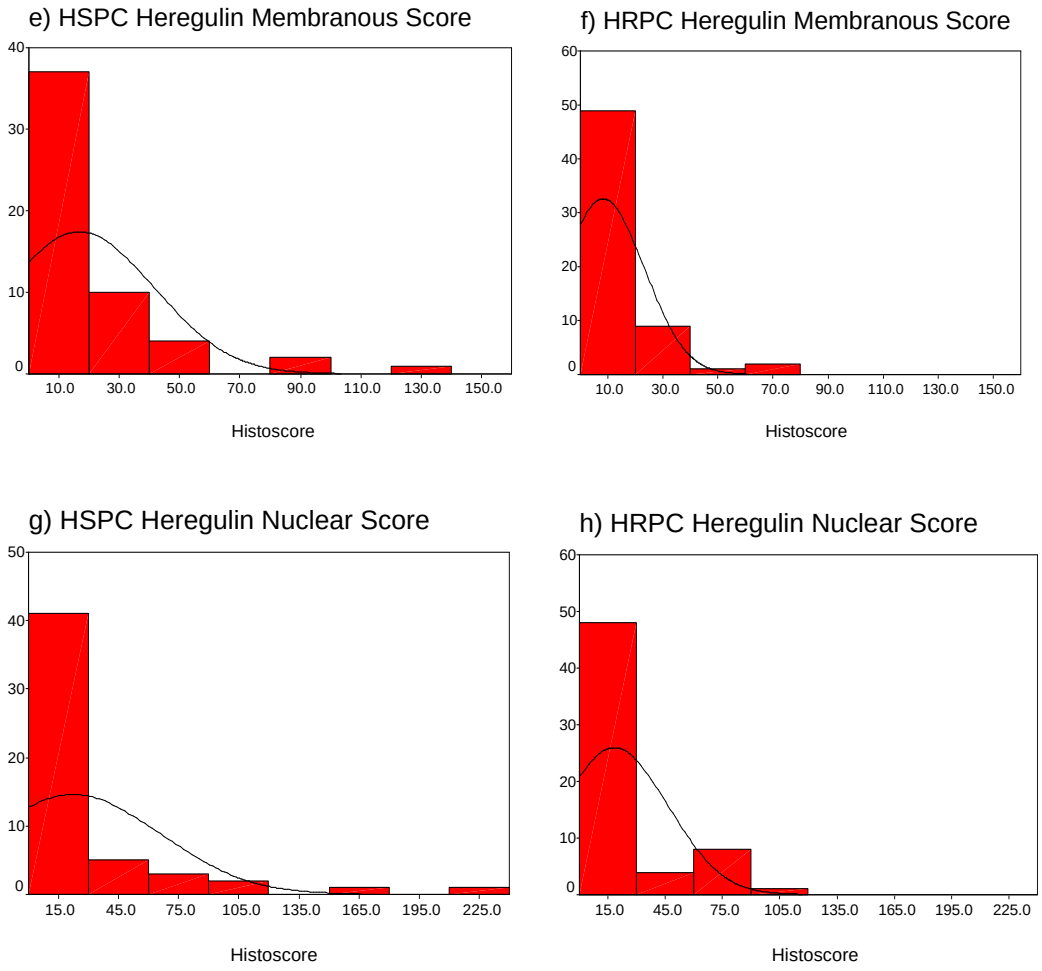


c) HSPC Heregulin Cytoplasmic Score



d) HRPC Heregulin Cytoplasmic Score





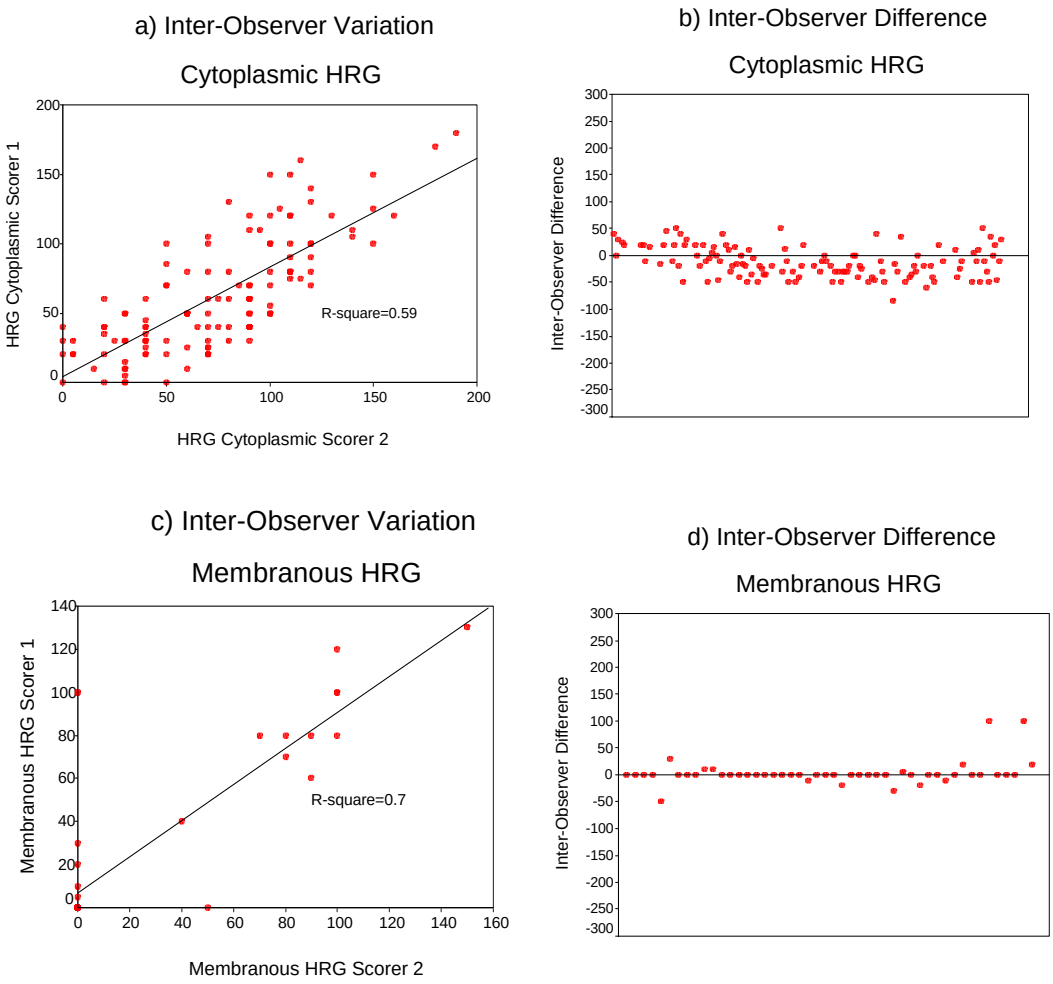
a1) Prostate Tumour Stained with HRG antibody demonstrating cytoplasmic and cell membrane staining. a2) Prostate Tumour Stained with HRG antibody demonstrating nuclear staining. b) Prostate Tumour Negative for HRG Staining
c) Histogram showing intensity of HRG cytoplasmic staining in HSPC specimens.
d) Histogram showing intensity of HRG cytoplasmic staining in HRPC specimens.
e) Histogram showing intensity of HRG membranous staining in HSPC specimens.
f) Histogram showing intensity of HRG membranous staining in HRPC specimens.
g) Histogram showing intensity of HRG nuclear staining in HSPC specimens.
h) Histogram showing intensity or HRG nuclear staining in HRPC specimens

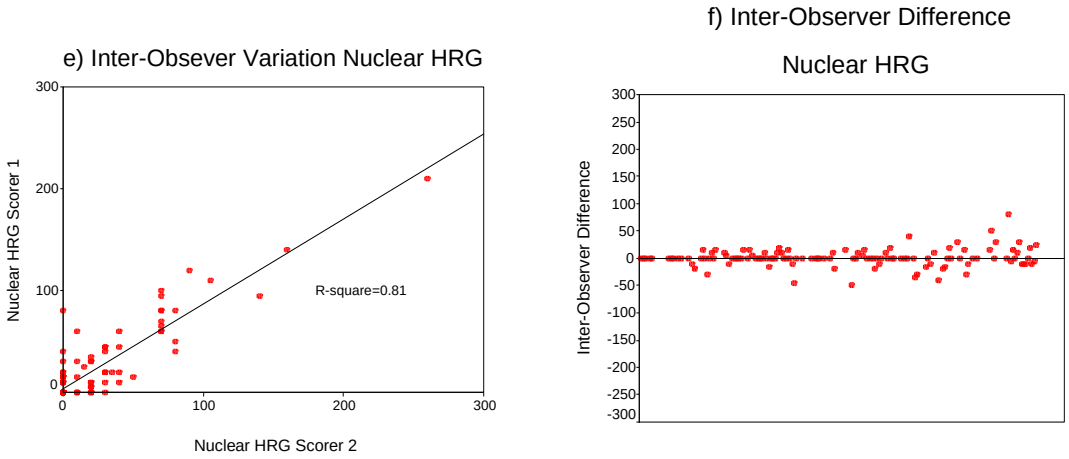
3.2.1.2 INTER-OBSERVER SCORING VARIATION

All tissue section stained for HRG were double scored by 2 independent observers. Cytoplasmic staining, the most commonly found, had the lowest ICCC of 0.72 (Pearson Coefficient 0.77) with membranous and nuclear staining giving higher ICCC values of

0.83 (Pearson 0.84) and 0.90 (Pearson 0.90) respectively. As all ICCC values were greater than 0.70 scoring of HRG in all areas was considered to be valid.

Figure 3.3: Inter-Observer Variation in Heregulin Staining between double scored tissue sections in Cohort 1





a)Scatter Graph Plot demonstrating Inter-Observer Variation in Cytoplasmic HRG Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Cytoplasmic HRG Staining. c) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous HRG Staining. d) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous HRG Staining. e) Scatter Graph Plot demonstrating Inter-Observer Variation in Nuclear HRG Staining. f) Bland-Altman Plot demonstrating Inter-Observer Variation in Nuclear HRG Staining.

3.2.2 KI-67

3.2.2.1 DESCRIPTION OF STAIN PROFILE

KI-67 was expressed only in the nucleus with no membranous or cytoplasmic expression. Positive and negatively stained nuclei were clearly distinguishable allowing assessment via nuclear counting method.

The IHC for KI67 in this study is demonstrated in appendix 1

3.2.2.2 INTER-OBSERVER SCORING VARIATION

All tissue sections stained for KI-67 were scored by one single observer with 40 specimens out of the 145 (27.6%) stained for KI-67 double scored by an independent observer to confirm accuracy of the first observer. ICCC score for double scored KI-67

sections was 0.95 (Pearson 0.96) confirming the accuracy of the first observer and reflecting the less subjective nature of nuclear counting compared to weighted histoscore. Graphs demonstrating this are in appendix 1

3.2.3 TUNEL ASSAY

3.2.3.1 DESCRIPTION OF STAIN PROFILE

As described in the ApopTag® instruction manual (Chemicon) the TUNEL assay primarily causes staining only in the nucleus although there was some minor non-specific background staining. Positive and negatively stained nuclei were clearly distinguishable allowing assessment via nuclear counting method. TUNEL IHC is demonstrated in appendix 1

3.2.3.2 INTER-OBSERVER SCORING VARIATION

All TUNEL Assay stained tissue sections and TMAs were viewed as a single group and scored by one observer with 10% double scored by an independent observer to ensure accuracy. Double scoring demonstrated an ICCC of 0.95 (Pearson 0.95) (see appendix 1)

3.2.4 SUMMARY OF COHORT 1 INTER-OBSERVER CORRELATIONS OF THIS STUDY AND THE PILOT

Table 3.3: ICCCs for Dual Scored Markers in this study and the pilot

Marker	2 Standard Deviations	Inter Class Correlation Coefficient	Pearson Coefficient
EGFR Cytoplasm*	26	0.87	0.89
EGFR Membrane*	28	0.89	0.89
HER2 Membrane*	26	0.91	0.90
HER3 Cytoplasm*	49	0.93	0.97
HER3 Membrane*	48	0.95	0.96
HER4 Cytoplasm*	47	0.90	0.91
HER4 Membrane*	32	0.91	0.93
EGFRvIII*	69	0.85	0.85
HRG Cytoplasm	57.2	0.72	0.77
HRG Membrane	46.9	0.83	0.84
HRG Nucleus	32.2	0.90	0.90
KI67	10.2	0.95	0.96
TUNEL	13.5	0.95	0.95

* Data from pilot study

3.3 MARKER EXPRESSION IN HORMONE SENSITIVE PROSTATE CANCER SPECIMENS

Levels of staining of HRG (cytoplasmic, membranous and nuclear), KI-67 and TUNEL assay were assessed separately in HSPC and HRPC samples. The results for HSPC samples are listed in table 3.4. Expression levels for each marker were then analysed to determine any association between expression and High Gleason Score (8-10), metastasis at presentation and patient outcome measures i.e. time to relapse, time to death from relapse and overall survival.

Table 3.4: Median and lower/upper quartile expression of HRG, KI67 and TUNEL assay in HSPC samples

Marker	HSPC Expression
HRG Cytoplasm	75 (50–100)
HRG Membrane	10 (0–30)
HRG Nucleus	7.5 (0-25)
KI67	2.9% (1.2-6.4)
TUNEL Assay	5.13% (2.0-17.7)

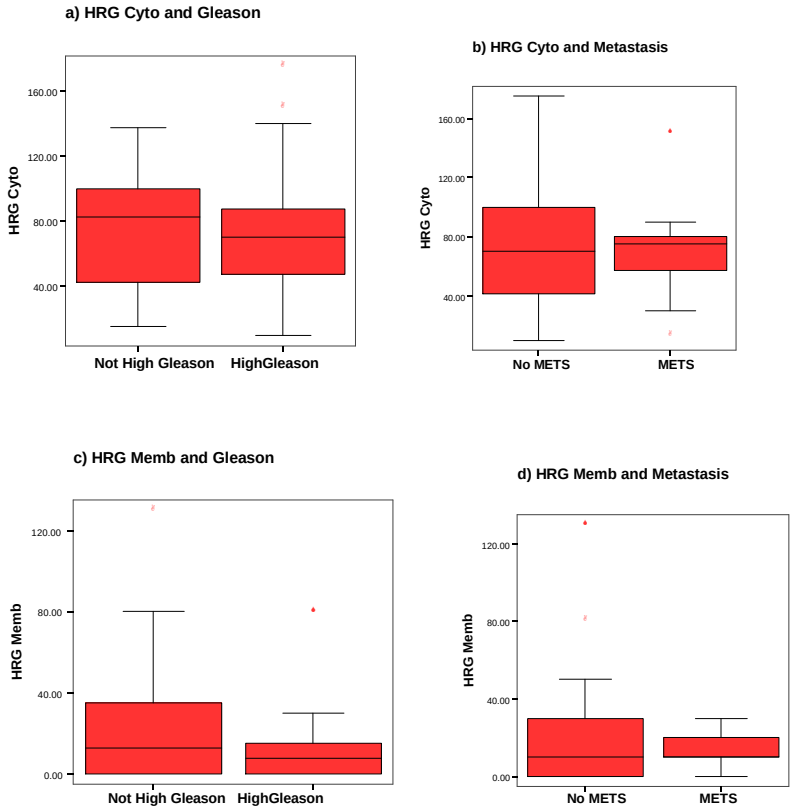
3.3.1 ASSOCIATION OF MARKER EXPRESSION IN HSPC WITH GLEASON SCORE AND METASTASIS

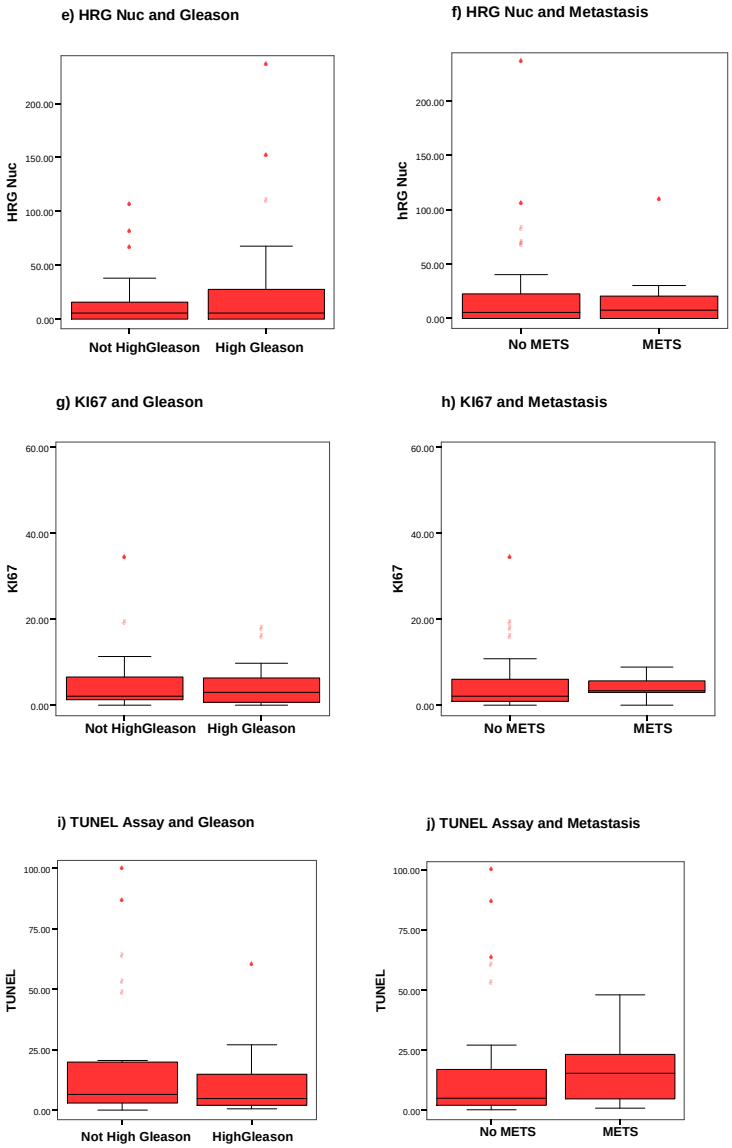
Association between expression and high Gleason score (8-10) or metastasis at presentation was assessed using Mann-Whitney analysis with $p<0.05$ representing significant association. No association was found in this group.

Table 3.5: Association of Markers in HSPC with high Gleason score and Metastasis

Marker	High Gleason Score P-value for Mann Whitney	Metastasis P-Value for Mann-Whitney
HRG Cytoplasm	0.665	0.929
HRG Membrane	0.112	0.732
HRG Nucleus	0.903	0.856
KI67	0.921	0.182
TUNEL	0.376	0.421

Figure 3.4: Boxplots of Marker expression in HSPC comparing patients with and without high Gleason score and Metastasis at presentation





a) Cytoplasmic HRG and Gleason Score, b) Cytoplasmic HRG and Metastasis, c) Membranous HRG and Gleason Score, d) Membranous HRG and Metastasis, e) Nuclear HRG and Gleason Score, f) Nuclear HRG and Metastasis g) KI67 and Gleason Score, h) KI67 and Metastasis, i) TUNEL Assay and Gleason Score, j) TUNEL Assay and Metastasis

3.3.2 IMPACT OF MARKER EXPRESSION IN HSPC ON TIME TO RELAPSE AND SURVIVAL

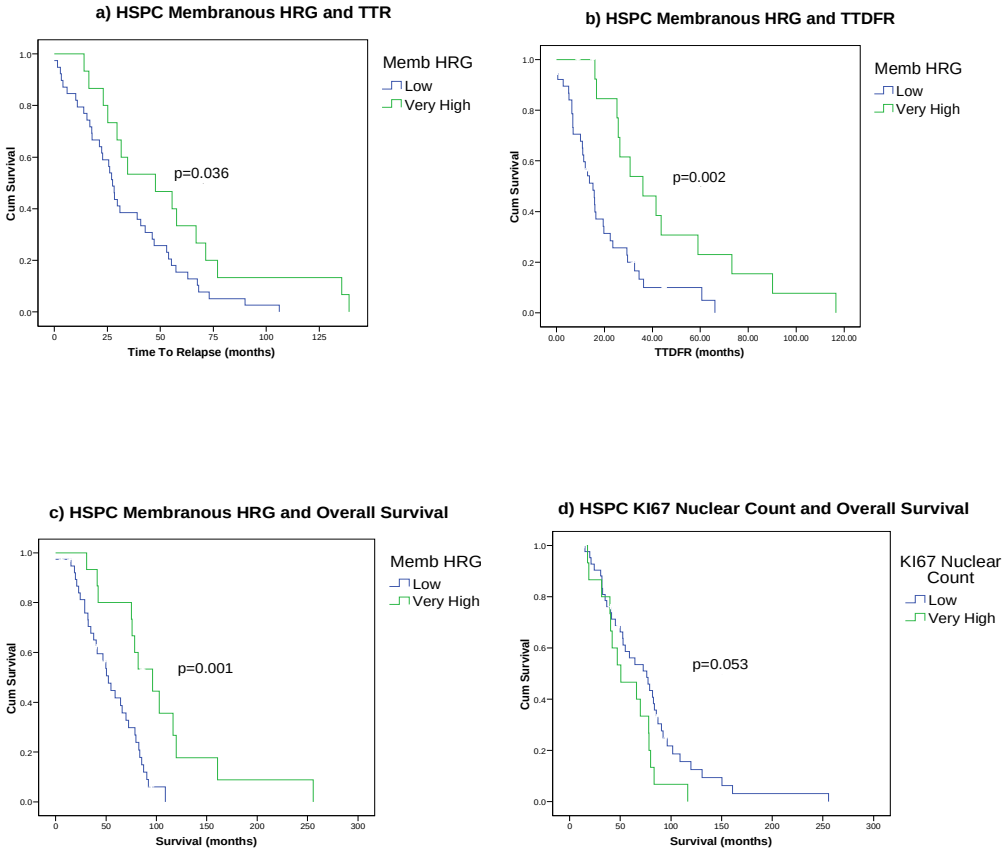
Associations between upper quartile (very high) marker expression in HSPC samples and TTR, TTDFR and OS were assessed using Kaplan-Meier analysis the p-values of which

are shown in table 3.6. Significant correlations were demonstrated between very high membranous HRG expression and increased TTR, TTDFR and OS. Patients with very high HRG membrane expression demonstrated a median time to relapse of 47.7 (27.4 – 69.1) months compared to 27.8 (16.4 – 48.5) months in those with low membranous HRG expression. Therefore very high expressers suffered biochemical relapse 20 months after low expressers. For patients with high HRG membranous expression median TTRFR was 30.7 (21 – 51.3) months compared to 13.3 (6.9 – 23.2) months in low expressers – a difference of 17 months. Median OS was 85.9 (75.6 – 109.7) months compared to 48 (29 – 74.1) months, a difference of nearly 46 months. KI67 was associated with reduced overall survival time but this did not quite achieve statistical significance (p=0.053).

Table 3.6. Association between marker expression in HSPC specimens and TTR, TTDFR and OS.

Markers	Time To Relapse Kaplan-Meier P- values	Time To Death From Relapse Kaplan-Meier P- values	Overall Survival Kaplan-Meier P- values
HRG Cytoplasm	0.169	0.233	0.121
HRG Membrane	**0.036**	**0.002**	**0.001**
HRG Nucleus	0.076	0.297	0.115
KI67	0.157	0.262	**0.053**
TUNEL	0.308	0.168	0.295

Figure 3.5: Kaplan-Meier Analyses of significant associations between HSPC Marker expression and study outcomes.



a) Membranous HRG and TTR, b) Membranous HRG and TTDFR, c) Membranous HRG and OS, d) KI67 and OS

3.4 MARKER EXPRESSION IN HORMONE RESISTANT PROSTATE CANCER SPECIMENS

All 5 markers are expressed in HRPC specimens

Table 3.7: Median and lower/upper quartile expression of HRG, KI67 and TUNEL assay in HSPC samples

Marker	HRPC Expression
HRG Cytoplasm	60 (35-100)
HRG Membrane	0 (0 – 15)
HRG Nucleus	5 (0-27.5)
KI67	7.7% (2.5-15.9)
TUNEL Assay	6.6% (3.4-31.4)

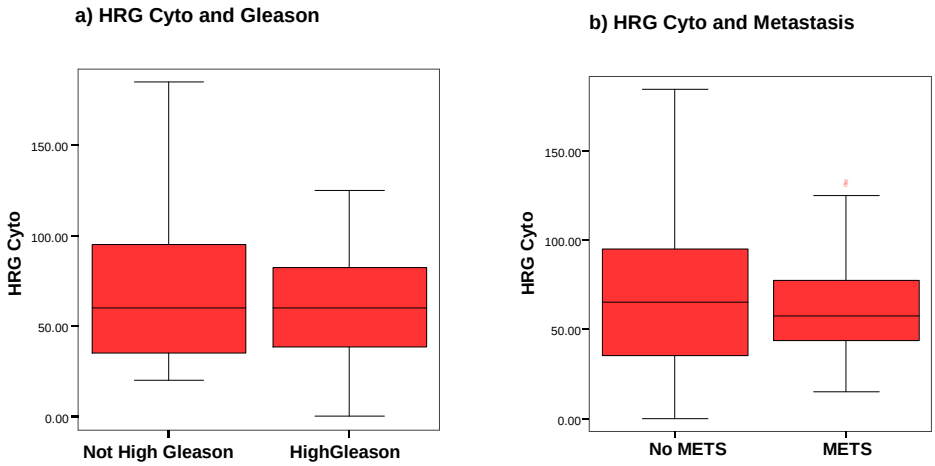
3.4.1 ASSOCIATION OF MARKER EXPRESSION IN HRPC WITH GLEASON SCORE AND METASTASIS

Association between expression and high Gleason score (8-10) or metastasis at presentation was assessed using Mann-Whitney analysis with $p<0.05$ representing significant association. One significant result was found with high HRG membranous expression associated with reduced metastasis at diagnosis. This means that patients with no metastasis had a significantly higher HRG membranous expression than those without metastasis.

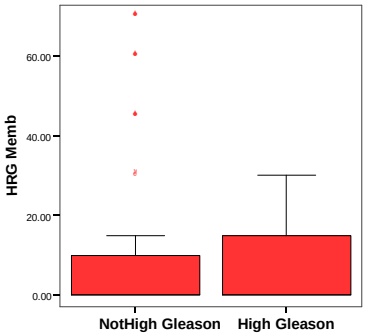
Table 3.8: Association of Markers in HRPC with high Gleason score and Metastasis

Marker	High Gleason Score P-value for Mann Whitney	Metastasis P-Value for Mann-Whitney
HRG Cytoplasm	0.924	0.781
HRG Membrane	0.799	**0.043**
HRG Nucleus	0.273	0.268
KI67	0.241	0.578
TUNEL	0.829	0.363

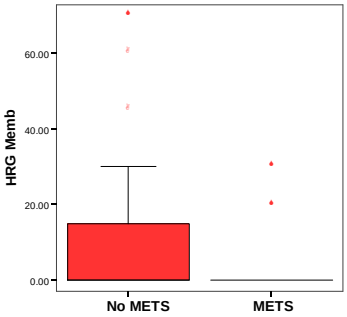
Figure 3.6: Boxplots of Marker expression in HSPC comparing patients with and without high Gleason score and Metastasis at presentation



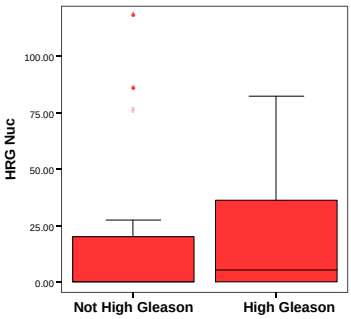
c) HRG Memb and Gleason



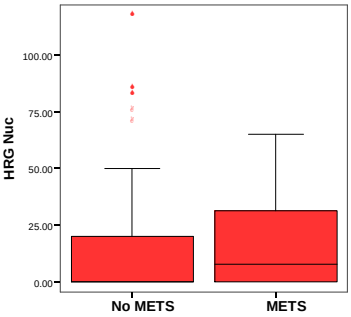
d) HRG Memb and Metastasis



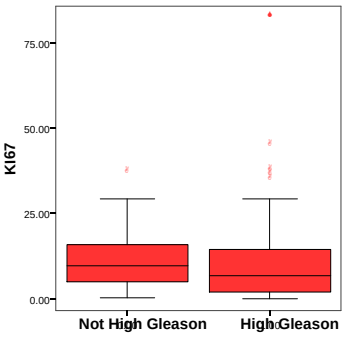
e) HRG Nuc and Gleason



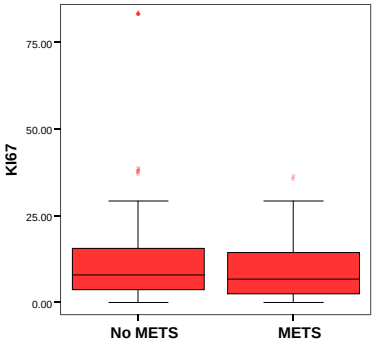
f) hRG Nuc and Metastasis

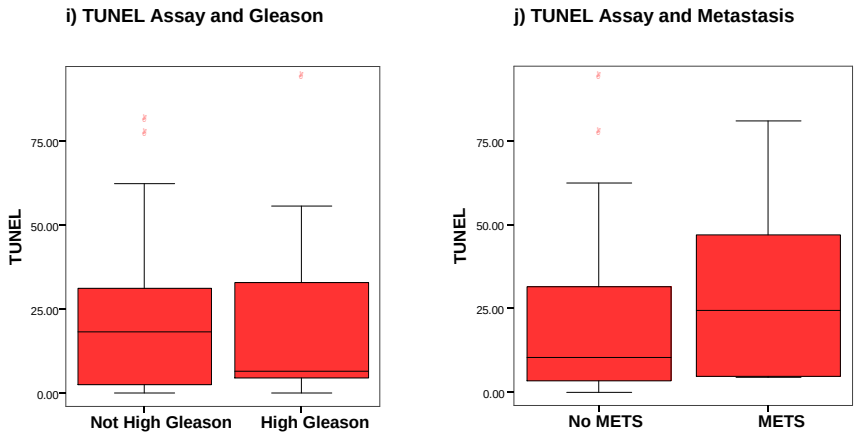


g) KI67 and Gleason



h) KI67 and Metastasis





a) Cytoplasmic HRG and Gleason Score, b) Cytoplasmic HRG and Metastasis, c) Membranous HRG and Gleason Score, d) Membranous HRG and Metastasis, e) Nuclear HRG and Gleason Score, f) Nuclear HRG and Metastasis g) KI67 and Gleason Score, h) KI67 and Metastasis, i) TUNEL Assay and Gleason Score, j) TUNEL Assay and Metastasis

3.4.2 IMPACT OF MARKER EXPRESSION IN HRPC ON TIME TO RELAPSE AND SURVIVAL

Associations between marker expression in HRPC samples and TTR, TTDFR and OS were assessed using Kaplan-Meier analysis the p-values of which are shown in table 3.9. No significant associations were demonstrated.

Table 3.9:Association between marker expression in HRPC specimens and TTR, TTDFR and OS.

Markers	Time To Relapse Kaplan-Meier P- values	Time To Death From Relapse Kaplan-Meier P- values	Overall Survival Kaplan-Meier P- values
HRG Cytoplasm	0.529	0.722	0.990
HRG Membrane	0.977	0.834	0.906
HRG Nucleus	0.997	0.802	0.943
KI67	0.665	0.086	0.425
TUNEL	0.417	0.106	0.219

3.5 COMPARISON OF PRE AND POST HORMONE ESCAPE EXPRESSION

Differences in pre and post hormone escape marker expression were assessed first by analysing the cohort as a whole then by determining the changes in individual sets of paired samples.

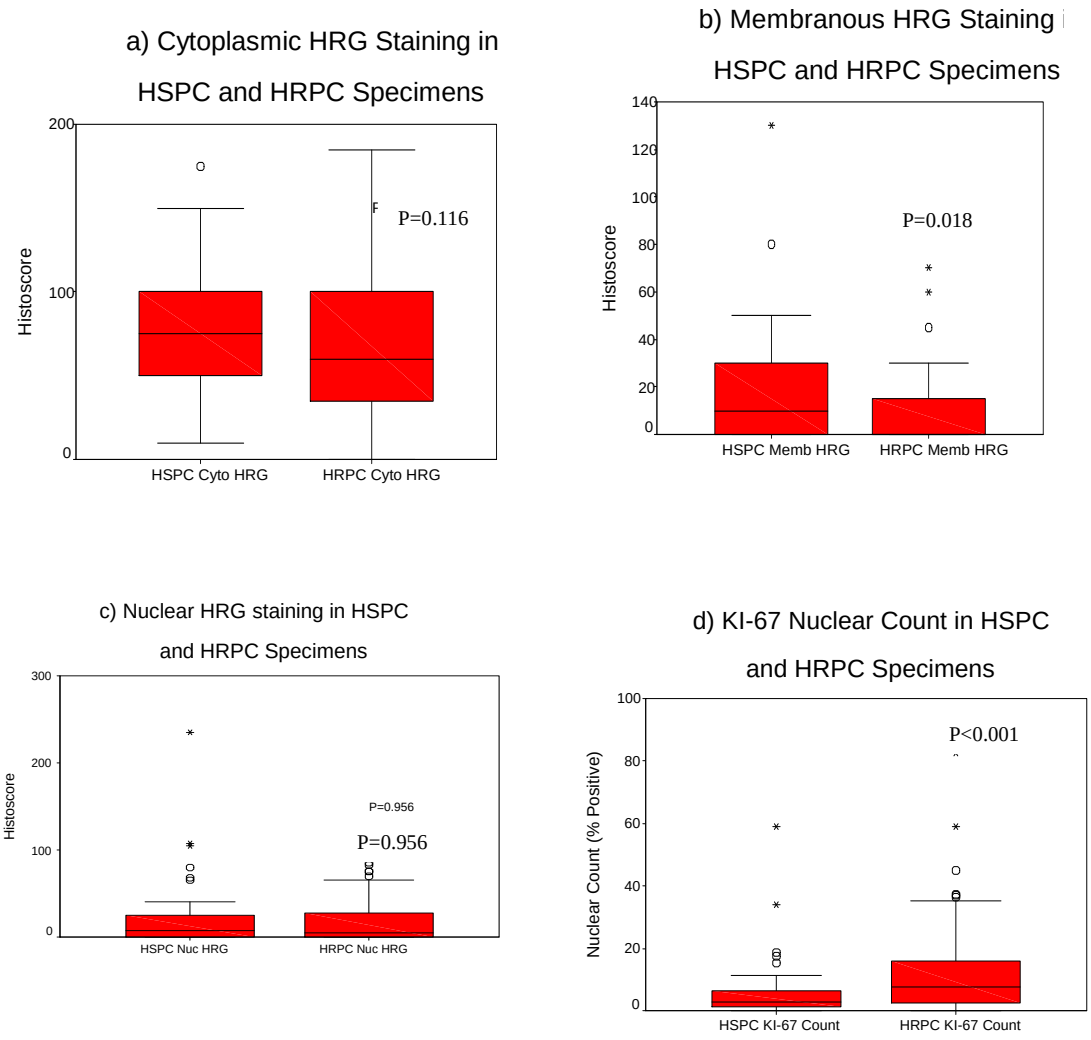
3.5.1 COMPARISON OF PRE AND POST HORMONE ESCAPE MARKER EXPRESSION IN FULL COHORT

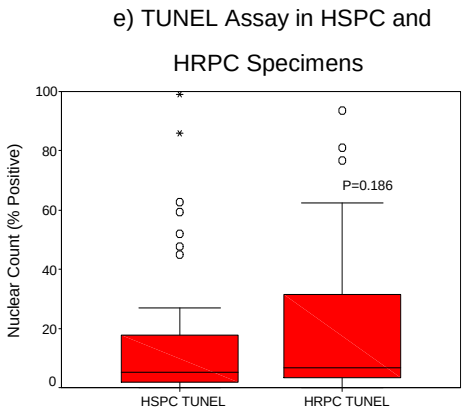
The mean histoscores/nuclear counts for pre and post hormone escape specimens in the 3 markers not in the pilot study HRG, KI-67 and TUNEL Assay were analysed to assess any significant rise or fall in expression in the cohort as a whole following hormone escape. Due to limited slide numbers remaining none of the markers were available in all 81 paired sets of pre and post hormone escape tissue. Paired stained samples were available for 45 patients in the case of Heregulin, 59 for KI67 and 45 for TUNEL. The Wilcoxen Signed Rank Test was used to determine if there had been a significant overall change in expression for each marker. HRG membrane staining was found to have fallen significantly ($p=0.012$) in HRPC tissue samples compared to HSPC conversely KI67 nuclear staining was significantly raised in post hormone escape tissue ($p<0.001$).

Table 3.10: Histoscores in Hormone Sensitive and Hormone Resistant Tumours for Study Markers not included in the Pilot Study.

Marker	HSPC	HRPC	Wilcoxen p-value
HRG Cytoplasm	75 (50–100)	60 (35-100)	0.116
HRG Membrane	10 (0–30)	0 (0 – 15)	**0.018**
HRG Nucleus	7.5 (0-25)	5 (0-27.5)	0.956
KI67	2.9% (1.2-6.4)	7.7% (2.5-15.9)	**<0.001**
TUNEL Assay	5.13% (2.0-17.7)	6.6% (3.4-31.4)	0.186

Figure 3.7: Variation in Overall Cohort Histoscore/Nuclear Count between HSPC and HRPC Sections





a) Box plot of HRG Cytoplasmic staining in pre and post hormone escape samples.
b) Box plot of HRG Membranous staining in pre and post hormone escape samples.
c) Box plot of HRG Nuclear staining in pre and post hormone escape samples.d) Box plot of KI-67 Nuclear Count in pre and post hormone escape samples. e) Box plot of TUNEL Assay Nuclear Count in pre and post hormone escape samples. a-e significance p-value determined using Wilcoxen Signed Rank Test

3.5.2 CHANGES IN EXPRESSION IN INDIVIDUAL PAIRED SAMPLES

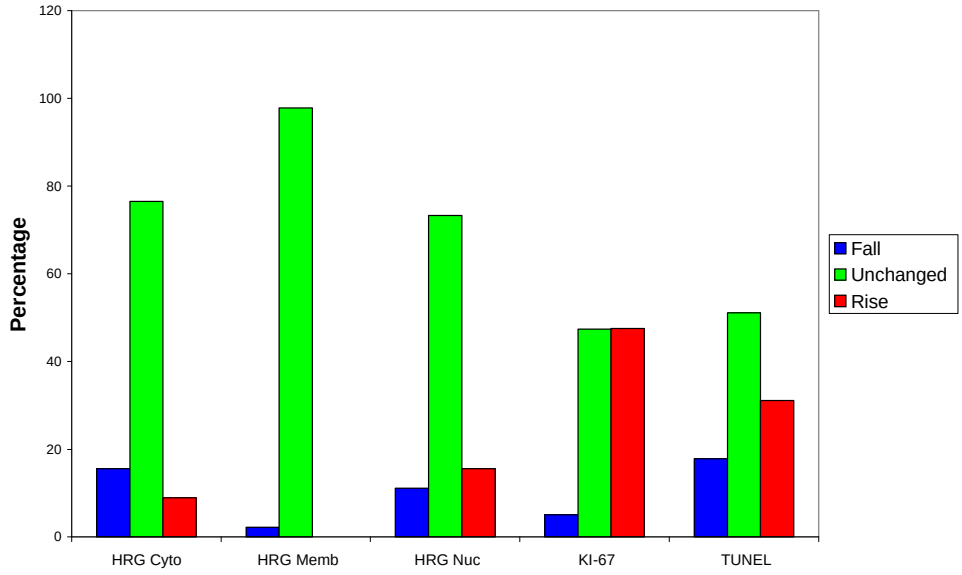
The cohort subjects were then subdivided according to whether there had been a significant rise or fall in expression, i.e. a change of greater than 2 times standard deviation of the ICCC, between the hormone sensitive and resistant samples in individual patients. Small changes in protein expression between paired ASPC and AIPC tumours could be due to random errors in the assessment of histoscores. To identify individual patients in whom there was strong evidence of a genuine rise or fall in protein expression, it was required that the change in expression exceed a threshold equal to two standard deviations of the inter-observer difference for that protein. This threshold was chosen because, if there was in reality no difference in protein expression between ASPC and AIPC tumours in a given patient, there would be only a 5% probability of an apparent difference being observed that exceeded the threshold due to random variation. This assumes that the random variation between two different observers assessing the same tumour is of a similar magnitude to the random variation that would affect a single

observer assessing two different tumours with the same level of protein expression. Changes in protein expression in individual patients that exceeded this threshold were termed significant.

Table 3.11: Subgroubs of Cohort 1 demonstrating significant change in Marker expression between HSPC and HRPC

Marker	2×SD ICCC	%Fall	%Unchanged	%Rise
HRG Cytoplasm	57.2	15.6%	76.5%	8.9%
HRG Membrane	46.9	2.2%	97.8%	0%
HRG Nucleus	32.2	11.1%	73.3%	15.6%
KI67	3.47	5.1%	47.4%	47.5%
TUNEL Assay	13.5	17.8%	51.1%	31.1%

Figure 3.8: Histogram Showing Proportions of Cohort Demonstrating Significant Difference in Marker Expression Between HSPC and HRPC Samples

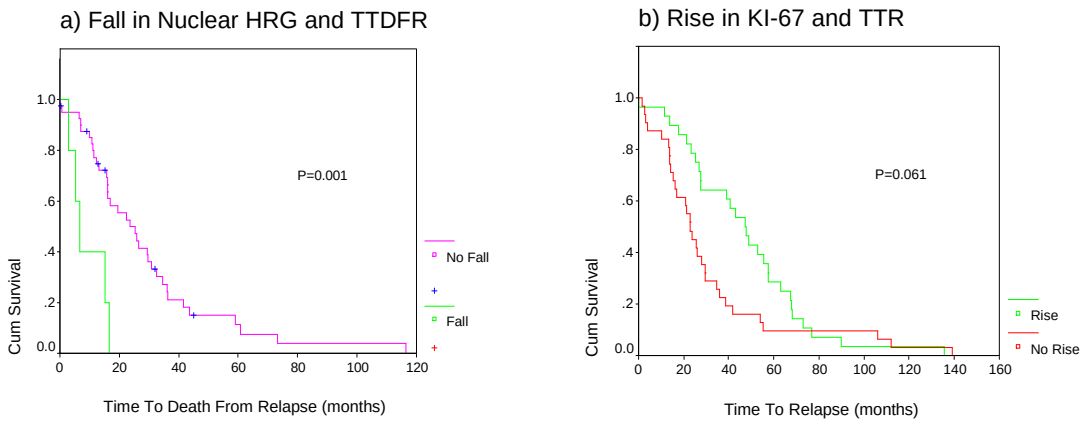


3.5.3 IMPACT OF DIFFERENTIAL EXPRESSION IN PRE AND POST HORMONE ESCAPE SAMPLES ON TIME TO RELAPSE AND SURVIVAL

Using Kaplan-Meier analysis the impact of rise or fall in staining following hormone escape in individual paired samples on time to relapse, time to death from relapse and overall survival were investigated. Analyses were carried out comparing these outcomes in patients with a significant rise in expression between pre and post hormone escape samples to those with no rise (i.e. no change or a fall in expression) and separately comparing those with a significant fall in expression between pre and post hormone expression samples to those with no change or a rise. No correlation was seen between rise or fall in HRG staining after hormone escape at the cytoplasm or membrane and time to relapse, time to death from relapse and overall survival. A significant fall in HRG nuclear staining between HSPC and HRPC samples was associated with a reduced time to death following relapse (P=0.001) but this did not translate to reduced overall survival. Neither time to relapse nor overall survival were associated with changes in nuclear HRG staining.

A rise in KI67 expression following hormone escape was associated with increased time to relapse but this did not achieve significance (P=0.061). Neither time to death following relapse nor overall survival were associated with KI67 rise or fall. There was no association between changes in TUNEL Assay and any of the outcome measures.

Figure 3.9: Changes in Pre and Post Hormone Escape Marker Expression that demonstrated significant impact on TTR, TTDFR or OS



a) Kaplan-Meier Graph showing Time To Death From Relapse Comparing Paired Samples with a significant fall in Nuclear HRG Expression following Hormone Escape to those with no change or a rise. b) Kaplan-Meier Graph showing Time To Biochemical Relapse Comparing Paired Samples with a significant Rise in KI67 Expression following Hormone Escape to those with no change or a fall

Table 3.12: Impact of changes in HRG, KI67 and TUNEL expression between Paired HSPC and HRPC samples on Time to Hormone Relapse (TTR)

Marker	Rise/Fall	Median TTR (mths)	P-value (Kaplan-Meier)
HRG Cytoplasm	Risers	51.3 (30.3-73.5)	0.518
	Non-Risers	30.26 (21.1-55.5)	
	Fallers	26.9 (17.1-47.5)	0.608
	Non-Fallers	32.7 (27.5-56.9)	
HRG Membrane	Risers	N/A	N/A
	Non-Risers	31.5 (21.1-57.4)	
	Fallers	77.0 (N/A)	0.367
	Non-Fallers	31.2 (21.1-60.0)	
HRG Nucleus	Risers	28.3 (20.2-50.7	0.822
	Non-Risers	31.5 (19.5-56.4)	
	Fallers	22.3 (16.8-47.1)	0.834
	Non-Fallers	31.5 (22.8-57,4)	
KI67	Risers	47.4 (26.5-64.1)	**0.061**
	Non-Risers	23.0 (14.0-35.1)	
	Fallers	22.8 (18.1-38.4)	0.414
	Non-Fallers	28.9 (17.6-55.3)	
TUNEL	Risers	32.1 (22.7-60.3)	0.733
	Non-Risers	27.4 (16.5-55.3)	
	Fallers	41.0 (23.0-49.7)	0.457
	Non-Fallers	27.5 (16.8-57.4)	

Median and interquartile ranges of changes in protein expression comparing risers to non-risers and fallers to non-fallers. P-values were calculated using Kaplan-Meier analysis.

Table 3.13: Impact of changes in HRG, KI67 and TUNEL expression between Paired HSPC and HRPC samples on Time To Death From Relapse (TTDFR)

Marker	Rise/Fall	Median TTDFR	P-value (Kaplan-Meier)
HRG Cytoplasm	Risers	11.1 (8.3-18.9)	0.266
	Non-Risers	16.4 (11.0-32.1)	
	Fallers	32.6 (15.9-39.1)	0.441
	Non-Fallers	16.0 (10.2-29.6)	
HRG Membrane	Risers	N/A	N/A
	Non-Risers	16.1 (10.9-32.1)	
	Fallers	8.9 (N/A)	0.653
	Non-Fallers	16.3 (11.0-32.2)	
HRG Nucleus	Risers	13.0 (6.0-38.7)	0.816
	Non-Risers	16.4 (10.9-30.2)	
	Fallers	6.7 (5.2-15.2)	**0.001**
	Non-Fallers	21.0 (11.9-33.1)	
KI67	Risers	16.4 (8.3-27.2)	0.118
	Non-Risers	24.3 (15.6-41.5)	
	Fallers	26.2 (21.1-43.4)	0.858
	Non-Fallers	19.2 (10.6-33.1)	
TUNEL	Risers	16.8 (9.2-36.3)	0.308
	Non-Risers	23.4 (15.1-33.3)	
	Fallers	28.0 (16.0-31.7)	0.821
	Non-Fallers	19.8 (12.9-36.4)	

Median and interquartile ranges of changes in protein expression comparing risers to non-risers and fallers to non-fallers. P-values were calculated using Kaplan-Meier analysis.

Table 3.14: Impact of changes in HRG, KI67 and TUNEL expression between Paired HSPC and HRPC samples on Overall Survival (OS)

Marker	Rise/Fall	Median OS (Mths)	P-value (Kaplan-Meier)
HRG Cytoplasm	Risers	77.4 (64.3-81.8)	0.758
	Non-Risers	59.1 (40.4-83.4)	
	Fallers	75.2 (40.3-86.0)	0.761
	Non-Fallers	61.8 (40.6-82.5)	
HRG Membrane	Risers	N/A	N/A
	Non-Risers	64.5 (40.4-83.4)	
	Fallers	85.9 (N/A)	0.228
	Non-Fallers	61.8 (40.4-82.8)	
HRG Nucleus	Risers	76.0 (32.1-88.8)	0.972
	Non-Risers	59.1 (40.3-82.3)	
	Fallers	32.0 (29.0-52.3)	0.234
	Non-Fallers	65.3 (41.0-83.9)	
KI67	Risers	75.5 (45.6-86.2)	0.368
	Non-Risers	49.8 (32.2-80.7)	
	Fallers	69.9 (54.8-76.6)	0.578
	Non-Fallers	57.0 (39.3-85.6)	
TUNEL	Risers	68.7 (46.3-81.7)	0.445
	Non-Risers	54.9 (38.4-85.0)	
	Fallers	63.7 (45.5-79.3)	0.879
	Non-Fallers	64.5 (40.5-86.0)	

Median and interquartile ranges of changes in protein expression comparing risers to non-risers and fallers to non-fallers. P-values were calculated using Kaplan-Meier analysis.

CHAPTER 4: RESULTS – COHORT 2

4.1 PATIENTS

This patient cohort includes the HSPC tumours from 81 patients from cohort 1 supplemented by 276 patients from 4 TMAs totalling 357 patients. Median age was 70.7 years (range 39.0 – 103.4 years) mean age was 70.4 years (SD ± 9.2). At diagnosis 65 (18.2%) of tumours were Stage T1, 53 (14.8%) T2, 86 (24.1%) T3, 30 (8.4%) T4 with the remainder of unknown/unrecorded stage. Gleason scores in HSPC specimens were 1-4 in 4 (1.1%), 5-7 in 206 (57.7%), 8-10 in 94 (26.3%) and not recorded in the remainder. 74 patients (20.7%) had known metastatic disease at diagnosis, 187 (52.4%) had no metastases with the metastatic status of the remainder unknown.

Patients within the cohort underwent a variety of treatment modalities. 227 patients (63.3%) including all those from the pilot study cohort underwent hormone therapy – antiandrogens, GnRH analogues, maximal androgen blockade or bilateral orchidectomy. At least 45 (12.6%) underwent radical retropubic prostatectomy. Over the recorded course of their disease 194 patients (54.3%) suffered biochemical relapse as defined above, 64 (17.9%) had no relapse and the relapse status was not recorded in 99 patients (27.7%). Mean time to relapse was 35.1 months (SD ± 32.3). At last known follow up 213 patients (59.7%) patients were deceased, 110 patients (31.9%) were alive with the status of the remainder unclear. Mean time to death/last follow up was 69.8 months (SD ± 54.0).

4.1.1 PATIENT DATA FOR COHORT 2

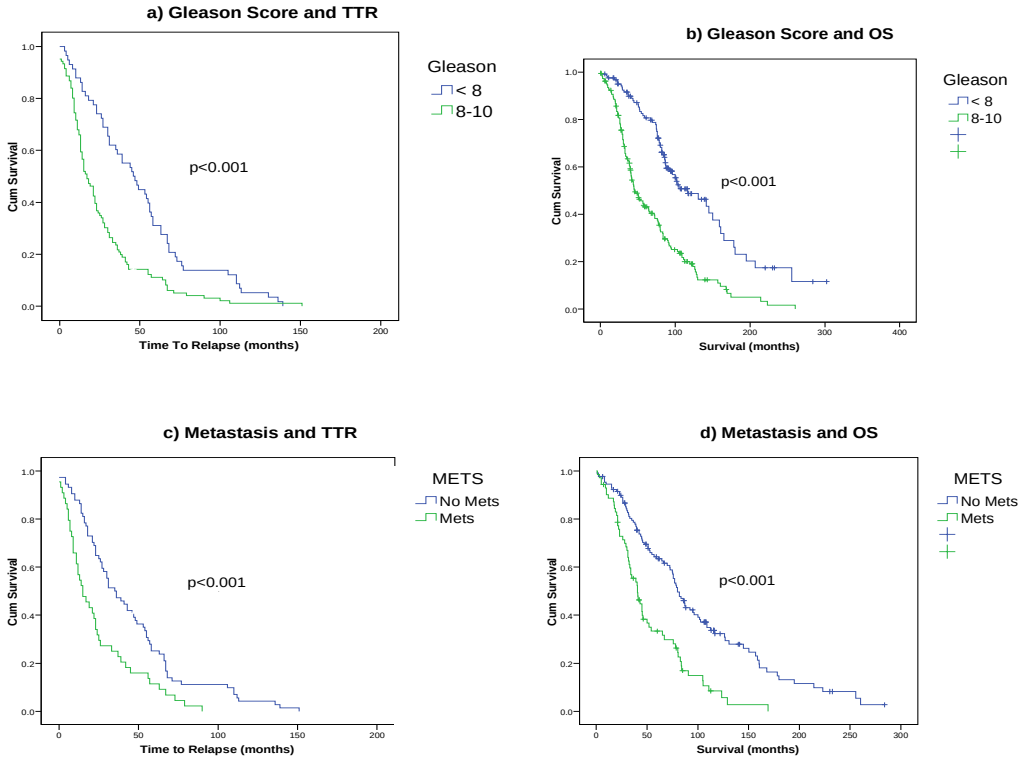
Table 4.1: Summary Statistics for All Cohort 2 Patients with Hormone Treated Subgroup

	HORMONE TREATED PATIENTS	ALL PATIENTS
N	227	357
Age	71.4 ± 8.6 (41.1 – 103.4)	70.4 ± 9.2 (39.0 – 103.4)
Gleason Score ▪ Low (2-4) ▪ Medium (5-7) ▪ High (8-10)	 4 116 69	 4 206 94
T Stage at Diagnosis ▪ T1 ▪ T2 ▪ T3 ▪ T4	 34 26 31 25	 65 54 86 30
Metastasis at Diagnosis ▪ Yes ▪ No ▪ Unknown	 57 110 60	 74 187 96
Biochemical Relapse ▪ Yes ▪ No ▪ Unknown	 182 36 12	 194 64 99
Time to Relapse	34.1 ± 32.1	35.1 months ± 32.3
Final Status ▪ Alive ▪ Deceased ▪ Unknown	 43 167 17	 110 213 34
Follow up	73.2 months ± 58.6	69.8 months ± 54.0

4.1.2 CORRELATION OF GLEASON SCORE AND METASTASIS WITH STUDY OUTCOMES IN COHORT 2

In this cohort High Gleason score (8-10) was associated with reduced time to biochemical relapse (P<0.001) and overall survival (P<0.001). Metastasis at presentation was associated with reduced time to relapse (P<0.001) and survival (P<0.001). These values are in accordance with known prostate cancer natural history and thus help validate the database.

Figure 4.1: Correlation of Gleason Score and Metastasis at Presentation with Time To Relapse and Overall Survival



a) Kaplan Meier Plot of Full Patient Cohort Comparing Patients with High Gleason Score (8-10) and those with Low-Medium (2-7) for Outcome Time to Biochemical Relapse. b) Kaplan Meier Plot of Full Patient Cohort Comparing Patients with High Gleason Score (8-10) and those with Low-Medium Score (2-7) for Outcome Overall Survival. c) Kaplan Meier Plot of Full Patient Cohort Comparing Patients with Distant Metastasis and No Metastasis for Outcome Time to Biochemical Relapse. d) Kaplan-Meier Plot of Full Patient Cohort Comparing Patients with Distant Metastasis and No Metastasis for Outcome Overall Survival

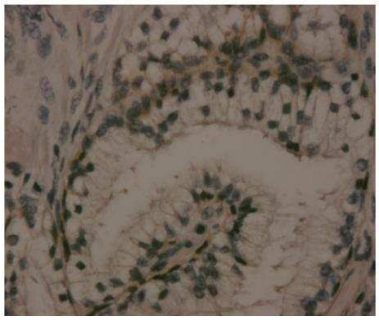
4.2 IMMUNOHISTOCHEMICAL EXPRESSION AND INTER-OBSERVER CORRELATIONS IN TISSUE MICROARRAYS

Staining of the TMAs revealed similar patterns to the single sample slides of the pilot group. EGFR staining was visible at the membrane and cytoplasm, HER2 at the membrane only, HER3 and HER4 at cytoplasm and membrane. Manufacture of EGFRvIII antibody had been discontinued at the time of this therefore we were unable to stain the additional samples for this antigen.. Heregulin staining was found predominantly in the cytoplasm but both nuclear and membrane staining were observed. As before KI67 and the TUNEL Assay are predominantly nuclear. The IHC and Inter-observer scoring graphs for EGFR are shown here as an example, the remainder are listed in appendix 2.

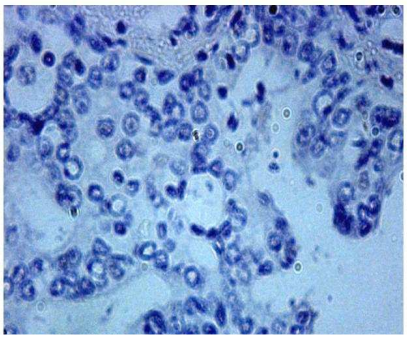
4.2.1 EGFR

As in the pilot specimens, relatively little EGFR staining was seen in the TMAs compared to HER3 and HER4. Both cytoplasmic and membranous staining was seen.

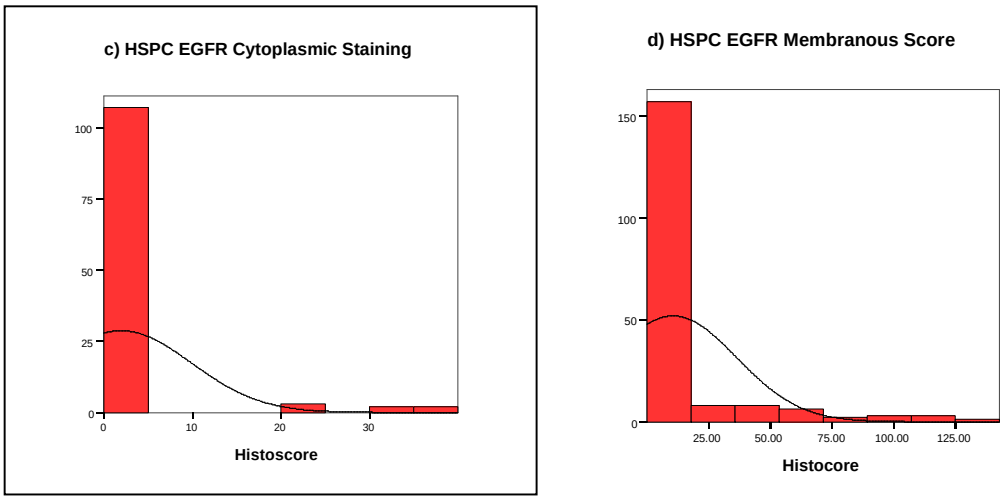
Figure 4.2: Immunohistochemistry of EGFR.



a) EGFR in Prostate Cancer



b) Negative Control for EGFR



a) Specimen of Prostate Adenocarcinoma demonstrating EGFR staining b) Negative control for EGFR staining c) Histogram showing intensity of EGFR cytoplasmic expression. d) Histogram showing intensity of EGFR membranous expression

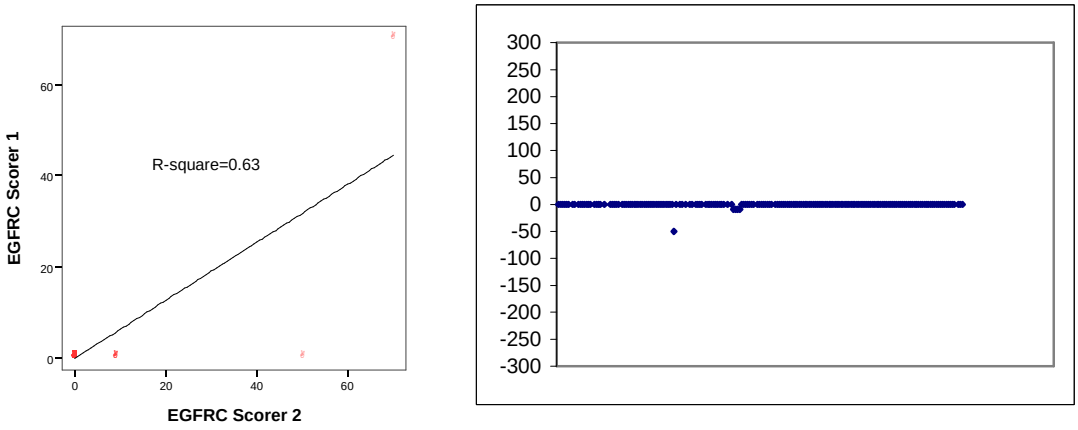
All TMA specimens were double scored for EGFR. ICCCs were 0.77 for cytoplasmic

EGFR and 0.99 for membranous EGFR

Figure 4.3: Inter-Observer Variation in Heregulin Staining between double scored tissue sections in Tissue Microarrays

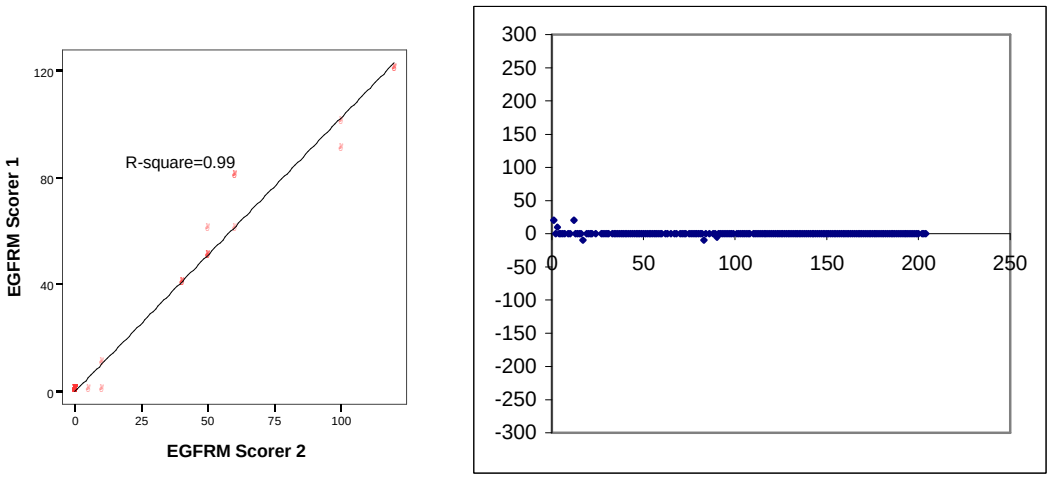
a) EGFR Cytoplasmic Staining

b) Inter-observer Difference Cytoplasmic EGFR



c) EGFR Membranous Staining

d) Inter-observer Difference Membranous EGFR



a) Scatter Graph Plot demonstrating Inter-Observer Variation in Cytoplasmic EGFR Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Cytoplasmic EGFR Staining. c) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous EGFR Staining. d) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous EGFR Staining.

4.2.2 HER2

As in the pilot only membranous staining of HER2 was observed and little of this was present in comparison to HER3 and HER4. All TMA specimens were double scored for HER2. ICCCs was 0.99 for membranous HER2.

4.2.3 HER3

Both membranous and cytoplasmic HER3 staining were observed on TMAs. No nuclear staining was observed. All TMA specimens were double scored for HER3. ICCCs were 0.99 for cytoplasmic HER3 and 0.93 for membranous HER3

4.2.4 HER4

Both membranous and cytoplasmic HER3 staining were observed on TMAs. No nuclear staining was observed. All TMA specimens were double scored for HER4. ICCCs were 0.90 for cytoplasmic HER3 and 0.96 for membranous HER4

4.2.5 HEREGULIN

As in the pilot study specimens stained for section 3 cytoplasmic, membranous and nuclear staining were seen in the TMAs with cytoplasmic most frequent and at greatest levels. 10% of TMA samples were double scored for HRG with ICCC >0.7 in each case taken as confirming the accuracy of the single scored specimens.

4.2.6 KI-67

KI-67 was expressed only in the nucleus with no membranous or cytoplasmic expression. Positive and negatively stained nuclei were clearly distinguishable allowing assessment via nuclear counting method. All tissue sections and TMAs stained for KI67 were scored as a whole by one single observer with 10% specimens stained for KI67 double scored by an independent observer to confirm accuracy of the first observer. All the double scored specimens were single tissue sections. ICCC score for double scored KI67 sections was 0.95 confirming the accuracy of the first observer and reflecting the less subjective nature of nuclear counting compared to weighted histoscore.

4.2.7 TUNEL ASSAY

As described in the ApopTag® instruction manual (Chemicon) the TUNEL assay primarily causes staining only in the nucleus although there was some minor non-specific

background staining. Positive and negatively stained nuclei were clearly distinguishable allowing assessment via nuclear counting method.

All TUNEL Assay stained tissue sections and TMAs were viewed as a single group and scored by one observer with 10% double scored by an independent observer to ensure accuracy. All the double scored specimens for TUNEL assay were on the TMAs rather than tissue sections. Double scoring demonstrated an ICCC of 0.95

Table 4.2: Summary of Inter-Class Correlation Coefficients

Marker	2 Standard Deviations	Inter class Correlation Coefficient	Pearson Coefficient
EGFR Cytoplasm	7.7	0.78	0.80
EGFR Membrane	4.9	0.99	0.99
HER2 Membrane	3.0	0.99	0.999
HER3 Cytoplasm	10.5	0.99	0.996
HER3 Membrane	31.9	0.93	0.93
HER4 Cytoplasm	19.4	0.96	0.96
HER4 Membrane	34.5	0.90	0.90
HRG Cytoplasm	46.4	0.75	0.80
HRG Membrane	46.9	0.83	0.83
HRG Nucleus	12.5	0.72	0.75
KI67	10.2	0.95	0.96
TUNEL Assay	13.5	0.95	0.95

4.3 CORRELATION OF MARKER EXPRESSION WITH GLEASON SCORE AND METASTASIS

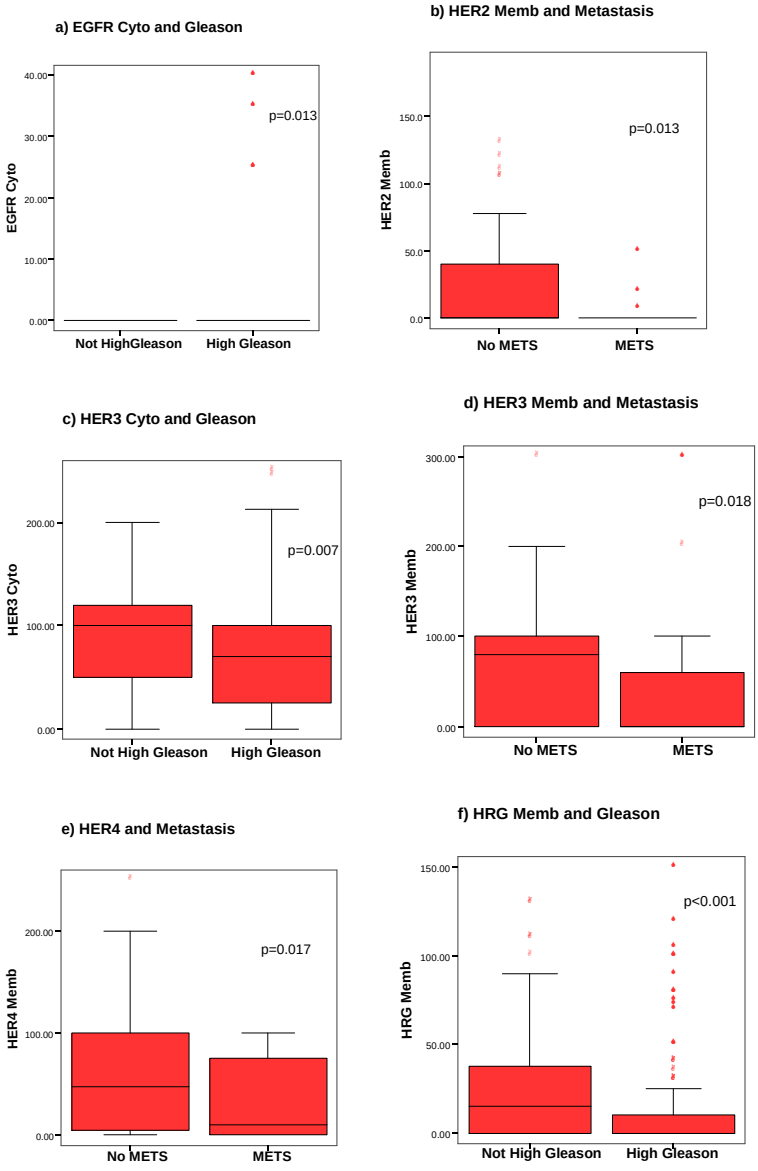
As with cohort 1 Mann-Whitney analyses were used to discern any association between expression of markers used in the study and high Gleason score (8-10)/metastasis at presentation in the full cohort 2. In this cohort high cytoplasmic HER3 and membranous HRG are associated with lower Gleason score. A statistically significant association is also seen with cytoplasmic EGFR but as there is very little positive staining of cytoplasmic EGFR the significance might not be a true observation. High KI67 is associated with high Gleason score.

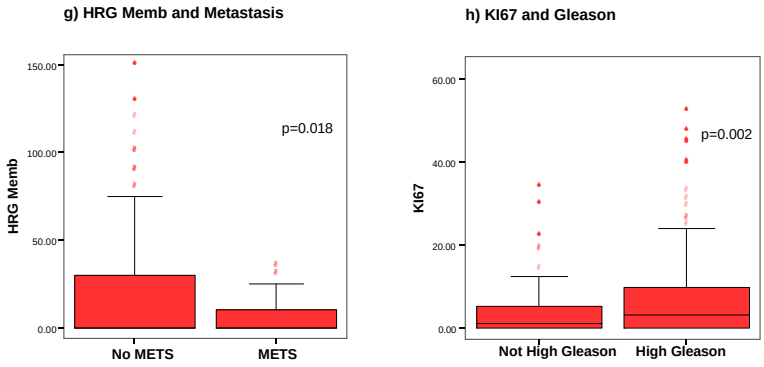
Membranous HER3, HER4 and HRG are all associated with reduced occurrence of metastasis at presentation. Higher TUNEL assay score is associated with increased rate of metastasis but this does not quite reach statistical significance ($p=0.051$).

Table 4.3: Associations between Marker Expression and Gleason Score/Metastasis at Presentation

Marker/Location	High Gleason Score Mann-Whitney P-value	Metastasis Mann-Whitney P-Value
EGFR – Cytoplasm	**0.013***	0.829
EGFR – Membrane	0.226	0.494
EGFR variant III	0.683	0.381
HER2 (Herceptest)	0.431	**0.013**
HER3 – Cytoplasm	**0.007**	0.147
HER3 – Membrane	0.381	**0.018**
HER3 – Nucleus	0.059	0.218
HER4 – Cytoplasm	0.608	0.926
HER4 – Membrane	0.430	**0.017**
HER4 – Nucleus	0.147	0.200
HRG – Cytoplasm	0.249	0.095
HRG – Membrane	**<0.001**	**0.018**
HRG – Nucleus	0.591	0.864
KI67 Count	**0.002**	0.333
TUNEL Assay	0.793	0.051

Figure 4.4: Boxplots of Associations between Marker Expression and High Gleason Score/Metastasis that indicated significance





a) Cytoplasmic EGFR and Gleason, b) Membranous HER2 and Metastasis, c) Cytoplasmic HER3 and Gleason, d) Membranous HER3 and Metastasis, e) Membranous HER4 and Metastasis, f) Membranous HRG and Gleason, g) Membranous HRG and Metastasis, h) KI67 and Gleason

4.4 IMPACT OF MARKER EXPRESSION IN PATIENT SUBCOHORT TREATED WITH ANDROGEN DEPRIVATION THERAPY

4.4.1 IMPACT OF SINGLE MARKER VALUES ON TIME TO RELAPSE AND SURVIVAL

Using a sub-cohort of all patients who had been treated with ADT as previously defined, Kaplan-Meier analyses were conducted to test if an associated with TTR or OS and expression of any of the markers studied was observed. Expression was divided into those with high and low expression (divided by the median) and those with very high expression (divided by the 3rd quartile).

Upper quartile (very high) membrane EGFR expression correlated with increased time to relapse (P=0.02) as did above the median (high) HER2 (P=0.02). Upper quartile (P=0.002) HER2, upper quartile HER4 (P=0.009), above the median (P=0.033) and upper quartile membrane (P=0.004) HRG and upper quartile nuclear HRG (P=0.005) were all associated with increased time to relapse. Upper quartile EGFRvIII expression was associated with reduced time to relapse (P=0.027).

Upper quartile EGFR membrane expression was correlated with increased overall survival (P=0.012) as were upper quartile HER2 (P=0.025), membrane HER4 (P=0.009) and membrane HRG (P=0.044). Above the median EGFRvIII expression was associated with reduced overall survival but this did not achieve significance (P=0.063) probably due to the unavailability of the antibody and consequent inability to expand the stained cohort.

To determine hazard ratios for all markers with a statistically significant influence COX regression analysis was performed for all markers with $p<0.05$ on Kaplan-Meier

analysis. Additionally a multivariate backwards: conditional COX analysis utilising Gleason score and Metastasis at presentation was performed for each of these to determine if they were independently significant.

Table 4.4: P-values for Kaplan-Meier Analyses of Time To Biochemical Relapse and Overall Survival for all Markers in HSPC samples from patients treated with ADT comparing those with high and low expression (divided by median)

Protein/Location	Time To Relapse Kaplan Meier P-Value	Overall Survival Kaplan Meier P-Value
EGFR – Cytoplasm (High)	0.548	0.407
EGFR – Membrane (High)	0.145	0.064
EGFR variant III (High)	0.998	0.962
HER2 (Herceptest) (High)	**0.02**	0.072
HER3 – Cytoplasm (High)	0.12	0.106
HER3 – Membrane (High)	0.282	0.47
HER3 – Nucleus (High)	0.8	0.783
HER4 – Cytoplasm (High)	0.986	0.361
HER4 – Membrane (High)	0.706	0.318
HER4 – Nucleus (High)	0.497	0.217
HRG – Cytoplasm (High)	0.519	0.679
HRG – Membrane (High)	**0.033**	0.858
HRG – Nucleus (High)	0.489	0.86
KI67 Count (High)	0.937	0.8334
TUNEL Assay (High)	0.295	0.227

Table 4.5: P-values for Kaplan-Meier Analyses of Time To Relapse and Overall Survival for all Markers in HSPC samples from patients treated with ADT comparing those with very high expression and those without (divided by third quartile)

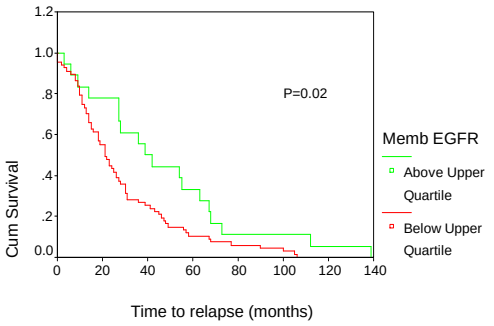
Protein/Location	Time To Relapse Kaplan Meier P-Value	Overall Survival Kaplan Meier P-Value
EGFR – Cytoplasm (Very High)	0.548	0.407
EGFR – Membrane (Very High)	**0.02**	**0.012**
EGFR variant III (Very High)	**0.027**	0.063
HER2 (Herceptest) (Very High)	**0.002**	**0.025**
HER3 – Cytoplasm (Very High)	0.843	0.779
HER3 – Membrane (Very High)	0.797	0.574
HER3 – Nucleus (Very High)	0.898	0.491
HER4 – Cytoplasm (Very High)	0.993	0.604
HER4 – Membrane (Very High)	**0.009**	**0.009**
HER4 – Nucleus (Very High)	0.57	0.916
HRG – Cytoplasm (Very High)	0.115	0.235
HRG – Membrane (Very High)	**0.004**	**0.044**
HRG – Nucleus (Very High)	**0.005**	0.218
KI67 Count (Very High)	0.995	0.728
TUNEL Assay (Very High)	0.944	0.526

It can be noted at this point that the upper quartile cut off gives a greater number of significant results both for TTR and OS indicating a gradation of increase likelihood of influence with increased expression of a given marker. It can also be seen that a greater

number of significant results are seen for the outcome TTR with only a portion of these translating into a significant effect on OS.

Figure 4.5: Correlations of Marker Expression with Time To Biochemical Relapse that show statistical significance in the Patient Subcohort treated with Androgen Deprivation Therapy

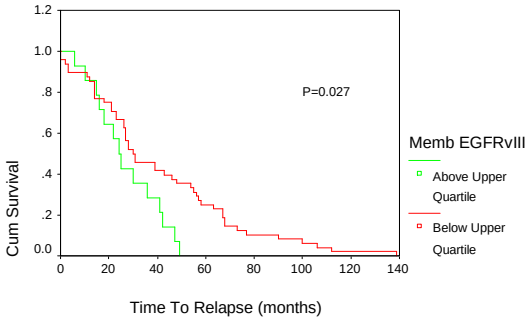
a) Memb EGFR (upper quartile) and TTR



COX Regression Hazard Ratio: 1.84 (1.05-3.20) p=0.03

Multivariate COX Regression: N/A

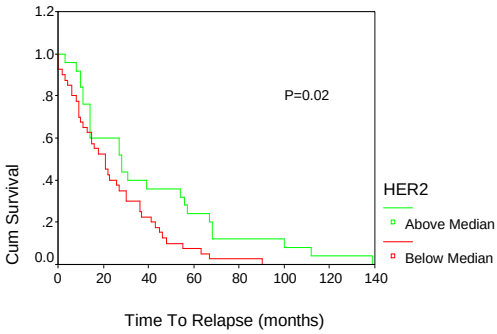
b) Memb EGFRvIII (upper quartile) and TTR



COX Regression Hazard Ratio: 0.46 (0.24-0.88) p=0.18

Multivariate COX Regression: N/A

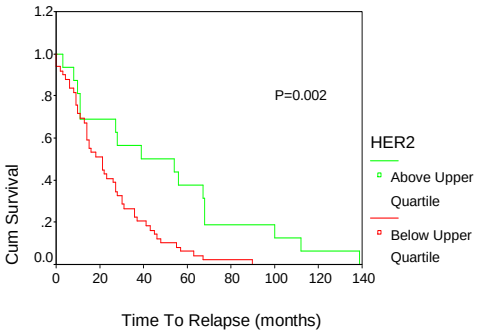
c) HER2 (Median) and TTR



COX Regression Hazard Ratio: 1.75 (1.01-3.02) p=0.044

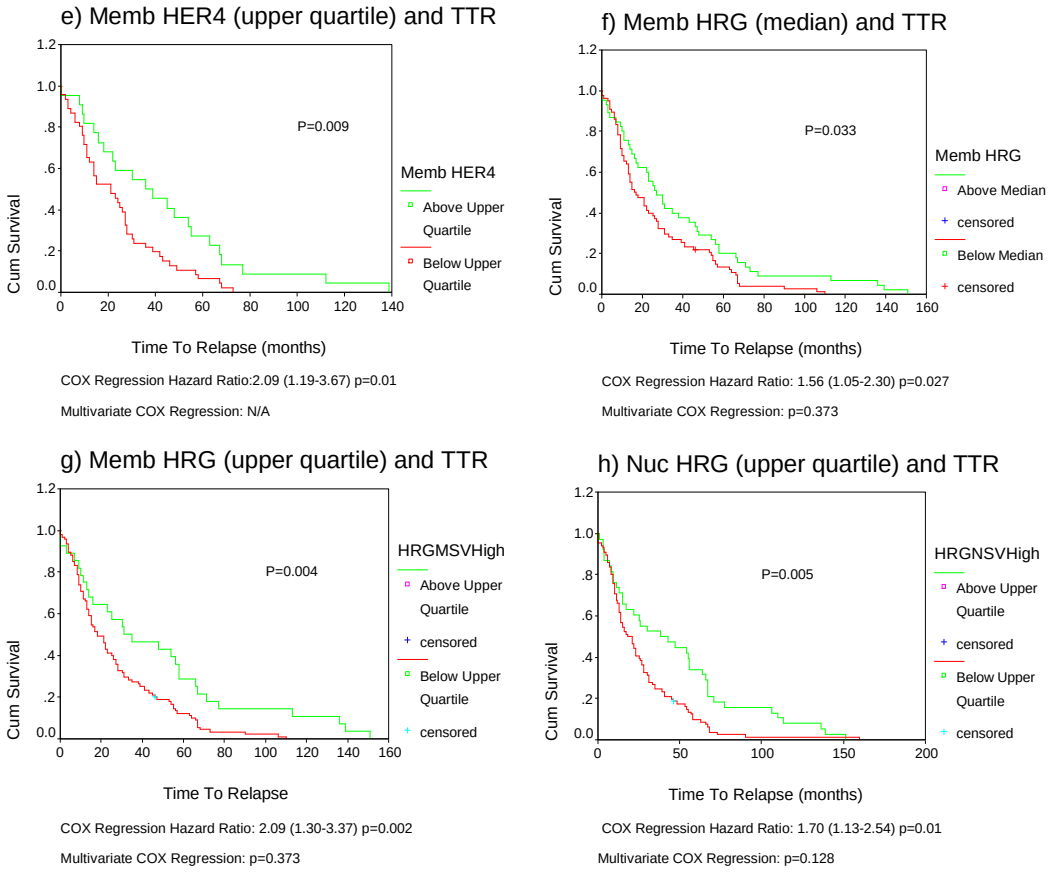
Multifactorial COX Regression: p=0.645

d) HER2 (upper quartile) and TTR



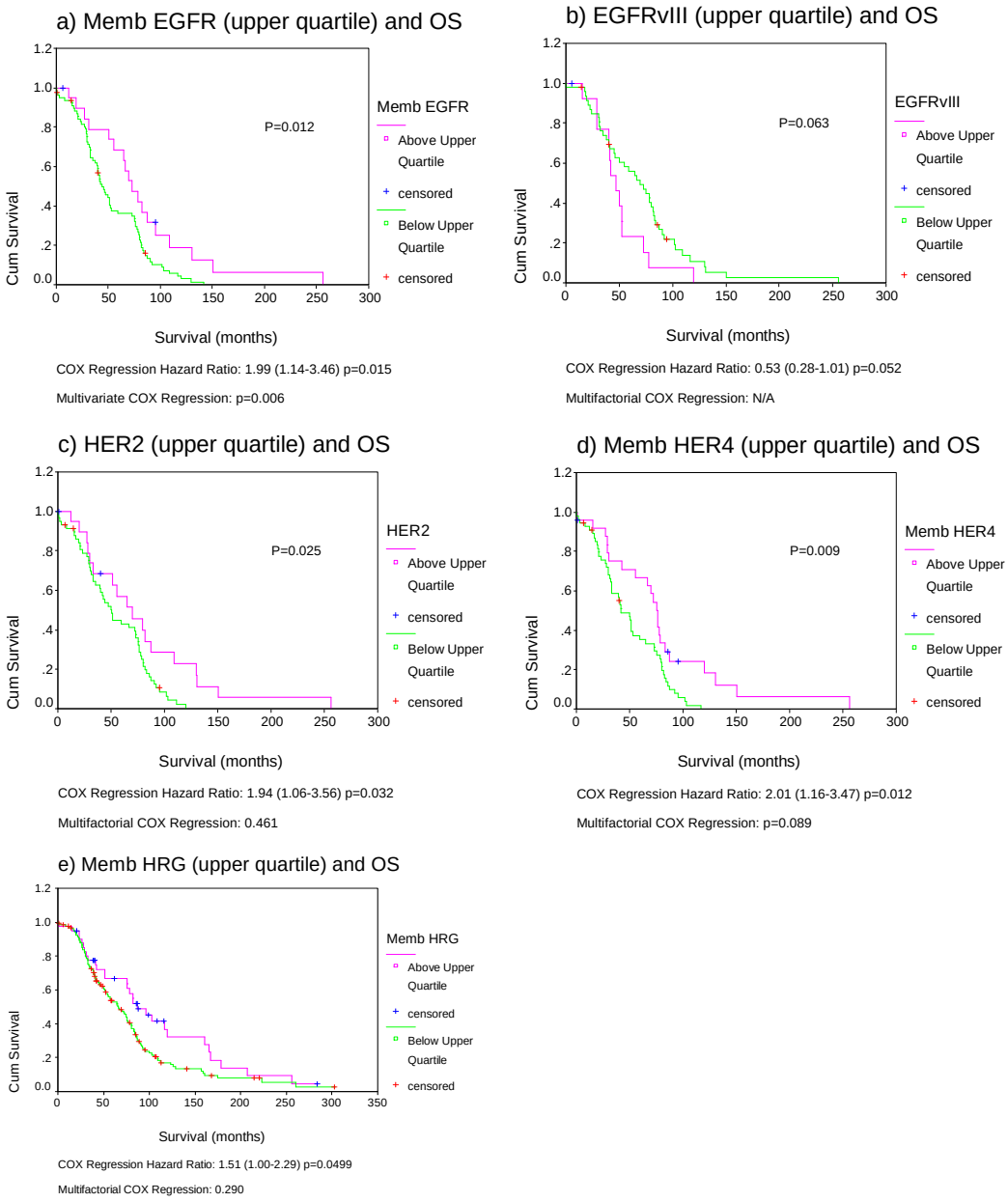
COX Regression Hazard Ratio: 2.53 (1.32-4.86) p=0.005

Multivariate COX Regression: p= 0.645



a) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of EGFR and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse. b) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of EGFRvIII and those without (divided by median) for outcome Time To Biochemical Relapse. c) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of HER2 and those without (divided by Median) for outcome Time To Biochemical Relapse. d) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of HER2 and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse e) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of HER4 and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse. f) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of HRG and those without (divided by Median) for outcome Time To Biochemical Relapse. g) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of HRG and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse. h) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Nuclear expression of HRG and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse

Figure 4.6: Correlations of Marker Expression with Overall Survival that show statistical significance in the Patient Subcohort treated with Androgen Deprivation Therapy



a) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of EGFR and those without (divided by Upper Quartile) for outcome Overall Survival. b) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of EGFRvIII and those without (divided by Upper Quartile) for outcome Overall Survival. c) Kaplan-Meier Plot comparing HSPC ADT patients with Very High Membrane expression of HER2 and those without (divided by Upper Quartile) for outcome Overall Survival. d) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of HRG and those without (divided by Upper Quartile) for outcome Overall Survival. e) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of HRG and those without (divided by Upper Quartile) for outcome Overall Survival

4.4.2 IMPACT OF MARKER COMBINATIONS ON TIME TO RELAPSE AND SURVIVAL

The impact of high expression (high and low divided by the median) of combinations of markers in the HSPC patient sub cohort subsequently treated with ADT was assessed first by grouping markers in pairs then by more general combinations – ≥1 marker, ≥2 markers, ≥3 markers and 4 markers. For a given marker, if expression had been demonstrated in more than 1 cell site (EGFR, HER3, HER4) markers were grouped with like site with like site (e.g. cytoplasmic EGFR and cytoplasmic HER3, membrane EGFR with membrane HER3). Where expression had been demonstrated in only one site (HER2, EGFRvIII) the marker was matched with all sites (e.g. HER2 with cytoplasmic HER3, HER2 with membrane HER3). The general analyses were performed on both HER1-3 only (given HER1-3 having different prognostic actions in breast cancer) and HER1-4. Analysis was repeated with EGFRvIII included i.e. high score in EGFR or variant III with others and again with HRG high score required in addition to other markers.

Increased time to relapse correlated with a number of HER family high expression combinations; memb EGFR/HER2 (P=0.008), memb EGFR/HER3 (0.03), memb EGFR/HER4 (0.013), HER2/cyto HER3 (0.006), HER2/memb HER3 (0.047), HER2/cyto HER4 (0.017), HER2/memb HER4, all 3 membranous markers from HER1-3 (0.006), all 4 membranous HER1-4 (0.001). If EGFRvIII was included any 1 cytoplasmic maker from HER1-4 (0.022), all membranous HER1-3 (0.021), and membranous (0.003) HER1-4 were correlated with increased time to relapse. If HRG and EGFRvIII were

included high expression of a single cytoplasmic marker was also correlated with increased time to relapse.

Increased overall survival was correlated with high memb EGFR/HER2 (0.012), memb EGFR/HER3 (0.015), memb EGFR/HER4 (0.023), HER2/cyto HER3 (0.019), HER2/memb HER3 (0.019), at least 2 cytoplasmic markers from HER1-3 (0.015), all 3 membranous HER1-3 (0.004), at least 3 cytoplasmic from HER1-4 (0.012), all 4 membranous HER1-4 (0.001). If EGFRvIII was included high expression of at least 2 cytoplasmic markers from HER1-3 (0.037), all 3 membranous (0.005) from HER1-3 and all 4 membranous HER1-4 (0.005) were correlated with increased overall survival.

To determine hazard ratios for all markers with a statistically significant influence COX regression analysis was performed for all markers with $p<0.05$ on Kaplan-Meier analysis. Additionally a multivariate backwards: conditional COX analysis utilising Gleason score and Metastasis at presentation was performed for each of these.

Table 4.6: P-values for Kaplan-Meier Analyses of Time To Relapse in HSPC samples from patients treated with ADT comparing those with high expression in 2 different markers to those without

	EGFRC	EGFRM	EGFR VIII	HER2	HER3C	HER3M	HER4C	HER4M	HRGC	HRGM	HRGN
EGFRC	ND	ND	ND	Nil	Nil	ND	0.078	ND	Nil	ND	ND
EGFRM		ND	ND	**0.008**	ND	**0.03**	ND	**0.013**	ND	0.399	ND
EGFR vIII			ND	0.513	0.908	0.072	0.944	0.879	0.51	0.569	0.385
HER2				ND	**0.006**	**0.047**	**0.017**	**0.046**	0.601	0.222	0.322
HER3C					ND	ND	0.228	ND	0.104	ND	ND
HER3M						ND	ND	0.084	ND	0.171	ND
HER4C							ND	ND	0.327	ND	ND
HER4M								ND	ND	0.958	ND

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Table 4.7: P-values for Kaplan-Meier Analyses of Time To Biochemical Relapse in HSPC samples from patients treated with ADT comparing those with high expression in different combinations of markers to those without

	Any1C	Any1M	Any2C	Any2M	Any3C	Any3M	4M
HER1-3	0.187	0.404	Nil	0.145	N/A	**0.006**	N/A
HER1-4	0.330	0.951	0.346	0.618	**Nil**	0.075	**0.001**
EGFR/vIII +HER2-3	0.062	0.092	0.192	0.098	N/A	**0.021**	N/A
EGFR/vIII +HER2-4	**0.022**	0.326	0.667	0.128	0.112	0.276	**0.003**
HER1-3 +HRG	0.123	0.445	Nil	0.303	N/A	0.076	N/A
HER1-4 +HRG	0.537	0.971	0.488	0.546	Nil	0.363	0.077
EGFR/vIII +HER2-3 +HRG	**0.018**	0.477	0.155	0.302	N/A	0.186	N/A
EGFR/vIII +HER2-4 +HRG	0.098	0.739	0.522	0.592	0.155	0.499	0.183

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Table 4.8: P-values for Kaplan-Meier Analyses of Overall Survival in HSPC samples from patients treated with ADT comparing those with high expression in 2 different markers to those without

	EGFRC	EGFRM	EGFR VIII	HER2	HER3C	HER3M	HER4C	HER4M	HRGC	HRGM	HRGN
EGFRC	ND	ND	ND	Nil	0.475	ND	0.803	ND	Nil	ND	ND
EGFRM		ND	ND	**0.012**	ND	**0.015**	ND	**0.01**	ND	0.29	ND
EGFR vIII			ND	0.288	0.437	0.564	0.341	0.951	0.276	0.183	0.272
HER2				ND	**0.023**	**0.019**	0.195	0.053	0.972	0.252	0.621
HER3C					ND	ND	0.682	ND	0.214	ND	ND
HER3M						ND	ND	0.219	ND	0.4	ND
HER4C							ND	ND	0.383	ND	ND
HER4M								ND	ND	0.539	ND

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

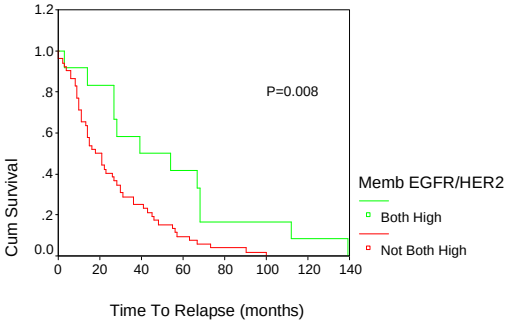
Table 4.9: P-values for Kaplan-Meier Analyses of Overall Survival in HSPC samples from patients treated with ADT comparing those with high expression in different combinations of markers to those without

	Any1C	Any1M	Any2C	Any2M	Any3C	Any3M	4M
HER1-3	0.155	0.365	0.472	0.083	N/A	**0.004**	N/A
HER1-4	0.521	0.269	0.868	0.431	0.499	0.07	**0.001**
EGFR/vIII +HER2-3	0.068	0.276	**0.037**	0.063	N/A	**0.005**	N/A
EGFR/vIII +HER2-4	0.140	0.247	0.415	0.09	0.241	0.153	**0.005**
HER1-3 +HRG	0.303	0.671	Nil	0.298	N/A	0.03*	N/A
HER1-4 +HRG	0.840	0.583	0.922	0.578	Nil	0.32	0.03*
EGFR/vIII +HER2-3 +HRG	0.172	0.417	**0.047**	0.186	N/A	0.107	N/A
EGFR/vIII +HER2-4 +HRG	0.433	0.515	0.544	0.219	0.284	0.439	0.109

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Figure 4.7: Correlations of Expression of combinations of Markers with Time To Biochemical Relapse that show statistical significance in the Patient Subcohort treated with Androgen Deprivation Therapy.

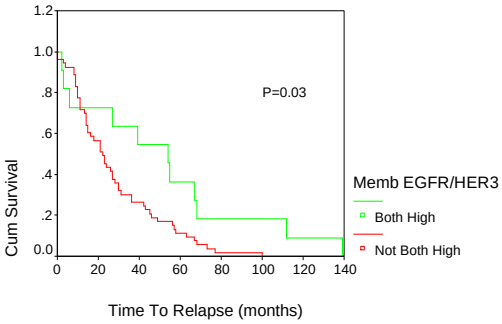
a) Memb EGFR/HER2 and TTR



COX Regression Hazard Ratio: 2.38 (1.19-4.78) p=0.014

Multifactorial COX Regression: N/A

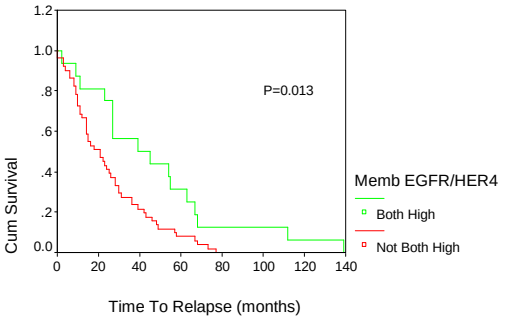
b) Memb EGFR/HER3 and TTR



COX Regression Hazard Ratio: 2.11 (1.02-4.36) p=0.072

Multivariate COX Regression: N/A

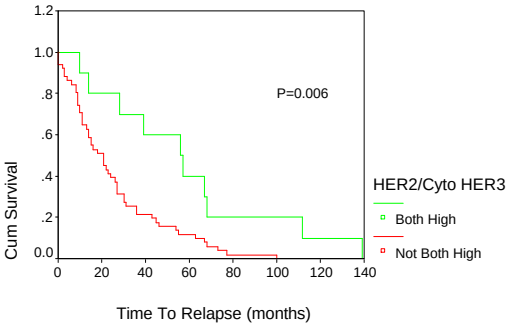
c) Memb EGFR/HER4 and TTR



COX Regression Hazard Ratio: 2.04 (1.11-3.73) p=0.021

Multivariate COX Regression: N/A

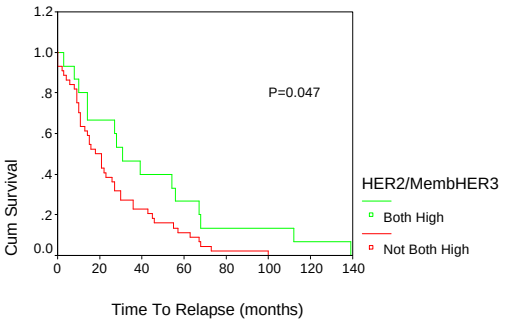
d) HER2/Cyto HER3 and TTR



COX Regression Hazard Ratio: 2.64 (1.23-5.67) p=0.013

Multivariate COX Regression: N/A

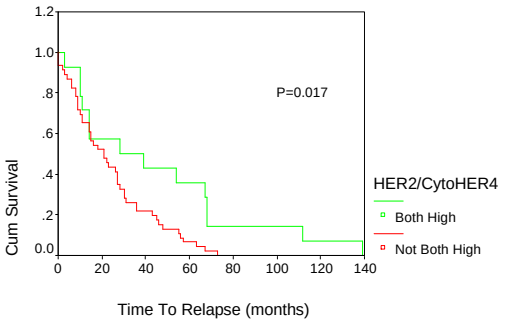
e) HER2/Memb HER3 and TTR



COX Regression Hazard Ratio: 1.76 (0.93-3.32) p=0.08

Multivariate COX Regression: N/A

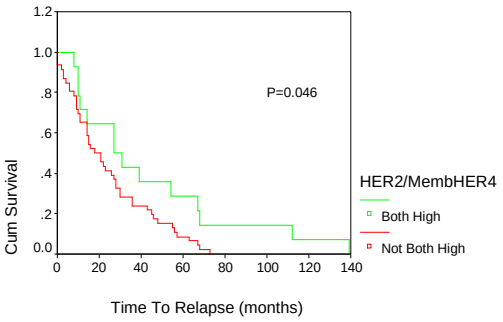
f) HER2/Cyto HER4 and TTR



COX Regression Hazard Ratio: 2.10 (1.07-4.12) p=0.032

Multivariate COX Regression: N/A

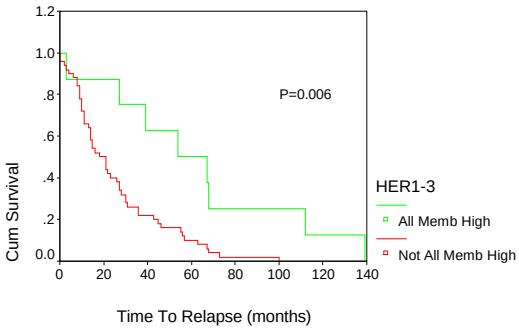
g) HER2/Memb HER4 and TTR



COX Regression Hazard Ratio: 1.81 (0.94-3.47) p=0.077

Multivariate COX Regression: N/A

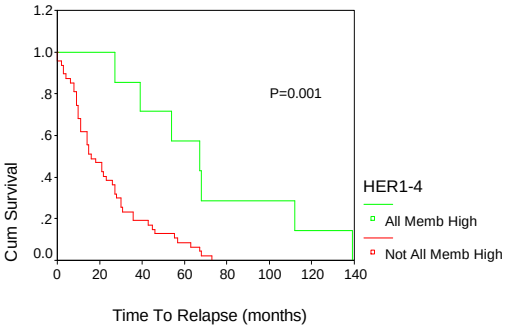
h) HER1-3 and TTR



COX Regression Hazard Ratio: 3.09 (1.29-7.38) p=0.011

Multivariate COX Regression: N/A

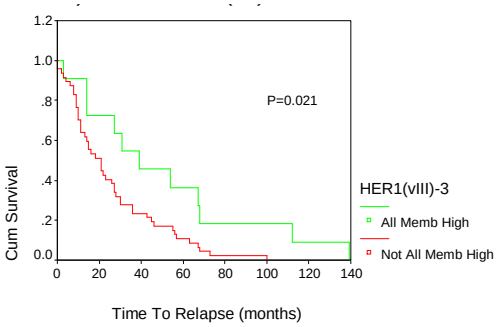
i) HER1-4 and TTR



COX Regression Hazard Ratio: 4.07 (1.57-10.52) p=0.004

Multivariate COX Regression: N/A

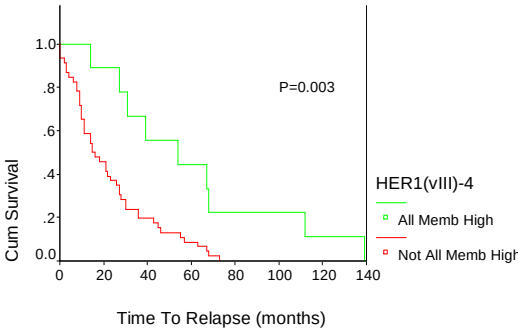
j) Memb HER1(vIII)-3 and TTR



COX Regression Hazard Ratio: 2.22 (1.07-4.60) p=0.033

Multivariate COX Regression: N/A

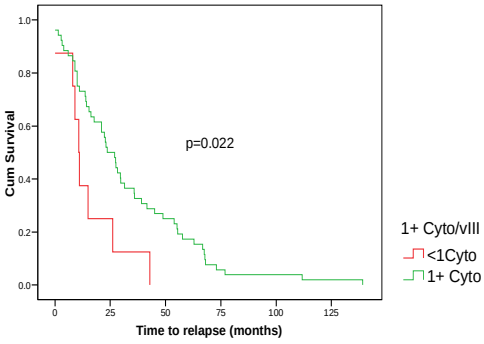
k) Memb HER1(vIII)-4 and TTR



COX Regression Hazard Ratio: 3.08 (1.36-7.00) p=0.007

Multivariate COX Regression: N/A

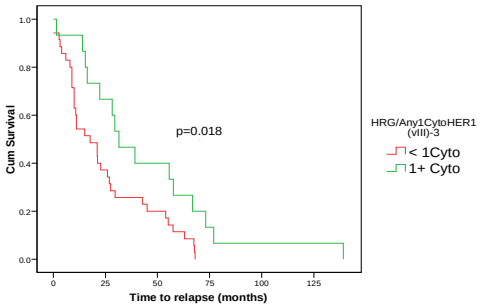
l) 1+ Cytoplasmic HER1(vIII)-4 and TTR



COX Regression Hazard Ratio: 2.39 (1.21-3.09) p=0.04

Multivariate COX Regression: N/A

m) HRG/Any 1 Cyto HER1(vIII)-3 and TTR

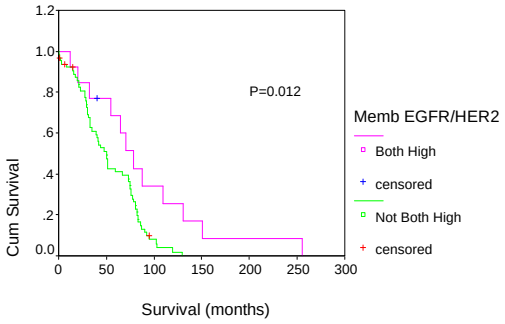


COX Regression Hazard Ratio: 2.8 (1.41 -4.1) p=0.021
Multivariate COX Regression: N/A

a) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of both EGFR and HER2 and those without for outcome Time To Biochemical Relapse. b) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of both EGFR and HER3 and those without for outcome Time To Biochemical Relapse. c) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of both EGFR and HER4 those without for outcome Time To Biochemical Relapse. d) Kaplan-Meier Plot comparing HSPC ADT patients with High expression of both HER2 and Membrane HER3 and those without for outcome Time To Biochemical Relapse. e) Kaplan-Meier Plot comparing HSPC ADT patients with High Membrane expression of both HER2 and HER3 and those without for outcome Time To Biochemical Relapse. f) Kaplan-Meier Plot comparing HSPC ADT patients with High expression of both HER2 and Membrane HER4 and those without for outcome Time To Biochemical Relapse. g) Kaplan-Meier Plot comparing HSPC patients with High Membrane expression of both HER2 and HER4 and those without for outcome Time To Biochemical Relapse. h) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous HER 1-3 proteins and those without for outcome Time To Biochemical Relapse. i) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of all 4 membranous HER 1-4 proteins and those without for outcome Time To Biochemical Relapse j) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of all membranous HER 1-3 proteins (where EGFR can be normal or variant III) and those without for outcome Time To Biochemical Relapse. k) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous HER 1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Time To Biochemical Relapse . l) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of any 1 cytoplasmic HER 1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Time To Biochemical Relapse. l) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of HRG and any 1 cytoplasmic HER 1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Time To Biochemical Relapse.

Figure 4.8: Correlations of Expression of combinations of Markers with Overall Survival that show statistical significance in the Patient Subcohort treated with Androgen Deprivation Therapy.

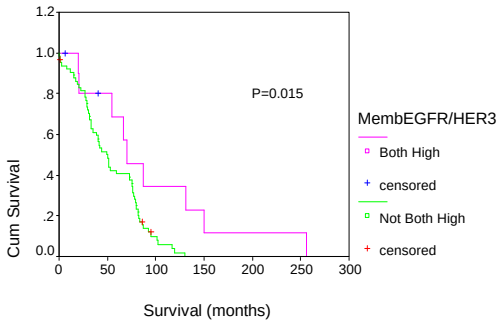
a) Memb EGFR/HER2 and OS



COX Regression Hazard Ratio: 2.43 (1.17-5.01) p=0.017

Multivariate COX Regression: p=0.006

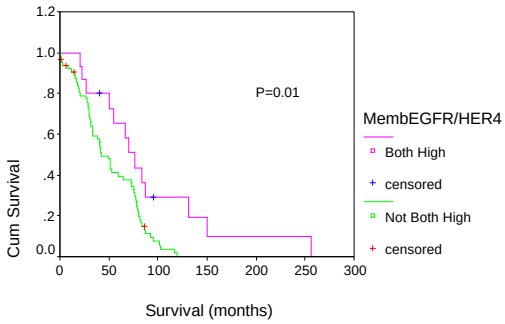
b) Memb EGFR/HER3 and OS



COX Regression Hazard Ratio: 2.78 (1.16-6.62) p=0.021

Multivariate COX Regression: N/A

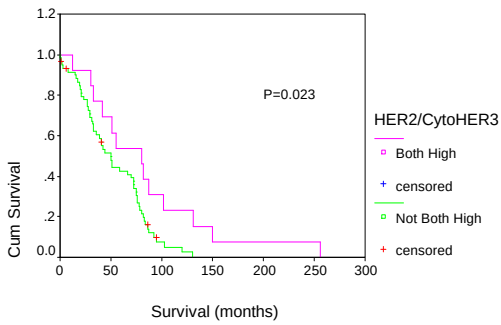
c) Memb EGFR/HER4 and OS



COX Regression Hazard Ratio: 2.37 (1.20-4.71) p=0.013

Multivariate COX Regression: p=0.783

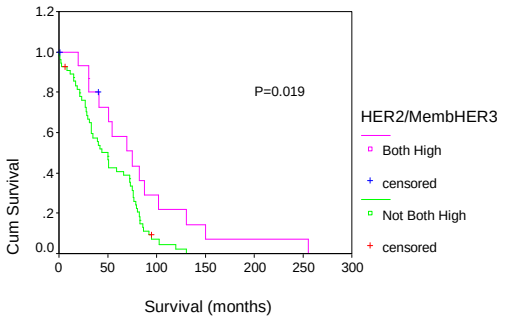
d) HER2/Cyto HER3 and OS



COX Regression Hazard Ratio: 2.17 (1.08-4.34) p=0.029

Multivariate COX Regression: p=0.015

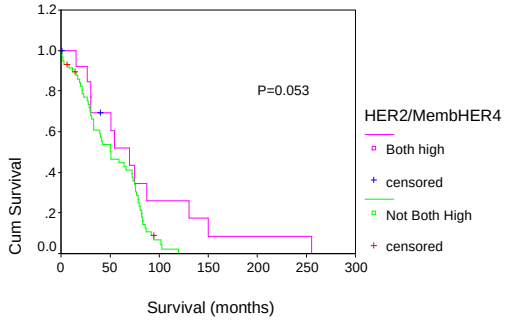
e) HER2/Memb HER3 and OS



COX Regression Hazard Ratio: 2.13 (1.10-4.15) p=0.025

Multivariate COX Regression: p=0.027

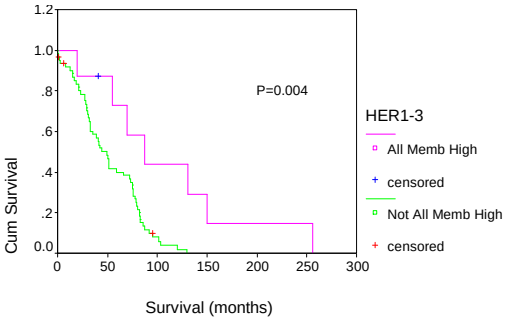
f) HER2/Memb HER4 and OS



COX Regression Hazard Ratio: 1.99 (0.96-4.11) p=0.064

Multivariate COX Regression: p=0.031

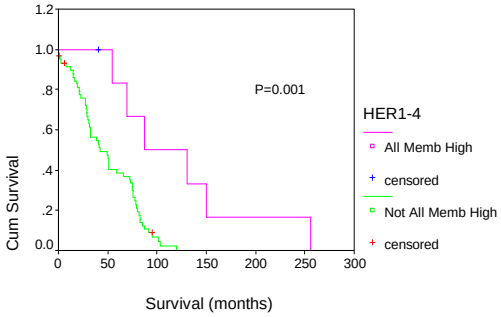
g) Memb HER1-3 and OS



COX Regression Hazard Ratio: 4.11 (1.44-11.69) p=0.008

Multivariate COX Regression: N/A

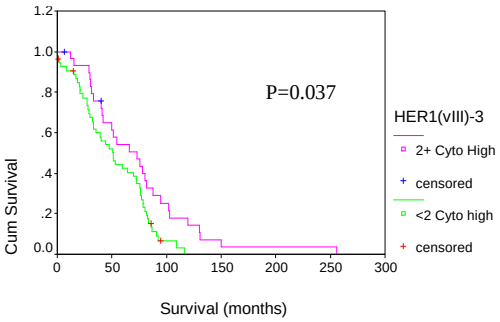
h) Memb HER1-4 and OS



COX Regression Hazard Ratio: 5.57 (1.69-18.41) p=0.005

Multivariate COX Regression: N/A

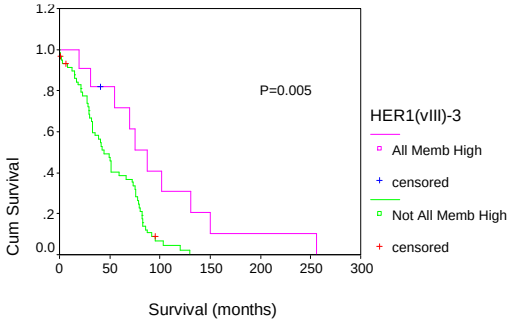
i) 2+ Cyto of HER1(vIII)-3 and OS



COX Regression Hazard Ratio: 1.80 (1.08-2.99) p=0.024

Multivariate COX Regression: p=0.664

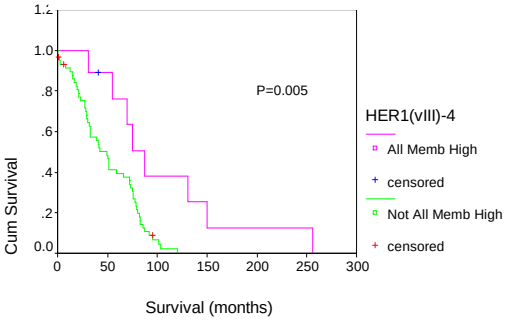
j) Memb HER1(vIII)-3 and OS



COX Regression Hazard Ratio: 2.97 (1.32-6.69) p=0.008

Multivariate COX Regression: N/A

k) Memb HER1(vIII)-4 and OS



COX Regression Hazard Ratio: 3.54 (1.38-9.08) p=0.009

Multivariate COX Regression: N/A

a) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous EGFR and HER2 and those without for outcome Overall Survival.
b) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous EGFR and HER3 and those without for outcome Overall Survival.
c) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous EGFR and HER4 and those without for outcome Overall Survival.
d) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of HER2 and membranous HER3 and those without for outcome Overall Survival. e) Kaplan-Meier

Plot comparing HSPC ADT patients with high expression of HER2 and membranous HER3 and those without for outcome Overall Survival. f) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of HER2 and membranous HER4 and those without for outcome Overall Survival. g) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of membranous HER1, 2 and 3 proteins and those without for outcome Overall Survival. h) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of all membranous HER1-4 proteins and those without for outcome Overall Survival. i) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of at least 2 cytoplasmic HER1-3 proteins (where EGFR can be normal or variant III) and those without for outcome Overall Survival. j) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of all membranous HER1-3 proteins (where EGFR can be normal or variant III) and those without for outcome Overall Survival. k) Kaplan-Meier Plot comparing HSPC ADT patients with high expression of all membranous HER1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Overall Survival.

4.5 IMPACT OF MARKER EXPRESSION IN FULL PATIENT COHORT

4.5.1 IMPACT OF SINGLE MARKER VALUES ON TIME TO RELAPSE AND SURVIVAL

Using the full patient cohort of Kaplan-Meier analyses were conducted correlating expression of each of the markers used in HSPC samples with time to relapse and overall survival comparing those with high and low expression (divided by the median) and those with very high expression and those without (divided by the 3rd quartile).

Upper quartile (Very high) membrane EGFR expression correlated with increased time to relapse (P=0.049) as were upper quartile HER2 (0.02), above median (High) cytoplasmic HER3 (0.006) and upper quartile HER4 (<0.001). Above median membrane HER4 was also associated with increased time to relapse but did not achieve significance (P=0.051). Upper quartile EGFRvIII expression was associated with reduced time to relapse (P=0.027).

Above median (0.03) and upper quartile HER3 (0.002) cytoplasm expression were correlated with increased overall survival as were upper quartile cytoplasmic HER4 (0.022), above median (0.008) and upper quartile (<0.001) membrane HER4 and membrane HRG (P=0.002). Upper quartile KI67 expression was associated with reduced overall survival (P=0.022).

To determine hazard ratios for all markers with a statistically significant influence COX regression analysis was performed for all markers with p<0.05 on Kaplan-Meier analysis. Additionally a multivariate backwards: conditional COX analysis utilising Gleason score and Metastasis at presentation was performed for each of these.

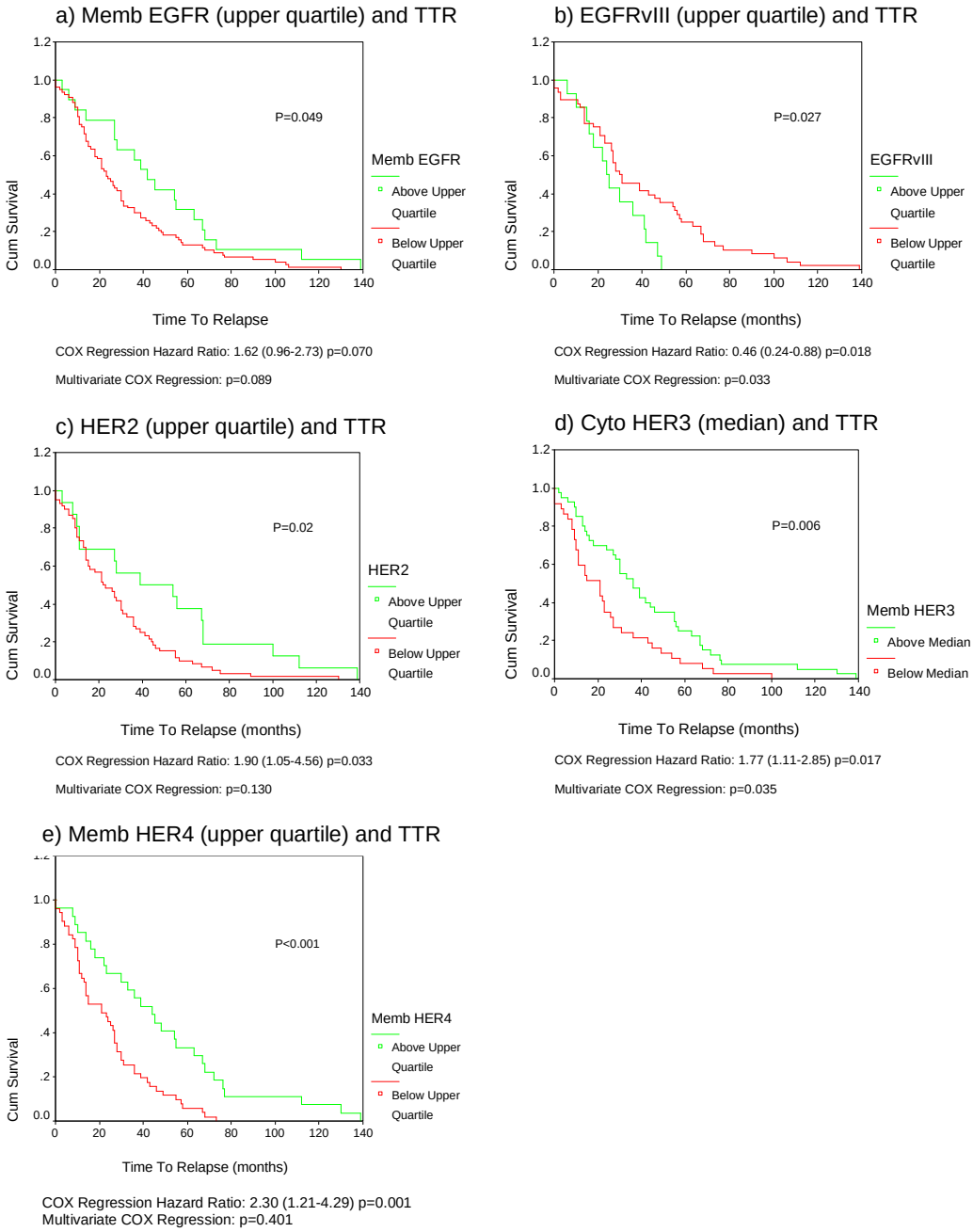
Table 4.10: P-values for Kaplan-Meier Analyses of Time To Biochemical Relapse and Overall Survival for all Markers in HSPC samples from all patients comparing those with high and low expression (divided by median)

Protein/Location	Time To Relapse Kaplan Meier P-Value	Overall Survival Kaplan Meier P-Value
EGFR – Cytoplasm (High)	0.211	0.096
EGFR – Membrane (High)	0.32	0.444
EGFR variant III (High)	0.998	0.962
HER2 (Herceptest) (High)	0.139	0.067
HER3 – Cytoplasm (High)	**0.006**	**0.03**
HER3 – Membrane (High)	0.448	0.783
HER3 – Nucleus (High)	0.8	0.783
HER4 – Cytoplasm (High)	0.322	0.753
HER4 – Membrane (High)	0.051	**0.008**
HER4 – Nucleus (High)	0.864	0.413
HRG – Cytoplasm (High)	0.214	0.385
HRG – Membrane (High)	0.063	0.101
HRG – Nucleus (High)	0.76	0.2
KI67 Count (High)	0.238	**0.022**
TUNEL Assay (High)	0.321	0.436

Table 4.11: P-values for Kaplan-Meier Analyses of Time To Relapse and Overall Survival for all Markers in All HSPC samples comparing those with very high expression to those without (divided by the Third Quartile)

Protein/Location	Time To Relapse Kaplan Meier P-Value	Overall Survival Kaplan Meier P-Value
EGFR – Cytoplasm (Very High)	0.211	0.096
EGFR – Membrane (Very High)	**0.049**	0.902
EGFR variant III (Very High)	**0.027**	0.063
HER2 (Herceptest) (Very High)	**0.02**	0.347
HER3 – Cytoplasm (Very High)	0.106	**0.002**
HER3 – Membrane (Very High)	0.86	0.321
HER3 – Nucleus (Very High)	0.898	0.491
HER4 – Cytoplasm (Very High)	0.093	**0.022**
HER4 – Membrane (Very High)	**<0.001**	**<0.001**
HER4 – Nucleus (Very High)	0.497	0.217
HRG – Cytoplasm (Very High)	0.078	0.434
HRG – Membrane (Very High)	0.094	**0.002**
HRG – Nucleus (Very High)	0.228	0.2
KI67 Count (Very High)	0.77	0.159
TUNEL Assay (Very High)	0.748	0.601

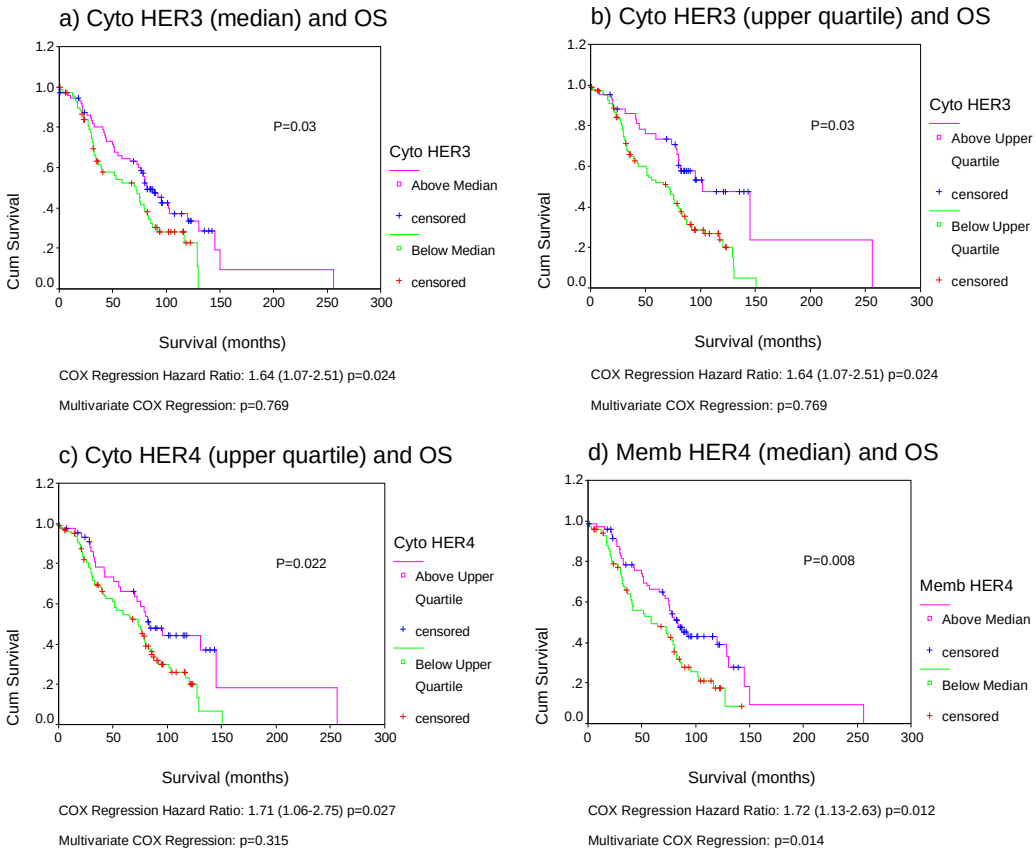
Figure 4.9: Correlations of Marker Expression with Time To Biochemical Relapse that show statistical significance in the Full Patient Cohort



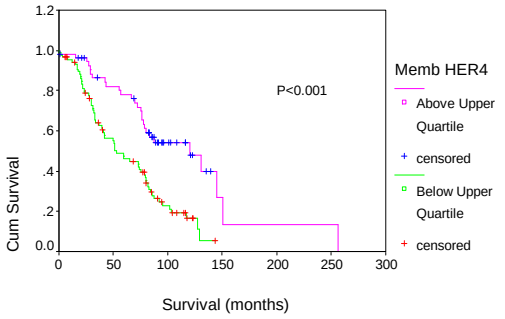
a) Kaplan-Meier Plot comparing all HSPC patients with very high membrane expression of EGFR and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse b) Kaplan-Meier Plot comparing all HSPC patients with very high membrane expression of EGFR variant III and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse. c) Kaplan-Meier Plot comparing all HSPC

patients with very high membrane expression of HER2 and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse
d) Kaplan-Meier Plot comparing all HSPC patients with high cytoplasm expression of HER3 and those without (divided by median) for outcome Time To Biochemical Relapse. e) Kaplan-Meier Plot comparing all HSPC patients with very high membrane expression of HER4 and those without (divided by Upper Quartile) for outcome Time To Biochemical Relapse

Figure 4.10: Correlations of Marker Expression with Overall Survival that show statistical significance in the Full Patient Cohort



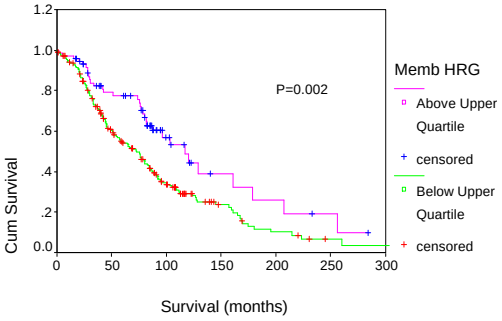
e) Memb HER4 (upper quartile) and OS



COX Regression Hazard Ratio: 2.52 (1.57-4.05) p<0.001

Multivariate COX Regression: p=0.414

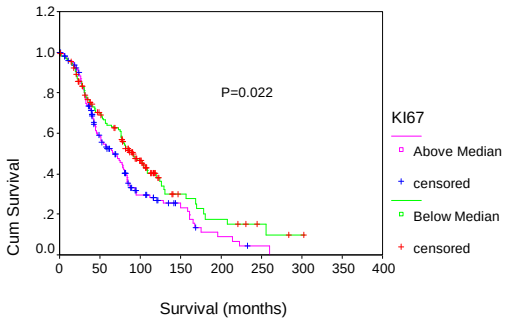
f) Memb HRG (upper quartile) and OS



COX Regression Hazard Ratio: 1.77 (1.22-2.58) p=0.003

Multivariate COX Regression: p=0.791

g) KI-67 (median) and OS



COX Regression Hazard Ratio: 0.71 (0.53-0.95) p=0.023

Multivariate COX Regression: p=0.411

a) Kaplan-Meier Plot comparing all HSPC patients with high cytoplasm expression of HER3 and those without (divided by median) for outcome Overall Survival b)Kaplan-Meier Plot comparing all HSPC patients with very high cytoplasm expression of HER3 and those without (divided by upper quartile) for outcome Overall Survival.
c) Kaplan-Meier Plot comparing all HSPC patients with very high cytoplasm expression of HER4 and those without (divided by upper quartile) for outcome Overall Survival d) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of HER4 and those without (divided by median) for outcome Overall Survival. e) Kaplan-Meier Plot comparing all HSPC patients with very high membrane expression of HER4 and those without (divided by upper quartile) for outcome Overall Survival. f) Kaplan-Meier Plot comparing all HSPC patients with very high membrane expression of HRG and those without (divided by upper quartile) for outcome Overall Survival. g) Kaplan-Meier Plot comparing all HSPC patients with high expression of KI67 and those without (divided by median) for outcome Overall Survival.

4.5.2 IMPACT OF MARKER COMBINATIONS ON TIME TO RELAPSE AND SURVIVAL

The impact of high expression (high and low divided by the median) of combinations of markers in the full HSPC patient cohort was assessed first by grouping markers in pairs then by more general combinations – ≥1 marker, ≥2 markers, ≥3 markers and 4 markers. For a given marker, if expression had been demonstrated in more than 1 cell site (EGFR, HER3, HER4) markers were grouped like site with like site (e.g. cytoplasmic EGFR and cytoplasmic HER3, membrane EGFR with membrane HER3). Where expression had been demonstrated in only one site (HER2, EGFRvIII) the marker was matched with all sites (e.g. HER2 with cytoplasmic HER3, HER2 with membrane HER3). The general analyses were performed on both HER1-3 only (given HER1-3 having different prognostic actions in breast cancer) and HER1-4. Analysis was repeated with EGFRvIII included i.e. high score in EGFR or variant III with others and again with HRG high score required in addition to other markers.

Increased time to relapse correlated with high memb EGFR/HER2 (0.0332, HER2/cyto HER3 (0.03), all 3 membranous markers from HER1-3 and all 4 membranous markers HER1-4 (0.001). If EGFRvIII was included high expression of all one cytoplasmic of HER1-3 (0.013), one of HER1-4 (0.004) or all 4 membranous (0.015) HER1-4 were correlated with increased time to relapse. No significant correlations were found if HRG was included.

Increased overall survival was correlated with high cyto HER3/HRG (0.025), high expression of at least 1 cytoplasmic marker from HER1-3 (0.036), HER1-4 (0.030) and with high expression of at least 1 memb marker from HER1-4. If EGFRvIII was included

high expression of at least 2 membranous markers of HER1-3 (0.026) was correlated with decreased overall survival. With HRG included again increased overall survival was correlated with at least one highly expressed cytoplasmic marker.

To determine hazard ratios for all markers with a statistically significant influence COX regression analysis was performed for all markers with $p<0.05$ on Kaplan-Meier analysis. Additionally a multivariate backwards: conditional COX analysis utilising Gleason score and Metastasis at presentation was performed for each of these.

Table 4.12: P-values for Kaplan-Meier Analyses of Time To Biochemical Relapse in HSPC samples from all patients comparing those with high expression in 2 different markers to those without

	EGFRC	EGFRM	EGFR VIII	HER2	HER3C	HER3M	HER4C	HER4M	HRGC	HRGM	HRGN
EGFRC		ND	ND	Nil	Nil	ND	0.5*	ND	Nil	ND	ND
EGFRM			ND	**0.033**	ND	0.069	ND	0.053	ND	0.459	ND
EGFR vIII				0.513	0.907	0.972	0.944	0.879	0.51	0.569	0.385
HER2					**0.02**	0.142	0.082	0.095	0.927	0.27	0.375
HER3C						ND	0.005	ND	**0.027**	ND	ND
HER3M							ND	0.074	ND	0.408	ND
HER4C								ND	0.738	ND	ND
HER4M									ND	0.724	ND

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Table 4.13: P-values for Kaplan-Meier Analyses of Time To Biochemical Relapse in HSPC samples from all patients comparing those with high expression in different combinations of markers to those without

	Any1C	Any1M	Any2C	Any2M	Any3C	Any3M	4M
HER1-3	0.109	0.791	**Nil**	0.337	N/A	**0.019**	N/A
HER1-4	0.189	0.355	0.124	0.814	**Nil**	0.113	**0.007**
EGFR/vIII +HER2-3	**0.035**	0.531	0.335	0.307	N/A	0.062	N/A
EGFR/vIII +HER2-4	**0.004**	0.055	0.317	0.596	0.135	0.384	**0.015**
HER1-3 +HRG	0.051	0.77	Nil	0.343	N/A	0.075	N/A
HER1-4 +HRG	0.438	0.901	0.468	0.692	Nil	0.402	0.076
EGFR/vIII +HER2-3 +HRG	**0.017**	0.891	0.136	0.368	N/A	0.214	N/A
EGFR/vIII +HER2-4 +HRG	0.114	0.748	0.463	0.817	0.210	0.561	0.209

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Table 4.14: P-values for Kaplan-Meier Analyses of Overall Survival in HSPC samples from all patients comparing those with high expression in 2 different markers to those without

	EGFRC	EGFRM	EGFR VIII	HER2	HER3C	HER3M	HER4C	HER4M	HRGC	HRGM	HRGN
EGFRC	ND	ND	ND	0.031*	0.177	ND	0.619	ND	0.196	ND	ND
EGFRM		ND	ND	0.7135	ND	0.95	ND	0.745	ND	0.251	ND
EGFR vIII			ND	0.288	0.437	0.564	0.341	0.951	0.276	0.183	0.272
HER2				ND	0.776	0.435	0.21	0.673	0.164	0.76	0,427
HER3C					ND	ND	0.511	ND	**0.025**	ND	ND
HER3M						ND	ND	0.404	ND	0.662	ND
HER4C							ND	ND	0.74	ND	ND
HER4M								ND	ND	0.108	ND

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

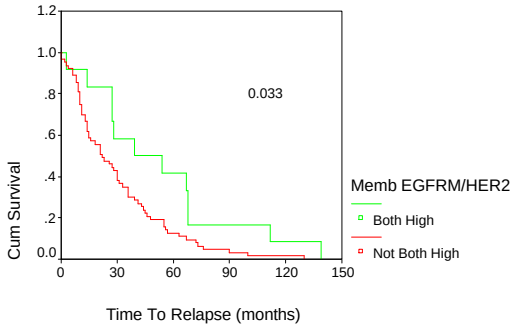
Table 4.15: P-values for Kaplan-Meier Analyses of Overall Survival in HSPC samples from all patients comparing those with high expression in different combinations of markers to those without

	Any1C	Any1M	Any2C	Any2M	Any3C	Any3M	4M
HER1-3	**0.036**	0.913	0.177	0.087	N/A	0.486	N/A
HER1-4	**0.030**	**0.044**	0.677	0.542	0.516	0.462	0.322
EGFR/vIII +HER2-3	0.215	0.206	0.312	**0.026**	N/A	0.819	N/A
EGFR/vIII +HER2-4	0.062	0.233	0.716	0.417	0.581	0.151	0.598
HER1-3 +HRG	**0.031**	0.848	Nil	0.277	N/A	0.113	N/A
HER1-4 +HRG	0.050	0.348	0.945	0.835	Nil	0.843	0.112
EGFR/vIII +HER2-3 +HRG	0.106	0.448	0.772	0.243	N/A	0.536	N/A
EGFR/vIII +HER2-4 +HRG	0.067	0.5	0.753	0.63	0.775	0.605	0.518

Key
ND – Not Done – Markers not paired with themselves, staining sites (cytoplasm/membrane) paired like with like where possible
N/A – Not possible
Nil – No patients had relevant combination
* - Statistical significance but too few patients had relevant combination for real significance

Figure 4.11: Correlations of Expression of combinations of Markers with Time To Biochemical Relapse that show statistical significance in the Full Patient Cohort.

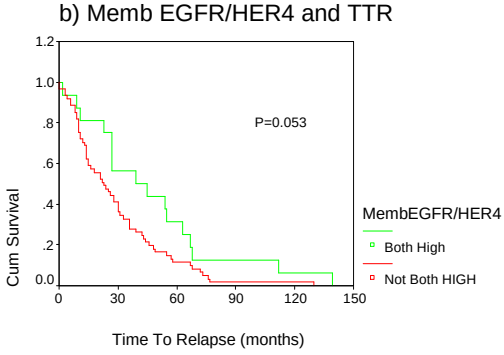
a) Memb EGFR/HER2 and TTR



COX Regression Hazard Ratio: 1.95 (1.01-3.76) p=0.045

Multivariate COX Regression: p=0.106

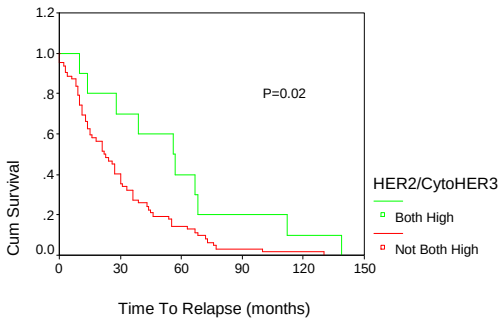
b) Memb EGFR/HER4 and TTR



COX Regression Hazard Ratio: 1.70 (0.95-3.01) p=0.071

Multivariate COX Regression: p=0.235

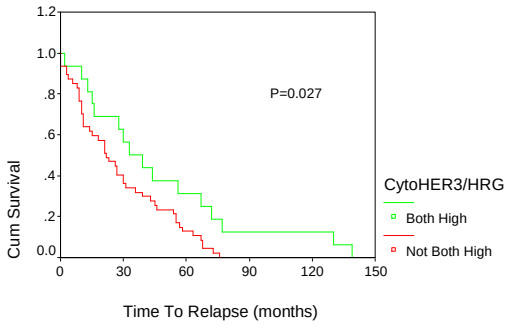
c) HER2/Cyto HER3 and TTR



COX Regression Hazard Ratio: 2.19 (1.07-4.48) p=0.032

Multivariate COX Regression: p=0.049

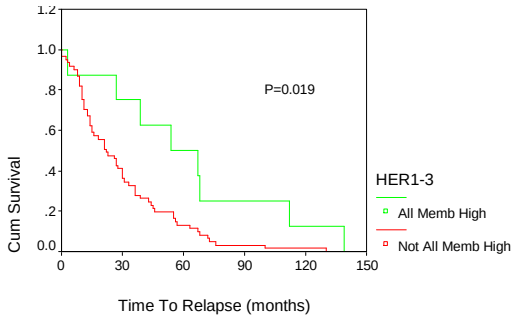
d) Cyto HER3/HRG and TTR



COX Regression Hazard Ratio: 1.90 (1.01-3.57) p=0.048

Multivariate COX Regression: p=0.807

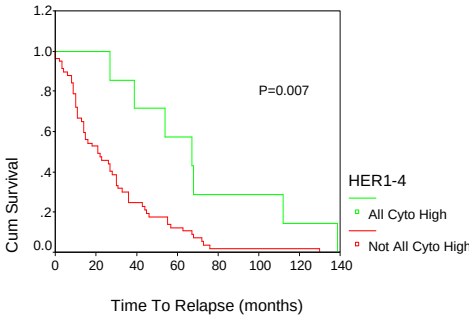
e) Memb HER1-3 and TTR



COX Regression Hazard Ratio: 2.46 (1.10-5.50) p=0.028

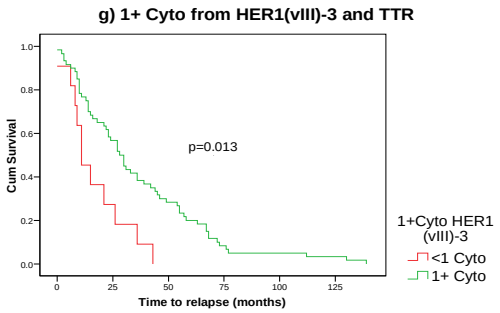
Multivariate COX Regression: p=0.041

f) Memb HER1-4 and TTR

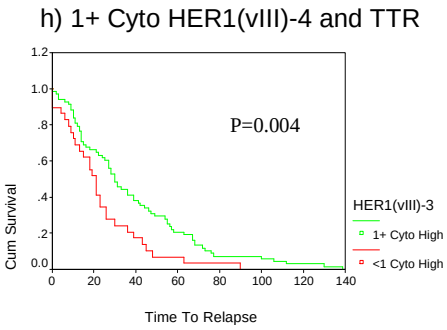


COX Regression Hazard Ratio: 2.99 (1.26-7.08) p=0.013

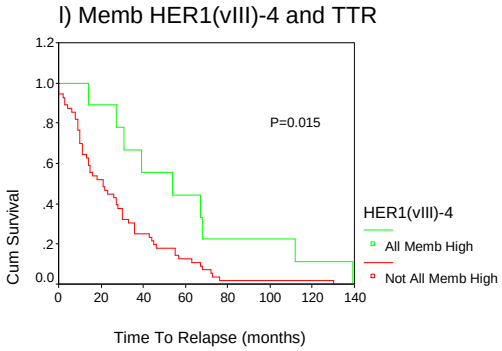
Multivariate COX Regression: p=0.045



COX Regression Hazard Ratio: 2.10 (1.01-4.09) p=0.03
Multivariate COX Regression: p=0.521



COX Regression Hazard Ratio: 1.69 (1.06-2.70) p=0.028
Multivariate COX Regression: p=0.569

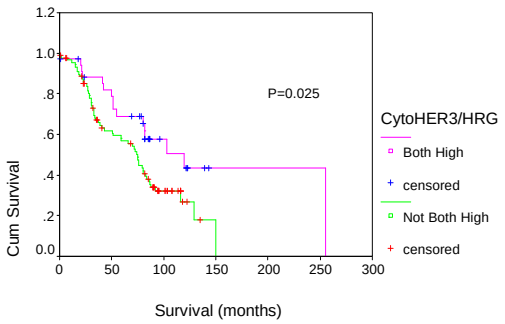


COX Regression Hazard Ratio: 2.37 (1.11-5.06) p=0.026
Multivariate COX Regression: p=0.598

a) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of EGFR and HER2 and those without for outcome Time To Biochemical Relapse.
b) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of EGFR and HER4 and those without for outcome Time To Biochemical Relapse
c) Kaplan-Meier Plot comparing all HSPC patients with high expression of HER2 and cytoplasmic HER3 and those without for outcome Time To Biochemical Relapse.
d) Kaplan-Meier Plot comparing all HSPC patients with high cytoplasmic expression of HER3 and HRG and those without for outcome Time To Biochemical Relapse
e) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of all HER1-3 proteins and those without for outcome Time To Biochemical Relapse. f) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of all HER1-4 proteins and those without for outcome Time To Biochemical Relapse. g) Kaplan-Meier Plot comparing all HSPC patients with high expression of at least 1 cytoplasmic HER1-3 proteins (where EGFR can be normal or variant III) and those without for outcome Time to Biochemical Relapse. h) Kaplan-Meier Plot comparing all HSPC patients with high expression of at least 1 cytoplasmic HER1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Time to Biochemical Relapse i) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of all HER1-4 proteins (where EGFR can be normal or variant III) and those without for outcome Time To Biochemical Relapse.

Figure 4.12: Correlations of Expression of combinations of Markers with Overall Survival that show statistical significance in the Full Patient Cohort.

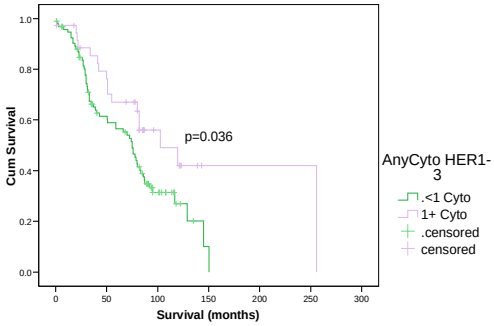
a) Cyto HER3/HRGC and OS



COX Regression Hazard Ratio: 1.87 (1.06-3.34) p=0.032

Multivariate COX Regression: p=0.270

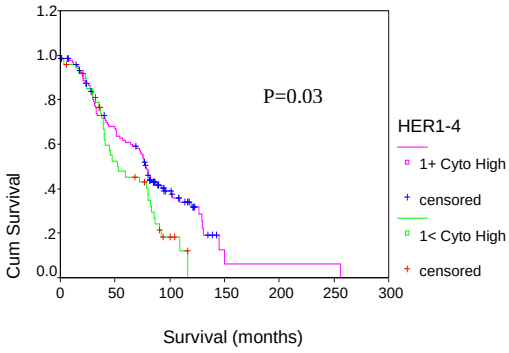
b) 1+ Cyro from HER1-3 and OS



COX Regression Hazard Ratio: 1.81 (1.01 - 3.52) p=0.049

Multivariate COX Regression p= 0.423

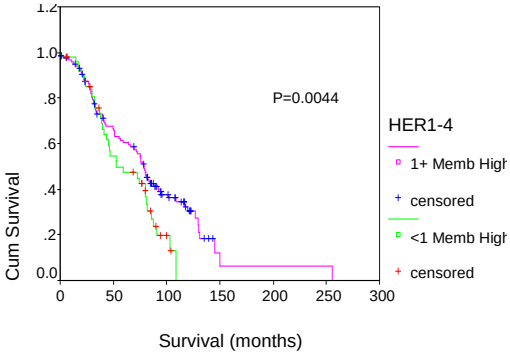
c) 1+ Cyto of HER1-4 and OS



COX Regression Hazard Ratio: 1.61 (1.07-2.41) p=0.023

Multivariate COX Regression: p=0.739

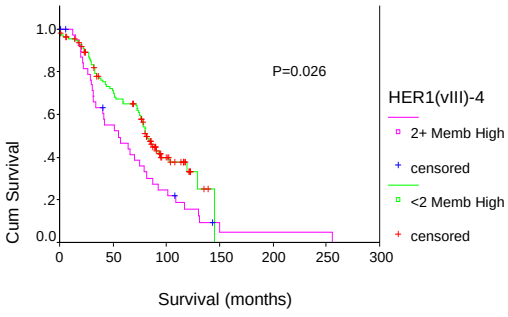
d) 1+Memb of HER1-4 and OS



COX Regression Hazard Ratio: 1.55 (1.02-2.35) p=0.038

Multivariate COX Regression: p=0.051

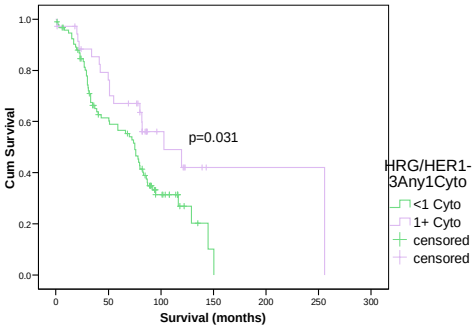
e) 2+ Memb of HER1(vIII)-3 and OS



COX Regression Hazard Ratio: 0.61 (0.39-0.93) p=0.023

Multivariate COX Regression: p=0.164

f) HRG/1+ Cyto from HER1-3 and OS



COX Regression Hazard Ratio: 2.02 (1.01 - 4.14) p=0.04

Multivariate COX Regression p= 0.821

a) Kaplan-Meier Plot comparing all HSPC patients with high cytoplasmic expression of HER3 and HRG and those without for outcome Overall Survival. b) Kaplan-Meier Plot comparing all HSPC patients with high expression of at least 1 cytoplasmic HER1-3 protein and those without for outcome Overall Survival. c) Kaplan-Meier Plot comparing all HSPC patients with high expression of at least 1 cytoplasmic HER1-4 protein and those without for outcome Overall Survival. d) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of at least 1 HER1-4 protein those without for outcome Overall Survival. e) Kaplan-Meier Plot comparing all HSPC patients with high membrane expression of at least 2 HER1-3 proteins (where EGFR can be normal or variant III) and those without for outcome Overall Survival. f) Kaplan-Meier Plot comparing all HSPC patients with high expression of HRG and at least 1 cytoplasmic HER1-4 protein and those without for outcome Overall Survival.

4.6 CORRELATIONS OF DIFFERENT MARKER EXPRESSION IN FULL COHORT

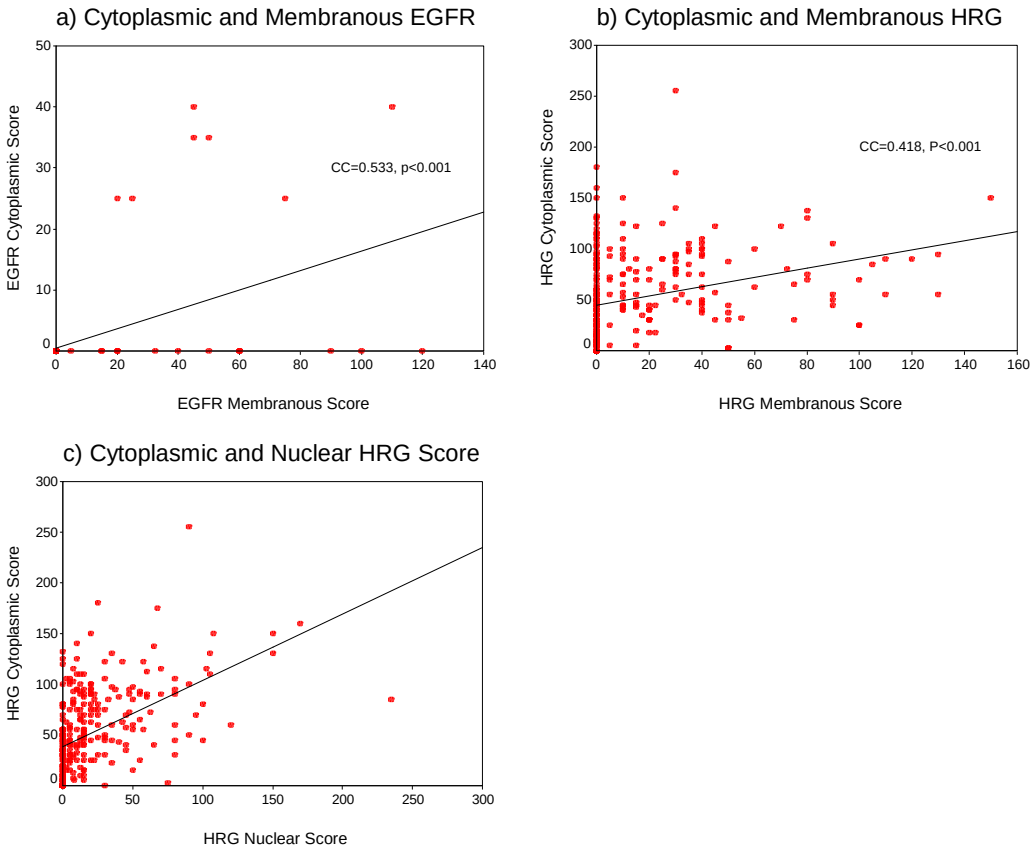
Correlation analyses were performed comparing expression of each marker with all others in all HSPC samples. A correlation coefficient of >0.4 and $p<0.05$ were taken as indicating a statistically significant correlation.

In this cohort statistically significant correlations were found between EGFR membrane and cytoplasmic expression, HRG cytoplasmic and HRG membranous expression, HRG cytoplasmic and HRG nuclear expression.

Table 4.16: Correlation Coefficients and P-values for Inter-Marker Expression Correlation Analyses

	EGFRM	EGFRvIII	HER2	HER3C	HER3M	HER4C	HER4M	HRGC	HRGM	HRGN	KI67	TUNEL
EGFRC	**.533 <.001**	N/A	-.009 929	.018 .852	.036 .712	.156 .117	.002 .985	-.193 .047	-.073 .455	-.042 .666	.052 .597	.041 .694
EGFRM		-.25 .846	.358 .000	.079 .329	.116 .15	.222 .006	.115 .154	-.56 .491	-.224 .005	-.168 .038	.115 .146	.042 .613
EGFRvIII			-.126 .401	.081 .583	-.121 .415	-.043 .764	-.115 .423	.061 .686	.016 .914	.233 .118	.083 .567	-.068 .652
HER2				.162 .048	.220 .007	.225 .006	.109 .188	.166 .045	-.137 .099	.024 .775	.087 .299	-.044 .612
HER3C					.388 .000	.179 .031	.147 .077	.207 .015	.109 .206	.083 .336	.06 .483	.006 .947
HER3M						.238 .004	.3 <.001	.099 .249	-.114 .186	-.082 .34	.08 .35	-.026 .77
HER4C							.216 .006	-.001 .992	.000 .997	.049 .573	.055 .521	-.145 .099
HER4M								.115 .18	.083 .334	-.025 .775	-.059 .485	.008 .925
HRGC									**.418 <.001**	**.601 <.001**	-.055 .355	-.088 .156
HRGM										.329 <.001	-.219 <.001	-.048 .443
HRGN											.015 .797	.122 .05
KI67												.036 .556

Figure 4.13: Significant Inter-Marker Expression Correlations



a) Scatter Graph of EGFR cytoplasmic and EGFR Membranous Expression in Full Patient Cohort. b) Scatter Graph of HRG cytoplasmic and HRG Membranous Expression in Full Patient Cohort c) Scatter Graph of HRG cytoplasmic and HRG Nuclear Expression in Full Patient Cohort

It should be noted that the EGFR scatter graph is not as convincing as the other 2 given the fewer number of stained specimens and the large proportion which are 0 value and the wide spacing of the remainder.

4.7 MULTIVARIATE COX REGRESSION ANALYSIS IN MARKERS SHOWING SIGNIFICANT IMPACT ON TIME TO RELAPSE OR SURVIVAL

4.7.1 PATIENT SUBCOHORT ON ANDROGEN DEPRIVATION THERAPY

Summarising the individual multivariate COX analyses with outcome time to biochemical relapse no factor was found to have a significant influence independent of Gleason score and metastasis at presentation.

Several markers/marker combinations had an independent influence on outcome Overall Survival in multivariate COX analysis with Gleason Score and Metastasis at presentation. These were very high Membrane expression of EGFR, high expression membranous EGFR and HER2, high expression HER2 and Cytoplasmic HER3, high expression HER2 and membranous HER3, high expression HER2 and membranous HER4.

b

4.7.2 FULL PATIENT COHORT

Several markers/marker combinations had and independent influence on outcome Time to Relapse in multivariate COX analysis with Gleason Score and Metastasis at presentation. These were very high membrane expression of EGFR variant III, high cytoplasm expression of HER3, high expression HER2 and cytoplasmic HER3, high expression HER1, 2 and 3 proteins, high membrane expression of HER1, 2, 3 and 4 proteins. A multivariate analysis including Gleason score, metastases, high HER3 and very high EGFRvIII indicates EGFRvIII ($p=0.038$) is a greater influence than HER3 ($p=0.051$)

High membrane expression of HER4 was the only factor significantly influencing overall survival independently of Gleason score and Metastasis at presentation in this cohort.

4.8 SUMMARY

Univariate analysis of an expanded cohort of HSPC patients with outcomes TTR and OS has yielded a large number of statistically significant results with a clear trend towards positive prognosis with increased marker expression. Several trends within this data are apparent e.g. greater influence of membranous staining, a larger number of significant results in the ADT subgroup and more significant p-values with multiple markers expressed concomitantly – these will be reviewed in the discussion. There are markedly fewer significant results following multivariate analysis with Gleason score and metastasis at presentation but some independent predictors remain.

5. DISCUSSION

5.1 RATIONALE FOR STUDY

With the rising incidence and high contribution to cancer mortality, Prostate Adenocarcinoma is an important health issue in the UK and elsewhere. It has become a significant priority to distinguish those patients with disease that is likely to progress and metastasise from those whose disease will remain quiescent throughout their lifetime allowing radical therapy, with all its associated side effects and complications to be appropriately utilised.

Hormone therapy with antiandrogens and GnRH analogues have long been a mainstay of treatment of both locally advanced and metastatic prostatic adenocarcinoma but while initial tumour response is good in the majority of patients eventual development of hormone resistance is common and heralds further progression, metastasis and death. It thus becomes clinically advantageous to discern which patients will have a poor initial hormonal response and which will undergo hormone relapse/clinical progression early. Such patients could thus be targeted with adjuvant therapies earlier including radiotherapy, targeted biological therapies and newer/currently evolving chemotherapeutic agents.

While chemotherapy trials are ongoing agents used in hormone refractory disease are still in their infancy. The immunotherapeutic agent Herceptin (Trastuzamab) targeting HER2 in breast cancer has been used in widespread clinical practice and multiple similar agents have been used in clinical trials however no such agent is currently available for CaP

whether hormone sensitive or refractory. There is a clinical need for such agents particularly in HRPC which has a poor prognosis and limited clinical options.

As explored in the background a multitude of extracellular, transmembrane and intracellular signalling molecules have been investigated for their potential as prognostic markers and treatment targets in CaP, the HER family is one of many investigated in this context.

5.2 COMPARISON OF RESULTS WITH PREVIOUS LITERATURE

5.2.1 EGFR

In this study univariate analysis of expression of EGFR in HSPC specimens was performed in cohort 2. High membranous EGFR expression was correlated with positive outcomes in both the full cohort and the ADT subcohort. This contrasts with existing literature where EGFR expression in CaP has been linked to either negative outcome or having no effect. The pilot study for this project showed no influence of EGFR expression on TTR/OS but this was limited by a relatively small patient cohort. (2004) et al. which similarly used IHC demonstrated a significant rise in expression following hormone escape but no correlation between expression and clinical outcome. Bartlett et al. utilised IHC and FISH to demonstrate a negative impact on prognosis of EGFR gene copy number an HRPC expression. Again no impact was seen with HSPC expression. Di Lorenzo et al (2002) demonstrated decreased time to hormone escape and relapse with high expression of EGFR. Multiple single marker EGFR studies –Maddy (1989), Glynne-Jones (1996), Myers 1997, Fowler (1998), De Miguel (1999), Zellweger (2005) have demonstrated increased expression post hormone escape and/or a negative impact on prognosis of increased EGFR expression

The reason for the difference between this study and others may involve subject numbers – the most closely related studies showing no influence had significantly fewer subjects and, like the pilot, a larger patient cohort may be required to demonstrate a trend. Differences in technique may also have influenced the disparity, this study unlike some others has clearly divided membranous and cytoplasmic expression and increased cytoplasmic expression has not shown an influence. Additionally this study has focussed

on HSPC whereas negative effects have rather been seen in HRPC in some previous studies. Given previously noted significant increases in EGFR expression following hormone escape increased expression may have different implications for HSPC and HRPC tissue.

In CaP and other solid tumours EGFR has been associated with negative prognosis and its activity within the signal transduction network particularly via MAP kinase and PI3K-Akt pathways to stimulate cell proliferation and decrease apoptosis are in keeping with an oncogenic role. The results of this study are at odds with this and counter-intuitive. One possible explanation is suggested by the clear demonstration in this study that in CaP EGFR and HER2 expression is low and infrequent compared to HER3, HER4 and HRG and the fact that expression of multiple HER family members is correlated with improved outcome most particularly in the ADT subcohort where expression of all 4 HER family members improves outcome to a greater degree of significance than EGFR alone. This importance of co-expression was noted in the pilot where high expression of 3+ markers gave a significantly longer TTR ($p=0.012$) than 2 or fewer. It may be that those tumours in which EGFR is detectable represent a subset in which HER family expression is generally higher but the influence of HER4 which has a previously demonstrated antiproliferative action (see below) is the most dominant, noting that in the full cohort high HER4 expression gives the lowest p-values. In effect EGFR is acting as a surrogate for very high HER4 activity. However it should be noted that no correlation was found between HER4 expression and that of other markers.

5.2.2 HER2

In this study the correlations between high HER2 expression and prognosis were determined in cohort 2. Univariate analysis indicated high membranous HER2 expression had a positive impact on prognosis in both the full cohort and ADT subcohort. This conflicts with previous studies which have shown a negative impact or no effect. In Hernes (2004) high HER2 expression in HRPC samples significantly associated with outcome – reduced time to death from relapse. Bartlett et al. demonstrated correlations between increased HER2 gene copy number and reduced survival and reduced time to death from relapse with a rise in HER2 following hormone escape. In this study an increased significance was noted in reduced TTDFR if both EGFR and HER2 expression were increased in the HRPC specimen.

Multiple studies have demonstrated greater HER2 expression in HRPC than HSPC in non-paired samples Xie (1995), Signoretti (2000), Shi (2001), Di Lorenzo (2002). Alternatively Lara et al. (2002) demonstrated no link between HER2 overexpression and hormone escape. Agus et al (1999) demonstrated greater HER2 expression in androgen independent compared to androgen dependent human CaP cells. HER2 expression has been correlated with poor prognosis in multiple studies Zhau (1992), Sadasivan (1993), Di Lorenzo (2004), Okegawa (2006) and Morote (1999) the last demonstrating HER2 expression as an independent predictor of worse prognosis on multivariate analysis Other studies have shown no relationship of HER2 with prognosis – Ware 1991, Mellon 1992, Ross 1997.

Possible explanations for this disparity are similar to those for EGFR – limited numbers, differing techniques, including HRPC specimens in other studies.

Like EGFR, where HER2 has been found to influence prognosis in CaP and other tumour types this influence has been negative the most prominent example being in breast CA. Like EGFR, HER2 is involved in signalling pathways that generally result in increased proliferation/decreased apoptosis. How then can the positive influence demonstrated in this study be explained? One possible explanation is involvement in alternative pathways having the opposite effect e.g. HER2 activating apoptosis via a caspase independent mechanism (Tikhomirov). However, as noted for EGFR, in this study HER2 expression is low and infrequent compared to HER3, HER4 and HRG and it is also noted that expression of multiple HER family members is correlated with improved outcome most particularly in the ADT subcohort where expression of all 4 HER family members improves outcome to a greater degree of significance than HER2 alone. In a similar fashion to EGFR tumours where HER2 is detectable represent a subset in which HER family expression is generally higher but the anti-oncogenic influence of HER4 is the most dominant (noting that in the full cohort high HER4 expression gives the lowest p-values);- HER2 acting as a surrogate for very high HER4 activity. Again it should be noted that no correlation was found between HER4 expression and that of other markers.

5.2.3 HER3

Univariate analysis of HER3 expression in cohort 2 demonstrated improved outcome with high cytoplasmic expression in the full cohort only not the ADT subcohort. There is

a good deal less previous literature regarding HER3 in CaP compared to EGFR/HER2. HER3 expression in HRPC cell line has been shown to be significantly higher than that of HSPC tissue (Koumakpayi 2006). In this study higher HER3 nuclear expression was correlated with higher Gleason score indicating poorer prognosis. Leung 1997 showed HER3 expression in abut not benign prostate tissue. Western blotting showed greater nuclear HER3 expression in HSPC cell lines as than HRPC contradicting IHC results. Hernes et al demonstrated no significant influence of HER3 expression on outcome. In Gregory et al. (2005) HER2 and HER3 expression, stimulated by HRG, was shown to increase AR transactivation and tumour proliferation in a recurrent CaP cell line in the absence of androgen – a possible route of hormone escape. Leung et al demonstrated HER3 expression linked to poor response to androgen therapy and decreased survival, however more recently Koumakpayi et al (2006) have demonstrated low nuclear HER3 as a predictor of biochemical recurrence in CaP. There is therefore no consistent position in terms of positive or negative prognosis in high HER3 expression even within the small number of existing studies however this is the first study to cytoplasmic expression so strongly to positive outcome. This may be due to higher patient numbers and the few studies using similar IHC methods targeting HER3.

In explaining this studies positive results it can be noted that HER3 along with HER4 and HRG has been shown to be positively prognostic in bladder Ca and Koumakpayi has indicated that low nuclear HER3 is negatively prognostic in CAP) therefore it is reasonable to conclude that HER3 is involved in antioncogenic signal transduction. Koumakpayi suggests HER3 interaction with Erb3 binding protein 1 (EBP1) which

suppresses AR mediated gene transcription. In this study while it is usually membranous expression of HER markers that has the greatest influence on outcome it is cytoplasmic rather than membranous HER3 expression that is linked to improved TTR/OS in the full cohort. This may reflect that only HER3 in a heterodimer with HER4 is anti-oncogenic although there is no correlation between HER3 and HER4 expression and it is membranous HER4 that is efficacious. It may be that membranous HER4 and cytoplasmic HER3 are both surrogate markers for the apoptotic activity of the HER4 intracellular domain.

5.2.4 HER4

Univariate analysis of HER4 expression and outcome in cohort 2 demonstrated strong correlations of high expression of membranous HER4 and improved outcome in both the full cohort and the ADT subcohort. This is in keeping with Hernes (2004) which demonstrated high expression of HER4 in the HRPC sample was correlated with improved 2 year survival to a degree nearly achieving statistical significance ($p=0.054$). This was in contrast to prognosis HER1-3 in Hernes as expression of these was associated with worse prognosis (although of these statistical significance occurred only with HER2). There is little else in the way of IHC studies of HER4 in CaP.

HER4 has previously been shown to be positively prognostic in cancers including breast (Tovey 2004) and bladder (Memon et al. 2004) with Hernes et al. (2004) noting a positive effect on survival that nearly reached significance. HER4 transfection results in reduced proliferation/increased apoptosis in breast Ca cells (Earp et al. 2003, Barnes et al

2005) and growth arrest in prostate Ca lines (Williams 2003). In fact HER4 involvement in signal transduction has been defined as antiproliferative in breast Ca studies with the HER4 intracellular domain released following HER4 degradation and accumulating in mitochondria to induce apoptosis (Vidal 2005). The HER4 results in this study are therefore in keeping with the majority of other literature – HER4 expression correlated to improved outcome due to its involvement in antiproliferative pathways.

5.2.5 EGFRvIII

As mentioned above EGFRvIII commercial antibody was unavailable at the time of this study but data from the pilot study was used in the analysis. Single marker EGFRvIII as in the pilot data was associated with poor prognosis. In combinations EGFRvIII was associated with some significant positive outcomes but in most cases the same combination without EGFRvIII was also significant with a smaller p-value again indicating a negative influence on prognosis. This is in keeping with previous literature where EGFRvIII gives a non-conflicted message. It has been detected only in malignant and not in benign prostate tissue with greatest expression in metastatic and high grade disease. EGFRvIII expression is greater in HRPC than HSPC and associated with high serum PSA and disease progression.

5.2.6 HRG

In this study HRG expression was analysed in cohort 1 and cohort 2. In both cohorts increased membranous HRG expression in HSPC is correlated with improved outcome with no effect noted in the HRPC specimens in cohort1. While there is a paucity of IHC

based studies of HRG in prostate CA these results are in keeping with the previous observations that HRG stimulation of HSPC cell lines is associated with antiproliferation (Lyne 1997) and that high levels of HRG are associated with growth inhibition (Sartor 2001). This study would appear to support the hypothesis that high HRG expression is positively prognostic. The fall in HRG membranous staining has not been noted specifically in previous literature but may tie in with previously noted greater expression in benign than malignant tissue i.e. greater malignancy – lesser expression (Lyne 1997).

5.2.7 MULTIPLE MARKERS

In both the ADT subcohort and full cohort 2 high expression of multiple marker combinations showed significant correlation with improved outcome as listed in chapter 4. The most notable combination was high expression of HER 1-4 combined significantly correlated with both TTR and OS in the ADT subcohort and with TTR alone in the full cohort. The results borne out in univariate COX analysis. A small number of combinations with positive correlation involved EGFRvIII however in most such cases the same combination without EGFRvIII (involving EGFR-WT only) also showed a similar correlation with a more significant p-value confirming the negative influence of EGFRvIII. There are few studies in the literature involving HER marker combinations for comparison. Hernes (2004) used HER1-4 as markers but did not explore the effect of high expression of combinations of theses markers. Bartlett et al had noted significantly reduced TTDFR in patients with increased EGFR or HER2 following hormone escape but for patients with raised EGFR and HER2 the significance of correlation with reduced TTDFR was increased indicating an additive effect. Di Lorenzo (2002) demonstrated

decreased time to hormone escape and relapse with high expression of EGFR also with an additive effect of increased HER2 expression. While these papers indicate EGFR/HER2 as having the converse effect to the present study they agree that increased expression of more than one marker together has an additive effect.

5.3. MULTIVARIATE ANALYSIS

All markers and marker combinations significant on univariate COX regression were analysed with multivariate COX regression including tumour grade and metastasis at diagnosis both for the ADT subcohort and full patient cohort 2. Only a relatively small proportion of the single markers and marker combinations found to be significant on univariate analysis and these are listed in chapter 4.

The most obvious reason for this reduction of significant associations on multivariate analysis would be that the significant results on univariate analysis were due to association between marker expression and Gleason/metastasis and indeed, within cohort 2 several of these associations are seen most notably high HER3, HER4 and HRG are all associated with either low/medium Gleason score or absence of metastases. Additionally there is the effect of attrition of subjects for each multivariate analysis making significance harder to achieve e.g. for a subject to be included in analysis for a given marker/TTR and metastasis in the ADT subcohort a subject must have 1) been stained for the correct marker 2) been treated with ADT, 3) had a biochemical relapse, 4) have the information available regarding metastasis at presentation; even with a starting cohort of over 350 subjects this number of requirements can reduce the pool of subjects to such low levels that significance is hard to achieve.

5.4 ANSWERS TO STUDY QUESTIONS

1. Are expression of HER 1-4 and EGFRvIII correlated with response to therapy/ time to relapse/time to death in prostate cancer?

Within this study there are multiple instances of expression of HER family members correlating with outcomes TTR and OS on Kaplan-Meier analysis with most of these also significant on univariate COX regression and a small proportion of these on mutivariate analysis. Not all HER family marker expressions investigated are significantly associated with outcomes and some trends are apparent

- High/Very High HER1-4 expression are associated with positive outcomes, EGFRvIII with negative
- Very High (Upper Quartile) expression is more likely to be associated with a significant outcome than high expression (above median)
- The most significant results on univariate analysis are seen with HER4 expression and a combination of all 4 main markers On multivariate analysis both HER4 and HER1-4 gave significant results for OS and TTR respectively in the full cohort.
- The majority of single Markers that give significant results are membranous staining rather than cytoplasmic or nuclear. As detailed in the background the membrane is the primary site where HER family receptors are activated before dimerisation and internalisation/signal transduction therefore membrane. Membrane expression could represent level of the receptor available for signal transduction hence a surrogate for activity. Alternatively high membrane expression could represent a lack of internalisation and therefore pathway activity. While few correlations in marker expression were seen the fact all correlations between membrane and cytoplasmic

expression that were demonstrated were positive rather than negative would suggest the former explanation rather than the latter. A nuclear role for HER3 have been noted earlier in this discussion (Koumakpayi 2006) but few correlations between prognosis and nuclear expression were seen in this study.

- Particularly with regard to Marker combinations there are a greater number of significant results within the ADT subcohort than in the full cohort 2. As detailed in the background HER family affects cellular activity via both pathways that interact with AR (Yeh 1999, Rochette-Egly 2003, Mellinghoff 2004) and those that do not (Lin 1999). One could hypothesise that given the increased time to biochemical recurrence and greater action in the ADT subcohort family pathway is acting to potentiate ADT or that it acts to block mechanisms that effect hormone escape.
- With outcomes TTR and OS in the ADT subgroup and TTR in the full cohort there is a general trend towards falling p-values with increased numbers of highly expressed HER family members. Notably the lowest p-values are seen where there is high membranous expression of all 4 HER family members. This gives a general indication of increased significance and an additive effect in terms of positive outcome the more HER family members are highly expressed. Additive effects of multiple HER family members have been noted in Bartlett (2005) and Di Lorenzo (2003). Witton et al. (2003) noted that in breast cancer patients HER1-3 were often co-expressed but rarely co-expressed with HER4. In ER positive patients with high expression of HER1-3 had a significantly worse prognosis than those expressing HER4 – HER1-3 acting in concert but in opposition to HER4 differing with this study which demonstrated all 4 markers correlated with positive outcome .

Heterodimerisation as a precursor to cellular action implies different HER family member expression can be associated with similar pathway activation and cellular effects hence increased expression of any is associated with increased pathway activity.

2. Is Heregulin (HRG) involved in mechanisms by which HER family proteins affect cancer progression?

Membranous HRG and in one experiment Nuclear HRG expression are associated with some positive outcomes in a similar fashion to HER1-4 however there is no correlation between HRG and individual HER family member expression. In cohort 1 high HRG expression in HSPC but not in HRPC is associated with improved outcome in terms of TTR/TTDFR/OS. In the full cohort 2 a combination of cytoplasmic HER3 and HRG improved outcome in terms of both TTR and OS. HRG is a primary ligand of HER3 and HER4 which are both associated with positive outcome as well. The greater significance values for HER4 would suggest that this is the most important factor and suggests it is via this that HRG has its positive effect. It could be hypothesised that the HRG-HER4 pathway acts to block mechanisms that bypass ADT but once this itself in bypassed hormone escape can occur and HRG-HER4 no longer has a protective effect.

3. Do HER family proteins effect oncogenesis via cell proliferation or reduced cell death?

In contrast to previous research, aside from EGFRvIII, the HER family members do not increase oncogenesis however there are significant correlations with positive outcomes. In the regression analyses neither KI67 nor TUNEL assay correlate with expression of any of the HER family members, additionally there is only one instance of expression of KI67 or TUNEL being associated with outcome and this was a negative one (High KI67

expression associated with **decreased** overall survival). Therefore there is no evidence within this study indicating that HER family members exert their influence primarily by either proliferation or apoptosis. This could be due to problems with the staining/scoring of the proliferation or apoptotic measures; – although the KI67 staining does give some expected results the TUNEL assay gave no significant results whatsoever indicating no influence of apoptosis on outcomes or that the assay itself was inaccurate. Alternatively it could be that proliferation and apoptosis as endpoints are too general to pick up subtle cellular effects of the HER family or that the HER pathways act via other cell mechanisms entirely.

4. Are trends seen in the pilot study borne out with a larger patient base?

The general trends of the pilot study within HSPC patients – positive outcomes associated with high HER2 and HER4 expression with negative outcomes associated with EGFRvIII are borne out in the larger patient base. In fact high expression of other family members EGFR and HER3 was also correlated with positive outcomes as well as some combinations of family members. In terms of specific conclusions some are carried over but the most notable HER 2 as an independent positive predictive marker of time to relapse in hormone sensitive tumours was not confirmed on multivariate analysis (although significant on Kaplan-Meier, univariate COX and in combination with cytoplasmic HER3 which was an independent predictor in this study).

5.5 IMPLICATIONS OF RESULTS

As was suggested by the pilot study HER family members are associated with positive outcomes in hormone sensitive prostate cancer and as such are not suitable for targeting with immunotherapy in this patient group. This also provides an explanation as to why no antitumour agents targeting HER family members have emerged as clinically viable in CaP. EGFRvIII remains the only negatively prognostic family member but given the lack of EGFRvIII staining possible in this study, it is difficult to draw conclusions regarding EGFRvIII targeting. Where HRG expression is associated with significant results high expression is positively prognostic therefore might be involved with activation of the HER family in this scenario.

High expression of HER family members is positively prognostic and there is evidence within this study that there is an additive effect with increased numbers of HER protein markers positively expressed. This effect is more profound in patients who have undergone ADT. Membranous expression of all 4 HER family members is an independent predictor of delayed TTR. Individual markers that have emerged as independent predictors within this study are

- Very High Membrane expression of EGFR in ADT patients
- Very high membrane expression of EGFRvIII, High cytoplasm expression of HER3, High membrane expression of HER4 in all patients

The possibility is raised for HER family member expression to be used as a prognostic test to indicate those most likely to respond well to ADT and therefore not require other treatments.

5.6 LIMITATIONS OF STUDY

The principle limitations to this study are incomplete staining of the full cohort and incomplete patient information. While the full cohort number of n=357 is high for a tissue based study, limitations in availability of all TMAs and commercial antibodies reduce the patient numbers for each specific marker reducing the statistical power of the study below its apparent level.

Despite rigorous data collection it is not possible to obtain all relevant information for each patient due to the limitations of availability of patient records, the large number of patients, the extensive duration over which original data was collected given the prolonged course of CaP and inconsistent completeness of computer records the further back in history you go. The incompleteness of information is apparent in the recorded demographics of the patient cohorts in this study where a proportion of patients have no recorded stage, Gleason score, metastasis status, hormone escape stage etc. again reducing the statistical power of the conclusions based on this data. The high number of subjects is, in itself, an attempt to compensate for the anticipated gaps in patient data.

The heart of this study is the modified histoscore – a subjective assessment of staining. The methods by which this subjective method is addressed and compensated for are detailed in this and previous studies (Kirkegarrd 2006).

5.7 CONCLUSION

In this study high expression of EGFR, HER2-4 and HRG in Hormone Sensitive Prostate Cancer were correlated with improved prognosis in terms of time to biochemical relapse and overall survival. EGFRvIII was correlated with poor prognosis. Several marker expression combinations were also correlated with improved prognosis including high expression of all 4 main family members together. Significant results are more common in a subcohort of patients treated with ADT. High expression of HER4 and high expression of HER1-4 together give the most significant results. While HER4 and HRG have previously been associated with positive outcome this study contradicts previous literature regarding EGFR and HER2 which have previously been associated with poor prognosis. The possibility is raised that HER family member expression be used as a prognostic test to indicate those most likely to respond well to ADT.

REFERENCES

1. Aguilar, Z. Akita, R.W. Finn, R.S. et al**. (**1999) Biologic effects of heregulin/neu differentiation factor on normal and malignant human breast and ovarian epithelial cells. *Oncogene*, **18**, 6050
2. Agus, D.B. Scher, H.I. Higgins, B. Fox, W.D. et al. (1999) Response of prostate cancer anti HER 2/neu antibody in androgen dependent and independent human xenograft models. *Cancer*, **59**: 4761-4
3. Angelucci, A. Gravina, G.L. Rucci, N. et al. (2006) Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice. *Endocrine-Related Cancer Res*.;**13(1)**:197-210
4. Azios NG, Romero FJ, Denton MC, Doherty JK, Clinton GM. (2001) Expression of Herstatin, an autoinhibitor of HER2/neu, inhibits transactivation of HER3 by HER2 and blocks EGF activation of the EGF Receptor. *Oncogene*;**20**:5199-209.
5. Bacus SS, Gudkov AV, Zelnick CR, et al. (1993) Neu differentiation factor (heregulin) induces expression of intercellular adhesion molecule 1: implications for mammary tumors. *Cancer Res*.**53:**5251
6. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. (2003) Constitutive activation of the ras/mitogen activated protein kinase signalling pathway promotes androgen hypersensitivity in LNCAP prostate cancer cells. *Cancer res*. **55**:1981-9
7. Barnes NL, Khavari S, Boland GP, et al. (2005) Absence of HER4 expression predicts recurrence of ductal carcinoma *in situ* of the breast. *Clinical Cancer Results*; **11**:2163–8
8. Bartlett JM, Edwards J et al. (2005) Type I receptor tyrosine kinases are associated

with hormone escape in prostate cancer. *Journal of Pathology*, **205**: 522-9

9. Bellezza I, Bracardo S, Caserta C, Minjelli A. (2006) Targetting of EGFR tyrosine kinase by ZD1839 in androgen responsive prostate cancer in vitro. *Molecular Genetics and Metabolism*.**88**:114-22
10. Ben-levy R, Paterson HF, Marshall CJ et al (1994). A single autophosphorlation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO Journal*;**13**:3302-11
11. Berger R, Lin DI, Nieto M, et al. (2006) Androgen-dependent regulation of Her2/neu in prostate cancer cells. *Cancer Res* ;**66**:5723-8
12. Bhowmick,N.A., Chytil,A., Plieth,D. et al. (2004) TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*,**303**:848–851
13. Blackard C, Doe RP, Mellinger GT et al. (1970) Incidence of Cardiovascular disease and death in patients receiving diethylstilbestrol for carcinoma of the prostate. *Cancer*; **26**:249-56
14. Blanco-Aparicio C, Molina MA, Fernandez-Salas E, et al.(1998) Potato Carboxypeptidase inhibitor, a T knot protein, is an epidermal growth factor antagonist that inhibits tumor cell growth. Journal of Biological Chemistry;**273**:12370-7
15. Bonaccorsi L, Carloni V, Muratori M, et al. (2004) EGF Receptor signalling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between EGFR and androgen receptor. *International Journal of Cancer*;**112**:78-86.
16. Bonaccorsi L, Marchiani, Muratori M, Forti G, Baldi E. (2004) Gefitinib (‘IRESSA’, ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing

PI3K/AKT activation. Journal Cancer Res Clinical Oncology;**130**:604-15

17. Bookstein R, Rio P, Madreperla S, et al.(1990) Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. *Proceedings of National Academy Science. USA*;**87**:7762-7
18. Borg A et al. (1994) ErbB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Letters*.**;81**:137-44
19. Brennan PJ, Kumagai T, Berezov A, et al. (2000) HER2/neu mechanisms of dimerisation/ oligomerisation . *Oncogene*;**19**:6093-101
20. Brown RSD, Edwards J, Dogan A, et al. (2002) Amplification of the androgen receptor gene in bone metastases from hormone refractory prostate cancer. *Journal of Pathology* ;**198**:237-44
21. Burden S, Yarden Y. (1997) Neuregulins and their receptors a versatile signalling module in organogenesis and oncogenesis. *Neuron* ;**18:**847-55
22. Calvo BF, Levine AM, Marcos M, et al. (2003) Human epidermal receptor-2 expression in prostate cancer. *Clinical Cancer Res*;**9**:1087–97
23. Cancer Research UK: UK Prostate Cancer Statistics. (2007) 09/05/2007, Available form world wide web http://info.cancerresearchuk.org/cancerstats/types/prostate/ ?a=5441
24. Canil CM, Moore MJ, Winquist E, et al. (2005) Randomised phase II study of two doses of gefitinib in hormone refractory prostate cancer. *Journal of Clinical Oncology*;**23**:455-60
25. Carpenter G. (2001) Employment of the epidermal growth factor receptor in growth factor independent signalling pathways. *Journal of Cell Biology*;**146**:697-702

26. Carraway KL, Sweeney C. (2001) Localization and modulation of ErbB receptor tyrosine kinases. *Current Opinion Cell Biology*;**13**:125-30

27. Carter BS, Beaty TH, Steinberg GD, et al. (1992) Mendelian inheritance of familial prostate cancer. *Proceedings National Academy Science*;**89**:3367-71

28. Chen CD, Welbsie DS, Tran C, et al. (2000) Molecular determinants of resistance to antiandrogen therapy. *Nature Medicine*;**10**:33-9

29. Chen,G., Shukeir,N., Potti,A, et al. (2004) Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer*, **101**:1345–1356.

30. Chen T, Cho RW, Stork PJ, Weber MJ. (1999) Elevation of cyclic adenosine 3,5-monophosphate potentiates activation of mitogen activated protein kinase by growth factors in LNCaP prostate cancer cells. **59**:2000-6

31. Chen X, Yeung TK, Wang Z**. (**2000)Enhanced drug resistance in cells coexpressing ErbB2 with EGF receptor or ErbB3 *Biochem Biophys Res Commun*;**277**:757

32. Chung, L.W., Baseman,A., Assikis,V. Zhau,H.E. (2005) Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J. Urol*;**173**: 10–20

33. Craft N, Chhor C, Tran C et al. (1999) Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumours through a 2 step process, *Cancer Res*;**53**:5030-6

34. Craft N, Shostak Y, Carey M, Sawyers CL. (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signalling by the HER2/neu tyrosine kinase. *National Medicine*;**5**:280-5

35. Culig Z, Klocker H, Bartsch G, Hobisch A, (2002) Androgen Receptors in prostate cancer. Endocrine-related cancer;**9**:155-70

36. Daly JM, Jannot CB, Beerli RR et al. (1997) Neu differentiation factor induces ErbB2 down-regulation and apoptosis of ErbB2-overexpressing breast tumor cells. *Cancer Res*;**57**:3804

37. Davies MA, Koul D, Dhesi H. et al. (1999) Regulation of Akt/PKB activity, cellular growth and apoptosis in prostate carcinoma cells by MMAC/PTEN. *Cancer Res*;**59**:2551-6

38. Debes JD, Tindall DJ. (2002) The Role of Androgens and the androgen receptor in prostate cancer. *Cancer Letters*;**187**:1-7

39. De Bono JS, Rowinsky EK. (2002) The ErbB Receptor family: a therapeutic target for cancer. *Trends in Molecular Medicine*;**8**:S19-26

40. De Miguel MP, Royuela M, Bethencourt FR, et al. (1999)Immunohistochemical comparative analysis of transforming growth factor α, epidermal growth factor receptor in normal, hyperplastic and neoplastic human prostates. *Cytokine*;**11**:722-7

41. Diamonti AJ, Guy PM, Ivanof C et al. (2002) An RBCC protein implicated in maintenance of steady state neuregulin receptor levels. *Proceedings National Academy Science USA*;**99**:2866-71

42. Di Lorenzo G, Autorino R, De Laurentiis M, et al. (2004) Her2/neu receptor in prostate cancer development and progression to androgen independence. *Tumori*;**90**:163-70

43. Di Lorenzo G, Bianco D, Tortora G et al. (2003) Involvement of Growth factor receptors of the epidermal growth factor receptor family in prostate cancer

development and progression to androgen independence. *Clinical Prostate Cancer* ;**2**:50-57

44. Di Lorenzo G, Tortora G, Armiento FP, et al. (2002) Expression of epidermal growth factor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clinical Cancer Research* ;**8**:3438-44
45. Doherty JK, Bond C, Jardim A, Adelman JP, Clinton GM. (1999) The HER-2/neu receptor tyrosine kinase gene encodes a secreted autoinhibitor. *Proceedings National Academy Science USA*;**96**:10869-74
46. Duan L, Miura Y, Dimri M, et al. (2003) Cbl mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. *Journal Biological Chemistry*;**278**:228950-60
47. Earp HS, III, Calvo BF, Sartor CI. (2003). The EGF receptor family: multiple roles in proliferation, differentiation, and neoplasia with an emphasis on HER4. *Trans Am Clin Climatol Assoc*;**114**:315–3
48. Edwards J, Bartlett J et al. (2004). HER2 and COX2 expression in human prostate cancer. *European Journal of Cancer* **40**:50-5
49. Edwards J, Krishna NS, Bartlett JMS. (2004) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. *British Journal of Cancer*;**89**:552-6
50. Edwards J, Krishna NS, Mukherjee R, Bartlett JMS. (2004) The role of c-Jun and c-Fos in androgen-independent prostate cancer. *Journal of Pathology* ;**204**:153-8
51. Edwards J, Krishna NS, Witton CJ, Bartlett JMS. (2003) Gene amplifications associated with the development of hormone resistant prostate cancer. *Clinical*

Cancer Research;**9**:5271-81

52. Edwards J, Mukherjee R, Munro AF, et al. (2004) HER2 and COX2 expression in human prostate cancer. *European Journal of Cancer*;**40**:50-55
53. Edwards J. Traynor P. Munro AF. Pirret CF. Dunne B. Bartlett JM. (2006) The role of HER1-HER4 and EGFRvIII in hormone-refractory prostate cancer. *Clinical Cancer Research*, **12(1),** 123-30
54. Elson SD, Browne CA, Thorburn CG. (1984) Identification of epidermal growth factor-like activity in human male reproductive tissue and fluids. *Journal Clinical Endocrinology and Metabolism*, **48**, 673-9
55. Emmett-Buck MR, Vocke CD, Pozzatti RO, et al. (1995) Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia. *Cancer Research*., **55**, 2959-62
56. Ennis BW, Lippman ME, Dickson RB. 1991 The EGF Receptor system as a target for antitumour therapy. *Cancer investigation*, **9**, 241-50
57. Falls DL. (2003) Neuregulins: functions, forms, and signaling strategies. *Experimental Cell Research*, **284**:14
58. Feldman BJ, Feldman D. (2001) The development of androgen-independent prostate cancer. *National Review Cancer*, **1**:3-45
59. Ferlay J. et al. GLOBOCON (2002). Cancer Incidence, Mortality and Prevalence Worldwide. IARC Cancerbase No. 5 Version 2.0. Iarcpress, Lyon
60. Ferretti, G, Felici A, Papaldo P, Fabi A, Cognetti F. (2007) HER2/neu role in breast cancer: from a prognostic foe to a predictive fiend. *Current Opinion Obstetrics Gynaecology*, **19**:56-62

61. Feuer E, Merrill R, Hankey B. (1999) Cancer Surveillance Series: Interpreting Trends in Prostate Cancer-Part II. Cause of Death Misclassification and the Recent Rise and Fall in Prostate Cancer Mortality. Journal of the National Cancer Institute. **91**:1025-32

62. Fong CJ, Sherwood ER, Mendleson J, Lee C, Kozlowski JM. (1992) Epidermal growth factor receptor monoclonal antibody inhibits constitutive receptor phosphorylation, reduces autonomous growth and sensitized androgen-independent tumor necrosis factor. *Cancer Research*, **52**:5887-92

63. Formento P, Hannoun-Levi JM, Fischel JL, et al. (2004)Dual HER1-2 targetting of hormone refractory prostate cancer by ZD1839 and trastuzamab *European Journal Cancer* **40**:2837-44

64. Formento P, Hannoun-Levi JM, Gerard F, et al. (2005) Geftinib-tratuzamab combination in hormone-refractory prostate cancer xenografts. *European Journal Cancer*,**1**:167-73

65. Foster,C.S., Dodson,A., Karavana,V., Smith,P.H. Ke,Y. (2002) Prostatic stem cells. *Journal of Pathology.* 2002;197:551–565.

66. Fournier G, Latil A, Amet Y, et al. Gene Amplification in advanced-stage human prostate cancer Urol Res,**22**:343-47

67. Fowler Jr JE, Lau JLT, Ghosh L, Mills SE, Mounzar A. (1998) Epidermal growth and prostatic carcinoma: an immunohistochemical study. Journal of Urology, 139:87-61

68. Fromont G, Vallencian G, Validire P, Levillain P, Cusenot O. (2007) BCAR1 expression in prostate cancer;67:268-73

69. Garret TP, Mckern NM, Lou M et al. (2003) The crystal structure of a truncated

ErbB2 ectoderm reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell*;**11**:495-505

70. Gelfanov VM, Burgess GS, Litz-Jackson S, et al. (2001) Transformation of interleukin-3-dependent cells without participation of Stat5/bcl-xL: cooperation of akt with raf/erk leads to p65 nuclear factor kappa B mediated antiapoptosis involving c-IAP2. *Blood*, **;98**:2508-17
71. Genetics Home Reference. Dec 27. 2007. Available from world wide web http://ghr.nlm.nih.gov/ghr/.
72. Ghosh PM, Bedolla R, Mikhailova M, Kreisberg JI. (2002) RhoA-dependent murine prostate cancer cell proliferation and apoptosis: role of protein kinase Czeta. *Cancer Research*, **62**:2630-6
73. Ghosh PM, Malik S, Bedolla R, Kreisberg JI. (2003) Akt in prostate cancer: Possible role in androgen independence. *Current Drug Metabolism*,**310**:87-96
74. Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Lunec J. (1997) Prognostic significance of HER2 and HER4 expression in childhood medulloblastoma. *Cancer Research*, **57**:3272-80
75. Glynne-Jones E, Goddard L, Harper ME. (1996) Comparative analysis oh mRNA and protein expression for epidermal growth factor receptor and ligands relative to the proliferative index in human prostate tissue. *Human Pathology*, **27**:668-94
76. Graff JR. (1997) Emerging targets in the AKT pathways for treatment of androgen-independent prostatic Adenocarcinoma. Epxpert Opin Ther Targets. 2002;6:103-13
77. Grasso et al. ErbB kinases and NDF signalling in human prostate cancer cells. *Oncogene*, **15**: 2705-16

78. Graus Porta D, Beerli RR, Daly JM, Hynes NE. (1997) ErbB2, the preferred heterodimerisation partner of all ErbB receptors, is a mediator of lateral signalling. *EMBO Journal*, **16**:1647-55

79. Gregory CW, Whang YE. McCall W. et al. (2005) Heregulin-induced activation of HER 2 and HER 3 increases androgen receptor transactivation and CWR0R1 human recurrent prostate cancer cell growth. *Clinical Cancer Research*, **11**:1704-12

80. Guy PM Platko J, Cantley LC, Cerione RA, Carraway KL. (1994) Insect cell expressed p180ErbB3 possesses an impaired tyrosine kinase activity. *Proceedings National Academy Science USA*,**91**:8132-6

81. Hankey B et al. (1999) Cancer Surveillance Series: Interpreting Trends in Prostate Cancer-Part 1: Evidence of the Effects of Screening in Recent Prostate Cancer Incidence. Mortality and Survival Rates. *Journal of the national cancer institute.*, **91**:1017-24

82. Henry RY, O'Mahoney D. (1999) Treatment of Prostate Cancer. *Journal Clin Pharm Ther* **24**:93

83. Hernes E, Fossa SD. Berner A. Otnes B. Nesland JM. (2004) Expression of the epidermal growth factor family in prostate carcinoma before and after androgen-independence. *British Journal Cancer*, **90**: 449-54

84. Hilmy M, Campbell R, Bartlett JMS, McNicol A-M, Underwood MA, McMillan DC. (2006) The Relationship between the systemic inflammatory response, tumour proliferation activity, T-lymphocytic infiltration and COX-2 expression and survival in patients with transitional cell carcinoma of the urinary bladder. *British Journal Cancer*,**95**:1234-8

85. Holmes WE, Sliwkowski MX, Akita RW, et al. (1992) Identification of Heregulin, a specific activator of p185ErbB2. *Science,* **256**:120-10

86. Hudziak RM, Schlessinger J, Ullrich A. (1987) Increased expression of the putative growth factor receptor p185 HER2 causes transformation and tumourigenesis of HIH 3T3 cells. *Proceedings National Academy Science USA*,**84**:719-63.

87. Huggins C, Hodges CV. Studies on Prostate Cancer I: (1941) The effect of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Research,* **43**:209

88. Huggins C, Stevens RE, Hodges CV. (1941) Studies on Prostate Cancer II: The effects of castration on advanced carcinoma of the prostate gland. *Archives Surgery*,**1**:293-7

89. Hung MC. Hortobagyi GN. Ueno NT, (2000) Development of clinical trial of E1A gene therapy targeting HER-2/neu-overexpressing breast and ovarian cancer. *Adv. Exp. Med. Biol,* **465**:171–80

90. Irvine RA, Yu MC, Ross RK, Coetzee GA. (1995). The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer. *Cancer Research,* **55**:1937-40

91. Isaacs WB, Bova GS, Morton RA. (1995) Molecular genetics and chromosomal alterations in prostate cancer. *Cancer*, **75**:2004-12

92. Johannsson S, Ljunggren E. (1981) Prostatic carcinoma with hormonal treatment. Scandinavian Journal of Urology and Nephrology, 1:331-2

93. Jones JT, Akita RW, Silwkowski MX, (1999) Binding specificities and affinities of EGF domains for ErbB receptors. *FEBS Letters*, **447**:227-31

94. Kambhampati S, Ray G, Sengupta K et al. (2005) Growth Factors involved in prostate carcinogenesis. *Front. Biosci*,**10**:1355-67

95. Karhadkar,S.S., Bova,G.S., Abdallah,N et al. (2004) Hedgehog signaling in prostate regeneration, neoplasia and metastasis. *Nature*, **431**:707–712

96. Karunagaran D, Tzahar E, Beerli RR et al. (1996) ErbB2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO Journal*, **15**:254-64

97. Kim HG, Kassis I, Sorrto JC, Turner T, Wells A. (1999) EGF receptor signalling in prostate morphogenesis and tumorigenesis. Histology and Histopathology, 14:1175-82

98. King CR, Kraus CH, Aaoronson SA, (1985) Amplification of a novel v-ErbB gene in human mammary carcinoma. *Science*,**229**:974-6

99. Kirkegaard T, Edwards J, Tovey S, et al. (2006) Observer variation in immunohistochemical analysis of protein expression, time for a change? *Histopathology*, **48**:787-94

100. Klapper LN et al. (1999) The ErbB2/HER2 oncoprotein of human carcinoma may function as a shared coreceptor for multiple stoma derived growth factors. *Proceedings National Academy Science USA*, **96:**4995-5000

101. Koumakpayi IH, Diallo JS, Le Page C, et al. (2006) Expression and nuclear localization of ErbB3 in prostate cancer, *Clinical Cancer Research*, **12**:2730-7

102. Kuhn EJ, Kurnot RA, Sesterhenn IA, et al. **(1993)** Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma *Journal of Urology*, **150**:1427-33

103. Kumar VL, Majumder PK, Gujral S, Kumar V. (1998) Comparative analysis of

epidermal growth factor mRNA levels in normal, benign hyperplastic and carcinomatous prostate. *Cancer Letters*. **134**:177-880

104. Lara PN, Meyers FJ, Gray CR, et al. (2002) HER2/neu is overexpressed infrequently in patients with prostate carcinoma. *Cancer*, **94**:2584-9
105. Lee H, Akita RW, Sliwlowsli MX, Maihie NJ. (2001) A naturally occurring secreted human ErbB3 receptor isoforms inhibits Heregulin stimulated activation of ErbB2, ErbB3 and ErbB4. *Cancer Research*, **61**:4467-71
106. Lee HJ, Chang C. (2003) Recent Advances in androgen receptor action. Cellular and *Molecular Life Sciences*, **60**:1613-22
107. Lee SO, Lou W, Hou W, et al. (2003) Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clinical Cancer Research*, **9**:370-6
108. Lenferink AE, Pinkas-Kramarski R, van de Poll ML, et al. (1998) Differential endocytic routing of homo and heterodimeric ErbB tyrosine kinases confers signalling superiority to receptor heterodimers. EMBO Journal. **17(12):**3385-97
109. Leung HY, Weston J, Gullick WJ, Williams G. (1997) A potential autocrine loop between Heregulin alpha and erbB-3 receptor in human prostate Adenocarcinoma. Be *Journal of Urology*, **79**:212-6
110. Levi F, Negri E, LaVecchia S. (2000) Recent trends in prostate cancer mortality in the European Union. *Epidemiology*, **11**:612
111. Levkowitz G, Klapper LN, Tzahar E, et al. (1996) Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins. *Oncogene*, **12**:1117-25
112. Levkowitz G, Waterman H, Ettenberg SA, et al. (1999) Ubiquitin ligase activity and

tyrosine phosphorylation underlie suppression of growth factor signalling by c-Cbl/Sli-1. *Mol Cell*, **4**:1029-40

113. Li J, Yen C, Liaw D, et al. (1997) PTEN, a Putative Tyrosine Phosphatase Gene Mutated in Human Brain, Breast and Prostate Cancer. *Science*, **275**:1943-47
114. Li Y, Cozzi PJ, Qu CF, et al. (2004) Cytotoxicity of human prostate cancer cell lines in vitro and induction of apoptosis using 213Bi-Herceptin alpha-conjugate. *Cancer Letters*, **205**:161-71
115. Liao YD, Grobholz R Abel U et al. (2003) Increase of Akt/PKB expression correlates with Gleason pattern in human prostate cancer. *International Journal Cancer*, **107**:676-80
116. Lin J, Adam RM, Santiestevan E, Freeman MR. (1999) The phosphstidylinositol 3-kinase pathway is a dominant growth factor activated cell survival pathway in LNCaP human prostate Adenocarcinoma cells. *Cancer Research*, **9**:2891-7
117. Lyne JC Melhem MF. Finley GG et al. (1997) Tissue expression on neu differentiation factor/heregulin and its receptor complex in prostate cancer and its biologic effects on prostate cancer cells in vitro. *Cancer journal from Scientific American*, **3**:21-30
118. Macdonald A, Habid FK.(1992) Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *British Journal of Cancer*, **65**:177-82
119. Macoska JA, Trybs TM Sakr WL et al. (1994) Fluorescence in situ hybridisation analysis if 8p allelic loss and chromosomal instability in human prostate cancer. *Cancer Research*, **85**:382-30

120. Maddy SQ, Chisholm GD, Busuttil A, Habib FK. (1989) Epidermal growth factor receptors in prostate cancer: correlation with histological differentiation of the tumour. *British Journal Cancer*, **60**:41-44

121. Magne N, Pivot X, Bensdoun RJ, et al. (2001) The relationship of epidermal growth factor receptor levels to the prognosis of unresectable pharyngeal cancer patients treated by chemo-radiotherapy. *European Journal Cancer*, **37**:2169-77

122. Majeed A. et al. (2000) Trends in prostate cancer incidence, mortality and survival in England and Wales 1971-98. *British Journal of Urology*, **85**:1058-62

123. Marinissen MJ, Gutkind JS. (2001) G-protein coupled receptors and signalling network: emerging paradigms. *Trends in Pharmacology*, **22**:368-76

124. Mark HFL, Feldman D, Das S, et al. (1999) Fluorescence in situ hybridization study of HER2/neu oncogene amplification in prostate cancer. *Exp Molecular Pathology*, **66**:170-8

125. Mass RD(2004) The HER receptor family: a rich target for therapeutic development. *International Journal Radiation Oncology Biology Physics*, **58**:932-940

126. Maygarden SJ, Novotny DB, Moul JL, et al. (1994) Evaluation of cathepsin D and epidermal growth factor receptor in prostate carcinoma. *Modern Pathology*, **7**:930-6

127. McCall P, Gemmell L, Mukherjee R, Bartlett JMS, Edwards J. (2008) Phosphorylation of Androgen Receptor is associated with decreased survival in hormone refractory prostate cancer patients. Accepted by *British Journal of Cancer*

128. McCarty KS, Szabo E, Flowers JL, et al. (1986) Use of monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumours. *Cancer Research*,**46S**:4244-8

129. Mellinghoff IK, Vivanco I, Kwon A, et al. (2004) HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell*, **6**:517-27

130. Mellon K et al. (1992) P53, c-erbB-2 and the EGFR in the benign and malignant prostate. *Journal of Urology*, **147**: 496-9

131. Memon AA. Sorensen BS. Melgard P. Fokdal L. Thykjaer T. Nexo E. (2004) Expression of HER3, HER4 and their ligand heregulin-4 is associated with better survival in bladder cancer patients *British Journal of Cancer*.**91(12)**:2034-41

132. Mendoza N, Phillips GL, Silva J, Schwall R, Wickramasinghe D. (2002) Inhibition of Ligand-Mediated HER2 Activation in Androgen-independent Prostate Cancer. *Cancer Research*, **62**:5485-8

133. Mimeault M, Batra SK. (2006) Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies. *Carcinogenesis*, **27**:1-22

134. Mimeault M, Pommery N, Henichart J-P. (2003) New Advances on Prostate Carcinogenesis and Therapies: Involvement of EGF-EGFR Transduction System. Growth Factors **21**:1-14

135. Morote J, de Torres I, Carne C, et al. (1999) Prognostic value of immunohistochemical expression of the c-erbB-2 oncoprotein in metastatic prostate cancer. *International Journal of Cancer*, **84**:421-5

136. Morris MJ, Reuter VE, Kelly WK et al. (2002) HER-2 profiling and targeting in prostate carcinoma. *Cancer*, **94**:980-6

137. Moscatello K, Holga-Madruga M, Godwin A, et al. (1995) Frequent expression of a

mutant epidermal growth factor in multiple human tumours. *Cancer Research*, **55**:5536-9

138. Mosesson Y, Shtiegman K, Katz M, et al. (2003) Endocytosis of receptor tyrosine kinases is driven by monoubiquitinylation, not polyubiquitinylation. *J Biol Chem*, **278**:21323-6
139. Moul JW, Maygarden SJ, Ware JL, et al. (1996) Cathepsin D and epidermal growth factor receptors immunohistochemistry does not predict outcome of prostate cancer patients undergoing radical prostatectomy. *Journal of Urology*, **155**:982-5
140. Murphy GP, Slack NH. (1980) Response criteria of the prostate of the USA National Prostate Cancer Project. Prostate, **1**:375-82
141. Myers RB, Grizzle WE. (1997) Changes in biomarker expression in the development of prostatic Adenocarcinoma. *Biotech Histochem*, **72**:86-95
142. Myers RB, Srivastava S, Oelschlager DK, Grizzle WE. (1994) Androgenic regulation growth factor receptor expression, **86**:1140-5
143. Nasu K, Tanji N, Nishioki R. (2006) Expression of ErbB Proteins in Human Prostate. *Archives of Andrology*, 52:185-90
144. Navone NM, Trincoso P, Pisters LL, et al. (1993) P53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *Journal of the National Cancer Institute*, **8**:1657-69
145. Nesbit RM, Baum WC, (1950) Endocrine Control of Prostate Carcinoma. *Journal of the American Medical Association*, **143**: 1317-20
146. Nicholson R, Gee JM, Harper ME (2001). EGFR and cancer prognosis. *European Journal of Cancer*, **37**:S9-15

147. Normanno N. Kim N, Wen D et al. (1995) Expression of messenger RNA for amphiregulin, Heregulin and cripto-1, three new members of the epidermal growth factor family, in breast carcinomas. *Breast Cancer Research and Treatment*,**35**:293-7.

148. Okegawa T, Kinjo M, Nutahara K, Higashihara E. (2006) Pretreatment serum level of HER2/neu as a prognostic factor in metastatic prostate cancer patients about to undergo endocrine therapy. *International Journal Urology*,**13**:1197-201

149. Olapade-Olaopa EO: (2001) The expression of a mutant epidermal growth factor receptor in prostatic tumours. *British Journal Urology International*, **87**:224-6

150. Olapade-Olaopa EO, Moscatello DK, Mackay EH, et al.(2000) Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. *British Journal Cancer*,**82**: 184-194

151. Oosterhoff JK, Kuhne LC, Grootegoed JA, et al. (2005) EGF signalling in prostate cancer cell lines is inhibited by a high expression level of the endocytosis protein REPS2. *International Journal Cancer*, **113**:561-7

152. Osman I, Mikhail M, Schuch B, et al. (2000) Serum levels of shed HER2/neu protein in men with prostate cancer correlate with disease progression. *Journal of Urology*; 174:2174-7

153. Paik S, Liu E. (2000) HER2 as predictor of therapeutic response in breast cancer. *Breast Disease*, **11**:91-102

154. Peles E, Bacus SS, Koski RA, et al. (1992) Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells *Cell*, **69**:205

155. Penuel E, Akita RW, Sliwkowski MX. (2002) Identification of a region within the

ErbB2/HER2 intercellular domain that is necessary for ligand-independent association. Journal of Biological Chemistry 2002;277:28468-73

156. Pfeil K, Eder IE, Putz T, et al. (2000) Long term androgen ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. *Prostate* **58**:2004
157. Poller DN, Spendlove I, Baker C, et al. (1992) Production and characterization of a polyclonal antibody to the c-erbB-3 protein: examination of c-ernN-3 protein expression in adenocarcinomas. *Journal of Pathology*, **168**:275-80
158. Polychronis A, Simmet HD, Hadjiminas D et al. (2005) Preoperative gefitinib versus gefitinib and anastrazole in postmenopausal patients with oestrogen-receptor positive an RGFR positive primary breast cancer, a double-blind placebo-controlled phase II randomised trial. *Lancet Oncology*, **6**:383-91
159. Press MF et al. HER2/neu gene amplification characterised by fluourescence in situ hybridization: poor prognosis in node negative breast carcinomas. J Clin Oncol. 1997;15:289-904
160. Prigent SA, Lemoine NR, Hughes CM, et al. (1992**)** Expression of the c-erbB-3 protein in normal human adult and fetal tissues *Oncogene*, **7**:1273-8
161. Pritchard KI, Shepherd LE, O'Malley FP, et al. (2006) HER2 and responsiveness of breast cancer to adjuvant chemotherapy. *New England Journal Medicine*,**354**:2103-11
162. Qiu Y, Ravi L, Kung HJ. (1998) Requirement of ErbB2 for signaling by interleukin-6 in prostate carcinoma cells. *Nature*, **393**: 83-85
163. Qui XB, Goldberg AL. (2002) Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member ErbB3. *Proceedings National Academy Science USA*, **99**:14843-8

164. Rana S, Sen R, Kalra R, et al. (2006) Immunohistochemical study of the expression of epidermal growth factor receptor in benign prostatic hypertrophy, prostatic intraepithelial neoplasia and prostatic carcinoma. *Indian Journal of Pathology and Microbiology*, **9**:495-9

165. Reese DJ, van Raj TM, Plowman GD, Andrews GC, Stern DF. (1995) The cellular response to neuregulins is governed by complex interactions of the ErbB receptor family. *Molecular Cell Biology*, **15**:5770-6

166. Rio C, Buxbaum JD, Peschon JJ, Corfas C. (2000) Tumor Necrosis Factor-α-converting Enzyme Is Required for Cleavage of erbB4/HER4. Journal of Biological Chemistry, 14:10379-87

167. Rochette-Egly C. (2003) Nuclear receptors. Integration of multiple signaling pathways through phosphorylation. *Cellular Signaling* **15**:3850-66

168. Rosenthal M, Toner G. (2003) Inhibition of epidermal growth factor in HRPC. Initial results of a phase II trial of gefitinib. *Proceedings American Society Clinical Oncology*;**21**:416

169. Ross JS, Fletcher JA. (1998) The HER2/neu oncogene in breast cancer: prognostic factor, predictive factor and target for therapy. *Stem Cells,* **16**:413-28

170. Ross JS, Sheehan CE, Hayner-Buchan AM, et al. (1997) Prognostic significance of HER2/neu gene amplification status by fluorescence in situ hybridization of prostate carcinoma. *Cancer*, **79**:2162-70

171. Rubenstein M. Mirochnik Y. Chou P. Guinan P. (1996) Antisense oligonucleotide intralesional therapy for human PC-3 prostate tumors carried in athymic nude mice. *Journal of Surgical Oncology*, **62(3):**194-200

172. Sadar MD. (1999) Androgen independent induction of prostate specific antigen gene expression via crosstalk between the androgen receptor and protein kinase A signal transduction pathways. *Journal Biological Chemistry*, **27**:7777-83

173. Sadasivan R, Morgan R, Jennings S, et al. (1993) Overexpression of Her 2/neu may be an indicator of poor prognosis in prostate cancer. *Journal of Urology* **150**:126-31

174. Saloman DS, Brandt R, Ciardello, Normamo N. (1995) Epidermal growth factor related peptides and their receptors in human malignancies. *Critical Review Oncology, Haematology,* **19**:183-232

175. Sartor CI, Zhou H, Kozlowska E, et al. (2001) HER 4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells. *Molecular Cell Biology*, **21**: 4265-75

176. Sato B. (1999) Can an autocrine loop explain sex hormone dependent tumor growth? A brief overview. *Oncology*, **57**:S3-6

177. Savainan KJ, Saramaki OR, Linja MJ, et al. (2002) Expression and gene copy number analysis of ErbB2 oncogene in prostate cancer. *American Journal of Pathology*. 1**60**:339-45

178. Schally AV, Caomaru-Schally AM, Gonzalez-Barcena D. (1992) Present status of agonistic and antagonistic analogs of LH-RH in the treatment of advanced prostate cancer. *Biomedical Pharmacotherapy*, **46**:465-71

179. Schechter AL, Stern DF, Vaidyenathen L, et al. (1984) The neu oncogene: an erb-B related gene encoding a 185 000 MR tumour antigen. *Nature*.**312**:513-16

180. Schiff R, Massarweh S, Hou J et al. (2003) Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response.

Clinical Cancer Research, **9:**S447-54

181. Schwartz Jr S, Caceres C, Morote J, et al. 1999 Gains of the relative genomic content of erbB-1 and erbB-2 in prostate carcinoma and their association with metastasis. *International journal of oncology*, **14**:367-71
182. Selley S et al. (1997) Diagnosis, Management and Screening of early localised prostate cancer. *Health Technol Assess*. **1**(2)
183. Shah GV, Rayford W, Noble MJ, et al. (1994) Calcitonin stimulates growth of human prostate cancer cells through receptor mediated increase in cyclic adenosine 3/5-monophosphates and cytoplasmic CA2+ transients. *Endocrinology*,**134**:96-102
184. Shah RB, Ghosh D, Elder JT.(2006)Epidermal growth factor receptor (ErbB1) expression in prostate cancer progression: correlation with androgen independence. *Prostate*, **66**:1437-44
185. Sherwood ER, Van Dongen JL, Wood CG, et al. (1998)Epidermal growth factor receptor activation in androgen-independent but not androgen stimulated growth of human prostatic carcinoma cells. *British Journal Cancer*, **77**:855-61
186. Shi Y, Brands FH, Chatterjee S, et al. (2001) HER2/Neu expression in prostate cancer:: High level of expression associated with exposure to hormone therapy and androgen independent disease. *Journal of Urology*, **166**:1514-19
187. Shtiegman, Yarden Y. (2003) The role of ubiquitylation in signalling by growth factors: implications to cancer. *Seminars in Cancer Biology* **13**: 29-40
188. Signoretti S, Montironi, Manolo J, et al.(2000) Her-2-neu expression and progression toward androgen independence in human prostate cancer. *Journal National Cancer Institute*. **92**:1918-25

189. Sirotnak FM, She Y, Lee F, Chen J, Scher HI. (2002) Studies with CWR22 xenografts in nude mice suggest that ZD1839 may have a role in the treatment of both androgen-dependent and androgen-independent prostate cancer, *Clinical Cancer Research*. **8**:3870-6

190. Slamon DJ, Clark GM, Wong SG, et al. (2003) Human breast cancer: correlation of relapse and survival with amplification of the HER2/neu oncogene.*Science*235:177-

191. Slamon DJ, Leyland-Jones B, Shak S, et al. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine*, **344**:783-92

192. Slikowski MX, Schaefer G, Akita RW, et al. (1994) Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for Heregulin. *Journal Biological Chemistry*;**269**:14661-5

193. Soltoff SP, Cantley LC. (1996) P120cbl is a cytosolic adaptor protein that associates with phosphoinositide-3-kinase in response to epidermal growth factor in PC12 and other cells. *Journal of Biological Chemistry*, **271**:63-7

194. Spector NL Xia W, Burris H, et al. (2005) Study of the biologic effects of lapitinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumour growth and survival pathways in patients with advanced malignancies. *Journal Clinical Oncology* **23**:2502-12

195. Suzuki H, Ueda T, Ichikawa T, Ito H. (2003) Androgen Receptor involvement in the progression of prostate cancer. *Endocrine-related Cancer*, **10:**209-16

196. Sweeney C, Carraway KL, (2004) Negative regulation of ErbB family receptor tyrosine kinases. *British Journal Cancer* **90**: 289-293

197. Swerdlow AJ, Dos Santos Silva I, Doll R. (2001) Cancer Incidence &Mortality in England and Wales: trends and risk factors. Oxford;Oxford University Press:

198. Tam L. McGlynn LM. Traynor P. Mukherjee R. Bartlett JM. Edwards J. (2007) Expression levels of the JAK/STAT pathway in the transition from hormone-sensitive to hormone-refractory prostate cancer. *British Journal of Cancer*,**97(3)**:378-83

199. Tanagho EA, McAninch JW. (Eds). (2004) Smiths General Urology. 16th Ed. London;Lange Medical Books/McGraw-Hill. Chapter 22

200. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. (1989) HER2/neu oncogene protein and prognosis in breast cancer. *Journal Clinical Oncology*.**7**:1120-8

201. Tannock IF, de Wit R, Berry WR, et al. (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. New England Journal Medicine 351:1502-12

202. Tarone R, Chu K, Brawley O. (2000) Implications of Stage-Specific Survival Rates in Assessing Recent declines in Prostate Cancer Mortality Rates. *Epidemiology*,**11**:167

203. Taplin ME, Bubley GJ, Ko YJ, et al. (1999) Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Research 59:2511-5

204. Taplin ME, Rajeshkumar B, Halabi S, et al. (2003) Androgen receptor mutations in androgen-independent prostate cancer. Cancer and Leukaemia Group B Study 9663. *Journal Clinical Oncology* ***21***:2673-8

205. Tikhomirov O, Dikov M, Carpenter G. (2005) Identification of proteolytic fragments from ErbB-2 that induce apoptosis. *Oncogene* **24**:3906–13

206. Torring N, Hansen FD, Sorenson BS, Orntoft TF, Nexo. (2005) Increase in

amphiregulin and epiregulin in prostate cancer xenografts after androgen deprivation-impact of specific HER1 inhibition. *Prostate*, **64**:1-8

207. Tovey SM. Witton CJ. Bartlett JM. (2004) Outcome and human epidermal growth factor receptor (HER) 1-4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling. Breast Cancer Research. 2004;6(3):R246-51
208. Trachtenberg J, Blackledge G. (2202) Looking to the future. Advances in the management of hormone refractory prostate cancer. European Urological Supplement 2002;1:44-53
209. Tsutsui S, Kataoka A, Ohno S, et al. (2002) Prognostic and predictive value of epidermal growth factor receptor in recurrent breast cancer. **8**:3454-60
210. Twillie MD, Eisenberger MA, Carducci MA, et al. (1995) Interleukin-6: A candidate mediator of human prostate cancer morbidity. *Urology*, **5**:542-9
211. Ullen A, Lennartsson L, Harmenberg U, et al. (2005) Prostate cancer cell lines lack amplification: overexpression of HER2. *Acta Oncologica*, ;44:90-5
212. Umbas R, Isaacs WB, Bringuier PP et al. (1994) Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. **554**:3929-33
213. Uzgare AR, Kaplan PJ, Greenberg NM. (2003) Differential expression and/or activation of P38MAPK, Erkl/2 and Jnk during the initiation and progression of prostate cancer. *Prostate* **55**:128-39
214. Van der Kwast TH, Schalken J, Ruizvald de Winter JA, et al. (1991) Androgen Receptors in endocrine-therapy resistant human prostate cancer. *International Journal of Cancer* **48**:189-93

215. Veltri RW, Partin AW, Epstein JE et al. (1994) Quantitative nuclear morphology. Markovian texture descriptors and DNA content captured on CAS-200 image analysis system combined with PCNA and HER2/neu IHC for prediction of prostate cancer prediction. *Journal Cell Biochemistry*, **10(**Suppl):249-58

216. Vicentini C, Festuccia C, Gravina GL, et al. (2003) Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in vitro on human cell lines and primary cultures. *Journal Cancer Research Clinical Oncology* **129**:165-74

217. Vidal GA, Naresh A, Marrero L, Jones FE. (2005) Presenilin-dependent γ-secretase processing regulates multiple ERBB4/HER4 activities. *Journal Biological Chemistry*, **280**:19777–83

218. Wallen MJ, Linja M, Kaartinen K, Schleutker J, Visakorpi T. (1999) Androgen receptor gene mutations in hormone refractory prostate cancer. *Journal of Pathology* 189:559-63

219. Ware JL, Maygarden SJ, Koontz WW, Strom SC. (1991) Immunohistochemical detection of c-erbB-2 protein in human benign and neoplastic prostate. *Human pathology*, **22**:254-8

220. Warren CM, Landgraf R. (2006) Signalling through ErbB recepeptors: Multiple layers of diversity and control. *Cellular Signaling*, **18**:923-33

221. Wein AJ, Kavoussi LR, Vovick AC, Partin AW, Peters AC. (Eds).(2007) Campbell-Walsh Urology. 9th Ed. Saunders Ellsevier, Philadelphia. Chapter 104

222. Wellington K, Keam SJ. (2006) Bicalutamide 150mg: a Review of its use in the treatment of Locally Advanced Prostate Cancer. *Drug*, **66**:837-50

223. Wen Y, Hu MC, Makino K, et al. (2000) HER-2/neu promotes androgen independent survival and growth of prostate cancer cells through Akt pathway. *Cancer Research*. **60**:6841-5

224. Wikstrand CJ, Reist CJ, Archer GE, Zalutsky MR, Bigner DD. (1998) The class III variant of the epidermal growth factor receptor, characterisation and utilisation as an immunotherapeutic target. *Journal Neurovirology*,**4**:18-58

225. Wilkinson JC, Staros JV. (2002) Effects of ErbB2 coexpression on the kinetic interactions of epidermal growth factor with it receptors in intact cells. *Biochemistry*, **41**:8-14

226. Williams EE, Trout LJ, Gallo RM, et al. (2003) A constitutively active ErbB4 mutant inhibits drug-resistant colony formation by the DU-145 and PC-3 human prostate tumor cell lines. *Cancer Letters*, **192**:67–74

227. Witton CJ, Reeves JR. Going JJ. Cooke TG. Bartlett JM. (2003) Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. *Journal of Pathology* **200(3).**290-97.

228. Witton CJ, Hawe SJ, Cooke TG, Bartlett JM, (2004) COX2 expression is associated with poor outcome in ER-negative, but not ER-positive, breast cancer. *Histopathology*, **45**:552-6

229. Wong A, Ruppert J, Bigner S, et al. (1990) Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proceedings National Academy Science USA*, **87**:4207-11

230. Xia W et al. (1999) Combination of EGFR, HER2/neu and HER3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family

members. *Clinical Cancer Research,* **5**:4164-74

231. Xie H, Turner T, Wang MH, et al. (1995) In vitro invasiveness of DU-15 human prostate carcinoma cells is modulated by EGF receptor mediated signals. *Clinical and Experimental Metastasis*, **13**:407-19

232. Yarden Y, Sliwkowski NX. (2001) Untangling the ErbB Signalling Network. *Molecular Cell Biology*,**2**:127-36

233. Yeh S, Chang C. (1996) Cloning and characterization of a specific coactivator ARA70 for the androgen receptor in human prostate cells. Proceedings National Academy Science USA. **93**:5517-21

234. Yeh S, Lin HK, Kang H, et al. (1999) Fro Her2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Biochemistry*,**96:**5458-63

235. Zellweger T, Ninck C, Bloch M, et al. (2005) Expression patterns of potential therapeutic targets in prostate cancer . *International Journal Cancer*, **113** :619-28

236. Zhang F, Yang Y, Smith T et al. (2003) Correlation between HER-2 expression and response to neoadjuvant chemotherapy with 5-fluorouracil, doxorubicin and cyclophosphamide in patients with breast carcinoma. *Cancer,* **97** :1748-65

237. Zhau HE, Wan DS, Zhou J, Miller GJ, von Eschenbach AC (1992). Expression of c-erb B2/neu proto-oncogene in human prostatic cancer tissues ands cell lines. Mol *Carcinogenesis*. **5**: 320-7

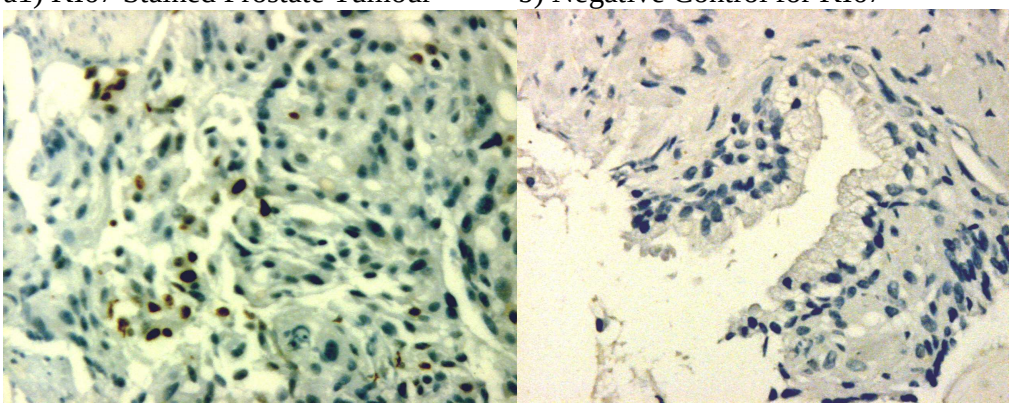
238. Zhou B, Hung M. (2003) Dysregulation of cellular signalling by HER2/neu in breast cancer. *Seminars in Oncology*, **30**:38-48

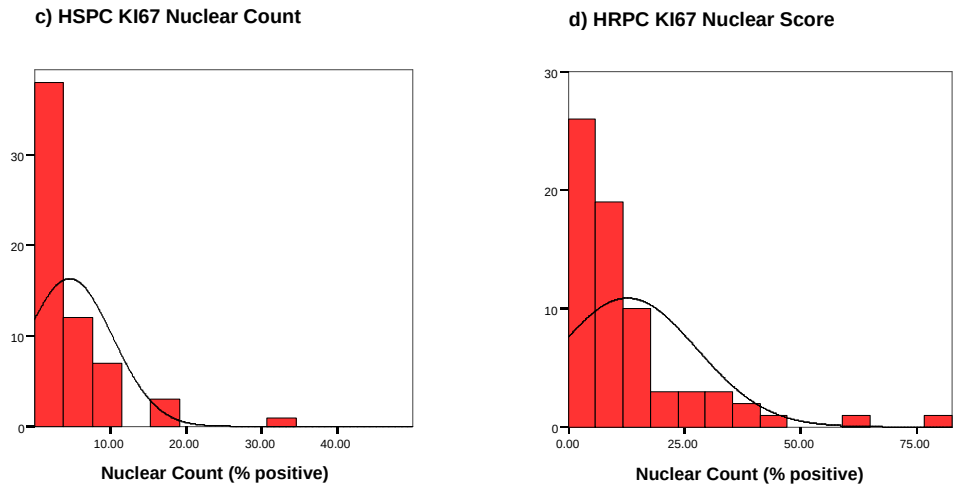
APPENDIX 1: IMMUNOHISTOCHEMISTRY AND INTER-OBSERVER SCORING FOR CHAPTER 3

AP1.1: KI-67

Figure AP1-1: KI-67 Immunohistochemistry

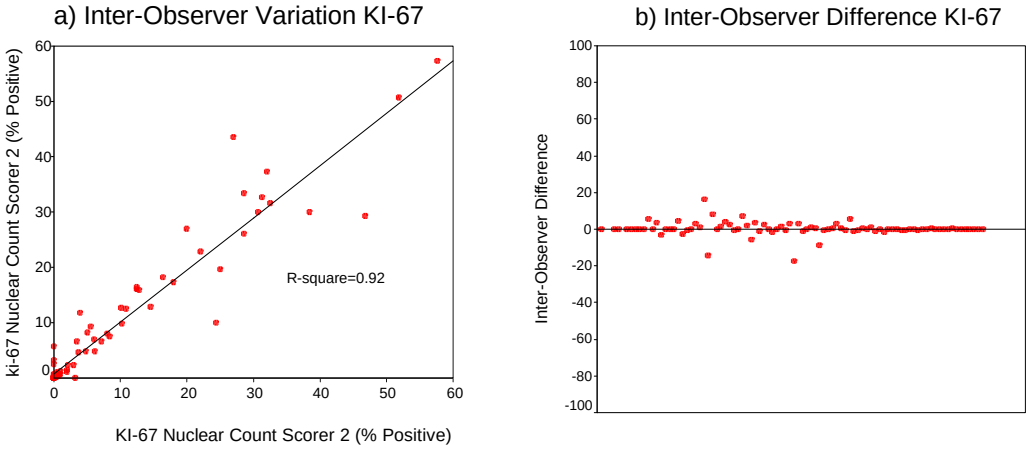
a1) KI67 Stained Prostate Tumour b) Negative Control for KI67





a1) Prostate Tumour stained with KI-67. b) Prostate Tumour Negative for KI-67 Staining c) Histogram showing distribution of KI-67 nuclear count in HSPC samples. d) Histogram showing distribution of KI-67 nuclear count in HRPC samples.

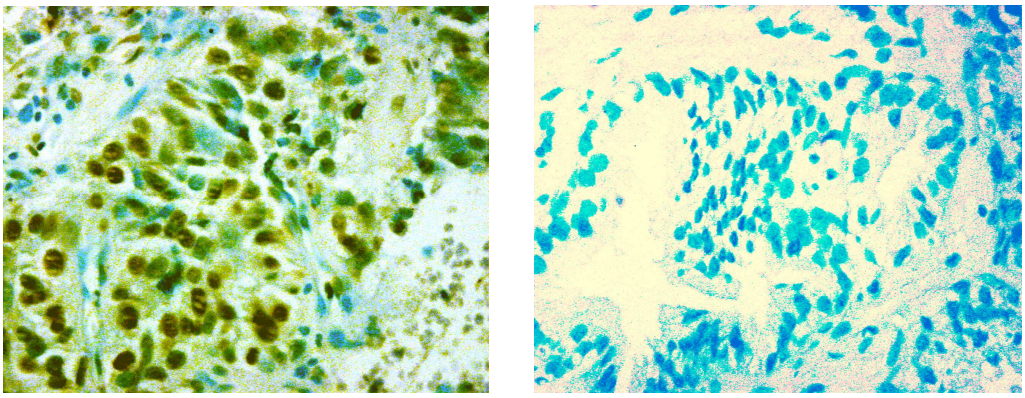
Figure AP1-2: Inter-Observer Variation in KI-67 Staining between double scored tissue sections in Cohort 1

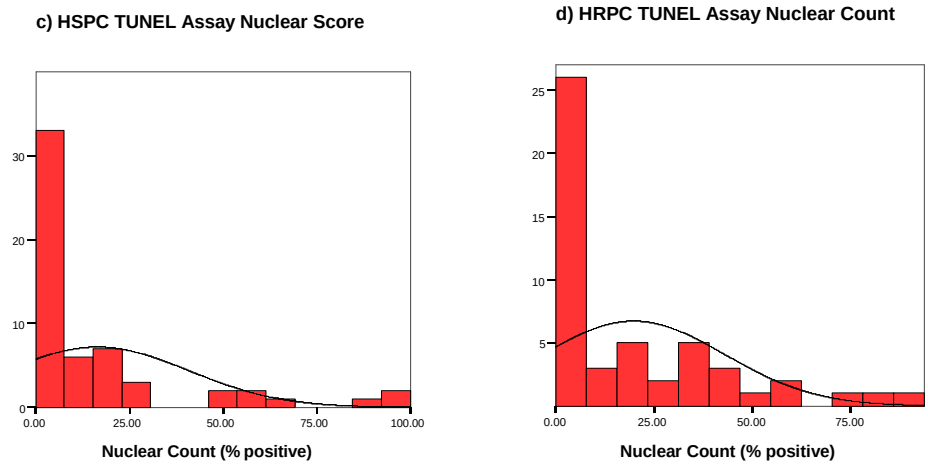


a) Scatter Graph Plot demonstrating Inter-Observer Variation in KI-67 Nuclear Count b) Bland-Altman Plot demonstrating Inter-Observer Variation in KI-67 Nuclear Count

AP1.2: TUNEL ASSAY

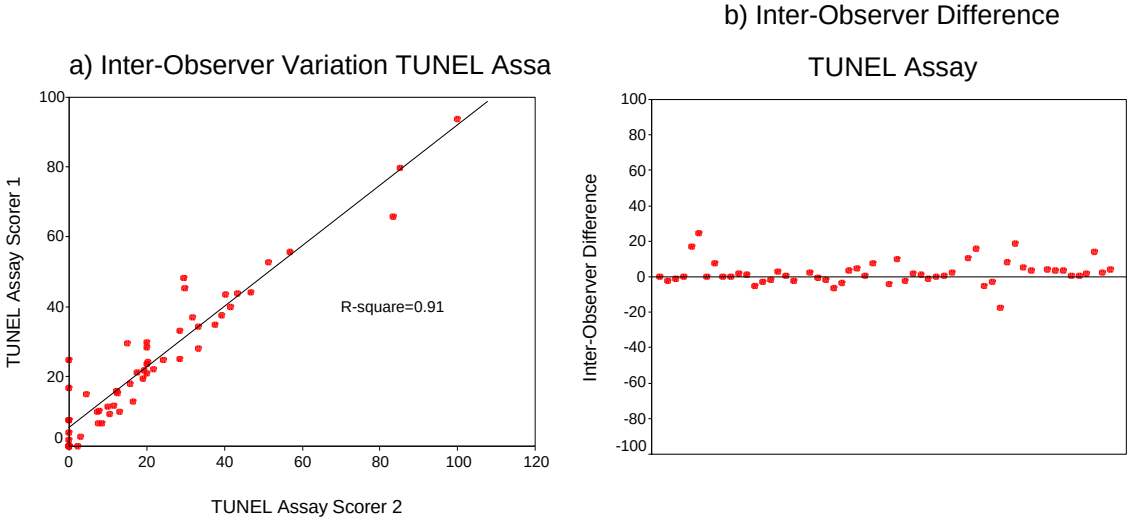
Figure AP1-3: TUNEL ASSAY Immunohistochemistry
a1) TUNEL Assay Staining in Prostate cancer b) Negative control for TUNEL Assay





a1) Prostate Tumour stained with TUNEL Assay. b) Prostate Tumour Negative for TUNEL Assay Staining c) Histogram showing distribution of TUNEL Assay nuclear count in HSPC samples. d) Histogram showing distribution of TUNEL Assay nuclear count in HRPC samples

Figure AP1-4: Inter-Observer Variation in TUNEL Assay Staining between double scored tissue sections in Cohort 1

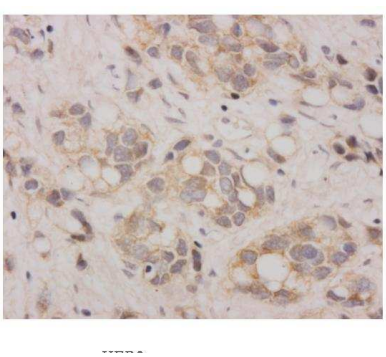


a) Scatter Graph Plot demonstrating Inter-Observer Variation in TUNEL Assay Nuclear count. b) Bland-Altman Plot demonstrating Inter-Observer Variation in TUNEL Assay Nuclear Count

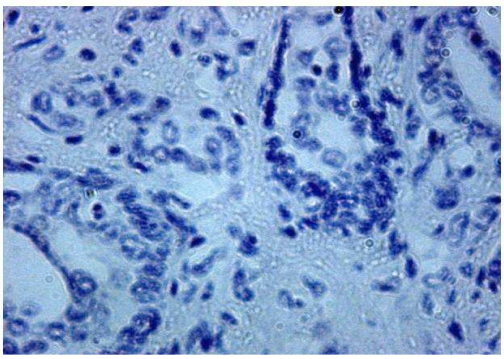
APPENDIX 2: IMMUNOHISTOCHEMISTRY AND INTER-OBSERVER SCORING FOR CHAPTER 4

AP2.1: HER2

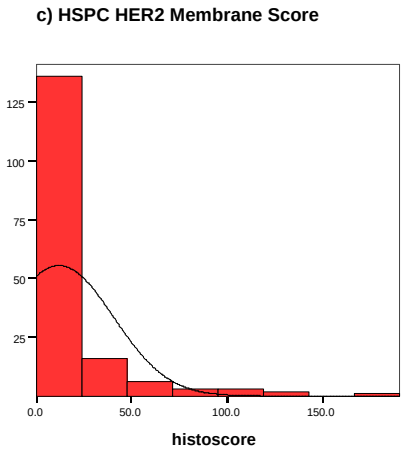
Figure AP2-1: Immunohistochemistry of HER2



a) HER2 in Prostate Cancer



b) Negative Control for HER2

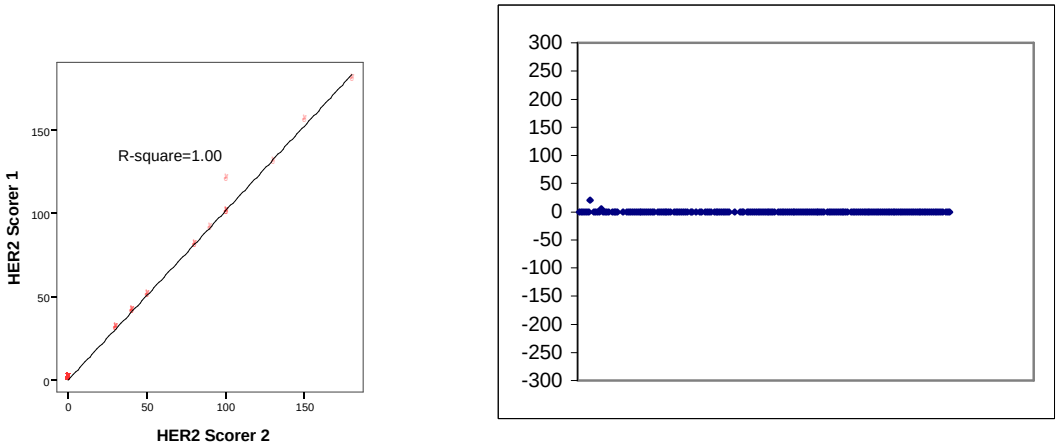


a) Specimen of Prostate Adenocarcinoma demonstrating HER2 staining b) Negative control for HER2 staining c) Histogram showing intensity of HER2 membranous expression.

Figure AP2-2: Inter-Observer Variation in Heregulin Staining between double scored tissue sections in Tissue Microarrays

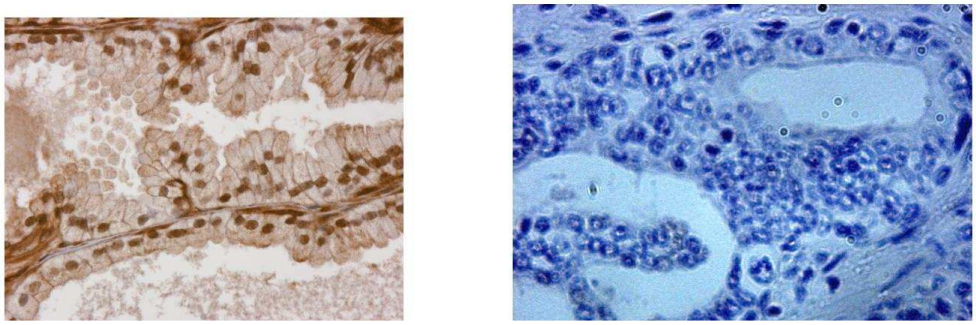
a) HER2 Membranous Staining

b) Inter-observer Difference HER2 Membranous



a) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous HER2 Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous HER2 Staining.

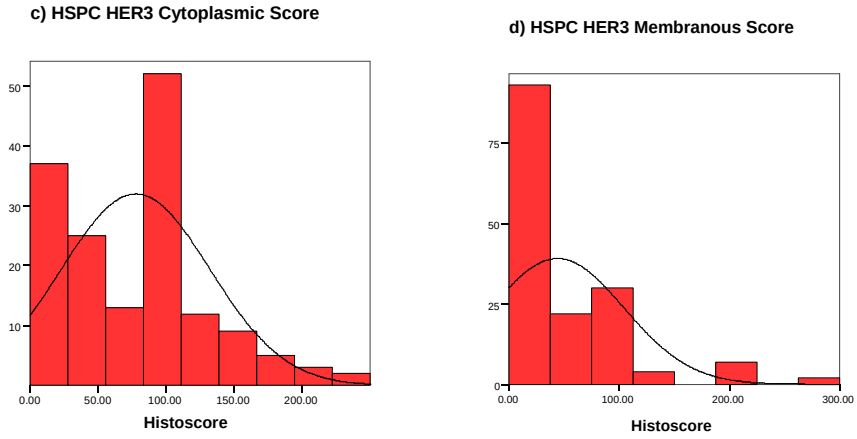
AP2.2 HER3



a) HER3 in Prostate Cancer

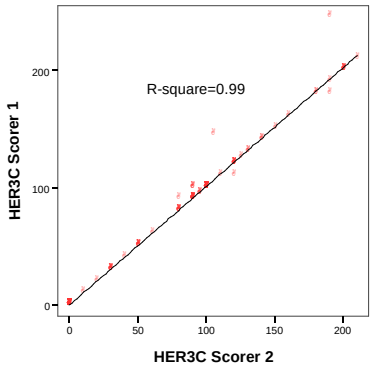
b) Negative Control for HER3

Figure AP2-3: Immunohistochemistry of HER3

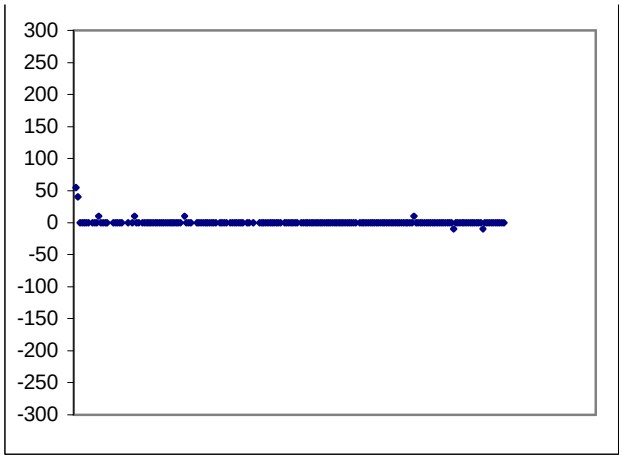


a) Specimen of Prostate Adenocarcinoma demonstrating HER3 staining b) Negative control for HER3 staining c) Histogram showing intensity of HER3 cytoplasmic expression. d) Histogram showing intensity of HER3 membranous expression.
Figure AP2-4: Inter-Observer Variation in Heregulin Staining between double scored tissue sections in Tissue Microarrays

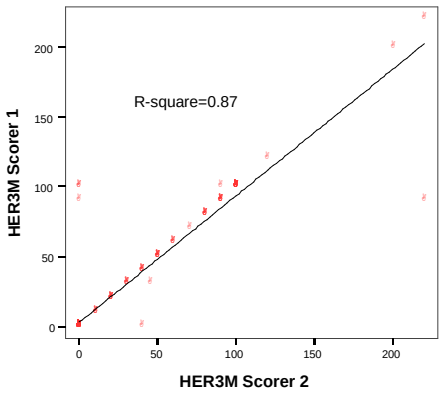
a) HER3 Cytoplasmic Staining



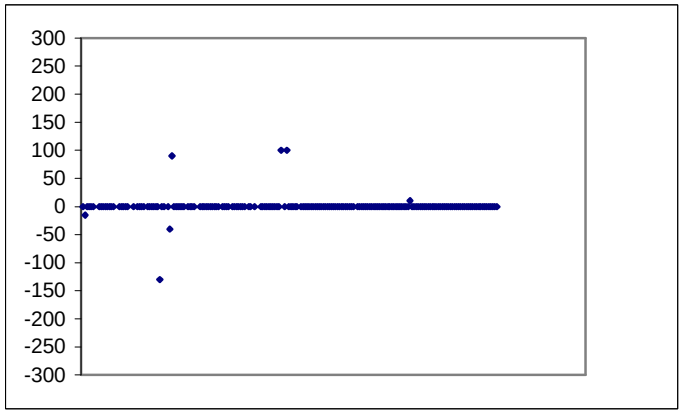
b) Inter-observer Difference EGFR Cytoplasmic



c) HER3 Membranous Staining



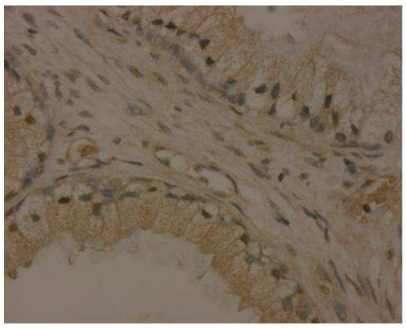
d) Inter-observer Difference HER3 Membranous



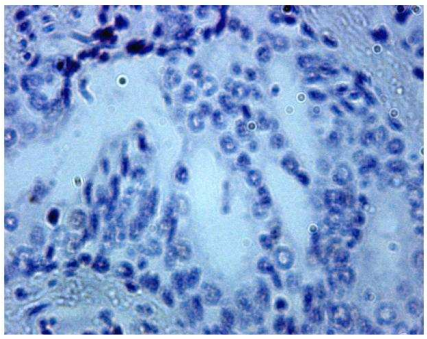
a) Scatter Graph Plot demonstrating Inter-Observer Variation in Cytoplasmic HER3 Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Cytoplasmic HER3 Staining. c) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous HER3 Staining. d) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous HER3 Staining.

AP2.3: HER4

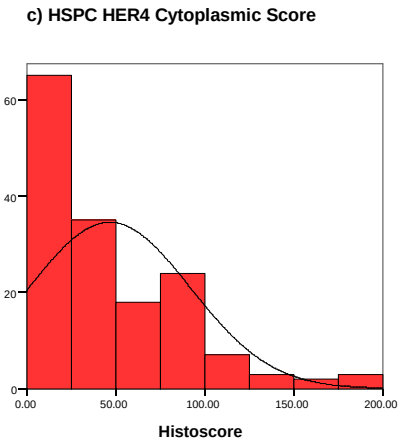
Figure AP2-5: Immunohistochemistry of HER

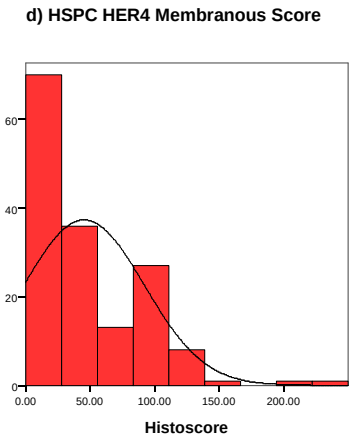


a) HER4 in Prostate Cancer



b) Negative Control for HER4

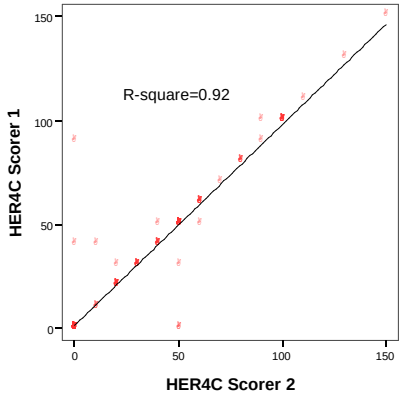




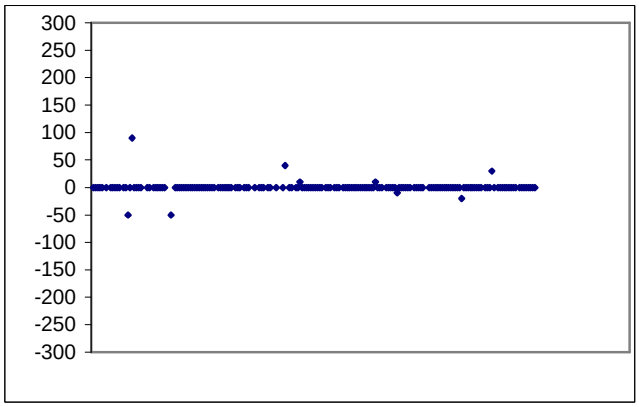
a) Specimen of Prostate Adenocarcinoma demonstrating HER4 staining b) Negative control for HER4 staining c) Histogram showing intensity of HER4 cytoplasmic expression. d) Histogram showing intensity of HER4 membranous expression.

Figure AP2-6: Inter-Observer Variation in HER4 Staining between double scored tissue sections in Tissue Microarrays

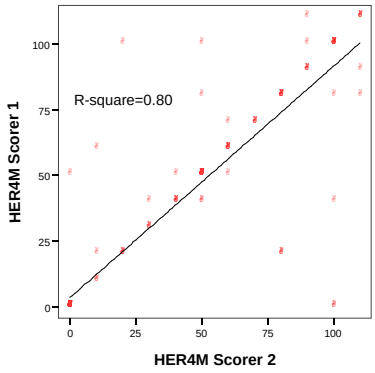
a) HER4 Cytoplasmic Staining



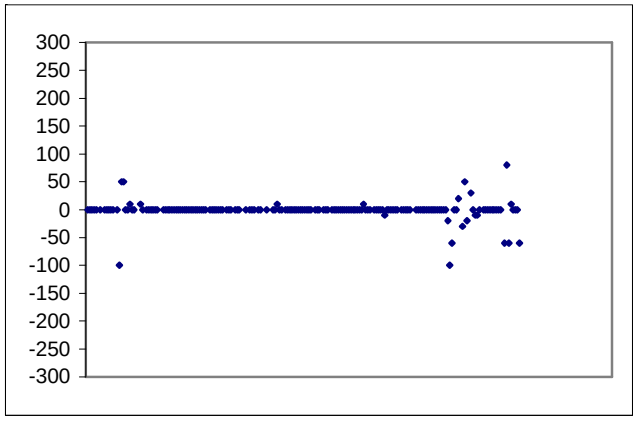
b) Inter-observer Difference HER4 Cytoplasmic



c) HER4 Membranous Staining



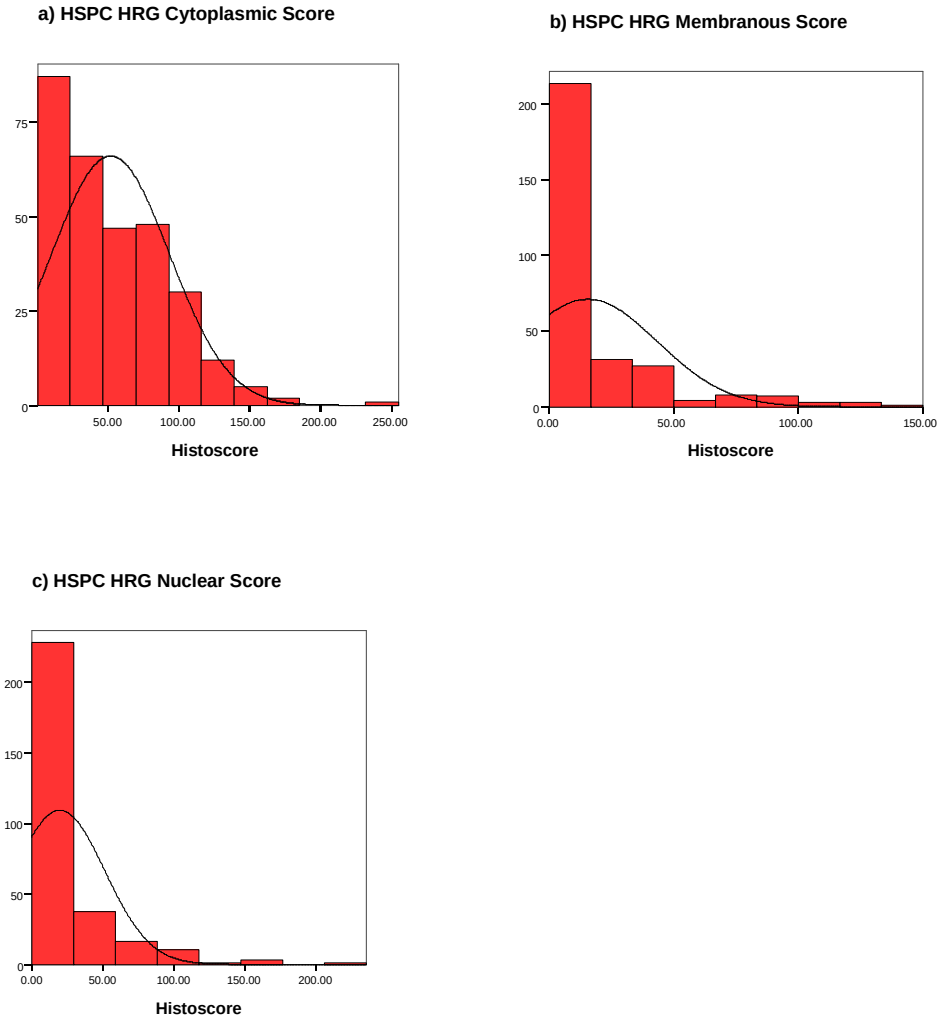
d) Inter-observer Difference HER4 Membranous



a) Scatter Graph Plot demonstrating Inter-Observer Variation in Cytoplasmic HER4 Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Cytoplasmic HER4 Staining. c) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous HER4 Staining. d) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous HER4 Staining.

AP2.4: Heregulin

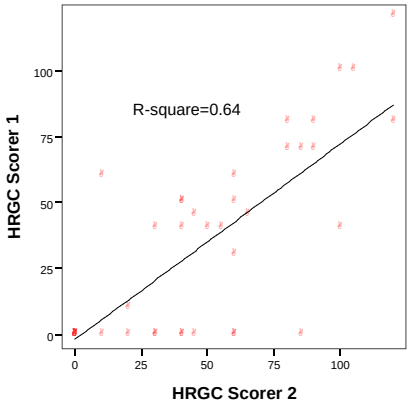
Figure AP2-7: Immunohistochemistry of HRG



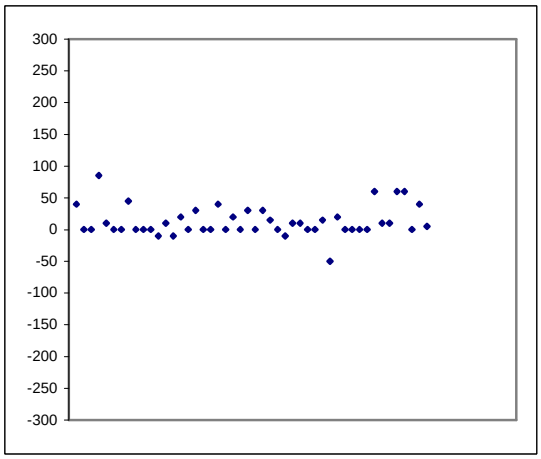
a) Histogram showing intensity of HRG cytoplasmic expression. b) Histogram showing intensity of HRG membranous expression. c) Histogram showing intensity of HRG Nuclear Expression

Figure AP2.8: Inter-Observer Variation in Heregulin Staining between double scored tissue sections in Tissue Microarrays

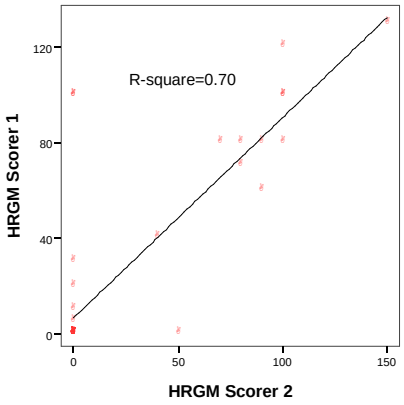
a) HRG Cytoplasmic Staining



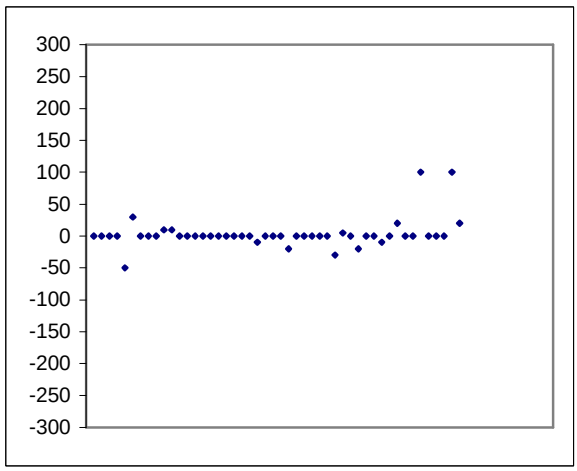
b) Inter-Observer Difference HRG Cytoplasmic



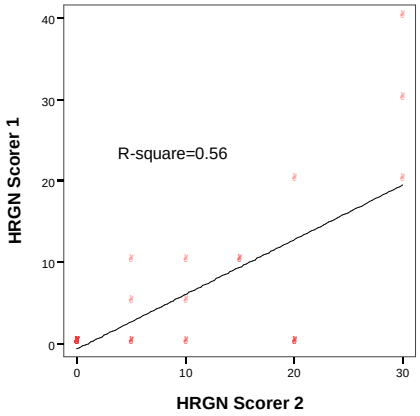
c) HRG Membranous Staining



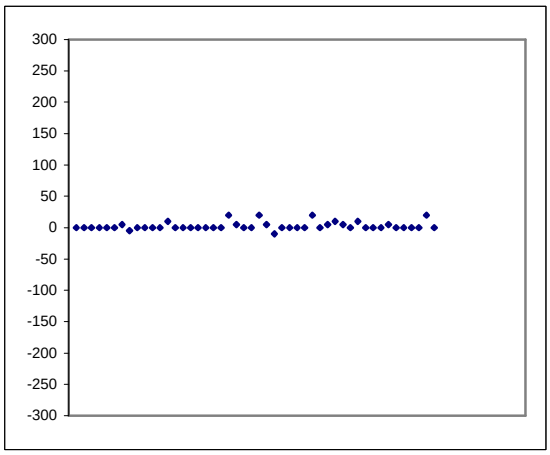
d) Inter-Observer Difference HRG Membranous



e) HRG Nuclear Staining



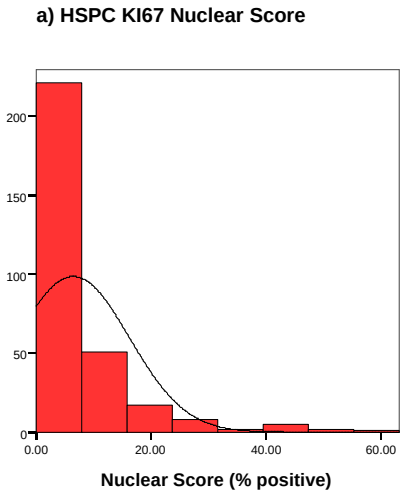
f) Inter-Observer Difference HRG Nuclear



a) Scatter Graph Plot demonstrating Inter-Observer Variation in Cytoplasmic HRG Staining. b) Bland-Altman Plot demonstrating Inter-Observer Variation in Cytoplasmic HRG Staining. c) Scatter Graph Plot demonstrating Inter-Observer Variation in Membranous HRG Staining. d) Bland-Altman Plot demonstrating Inter-Observer Variation in Membranous HRG Staining. e) Scatter Graph Plot demonstrating Inter-Observer Variation in Nuclear HRG Staining. f) Bland-Altman Plot demonstrating Inter-Observer Variation in Nuclear HRG Staining.

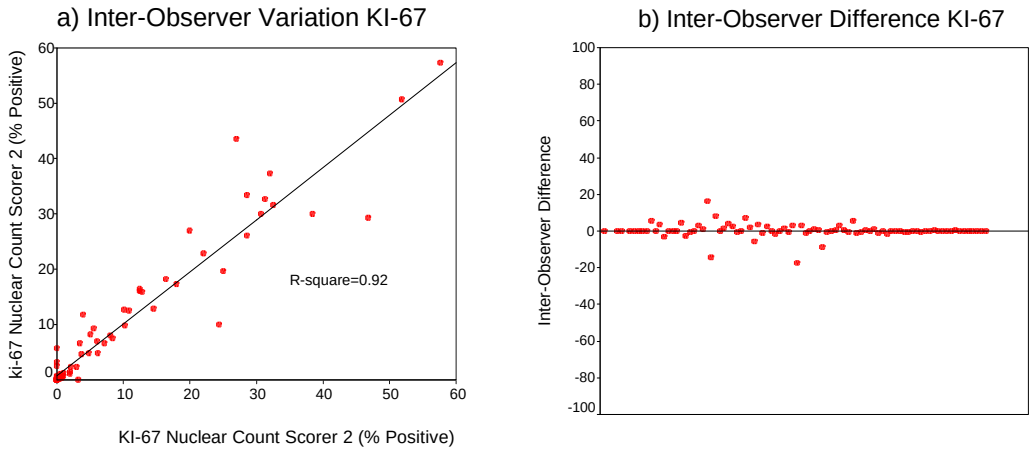
AP2.5: KI67

Figure AP2-9: KI67 Immunohistochemistry



a) Histogram showing distribution of KI67 nuclear count in Full Cohort.

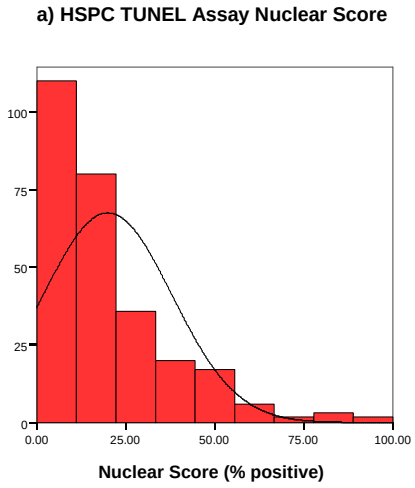
Figure AP2-10: Inter-Observer Variation in KI67 Staining between double scored tissue sections in Cohort



a) Scatter Graph Plot demonstrating Inter-Observer Variation in KI67 Nuclear Count b) Bland-Altman Plot demonstrating Inter-Observer Variation in KI67 Nuclear Count

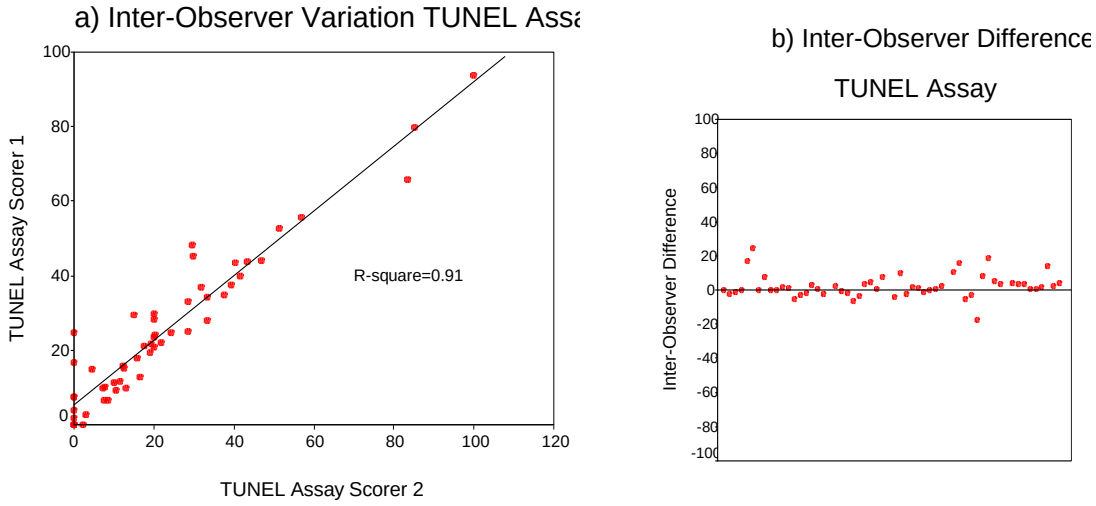
AP2.6: TUNEL ASSAY

Figure AP2-11: TUNEL ASSAY Immunohistochemistry



a) Histogram showing distribution of TUNEL Assay nuclear count in Full Patient Cohort

Figure AP2-12: Inter-Observer Variation in TUNEL Assay Staining between double scored tissue sections in Cohort 1
a) Scatter Graph Plot demonstrating Inter-Observer Variation in TUNEL Assay Nuclear count. b) Bland-Altman Plot demonstrating Inter-Observer Variation in TUNEL Assay Nuclear Count



SUMMARY

Prostate Adenocarcinoma is a significant issue facing UK healthcare today with incidence growing to the level that it is the most commonly diagnosed cancer in males. Research has focussed on signal transduction molecules that may underlie progression of CaP allowing the development of prognostic markers to guide therapy and anti-tumour therapies that may complement or replace current treatments - radical and hormonal therapy.

The HER tyrosine kinase family - EGFR, HER2, HER3 and HER4 - has proved fruitful in breast cancer research but currently evidence regarding influence of these receptors during disease progression in prostate adenocarcinoma is conflicting – both poor prognosis and no influence on outcome are reported. Few studies have considered the family as a whole. A small cohort pilot study of paired hormone sensitive (HSPC) and refractory (HRPC) specimens demonstrated HER2/HER4 as positively prognostic in HSPC. While HER4 has been demonstrated as positively prognostic in breast cancer previously, HER2 has been shown to have a negative influence in many cancer types and this apparent positive correlation is a rare finding. In accordance with previous studies the constitutively active variant EGFRvIII was seen in the pilot to negatively influence prognosis

Attention has also focused on Heregulin (a principle HER family ligand, which has previously been noted to have a differential effect on HSPC (decreased proliferation) and HRPC (increased proliferation) cell lines. This study determines influence of HER family and HRG in a larger HSPC cohort and whether downstream influence mechanisms involve proliferation or apoptosis.

Patients and Methods

Immunohistochemical staining for HRG, KI67 (proliferation), TUNEL (apoptosis) was performed on pilot study specimens with Further IHC for EGFR, HER2, HER3, HER4, HRG, KI67 and TUNEL was performed on HSPC tissue microarrays. Correlations between target protein expression and the outcomes time to biochemical relapse and overall survival were determined.

Result

On univariate analysis high expression of all 4 main HER was correlated with improved prognosis particularly in androgen deprivation treated subcohort (examples include high EGFR and longer time to relapse p=0.02, high HER2 and delayed relapse p=0.002, high HRG and delayed relapse p=0.004). Concurrent high expression of several marker combinations was also correlated with improved outcome and high expression of all 4 main members increased association significance (e.g. high HER1-4 and delayed relapse p=0.001).

Several trends were seen within the data;- if samples were divide into high and low expression by the upper quartile rather than the median more significant results were seen, membranous marker expression gave more significant correlations than other cellular locations and more significant results were seen in the subcohort of patients treated with Androgen Deprivation Therapy. Membranous HRG was seen to be positively prognostic if highly expressed in HSPC but not HRPC tissue.

With multivariate analysis a small number of these markers remained significant if the influence of Gleason Score and Metastasis at presentation were included. Markers

retaining significance on multivariate analysis included cytoplasmic HER3 (p=0.035), membranous HER4 (p=0.014) and membranous HER1-4 (p=0.045) combined

No correlations between HER/HRG expression and proliferation or apoptosis were seen.

Conclusion

The HER family and HRG are positively prognostic in prostate adenocarcinoma. In keeping with previous literature high HER4 appears to have the most significant positive influence but high expression of other markers is also seen to have a positive influence counter to previous studies. HRG positive influence is in keeping with its action as a HER4 ligand.

These results provide an explanation as to why efficacy of HER family based anti-tumour agents have had limited efficacy in CaP. These results have implications for the use of HER family as outcome predictors to guide management possibly in relation to predicting good response to Androgen Deprivation Therapy.