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BIOLOGICAL SIGNIFICANCE OF HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2 (HER2) IN BREAST CANCER: EFFECT OF OESTROGEN RECEPTOR

by

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Abstract

Background:

HER2 gene amplification and protein overexpression defined as HER2 positivity (HER2+) breast cancer (BC) is encountered in 15-25% of cases and is characterised by an aggressive behaviour and poor outcome. Despite the high clinical efficacy of anti-HER2 targeted therapy, the response and clinical behaviour of HER2+ tumours is variable. There is evidence indicating that the response of HER2+ BC to anti-HER2 targeted therapy and chemotherapy is related to Oestrogen Receptor (ER) expression. In addition global gene expression profiling studies have demonstrated that ER and HER2 are the main determinant of BC molecular profiles and that HER2+/ER+ (luminal B) are molecularly distinct from HER2+/ER- (HER2 positive) tumours. It is hypothesised that ER+/HER2+ BC is also a distinct molecular class when compared to tumours with single positive or double negative expression. Therefore, this study aimed to investigate the biological impact of ER expression in HER2+ BC with consideration of the molecular classification of BC and key pathways related to expression and behaviour of both proteins in an attempt to understand their variable biological significance and relationship to treatment response and potentially to identify new therapeutic targets.

Methods:

Methods included assessment of proteins with known associations with HER2 and ER status and correlating their expression with clinicopathological variables, molecular classes, different key BC proteins and outcome. For this purpose, Immunohistochemistry (IHC) was used to stain a number of key targets, including Mitogen Activated Protein Kinases (MAPKs), Phosphatidylinositol 3 kinase (PI3K)/Akt/mammalian target of Rapamycin (mTOR) pathway members and other proteins related to HER2 and ER and proliferation in a large well-characterised uniformly treated and annotated cohort of 1835 patients with primary BC. In addition, a cohort of 197 primary BC patients treated with Trastuzumab between 2003 and 2012, were also included. Reverse Phase Protein Array (RPPA) was used to quantify protein expression in six BC cell lines. To assess the effect of HER2 on cell lines with and without ER expression, two HER2 negative cell lines (MCF-7 and MDA-MB-231) were transfected with HER2.

Results:

The majority of MAPKs pathway members (pan Extracellular Signal- Regulated Kinase (ERK1/2), nuclear phosphorylated (p)-ERK1/2, p-c-jun-N terminal Kinase (JNK1/2), pan p38, p-p38 and p-ATF2 and p-C-JUN) showed positive associations with good prognostic variables and longer survival in the whole (unselected) cohort and in ER+ tumours but many of these associations were lost with HER2 co-expression. Such associations were infrequently observed within ER-HER2+ cases. HER2 overexpression was associated with downregulation of phosphorylated MAPKs within the whole cohort and within ER+ BC (significant for nuclear p-ERK1/2, p-ATF2 and p-p38), but ERK1/2 and p-p38 were associated with HER2 positivity within ER- tumours implying their context specific function. In addition, pan ERK1/2, p-p38 and p-ATF2 were independent predictors of better survival in BC and in ER+ BC. RPPA confirmed the IHC findings and showed similar association where the expression of MAPKs was different in ER+HER2+ cell lines compared to ER-HER2+ and ER+HER2- ones.

Regarding the PI3K/Akt/mTOR pathway, p-mTORC1 and Phosphatase and Tensin homolog (PTEN) were negatively associated with HER2 overexpression in ER+ tumours but were (in addition to Akt and PI3K) positively associated with HER2 in ER- tumours. Meanwhile, mTOR exhibited positive associations with favourable prognostic factors within ER+ BC which were decreased with HER2 co-expression and with ER loss. Additionally, p-mTORC1 was associated with prolonged breast cancer specific survival (BCSS) within Akt+ tumours but not within the whole cohort or other subgroups. In this study, using RPPA, mTOR and PTEN were positively associated with ER and negatively with HER2 in ER+ cell lines and p-mTORC1 was positively associated with HER2 in ER- cell lines in addition to other members. Importantly, PI3K, Akt, p-mTORC1 and its downstream p-S6K showed increased expression within ER+HER2+ cell lines compared to ER-HER2+ cell lines but PTEN expression was increased in ER-HER2+ vs ER+HER2+ cell lines.

When the biological significance of HER2 and KI67-LI was investigated in ER+ tumours, both HER2 and KI67-LI were associated with poor prognostic variables and adverse outcome in the ER+ tumours. Although KI67-LI rather than HER2 was associated with downregulation of luminal associated biomarkers, HER2 positivity was associated with worse outcome in ER+ tumours, indicating that

HER2+ BC are distinct aggressive tumours regardless of their proliferative activity.

Investigation of other proteins related to HER2 and ER pathways revealed that nuclear form of both the Carboxyl-terminus of Hsp-70-Interacting Protein (CHIP) and the stem cell protein, Sry-Related HMG Box 9 (SOX9), were negatively associated with HER2. CHIP was positively associated with ER, ER-associated proteins and prolonged outcome in the unselected BC and in ER+ BC but not in ER+/HER2+ and ER-/HER2+ tumours. The phosphorylated form of ER at Serine (SER) 118 was positively associated with good prognostic variables and negatively with HER2 in the unselected series and in the ER+ BC group with an observed decrease in these associations within ER+/HER2+ tumours. Increased loss of association was encountered and even some unfavourable associations were observed within ER-HER2+. Furthermore, it was associated with prolonged survival in ER+ tumours and was a predictor of prolonged survival in patients receiving tamoxifen therapy.

Clustering analysis to predict class memberships based on HER2 and ER expressions using a large panel of biomarkers related to ER, HER2 and key pathways' proteins, generated a decision tree that could be a future model for patients' stratification which indicated the overwhelming driving effect of HER2 expression.

Conclusions:

ER+/HER2+ BC is a distinct biological group, having some luminal features but is associated with worst outcome owing to the co-expression of HER2 independently from ER influence. The investigation of MAPKs, PI3K/Akt/mTOR pathways and other proteins highlighted their differential expressions and associations (with key proteins related to ER and HER2) within different BC subgroups based on ER and HER2 expressions indicating that ER+HER2+ stands as a group with unique features from those with single positive or double negative expression. Finally, development of a decision tree is a potentially promising tool for patients' stratification. Breast cancer cell line studies using the high throughput technique, RPPA, showed good concordance with IHC results, implying that further *in vitro* studies using relevant cell line models could be possible.

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Abstracts and Publications

Presented abstracts at national and international conferences

1-Biological significance of proliferation and HER2 overexpression in the luminal/ oestrogen receptor-positive breast cancer

Dena Jerjees, Andrew Green, Ahmed Benhasouna, Alaa Alshareeda, Rezvan Abduljabbar, Fabricio Barros, Christopher Nolan, Ian Ellis and Emad Rakha

Has been presented as an oral presentation at the 24th European Congress of Pathology, 8th – 12th Sep /2012, Prague

2-The biological and clinical significance of p-JNK1-2 in human breast cancer

Dena Jerjees, Rezvan Abduljabbar, Andrew Green, Ian Ellis and Emad Rakha

Has been presented as an oral presentation at the Scientific Meeting of the Pathological Society of Great Britain & Ireland, 18th -21st June/ 2013, Edinburgh, United Kingdom

3-Liver Receptor Homolog 1 Expression and its Correlation to the Breast Biomarkers in a Large Cohort of Breast Cancer Patients

Rezvan Abduljabbar; Emad Rakha; Dena Jerjees; Chris Nolan; f Lai; L Buluwela; Simak Ali; Ian Ellis.

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4-Oestrogen receptor and HER2 differentially activate ERK/MAPK signaling pathway in breast cancer. A large cohort study

Dena Jerjees, Rezvan Abduljabbar, Chris Nolan, Andy Green, Ian Ellis, Emad Rakha

Poster presentation, 25th European Congress of Pathology, 31st Aug- 4th Sep, 2013, Lisbon, Portugal

5-Retinoic acid receptor-Alpha (RARα) is an independent prognostic marker in breast cancer and it plays different roles in ER-positive and HER2 tumours

Rezvan Abduljabbar; Emad Rakha; Dena Jerjees; Chris Nolan; f Lai; L Buluwela; Simak Ali; Ian Ellis.

Poster presentation, 25th European Congress of Pathology, 31st Aug- 4th Sep, 2013, Lisbon, Portugal

6-ERK1/2 expression in breast cancer: The impact of subcellular localisation

Dena Jerjees, Ola Negm, Sameer Mirza, Rezvan Abduljabbar, Nouf Alsubhi, Christopher Nolan, Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

7-The biological and clinical significance of phosphorylated mTOR (PI3K/AKT pathway related) and its association with oestrogen receptor and HER2 pathways

Dena Jerjees, Ola Negm, Sameer Mirza, Rezvan Abduljabbar, Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

8-p38 MAPK is a marker of good prognosis in breast cancer and is an independent predictor of better outcome in the luminal class

Dena Jerjees, Rezvan Abduljabbar, Methaq Al-Kaabi, Chris Nolan, Andrew Green, Ian Ellis and Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

9-Activated c-Jun is a luminal-related marker and is associated with better outcome in breast cancer

Dena Jerjees, Rezvan Abduljabbar, Abir Muftah, Chris Nolan, Andrew Green, Ian Ellis and Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

10-Proteomics of Breast Cancer

Ola Negm, Dena Jerjees, Alaa Alshareeda, Andy Green, Vimla Band, Sameer Mirza, Patrick Tighe, Ian Ellis, Emad Rakha

oral presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

11-Androgen receptor is an independent predictor of outcome in breast cancer that performs better than progesterone receptor

Rezvan Abduljabbar, Andy Green, Dena Jerjees, Chris Nolan, L Buluwela, Simak Ali, Ian Ellis, Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

12-Peroxisome proliferator-activated receptor gamma expression is useful predictive and prognostic tool in luminal breast cancer

Rezvan Abduljabbar, Dena Jerjees, Andy Green, Chris Nolan, L Buluwela, Simak Ali, Emad Rakha, Ian Ellis

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

13-The proliferation marker Ki67 in breast cancer: A reliability study to assess its readiness for routine clinical management

Abir Abdelhadi Muftah, Meethaq Al-Kaabi, Mohammed Aleskandarany, Dena Jerjees, Nouf Alsubhi, Rezvan Abduljabbar, Chris. Nolan, Andy Green, Ian Ellis, Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

14-Deciphering the role of PTEN and its role in the DNA damage response in breast cancer

Nouf Alsubhi, W. Flesher, Alaa Alshareeda, Abir Muftah, Dena Jerjees, Rezvan Abduljabbar, Meethaq Al-Kaabi, Andy Green, Srinivasan Madhusudan, Ian Ellis, Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

15-Checkpoint Kinase1 (CHK1) is an important biomarker in breast cancer related to DNA damage repair mechanism and response to chemotherapy

Methaq Al-Kaabi, Alaa Alashareeda, Dena Jerjees, Abir Muftah, Nouf Alsubhi, Andy Green, Stephen Chan, E. Cornford, Srinivasan Madhusudan, Ian Ellis, Emad Rakha

Poster presentation, 26th European Congress of Pathology, 30th Aug – 3rd Sep, ExCel, London, United Kingdom

16-Mitogen activated protein kinase signalling proteins are associated with good prognosis in breast cancer and are mainly related to oestrogen receptor

Dena Jerjees, Ola H Negm, M. Alabdullah, Mohammed Aleskandarany, Sameer Mirza, Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

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17-Reverse phase protein array is a useful high throughput technique for assessment of multiple proteins in breast cancer

Dena Jerjees, Ola Negm, M. Alabdullah, Methaq Alkaabi, Sameer Mirza, Rezvan Abduljabbar, Abir Muftah , Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

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Publications

1-Prognostic and biological significance of proliferation and HER2 expression in the luminal class of breast cancer

Dena Jerjees, M Alabdullah, Andrew Green, Alaa Alshareeda, R. Douglas Macmillan, Ian Ellis, and Emad Rakha

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2-ERK1/2 is related to oestrogen receptor and predicts outcome in hormone-treated breast cancer

Dena Jerjees, M. Alabdullah, Methaq Alkaabi, Rezvan Abduljabbar, Abir Muftah, Chris Nolan, Andrew Green, Ian Ellis, Emad Rakha

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3-The mammalian target of rapamycin complex 1 (mTORC1) in breast cancer: The impact of oestrogen receptor and HER2 pathways.

Dena Jerjees, Ola Negm, M. Layth Alabdullah, Sameer Mirza, Methaq Alkaabi, Mohamed Hamed, Rezvan Abduljabbar, Abir Muftah, Chris Nolan, Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

Breast Cancer Res Treat, 150: 91-103

4-Checkpoint kinase1 (CHK1) is an important biomarker in breast cancer having a role in chemotherapy response

Meethaq Al-kaabi, Alaa Alshareeda, Dena Jerjees, Abir Muftah, Andy Green, Nouf Alsubhi, Chris Nolan, Stephen Chan, E Cornford, Srinivasan Madhusudan, Ian Ellis and Emad Rakha

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5-Clinical and biological significance of glucocorticoid receptor (GR) expression in breast cancer

Rezvan Abduljabbar, Ola Negm, Chun-Fui Lai, Dena Jerjees, Methaq Al-Kaabi, Mohamed Hamed, Patrick Tighe, Lakjaya Buluwela, Abhik Mukherjee, Andrew Green, Simak Ali, Emad Rakha, Ian Ellis

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6-Prognostic and biological significance of peroxisome proliferator-activated receptor-gamma in luminal breast cancer

Rezvan Abduljabbar, Methaq Mueen Al-Kaabi, Ola H. Negm, Dena Jerjees, Abir A. Muftah, Abhik Mukherjee, Chun F. Lai, Laki Buluwela, Simak Ali, Patrick J. Tighe, Andrew Green, Ian Ellis, Emad Rakha

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7-Mitogen activated protein kinase signalling proteins are associated with good prognosis in oestrogen receptor positive breast cancer

Dena Ahmad, Ola Negm, M. Layth Alabdullah, Sameer Mirza , Mohamed Hameed, Andrew Green, Patrick Tighe, Vimla Band, Ian Ellis and Emad Rakha

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8-Clinical utility of reverse phase protein array for molecular classification of breast cancer

Ola Negm, Abir Muftah, Mohammed Aleskandarany, Mohamed Hamed, Dena Jerjees, Christopher Nolan, Maria Diez-Rodriguez, Patrick Tighe, Ian Ellis, Emad Rakha and Andrew Green.

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Abbreviations

4 E- BP1	Eukaryotic initiation Factor 4 E Binding Protein1
AF-1	Activation Function- 1
AF-2	Activation Function- 2
AIB1	Amplified in Breast Cancer 1
ARF	Alternative Reading Frame
BC	Breast Cancer
BCL2	B- Cell CLL/Lymphoma 2
BCSS	Breast Cancer Specific Survival
CAF	Cyclophosphamide, Adriamycin and Fluorouracil
CARM1	Coactivator Associated Arginine Methyl-transferase-1
CD71	Transferrin Receptor
CDKs	Cyclin Dependent Kinases
CEP	Chromosome Enumeration Probe
CHIP	C Terminus of Hsc -70 Interacting Protein
DAB	3,3- Diaminobenzidin
DAPK	Death Associated Protein Kinase
DCIS	Ductal Carcinoma Insitu
DFI	Disease Free Interval
DW	Distilled Water
ECD	Ecdysoneless
EDTA	Ethylenediaminetetraacetic acid
Endo G	Endonuclease G
EPG	Excellent Prognostic Group
ER	Oestrogen Receptor
EREs	Oestrogen Responsive Elements
ERK	Extracellular Signal Regulated Kinase
FOXA	Forked Head Protein A1
GATA3	Transacting T-Cell- specific Transcription Factor
GCDFP-15	Gross Cystic Disease Fluid Protein
GEFs	Guanine Nucleotide Exchange factor
GEP	Gene Expression Profiling
GPG	Good Prognostic Group
HER	Human Epidermal Growth Factor Receptor
HMG	Homologus High Motility Group
HRs	Hormone Receptors
IGF-1R	Insulin-Like Growth Factor- 1 Receptor
IRS1	Insulin Receptor Substrate 1
JNK	C-jun-NH2 Kinase
LN	Lymph Node
LVI	Lymphovascular Invasion
MA Scs	Mammary Stem Cells
MAPK	Mitogen Activated Protein Kinase
MCF-7	Michigan Cancer Foundation- 7
MEK	Mitogen Activated Protein Kinase/ ERK Kinase
MPG	Moderate Prognostic Group
mTORC1	Mammalian Target of Rapamycin Complex- 1
mTORC2	Mammalian Target of Rapamycin Complex- 2
NPI	Nottingham Prognostic Index
NR2E3	Nuclear Receptor Subfamily- 2
NST	No Special Type

P70 S6K	Ribosomal p70 S6 Kinase
PBS	Phosphate Buffer Saline
PELP1	Proline Glutamate and Leucin Rich Protein
PgR	Progesterone
PI3K	Phosphatidyl Inositol 3 Kinase
PIP2	Phosphatidyl Inositol- 4, 5 Biphosphate
PIP3	Phosphatidyl Inositol- 4, 5 Triphosphate
PPG	Poor Prognostic Group
Rb	Retinoblastoma
RPPA	Reverse Phase Protein Array
RTKs	Receptor Tyrosin Kinases
SDS	Sodium Dodecyl Phosphate
SER	Serine
SOX9	Sry -Related HMG Box 9
SRC3	Steroid Receptor Coregulator- 3
T	Transfected
TBS	Tris Buffered Saline
TDLU	Terminal Duct Lobular Unit
TFEB	Transcription Factor EB
TFF1	Trefoil Factor- 1
TFF3	Trefoil Factor- 3
TN	Triple Negative
TPBS	Tween-20 PBS
VEGR	Vascular Endothelial Growth Factor Receptor
VPG	Very Poor Prognostic Group
W	Wild

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1 General Introduction

1.1 Breast

1.1.1 The normal development of the breast, anatomical and histological overview

The development of the breast system is a complex process involving different processes of: proliferation, apoptosis, differentiation and migration. Although the mammary tissue appears during the embryonic period, the morphogenesis required for the proper development of the ductal system is mainly noticed at puberty (Wiseman and Werb, 2002). The breast is comprised of 12-20 lobes, arranged like a daisy petals, each of which is subdivided into several thousands of lobules and each in turn can have up to 20-200 sac-like acini structures which functions to secrete milk. Ducts from these acini unite together forming the terminal duct and with associated lobule forms the basic structure of the breast: the Terminal Duct Lobular Unit (TDLU). Interestingly, this structure is the focus of investigation of many scientists with an overall concept that it is the site of emergence of breast neoplasm. Of worth, these TDLUs reunite with the sub-segmental ducts, segmental, lactiferous duct which then open into lactiferous sinus which opens in the nipple. This branching structure is subjected to hormonal changes especially after puberty and with increasing age, is thought to be a source of neoplasm; thus it has been proposed that stem cells are contained within this duct system. Based on these data, it has been postulated that there are three types of cells in the ducts: luminal, basal and the stem cells (Birnbaum et al., 2004).

1.1.2 Breast cancer (BC)

Breast cancer is a disease that generally affects middle aged and elderly women. Diverse variety of histological entities, clinical behaviour, outcome, and response to therapy are encountered with breast tumours. Importantly, despite the efforts spent on subclassifying and diagnosing this disease and the availability of different treatment options, considerable relapses and/or mortalities are encountered (Levi et al., 2007, Berry et al., 2005).

1.1.3 Breast cancer: Incidence and mortality

Breast cancer is by far, an alarming health issue worldwide (Sørlie et al., 2003). Apart from non-melanoma skin cancer, BC is considered the most common cancer in women. Moreover, in 2008, it accounted for 458,503 deaths worldwide (Boyle and Levin, 2008). Approximately 230,480 new cases of invasive BC together with 39,520 deaths due to BC were recorded in 2011 in the USA (DeSantis et al., 2011). Recently, BC was reported to cause 1.68 million new cases and 522 000 deaths annually all over the world (J et al., 2013). It has been agreed that BC is not a disease of uniform portrait but rather a heterogeneous one having different histological types; however, even morphologically similar tumours can have different behaviour and there are efforts to subclassify BC cases based on molecular features that could imply the actual different behaviour between its subsets (Perez, 2011).

1.1.4 Theories behind the origin of breast cancer

In general, cancer initiation and progression are the product of seven crucial changes: Growth signals' self-sufficiency, non-responsiveness to growth inhibitory stimuli, obtaining continuous replicative ability, evasion of apoptosis, impaired DNA repair pathways, enhanced angiogenesis, and obtaining the ability to metastasise, all of these changes are integrated together to enhance cancer development (Hanahan and Weinberg, 2000, Sledge and Miller, 2003). Ultimately, overwhelming increased cell production along with genetic instability are also determinant factors for cancer evolvement (Beckmann et al., 1997).

Regarding the cells of origin of BC, two theories have been suggested. The first proposed is that BC stems from common epithelial stem cells and the resultant phenotype is determined by the subsequent genetic alteration known as the hierarchical model. Secondly, distinct cancer stem cells and progenitor cells are the cause and that the phenotype is influenced by the cells of origin which still possesses its markers' signature and its specific criteria and these cells could be at any maturation or differentiation stage known as the stochastic model (Singh et al., 2004, Yue et al., 2003, Molyneux et al., 2007, Dumitrescu and Cotarla, 2005, Birnbaum et al., 2004).

1.1.5 Risk factors for breast cancer

There are many risk factors related to BC which were grouped as either hereditary or environmental factors. In spite of the fact that many risk factors have been reported; the actual causes in the majority of BCs remains unclear. Importantly, hereditary factors account for only 5% of the causes of BC and the environmental factors are responsible for the other causes (Madigan et al., 1995). Hormonal factors are considered the main causative factors; nevertheless, the following factors are also important risk factors (Key and Verkasalo, 1999).

1.1.5.1 Hormone factors

Oestrogen and progesterone have been reported to increase the incidence of BC. Interestingly, the incidence of BC is supposed to decrease after menopause due to cessation of hormone synthesis; however, BC occurrence in these patients indicates that they remain under hormonal influence (Kwong et al., 2008, Garcia-Closas et al., 2006). In a study conducted to reveal the effect of taking combined oestrogen and progesterone hormone replacement therapy versus placebo, more BC cases were diagnosed in the former group and those tumours were larger and of more advanced stage. Moreover, after one year, substantially greater abnormal mammograms were observed in the former group compared to those with placebo therapy (Chlebowski et al., 2003). Furthermore, oestrogen and progesterone were found to collaborate with proto-oncogene and growth factors *in vi*tro to enhance BC proliferation (Pike et al., 1993).

Regarding lactation, a report which analysed the data from 47 epidemiological studies performed in 30 countries, indicated that the women with more births and had a longer time for breast feeding than others (approximately 15 months) have gained a two third protection from BC (2002). In addition, other factors like early menarche and late menopause are also BC risk factors (Hankinson et al., 1998, Cauley et al., 1999).

1.1.5.2 Age

It is well established that the liability of woman to have BC increases with age. The risk of having this disease doubles every ten years until menopause (Key et al., 2001). Age adjusted prevalence and death rates differ between communities

and western societies have fivefold higher incidence rate than Far East countries (Fregene and Newman, 2005, Adebamowo et al., 2008).

1.1.5.3 Family history and genetic predisposition

Germ line mutations of BRCA1 and BRCA2 are primarily responsible for hereditary BC. Carriers of these defective genes render a double risk of BC (Clamp et al., 2002). However, BC due to BRCA1 mutation accounts for less than 2% of cases in the UK (Peto et al., 1999). Results from one study indicated that BRCA1 mutation is associated with early onset BC (Jeon et al., 2015). Similarly, a study that has been conducted by analysing the data from 22 studies indicated that the average cumulative risk in carriers of BRCA1 mutation is 65% at the age of 70 compared to 39% carriers of BRCA2 at the same age (Antoniou et al., 2003). Many researchers have investigated the correlation between germ line mutation associated BC and survival and while some indicated that there is no difference (Schouten et al., 1997, Russo et al., 2002, Chang et al., 2009), others observed an association with short survival (Slattery et al., 1993, Stratton and Rahman, 2008). On the other hand, other investigators had different views and they observed better association with survival (Thalib et al., 2004, Mohammed et al., 1998).

1.1.5.4 Alcohol intake and dietary factors

Alcohol consumption has been linked to development of BC (de Menezes et al., 2013, Roswall and Weiderpass, 2015). Moreover, high fat intake has also been reported to be a risk factor for BC (Boyd et al., 2003). In addition, an association between BC and smoking, has not yet been confirmed (Nyante et al., 2014).

1.1.5.5 Exposure to radiation

It has been indicated that the magnitude of risk of radiation on BC is dose dependent; however, other factors are implemented together with radiation itself in terms of age of exposure, parity, time of first full term birth (Ronckers et al., 2005).

1.1.5.6 Benign breast conditions

Although some benign breast lesions could predispose to BC; nevertheless, only the proliferative conditions with atypia are encountered to be a risk as atypical ductal hyperplasia and atypical lobular hyperplasia. The relative risk for

proliferative breast conditions with atypia are more than those lacking atypia and the relative risk in non proliferative conditions is much less likely to be a risk factor (Hartmann et al., 2005).

1.1.6 Prognostic and predictive factors in breast cancer

1.1.6.1 Prognostic factors

Several clinical and pathological factors have been well recognised to assess the prognosis and help in treatment decision (Schnitt, 2010). Using a combination of prognostic factors are more valuable than using an individual one and this was the base of creating certain formulas that is indicative of powerful prognosis, one of the most important is Nottingham Prognostic Index (NPI), (Galea et al., 1992). The prognostic factors in BC involve the following:

1.1.6.1.1 Histological type

Histological type of BC is not regarded as a powerful prognostic factor; yet, its determination is mandatory in the clinical practice (Rakha et al., 2010a). Invasive ductal No Special Type (NST) is regarded as the most common type and constitutes up to 75% of BC. It has no defining characteristics or constitutes less than 50% of the total components. This type ultimately has intermediate prognosis and it depends on many factors including: tumour size, histological grade, Lymph Node (LN) stage prognosis, Hormone receptors (HRs) and HER2 status (Arpino et al., 2004). In addition, tubular carcinoma merits the best prognosis. Indeed, other special (where special components should constitute >90% of the tumour) and mixed histological types (where special components constitute >50% and <90%) lie between these two prognostic ranges (Ellis et al., 1992). The pure types include certain distinct types including: 1) Infiltrating lobular carcinoma 2) Invasive cribriform carcinoma, 3) Medullary-Like carcinoma, 4) Mucinous carcinoma and 5) Infiltrating papillary and micropapillary carcinoma (Rakha et al., 2010a, Venable et al., 1990, Orvieto et al., 2008, Ellis et al., 2005, Pal et al., 2010).

1.1.6.1.2 Tumour grade

Tumour grade is a powerful prognostic factor and is incorporated into the NPI. It is a three tier system having main three components: levels of tubule formation (1: majority of tumour, (> 75), 2: moderate degree (10-75%) and 3: little or

none (<10%)), nuclear pleomorphism (1: small, regular uniform cells, 2: moderate increase in size and variability and 3: marked variation) and mitosis (1: 1-9. 2: 10-19 and 3: ≥20, and this number is assessed in 10 high power field by light microscopy with a field diameter of 0.59 mm). As each one of the latter scores from 1-3, by sum up score from all components, grade is assigned for each case, where grade I is given to the sum of 3-5, grade II is for 6-7 and finally grade III is given for the sum from 8-9 (Elston and Ellis, 1991). Grade represents a reflection of the biological aggressiveness of the tumour by determining the degree of its similarity to the tissue of origin, therefore, grade I reflects good prognosis, grade II reflects moderate while grade III reflects the worst prognosis (Rakha et al., 2008). Of worth, grade has similar weighting as LN stage in NPI (Ellis et al., 1992) and it has gained more importance after inclusion of mammographic screening as this has raised the self awareness and early detection of the disease, since it has been indicated that grade III tumours are associated with high grade Ductal Carcinoma Insitu (DCIS) as manifested by calcification which is obviously seen in mammography and these DCIS foci are observed surrounding the tumour which is usually of more than 10 mm diameter (Anttinen et al., 2006, Shen et al., 2005, Hanrahan et al., 2006).

1.1.6.1.3 Tumour size

As a concept, it is well known that the patients who present with large tumours will have shorter survival compared to those with smaller tumours (Elston and Ellis, 1991). Size is regarded as one of the most powerful and significant parameters in predicting tumour behaviour and patient survival (Mahmood et al., 2015). To reveal the association between tumour size and LN stage, a study has been carried out revealing that those patients with tumour size of less than 1cm, 10-20% of them displayed axillary nodal involvement (Carter et al., 1989).

1.1.6.1.4 Lymph node stage

LN stage by far, is regarded as the most powerful indicator of Disease Free Interval (DFI) and overall BC survival (Arriagada et al., 2006, Vorgias et al., 2001, Weiss et al., 2003). It has been reported that BC in patients younger than 36 years will have more advanced LN stage (Goksu et al., 2014) and different studies were implicated to evaluate the extent of LN stage and one of these studies reported that an independent correlation has been revealed by LN stage

in causing lethality of BC patients and this lethality increases with more LN involvement (Michaelson et al., 2003). It is worth mentioning that the pathological system used to reveal staging is divided into three categories: 1) Stage 1=0 LNs, 2) Stage 2= 1-3 LNs and 3) Stage 3 > 3 LNs (Galea et al., 1992).

1.1.6.1.5 Nottingham Prognostic Index

Galea et al regarded NPI as a tool for indicating patients' prognosis and stratification (Galea et al., 1992). It comprises three powerful clinicopathological parameters which are; tumour grade (scores as in section 1.1.6.1.2, page 5), LN stage (relating to number of positive LNs and not to be confused with TNM staging): (1-3, 1: 0 LNs, 2: 1-3 LNs and 3: > 3 LNs) and tumour size with maximum diameter (0.2 × size in CM) and their contribution to predict prognosis is better than using each parameter individually. According to this index, three prognostic groups are determined by the sum up of the results of these three components. The first group assigns NPI of $\leq$3.4 and this group is the most favourable one. Next is the moderately prognostic group whose NPI falls between 3.41-5.4 and finally the worst prognostic group is the one which assigns >5.4 NPI (Lee and Ellis, 2008).

Later on, Blamey et al expanded these prognostic groups to six, giving a range of NPI from 2.08 (LN negative, grade 1, 0.4 CM) to 6.8 (LN Stage 3, grade 3, size 4.9 CM). These groups include: An Excellent Prognostic Group (EPG) with an observed NPI range of 2.08–2.4, Good (GPG) 2.42 to 3.4; Moderate I (MPG I) 3.42 to 4.4, Moderate II (MPG II) 4.42 to 5.4, Poor (PPG) 5.42 to 6.4 and Very Poor (VPG) 6.5–6.8 (Blamey et al., 2007)

1.1.6.1.6 Lymphovascular invasion

Lymphovascular Invasion (LVI) is related to local, distant recurrence and even BCSS. This parameter has proved of limited value in LN positive disease; yet, its independent prognostic value has been reported in those with node negative disease (Lee et al., 2006, Mohammed et al., 2007).

1.1.6.2 Predictive factors

These factors are fundamental in predicting the response of tumour to treatment and can be useful in determining patients stratification (DeVita et al., 2008). These factors include the followings:

1.1.6.2.1 Hormone receptors

According to the Guidelines from the American Society of Clinical Oncology/College of American Pathologists, they recommended that detection of oestrogen receptor (ER) and progesterone receptor (PgR) is valuable from 1% of tumour nuclei (Hammond et al., 2010). ER is a powerful indicator for prediction of hormonal therapy after surgery (Al-Mubarak et al., 2014), in addition, the co-expression of PgR will render ER superior predictive ability (Mohsin et al., 2004) and it is widely perceived that stratification of BC cases according to the expression of ER and PgR from a clinical standing point is a helpful strategy for optimal treatment of patients (Colditz et al., 2004). The predictive potential of ER and PgR indicates that the response to hormonal therapy is 80% when both receptors are co-expressed while the response decreases to 50% in patients harbouring ER alone. Additionally, from a practical point of view, ER positivity is mainly encountered in Post-menopausal women rather than younger patients who usually display more aggressive type of the disease (Rakha et al., 2010b).

1.1.6.2.2 HER2/neu

The HER2 gene (ERBB2) encodes a transmembrane tyrosine receptor kinase (TRK) protein (c-erbB-2; CD340; proto-oncogene Neu) which is a trans-signaling molecule that mediates critical function in normal and BC epithelium (Yarden and Sliwkowski, 2001b). In general, alterations in form of either amplification of the gene or overexpression of its protein product are reported in approximately 20-25% of BC patients (Slamon et al., 1987a, Koninki et al., 2009). Preclinical data reveals an unfavourable clinical picture with HER2 where fundamental changes alters the biological environment of BC including: enhancing the proliferation and motility potential, downregulation of apoptosis, sustained angiogenesis, rendering epithelial cells able to invade and finally hormone independence (Slamon et al., 1987a, Slamon et al., 1989). Clinically, HER2 is associated with higher tumour grade, LN metastasis and thus shorter outcome (De Luca et al., 2008, Feigin and Muthuswamy, 2009, Marcotte and Muller, 2008). Importantly, the targeted treatment for HER2+ by using immunotherapy; Herceptin (Trastuzumab) and systemic chemotherapy has been shown effective (Slamon et al., 2001).

1.1.7 Molecular classification of breast cancer

Breast cancer is a well-established phenotypically and genotypically heterogeneous disease and its molecular biology is quite complex owing to the fact that morphologically similar tumours do not have the same behaviour (Alizadeh et al., 2001). Therefore, the current reliance of clinicians on traditional systems of prognosis which use histological grade, tumour size and LN metastasis is less than satisfactory (Alizadeh et al., 2001). This issue necessitates the availability of a modern system which might simplify the process of carcinogenesis of such tumours and perhaps can aid in the refined categorisation of patients into prognostic groups with distinct molecular characteristics as a step to offer more personalised treatment choice for patients. Furthermore, to avoid the subjectivity with the traditional methods and for this reason, different techniques have been emerged (Bertucci et al., 2000).

1.1.7.1 Gene Expression Profile

Gene Expression Profiling (GEP) has enabled thorough molecular classification of BC; it relies on the sub-classification of BCs according to their similarity of expression of certain genes and it has changed the way BC is perceived in. Initially, Perou et al used 1,753 genes to distinguish between forty BC tumours of different histological types, and were able to recognise two main groups: Luminal and basal like BC subgroups. The first group showed expression of ER and biomarkers characteristic of luminal epithelium, while the other group exhibited genes expressed in normal breast epithelium but showed relatively high expression for genes related to fat cells and other mesenchymal cells while deficient of those defining luminal category (Perou et al., 2000a).
Later on, Sorlie et al refined the latter list of genes into 456 and used 87 BC cases aiming to further subclassify the luminal group where he noticed three subgroups emerged A, B and C (Sorlie et al., 2001). The same investigators in another study modified their results by using 534 genes and reported that luminal group has only A and B subclasses and that class C was redistributed between luminal B and the basal like one (Sorlie et al., 2003b), (Figure 1-1).

Furthermore, Hu et al (Hu et al., 2006b) used a novel set of 1,300 genes to obtain a final shared gene signature of 306 genes that finalised BC into five subclasses: Luminal (A& B), HER2 enriched (ER-HER2+), basal-like and normal-

like. From the practical point of view, although GEP is a useful technique, still its use is criticised by sampling method, reproducibility, cost issues and even the incompatibility among different data sets used by different researchers (Correa Geyer and Reis-Filho, 2009, Geyer et al., 2009).

The clinical utility of GEP appears in distinguishing between patients who will benefit from chemotherapy and those who might not benefit particularly those who receive adjuvant tamoxifen. Such tests include Oncotype DX and Mammaprint (Marchionni et al., 2007). Traditionally, recurrence risk assessments are based on tumour size, grade and nodal involvement in addition to HR status (Marchionni et al., 2007, Persing and Grosse, 2007). Importantly, GEP measures the expression of prognostically relevant sets of genes for prediction of distant recurrence liability at 10 years and the probable benefit from chemotherapy (van de Vijver et al., 2002b). In the same context, current guidelines for the management of early BC have indicated that thousands of women receive chemotherapy with no benefit (Sparano and Paik, 2008). Interestingly, a study conducted to determine the utility of chemotherapy in 22,600 Canadian patients with BC (HR+, HER2- and node -), according to guidelines, 90% of patients had to have chemotherapy (Sparano and Paik, 2008, Marchionni et al., 2007). Nevertheless, it was estimated that only 15% of the patients will have recurrence, outlining that 8500 cases will have no benefit from chemotherapy (Marchionni et al., 2007, Persing and Grosse, 2007).

1.1.7.2 Multi Gene Signature

This encompasses a range of prognostic signatures that are used to predict recurrence or metastasis in BC patients relying on the expression of certain genes of metastasis, invasion or angiogenesis. Some of these assays have been widely investigated, for instance, the MammaPrint™ is a fully commercialised microarray-based multigene assay that can predict metastasis ability in LN negative BC patients who are either ER+ or ER- using 70 genes (van de Vijver et al., 2002a). Similarly, Oncotype DX Breast Cancer Assay which is known as the recurrence score is based on the use of 16 cancer genes and 5 reference genes. It is usually used to predict recurrence and response to chemotherapy (Paik et al., 2004). Additionally, there is a Genomic Grade Index (GGI) assay Map Quant DX, which categorises ER+ tumours into high and low grades depending on their expression signature. A study has been conducted in this regard, in which the

microarray analysis of patients with known histopathological grading was performed. When correlating the data, GGI was strongly correlated to grade I and III tumours. Meanwhile, grade II was split into two distinct subgroups equivalent in their outcome to grade I and III respectively (Sotiriou et al., 2006a).

1.1.7.3 Immunohistochemistry

By using Immunohistochemistry (IHC), categorisation of BC cases is possible, and there is a consensus that using a combination of markers instead of a single one is more beneficial as the reliance on an individual protein may not indicate the behaviour of a certain group and can tailor the optimal therapy (Abd El-Rehim et al., 2005). Using IHC for 25 relevant markers on 1,076 invasive BCs, classifies two luminal groups which were positive for HRs (ER and PgR) and showed positivity of luminal CKs (7/8, 18, 19) and positivity of MUC1 but negativity for basal epithelial markers. Additionally, the first luminal group revealed high expression of HER3 and HER4 but low mean of expression of BRCA1 protein vs the second luminal group (Abd El-Rehim et al., 2005).

Others have also identified subgroups using IHC: The first two were luminal (ER+, PgR+) with luminal A group being HER2- and luminal B group is HER2+. The third group is HER2 enriched (ER-/PgR -/HER2+), basal like (ER-/PgR-, HER2-/ (CK 5/6)+ and/or EGFR positive), (Carey et al., 2006). The results from other investigators were in line with Carey et al for its classification of the luminal subgroup, where they had a consensus on classifying luminal A group as being HRs+ and HER2- vs luminal B subgroup (Matos et al., 2005, Kurebayashi et al., 2007, Ihemelandu et al., 2007, Onitilo et al., 2009). Interestingly, Cheang et al identified 3 subgroups the first one is HRs positive with low HER2 and KI67, while the other group differs from the first by HER2 negativity and the final group was ER+ enriched with HER2 regardless of KI67-LI (Cheang et al., 2009a). Nearly parallel with the latter, Hugh et al identified two luminal groups based on low and high HER2 and KI67(Hugh et al., 2009a), (Table 1-1).

Table 1-1: immunohistochemical classification of breast cancer, modified from (Habashy et al., 2012)

Studies	Group 1 (luminal-A)	Group 2 (luminal-B)	Others
Abd El-Rehim et al (Abd El-Rehim et al., 2005)	ER+/PgR+/ luminal CKS+/HER3,4, low BRCA1, MUC-1+	ER+/PgR+/high BRCA1, MUC-1+	-
Matos et al (Matos et al., 2005)	ER+/HER2+(0,+1,+2)	ER+/HER2+ (+3)	-
Carey et al (Carey et al., 2006)	ER+ and/or PgR+/HER2-	ER+ and/or PgR+/ HER2+(100%), p53+(23%)	
Kurebayashi et al (Kurebayashi et al., 2007)	ER+ and/or PgR+/HER2-	ER+ and/or PgR+/HER2+	-
Ihemelandu et al (Ihemelandu et al., 2007)	ER+ and/or PgR+/HER2-	ER+ and/or PgR+/HER2+	-
Onitilo et(Onitilo et al., 2009)	ER+/PgR+/HER2-	ER+/PgR+/HER2+	
Hugh et al (Hugh et al., 2009a)	ER+/low HER2 and KI67	ER+/ high HER2 and KI67	-
Cheang et al (Cheang et al., 2009a)	**ER+/PgR+/low KI67-LI/HER2-**	ER+ and /or PgR (+/-), high KI67, HER2-	ER+ HER2+
Green et al (Green et al., 2013)	**Positive for ER, PgR, HER3 and HER4**	Positive for ER, HER3, HER4; Negative for PgR	Luminal N: Positive for ER, PgR, Negative for HER3, HER4
Prat Et al1(Prat et al., 2013)	**ER+/HER2-/KI67-LI <14%/PgR >20%**	ER+/HER2-/KI67-LI <14%/PgR $\leq$20% or/ ER+/HER2+/KI67-LI $\geq$14%	

Recently, the results of Abd El-Rehim et al (Abd El-Rehim et al., 2005) were refined by Green et al who used a reduced panel of biomarkers which had similar biological classes as the previous study were observed in addition to two novel subgroups of luminal and basal tumours (Green et al., 2013), (Table 1-1, Figure 1-2). In addition, recently, Prat et al updated the definition of luminal A by including PgR of >20% and luminal B was defined of having <20 PgR in addition to other criteria (Table 1-1).

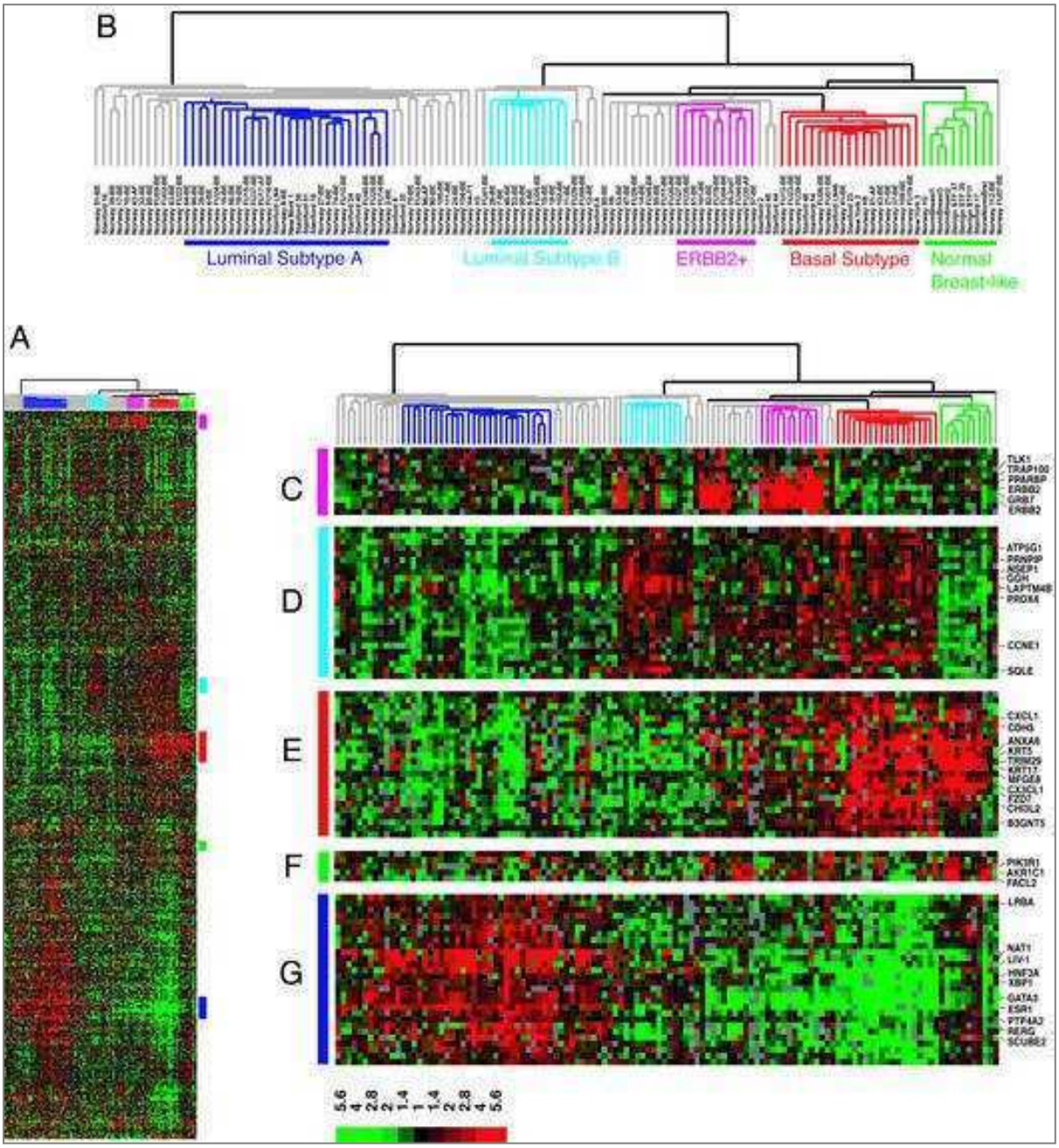

Figure 1-1: Hierarchical clustering of 122 tissue samples (115 BC cases and 7 non malignant) using intrinsic gene signature of 534 genes identified 5 subgroups, (Sorlie et al., 2003b)

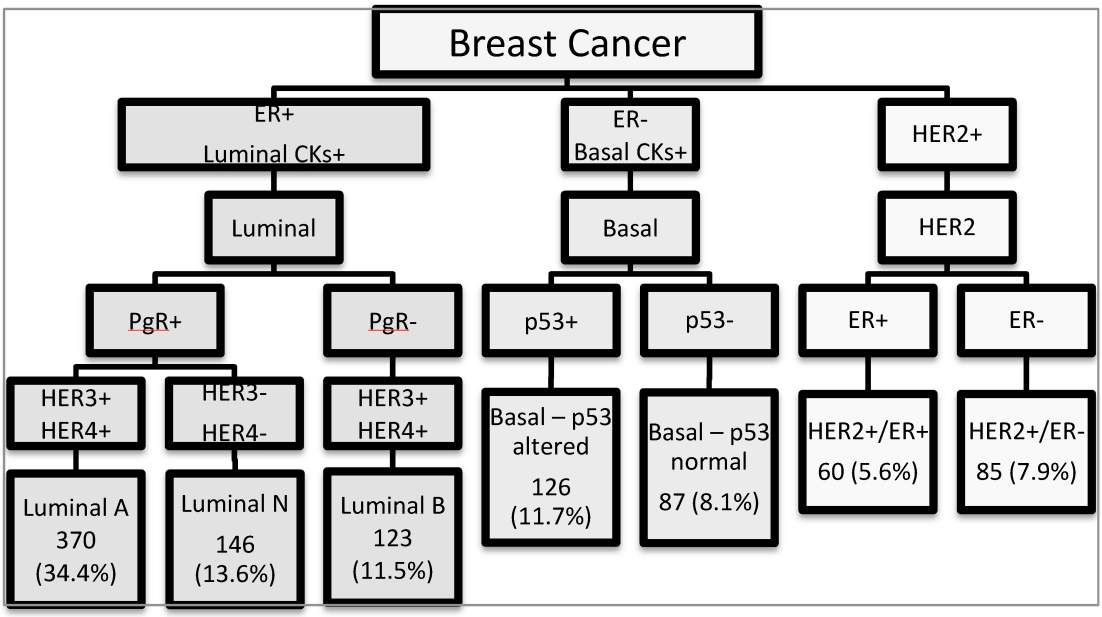

Figure 1-2: Biological BC subgroups according to (Green et al., 2013)

1.2 Receptor tyrosin kinase family type I (Human epidermal growth factor receptor family)

Human epidermal growth factor receptor family (HER) is composed of four members: HER1 (EGFR or c-erbB-1), HER2 (neu, p185 or c-erbB-2), HER3 (c-erbB-3) and HER4 (c-erbB-4) (Witton et al., 2003). This family comprises type I group of 20 families of Receptor Tyrosine Kinases (RTKs) that enhance several transduction reactions (Gschwind et al., 2004, van der Geer et al., 1994). The symbol, ERBB, is originally derived from the name of the avian viral erythroblastosis oncogene B-2 to which these receptors belong and these receptors are ubiquitously expressed in epithelial, mesenchymal and neuronal cells and even in their progenitors (Wain et al., 2002).

The HER family of protein kinases consists of an extracellular portion that encompasses four segments: domains I and III, that have role in ligand binding as they are leucine-rich segments. Next are domains II and IV, where the latter participate in disulfide bond formation having cysteine residues while the role of domain II seems to influence homo and heterodimer formation. A single transmembrane hydrophobic segment of 19–25 amino acid residues that include five amino acid residues in the α-helices with a fundamental role in dimerisation and creating a constant dimer is then followed and an intracellular portion of about 550 amino acid residues is the next segment that has three portions: (1)

a juxtamembrane segment, (2) a protein kinase domain, (3) a carboxyterminal tail (Riethmacher et al., 1997, Sternberg and Zvelebil, 1990, Stern et al., 1988, Weiner et al., 1989), (Figure 1-3). The importance of the carboxy tail appears in the availability of tyrosine residues that are the sites of phosphorylation, which react under ligand-monomer/monomer interactions (Honegger et al., 1990), (Figure 1-3). Exceptionally, HER family members irrespective of other members from other RTK families, can form heterodimers and enhance cell signaling (Dawson et al., 2005). Moreover, they are the only members from the whole RTK group that their dimerisation can enhance their downstream signaling in an allosteric manner where the C-terminus of one lobe reacts with the N-terminus of the other to activate the kinase domain which will subsequently phosphorylate tyrosine residues (Zhang et al., 2006), (Figure 1-4).

Importantly, the structure of these RTKs allows a connection between intracellular and extracellular portions, consequently, docking sites for cellular complexes will be triggered and elaborated to the cytoplasm to control several cellular fates (Yarden and Sliwkowski, 2001b). The twenty families of RTK have functional and structural identities of segments shared in common (intra and extracellular portions), (Ullrich and Schlessinger, 1990, Yarden, 2001) and HER family members have a range of structural similarities amongst each other but the most important is the highest similarity which is recognised in protein kinase domains of all these HER receptors (Jorissen et al., 2003).

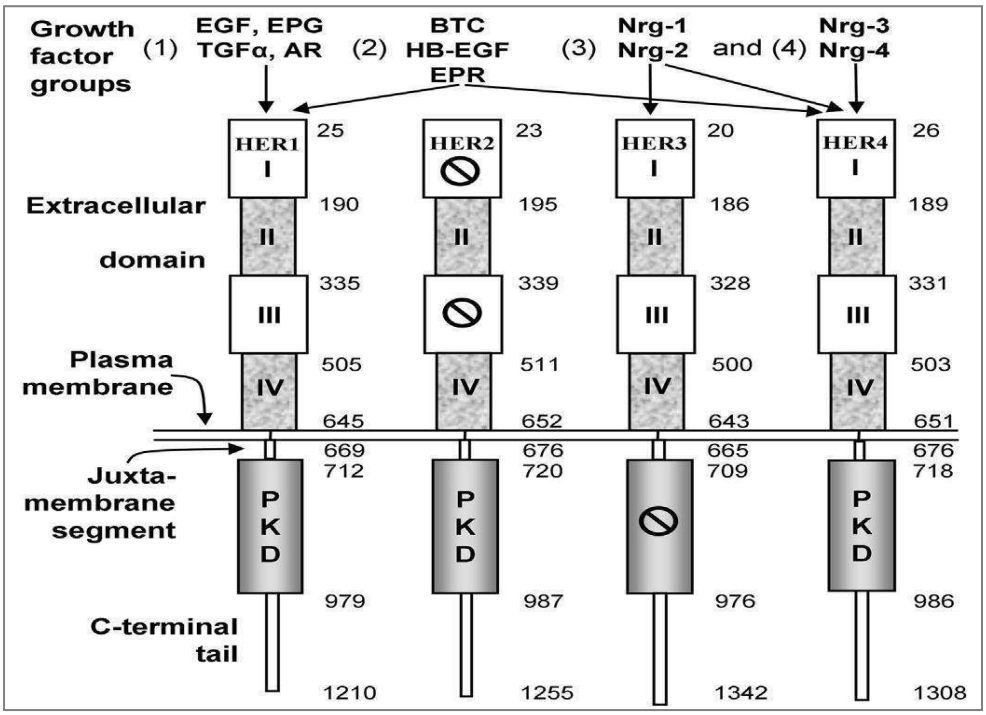

Figure 1-3: The structure of HER proteins. The stop symbol represented in HER2 and HER3 indicate the impaired correspondent areas. JM, juxtamembrane; PKD, protein kinase domain; EGF, epidermal growth factor; EPG, epigen; TGF, transforming growth factor-; AR, amphiregulin; BTC, betacellulin; HB-EGF, heparin-binding epidermal growth-factor like growth factor; EPR, epiregulin; Nrg-1/2/3/4, neuregulin-1/2/3/4. The numbers shown in the figure represent the amino acid sequences of each subsegment and relatively reflect some similarities between these proteins, (Roskoski, 2014).

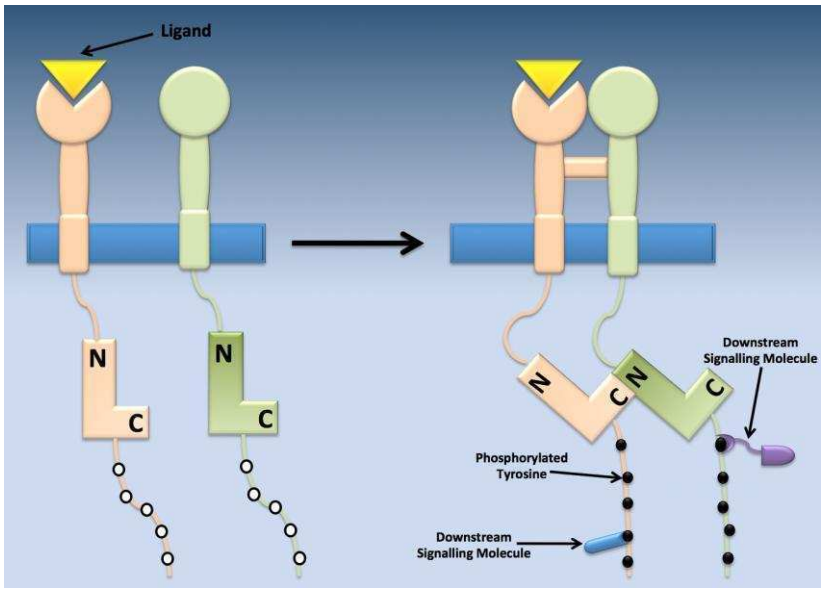

Figure 1-4: Ligand receptor interaction with subsequent allosteric activation of monomers of HER proteins, (Barros et al., 2010)

1.2.1 HER2 receptor

HER2 is the most fundamental protein to be studied from RTK families. Its importance in BC and other cancers is related to its critical involvement in different cellular functions and most importantly, it is a therapeutic target.

Schechter et al was the first to observe this protein in 1984 when he reported a 185 kDa transmembrane protein with its gene is located on chromosome 17q21 (Schechter et al., 1984, Yamamoto et al., 1986). The normal frequency of HER2 expression per single breast epithelial cell was suggested to be between 20,000-50,000. Nevertheless, this frequency in carcinoma per cell can be encountered in excess of this number of receptors (Cersosimo, 2003). In addition, HER2 can be activated via a mutation of HER2 gene at codon 655, inducing conformational changes which leaves HER2 active and ready to form heterodimers (Papewalis et al., 1991). Eventually, BC cells with gene amplification or mutation can enhance cellular liability for uncontrolled growth, invasion, metastasis, impaired apoptosis and thus an aggressive phenotype (Xie et al., 2000).

It is necessary to infer that HER2 can either react by itself or form dimers with other members of its family and it is well known that its ectodermal domain has no liability to bind with ligands as its conformational arm is exposed in a competent form, actively ready to interact and form dimers with other monomers of HER family and enhance subsequent downstream signalling (Zwick et al., 2001, Holbro et al., 2003).

Basically, HER2 enhances cellular proliferation by transduction of signals to its main downstream pathways: RAS-MAPK and PI3K/Akt pathways (Yarden and Sliwkowski, 2001b). The latter pathway appears to influence cell cycle enhancement by stimulating cyclin D1 and inhibition of p27 (Lee et al., 2000). In addition, this pathway inhibits apoptosis by either inhibiting Phosphatase and Tensin homolog (PTEN) or by interaction of p21 (inactive) with apoptosis-signal-regulating kinase 1 (ASK1), rendering apoptosis inactive (Zwick et al., 2001, Sherr and Roberts, 1999). Furthermore, sustained angiogenesis becomes evident with HER2 as it stimulates Vascular Endothelial Growth Factor (VEGF) protein and even HER2 overexpressing tumours show high expression of KI67 which might indicate more aggressive behaviour (Yen et al., 2000, Zhou et al., 2001).

1.3 Cross talk between ER and HER2 in breast cancer

It is well known that ER in the nucleus functions as a transcriptional factor that regulates specific genes (Parker, 1993, Osborne et al., 2000). This receptor has ligand binding domain which is known as Activation Function-2 (AF-2) located at the carboxy terminal end and non-ligand domain which is known as Activation Function-1 (AF-1) located at the N-terminus, both these domains flank from either sides of the DNA-binding domain that can bind to oestrogen Responsive Elements (EREs) in the promoter region of target genes and finally, it has transcription activation domains (Parker, 1993, Osborne et al., 2000). When oestrogen binds to ER, this will trigger changing its conformation and subsequent dimerisation which facilitates the binding of this complex to promoter areas of the target genes and is facilitated by the availability of coactivators including AIB1 and acetyl transferases (McKenna et al., 1999). Alternatively, growth factors like HER2 can enhance activation of different signaling molecules; for instance, PI3K/Akt proteins and MAPKs can phosphorylate ER in its ligand independent binding domain at certain sites known as SER 118 and SER 167 where ER can be phosphorylated enhancing transcriptional activity (Campbell et al., 2001, Clark et al., 2001)

It has been reported that ER has different modes of action: 1) the genomic mode of stimulation which includes the classical and the non-classical pathways, 2) the non-genomic mode of stimulation and 3) The ligand independent pathway. The classical way includes the binding of ER after stimulation immediately to EREs in the promoter region of target genes. On the other hand, the non-classical way includes the binding of ER (when there are no EREs in the target genes) to other transcription factors and this complex in turn will bind to the specific promoter regions of the genes to be transcripted. Both of these pathways rely on the nuclear function of ER (Kushner et al., 2000, Safe, 2001). On the contrary, the mechanism of action of ER in the non-genomic way is totally different from the genomic action in that ER activity is encountered in the membranous or cytoplasmic portions of the cells. In this respect, ER has been shown to interact with several kinases including the Insulin-like Growth Factor-1 Receptor (IGF-1R), SRC, phosphatidylinositol 3-kinase (PI3K), MAPK, Epidermal Growth Factor Receptor (EGFR) and HER2 (Wong et al., 2002, Schiff et al., 2004, Shou et al., 2004), (Figure 1-5). The interaction between ER and these

factors can induce rapid physiological action in short time before transcription of target genes that can be initiated by nuclear ER activation. Finally, the ligand independent binding involves the binding of MAPK or Akt to ligand independent binding domain of ER and enhance its nuclear action (Normanno et al., 2005, Yang et al., 2004).

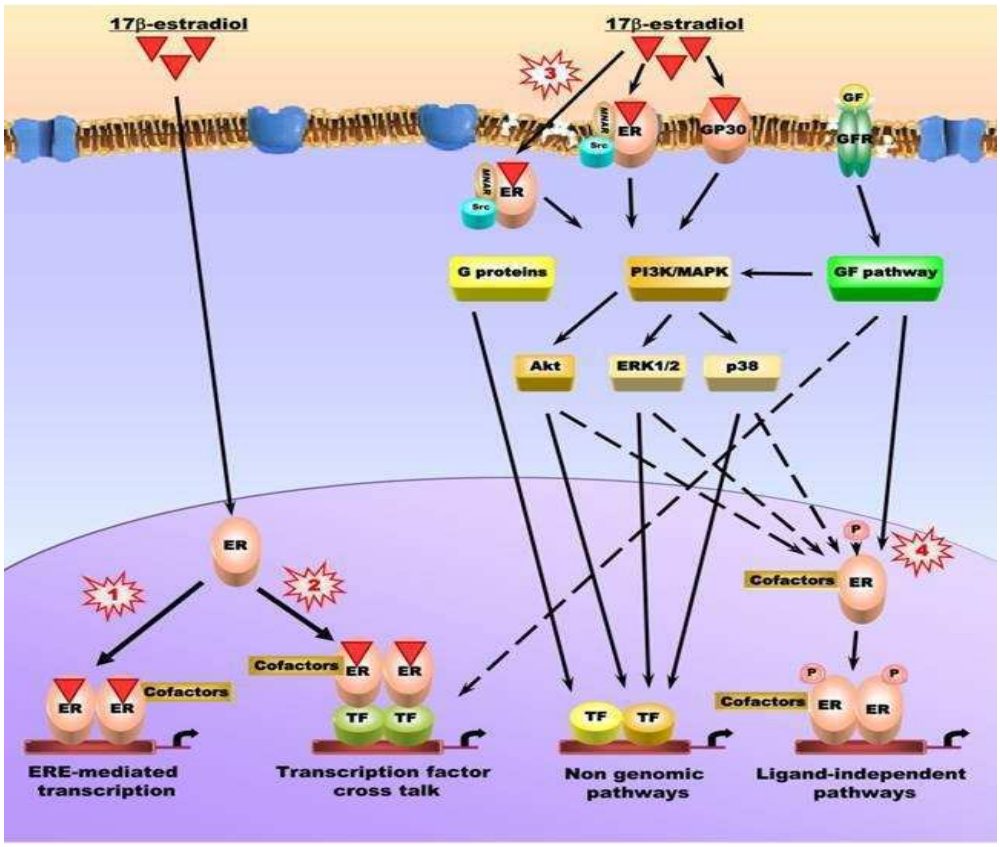

Figure 1-5: Different modes of activation of ER and its interaction with growth factors. Pathway 1: classical estrogen signaling pathway (estrogen response element (ERE)-dependent). Pathway 2: Non classical or non-ERE estrogen signaling pathway - ligand-bound ERs interact with other transcription factors, such as activator protein (AP)-1, NF-κB and Sp1, forming complexes that mediate the transcription of genes whose promoters do not harbour EREs. Co-regulator molecules regulate the activity of the transcriptional complexes. Pathway 3: non-genomic estrogen signaling pathways - ERs and GP30 localized at or near the cell membrane might elicit the rapid response by activating the phosphatidylinositol-3/Akt (PI3K/Akt) and/or protein kinase C/mitogen activated protein kinase (PKC/MAPK) signal transduction pathways. Pathway 4: ligand-independent pathways - ERs can be stimulated by growth factors and other proteins in ligand independent site (Roman-Blas et al., 2009)

1.4 Resistance to hormonal therapy and the interaction with HER2 in ER+/HER2+ tumours

Approximately, 75% of BC tumours are ER and/or PgR positive. HR status is the reliable predictive measure for selecting adjuvant hormonal therapy for those with early and metastatic BC (Goldhirsch et al., 2006, Jordan, 2002, Buzdar, 2001). HER2 protein overexpression or amplification of its gene occurs in approximately one fifth of BCs and is associated with shortened disease free interval and overall survival (Wolff et al., 2014). Interestingly, half of HER2+ primary BCs and metastatic BCs are also HR+ (Untch et al., 2008, Brufsky et al., 2005) while only one tenth of HR+ tumours are HER2+ (Dowsett et al., 2008, Mauriac et al., 2007).

It has been hypothesised that a stepwise mode occurs in the resistance to endocrine therapy. Firstly, the BC cells are dependent on oestrogen, after that they become responsive to endocrine therapy then move to a state of non responsiveness to finally reach the oestrogen independent status. Meanwhile, it is likely that there is an associated response from tumour cells for each of the previous stages. For instance, at the beginning, there is hypersensitivity to oestrogen which is associated with increase its transcriptional activity or enhancement of its non-genomic mode of action. Later on, such action will be changed to oestrogen super sensitivity and increase growth factor signaling, this is to be followed by a state of oestrogen independence (Normanno et al., 2005)

From the clinical point of view, almost all BC patients in western societies receive tamoxifen either as an adjuvant treatment after surgery or as the first treatment choice for those with advanced disease. However, 50% of patients with advanced disease do not respond, almost all those with metastatic disease and 40% of those taking tamoxifen as an adjuvant therapy, experience relapses and subsequently die. Taking into account all the above mentioned evidences, there is a strong indication that de novo or acquired resistance to hormonal therapy is inevitable and might be encountered at any stage (Gradishar, 2004). In clinical practice, using ER selective modulators such as tamoxifen, enhances the recruitment of corepressors and deacetylases that inhibit the transcriptional activity of ER (Shou et al., 2004, Smith et al., 1997). Importantly, tamoxifen

exhibits partial agonist-antagonist activities in different cells and this depends on the level of corepressors and coactivators (Smith et al., 1997).
It has been observed that in Michigan Cancer Foundation- 7 (MCF-7) /HER2-18, which has been transfected with HER2 amplification, tamoxifen functions as an oestrogen agonist and stimulates tumour growth, whereas oestrogen deprivation therapy remains effective and inhibits growth in this and MCF-7 cells (Ellis et al., 2001). For this reason, there is a growing body of evidence that aromatase inhibitors are superior to tamoxifen especially in Post-menopausal women as the production of oestrogen in these patients occurs in peripheral tissue or the tumour (Johnston and Dowsett, 2003, Lonning, 2004); nevertheless, figures indicated that the response rate is not much higher than that encountered by tamoxifen in those with advanced disease due to associated resistance (Strasser-Weippl and Goss, 2005, Gradishar, 2004). It has been reported that increased growth factor signaling might increase the ER activity through the non-genomic action which is observed after binding of tamoxifen to ER (Cui et al., 2003).

1.5 Breast cancer therapy for HER2

It has been well documented in clinical practice that tumours with ER+ and HER2+ phenotype are resistant to tamoxifen and its effect is inversely correlated with patients' outcome (Dowsett and Grp, 2003, De Placido et al., 2003). Furthermore, the adverse effect observed when combined tamoxifen and Cyclophosphamide, Adriamycin and Fluorouracil (CAF) is not encountered (Ravdin et al., 1998). In addition, aromatase inhibitors, such as letrozole, have a superior effect as a hormonal therapy compared to tamoxifen in the context of ER+/HER2+ BC (Ellis et al., 2001). Importantly, using an adjuvant chemotherapy based on anthracyclins compared to the regimen of CAF has been considered as a favourable therapeutic option; therefore, targeting HER2 appears to be mandatory either when used with hormonal therapy or chemotherapy (Paik et al., 2000, Sparano and Rajdev, 2000).

1.5.1 Trastuzumab (Herceptin^{tm}): Mechanisms of action and causes of resistance

This drug is already approved by the US Food and Drug Administration (Weinberg, 2007) and also it is an established drug for the treatment of HER2+ BC cases in the UK (Rakha et al., 2015, Strasser-Weippl et al., 2015). It is used as a monoclonal therapy against HER2 and is effective therapy for HER2+ tumours irrespective of the hormonal receptor status (Untch et al., 2008, Wolff et al., 2014). Trastuzumab was initially developed as a mouse monoclonal antibody directed against HER2 having the characteristic of targeting HER2 only when it exceeds 10-100 times its normal level and it acts to inhibit homo- and hetero-dimer formation. To avoid adverse effects, the constant region of this antibody was humanised (Weinberg, 2007), (Figure 1-6).

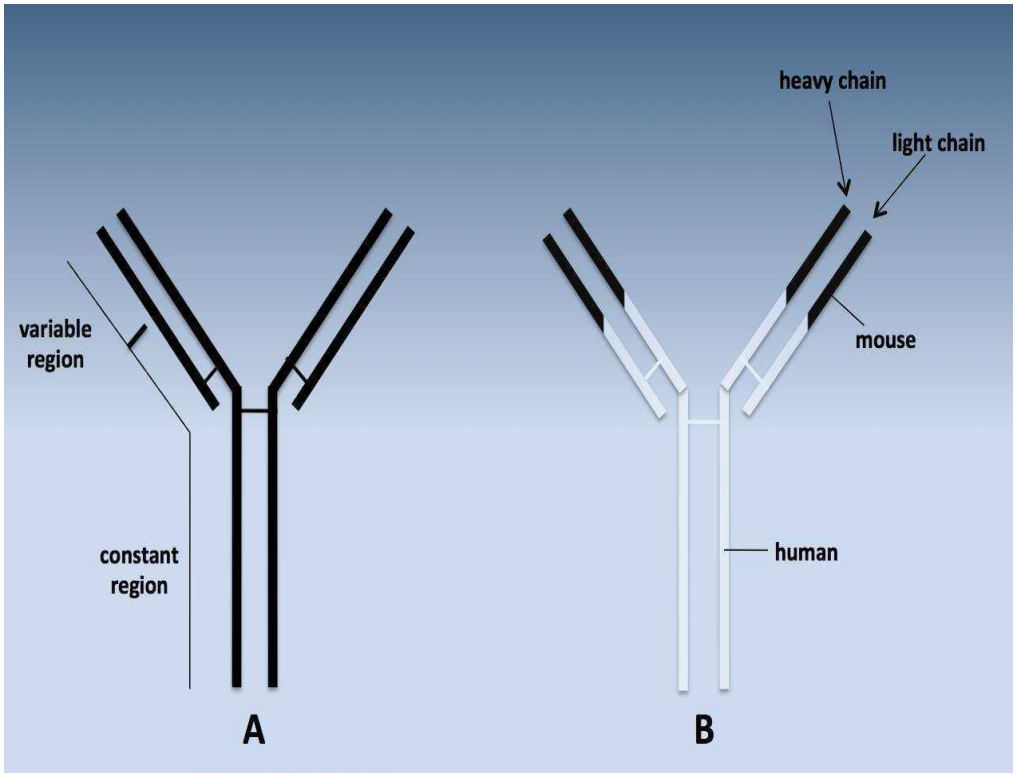

Figure 1-6: Humanised Trastuzumab; A: mouse monoclonal antibody, B: humanised monoclonal antibody, (Barros et al., 2010)

The benefit of Trastuzumab is at its maximum when Hercept test (protein measurement using IHC) =+3, when gene amplification is high using Fluorescent in situ hybridisation technique where the HER2/ Chromosome Enumeration Probe (CEP) 17 ratio is >2 (HER2/CEP17 >2 and HER2 gene copy numbers are $\geq$6) or even by using other techniques (Hudis, 2007). Meanwhile, 2+ (protein measurement) cases with gene amplification, do benefit from this treatment (Seidman AD, 2004).

Interestingly, the mechanism of action of Trastuzumab can be demonstrated in different ways: 1) Binding of this drug to HER2 will enhance internalisation, denaturalisation and destruction of this complex (Menard et al., 2003, Rubin and Yarden, 2001) or a downregulation of the PI3K/Akt pathway (Le et al., 2005), 2) Trastuzumab can enhance the immune system to initiate apoptosis by inducing antibody dependent cellular cytotoxicity (Rita Nahta, 2006), 3) Trastuzumab can function to inhibit angiogenesis and limits metastasis (Yotaro Izumi, 2002), 4) promoting cell cycle arrest by inducing the expression of $p27^{KIP1}$gene which is a potent cell cycle repressor (Lane et al., 2001), and its action can be augmented if it is combined with chemotherapy (Toyoshima and Hunter, 1994).

In this respect, it is important to consider that although Trastuzumab is an effective drug in this context, resistance to its action can still to be a possibility and it is indicated to be proved effective in only 35% of cases if used individually (Charles L. Vogel, 2002); nevertheless, when used with chemotherapy, its success rate improves to reach 50-84% (Burris et al., 2004). In spite of using trastuzumab alone or in combination with other therapy, resistance seems to be shortly evident and this, in particular suggests the possibility of other mechanisms causing resistance (Burris et al., 2004). Probably, one of these mechanisms is that Trastuzumab is ineffective in preventing dimerisation when ligands are expressed in high levels (Andrea B. Motoyama, 2002). Another possibility is the presence of truncated HER2 (p95 HER2) which does not have an ectodomain, the site for attachment of trastuzumab, hence, the latter will not destroy HER2 which will be constantly active and will form dimers with HER3 which is a well known potent activator of PI3K/Akt (Miguel A. Molina, 2001). Moreover, PTEN, a well-known inhibitor of the latter pathway is highly correlated with trastuzumab response which can compete with Steroid Receptor Co-regulator (SRC) for binding with HER2 and enhance the function of PTEN (Yoichi Nagata and Mien-Chie Hung, 2004).

In addition, EGFR is another potential factor likely to cause resistance of Trastuzumab if it is expressed in high levels in HER2+ tumours based on evidence from *in vitro* study (Rajiv Dua, 2010). Indeed, it has been shown that long exposure to this drug increases the expression of EGFR and this can be inhibited by using EGFR inhibitors as gefitinib (Narayan et al., 2009). Likewise, HER3 is not exempted from causing impaired function of Trastuzumab since the latter is thought to disturb the HER2/HER3 dimer for few hours only which is not sufficient to decrease its downstream effects (Sergina et al., 2007).

1.6 Current management of breast cancer

The management of early BC in the UK involves choices between surgery, radiotherapy, endocrine therapy, chemotherapy and anti-HER2 treatment based on the NPI, BC subtype, HRs and the menopausal status. Although the risk of loco-regional relapse is related to the biological aggressiveness of the disease as reflected in its intrinsic subtype, there is no evidence that more extensive surgery will overcome this risk (Morrow, 2013). Effective systemic therapy decreases loco-regional recurrence (Kiess et al., 2012). Regarding radiotherapy, clinical trial evidence supports the validity of hypo fractionated radiotherapy such as 40 Gy in 15 or 42.5 Gy in 16 fractions in many patients (Whelan et al., 2010) . Such short course whole breast radiation therapy has obvious benefits with regard to patient convenience and cost. For women with Pre-menopausal BC (NPI>3.4), tamoxifen is given for 5 years (Goldhirsch, 2013). Recent evidence from the ATLAS trial suggests that durations of tamoxifen >5 years may be appropriate (Davies et al., 2013). In addition, Post-menopausal women with endocrine responsive disease, letrozole therapy administered after 5 years of tamoxifen is effective(Goss et al., 2003); however, adverse effects of aromatase inhibitors limit their use in a substantial proportion of women, and particular concern may exist for those with pre-existing ischaemic cardiovascular disease (Ingle, 2013). For HER2 positive BC, clinical trial results support a standard duration of adjuvant Trastuzumab of one year rather than longer (Goldhirsch et al., 2013) or shorter (Pivot et al., 2013). A major unresolved question is the threshold for use of adjuvant cytotoxic chemotherapy for patients with luminal A or luminal B disease. In prospective/retrospective studies, the 21- gene

recurrence score identifies groups with no benefit from the addition of chemotherapy in node-negative (Paik et al., 2006) or node-positive (Albain et al., 2010) disease. In both these studies based on randomised trials, chemotherapy benefit was limited to the group with high 21-gene recurrence score. For BC patients with triple-negative disease, optimal chemotherapy regimens have not yet been defined; however, evidence supports the use of anthracyclins and taxanes, but not bevacizumab, platinum salts, capecitabine, or gemcitabine (Burstein, 2013).

1.7 The clinical differences between ER+/HER2+ and ER-HER2+ breast cancer subgroups

Although luminal BC is associated with favourable outcome compared to other subgroups of BC, there is a clinical difference between subclasses of the luminal groups. ER+/HER2+ group is clinically different from luminal A in terms of response to therapy and outcome (Sotiriou and Pusztai, 2009). Importantly, this subset is associated with poor DFI and increased risk of early relapse. In addition, it is characterised by insensitivity to endocrine therapy compared to the luminal A subclass. From another point, this group seems to reveal relative insensitivity to chemotherapy compared to ER-HER2+ tumours (Tran and Bedard, 2011). Interestingly, the overall survival of untreated ER+HER2+ group is comparative to that of ER-HER2+ (Perou et al., 2000b) and the increased relaps risk which is associated with ER+HER2+ group is only limited to the early period after surgery and not surprisingly it is recurrence liability which increases only in the first 5 years after diagnosis (Ignatiadis et al., 2009).

For the predilection to the metastasis site, ER+HER2+ BC shows a liability to pleura and bone metastasis contrary to ER-HER2+ tumours which show a predilection to brain metastasis (Leyland-Jones, 2009, Gabos et al., 2006). Several studies have indicated that ER+HER2+ BC is relatively insensitive to endocrine therapy and chemotherapy compared to luminal A and ER-HER2+ groups respectively and that the pathological complete response after neoadjuvant chemotherapy is constantly low in ER+HER2+ group compared to ER-HER2+ (Esserman et al., 2009, de Ronde et al., 2010, Carey et al., 2007). Moreover, another study reported the impact of complete pathological response

after neoadjuvant anthracycline in ER-HER2+ group relative to the double positive group (von Minckwitz et al., 2012).

1.8 Signalling pathways associated with HER2 and ER

ER and HER2 are key proteins that have great effect in BC fate and their interaction with different pathways and downstream proteins made it substantially difficult to address the mere effect of these proteins without a simultaneous effect from their surrounding upstreams and downstream factors. Therefore, studying different pathways associated with these proteins could unravel the biological significance of these proteins especially when they co-express together (Figure 1-7).

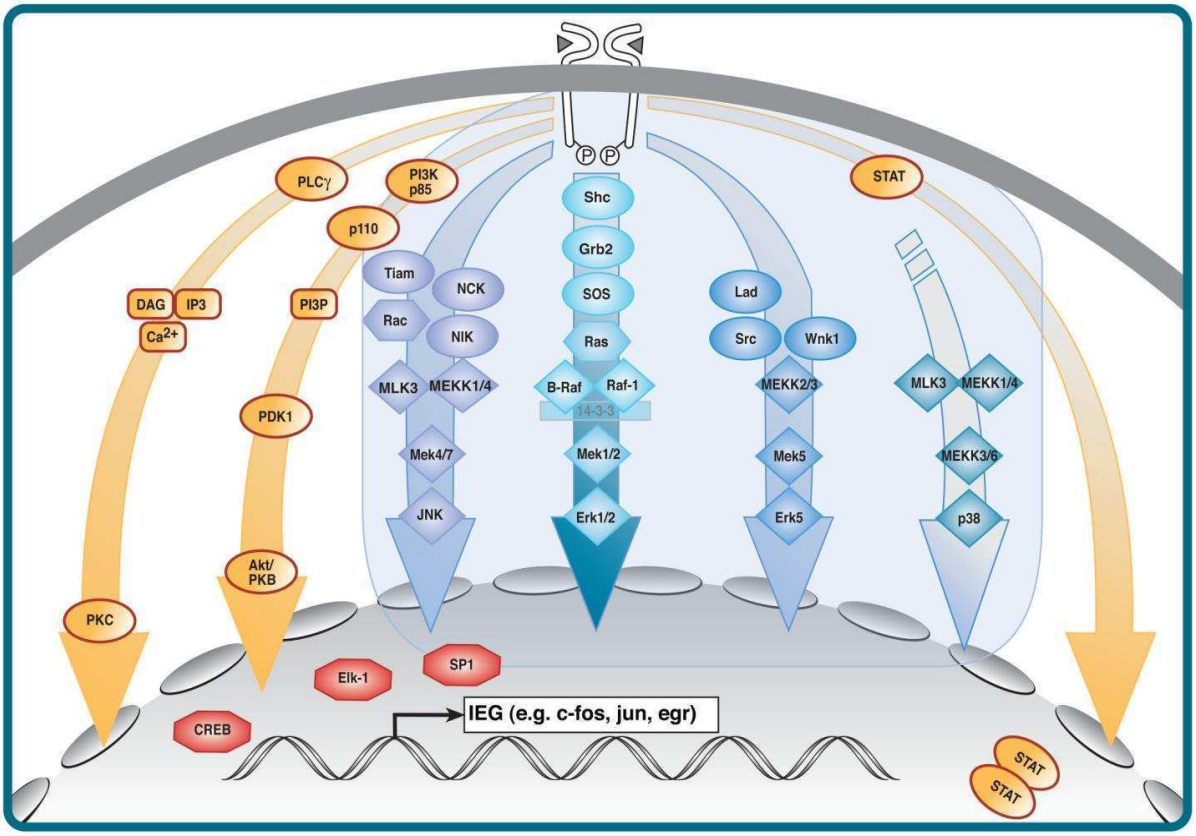

Figure 1-7: : schematic representation of the four downstream pathways ofHER2 protein and other growth factors through RTKs. Signaling pathways stimulated by growth factors and their receptor tyrosine kinases. Growth factor binding to and dimerization of transmembrane receptors is followed by trans-phosphorylation of the cytoplasmic portions of the receptors (P letters represent phosphate groups). The activated receptor physically recruits from the cytoplasm and from the plasma membrane a large variety of adaptors and enzymes, which subsequently put in motion several linear cascades, some of which are presented. The four canonical MAPK pathways are presented. Also shown are the PI3K-Akt, phospholipase C-PKC and the STAT pathways. All routes culminate in regulation of gene expression, such as rapid transcription of a group of immediate early genes (IEGs). (Katz et al., 2007)

1.8.1 Mitogen activated protein kinase pathway and its members

Mitogen Activated Protein Kinases (MAPKs) are evolutionary conserved enzymes which function as signal transduction pathways. Growth factors, cytokines or stress factors can stimulate this protein cascade and physiological or pathological responses can be subsequently revealed (Dhillon et al., 2007).
Importantly, MAPK pathway is a three layer signaling cascade that following binding of growth factors to their receptors, signals are transmitted through these three layers named MAPKKK (MAP3K), MAPKK (MAP2K) and finally to MAPKs (Figure 1-8). When the latter are phosphorylated, they form dimers and translocate to the nucleus to stimulate transcription factors that can enhance transcription of target genes responsible for cell growth and differentiation (Brunet et al., 1999).

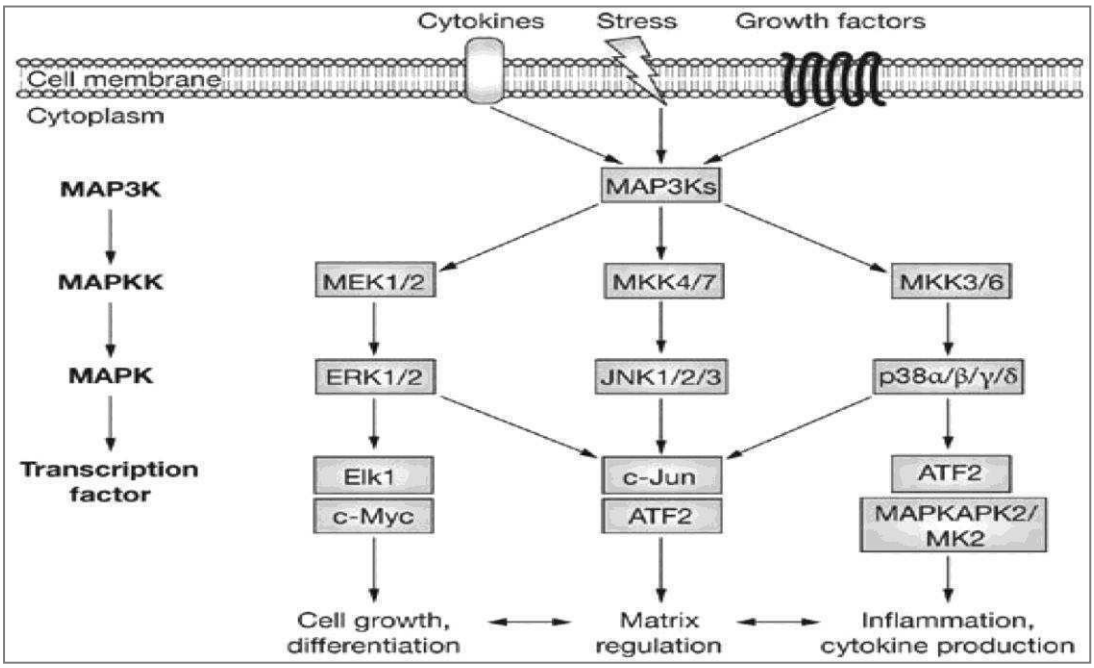

Figure 1-8: : A schematic representation of the three layer MAPK pathway and their upstream tier and downstream substrates, adapted from (Hammaker and Firestein, 2010)

The process of provoking MAPKs starts with stimulation of receptor tyrosine kinase (RTK) by one of the mentioned factors mainly growth factors. This can stimulate dimerisation, activation and transphosphorylation of these receptors. When the intracellular domains are phosphorylated, they attach to certain adaptor proteins that in turn can enhance the recruitment of substances called Guanine Nucleotide Exchange Factors (GEFs) in the cell membrane. GEFs can stimulate some small GTPase proteins like H-RAS, N-RAS and K-RAS which eventually regulate the switch from GDP to GTP and vice versa (McCubrey et al., 2007).

Interestingly, there are three main MAPKs: (1) Extracellular Signal-Regulated Kinase (ERK1/2), (2) c-jun-N terminal Kinase (JNK1/2) and (3) p38. ERK1/2 which is one of a small group of dual-specificity kinases that requires phosphorylation of both tyrosine and threonine in their motifs (Roskoski, 2012), is the main MAPK that is stimulated by growth factors and can have impact on cell proliferation, differentiation, apoptosis and migration (Katz et al., 2007). RAF proteins can directly phosphorylate mitogen activated protein kinase/ERK kinase (MEK1/2)(Ramos, 2008, Johannessen et al., 2010) which are tyrosine, serine/threonine and can activate ERK1/2 (Ramos, 2008) specifically. The latter can enhance the activation of different nuclear and cytoplasmic factors such as transcription factors, kinases and phosphatases (Ramos, 2008, McCubrey et al., 2007).

It is worth noting that due to either presence of feedback loops or interaction of RAS-ERK pathway with others, MEK inhibition or resistance can occur (De Luca et al., 2012). Importantly, many growth factor genes have binding sites for transcription factors which are located in the promoter region of these genes and by the activation of RAS-ERK pathway, there will be concomitant activation of these growth factor genes (Maurer et al., 2011).

1.8.1.1 The role of Mitogen Activated Protein Kinases in breast cancer

It has been well established that women with HER2+ BC have more aggressive phenotype, a higher likelihood of recurrence after treatment, and poorer outcome (Slamon et al., 1987b). HER2 overexpression may lead to increased receptor homodimerisation and heterodimerisation, which enhances receptor tyrosine kinase activity by inducing autophosphorylation utilising several signaling pathways, including the Ras/MAPK pathway (Creighton et al., 2008).
In different studies, the role of MAPKs has been investigated and HER2 is one of the factors known to affect this pathway (Yarden and Sliwkowski, 2001a). ER in turn, has many cross talks with HER2. Moreover, it has been reported that MAPK signalling has been considered one of the causes attributed to loss of ER expression, in particular in those cells expressing HER2 and that ER expression can be restored when MAPKs are inhibited (Creighton et al., 2006). Thus, it seems that there are many interactions and controversies with the above pathways but the challenge is still based on which one is the predominant factor that can orchestrates all of these behaviours of ER and HER2 (Oh et al., 2001).
Importantly, one of the mechanisms which reveals the interaction between ER and MAPKs has been illustrated by the activation of ER in a ligand-independent manner, via signal transduction pathways after HER2 stimulation (Kong et al., 2003). Studies indicated that co-expression of HER2 with ER in BC is one of the possible explanations of the obtained resistance after tamoxifen treatment as the latter switches to be an ER agonist in this context. Moreover, ER is a suitable target for MAPKs which they can activate at the AF-1 domain by inducing its phosphorylation at SER118. Less likely is phosphorylation at SER167 where several changes to ER can be determined with subsequent translocation to the nucleus (Chen et al., 2002, Kato et al., 1995a, Bunone et al., 1996, Joel et al., 1998a). Residues surrounding SER118 comprise the MAPK consensus sequence, Pro-Gln-Leu-Ser-Pro (Joel et al., 1998a). Research illustrates that the activation of ER via this route is rapid and short lived compared to that enhanced by oestrogen; nevertheless, it has not been revealed whether this activation is accomplished inside or outside the nucleus (McGlynn et al., 2013, Kurokawa and Arteaga, 2003).

In keeping with other views, Sivaraman et al. have suggested a potential role for MAPKs signaling in the initiation and pathogenesis of BC where they indicate that MAPK activity is elevated in primary and metastatic BC compared to the normal tissue (Sivaraman et al., 1997). Another report revealed that constitutive expression of RAF-1 kinase (an upstream mediator of MAPK) in MCF-7 human BC cell line resulted in growth which is independent of oestrogen (El-Ashry et al., 1997).

ER, along with other members of the steroid receptor superfamily, is functionally phosphorylated by MAPK (Kato et al., 1995b, Arnold et al., 1995, Bunone et al., 1996, Le Goff et al., 1994) which act via regulating the DNA-binding domain and subsequent transcription activation. ER phosphorylation by MAPKs is considered a step towards hormonal independence; however, the mechanisms underline these events are not clearly understood especially with persistent ER expression (Kato et al., 1995a, Bunone et al., 1996).

Other researchers suggested that although RAF/ MEK /ERK pathway was found to have a role in cancer progression, it can concomitantly enhance cell cycle arrest and growth inhibition (Cagnol and Chambard, 2010, Subramaniam and Unsicker, 2010). This growth arrest is referred to as oncogenic-induced senescence. The justification of the latter phenomenon is that innate tumour suppressor mechanisms will be triggered by aberrant growth signals. It is of note that ERK activity can enhance both intrinsic and extrinsic apoptotic pathways. Such pathways will be triggered either by inducing the release of mitochondrial cytochrome c, activating caspase 8, in addition to inducing permanent cell cycle arrest or autophagic vacuolisation (Cagnol and Chambard, 2010, Courtois-Cox et al., 2006). Furthermore, it has been indicated that RAS/RAF mutations can cause feedback inhibition of RAS or its upstream mediators and even more, these inhibitory signals will also inhibit Ras-PI3K arm and this is thought to be mediated by HDM2 and FOXO3 (Courtois-Cox et al., 2006)

Another study found that a sort of sequestration of cytoplasmic ERK induced by some proapoptotic molecules such as Death Associated Protein Kinase (DAPK) can augment the apoptotic action of DAPK which later on enhances cell death (Mebratu and Tesfaigzi, 2009). Apart from the above views, a plethora of studies referred to the role of MAPKs in having an inverse association with BC

progression and even implied their participation in inducing apoptosis (Milde-Langosch et al., 2005, Hsu et al., 2005, Altiok et al., 2007).

1.8.2 Phosphatidylinositol 3 kinase signalling pathway

Enhancing cells by growth factors and hormones initiates a coordinated series of events which can stimulate different cellular functions including: cell growth, proliferation, migration, and survival. One of the crucial mechanisms for extracellular growth signalling is Phosphatidylinositol-3-kinase (PI3K) pathway (Cantley, 2002), with its downstream arm, Protein kinase B (PKB/Akt), (Arcaro and Guerreiro, 2007). Lipid kinases of PI3K family, have been a matter of investigation and attracted researchers not only in BC but also in different tumours owing to their extraordinarily diverse effects they can exert, stem from their ability to stimulate downstream effectors especially Akt and the mammalian target of rapamycin (mTOR), (Jiang and Liu, 2008, Dillon et al., 2007).

The PI3K family has three main classes: Class I, Class II and Class III. This classification is based on primary structure, regulation, and the specificity of its lipid substrate *in vitro* (Leevers et al., 1999). PI3K is composed of a heterodimer which has catalytic and regulatory subunits; p110 and p85 respectively. p85 has other variants, designated p85α, p55α, p50α, p85β, and p55γ, the first three of which are expressed from one gene while the other two are from another. Moreover, p110 has three variants each of which is a product of a separate gene, these variants are designated as: p110α, β, or δ catalytic subunits (Carpenter et al., 1990). Recently, there has been a consensus that a mutation of transforming oncogene; PI3K catalytic subunit alpha (PIK3CA), (Maira et al., 2009) which encodes for α catalytic subunit, enhances its enzymatic activity which will proceed with cancer progression (Kang et al., 2005).

The PI3K pathway is an immediate downstream of HER2 and its aberrant stimulation is either because of mutations of the PIK3CA gene or due to loss of PTEN expression which have been found to affect the sensitivity to inhibitor–based therapies (Nagata et al., 2004a, Berns et al., 2007a, Eichhorn et al., 2008). Akt, which is a serine/threonine kinase, is activated in a multistep cascade-like process. The triggering starts by binding of receptors that have tyrosine-kinase activity with the growth factors which enhance the

autophosphorylation of the latter kinase (Jiang and Liu, 2009). Later on, RTK will activate PI3-kinase, which converts phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-4, 5-trisphosphate (PIP3), this in turn, recruits PH – containing proteins to the plasma membrane, leading to their stimulation. Of particular importance are the Phosphoinositide-dependent Kinase (Pdk1) and Akt1 protein which will be activated by the former at Threonine (Thr) 308, and later on at SER 473 by mTORC2 (Laplante and Sabatini, 2012, Jiang and Liu, 2008, Vogt et al., 1999, Woodgett, 2005).

Interestingly, PTEN is the negative regulator of Akt and its mutation or deletion confers the latter protein constitutive activation (Jiang and Liu, 2008). Additionally, an aberrant stimulation of Akt could be rendered from either the amplification of its gene (Staal, 1987) or alternatively the mutation to the pleckstrin homology (PH) domain of Akt1 which in turn can enhance its membrane localisation (Carpten et al., 2007). A compelling therapeutic goal though could be obtained by simultaneous targeting of the PI3K (which is currently considered as a transforming oncogene) pathway together with anti-HER2 agents (Maira et al., 2009, Samuels et al., 2004).

1.8.2.1 Preclinical indications that PI3K/Akt/mTORC1 pathway is a therapeutic target in breast cancer.

Despite the fact that the PI3k pathway can enhance proliferation and abolish apoptosis through variable mechanisms, a cross-talk between the ER and this pathway has been also revealed. Rapid non genomic action of membranous ER is encountered in triggering PI3K signalling (Stoica et al., 2003a, Stoica et al., 2003b), where growth factors as HER2 seems to play fundamental role but not the transcriptional activation of ER. Investigators have shown that ER binds to the p85 regulatory subunit of PI3k (Simoncini et al., 2000), while Akt and the mTOR can phosphorylate ER at serine 167, enhancing both its ligand-dependent and ligand-independent transcriptional activity (Campbell et al., 2001). Others focused on the role of constitutively active forms of Akt or PI3-kinase in establishing oestrogen independent growth in BC cell lines when they are forcedly expressed, in addition to endocrine resistance that was revealed through knocking out PTEN (Miller et al., 2009, Beeram et al., 2007).

Importantly, experiments carried out using ER+ cell lines have shown that long term depletion of oestrogen enhances the activation of PI3K pathway (Martin et

al., 2000, Cavazzoni et al., 2012) which can be further diminished by using inhibitors to such proteins and furthermore, BC cells regain their sensitivity to oestrogen (Boulay et al., 2005, Beeram et al., 2007, Miller et al., 2010). In contrast, other researchers had conflicting results in this context and they did not observe an improved response whilst using such inhibitors (Yue et al., 2007, Leung et al., 2011). An element of evidence has been revealed that Trastuzumab resistance in HER2+ BC can be attributed to the activation of PI3K and its downstreams that can be reversed by concomitant use of PI3-kinase, Akt, or mTOR inhibitors (Berns et al., 2007a, Lu et al., 2007).

1.8.2.2 The Mammalian Target of Rapamycin Complex 1 (mTORC1) as a downstream signalling member of PI3K pathway

The mammalian Target of Rapamycin Complex 1 (mTORC1) is an important member in PI3K/Akt signalling and it is central sensor for nutrient/energy availability, modulated through PI3K/Akt-dependent mechanisms. In the presence of mitogenic influence and sufficient nutrients and energy, mTORC1 acquires a positive signal to the translational machinery by stimulating its downstream effectors (Bjornsti and Houghton, 2004).

In mammalian tissues, two proteins have been identified: mTORC1 and mTORC2, both containing atypical serine /threonine kinases, which belong to the PI3K-like kinase family and this influences a variety of cellular functions. Such kinase is called mTOR/FRAP1 but with RAPTOR (a scaffolding protein) it forms the mTORC1 and with RICTOR (mTORC2 associated scaffolding protein) forms mTORC2, in addition to another mammalian stress-activated protein kinase interacting protein (Strimpakos et al., 2009, Reiling and Sabatini, 2006, Sabatini, 2006). Furthermore, p-mTORC1 is responsible for the phosphorylation of two important regulators of protein translation: Ribosomal p70S6 Kinase (p70S6K) and Eukaryotic initiation factor 4E binding protein (4E-BP), (Reiling and Sabatini, 2006, Zoncu et al., 2011), (Figure 1-9). Several factors can stimulate mTORC1 including growth factors which can enhance the upstream effecter PI3K either directly or indirectly through certain docking proteins like insulin receptor substrate or GRB2-associated binder. Other factors that can stimulate p-mTORC1 include: a status of energy, amino acids levels and cellular stress (Sengupta et al., 2010, Strimpakos et al., 2009).

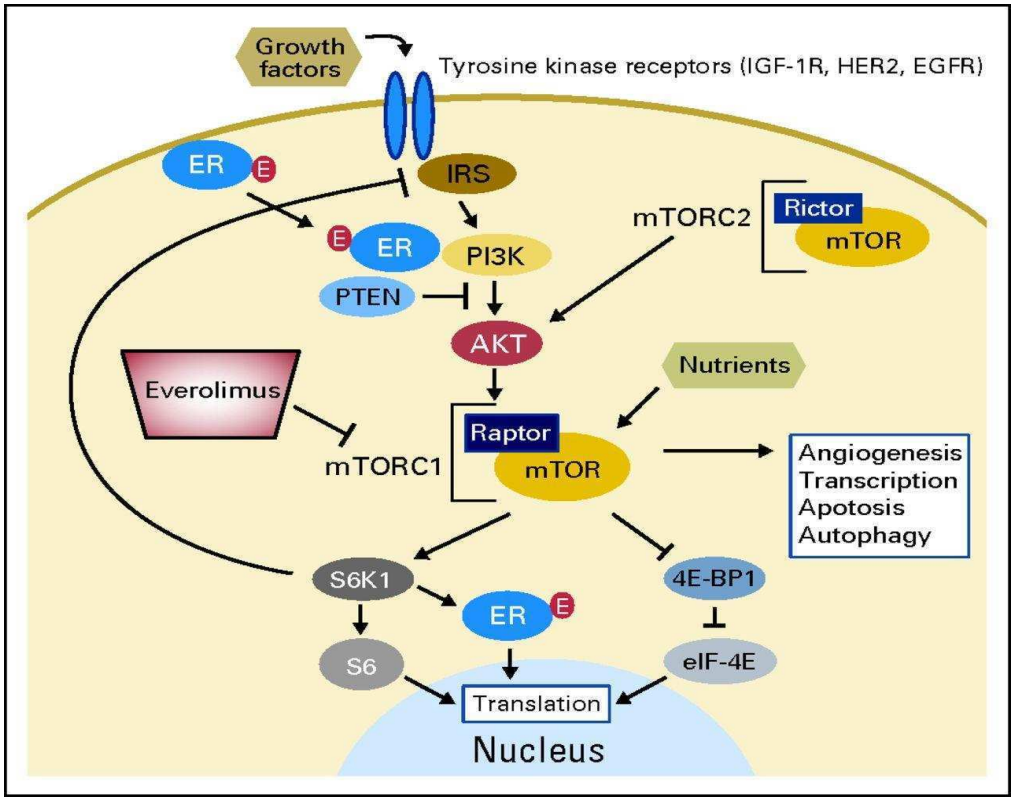

Figure 1-9: mTORC1 as a downstream signalling of PI3K pathway and its interactions with ER and HER2, (Rugo and Keck, 2012)

1.8.2.3 Association between mTORC1 signalling and ER and HER2

Recently, it has become evident that oestrogen/ER signalling is more complex than was anticipated, displaying diverse effects through its interactions with growth factor signalling pathways by non genomic pathway. For instance, in steroid-deprived MCF-7 breast carcinoma cells, ER is predominantly localised in the nucleus; however, upon oestrogen stimulation, a substantial proportion is translocated to the plasma membrane (Santen et al., 2002), contributing to growth factor receptor signalling (Johnston, 2005, Schiff et al., 2004). Importantly, oestrogen has been revealed to enhance mTOR pathway in oestrogen target tissues such as BC (Yin et al., 2007), (Figure 1-9). However, this activation is only demonstrated either by regulation of upstream or downstream mediators of mTOR rather than phosphorylation of the latter (Boulay et al., 2005).

Meanwhile, p-mTORC1 was shown to directly phosphorylate ER on SER 167 through its downstream target p70-S6k, and increase the transcriptional activity of ER. The possibility remains that increased mTOR activity may help drive ligand-independent ER signalling and short circuit the ligand-dependent one which is the main target of endocrine therapies (Yamnik et al., 2009). Interestingly, it is known that if growth and survival of a tumour depends on oestrogen and an intact signalling dependent on this hormone exists, it is more likely that this tumour will benefit from endocrine therapy (Osborne and Schiff, 2005).

1.8.3 Other signalling proteins associated with ER and HER2

Apart from investigating major signalling pathways in BC that are downstream of HER2 and have interactions with ER as MAPKs and PI3K/Akt pathways, it is reasonable to address the possible associations with other signalling proteins related to different pathways that might help in exploring the heterogeneous biological behaviour of HER2 in the presence or absence of ER expression. These biomarkers range between those related to protein quality control in the cell as Carboxyl-terminus of Hsp-70- Interacting Protein (CHIP) and those which are related to stem cells as Sry-Related HMG Box 9 (SOX9), transcription factors as SER 118 ER, Steroid Receptor Co-activator-3 (SRC-3)/ Amplified in Breast Cancer 1 (AIB1) and cell cycle regulators as Ecdysoneless (ECD). These biomarkers will be dealt with in a separate chapter later on (chapter 6) with relevant associations with HER2 and ER.

1.8.3.1 CHIP

CHIP functions to maintain protein quality control as it is well known for its protective mechanism from various cellular stresses where it targets different proteins for proteosomal degradation (Lee et al., 2013). Prolonged exposure of cells to oxidative stress promotes irreversible damage and cell death (Finkel and Holbrook, 2000, Klein and Ackerman, 2003). During a state of oxidative stress, cells protect themselves by modulating their redox state by elevating the level of O_2 species scavenging proteins and activate the ubiquitin-proteosome system to maintain protein homeostasis.

Of worth, CHIP act as E3 ligase, which is a protein that recruits an E2 ubiquitin-conjugating enzyme that has been loaded with ubiquitin, recognises a protein substrate, and assists or directly catalyses the transfer of ubiquitin from the E2 to the protein substrate, for misfolded or damaged proteins (Murata et al., 2001, Meacham et al., 2001). The latter function is performed through presenting the damaged proteins by Heat Shock Protein 70 (Hsp-70) which is a mediator protein between CHIP and mitochondrial protein endonuclease G (Endo G). Under normal conditions, CHIP inhibits the dissociation of Hsp-70 from Endo G protein, inhibiting the stabilisation and translocation of the latter to the nucleus; however, under oxidative stress, Endo G protein is released from the effect of

Hsp-70, translocate to the nucleus and degrades chromosomal DNA and cause cell death and this is maintained under the effect of CHIP protein (Murata et al., 2001, Meacham et al., 2001), (Figure 1-10). Moreover, recent reports have shown that Endo G is a necessary effecter of caspase independent cell death during stress (Chinnathambi et al., 2008, Ishihara and Shimamoto, 2006, Nielsen et al., 2009, Apostolov et al., 2011).

The mechanisms on how CHIP can maintain homeostasis in oxidative stress is still to be determined (Lee et al., 2013) and its overall action is to direct for degrading damaged proteins and maintain protein homeostasis (Qian et al., 2006, Ross and Poirier, 2005, Dickey et al., 2007, Min et al., 2008)

Importantly, it has been shown that CHIP level is negatively correlated with BC progression and that there are some interactions between CHIP and HER2 and ER (Lee et al., 2013) so we thought to study this biomarker with relevance To HER2 and ER and the possibility of being a therapeutic target.

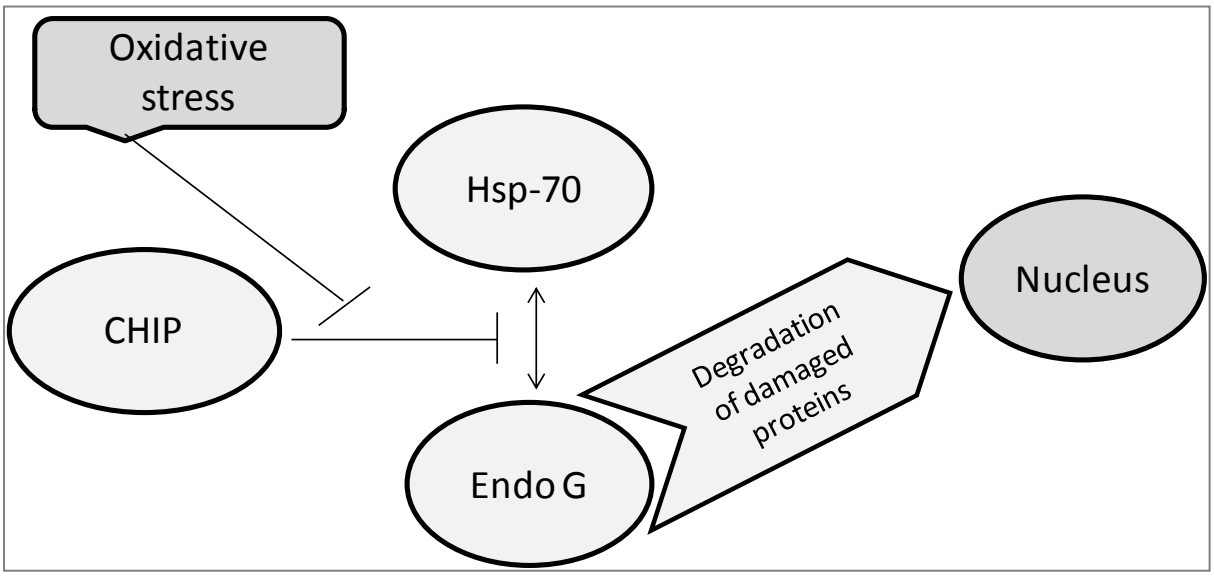

Figure 1-10: Schematic representation of the function of CHIP and the role of oxidative stress, adapted from (Murata et al., 2001) and (Meacham et al., 2001)

1.8.3.2 SOX9

SOX9 is related to the SOX family of transcription factors which share a homologus high motility group (HMG) box DNA binding domain and can regulate different developmental processes (Schepers et al., 2002). Stem cell state recognition is widely regulated by transcription factors that can orchestrate several cellular networks; nevertheless, which transcription factors are involved in the regulation of adult stem cells is still to be investigated (Guo et al., 2012). Of note, GEP has considered SOX9 as a member of the basal-like signature genes used to classify BC classes (Perou et al., 2000a, Sørlie et al., 2001, Rakha and Reis-Filho, 2009)

Importantly, two transcription factors have been identified as determinants of Mammary Stem Cells (MaSCs): SOX9 and Slug where the blockade of these proteins blocks MaSCs activity while collaboration of both proteins is suitable to convert various luminal cells into MaSCs having long term constituting ability. Moreover, the cooperation between these two proteins initiates tumurogenesis and metastasis in BC and even is related to poor prognosis (Guo et al., 2012).

Interestingly, studies have revealed that the mammary tissue is very useful model to study epithelial stem cells regulation as they have small subpopulation of cells which has robust stem cell activity. In addition, these studies have also reported that implantation of even a single murine MaSC into the murine mammary fat pad which is a stromal component, is sufficient enough for the induction of a whole mammary tree (Kordon and Smith, 1998, Shackleton et al., 2006, Stingl et al., 2006). For this reason, we considered the investigation of this protein in relation to HER2 and with possible interactions with ER especially some unpublished cell line work indicated an association of SOX9 with HER2.

1.8.3.3 SRC3/ AIB1

SRC3/ AIB1 protein is the only member of the steroid receptor co activator family that is amplified and overexpressed in numerous human epithelial neoplasias, such as breast and prostate cancer (Anzick et al., 1997, List et al., 2001, Gnanapragasam et al., 2001). SRC3 is expressed in approximately 30% of BC (Anzick et al., 1997, List et al., 2001, Bautista et al., 1998). Moreover, it possesses an important role in inducing HER2 signalling (Ma et al., 2011). Therefore, both have major impact on short BC outcome (Thorat et al., 2008,

Alkner et al., 2010, Osborne et al., 2003b). Furthermore, in mice, studies found that it is associated with mammary carcinoma (Tilli et al., 2005, Torres-Arzayus et al., 2004). Decreased SRC3 also decreases IGF-I signalling in MCF-7 cell lines which can affect HER2 signalling via decreasing EGF signalling of EGFR and HER2 (Oh et al., 2004). Evidence is obvious from the above data that AIB1/SRC-3 can enhance the oncogenic activity of HER2/neu in the breast, making it a potential attractive therapeutic target in HER2/neu expressing BC.

1.8.3.4 ECD

Human ECD protein (human ortholog of Drosophila Ecdysoneless, hereafter called ECD) is regarded as a novel mammalian cell cycle promoter. It functions to release the inhibitory effects of Retinoblastoma (Rb)-family tumour suppressors on E2F transcription factors (Zhao et al., 2012) which play a crucial role to turn on the expression of a large panel of genes necessary for cell cycle progression (Malumbres and Barbacid, 2005). E2F transcription factors are held in a repressive complex with hypophosphorylated form by members of the Rb protein family (Hinds et al., 1992). Importantly, Cyclin-dependent Kinases (CDKs) can phosphorylate Rb proteins (Ewen et al., 1993, Hwang and Clurman, 2005, Resnitzky et al., 1995) thus releasing the Rb proteins from E2Fs which can enhance transcription of target genes (Hwang and Clurman, 2005, Du and Pogoriler, 2006)

Consistent with this basic paradigm, it is not infrequent to notice that machinery components of cell cycle can be genetically altered (Burkhart and Sage, 2008, Mammas et al., 2008, Kim et al., 2009). Interestingly, a hallmark of cancer is uncontrolled growth and that drivers of BC oncogenesis can enhance in certain way the function of genes regulating cell cycle progression (Sanchez and Dynlacht, 2005). Delineating the pathways that can have major impact on human BCs to recognise novel prognostic markers that might be useful in treatment decision is therefore considered a research priority (Parkin et al., 2005). For this reason, analyses of novel markers related to cell cycle are a useful opportunity to discover new prognostic markers in BC, especially in the context of association with HER2 and ER.

1.8.3.5 Serine (SER) 118 ER

ER is a transcription factor which is activated by a ligand (estradiol) and can be phosphorylated at several sites (Lannigan, 2003). Activation of this receptor can be stimulated by estradiol binding and even through activation of other signal transduction molecules (Lannigan, 2003). A well recognised phosphorylation site on ER is SER 118 (Lannigan, 2003), (Figure 1-11). Both oestrogen and growth factors, such as epidermal growth factor (EGF), can enhance phosphorylation of SRE 118 ER (Lannigan, 2003, Joel et al., 1998b, Chen et al., 2002), and direct phosphorylation of SER 118 ER by mitogen-activated protein kinase (MAPK; ERK1/2) is feasible *in vitro* (Kato et al., 1995a). In addition, it has been reported that SER 118 ER is activated by Ras-Raf-MAPK-ERK1/2 pathway *in vivo* (Joel et al., 1998b, Bunone et al., 1996, Migliaccio et al., 1996), and this process can be ligand independent (Migliaccio et al., 1996, Chen et al., 2002).

Whilst endocrine therapy is useful for women with ER+ BC, currently there is no prognostic assay which is able to detect those patients who will benefit from endocrine therapy (Yamashita et al., 2008). Furthermore, a preclinical study has shown that phosphorylation of ER at SER 118 site is necessary for tamoxifen mediated inhibition of ER induced gene expression (Kok et al., 2009a).

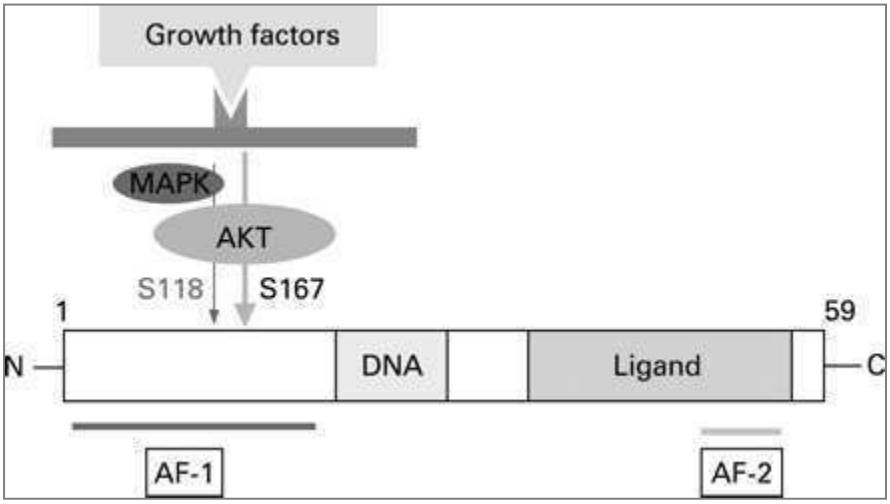

Figure 1-11: Other sites of ER phosphorylation at AF-1 domain; SER 118 ER and SER 167, (Badve and Nakshatri, 2009)

1.9 Hypothesis of the PhD

Breast cancer is a heterogeneous disease in terms of morphology, molecular profile, behavior and response to therapy. HER2 and ER are key driving biomarkers influencing BC biology and response to therapy. It was hypothesised that the interaction between HER2 and ER in BC is complex and that the effect of each biomarker is dependent on the other. Tumours showing HER2 amplification / protein overexpression and ER expression are unique class of BC that is different from single positive classes.

1.10 Aims of the study

The aims of the study were to focus on pathways directly related to these two proteins; HER2 and ER in order to understand the biology and clinical significance of HER2+/ER+ BC class and explore which of these key proteins merits to be dominantly functioning when co-expressed. Moreover, deciphering the molecular profiles and status of signalling pathways involved with each receptor, specifically the MAPKs, PI3K/Akt/mTOR, would help to understand the biology of different BC subgroups and possible biomarker interactions and identify potential therapeutic targets.

The specific aims were:

1- To analyse the expression of signalling pathways' members using IHC in BC from patients either receiving hormone therapy with or without trastuzumab therapy
2- To correlate the expression of the signalling pathways' members with clinicopathological variables and patients outcome.
3- To correlate the expression of the signalling pathways with other ER and HER2 related proteins and other biomarkers relevant to BC.
4- To determine the prognostic and predictive value of the relevant signalling pathways' key members using univariate and multivariate analysis.
5- To quantify the expression of signalling pathways related to ER and HER2, using RPPA.

2 Material and Methods

2.1 Patients' cohorts' characteristics

2.1.1 Primary series

The study cohort included 1902 unselected cases of female primary operable BC between 1986 and 1998. Clinicopathological information available included: patients' age at diagnosis, menopausal status, histological grade (and components: mitosis, tubule formation and pleomorphism), tumour type, LN stage, tumour size, LVI and NPI. Moreover, other information regarding adjuvant therapy including: surgical procedure performed, hormonal, radiotherapy and chemotherapy were available. The valid cases from the primary series after TMA construction were 1835 and the characteristics of this series are summarised in Table 2-1.

2.1.2 Patients' outcome

Survival data in this series was available where survival status was recorded (alive or dead), survival time (in months) and the cause of death (whether related to BC or not). Breast Cancer Specific Survival (BCSS) is defined as the time in months from surgery until the patient died from BC, (median=108 months). DFI is the period in months from the surgery until the patient develops loco-regional recurrence and /or distant metastasis (median=106 months). Finally, distant metastasis free survival (DMFS) is the time from the surgery until developing distant metastasis (median =114 months), the total follow up time for this study was 15 years (Table 2-1).

Table 2-1: The characteristics of the primary series

Clinicopathological characteristics	N (%)
Age	
≤50	631 (35)
>50	1194 (65)
Menopausal Status	
Pre-	702 (38)
Post-	1122 (62)
Tumour Size (cm)	
≤2.0	924 (51)
>2.0	889 (49)
Tumour Type	
Ductal (inc. mixed)	1472 (82)
Lobular	198 (11)
Medullary-like	37 (2)
Special types	96 (5)
NPI	
Good Prognostic Group (GPG)	582 (33)
Moderate Prognostic Group (MPG)	915 (53)
Poor Prognostic Group (PPG)	245 (14)
Stage	
1	1138 (63)
2	529 (29)
3	150 (8)
Grade	
1	326 (18)
2	610 (34)
3	881 (48)
Lymphovascular Invasion (LVI)	
Definite	573 (32)
Negative/Probable	1230 (68)
ER	
Negative	453 (25)
Positive	1360 (75)
PgR	
Negative	711 (41)
Positive	1015 (59)
HER2 status	
Negative	1509 (87)
Positive	224 (13)
Triple Negative status (TN)	
TN	294 (17)
Non-TN	1472 (83)
BCSS	
Alive	861(62)
Dead	525 (38)
Distant metastases	
Yes	598 (39)
No	938 (61)
Recurrence	
Yes	878 (55)
No	719 (45)
ER and HER2 based groups	
ER+/HER2-	1181 (69)
ER+/HER2+	116 (7)
ER-/HER2+	108 (6)
ER-HER2-	343(18)

2.1.3 Adjuvant therapy

In 1989; prognostic and predictive factors including: NPI, menopausal and ER status were used to decide patient's clinical management. If the NPI was $\leq$3.4 the patient received no adjuvant therapy as they were considered low risk, while if NPI was >3.4, in Pre-menopausal women with positive ER status, they received tamoxifen and CMF if they were fit enough to tolerate chemotherapy. For Post-menopausal patients with ER+ tumours, they received tamoxifen only. Additionally, CMF was the choice for ER- patients if tolerated. After 1990, the same treatment regimen was still applied for hormonal and chemotherapy but prophylactic irradiation to the axilla after surgery was added (Blamey, 2002).

2.1.4 Trastuzumab treated HER2+ series

This series includes 197 cases of primary operable HER2+ BC cases that presented in Nottingham during the period from 2003 to 2012. These patients received adjuvant Trastuzumab after surgery. The patient follow up of this series was performed in a prospective manner, the same as in the primary series. The follow up started three months after surgery, followed by another after six months and then at twelve months (only the survival data regarding overall and DFI were collected for this series). For the details of this series see Table 2-2.

Table 2-2: The characteristics of Trastuzumab treated HER2+ series

Clinicopathological characteristics	N (%)
Age	
≤50	72(42)
>50	98(58)
Menopausal Status	
Pre-	85(47)
Post-	94(53)
Tumour Size (cm)	
≤2.0	2 (1)
>2.0	175(99)
Stage	
1	109 (62)
2	57 (32)
3	10 (56)
Grade	
1	4 (2)
2	47 (26)
3	128 (72)
NPI	
GPG	14 (89)
MPG	96 (60)
PPG	49 (31)
Tubule formation	
1	0(0)
2	30 (17)
3	149 (83)
Pleomorphism	
1	0(0)
2	15 (8)
3	164 (92)
Mitosis	
1	46 (26)
2	54 (30)
3	79 (44)
LVI	
Definite	64 (36)
Negative/Probable	115 (64)
ER	
Negative	76 (43)
Positive	103 (57)
PgR	
Negative	88(60)
Positive	57 (39)
Overall survival	
Alive	162(90)
Dead	17 (10)
Recurrence	
Yes	153 (85)
No	26(15)
Distant metastasis	
Yes	90(87)
No	13 (13)

2.2 Data of available biomarkers in the group

Wide ranges of informative biological data that are clinically and biologically relevant to BC were available including HRs (ER, PgR and AR), cytokeratins (CKs); basal (CK5/6, CK14 and CK17) and luminal (CK7/8, CK18 and CK19), Gross cystic disease fluid protein (GCDFP-15), Fragile histidine triad protein (FHIT) and MUC1. Furthermore, tumour suppressor proteins and proapoptotic molecules (p53, B-cell CLL/lymphoma 2: BCL2), HER family proteins (HER1, HER2, HER3, HER4), E-Cadherin and P-Cadherin (Abd El-Rehim et al., 2005), a wide range of data regarding ER related proteins (some of published and others of unpublished work of Dr Hany Habashy) including Forkhead box protein A1 (FOXA1), Trefoil factor 3 (TFF3), Trefoil factor 1(TFF1), Trans-acting T-cell-specific transcription factor (GATA3), Co-activator associated arginine methyl transferase (CARM1) Proline, glutamate and leucin rich protein 1(PELP1)Transferrin receptor (resistance to endocrine treatment: CD71 and Brain expressed, X-linked-1 (BEX1) (Habashy et al., 2013, Habashy et al., 2008a, Habashy et al., 2010a, Habashy et al., 2010c) and proliferation related markers (KI67-LI, PI3K and Akt and N-Cadherin), (Aleskandarany et al., 2011, Aleskandarany et al., 2012), PTEN and HER2 dimers and dimers' combinations were assessed by Dr Fabricio Barros (Table 2-3). All the mentioned biomarkers were used for comparison for the primary series while for the Trastuzumab treated HER2+ data, some biomarkers of interest were only considered including: ER, PgR, CK7/8, CK18, BCL2 and p53 which were assessed by Prof. Chan group. Moreover, the relations with HER2 dimers and their combinations were considered in both series.

Table 2-3: Biomarkers used for comparison in this study for the Primary Series

Marker	Antibody [clone]	Dilution	Categorical cut points	Supplier	Pre Treatment	Reference
1-Hormone receptors (HRs)	ER [clone 1D5]	1:150	Negative (0), Positive (≥1)	DAKO	Microwave	Abd El-Rehim et al
	PgR[clone PgR 636]	1:100	Negative (0), Positive (>1)	DAKO	Microwave	Abd El-Rehim et al
	AR[clone F39.4.1]	1:30	Negative (0), Positive (>1)	Biogenex	Microwave	Abd El-Rehim et al
2-luminal cytokeratins (CKs)	Ck7/8 [clone CAM 5.2]	1:2	Negative/Low (<50), High (≥50)*	Becton Dickinson	Microwave	Abd El-Rehim et al
	Ck18 [clone DC10]	1:50	Negative/Low (<50), High (>50)*	DAKO	Microwave	Abd El-Rehim et al
	Ck19 [clone BCK 108]	1:100	Negative/Low (<50), High (>50)*	DAKO	Microwave	Abd El-Rehim et al
3- Basal CKs	Ck5/6 [clone D5/16134]	1:100	Negative (<10), Positive (≥10)	BoehringerBiochemica	Microwave	Abd El-Rehim et al
	Ck14 [clone LL002]	1:100	Negative (<10), Positive (≥10)	Novocastra	Microwave	Abd El-Rehim et al
	Ck17[clone E3]	1:20	Negative (<10), Positive (≥10)	Abcam	Microwave	Abd El-Rehim et al
3-Other ER related proteins & ER co-regulators	Trefoil factor 3(TFF3) [cloneAb57752]	3µg/ml	Negative (<90), Positive (≥90)*	ABCAM	Microwave	Unpublished work of Habashy et al.
	Trefoil factor 1(TFF1)[cloneAb17829]	1:2000	Negative (<100), Positive (≥100)*	ABCAM	Microwave	Unpublished work of Habashy et al.
	Forkhead box protein A1 (FOXA1) [clone 2F83]	1:2,000	Negative (<10), Positive (≥10)*	ABCAM	Microwave	(Habashy et al., 2008b)
	Trans-acting T-cell-specific transcription factor (GATA3) [HG3-31]	1:80	Negative/Low (<60), Positive (≥60)*	Santa Cruz Biotechnology	Microwave-	(Kouros-Mehr et al., 2006) Abd El-Rehim et al
	MUC1 [clone Ma695]	1:300	Negative/low(20), moderate(20), High (>200)*	Novocastra	Microwave-	Abd El-Rehim et al
	Gross cystic disease fluid protein (GCDFP-15)	1:30	Negative/low (<10), positive (≥10)	Novocastra	-	
	Co-activator associated arginine methyl transferase (CARM1)[clone NB100 -1817]	1:300	Negative/Low (<30), Moderate (30-149), High (150-300)	NOVUS	-	(Habashy et al., 2013)
	Proline, glutamate and leucin rich protein 1(PELP1)[clone NB100 -1749]	1:100	Negative/Low (0-4), Moderate (5-169), High (170-300)*	NOVUS	-	(Habashy et al., 2010a)
	Transferrin receptor (resistance to endocrine treatment: CD71[clone 10F11]	1:30	Negative (≤5), Positive (>5)	ABCAM	-	(Habashy et al., 2010b)
4- Proliferation and apoptosis related markers						
Makers of proliferation	Phosphatidylinositol-3 kinase [HPA009985]	1:50	Negative (<30), Low (30-100), High (101-300)*	Sigma	Microwave	(Aleskandarany et al., 2010b)
	Protein kinase B (Akt), [RB-10369-P1]	1:150	Negative (<65), Positive (≥65)*	Neomarker	-	(Aleskandarany et al., 2010a)
	Ki-67[clone MIB1]	1:100	Negative (<14), Positive (≥14)	DAKO	-	(Cheang et al., 2009a)
Apoptosis related markers	B-cell CLL/lymphoma -2 (Bcl2) (pro-apoptotic) [clone 124]	1:400	Negative/Low (<30), Positive (≥30)	DAKO	Microwave	Abd El-Rehim et al
6-Tumour suppressor proteins, cell adhesion molecules and mucins	p53 [clone DO7]	1:50	Negative (<5), Positive (≥5)	Novocastra	Microwave	Abd El-Rehim et al
	Brain expressed, X-linked-1 (BEX1) [Ab69032]		Negative (<100), Positive (>100)*	Abcam	Microwave	Unpublished work of Habashy et al.
	Fragile histidine triad protein (FHIT) [clone ZR44]	1:3500	Negative, (<5), positive (≥5)	Zymed Laboratories	Microwave	Abd El-Rehim et al
	Phosphatase and tensin homolog (PTEN) [6H2.1]	1:600 1:100	Negative (0), low (1-<10), moderate (≥10-<100), high (≥100)	DAKO	Microwave	(Aleskandarany et al., 2010a), data of DR Barros
Cell adhesion molecules and Mucins	E-Cadherin [clone HECD-1]	1:100	Negative/Low (<100), High (≥100)*	Zymed Laboratories	Microwave	Abd El-Rehim et al
	P-Cadherin [clone 56]	1:200	Negative (<5), Positive (≥5)	BD Biosciences	Microwave	Abd El-Rehim et al
	N-Cadherin C3865	4 ug/ml	Negative/Low (<100), High (≥100)*	Sigma	Microwave	(Aleskandarany et al., 2014)
7-HER family proteins	HER1 [clone EGFR.113]	1:10	Negative (<10), Positive (≥10)	Novocastra	Microwave	Abd El-Rehim et al
	HER2 [clone cerbB-2]	1:250	Negative (<10), Positive (≥10)	DAKO	-	Abd El-Rehim et al
	HER3[clone RTJ1]	1:20	Negative (<10), Positive (≥10)	Novocastra	Microwave	Abd El-Rehim et al
	HER4 [clone HER1]	6:4	Negative (<10), Positive (≥10)	Neomarkers	-	Abd El-Rehim et al

*** : H-score**

2.3 Construction of tissue microarray (TMA): For the primary and Trastuzumab treated HER2+ series

2.3.1 Preparation of donor blocks

Archival Formalin Fixed Paraffin Embedded (FFPE) blocks of BC tissue were retrieved from the Nottingham Health Science Biobank. Haematoxylin and Eosin stained sections from these blocks were taken and assessed for sufficient invasive malignant cells for sampling. The most representative tumour areas were marked together with the corresponding areas on the FFPE blocks, this marking of tumour areas in the slides and correspondent blocks was performed by a pathologist; Dr. Dena Ahmad. Marked blocks were loaded onto the TMA Grand Master® (3D HISTECH®, Budapest, Hungary).

2.3.2 Recipient block

Molten paraffin wax (55-58°C) was poured into a mould onto which a histopathological tissue cassette was mounted. Ice was used to solidify the paraffin and the mould removed. For TMA construction, blocks were trimmed.

2.3.3 TMA construction

TMA blocks were designed to hold a maximum of 150 cases. Marked areas of the tumours from the donor blocks were harvested using a 0.6 mm needle. TMA construction was performed by Dr. Dena Ahmad (PH.D student/ Pathology), Dr. Alaa Alshareeda (PH.D Oncology) Dr. Rezvan Abduljabbar (PH.D student) and Mr Glynn Donovan (an employee in the Biobank). These individuals shared retrieving blocks and slides from the Biobank, matching the slides with their correspondent blocks and shared TMA construction.

2.4 Immunohistochemistry

2.4.1 Steps of immunohistochemistry technique

IHC was performed manually where 4µm TMA sections on slides were incubated for 10 minutes on a hot plate (60 °C) and left to cool. Slides were dewaxed in xylene twice for 5 minutes. Slides were then rehydrated in methanol 3 times (2 minutes/each) followed by 2 minutes in washing water. Antigen retrieval was

performed as necessary (Table 2-4) in a Whirlpool JT359 microwave (1000 W) using a full power for 20 minutes with citrate buffer. Slides were cooled for 5 minutes under running tap water and then were rinsed with Tris Buffered Saline (TBS) with PH (7.6). Incubation of the slides was followed next with peroxidase block for 5 minutes, protein block for 5 minutes and primary antibodies that have been optimally diluted using Leica antibody diluent were added (Table 2-4) and each of these steps was followed by washing 2×5 minutes with TBS. Post primary block was incubated for 30 minutes followed by 2×5 minutes washing with TBS and then Novolink Polymer was added for 30 minutes and followed by the same washing. 3,3'-Diaminobenzidine (DAB) working solution (DAB chromogen and DAB substrate buffer of a ratio 1:20) was added for 5 minutes, 2×5 minutes washing then was followed by counterstaining with Haematoxylin for 6 minutes and again 2×5 minutes washing with TBS. The slides were dehydrated with alcohol and cleared with xylene. Finally, slides were mounted with Distyrene, a Plasticizer, and Xylene (DPX).

Table 2-4: Conditions of the antibodies that were stained in this study

Antibody	Supplier	species	Clone	Molecular weight (kDa)	Dilution	Incubation time	Positive control
ERK1/2[1]	Cell signalling	Rabbit polyclonal	-	42,44	1:400	1 hr	BC tissue
p-ERK1/2[2](PT185/pY187)	Cell signalling	Rabbit monoclonal	15H10L7	42,44	1:100	Overnight/4°C	BC tissue
JNK1/2[1]	Cell signalling	Mouse monoclonal	279Q38	46,54	1:300	1 hr	BC tissue
p-JNK1/2[2](T183/Y185)	Cell signalling	Rabbit monoclonal	D12H7L17	46,54	1:3000	Overnight/4°C	BC tissue
P38[1]	Cell signalling	Rabbit polyclonal	-	38	1:50	1 hr	BC tissue
p-p38[2](T180/Y182)	Cell signalling	Rabbit monoclonal	12F8	43	1:200	Overnight/4°C	Colon cancer tissue
p-ATF2[2] (Thr69/71)	Cell signalling	Rabbit monoclonal	-	70	1:100	Overnight/4°C	BC tissue
p-C-JNU[2](Ser 73)	Cell signalling	Rabbit monoclonal	D47G9	48	1:200	Overnight/4°C	BC tissue
p-mTORC1[2](Ser2448)	Cell signalling	Rabbit monoclonal	49F9	289	1:150	Overnight/4°C	BC tissue
p-SER 118 ER[2](Ser 118)	Cell signalling	Mouse monoclonal	16J4	66	1:350	Overnight/4°C	BC tissue
CHIP[2]	Thermo scientific	Rabbit polyoclonal	PA5-29024	35	1:2000	Overnight/4°C	BC tissue
SOX9	EMD	Rabbit polyoclonal	-	65	1:10000	Overnight/4°C	BC tissue
ECD[3]	-	Mouse monoclonal	4A8	75	1:2000	Overnight/4°C	BC tissue
SRC3	BD Biosciences	Mouse monoclonal	-	155	1:250	Overnight/4°C	BC tissue

[1]: These proteins are called pan, total or unphosphorylated forms, [2]: these proteins with (p) are the phosphorylated forms. [3] : generated by the Monoclonal Antibody Facility at the Lurie Cancer Centre, Northwestern University, Chicago

2.4.2 Assessment of immunoreactivity

High resolution digital images (0.45µm/pixel) were obtained using a NanoZoomer slide scanner (Hamamtsu Photonics, Welwyn Garden City, UK). Digital slides were accessed using a web based interface (Distiller, Leica Ltd, Newcastle, UK). Immunoreactivity was assessed at 20x magnification using a minimum of 24 high resolution screen (1920x1080).

The first step was to assess each TMA core whether invasive tumour/core occupied $\geq$15% of the total area of the core. If this was the case, the core was eligible for scoring. In this study, H-score, was the main scoring system used as it takes into consideration the intensity [0=negative, 1=weak, 2=moderate and 3=strong staining] and the percentage of stained tumour (0-100%). The intensity was multiplied by the percentage and added together to obtain the final H-score. Therefore, this score had a range from 0-300. To ensure reproducibility, at least 25% of the cases/ stained marker, were reassessed by an independent scorer with a concordance rate of approximately 80%.

2.4.3 Determination of cut-off points

Cut-off values for the dichotomisation of biomarkers were individually assessed and determined by several methods 1) Using cut-offs from other studies that assessed large number of data if available 2) Using either the mean or median according to the distribution of data using Shapiro-Wilk test either normally or not normally distributed respectively and 3) Using non commercial research tool; x-tile software (version 3.6.1, 2003-2005, Yale University, USA http://x-tile.software.informer.com. This programme randomly divides the total patient cohort in to two separate training and validation sets ranked by the patient follow up time. Checking the obtained cut off points to the validation set tested the statistical significance. Information regarding the stained antibodies in this study, their expression and their cut-offs are listed in Table 2-5.

Table 2-5: Antibodies stained in this study, their expression sites and cut-offs.

Antibodies	Stained series	IHC localisation	Cut-off points	Method
ERK1/2	Primary and HT series	Cytoplasmic	≥100(H-score)	X-Tile
p-ERK1/2	Primary and HT series	Nuclear/Cytoplasmic	≥140 (H-score)/ >30(H-score)	X-Tile
JNK1/2	Primary and HT series	Cytoplasmic	≥103 (H-score)	X-Tile
p-JNK1/2	Primary and HT series	Nuclear	≥124 (H-score)	X-Tile
P38	Primary and HT series	Cytoplasmic	≥112 (H-score)	X-Tile
p-p38	Primary and HT series	Nuclear	>110 (H-score)	X-Tile
p-ATF2	Primary and HT series	Nuclear	>70 (H-score)	X-Tile
p-C-JNU	Primary and HT series	Nuclear	>3 (percent)	X-Tile
p-mTORC1	Primary and HT series	Cytoplasmic	>30 (H-score)	X-Tile
SER 11 8 ER	Primary and HT series	Nuclear/Cytoplasmic	>140 (H-score)/>70(percent)	median
CHIP	Primary and HT series	Nuclear/Cytoplasmic	>30 (H-score)/ >140 (H-score)	median
SOX9	Primary series	Nuclear/Cytoplasmic	>80 (percent)/Cytoplasmic intensity(0,1,2,3)	X-Tile
ECD	HT series	Cytoplasmic	>80 (H-score)	X-Tile
SRC3	HT series	Nuclear	>130 (H-score)	median

2.5 Cell culture

In this study, cell culture was used to grow six BC cell lines reflecting variable expressions of ER and HER2. Then after, the protein lysate was prepared for Western Blot (WB) testing of the stained proteins and for the quantitative analysis of Reverse Phase Protein Array (RPPA).

2.5.1 Cell lines

Four wild (W)-type BC cell lines were used in this study:MCF-7 (ER+/HER2-), MDA-MB-231(ER-/HER2-), BT474 (ER+/HER2+) and SKBR3 (ER-HER2+), all apart from BT474 were a gift from Dr. SG Martin, School of Medicine, University of Nottingham originally supplied from the American Type Culture Collection (Manassas, USA).
BT474 was a gift from our collaborators the US: Dr. Sameer Mirza and Prof Vimla Band, (Department of Genetics, Cell Biology and Anatomy, University of Nebraska, USA)

2.6 Retroviral infections

Retroviral supernatants were generated by calcium phosphate–mediated cotransfection of the expression plasmids and the packaging plasmid pIK into the packaging cell line TSA54 (Dimri et al., 2007). The retroviral supernatants, collected 24 h after transfection, were used to infect subconfluent MCF-7 and MDA-MB-231 cells in three sequential 4-h incubations in the presence of 4 μg/mL polybrene (Sigma-Aldrich). Transductants were selected in puromycin 0.5 μg/mL; 48 h after infection. The generated transducants were always cultured in the selection media (Dimri et al., 2007). The transfection procedure was performed by our collaborators in the US: Dr Sameer Mirza (Department of Genetics, Cell biology and Anatomy, University of Nebraska, USA). Growing of the W and Transfected (T) cell lines and the preparation of the lysates from all the six BC cell lines were performed by: Dr Dena Ahmad. Importantly, the author assessed HER2 transfection using ICC. Additionally, HER2 and ER expressions were assessed by the author using WB (Figure 2-1 and Figure 2-2) in the six BC cell lines.

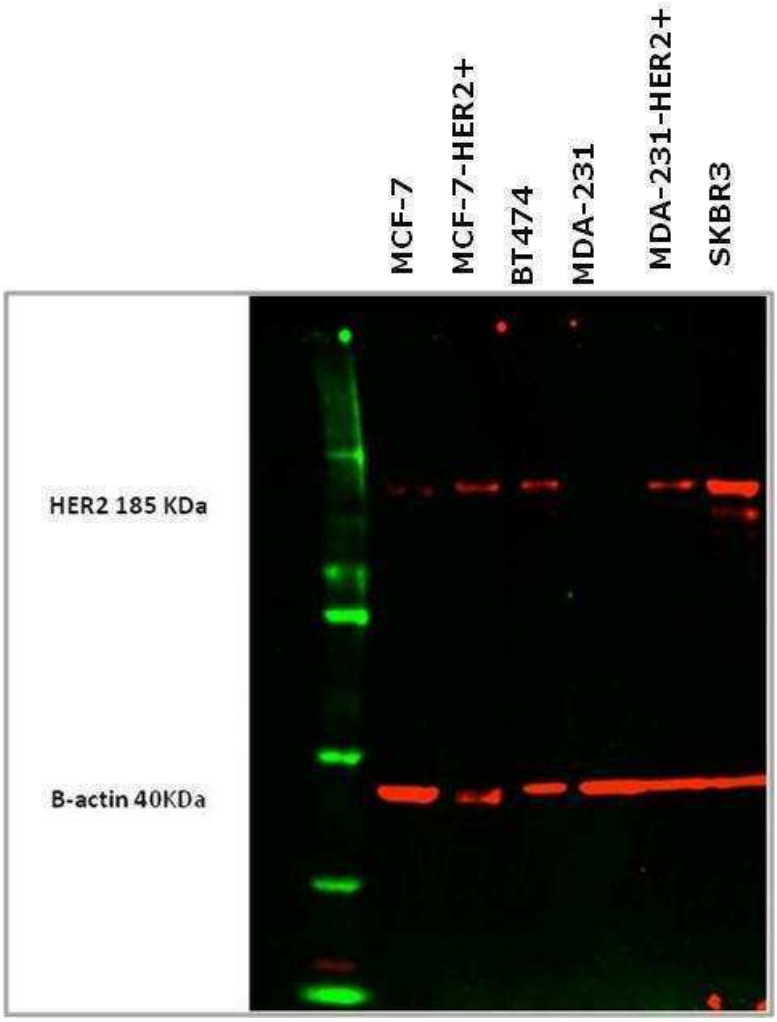

Figure 2-1: Western blot to assess HER2 expression in the six BC cell lines

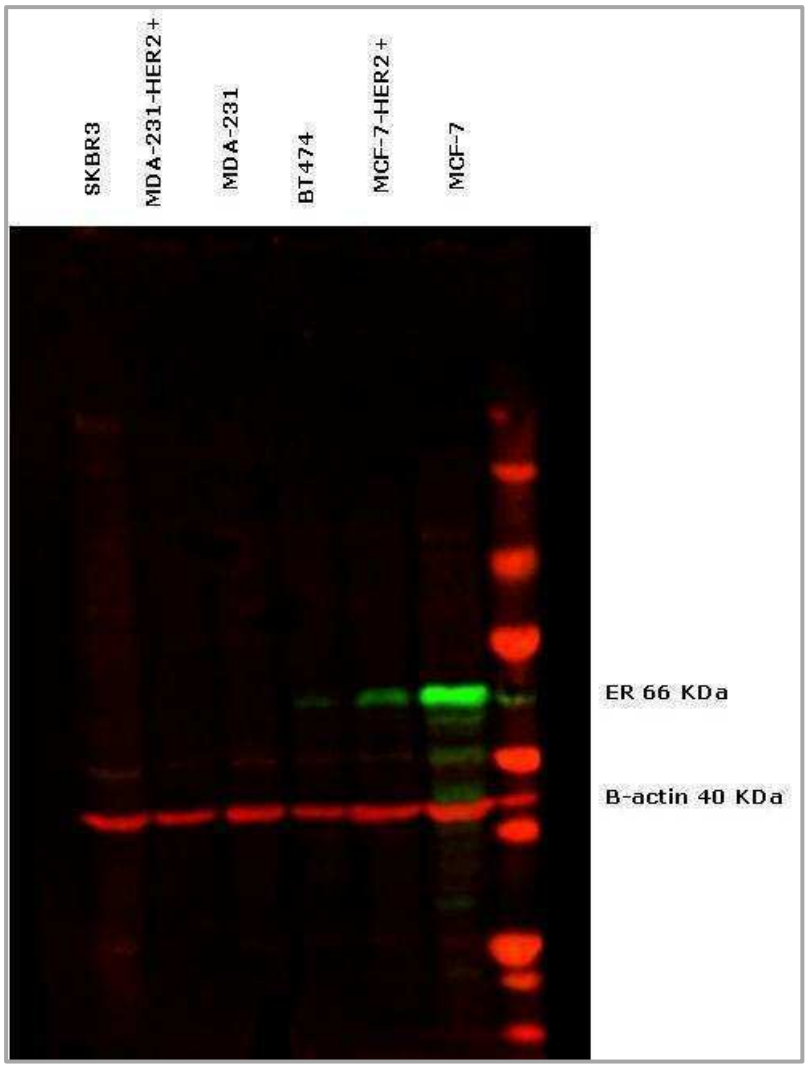

Figure 2-2: Western blot to assess ER expression in the six BC cell lines

2.7 Immunocytochemistry (ICC)

2.7.1 Immunocytochemistry procedure

Immunocytochemistry is a cytogenetic technique that is used to visualise certain proteins in the cell.

Positive control was added in each ICC run using the house keeping protein: rabbit polyclonal β_2 microglobuline (Dako) at a concentration of 1:2000. Negative control was used by adding only PBS.

In day 1: Cells from the target cell lines (MCF-7-HER2+ and MDA-MB-231-HER2+) and the positive control (SKBR3) were counted manually under light microscope using Neuber Haemocytometer. This was performed by preparing labelled eppendorfs with 50µl Trypan Blue (a substance that will stain the non viable cells rather than the healthy ones) and add 50µl (equal volume 1:1 ratio) of cell suspension. A mixture of this suspension will be loaded onto Neuber Haemocytometer. This is designed to have two chambers, each of which has 9 squares where cells (not blue) will be counted in all the squares of the small central grid (5x5 square grid) after applying a cover slip (square glass of 22 mm width) onto the chamber and put a drop of the mixture of cell suspension and Trypan blue between the chamber and the cover slip (Figure 2-3). The number of cells per ml will be counted by multiplying our cell count by $2e^4$ / total number of squares. Then, the total number of cells is calculated by multiplying the value obtained by the total volume of cell suspension. The explanation of this calculation is that each individual square has a surface area of 0.1 CM ×0.1 CM= 0.01 CM^2. The depth of the chamber is 0.01 CM and to calculate the volume of each square: 0.01 CM^2×0.01 CM= 0.0001 CM^3. So we are actually counting cell in 0.0001 ML rather than in 1 ML, for this reason we multiplied × e^4 and we multiplied by another 2 to overcome the dilution effect of Trypan blue.

After knowing the number of cells in our sample, we can seed the optimal amount we need. In this procedure, we seeded cells at 5×10^4/ cover slip in a pre prepared 6-well plate. Firstly, a few drops of sterilised Distilled Water (DW) were added to the base of each well and a piece of paraffin film was applied and by using a sucker, firmly adhering the film to the base. Next, a few drops of the sterilised DW were added onto the paraffin film and a cover slip was placed onto

the film. Then after, few drops of media were added on each cover slip making sure that it will not spill around and then is incubated at 37 °C.

Next day, Cells were checked for adherence to the cover slips. Media was aspirated and cover slips were washed with Phosphate Buffer Saline (PBS) for 5 minutes. Then cells were fixed with 4% paraformaldehyde in PBS for 10 minutes. Later on, covers slips were washed in PBS three times for 5 minutes. Methanol (at -20°C) was added over each cover slip and incubated for 5 minutes at -20°C. After washing with PBS for 5 minutes, detection of antigens was determined using the same procedure for IHC (Section 2.4.3.2). Finally, for mounting; glycerol DPX (Sigma) was used where edges of the cover slips were fixed with colourless nail varnish and left to dry for 48 hours.

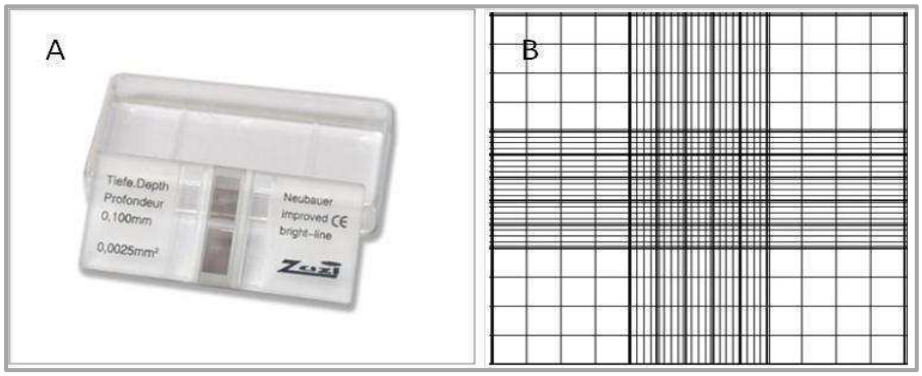

Figure 2-3: Neuber Haemocytometer for cell counting, A: showing the 2 chambers, upper and lower ones, B: illustrating the highlighted central grids inside which viable cells are counted, (Oscar)

2.7.2 Assessment of staining

Assessment was performed manually under the microscope by determining sub-cellular localisation of immunoreactivity (nuclear, cytoplasmic or membranous) and in case of HER2, it was membranous (Figure 2-4).

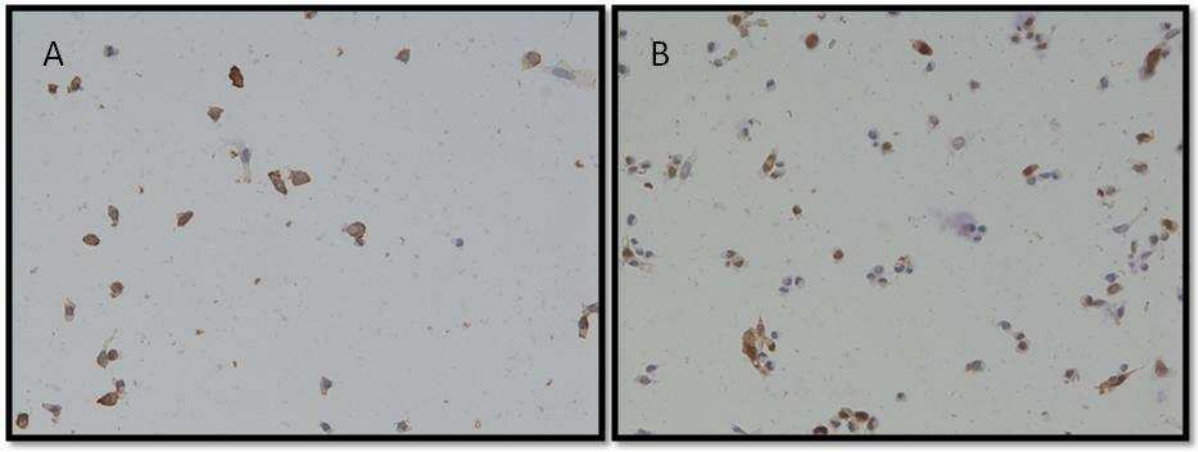

Figure 2-4: ICC of HER2 confirming its expression in transfected cell lines; A: MCF-7-HER2+ and B: MDA-231-MB-HER2+

2.8 Growing of cells and preparation of cell lysates

MCF-7, MDA-MB-231 and BT474 were grown using RPMI media (Sigma) supplied with 10% Foetal Bovine Serum (FBS) (Sigma). SKBR3 cell line was grown in McCoy's media (Sigma) which was also supplied with 10% FBS. For the T cell lines, they were grown in MEMα media (Sigma) supplied with 10% FBS and 0.5 µg/ml puramycin (Invitrogen). Nutrient materials were added to the media including 2 mM glutamine (Sigma), 1mM sodium pyrovate (Sigma), 1×MEM non essential amino acids (Sigma), 10 mM HEPES (Sigma), 10 µg/ml insulin (Sigma) and finally10 µg/ml gentamycin (Sigma).

Cells were grown until reached 80% confluency (which took 2-3 days for all cell lines apart from BT474 which required 4-5 day) after which they were washed with sterile PBS and incubated with 10% trypsin/EDTA (Sigma) , pre-warmed to 37ºC, for 5 minutes, to disrupt the cell monolayer. Fresh media was used to neutralise the trypsin. Then, cells were centrifuged at 500*g* for 5 minutes and suspended with media later on and counted (the aim is 1×10^6 cells/ML). After centrifugation, the cell pellet was washed in PBS and lysed using RIPA buffer (25mM Tris, 150mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% Sodium Dodecyl Sulfate (SDS) (Thermo Scientific) and a phosphatase and protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, UK). In addition, 5 µl benzonase (Benzonase Nuclease ultrapure, Sigma) in benzonase buffer (1 µl of benzonase + 250 µl of benzonase buffer) was added to break the DNA strands so that the protein will be less viscous and can be pipetted easily. After 15 minutes of incubation on ice cells were centrifuged 20 minutes at 4°C using the maximum speed of centrifuge to facilitate the removal of cell debris and the supernatant was stored at -80 °C until use. Three independent procedures were performed and two replicates were prepared from each one.

2.9 Western blot technique

Western blot was performed in this study to check the specificity of the antibodies to be assessed in IHC and in RPPA. For this purpose, a mixture of cell lysates from 6 BC cell lines (MCF-7, MCF-7-HER2+, BT474, MDA-MB-231, MDA-MB-231-HER2+ and SKBR3) was used.

2.9.1 Preparation of samples, electrophoresis and transfer

The samples were prepared in a maximum volume of 20 µL; X (13) µL of cell lysate, 5 µL of NuPAGE LDS Sample buffer × 4, 2 µL of NuPAGE reducing agent, and X µL D.W (in case needed to complete the volume up to 20 µL), (all reagents are from Novex Life Technologies), then this mixture was heated for 5 minutes at 100 °C and left to cool on ice. The prepared sample was loaded onto NuPAGE Bis-Tris Mini Gel (4-12%) (Novex Life Technologies) in 20x MOPS running buffer (Novex Life Technologies). The pre-stained 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard and gels were run at 150 volt for 90 minutes.

Proteins were transferred to a Hybond ECL Nitrocellulose (GE Healthcare) by wet transfer and electro-blotting at 30 volt for 1 hour in transfer buffer (20% (v/v) methanol, 50mM Tris-HCl and 380mM glycine). For blocking, the membranes were incubated in PBS (Sigma) containing 0.1% Tween-20 (Dako) and 5% non-fat dry milk for 60 minutes. The membrane was washed three times with PBS Tween-20 (TPBS) and incubated with the primary antibody overnight at 4°C using the optimal concentration as instructed by the manufacturer (Table 2-6).

Membranes were washed with TPBS (3 x 5 minutes) and incubated with the secondary antibody; Horseradish peroxidase-linked (HRP) secondary antibody (Anti-rabbit or anti mouse monoclonal horseradish peroxidase linked, Sigma-Aldrich) at a concentration of 1:2000 diluted with blocking solution for 1 hour in a rocker at room temperature then was washed with TPBS (3 x 5 minutes). While for the florescent WB, florescent secondary antibody was used (anti mouse or anti rabbit, Licor Biosciences) at a concentration of 1:15000 µL. The membrane was developed using the Enhanced Chemiluminescence Detection reagent (ECL, Amersham, UK), exposed to film (Kodak, Sigma Aldrich, UK) and developed, for fluorescence, the membrane was visualised using Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

The primary antibody for β-actin was used at a concentration of 1:1000, while for the secondary; it was used at a concentration of 1:2000 for HRP and 1:15000 µL for the florescence WB). ECL WB was performed by Dr. Ola Negm, School of Life Sciences, University of Nottingham) and the florescence WB was performed by the author.

Table 2-6: The antibodies used in Western blot and Reverse Phase Protein Array

Antibody	Supplier	Species	Molecular weight(KDa)	Concentration	Purpose
1-MAPKs					
p-c-RAF(Ser259)	Cell Signaling	rabbit	74	1:1000	Specificity*
p-MKK1/2 (S217/221)	Cell Signaling	rabbit	45	1:1000	specificity
p-MKK3(ser189/6(ser207)	Cell Signaling	rabbit	40	1:1000	specificity
p-MKK7(S217/221)	Cell Signaling	rabbit	48	1:1000	specificity
ERK1/2	Cell Signaling	rabbit	42,44	1:1000	specificity
p-ERK1/2 (Pt185/pY187)	Cell Signaling	rabbit	42,44	1:1000	Specificity/expression**
JNK1/2	Cell Signaling	mouse	46,54	1:1000	specificity
p-JNK1/2(T183/Y185)	Cell Signaling	rabbit	46,54	1:1000	Specificity/expression
P38	Cell Signaling	rabbit	38	1:1000	specificity
p-p38 (T180/Y182)	Cell Signaling	rabbit	43	1:1000	Specificity/expression
p-ATF2 (Thr69/71)	Cell Signaling	rabbit	70	1:1000	Specificity/expression
C-JUN	Cell Signaling	rabbit	48	1:1000	specificity
p-C-JNU (Ser 73)	Cell Signaling	rabbit	48	1:1000	Specificity/expression
P-SMAD3(Ser423/425)	Cell Signaling	rabbit	52	1:1000	specificity
p-STAT3	Cell Signaling	rabbit	54	1:1000	specificity
p-MSK2(DU1AU)	Cell Signaling	rabbit	85	1:1000	specificity
p-ELK1(Ser383)	Cell Signaling	rabbit	47	1:1000	specificity
2-PI3K pathway	Cell Signaling	rabbit			specificity
p-PI3K(p110)	Cell Signaling	rabbit	60,85	1:1000	specificity
p-Akt(S473)	Cell Signaling	rabbit	60	1:1000	specificity
p-mTORC1(Ser2448)	Cell Signaling	rabbit	289	1:1000	specificity
p-S6K	Cell Signaling	rabbit	46	1:1000	specificity
PTEN	DAKO	mouse	54	1:1000	specificity
p-PTEN	DAKO	mouse	54	1:1000	specificity
ER	Dako	rabbit	66	1:500	expression
HER2	Dako	rabbit	200	1:500	expression
***For specificity, lysate from a mixture of 6 BC cell lines mentioned in material and methods was used to determine the band.**					

2.10 Reverse phase protein array

The concentration of protein in the cell lysate which were prepared previously from the six BC cell lines was quantified using the Thermo Scientific™ Pierce™ BCA Protein Assay. According to this quantification, the proteins from each cell line were normalised and equal amount of protein from each cell line was put in the plate for microarray. Firstly, the proteins from the cell lysate were solubilised in 4x SDS sample buffer at a ratio of 1:3 respectively and heated at 95°C for 5 minutes. Then after, prepared samples were loaded onto 384 well plate (Genetix, UK) using three serial dilutions for each sample in 1x SDS buffer. Subsequently, robotic spotting of the samples in duplicates (each with three dilutions) onto nitrocellulose-coated glass slide (GraceBiolab, USA) was performed via a microarrayer (MicroGridII) by using silicon pins (Figure 2-5).

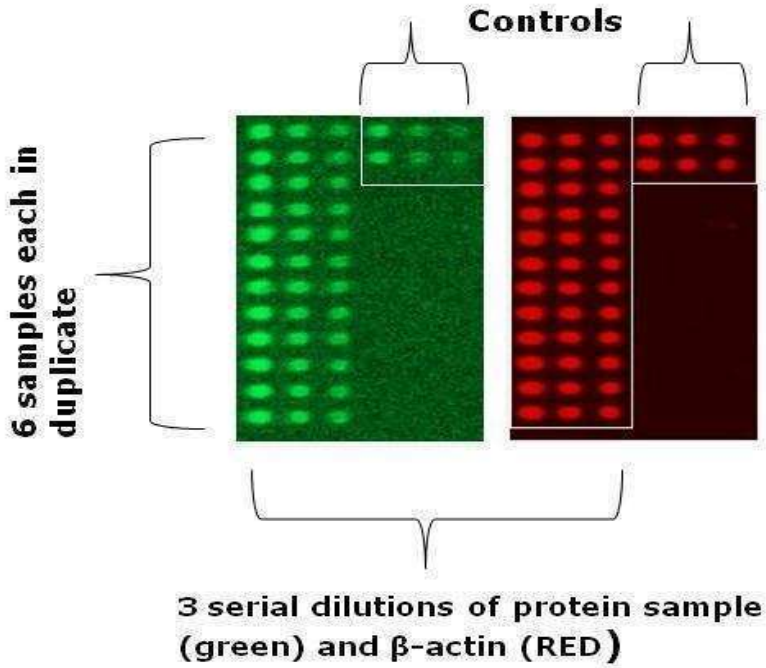

Figure 2-5: Schematic diagram of the nitrocellulose coated glass slide distribution of samples. 6 duplicate samples are arranged each with 3 serial dilutions for the protein sample and β-actin and controls were used for both.

Next, incubation of the samples containing slides with blocking solution (0.2% I-block (Tropix, Bedford, MA, USA), 0.1% Tween-20 in PBS) at 4°C overnight with shaking of the samples was considered. After three washings with TPBS x 5 minutes each, incubation with optimally diluted (diluent DAKO) antibodies was performed (Table 2-7). Rabbit or mouse β-actin (Sigma Aldrich, UK) was used as a positive control for loading of other proteins and was diluted, using DAKO diluent, at 1:1000 and slides were kept overnight at 4°C with shaking.

Following three washings with TPBS, slides were incubated for 30 minutes at room temperature with infrared secondary antibodies (diluted at 1:5000 in washing buffer in the dark with shaking); 800CW anti-rabbit and 700 CW anti-mouse antibodies (800 and 700 nm ranges of spectrum, respectively) from LI-COR, Biosciences. Then, washing with TPBS and drying of the slides was performed by centrifugation at 500g for 5 minutes, and slides were scanned with a Licor Odyssey scanner at 21 μm resolution at 800nm (green) and 700nm (red). The obtained TIFF images were processed with Axon Genepix Pro-6 Microarray Image Analysis software (Molecular Services Inc.) in order to obtain fluorescence data for each feature and to generate gpr files. The signals of protein were determined, after two important steps: subtraction of the background and normalisation to the internal housekeeping target, using RPPanalyzer, a module within the statistical language on the CRAN (http://cran.r-project.org/). Finally using Multi Experiment Viewer (MEV) software, the heat maps were created.

The RPPA technique and generation of raw data and bar charts was performed by Dr. Ola Negm, School of Life Sciences, University of Nottingham. The samples of protein lysates were prepared by the author and the analysis of the raw data to reveal significant associations between the six BC cell lines were performed by the author.

Table 2-7: Antibodies used for RPPA

Antibody	RPPA concentration
1-MAPKs	
p-c-RAF	1:500
p-MEK1/2	1:250
p-MKK3(ser189/6(ser207)	1:100
p-MKK7(S217/221)	1:100
ERK1/2	1:1000
p-ERK1/2 (PT185/pY187)	1:1000
JNK1/2	1:500
p-JNK1/2(T183/Y185)	1:500
P38	1:1000
p-p38 (T180/Y182)	1:1000
p-ATF2 (Thr69/71)	1:100
C-JUN	1:250
p-C-JNU (Ser 73)	1:250
P-SMAD3	1:500
p-STAT3	1:50
p-MSK2	1:500
p-ELK1	1:250
2-PI3K pathway	
p-PI3K(p110)	1:250
p-Akt(serine)	1:25
p-mTORC1(Ser2448)	1:250
p-S6K	1:500
PTEN	1:500
p-PTEN	1:500

2.11 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences SPSS v21 for Windows (Chicago, IL, USA). For the Decision tree algorithms, WEKA software was used and the analysis was performed by Daniele Soria (School of Computer Science, University of Nottingham, Advanced Data Analysis Centre).

2.11.1 Univariate analysis with clinicopathological variables and biological markers

To test the association between the biomarkers under investigation in this study and clinicopathological parameters and other biomarkers, Chi-Squared test (x^2) test was used for this purpose. For testing correlation between uncategorical data, Spearman's Rank correlation was used while Mann Whitney test was used to test categorical with uncategorical data and for these analysis, the threshold of p-value was decreased to <0.01 to be considered significant to compensate for multiple comparisons. In addition, Kruskal Wallis/ANOVA test was used to determine the difference in expression of proteins in different BC cell lines representing different BC classes in RPPA and the p-value was adjusted using bonferroni correction.

2.11.2 Univariate analysis for patients' outcome

Kaplan-Meier survival curve was drawn to determine the differences in the survival in the different biomarkers by plotting survival curves and log-rank test was used to find the significant associations. In this test, patients were censored either if their follow up was lost or they died due to causes other than BC.

2.11.3 Multivariate analysis

If a statistically significant association was found in the biomarkers with patients' outcome in Kaplan-Meier test, cox regression test for multivariate analysis can be applied which can help test for confounders and prognostic or predictive independency of the investigated biomarker. For this purpose, standard well known prognostic/predictive factors were used: tumour grade, tumour stage and tumour size.

2.12 Remark criteria

This retrospective study adheres to REMARK criteria (McShane et al., 2006).

2.13 Ethical approval

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetics classification of breast cancer" under the ethical approval number (RC2020313)

3 Role of Mitogen Activated Protein Kinases in Breast Cancer

3.1 Introduction

Mitogen activated protein kinases are a family of protein kinases involved in transmitting signals from a variety of stimuli from the cell membrane to the nucleus. The MAPKs are activated by mitogenic stimuli from growth factor receptors such as epidermal growth factor. Once MAPKs are activated, they phosphorylate a variety of proteins, including transcription factors, to exert certain function on gene expression. A cascade of protein kinases regulate and activate MAPKs via phosphorylation of both threonine and tyrosine residues (Seger and Krebs, 1995).

The function of MAPKs in BC is complex owing to the large number of cellular responses that they modulate and their interaction with different pathways in the malignant cells (Kyriakis and Avruch, 2001, Karin and Gallagher, 2005, Weston and Davis, 2007). In addition, their complex interaction with ER and HER2 could explain their diverse behaviour in BC (Figure 1-5); therefore, a full understanding is missing on how these proteins act either as tumour suppressor proteins or oncoproteins in different cell types.

Importantly, MAPKs have been investigated in BC including their interaction with HRs and HER2; however, conflicting results were reported and the exact role of MAPKs in BC and their interaction with ER and HER2 remains to be determined (Merlin et al., 2013, Huang et al., 2013, Kuo et al., 2013)

Interestingly, some investigators have explained a process of senescence that adds a new dimension in the understanding of the function of MAPKs. This process stems from the active RAS which activates two protein kinase cascades: RAF/MAPKs and PI3K. RAF activates a tumour suppressor protein known as Alternative Reading Frame (ARF) which stops the inhibitory effect of HDM2 on p53 (a potent stimulator of senescence), alternatively, RAF activates another kinase known as p38-regulated/activated protein kinase that will immediately phosphorylate p53. Independently, RAF can induce senescence without any assistance from other intermediates. Meanwhile, RAS can enhance its other cascade arm; the PI3K which functions to inhibit HDM2 and enhances p53 action. Interestingly, RAS can stimulate DNA replication which induces a sort of DNA imbalance and damage that can eventually result in p53 phosphorylation; however, RAS can enhance this task alone or by inducing certain intermediates

as reactive oxygen species (ROS), (Sun et al., 2007, Bartkova et al., 2006, Di Micco et al., 2006, Mallette et al., 2007), (Figure 3-1).

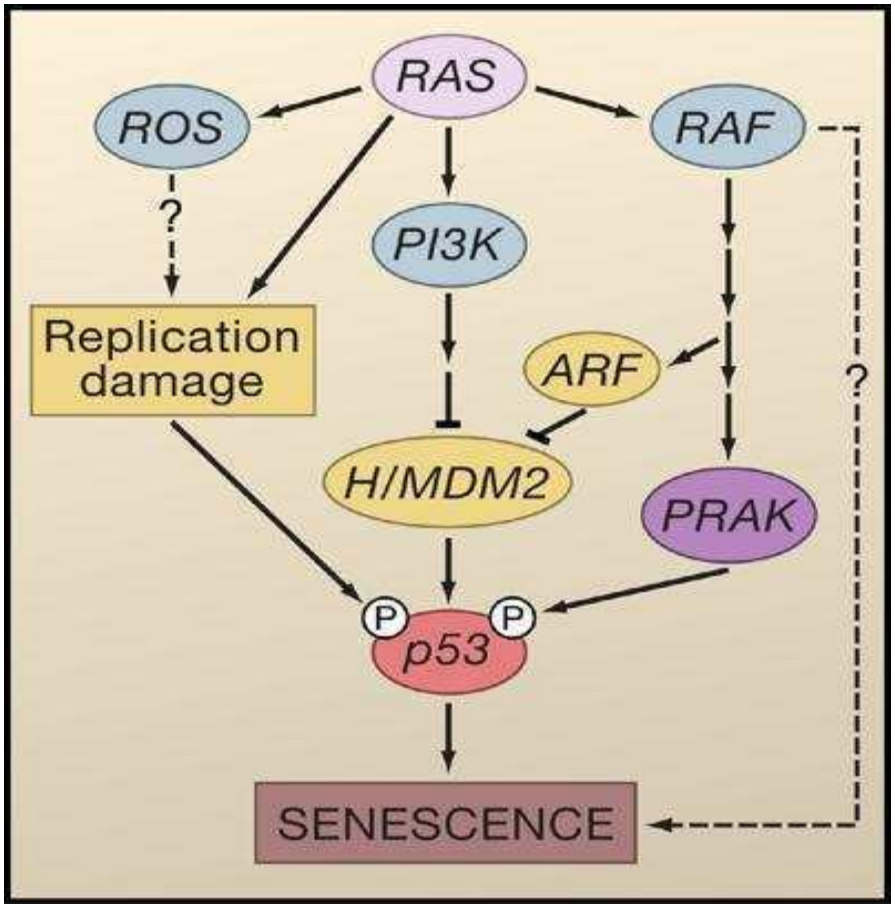

Figure 3-1: Multiple Pathways Mediate Oncogene-Induced Senescence, details of this pathway are highled in the text, (Yaswen and Campisi, 2007)

3.1.1 Hypothesis

MAPKs are important signalling transduction molecules that have diverse function in BC. It is hypothesised that MAPKs interact with ER and HER2 pathways resulting in divergent associations and outcome in BC subgroups based on HER2 and ER expressions indicating a biological difference in these subgroups mainly with respect to ER+HER2+ vs ER+HER2- and ER-HER2+ groups.

3.1.2 Aims

1- To determine the expression of MAPKs (ERK1/2, p-ERK1/2, JNK1/2, p-JNK1/2, p38, p-p38, p-ATF2 and p-C-JUN) in a large well characterised series of primary operable BC cases and to correlate their expression with clinicopathological variables and a range of biomarkers related to ER and HER2 pathways, apoptosis, p53 and EMT (in BC and in different BC subgroups) based on HER2 and ER expressions (ER+HER2-, ER+HER2+, ER-HER2+ and ER-HER2-) to illustrate the biological difference between these subgroups in terms of expressions of these MAPKs.

2- To assess the prognostic and predictive potential of MAPKs.

3- To determine the role of MAPKs in Trastuzumab treated (response and resistance) series.

4- To quantify the expression of MAPKs in BC using RPPA.

3.2 Methods

Tissue microarray form BC was prepared and immunohistochemical staining was performed as described, (section 2.4.1, page 50). The expression of MAPKs antibodies used in IHC was determined in the Primary and in Trastuzumab treated series. WB was used to check the specificity of the antibodies and RPPA was used to quantify the expression of MAPKs (section 2.10, page 64). For the details of concentrations used for WB and RPPA, refer to Table 2-6 and Table 2-7 in methodology chapter. In addition, a range of biological markers was used to compare with MAPKs in both series (Table 2-3) and for the details of MAPKs proteins used see Table 3-1.

Table 3-1: Details of MAPKs used in this study

Antibody	Species	Molecular weight(KDa)	Pre treatment	Primary Series		Trastuzumab Treated Series		Purpose
				Negative (IHC) N (%)	Positive(IHC) N (%)	Negative (IHC) N (%)	Positive (IHC) N (%)	
p-c-RAF	rabbit	74		-	-	-	-	WB,RPPA
p-MEK1/2	rabbit	45		-	-	-	-	WB,RPPA
p-MKK3(ser189/6(ser207)	rabbit	40		-	-	-	-	WB,RPPA
p-MKK7(S217/221)	rabbit	48		-	-	-	-	WB,RPPA
ERK1/2	rabbit	42,44	Microwave/Citrate	Cytoplasmic: 625 (52.3)	571 (47.7)	Cytoplasmic: 64 (59.3%)	44 (40.7%)	IHC,WB,RPPA
p-ERK1/2 (Pt185/pY187)	rabbit	42,44	Microwave/Citrate	Nuclear: 561 (48.4)	597 (51.6)	Nuclear: 84 (55.3%)	68 (44.7%)	IHC,WB,RPPA
				Cytoplasmic: 533 (46.5)	613 (53.5)	Cytoplasmic: 135 (88.8%)	17 (11.2%)	IHC,WB,RPPA
JNK1/2	mouse	46,54	Microwave/Citrate	Cytoplasmic: 573 (53.3%)	502 (46.7%)	Cytoplasmic: 78 (51.3)	74 (48.7)	IHC,WB,RPPA
p-JNK1/2(T183/Y185)	rabbit	46,54	Microwave/Citrate	Nuclear: 229 (21.7%)	827 (78.3%)	Nuclear: 85 (52.8%)	76 (48.2%)	IHC,WB,RPPA
P38	rabbit	38	Microwave/Citrate	Cytoplasmic: 703 (60.3%)	463 (39.7%)	Cytoplasmic: 78 (50.3)	77 (49.7)	IHC,WB,RPPA
p-p38 (T180/Y182)	rabbit	43	Microwave/Citrate	Nuclear: 941 (70.5%)	394 (29.5%)	Nuclear: 44(29.9)	103 (70.1)	IHC,WB,RPPA
p-ATF2 (Thr69/71)	rabbit	70	Microwave/Citrate	Nuclear: 958 (73.9%)	338 (26.1%)	Nuclear: 87 (58)	63 (42)	IHC,WB,RPPA
C-JUN	rabbit	48	-	-	-	-		WB,RPPA
p-C-JNU (Ser 73)	rabbit	48	Microwave/Citrate	Nuclear: 453 (35.3%)	831 (64.7%)	Nuclear: 65 (43.6)	84 (56.4)	IHC,WB,RPPA
P-SMAD3	rabbit	52	-	-	-	-	-	WB,RPPA
p-MSK2	rabbit	85	-	-	-	-	-	WB,RPPA
p-ELK1	rabbit	47	-	-	-	-	-	WB,RPPA

3.3 Results

3.3.1 Specificity of MAPKs

Western blot revealed that all MAPKs used in this study showed their specific band(s) at the right molecular weight(s) as instructed by the supplying company (all MAPKs were provided by Cell Signalling). Some antibodies as ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 have two epitopes so two bands are shown in their WB (Figure 3-2).

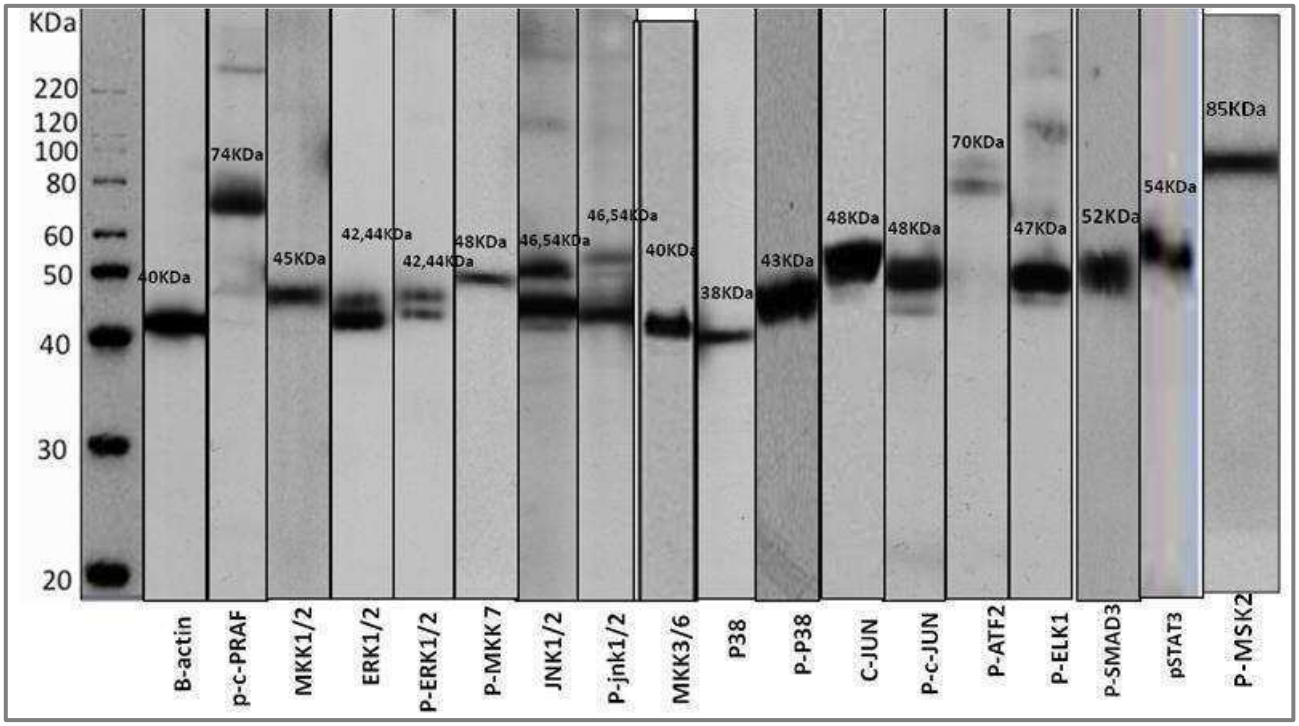

Figure 3-2: Western blot of MAPKs

3.3.2 Immunohistochemistry results

3.3.2.1 Expression of MAPKs in the primary breast cancer series

3.3.2.1.1 ERK1/2 (pan)

1196 cases were valid for the assessment of ERK1/2 (pan). The expression of this protein was cytoplasmic in the invasive tumour with variable intensities although there were some nuclei with faint staining which were not taken into consideration (Figure 3-3 A). Moreover, the same pattern of expression was observed in normal breast epithelial cell and DCIS which were within some TMA cores. The cut-off point was set at > 100 (H-score) using X-tile software where 625 (52.3%) cases were classified as negative/low, while 571 (47.7%) cases had high expression (Table 3-1).

3.3.2.1.2 p-ERK1/2

This biomarker revealed both nuclear and cytoplasmic expression in invasive breast tumour cells. A total of 1158 cases were available for assessment, and both forms were observed in normal breast tissue and DCIS within TMA cores (Figure 3-3 B). The cut-offs for nuclear and cytoplasmic forms were chosen at $\geq$140 (H-score) and $\geq$ 30 (H-score) using X-tile. For p-ERK1/2 nuclear expression, 561 (48.4%) cases were negative/low and 597 (51.6%) had high expression). Moreover, for cytoplasmic expression, 533 (46.5%) showed negative/low and 613 (53.5%) had high expression (Table 3-1). Furthermore, negative/low nuclear and cytoplasmic: 307 cases, high expression of nuclear without cytoplasmic: 90, high expression of cytoplasmic without nuclear: 118 and high expression of both: 355.

3.3.2.1.3 JNK1/2 (pan)

A total of 1075 cases were valid for assessing this protein from the primary series TMA. Its expression was mainly cytoplasmic which was likewise observed in the trapped DCIS foci and normal breast tissue in the cores (Figure 3-3 C). Importantly, the value of $\geq$103 (H-score) was set to be the cut-off using X-tile software. Out of the total valid cases, 573 (53.3%) were rendered negative/low while 502 (46.7%) deemed high expression (Table 3-1).

3.3.2.1.4 p-JNK1/2

A total of 1056 cases were valid for the assessment of this protein from the primary series TMA. The expression was only nuclear in the invasive tumour and in the trapped foci of DCIS and normal breast tissue in the cores (Figure 3-3 D). Of worth, the cut-off point chosen by using X-tile software was set at >124. Importantly, out of the total valid cases, 229(21.7%) were negative/low and 827(78.3%) had high expression (Table 3-1).

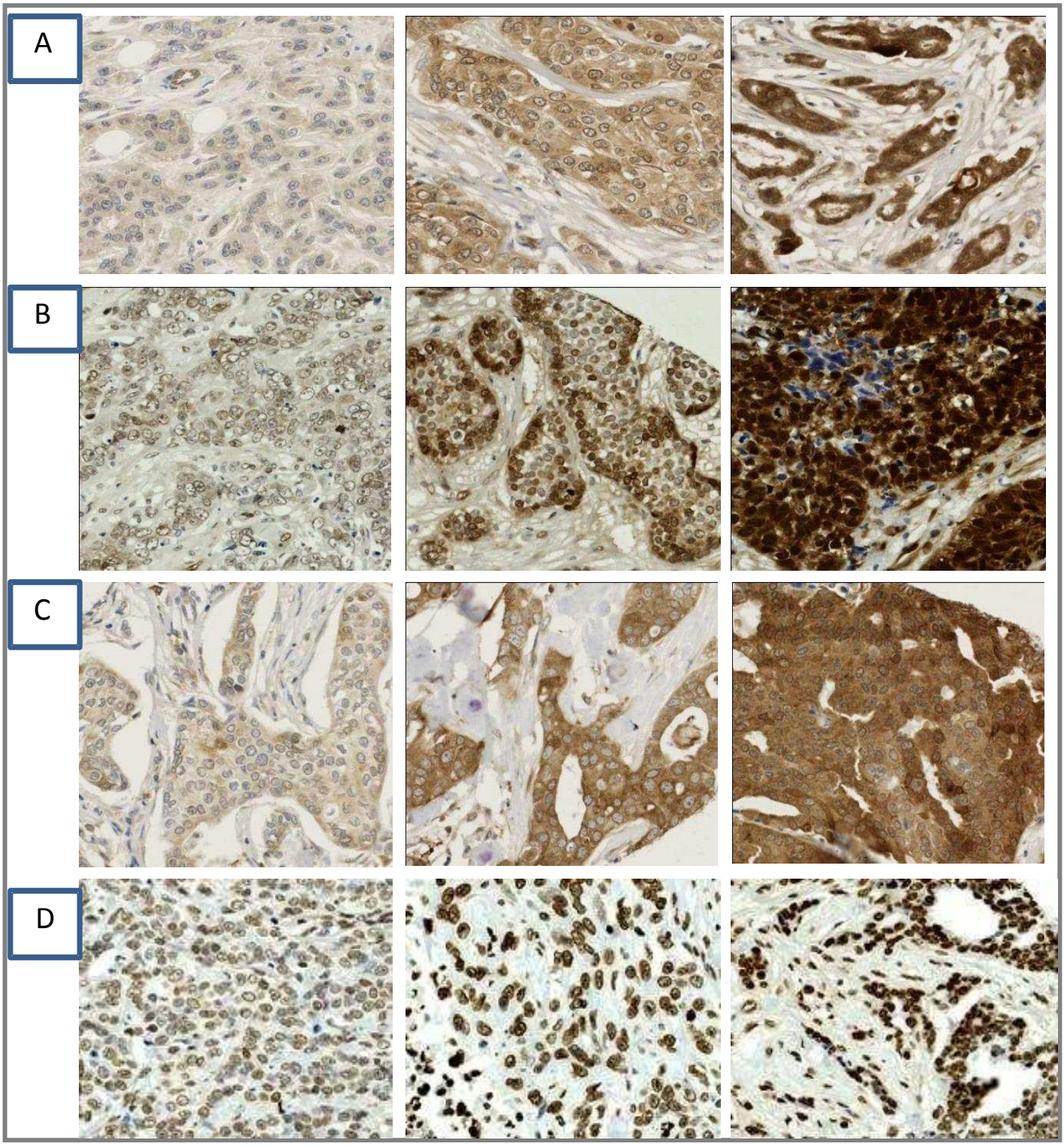

Figure 3-3: Different intensities of nuclear&/or cytoplasmic staining of MAPKs, from the left to right: Weak, moderate and strong intensities. A: ERK1/2, B: p-ERK1/2, C: JNK1/2 and D: p-JNK1/2. All pictures were taken using digital pathology system at x20

3.3.2.1.5 P38 (pan)

1166 were the total valid cases for the assessment of this protein from the primary series TMA. Its expression was cytoplasmic in the invasive tumour and in normal breast tissue and DCIS foci found within the TMA cores, (Figure 3-4 A). The cut-off point chosen was set at >112 using X-tile software. Out of the total, 703(60.3%) cases were negative/low while 463(39.7%) cases showed high expression (Table 3-1).

3.3.2.1.6 p-p38

A total of 1335 cases were available for assessing this protein in the primary series TMA. The expression of this protein was nuclear and even it was noticed in the foci of DCIS and normal breast tissue that have been trapped within the cores (Figure 3-4 B). In addition, cytoplasmic staining was also noticed but its median of H- score was not representative of a specific distribution that can split the cases into groups, so this cytoplasmic staining was not considered. The X-tile software was used to choose the cut-off value at >100 (H-score). 941 (70.5%) out of the total cases were found to be of negative/low expression while those deemed high expression were only 394(29.5%) cases (Table 3-1).

3.3.2.1.7 p-ATF2

1296 were the total valid cases for this protein to be assessed in the primary series TMA. Nuclear expression of this protein was observed in addition to its staining that has been observed in normal and DCIS foci found within the cores, (Figure 3-4 C). The cut-off at >70 (H-score) value could dichotomise the cohort into two categories by X-tile software. For this protein, two third of the total cases: Nuclear: 958(73.9%), revealed negative/low expression and the remaining one third had high expression (Table 3-1).

3.3.2.1.8 P-C-JUN

In total, 1284 cases were valid for the assessment of this transcription factor protein within the TMA of primary series. The expression was nuclear which was also noticed in trapped foci of normal breast tissue and DCIS within the cores, (Figure 3-4 D). The optimal cut-off point was >3 (percent) chosen by X-tile software. Out of the total valid cases, 453(35.3%) had negative/low expression and the majority: 831(64.7%) cases, showed high expression (Table 3-1).

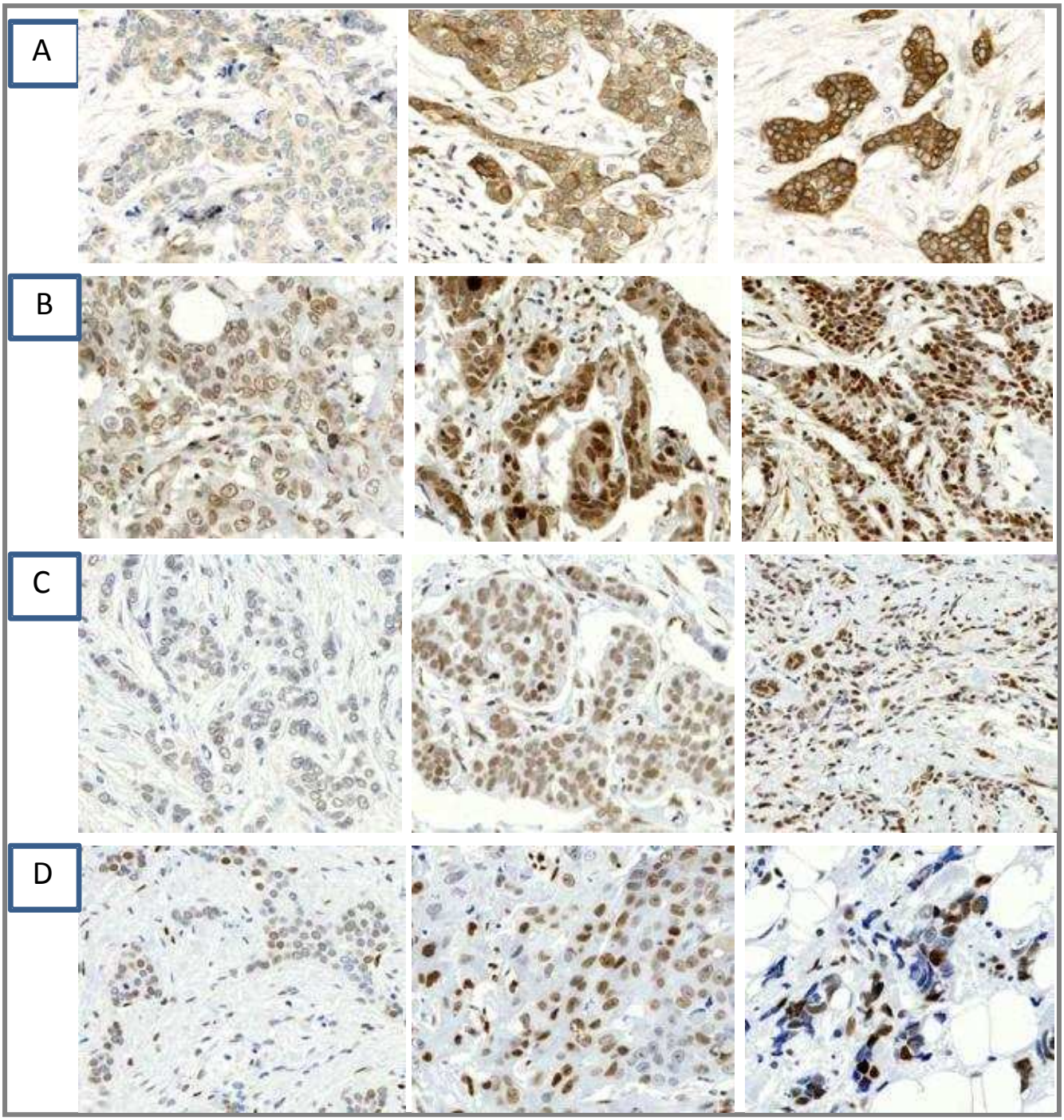

Figure 3-4: Different intensities of nuclear&/or cytoplasmic staining of MAPKs, from the left to right: Weak, moderate and strong intensities. A: pan p38, B: p-p38, C: p-ATF2 and D: p-C-JUN. All pictures were taken using digital pathology system at x20

3.3.2.2 The associations of MAPKs with each other

The expression of all MAPKs was positively associated with each other using continuous data It was observed that the phosphorylated forms (nuclear, cytoplasmic or both) of MAPKs were positively associated with their total forms except p-JNK: Spearman's rank correlations of all MAPKs were directly positively correlated (Table 3-2). Most of these associations were maintained within ER+HER2- cohort, (appendix table 1). Moreover, still many positive associations between these MAPKs were maintained within ER+HER2+ (appendix table 2) and fewer associations were maintained within ER-HER2+ cohorts (appendix table 3).

Table 3-2: The associations of MAPKs used in IHC with each other in the Primary breast cancer series

		p-JNK1/2	JNK1/2	N-p-ERK1/2	C-p-ERK1/2	ERK1/2	p38	N-p_p38	p-c-jun	p_ATF2
***[1]p-JNK1/2**	**Spearmann's Rank correlation**		.048	.588**	.371**	.111**	.097**	.430**	.280**	.381**
	p-value	-	**.226**	**.000**	**.000**	**.002**	**.009**	**.000**	**.000**	**.000**
	Number of cases		625	919	911	788	728	861	825	840
JNK1/2	**Spearmann's Rank correlation**	.048		.099*	.161**	.184**	.087*	.118**	.171**	.058
	p-value	**.226**	-	**.010**	**.000**	**.000**	**.019**	**.001**	**.000**	**.115**
	Number of cases	625		671	661	710	722	779	748	741
***[2]N-p-ERK1/2**	**Spearmann's Rank correlation**	.588**	.099*		.682**	.178**	.225**	.539**	.411**	.499**
	p-value	**.000**	**.010**	-	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**
	Number of cases	919	671		1145	871	816	957	923	938
***[3]C-p-ERK1/2**	**Spearmann's Rank correlation**	.371**	.161**	.682**		.183**	.181**	.443**	.390**	.361**
	p-value	**.000**	**.000**	**.000**	**-**	**.000**	**.000**	**.000**	**.000**	**.000**
	Number of cases	911	661	1145		860	806	950	916	931
ERK1/2	**Spearmann's Rank correlation**	.111**	.184**	.178**	.183**		.295**	.177**	.210**	.185**
	p-value	**.002**	**.000**	**.000**	**.000**	-	**.000**	**.000**	**.000**	**.000**
	Number of cases	788	710	871	860		927	971	942	955
p38	**Spearmann's Rank correlation**	.097**	.087*	.225**	.181**	.295**		.137**	.205**	.133**
	p-value	**.009**	**.019**	**.000**	**.000**	**.000**	-	**.000**	**.000**	**.000**
	Number of cases	728	722	816	806	927		914	882	902
N-p-p38	**Spearmann's Rank correlation**	.430**	.118**	.539**	.443**	.177**	.137**		.461**	.532**
	p-value	**.000**	**.001**	**.000**	**.000**	**.000**	**.000**	-	**.000**	**.000**
	Number of cases	861	779	957	950	971	914		1222	1230
p-c-jun	**Spearmann's Rank correlation**	.280**	.171**	.411**	.390**	.210**	.205**	.461**		.419**
	p-value	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	-	**.000**
	Number of cases	825	748	923	916	942	882	1222		1175
p-ATF2	**Spearmann's Rank correlation**	.381**	.058	.499**	.361**	.185**	.133**	.532**	.419**	
	p-value	**.000**	.115	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	-
	Number of cases	840	741	938	931	955	902	1230	1175	

(-) Represents the analysis of each marker with itself, (*[1]) is phosphorylated, (*[2]) is nuclear, (*[3]) is cytoplasmic, (*) is the correlation which is significant at the 0.05 level (2-tailed)and (**) is the correlation coefficient.

3.3.2.3 Associations of MAPKs with clinicopathological variables in the unselected primary breast cancer series and different breast cancer subgroups

3.3.2.3.1 Pan ERK1/2 and p-ERK1/2

High expression pan ERK1/2 was positively associated with smaller tumour size (p=0.029), ductal carcinoma type (p=0.003), lower tumour grade (p=0.031: borderline), more tubule formation (p<0.001) and with the GPG of the NPI (p=0.015: borderline, Table 3-3).

Within ER+HER2- tumours, association of pan ERK1/2 with ductal tumour type and more tubule formation were maintained (p=0.006 and p=0.027: borderline respectively; Table 3-3). In the ER+HER2+ tumours, high expression of pan ERK1/2 was observed in Pre-menopausal women (p=0.010: borderline) and in ER-HER2+ cohort, smaller size and early stage were associated with high expression (p=0.036 and p=0.023, both borderline, respectively, appendix table 4). For p-ERK1/2, its high nuclear expression was positively associated with smaller tumour size, lobular carcinoma, lower tumour grade, more tubule formation, less pleomorphism, lower mitotic count and better NPI score (all p<0.001; Table 3-4). When the analysis was restricted to ER+HER2- cases, similar associations were observed to those within the whole series except the patients were younger and Pre-menopausal (p=0.010 and p=0.034, respectively, Table 3-4). Moreover, the latter associations (young and Pre-menopausal) were also observed within ER+HER2+ cohort (p=0.031: borderline, p=0.009) respectively and a trend for smaller tumour size was observed within ER-HER2+ also in association with high nuclear p-ERK1/2 (p=0.050, appendix table 4).

For cytoplasmic p-ERK1/2, the same associations were observed in the whole series and in ER+HER2- cohorts, where high expression of this protein was positively associated with good prognostic clinicopathological parameters (Table 3-4).

In association with high cytoplasmic expression within ER+HER2+, less mitosis was observed (p=0.038: borderline). While within ER-HER2+, a trend for moderate NPI score and for younger age patients were observed in association with high expression (p=0.025, p=0.050) respectively. No associations were

observed within ER-HER2- tumours for these proteins apart from moderate NPI score for cytoplasmic p-ERK1/2 (p=0.026: borderline, appendix table 4).

3.3.2.3.2 Pan JNK1/2 and p-JNK1/2

Apart from an association between high pan JNK1/2 expression and lower tumour size within ER+HER2- (p=0.013: borderline) cohort, pan JNK1/2 did not reveal other associations (Table 3-5). However, high expression of p-JNK1/2 was positively associated with good prognostic clinicopathological variables including smaller tumour size (p=0.003), lobular carcinoma (p=0.004), lower tumour grade, less pleomorphism (p<0.001for both) and lower NPI score (p=0.002), (Table 3-6). Some of these associations were highly significant and were maintained within ER+HER2- tumours, including smaller tumour size (p=0.002), lower tumour grade and less pleomorphism (p<0.001 for both, Table 3-6).
Within the ER+HER2+ cohort, high p-JNK1/2 expression was associated with Pre-menopausal patients (p=0.028: borderline), while within the ER-HER2+ cohort, it was associated with a trend for stage II disease, absent LVI and moderate NPI score (p=0.013, p=0.014 and p=0.027) respectively. No associations were found within ER-HER2- cohort, (appendix table 4).

3.3.2.3.3 Pan p38 and p-p38

High expression of pan p38 was associated with lower tumour grade and less mitosis (p=0.002, both), more tubule formation (p<0.002) and good NPI score (p=0.011: borderline, Table 3-7). The preferable associations regarding grade and tubule formation were maintained within ER+HER2- cohort (p=0.041, p=0.011, both borderlines, respectively) but there was an association with high expression of this protein and ductal tumour type (p=0.018: borderline, Table 3-7). Moreover, high expression of this protein within ER+HER2+ cohort was positively associated with younger age and Pre menopausal patients (p=0.04, p=0.012: borderline) respectively, moderate LN stage (p=0.003) and more pleomorphism (p=0.032: borderline, appendix table 5).
Regarding the phosphorylated form of this protein (p-p38), its high expression was positively associated with almost all clinicopathological variables in the whole cohort including, smaller tumour size, lobular carcinoma, lower tumour grade and better NPI score (all p<0.001), in addition to an association with an early stage (p=0.008) and absent LVI (p=0.002, Table 3-8).

All these associations were highly significant and were maintained within ER+HER2- cases (Table 3-8). Only a trend for small tumour size (p=0.068) was observed within ER-HER2+ cohort. Within ER-HER2- tumours, p-p38 was only associated with moderate pleomorphism (p=0.004) and a trend for smaller tumour size (0.067, appendix table 5).

3.3.2.3.4 p-ATF2

High expression of p-ATF2 was positively associated with lobular carcinoma type, lower tumour grade, less pleomorphism and mitosis and better NPI score within the whole series, (p<0.001), furthermore, it was associated with more tubule formation (p=0.001) and absent LVI (p=0.032, Table 3-9). Meanwhile, some of these associations were maintained within ER+HER2- cases (grade and mitosis: p<0.001, pleomorphism: p=0.002 and NPI: p=0.019, Table 3-9). When the cohort was selected to ER-HER2+, p-ATF2 was associated with higher tumour grade (p=0.023: borderline) while within ER-HER2- BC, p-ATF2 was associated with less tubule formation, moderate pleomorphism and moderate mitotic count (p=0.001, p=0.002 and p=0.009, respectively, appendix table 5)

3.3.2.3.5 p-C-JUN

p-C-JUN, was positively associated with good prognostic clinicopathological variables including lower tumour grade (p<0.001), more tubule formation (p=0.020: borderline), less mitosis, better NPI (both: p=0.001) and absent LVI (p=0.015: borderline) within the whole series (Table 3-10). Moreover, shifting towards Pre-menopausal patients (p=0.005), lower tumour grade and less mitosis (p<0.001, p=0.002), respectively and absent LVI (p=0.035: borderline) were all maintained within ER+HER2- cohort in association with high p-C-JUN expression (Table 3-10). No other associations were found within other BC subgroups but a borderline association with increased mitotic count within ER+HER2+ cohort, (p=0.031, appendix table 5).

Table 3-3: The associations between pan ERK1/2 and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.404			0.297
≤50	230(37)	198(34)	(0.69)	120(32)	115(28)	(1.08)
>50	396(63)	377(67)		259(68)	292(72)	
Menopausal Status			0.456			0.809
Pre-	252(40)	219(38)	(0.555)	131(35)	137(34)	(0.05)
Post-	374(60)	355(62)		248(65)	269(66)	
Tumour Size (cm)			**0.029**			0.211
≤2.0	290(47)	301(53)	**(4.77)**	195(52)	227(56)	(1.56)
>2.0	334(53)	269(47)		183(48)	178(44)	
Stage			0.084			0.301
1	379(61)	343(60)	(4.93)	242(64)	247(61)	(2.39)
2	180(29)	188(33)		106(28)	133(33)	
3	63(10)	40(7)		29(8)	25(6)	
Tumour Type			**0.003**			**0.006**
Ductal	513(83)	488(86)	**(13.72)**	290(77)	339(84)	**(12.52)**
Lobular	81(13)	43(7)		72(19)	42(10)	
Medullary-like	11(2)	9(2)		1(0)	2(1)	
Special-type	14(2)	26(5)		14(4)	21(5)	
Grade			**0.031**			0.463
1	85(14)	99(17)	**(6.93)**	75(20)	92(23)	(1.54)
2	199(32)	203(36)		166(44)	181(45)	
3	338(54)	268(47)		136(36)	131(32)	
Tubules			**<0.001**			**0.027**
1	23(4)	33(6)	**(17.14)**	21(6)	28(7)	**(7.19)**
2	167(28)	209(37)		127(35)	170(43)	
3	410(68)	316(57)		216(59)	195(50)	
Pleomorphism			0.214			0.139
1	16(3)	10(2)	(3.08)	15(4)	7(2)	(3.59)
2	214(36)	223(40)		183(50)	210(53)	
3	370(61)	323(58)		166(46)	176(45)	
Mitosis			0.067			0.675
1	194(32)	203(36)	(5.41)	173(48)	185(47)	(0.78)
2	106(18)	114(21)		78(21)	94(24)	
3	300(50)	241(43)		113(31)	114(29)	
LVI			0.433			0.576
Probable/Negative	399(65)	381(67)	(615)	252(67)	279(69)	(0.31)
Definite	219(35)	190(33)		124(33)	126(31)	
NPI			**0.015**			0.475
GPG	170(28)	183(34)	**(8.41)**	148(41)	161(42)	(1.48)
MPG	324(54)	293(54)		176(48)	190(50)	
PPG	106(18)	66(12)		40(11)	32(8)	

Significant p-values are highlighted in bold in this table and in the other tables. LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-4: The associations between Nuclear and Cytoplasmic p-ERK1/2 and clinicopathological variables

	Whole series (nuclear p-ERK1/2)			ER+HER2- tumours (nuclear p-ERK1/2)			Whole series (cytoplasmic p-ERK1/2)			ER+HER2- tumours(cytoplasmic pERK1/2)		
	Neg/low(N) N (%)	High(N) N (%)	*p*-value (χ^2)	Neg/low(N) N (%)	High(N) N (%)	*p*-value (χ^2)	Neg/low(C) N (%)	High(C) N (%)	*p*-value (χ^2)	Neg/low(C) N (%)	High(C) N (%)	*p*-value (χ^2)
Age			136			**0.10**			0.092			**0.014**
≤50	188(33)	224(37)	(2.22)	81(26)	152(35)	**(6.71)**	175(33)	230(38)	(2.82)	88(26)	141(35)	**(6.07)**
>50	376(67)	373(63)		234(74)	288(65)		360(67)	384(62)		249(74)	268(65)	
Menopausal Status			0.256			**0.034**			0.405			0.150
Pre-	211(37)	242(41)	(1.29)	96(31)	167(38)	**(4.49)**	202(38)	245(40)	(694)	108(32)	151(37)	(2.07)
Post-	352(63)	352(59)		218(69)	272(62)		333(62)	365(60)		229(68)	256(63)	
Tumour Size (cm)			**<0.001**			0.052			**<0.001**			**<0.001**
≤2.0	242(43)	324(54)	**(15.00)**	154(49)	246(56)	(3.76)	222(42)	336(55)	**(20.02)**	148(44)	245(60)	**(18.60)**
>2.0	321(57)	272(46)		161(51)	193(44)		312(58)	277(45)		188(56)	164(40)	
Stage			0.873			0.351			0.170			0.910
1	343(61)	118(20)	(0.27)	197(63)	267(61)	(2.09)	313(58)	384(63)	(3.54)	206(61)	255(63)	(0.18)
2	170(30)	241(41)		98(31)	132(30)		164(31)	178(29)		104(31)	120(30)	
3	49(9)	235(39)		19(6)	39(9)		57(11)	48(8)		26(8)	32(8)	
Tumour Type			**<0.001**			**0.010**			0.269			0.163
Ductal	488(88)	469(79)	**(29.89)**	265(84)	333(76)	**(11.24)**	439(83)	507(83)	(3.57)	259(78)	330(81)	(5.11)
Lobular	44(8)	85(14)		42(14)	77(18)		65(12)	64(11)		63(19)	56(14)	
Medullary-like	17(3)	8(1)		1(0)	1(0)		11(2)	13(2)		1(0)	1(0)	
Special-type	8(1)	32(6)		6(2)	27(6)		13(3)	27(4)		11(3)	22(5)	
Grade			**<0.001**			**<0.001**			**0.001**			**<0.001**
1	54(9)	118(20)	(64.49)	47(15)	109(25)	**(26.03)**	52(10)	119(20)	**(29.83)**	48(14)	107(26)	**(23.76)**
2	156(28)	241(41)		131(42)	213(49)		172(32)	220(36)		149(44)	189(47)	
3	352(63)	235(39)		136(43)	116(26)		310(58)	271(44)		139(42)	111(27)	
Tubules			**<0.001**			**0.001**			**<0.001**			**<0.001**
1	10(2)	38(7)	**(28.59)**	9(3)	33(8)	**(13.16)**	13(2)	35(6)	**(20.45)**	12(4)	30(8)	**(21.16)**
2	147(27)	204(35)		103(34)	172(40)		139(27)	208(35)		100(31)	171(43)	
3	387(71)	334(58)		191(63)	219(52)		369(71)	346(59)		214(65)	193(49)	
Pleomorphism			**<0.001**			**<0.001**			**<0.001**			**0.001**
1	8(1)	20(4)	**(48.35)**	8(3)	16(4)	**(17.37)**	8(2)	20(3)	**(24.74)**	8(3)	16(4)	**(14.99)**
2	161(30)	276(48)		135(44)	250(59)		169(32)	264(45)		150(46)	231(59)	
3	374(69)	279(48)		160(53)	158(37)		344(66)	303(52)		168(51)	147(37)	
Mitosis			**<0.001**			**<0.001**			**0.001**			**0.030**
1	133(24)	253(44)	**(53.34)**	119(39)	232(55)	**(17.34)**	157(30)	229(39)	**(13.52)**	144(44.2)	206(52.3)	**(6.99)**
2	107(20)	112(19)		77(26)	87(20)		96(19)	120(20)		72(22)	89(23)	
3	304(56)	211(37)		107(35)	105(25)		268(51)	240(41)		110(34)	99(25)	
LVI			0.193			0.135			**0.001**			**0.002**
Probable/Negative	357(64)	399(67)	(0.169)	203(65)	306(70)	(3.23)	322(61)	425(70)	**(11.59)**	208(62)	295(73)	**(9.40)**
Definite	202(36)	192(33)		110(35)	131(30)		210(39)	181(30)		127(38)	111(27)	
NPI			**<0.001**			**0.016**			**<0.001**			**0.001**
GPG	121(23)	209(37)	**(28.67)**	104(35)	186(44)	**(8.32)**	122(24)	205(35)	**(27.32)**	110(35)	177(45)	**(14.74)**
MPG	314(59)	293(51)		152(51)	198(47)		286(56)	317(54)		161(50)	185(47)	
PPG	101(19)	70(12)		42(14)	38(9)		104(20)	64(11)		49(15)	30(8)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group. N: nuclear , C: cytoplasmic.

Table 3-5: The associations between pan JNK1/2 and clinicopathological variables.

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.262			0.222
≤50	202(35)	160(32)	(1.25)	124(33)	87(28)	(1.49)
>50	370(65)	339(68)		258(67)	222(72)	
Menopausal Status			0.484			0.481
Pre-	243(38)	209(36.0)	(0.49)	139(36)	105(34)	(0.49)
Post-	398(62.1)	372(64.0)		246(64)	208(66)	
Tumour Size (cm)			0.079			**0.013**
≤2.0	283(50)	274(55)	(3.09)	196(51)	188(61)	**(6.10)**
>2.0	286(50)	223(45)		185(49)	121(39)	
Stage			0.993			0.470
1	398(65)	356(65)	(0.10)	244(63)	209(67)	(1.51)
2	169(8)	150(27)		114(30)	79(26)	
3	46(7)	42(8)		26(7)	22(7)	
Tumour Type			0.728			0.860
Ductal	458(81)	405(82)	(1.30)	291(77)	238(77)	(0.75)
Lobular	63(11)	47(9)		58(15)	42(14)	
Medullary-like	12(2)	14(3)		2(1)	2(1)	
Special-type	30(6)	28(6)		27(7)	26(8)	
Grade			0.190			0.070
1	100(17)	104(19)	(3.31)	84(22)	92(30)	(5.31)
2	216(36)	168(32)		175(46)	129(1)	
3	277(47)	260(49)		124(32)	90(29)	
Tubules			0.874			0.520
1	36.0(7)	35(7)	(0.27)	31(8)	33(11)	(1.30)
2	187(33)	162(31)		146(40)	117(39)	
3	345(61)	320(62)		193(52)	151(50)	
Pleomorphism			0.263			0.083
1	10(2)	17(3)	(2.67)	9(2)	16(5)	(4.97)
2	239(42)	212(41)		199(54)	16(56)	
3	320(56)	285(56)		162(44)	116(39)	
Mitosis			0.497			0.853
1	218 (20)	18135)	(1.40)	179(48)	152(50)	(0.31)
2	24138)	111(22)		87(24)	69(23)	
3	111 (42)	225(43)		104(28)	80(27)	
LVI			0.371			0.248
Probable/Negative	376(67)	344(69)	(0.80)	261(69)	224(73)	(1.33)
Definite	187(33)	152(31)		119(31)	84(27)	
NPI			0.852			0.060
GPG	192(33)	181(35)	(0.32)	152(41)	150(50)	(5.61)
MPG	303(53)	266(52)		179(48)	125(42)	
PPG	79(14)	69(13)		40(11)	25(8)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-6: The associations between p-JNK1/2 and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.424			0.139
≤50	76(33)	297(36)	(0.64)	27(24)	168(30)	(2.19)
>50	155(67)	534(64)		88(76)	385(70)	
Menopausal Status			0.257			0.063
Pre-	81(35)	326(39)	(1.28)	29(25)	190(34)	(3.45)
Post-	149(65)	503(61)		85(75)	362(66)	
Tumour Size (cm)			**0.003**			**0.022**
≤2.0	93(40)	427(52)	**(8.93)**	50(44)	307(56)	**(5.25)**
>2.0	137(60)	401(48)		64(56)	245(44)	
Stage			0.757			0.546
1	140(62)	498(60)	(0.55)	72(65)	338(61)	(1.21)
2	66(29)	260(32)		30(27)	177(32)	
3	21(9)	69(8)		9(8)	37(7)	
Tumour Type			**0.004**			0.125
Ductal	194(85)	678(82)	**(13.13)**	90(80)	437(79)	(5.74)
Lobular	14(6)	102(12)		14(12)	93(17)	
Medullary-like	11(49)	15(2)		1(1)	1(0)	
Special-type	9(4)	28(4)		8(7)	19(4)	
Grade			**<0.001**			**0.001**
1	26(11)	131(16)	**(34.84)**	23(21)	118(22)	**(14.29)**
2	45(20)	308(37)		35(31)	267(48)	
3	156(69)	388(50)		53(48)	167(30)	
Tubules			0.280			0.298
1	11(5)	42(5)	(2.54)	11(10)	35(7)	(2.42)
2	57(26)	250(31)		34(32)	198(37)	
3	153(69)	510(64)		61(58)	303(56)	
Pleomorphism			**<0.001**			**<0.001**
1	8(4)	13(2)	**(38.55)**	8(8)	10(2)	**(21.65)**
2	45(20)	344(43)		38(36)	304(57)	
3	167(76)	443(55)		59(56)	222(41)	
Mitosis			**<0.001**			0.123
1	49(22)	299(37)	**(27.10)**	43(40)	270(50)	(4.18)
2	35(16)	161(20)		25(24)	121(23)	
3	137(62)	342(43)		38(36)	145(27)	
LVI			0.752)			0.774
Probable/Negative	153(67)	540(66)	(0.10)	75(66)	372(68)	(0.08)
Definite	76(33)	282(34)		38(34)	177(32)	
NPI			**0.002**			0.359
GPG	47(21)	255(32)	**(11.99)**	39(36)	226(43)	(2.04)
MPG	132(60)	435(55)		57(52)	254(48)	
PPG	42(19)	103(13)		13(12)	49(9)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-7: The associations between pan p38 and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.142			0.215
≤50	237(34)	175(38)	(2.08)	126(29)	108(33)	(1.53)
>50	467(66)	288(62)		308(71)	217(67)	
Menopausal Status	277(37)	207(41)	0.161			0.320
Pre-	472(63)	299(59)	(1.96)	146(34)	122(37)	(8.90)
Post-				287(66)	206(63)	
Tumour Size (cm)	323(46)	232(50)	0.170			0.563
≤2.0	376(54)	229(50)	(1.88)	222(51)	173(53)	(0.33)
>2.0				211(49)	151(47)	
Stage			0.288			0.546
1	458(63)	311(63)	(2.48)	272(63)	210(65)	(1.21)
2	203(28)	145(30)		126(29)	95(29)	
3	67(9)	33(7)		34(8)	20(6)	
Tumour Type			0.315			**0.018**
Ductal	567(82)	392(85)	(1.00)	329(76)	266(82)	**(10.01)**
Lobular	81(12)	35(8)		76(18)	33(10)	
Medullary-like	21(3)	6(1)		3(1)	1(0)	
Special-type	23(3)	26(6)		22(5)	24(8)	
Grade			**0.002**			**0.041**
1	102(14)	100(21)	**(12.43)**	84(20)	87(27)	**(6.39)**
2	230(32)	164(34)		191(44)	139(43)	
3	392(54)	219(45)		157(36)	99(30)	
Tubules			**<0.001**			**<0.011**
1	36(5)	31(7)	**(21.72)**	30(7)	28(9)	**(15.89)**
2	194(28)	185(40)		145(35)	150(47)	
3	470(67)	250(53)		246(58)	138(44)	
Pleomorphism			0.565			0.995
1	17(2)	11(2)	(1.14)	13(3)	10(3)	(0.01)
2	252(36)	182(39)		212(50)	158(50)	
3	430(62)	272(59)		196(47)	148(47)	
Mitosis			**0.002**			0.079
1	222(32)	194(42)	**(12.79)**	194(46)	172(55)	(5.08)
2	136(19)	86(18)		99(24)	61(19)	
3	342(49)	186(40)		128(30)	83(26)	
LVI			0.781			0.315
Probable/Negative	459(66)	305(67)	(0.07)	289(67)	226(70)	(1.00)
Definite	237(34)	152(33)		144(33)	96(30)	
NPI			**0.011**			0.179
GPG	208(30)	172(38)	**(9.08)**	168(41)	146(47)	(3.44)
MPG	377(54)	229(50)		199(48)	135(44)	
PPG	114(16)	56(12)		47(11)	28(9)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-8: The associations between p-p38 and clinicopathological variables.

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.779			**0.017**
≤50	319(34)	136(35)	(0.79)	157(27)	97(34)	**(5.66)**
>50	627(66)	258(65)		436(73)	186(66)	
Menopausal Status			0.925			0.088
Pre-	359(38)	150(38)	(0.09)	186(31)	105(37)	(2.90)
Post-	586(62)	242(62)		406(69)	177(63)	
Tumour Size (cm)			**<0.001**			**0.007**
≤2.0	423(45)	225(58)	**(17.39)**	295(50)	168(60)	**(7.32)**
>2.0	517(55)	166(42)		295(50)	113(40)	
Stage			**0.008**			**0.009**
1	550(59)	261(67)	**(9.54)**	344(59)	192(68)	**(9.31)**
2	310(33)	96(24)		202(34)	68(24)	
3	78(8)	34(9)		42(7)	21(8)	
Tumour Type			**<0.001**			**0.001**
Ductal	813(87.3)	298(76)	**(39.44)**	496(85)	205(73)	**(17.05)**
Lobular	68(7.3)	62(16)		66(11)	54(19)	
Medullary-like	23(2.5)	3(1)		2(0)	0(0)	
Special-type	27(2.9)	27(7)		24(4)	21(8)	
Grade			**<0.001**			**<0.001**
1	120(13)	82(21)	**(43.48)**	108(18)	75(27)	**(24.50)**
2	293(31)	166(43)		253(43)	143(51)	
3	525(56)	142(36)		227(39)	62(22)	
Tubules			0.104			0.652
1	39(4)	25(7)	(4.52)	37(6)	21(8)	(0.85)
2	303(33)	131(35)		239(42)	104(39)	
3	572(63)	215(58)		298(52)	140(53)	
Pleomorphism			**<0.001**			**<0.001**
1	12(1)	15(4)	**(37.77)**	11(2)	13(5)	**(19.86)**
2	309(34)	179(49)		275(48)	159(60)	
3	593(65)	175(47)		288(50)	93(35)	
Mitosis			**<0.001**			**<0.001**
1	263(29)	172(46)	**(48.86)**	235(41)	157(59)	**(27.54)**
2	171(19)	80(22)		140(24)	56(21)	
3	480(52)	119(32)		199(35)	52(20)	
LVI			**0.002**			**0.012**
Probable/Negative	602(65)	284(73)	**(9.47)**	382(65)	206(74)	**(6.26)**
Definite	332(35)	104(27)		205(35)	74(26)	
NPI			**<0.001**			**<0.001**
GPG	235(26)	159(43)	**(35.98)**	205(36)	139(52)	**(20.79)**
MPG	508(57)	177(47)		285(51)	112(42)	
PPG	156(17)	38(10)		73(13)	17(6)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-9: The associations between p-ATF2 and clinicopathological variables.

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.959			0.318
≤50	331(34)	116(34)	(0.03)	170(29)	82(32)	(0.99)
>50	632(66)	223(66)		424(71)	174(68)	
Menopausal Status			0.665			0.188
Pre-	364(38)	132(39)	(0.18)	191(32)	94(37)	(1.73)
Post-	598(62)	205(61)		402(68)	161(63)	
Tumour Size (cm)			0.150			0.188
≤2.0	451(47)	173(52)	(2.07)	306(52)	139(55)	(1.73)
>2.0	507(53)	162(48)		285(48)	115(45)	
Stage			109			0.431
1	571(60)	208(62)	(4.24)	360(61)	157(62)	(0.61)
2	311(32)	92(28)		193(33)	74(29)	
3	74(8)	35(10)		36(6)	23(9)	
Tumour Type			**<0.001**			0.292
Ductal	822(87)	261(79)	**(24.62)**	490(83)	194(77)	(2.95)
Lobular	76(8)	49(15)		72(12)	44(17)	
Medullary-like	22(2)	1(0)		2(1)	0(0)	
Special-type	29(3)	20(6)		24(4)	16(6)	
Grade			**<0.001**			**<0.001**
1	114(12)	81(24)	**(56.19)**	104(18)	70(28)	**(20.68)**
2	303(32)	139(42)		256(43)	122(48)	
3	539(56)	114(34)		229(39)	61(24)	
Tubules			**0.001**			0.338
1	36(4)	26(8)	**(13.77)**	33(6)	21(8)	(2.16)
2	298(32)	122(37)		233(41)	100(41)	
3	592(64)	177(55)		304(53)	125(51)	
Pleomorphism			**<0.001**			**0.002**
1	11(1)	14(4)	**(39.41)**	10(2)	11(4)	**(12.00)**
2	313(34)	159(49)		276(48)	140(57)	
3	601(65)	152(47)		284(50)	95(39)	
Mitosis			**<0.001**			**<0.001**
1	265(29)	155(48)	**(57.43)**	236(41)	140(57)	**(21.92)**
2	168(18)	74(23)		131(23)	56(23)	
3	493(53)	96(29)		203(36)	50(20)	
LVI			**<0.032**			0.090
Probable/Negative	611(64)	234(71)	**(4.62)**	382(65)	179(71)	(2.86)
Definite	344(36)	98(29)		208(35)	74(29)	
NPI			**<0.001**			**0.019**
GPG	240(26)	132(41)	**(62.43)**	209(37)	114(47)	**(7.88)**
MPG	530(58)	143(45)		292(52)	102(43)	
PPG	149(16.)	44(14)		65(11)	24(10)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 3-10: The associations between p-C-JUN and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.128			0.076
≤50	145(32)	302(36)	(2.32)	70(26)	178(32)	(3.14)
>50	309(68)	533(64)		203(74)	385(68)	
Menopausal Status			**0.013**			**0.005**
Pre-	154(34)	341(1)	**(5.19)**	74(27)	207(37)	**(7.88)**
Post-	300(66)	491(59)		199(73)	354(63)	
Tumour Size (cm)			0.08			0.400
≤2.0	206(46)	421(51)	(3.04)	140(51)	304(54)	(0.70)
>2.0	245(54)	408(49)		133(49)	255(46)	
Stage			0.453			0.676
1	266(59)	517(62)	(1.58)	172(64)	347(62)	(0.78)
2	143(32)	251(30)		84(31)	172(31)	
3	40(9)	61(8)		15(6)	40(7)	
Tumour Type			0.077			0.593
Ductal	383(86)	691(84)	(6.83)	226(84)	451(81)	(1.90)
Lobular	34(8)	84(10)		32(12)	76(14)	
Medullary-like	14(3)	13(2)		0(0)	2(0)	
Special-type	14(3)	37(4)		12(4)	30(5)	
Grade			**<0.001**			**<0.001**
1	62(14)	135(16)	**(23.07)**	55(20)	122(22)	**(25.11)**
2	119(26)	313(38)		93(34)	277(50)	
3	268(60)	380(46)		123(46)	159(28)	
Tubules			**0.020**			**0.063**
1	14(3)	46(5)	**(7.81)**	14(5)	40(7)	**(5.53)**
2	132(31)	288(36)		95(37)	236(44)	
3	283(66)	474(59)		150(58)	267(49)	
Pleomorphism			0.061			0.285
1	6(1)	22(2)	(8.22)	6(2)	18(3)	(2.51)
2	141(33)	319(40)		124(48)	285(53)	
3	281(66)	467(58)		129(50)	240(44)	
Mitosis			**0.001**			**0.002**
1	118(27)	293(36)	**(17.30)**	103(40)	266(49)	**(12.96)**
2	75(18)	170(21)		55(21)	133(25)	
3	236(55)	345(43)		101(39)	144(26)	
LVI			**0.015**			**0.035**
Probable/Negative	278(62)	566(69)	**(5.96)**	169(62)	387(70)	**(4.46)**
Definite	171(38)	258(31)		103(38)	170(30)	
NPI			**0.001**			0.138
GPG	114(26)	264(33)	**(13.01)**	96(36)	233(44)	(3.96)
MPG	238(55)	426(54)		136(52)	247(46)	
PPG	84(19)	100(13)		31(12)	52(10)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

3.3.2.4 The associations between MAPKs and biological markers within the unselected primary breast cancer series and different breast cancer subgroups

3.3.2.4.1 Pan ERK1/2 and p-ERK1/2

Conducting the analysis within the whole series revealed that high pan ERK1/2 and nuclear p-ERK1/2 expressions were positively associated with HRs: ER, PgR and AR (all $p<0.001$) and also a highly significant association was observed between their high expressions and positivity of luminal cytokeratins: CK7/8, CK18 and CK19 (Table 3-11and Table 3-12). In addition, high expression of pan ERK1/2 and nuclear p-ERK1/2 were positively associated with other ER related proteins: FOXA1, BEX1 and GATA3. In addition, pan ERK1/2 and nuclear p-ERK1/2 were also positively associated with E-cadherin and BCL2 ($p<0.001$), (Table 3-11and Table 3-12). Within ER+HER2- cohort, the positive associations between pan ERK1/2 high expression and positivity of luminal CKs, E-cadherin and BCL2, were all maintained in addition to an association with higher expression of PELP1 and HER4 proteins ($p=0.02$, $p=0.046$, both borderline, respectively, Table 3-11).

Moreover, there was an association with high expression of AR ($p=0.001$) with high pan ERK1/2 expression when the analysis was restricted to ER+HER2+ tumours but there was only an association for high expression of BEX1 but a trend for high KI67-LI within ER-HER2+ cohort ($p=0.002$, $p=0.056$, respectively) and no associations were revealed in ER-HER2- BC (appendix table 6).

In addition to the associations in common with pan ERK1/2, high expression of nuclear p-ERK1/2 was positively associated with TFF3 positivity ($p=0.047$: borderline) but was negatively associated with CD71 positivity ($p=0.022$) and HER4 positivity ($p=0.005$: borderline). Meanwhile, the high expression of the cytoplasmic form was positively associated with TFF1 and CD71 positivity (E-Cadherin: $p=0.020$: borderline, P-Cadherin: $p=0.007$) respectively, in addition to its positive associations with HRs, luminal CKs, FOXA1, BEX1, GATA3 and E-Cadherin similar to those as with pan ERK1/2 and nuclear p-ERK1/2 (Table 3-12 and Table 3-13).

The high expression of the nuclear form was associated with low KI67-LI and HER2 ($p<0.001$) and the association for KI67-LI was maintained within

ER+HER2- cohort. The cytoplasmic form high expression was associated with high E-cadherin and P-cadherin but low KI67-LI (p=0.018: borderline, p=0.003) respectively, and interestingly the same associations were found while restricting the analysis within ER+HER2- tumours, in addition to its positive associations with AR, FOXA1, BEX1 and E-Cadherin (Table 3-12, Table 3-13).

Within ER+HER2+ tumours, only cytoplasmic p-ERK1/2 high expression was associated with negativity of EGFR (p=0.048: borderline) and it was associated with increased expression of BEX1 and CD71 (p=0.009 and p=0.040: borderline) but the nuclear form high expression was only associated with high FOXA1 (p=0.031: borderline). No associations were found within ER-HER2+ while Within ER-HER2-, the cytoplasmic form was associated with increased expression of p-Cadherin (p=0.043: borderline, appendix table 6).

3.3.2.4.2 Pan JNK1/2 and p-JNK1/2

High Pan JNK1/2 expression was associated with ER, PgR and BCL2 negative tumours (p=0.015, p=0.017, both borderline, p=0.004); however, it was associated with high BEX1 (trend) and CD71 (p=0.024, p<0.001 respectively) and the latter associations were maintained within ER+HER2- cohort (Table 3-14). In addition, within ER+HER2+ cohort, high pan JNK1/2 expression was associated with AR (p=0.035: borderline) positive tumours and was associated with p53 positivity within ER-HER2+ tumours (p=0.002, Table 3-14).

The high expression of p-JNK1/2, was highly significantly associated with positive expression of HRs (p<0.001), luminal CKs: CK7/8, CK18 and CK19 (p<0.001, p=0.001, p=0.040: borderline) and ER related proteins: FOXA1, BEX1, TFF1, TFF3 and GATA3 (p=0.001, p<0.001, p=0.025: borderline, p=0.003 and p<0.001) respectively (Table 3-15). In addition, it was positively associated with E-cadherin and BCL2 (p=0.008, p=0.006) but negatively with KI67-LI (p=0.002); however, only the association with BEX1 was maintained in ER+HER2- tumours (Table 3-15). When the analysis was restricted to ER+HER2+, it was associated with increased expression of BEX1 (p=0.009). No other associations were noticed within ER-HER2- subgroup (appendix table 6)

3.3.2.4.3 Pan p38 and p-p38

Similar to p-JNK1/2 protein, pan p38 with its high expression was positively associated with the same proteins as those with p-JNK1/2. These include, HRs

(p<0.001), luminal CKs: CK7/8, CK18 and Ck19 (p=0.006, p=0.009, p<0.001) respectively, ER related proteins and others: FOXA1 (p=0.005), TFF3 (p=0.003), BEX1, GATA3, E-cadherin, BCL2 (all p<0.001) but low P-cadherin (p=0.002). Many of these associations (including AR, FOXA1, BEX1 and E-Cadherin) were still observed within ER+HER2- tumours in addition to an association with CARM1 (p=0.021: borderline, Table 3-16). In addition, high pan p38 expression was associated with a trend of PgR positivity and high KI67-LI in the ER+ HER2+ cohort (p=0.018, p=0.061, respectively) and with increased FOXA1 and BEX1 expressions in ER-HER2+ tumours (p=0.005 and p=0.021: borderline, respectively, appendix table 6).

The high expression of p-p38 was positively associated with positivity of HRs: ER (p=0.002), PgR and AR (p<0.001), CK18 (p=0.008), CK19 (p=0.017: borderline), TFF1 (p=0.016: borderline), TFF3 (p=0.001), FOXA1, BEX1, GATA3 (all p<0.001) and BCL2 (p=0.040: borderline) but was negatively associated with KI67-LI positivity (p<0.001) (Table 3-17). Interestingly, PgR and FOXA1 positivity was associated with high expression of p-p38 within ER+HER2+ tumours (p=0.044, p=0.013, both borderline) but was negatively associated with a trend of BCL2 expression (p=0.028) in ER-HER2+. No associations were revealed within ER-HER- tumours (appendix table 6).

3.3.2.4.4 P-ATF2

Similar to other phosphorylated MAPKs, the high expression of the transcription factor p-ATF2 was positively associated with HRs (p<0.001), luminal CKs18 and 19 (p=0.005, p=0.043: borderline), ER related proteins: BEX1 (p=0.006), FOXA1, GATA3 (p<0.001) and BCL2 (p=0.010: borderline) significantly; however, it was negatively associated with negative CD71 (p=0.007), p53 (p=0.027: borderline), KI67-LI (p<0.001), HER2 and HER4 proteins (p=0.001, p=0.005) and association with HRs, ER related proteins (CK18, FOXA1, BEX1 and GATA3), KI67-LI and HER4 were all observed within ER+HER2- tumours (Table 3-18). Of worth, within ER+HER2+ cohort, high expression of this protein was positively associated with AR and PELP1 (p=0.035, p=0.039, both are trends). Moreover, p-p38 was negatively association with high CD71 expression (p=0.010) but was positively association with PELP1 (p=0.012: borderline) within ER-HER2+ tumours and similarly, p-ATF2 was positively associated with FOXA1 (p=0.024: borderline) in ER-HER2- BC (appendix table 6).

3.3.2.4.5 P-C-JUN

For p-C-JUN high expression within the whole series, similar associations to other MAPKs were found since it was positively associated with HRs: ER (p=0.004), PgR and AR (p<0.001), luminal CKs: CK7/8, CK18 and 19 (p=0.032: borderline, p=0.005, p<0.001), ER related proteins including FOXA1 (p=0.001), BEX1, GATA3 and CARM1 (p<0.001). Moreover, high expression was also associated with E-cadherin but associated with low KI67-LI (p=0.043: borderline, p<0.001) respectively. The associations regarding AR, CK19, FOXA1, BEX1, GATA3, CARM1 and KI67-LI, were all remained significant while restricting the analysis within ER+HER2- tumours (Table 3-19). No significant associations were observed within ER+HER2+ tumours however, high expression of p-C-JUN was negatively associated with AR but positively with P-cadherin (p=0.049, p=0.039, both are trends, respectively) within ER-HER2+ BC and an association with decreased CARM1 expression (p=0.008) was observed within ER-HER2- BC (appendix table 6).

In addition to all above, it is important to highlight that within ER+ tumours, nuclear p-ERK1/2, p-p38 and p-ATF2 were all negatively associated with HER2 expression (p=0.005, p=0.001, p=0.010: borderline) respectively. In contrast, p-p38 and ERK1/2 were positively associated with HER2 within ER- BC (p=0.003, p=0.005) respectively. In addition, no associations were noticed regarding MAPKs with HER2 dimers.

Table 3-11: The associations between pan ERK 1/2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	176(28)	112(20)	**(12.59)**			
Positive	444(72)	460(80)				
PgR			**<0.001**			0.071
Negative	264(45)	192(35)	**(12.80)**	82(22)	68(17)	(3.25)
Positive	325(55)	365(65)		290(78)	334(83)	
AR			**<0.001**			0.236
Negative	229(41)	151(30)	**(15.54)**	86(25)	77(21)	(1.40)
Positive	324(59)	356(70)		265(75)	293(79)	
CK7/8			**0.006**			0.298
Negative	17(3)	4(1)	**(7.45)**	1(0)	0(0)	(1.08)
Positive	571(97)	551(99)		371(100)	402(100)	
CK18		46(9)	**<0.001**			**0.005**
Negative	101(19)	456(91)	**(18.89)**	22(6)	8(2)	**(7.76)**
Positive	445(81)			323(94)	359(98)	
CK19			**<0.001**			**0.014**
Negative	73(13)	29(5)	**(18.6)**	25(7)	12(3)	**(5.98)**
Positive	506(87)	516(95)		340(93)	384(97)	
FOXA1			**0.004**			0.138
Negative	223(60)	186(49)	**(8.50)**	111(50)	115(43)	(2.19)
Positive	149(40)	191(51)		112(50)	152(57)	
BEX1			**0.002**			0.121
Negative	145(40)	100(29)	**(9.53)**	80(35)	74(29)	(2.39)
Positive	221(60)	249(71)		147(65)	184(71)	
TFF1			0.240			0.506
Negative	171(53)	156(48)	(1.38)	94(49)	107(46)	(0.44)
Positive	155(47)	170(52)		98(51)	127(54)	
TFF3			0.488			0.341
Negative	162(49)	154(46)	(0.48)	75(38)	104(42)	(0.90)
Positive	171(51)	181(54)		125(62)	144(58)	
GATA3			**0.009**			0.148
Negative	222(66)	184(56)	**(6.92)**	103(52)	107(45)	(2.09)
Positive	115(34)	145(44)		97(48)	133(55)	
CD71			0.976			0.383
Negative	171(45)	176(45)	(0.001)	128(55)	143(51)	(0.76)
Positive	208(55)	215(55)		105(45)	137(49)	
CARM1			0.231			0.199
Negative	99(26)	87(24)	(2.93)	78(34)	74(28)	93.22)
Moderate	186(49)	204(55)		109(48)	148(56)	
High	93(25)	78(21)		41(18)	44(16)	
PELP1			0.294			**0.020**
Negative	74(19)	58(15)	(2.45)	60(24)	42(15)	**97.82)**
Moderate	248(64)	260(67)		161(66)	201(72)	
High	66(17)	71(18)		24(10)	36(13)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**<0.001**			**0.001**
Negative	235(41)	164(30)	**(14.16)**	147(41)	117(30)	**(10.16)**
Positive	342(59)	382(70)		216(59)	280(70)	
p-Cadherin			0.088			**0.004**
Negative	231(47)	236(52)	(2.90)	199(64)	207(63)	**(8.50)**
Positive	264(53)	216(48)		113(36)	119(37)	
p53			0.064			0.093
Negative/low	400(68)	405(73)	(3.42)	295(80)	340(84)	(2.82)
Positive	185(32)	147(27)		75(20)	63(16)	
KI67-LI			0.050			0.436
Negative/low	200(39)	208(46)	(3.85)	166(53)	184(56)	(0.60)
High	307(61)	247(54)		148(47)	145(44)	
BCL2			**<0.001**			**0.035**
Negative/low	199(47)	134(32)	**(19.25)**	74(28)	64(21)	**(4.43)**
High	225(53)	283(68)		190(72)	248(80)	
HER1			0.959			0.068
Negative	465(79)	435(79)	(0.003)	331(90)	337(85)	(3.34)
Positive	126(21)	117(21)		38(10)	58(15)	
HER2			0.985	-	-	-
Negative	516(87)	480(87)	(<0.001)			
Positive	75(13)	70(13)				
HER3			0.296	39(12)	31(8)	0.145
Negative	55(10)	43(8)	(1.09)	297(88)	341(92)	(2.12)
Positive	481(90)	470(92)				
HER4			**0.013**			**0.046**
Negative	97(17)	63(12)	**(6.20)**	71(19)	55(14)	**(3.99)**
Positive	485(83)	485(88)		296(81)	339(86)	

Table 3-12: The associations between nuclear p-ERK 1/2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	186(33)	102(17)	**(38.46)**			
Positive	373(67)	487(83)				
PgR			**<0.001**			0.304
Negative	262(48)	183(32)	**(28.77)**	64(21)	76(18)	(1.05)
Positive	283(52)	385(68)		247(79)	356(82)	
AR			**<0.001**			**0.001**
Negative	235(50)	140(27)	**(42.98)**	86(30)	74(19)	**(11.91)**
Positive	267(53)	377(73)		199(70)	320(81)	
CK7/8			**0.022**	-	-	**-**
Negative	13(2)	4(1)	**(5.27)**			
Positive	531(98)	565(99)				
CK18			**<0.001**			0.198
Negative	94(19)	55(11)	**(12.14)**	19(7)	17(4)	(1.66)
Positive	409(81)	454(89)		265(93)	368(96)	
CK19			0.053			0.498
Negative	60(11)	43(8)	(3.45)	18(6)	20(5)	(0.45)
Positive	477(89)	512(92)		288(94)	401(95)	
FOXA1			**<0.001**			**<0.001**
Negative	238(66)	157(41)	**(48.84)**	116(58)	97(35)	**(26.34)**
Positive	122(34)	231(59)		83(42)	183(65)	
BEX1			**<0.001**			**0.001**
Negative	145(42)	88(24)	**(28.45)**	85(44)	52(19)	**(33.74)**
Positive	197(58)	286(76)		108(56)	220(81)	
TFF1			0.065			0.065
Negative	221(69)	205(62)	(3.39)	95(52)	100(43)	(3.41)
Positive	99(31)	124(38)		89(48)	135(57)	
TFF3			**0.047**			0.328
Negative	166(51)	152(43)	**(3.95)**	77(43)	97(38)	(0.95)
Positive	159(49)	198(57)		104(57)	159(62)	
GATA3			**<0.001**			**<0.001**
Negative	251(75)	169(51)	**(39.29)**	114(62)	101(42)	**(16.27)**
Positive	84(25)	160(49)		71(38)	140(58)	
CD71			**<0.022**			0.750
Negative	150(39)	183(47)	**(5.26)**	113(51)	149(52)	(0.10)
Positive	239(61)	209(53)		110(49)	137(48)	
CARM1			0.061			**0.003**
Negative	106(29)	84(22)	(5.59)	77(38)	71(25)	**(11.40)**
Moderate	181(50)	202(53)		97(48)	149(53)	
High	74(21)	94(25)		27(14)	61(22)	
PELP1			0.053			**0.002**
Negative	60(16)	66(17)	(5.87)	44(19)	53(18)	**(12.90)**
Moderate	283(74)	263(68)		171(76)	194(67)	
High	38(10)	60(15)		11(5)	42(15)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**<0.034**			**0.024**
Negative	216(41)	192(35)	**(4.49)**	125(41)	140(33)	**(5.12)**
Positive	312(59)	362(65)		177(59)	282(67)	
p-Cadherin			0.723			**0.003**
Negative	209(47)	220(48)	(0.12)	174(69)	197(57)	**(8.93)**
Positive	239(53)	240(52)		79(31)	150(43)	
p53			0.036			0.685
Negative/low	361(68)	416(74)	(4.41)	250(82)	347(80)	(0.16)
Positive	172(32)	149(26)		56(18)	84(20)	
KI67-LI			**<0.001**			**0.006**
Negative/low	153(34)	224(47)	**(16.61)**	125(48)	209(60)	**(7.56)**
High	304(66)	250(53)		137(52)	146(41)	
BCL2			**0.001**			**0.009**
Negative/low	113(37)	77(24)	**(21.37)**	79(32)	72(23)	**(6.77)**
High	196(63)	239(76)		165(68)	247(77)	
HER1			<0.246			0.236
Negative	420(78)	461(81)	(1.34)	270(89)	367(86)	(1.40)
Positive	117(22)	108(19)		33(11)	59(14)	
HER2			**<0.001**	-	-	-
Negative	443(83)	516(90)	**(11.90)**			
Positive	93(17)	58(10)				
HER3			<0.627			0.981
Negative	42(8)	48(9)	(0.23)	28(10)	38(10)	(0.001)
Positive	457(92)	469(91)		257(90)	351(90)	
HER4			**0.005**			**0.015**
Negative	60(11)	97(17)	**(7.81)**	40(13)	85(20)	**(5.93)**
Positive	473(89)	467(83)		265(87)	340(80)	

Table 3-13: The associations between cytoplasmic p-ERK 1/2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			0.074	-	-	**-**
Negative	146(28)	139(23)	(3.18)			
Positive	385(72)	468(77)				
PgR			**0.037**			0.153
Negative	225(44)	216(37)	**(4.35)**	71(21)	68(17)	(2.04)
Positive	294(56)	365(63)		264(79)	331(83)	
AR			**0.001**			**0.012**
Negative	203(42)	166(32)	**(11.74)**	86(28)	71(20)	**(6.38)**
Positive	281(58)	359(68)		223(72)	292(80)	
CK7/8			0.321	-	-	**-**
Negative	10(2)	7(1)	(0.87)			
Positive	507(98)	577(99)				
CK18			**0.002**			0.061
Negative	88(18)	59(11)	**(9.61)**	22(7)	14(4)	(3.50)
Positive	391(82)	463(89)		282(93)	343(96)	
CK19			**0.017**			0.123
Negative	60(12)	42(7)	**(5.74)**	22(7)	16(4)	(2.38)
Positive	450(88)	529(93)		307(93)	374(96)	
FOXA1			**<0.001**			**0.017**
Negative	198(61)	194(47)	**(13.70)**	105(51)	107(40)	**(5.65)**
Positive	127(39)	218(53)		100(49)	159(60)	
BEX1			**<0.001**			**<0.001**
Negative	135(42)	96(25)	**(23.03)**	81(39)	55(22)	**(16.83)**
Positive	186(58)	290(75)		125(61)	198(78)	
TFF1			**0.020**			0.099
Negative	155(54)	158(45)	**(5.36)**	94(51)	97(43)	(2.71)
Positive	130(46)	192(55)		90(49)	129(57)	
TFF3			0.122			0.734
Negative	150(50)	161(44)	(2.39)	77(40)	92(39)	(0.11)
Positive	150(50)	205(56)		115(60)	147(61)	
GATA3			**0.008**			0.150
Negative	21(69)	203(58)	**(7.03)**	104(54)	110(47)	(2.07)
Positive	98(31)	144(42)		87(46)	122(53)	
CD71			**0.007**			0.403
Negative	159(45)	170(40)	**(1.80)**	120(54)	138(50)	(0.69)
Positive	193(55)	251(60)		104(46)	139(50)	
CARM1			0.199			0.090
Negative	96(29)	92(23)	93.22)	75(36)	72(27)	(4.80)
Moderate	167(50)	209(53)		101(48)	139(52)	
High	71(21)	97(24)		33(16)	55(21)	
PELP1			0.370			**0.020**
Negative	59(17)	64(16)	(1.98)	49(21)	46(16)	**(7.85)**
Moderate	255(72)	285(70)		168(72)	194(70)	
High	39(11)	59(14)		15(7)	38(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.018**			**0.017**
Negative	211(42)	195(35)	**(5.62)**	136(42)	129(33)	**(5.68)**
Positive	296(58)	369(65)		190(58)	261(67)	
p-Cadherin			**0.018**			**0.019**
Negative	222(51)	203(43)	**(5.62)**	185(67)	182(57)	**(0.55)**
Positive	211(49)	265(57)		92(33)	135(43)	
p53			0.172			0.933
Negative/low	351(69)	416(72)	(1.86)	267(81)	323(81)	(0.0)
Positive	160(31)	158(28)		63(19)	751(9)	
KI67-LI						**0.003**
Negative/low	159(37)	233(47)	**0.003**	131(48)	202(60)	**(8.69)**
High	266(63)	263(53)	**(8.56)**	142(52)	135(40)	
BCL2			0.141			0.102
Negative/low	174(46)	183(41)	(2.16)	76(30)	73(24)	(2.67)
High	208(54)	269(59)		175(70)	230(76)	
HER1			0.544			0.3056
Negative	408(80)	461(79)	(0.36)	292(90)	336(85)	(3.66)
Positive	101(20)	125(21)		33(10)	59(15)	
HER2			0.936	-	-	-
Negative	442(86)	506(86)	(0.00)			
Positive	71(14)	79(14)				
HER3			0.416			0.946
Negative	38(8)	50(9)	(0.66)	29(10)	35(10)	(0.0)
Positive	438(92)	480(91)		275(90)	326(90)	
HER4			0.358			0.557
Negative	67(13)	88(15)	(0.84)	53(16)	70(18)	(0.34)
Positive	439(87)	491(85)		275(84)	323(82)	

Table 3-14: The associations between pan JNK1/2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**0.015**	-	-	**-**
Negative	130(22)	149(28)	**(5.88)**			
Positive	466(78)	382(72)				
PgR			**0.017**			578
Negative	200(37)	215(44)	**(5.66)**	72(19)	64(21)	(0.31)
Positive	339(63)	269(56)		303(81)	242(79)	
AR			0.895			578
Negative	183(37)	165(38)	(0.01)	81(24)	62(22)	(0.31)
Positive	306(63)	271(62)		262(76)	218(78)	
CK7/8			0.500	-	-	-
Negative	8(2)	5(1)	(0.45)			
Positive	527(98)	484(99)				
CK18			0.705			594
Negative	68(14)	58(13)	(0.14)	15(5)	15(5)	(0.28)
Positive	412(86)	378(89)		321(95)	263(95)	
CK19			0.699			946
Negative	56(11)	48(10)	(0.14)	21(6)	17(6)	(0.0)
Positive	463(89)	430(90)		343(94)	284(94)	
FOXA1			0.875			634
Negative	182(53)	176(54)	(0.02)	110(46)	85(43)	(0.28)
Positive	160(47)	151(46)		131(54)	111(57)	
BEX1			**0.024**			**0.008**
Negative	118(36)	89(28)	**(5.07)**	82(36)	48(24)	**(7.05)**
Positive	212(64)	234(72)		148(64)	153(76)	
TFF1			0.907			0.479
Negative	136(46)	136(47)	(0.01)	94(45)	72(41)	(0.50)
Positive	157(54)	154(53)		115(55)	102(59)	
TFF3			0.381			0.274
Negative	141(47)	125(43)	(0.76)	84(40)	62(34)	(1.19)
Positive	161(53)	165(57)		127(60)	118(66)	
GATA3			0.324			0.827
Negative	176(62)	184(66)	(0.97)	94(50)	91(51)	(0.04)
Positive	107(38)	94(34)		93(50)	86(49)	
CD71			**<0.001**			**0.002**
Negative	178(49)	116(35)	**(14.57)**	148(59)	88(44)	**(10.01)**
Positive	187(51)	220(65)		104(41)	113(56)	
CARM1			0.728			0966
Negative	92(26)	74(24)	(0.63)	75(32)	56(31)	(0.06)
Moderate	186(53)	157(52)		120(51)	95(52)	
High	73(21)	70(23)		41(17)	32(17)	
PELP1			0.492			0.339
Negative	68(19)	51(16)	(1.42)	58(23)	36(18)	(2.16)
Moderate	242(68)	232(71)		166(66)	146(71)	
High	48(13)	43(13)		29(11)	22(11)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			0.493			0.776
Negative	201(39)	174(37)	(0.46)	130(36)	111(37)	(0.08)
Positive	320(61)	303(63)		233(64)	190(63)	
p-Cadherin			0.391			0.700
Negative	203(47)	168(44)	(0.73)	177(59.6%)	142(58.0%)	(0.14)
Positive	228(53)	213(56)		120(40.4%)	103(42.0%)	
p53			0.099			0.711
Negative/low	394(74)	331(69)	(2.72)	308(82)	247(81)	(0.13)
Positive	141(26)	149(31)		66(18)	57(19)	
KI67-LI			0.807			0.578
Negative/low	199(44)	172(45)	(0.06)	168(55)	141(57)	(0.31)
High	255(56)	213(55)		139(45)	106(43)	
BCL2			**0.004**			0.361
Negative/low	150(38)	183(48)	**(8.36)**	68(24)	64(28)	(0.83)
High	246(62)	197(52)		216(76)	169(72)	
HER1			0.429			0.612
Negative	411(77)	378(79)	(0.62)	310(84)	258(85)	(0.25)
Positive	125(23)	102(21)		59(16)	44(15)	
HER2			0.092	-	-	-
Negative	478(88)	406(85)	(2.84)			
Positive	63(12)	73(15)				
HER3			0.620			0.980
Negative	50(10)	49(11)	(0.24)	42(12)	35(123)	(0.0)
Positive	440(90)	388(89)		297(88)	246(87)	
HER4			0.401			0.798
Negative	84(16)	65(14)	(0.70)	70(19)	54(18)	(0.06)
Positive	447(84)	402(86)		298(81)	242(82)	

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Table 3-15: The associations between p-JNK1/2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	94(41)	189(23)	**(28.31)**			
Positive	136(59)	633(77)				
PgR			**<0.001**			0.172
Negative	125(56)	301(38)	**(21.60)**	27(24)	98(18)	(1.86)
Positive	100(44)	491(62)		88(76)	447(82)	
AR			**<0.001**			<0.001
Negative	127(61)	230(32)	**(58.00)**	43(41)	103(21)	(19.95)
Positive	82(39)	491(68)		62(59)	400(79)	
CK7/8			**<0.001**	-	-	**-**
Negative	8(4)	5(1)	**(12.18)**			
Positive	213(96)	787(99)				
CK18			**0.001**			0.229
Negative	45(22)	91(13)	**(10.96)**	7(7)	20(4)	(1.744)
Positive	160(78)	624(87)		97(93)	475(96)	
CK19			**0.040**			0.936
Negative	29(13)	67(9)	**(4.22)**	5(5)	25(5)	(0.0)
Positive	190(87)	708(91)		106(95)	509(95)	
FOXA1			**0.001**			0.085
Negative	97(66)	273(51)	**(10.99)**	38(54)	153(43)	(2.95)
Positive	49(34)	265(49)		32(46)	202(57)	
BEX1			**<0.001**			**0.001**
Negative	66(47)	147(28)	**(16.56)**	29(43)	97(28)	**(6.62)**
Positive	75(53)	372(72)		38(57)	255(72)	
TFF1			**0.025**			0.512
Negative	93(68)	294(63)	**(5.04)**	33(49)	136(44)	(0.43)
Positive	44(32)	169(37)		35(51)	172(56)	
TFF3			**0.003**			0.503
Negative	82(61)	224(46)	**(8.95)**	28(44)	128(39)	(0.44)
Positive	53(39)	261(54)		36(56)	198(61)	
GATA3			**<0.001**			0.036
Negative	110(77)	282(60)	**(13.70)**	46(63)	154(49)	(4.41)
Positive	32(23)	186(40)		27(37)	158(51)	
CD71			0.503			0.967
Negative	64(39)	229(42)	(0.44)	44(52)	187(51)	(0.0)
Positive	102(61)	320(58)		41(48)	176(49)	
CARM1			0.160			0.032
Negative	47(31)	122(24)	(3.65)	31(41)	97(28)	(6.90)
Moderate	73(48)	268(51)		35(47)	177(50)	
High	33(21)	132(25)		9(12)	76(22)	
PELP1			0.708			0.217
Negative	21(14)	85(16)	(0.69)	16(19)	64(17)	(3.05)
Moderate	114(73)	365(67)		62(76)	268(72)	
High	20(13)	92(17)		4(5)	42(11)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.008**			0.681
Negative	101(46)	282(37)	**(7.08)**	43(39)	198(37)	(0.16)
Positive	116(54)	489(63)		67(61)	337(63)	
p-Cadherin			0.195			**0.545**
Negative	73(41)	295(46)	(1.86)	57(63)	263(60)	**(0.36)**
Positive	107(59)	343(54)		33(37)	176(40)	
p53			0.332			0.269
Negative/low	149(68)	563(72)	(0.94)	95(85)	437(80)	(1.22)
Positive	70(32)	222(28)		17(15)	107(20)	
KI67-LI			**0.002**			0.101
Negative/low	58(31)	292(44)	**(9.89)**	44(47)	253(57)	(2.68)
High	127(69)	368(56)		49(53)	194(43)	
BCL2			**0.006**			0.348
Negative/low	90(52)	247(41)	**(7.57)**	27(29)	102(25)	(0.87)
High	82(48)	368(59)		65(71)	312(75)	
HER1			0.052			0.471
Negative	159(74)	634(80)	(3.78)	97(89)	465(86)	(0.52)
Positive	57(26)	161(20)		12(11)	73(11)	
HER2			0.198	-	-	-
Negative	184(83)	690(87)	(1.65)			
Positive	37(17)	103(13)				
HER3			0.608			0.600
Negative	19(9)	74(10)	(0.26)	11(10)	60(12)	(0.27)
Positive	190(91)	641(90)		95(90)	432(88)	
HER4			0.361			
Negative	26(12)	113(5)	(0.83)	15(14)	96(18)	0.260
Positive	191(88)	668(85)		96(86)	439(82)	(1.26)

Table 3-16: The associations between pan p38 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	221(31)	89(19)	**(23.03)**			
Positive	492(69)	387(81)				
PgR			**<0.001**			**0.002**
Negative	328(49)	134(30)	**(38.32)**	102(24)	48(15)	**(9.22)**
Positive	346(5)	310(70)		324(76)	273(85)	
AR			**<0.001**			**0.035**
Negative	260(4)	112(28)	**(19.54)**	101(25)	55(19)	**(4.46)**
Positive	369(59)	292(72)		300(75)	243(81)	
CK7/8			**0.006**			0.386
Negative	18(3)	2(1)	**(7.45)**	1(0)	0(0)	(0.75)
Positive	658(97)	442(99)		427(99)	321(100)	
CK18			**0.009**			**0.017**
Negative	99(16)	42(10)	**(6.84)**	22(6)	6(2)	**(0.75)**
Positive	518(84)	367(90)		369(94)	293(98)	
CK19			**<0.001**			0.060
Negative	91(14)	25(56)	**(17.91)**	28(7)	11(3)	(3.53)
Positive	576(86)	416(94)		396(93)	305(97)	
FOXA1			**0.005**			0.209
Negative	262(61)	146(50)	**(7.76)**	133(50)	89(45)	(1.57)
Positive	170(39)	145(50)		131(50)	111(55)	
BEX1			**<0.001**			**<0.001**
Negative	167(39)	59(22)	**(23.51)**	103(38)	36(18)	**(21.47)**
Positive	260(61)	212(78)		166(62)	160(82)	
TFF1			0.237			0.479
Negative	193(52)	121(47)	(0.41)	105(46)	84(48)	(0.50)
Positive	179(48)	137(53)		122(54)	90(52)	
TFF3			**0.003**			0.375
Negative	207(53)	106(42)	**(8.55)**	99(42)	69(38)	(0.78)
Positive	181(47)	149(58)		138(58)	115(62)	
GATA3			**<0.001**			**0.020**
Negative	266(69)	134(54)	**(14.42)**	123(53)	72(41)	**(.43)**
Positive	122(31)	115(46)		110(47)	103(58)	
CD71			0.391			0.742
Negative	197(44)	139(47)	(0.97)	148(53)	111(55)	(0.10)
Positive	250(56)	156(53)		129(47)	91(45)	
CARM1			0.342			**0.021**
Negative	115(27)	64(23)	(2.14)	91(35)	47(25)	**(0.77)**
Moderate	220(52)	146(53)		133(51)	102(54)	
High	88(21)	68(24)		35(14)	40(21)	
PELP1			0.470			0.517
Negative	86(19)	51(18)	(1.50)	68(23)	38(19)	(1.31)
Moderate	289(63)	188(66)		190(65)	137(70)	
High	86(18)	47(16)		34(12)	21(11)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**<0.001**			**0.006**
Negative	265(40)	130(29)	**(12.29)**	164(39)	93(29)	**(7.58)**
Positive	401(60)	312(71)		259(61)	227(71)	
p-Cadherin			**0.002**			0.102
Negative	252(45)	197(55)	**(9.42)**	215(60)	177(67)	(2.67)
Positive	314(55)	163(45)		141(40)	88(33)	
p53			0.052			0.404
Negative/low	460(69)	331(74)	(3.76)	348(82)	274(85)	(0.69)
Positive	205(31)	113(26)		75(18)	50(15)	
KI67-LI			0.053			0.463
Negative/low	594(88)	385(87)	(3.47)	194(54)	152(57)	(0.53)
High	81(12)	59(13)		164(46)	114(43)	
BCL2			**<0.001**			0.075
Negative/low	224(46)	114(34)	**(12.43)**	85(27)	50(21)	(3.16)
High	259(54)	221(66)		225(73)	190(79)	
HER1			0.918			0.277
Negative	526(78)	346(79)	(0.01)	371(88)	269(85)	(1.18)
Positive	144(22)	93(21)		50(12)	46(15)	
HER2			0.588	-	-	-
Negative	594(88)	385(87)	(0.29)			
Positive	81(12)	59(13)				
HER3			0.491			0.261
Negative	52(9)	40(10)	(0.47)	33(9)	33(11)	(1.26)
Positive	560(91)	371(90)		353(91)	264(89)	
HER4			0.219			0.405
Negative	83(13)	65(15)	(1.51)	65(16)	56(18)	(0.69)
Positive	82(87)	367(85)		355(84)	259(82)	

Table 3-17: The associations between nuclear p-p38 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**0.002**	-	-	**-**
Negative	246(26)	70(18)	**(9.81)**			
Positive	692(74)	317(82)				
PgR			**<0.001**			**0.019**
Negative	399(44)	122(33)	**(12.95)**	134(23)	44(16)	**(5.49)**
Positive	509(56)	248(67)		450(77)	231(84)	
AR			**<0.001**			**0.018**
Negative	357(2)	83(26)	**(25.93)**	144(26)	46(19)	**(5.61)**
Positive	496(58)	239(74)		404(74)	202(81)	
CK7/8			0.075			0.144
Negative	17(2)	2(1)	(3.17)	0(0)	1(0)	(2.13)
Positive	891(98)	367(99)		584(100)	273(100)	
CK18	1		**0.008**			0.369
Negative	32(16)	30(10)	**(7.13)**	28(5)	9(4)	(0.72)
Positive	713(84)	285(90)		514(95)	230(96)	
CK19			**0.017**			0.060
Negative	98(10)	24(7)	**(5.66)**	32(6)	11(4)	(3.53)
Positive	791(90)	338(93)		537(94)	259(96)	
FOXA1			**<0.001**			**<0.001**
Negative	373(61)	89(37)	**(40.28)**	198(53)	51(29)	**(27.33)**
Positive	236(39)	151(63)		174(47)	123(71)	
BEX1			**<0.001**			**<0.001**
Negative	221(37)	45(21)	**(18.74)**	133(35)	30(19)	**(14.25)**
Positive	380(63)	173(79		248(65)	131(81)	
TFF1			**0.016**			**0.045**
Negative	288(53)	88(44)	**(5.83)**	173(51)	60(41)	**(4.02)**
Positive	251(47)	114(56)		164(49)	85(59)	
TFF3			**0.014**			0.064
Negative	286(52)	92(42)	**(6.06)**	157(45)	59(36)	(3.42)
Positive	268(48)	128(58)		191(55)	103(64)	
GATA3			**<0.001**			**0.001**
Negative	376(68)	101(49)	**(23.55)**	184(54)	55(38)	**(11.06)**
Positive	173(32)	104(51)		154(46)	90(62)	
CD71			0.084			0.436
Negative	258(41)	117(47)	(2.98)	94(50)	94(53)	(0.60)
Positive	373(59)	130(53)		195(50)	82(47)	
CARM1			0.100			**0.015**
Negative	166(28)	50(21)	(4.60)	126(34)	40(23)	**(15.34)**
Moderate	303(51)	132(55)		187(50)	97(55)	
High	128(21)	59(24)		58(16)	39(22)	
PELP1			0.580			0.282
Negative	99(16)	37(16)	(1.08)	75(19)	29(17)	(2.53)
Moderate	417(66)	148(63)		279(71)	120(68)	
High	111(18)	50(21)		41(10)	26(15)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			0.573			0.972
Negative	336(38)	127(36)	(0.31)	205(36)	95(36)	(0.0)
Positive	559(62)	227(64)		371(64)	171(64)	
p-Cadherin			0.231			0.642
Negative	361(47)	143(51)	(1.43)	310(63)	130(61)	(0.21)
Positive	406(53)	136(49)		183(37)	83(39)	
p53			0.291			0.832
Negative/low	637(70)	267(73)	(1.11)	480(82)	226(82)	(0.04)
Positive	269(30)	97(27)		102(18)	50(18)	
KI67-LI			**<0.001**			**0.001**
Negative/low	292(39)	161(53)	**(18.32)**	246(50)	139(63)	**(10.56)**
High	463(61)	142(47)		243(50)	80(37)	
BCL2			**0.040**			**0.019**
Negative/low	297(43)	99(36)	**(4.23)**	126(28)	39(20)	**(5.42)**
High	391(57)	177(64)		319(72)	160(80)	
HER1			0.676			0.195
Negative	722(80)	301(81)	(17)	510(88)	234(85)	(1.67)
Positive	184(20)	72(19)		66(12)	40(15)	
HER2			0.192	-	-	-
Negative	777(86)	329(89)	(1.70)			
Positive	128(14)	42(11)				
HER3			0.294			0.438
Negative	65(8)	32(10)	(110)	47(9)	26(10)	(0.60)
Positive	776(92)	301(90)		492(91)	223(90)	
HER4			0.052			0.048
Negative	102(11)	56(15)	(3.76)	75(13)	49(18)	(3.90)
Positive	797(89)	309(85)		502(87)	221(82)	

Table 3-18: The associations between nuclear p-ATF2 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	260(27)	51(15)	**(18.85)**			
Positive	693(73)	281(85)				
PgR			**<0.001**			**0.037**
Negative	410(45)	98(30)	**(20.16)**	134(23)	42(17)	**(4.33)**
Positive	510(55)	226(70)		451(77)	212(83)	
AR			**<0.001**			**<0.001**
Negative	366(43)	67(23)	**(38.08)**	151(28)	36(16)	**(13.12)**
Positive	483(60)	226(77)		393(72)	195(84)	
CK7/8			0.844			0.512
Negative	16(2)	5(2)	(0.03)	1(0.0)	0(0.0)	(3.40)
Positive	906(98)	313(98)		581(100)	250(100)	
CK18			**0.005**			**0.007**
Negative	134(16)	27(9)	**(7.56)**	34(6)	4(2)	**(7.27)**
Positive	702(84)	261(91)		496(94)	224(98)	
CK19			**0.043**			0.247
Negative	96(11)	21(7)	**(4.09)**	32(6)	9(4)	(1.33)
Positive	809(89)	292(93)		539(94)	236(96)	
FOXA1			**<0.001**			**<0.001**
Negative	373(60)	81(38)	**(29.98)**	193(51)	55(34)	**(14.35)**
Positive	249(40)	131(62)		184(49)	109(66)	
BEX1			**0.006**			**0.012**
Negative	209(35)	49(24)	**(7.67)**	126(33)	36(23)	**(6.36)**
Positive	390(65)	153(76)		251(67)	124(77)	
TFF1			0.378			0.595
Negative	282(52)	85(48)	(0.77)	165(49)	63(46)	(0.28)
Positive	266(48)	93(52)		174(51)	74(54)	
TFF3			0.643			0.445
Negative	281(50)	95(48)	(0.21)	148(42)	70(46)	(0.58)
Positive	285(50)	104(52)		206(58)	84(54)	
GATA3			**<0.001**			**0.005**
Negative	383(68)	84(48)	**(23.18)**	185(54)	53(40)	**(7.77)**
Positive	179(32)	91(52)		157(46)	80(60)	
CD71			**0.007**			0.634
Negative	263(40)	107(51)	**(7.21)**	200(50)	85(53)	(0.22)
Positive	389(60)	103(49)		198(50)	77(47)	
CARM1			0.504			0.157
Negative	161(26)	52(26)	(1.37)	119(31)	43(27.2)	(3.75)
Moderate	327(52)	98(48)		201(53)	78(49)	
High	137(22)	52(26)		63(16)	37(24)	
PELP1			0.123			0.360
Negative	100(15)	36(18)	(4.19)	74(18)	31(20)	(2.04)
Moderate	442(68)	115(59)		292(71)	102(66)	
High	111(17)	44(23)		43(11)	22(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			0.488			0.401
Negative	339(38)	112(36)	(0.48)	212(37)	84(34)	(0.70)
Positive	560(62)	203(64)		362(63)	164(66)	
p-Cadherin			0.060			0.909
Negative	344(46)	140(53)	(3.53)	291(61)	129(62)	(0.01)
Positive	406(54)	126(47)		184(39)	80(38)	
p53			**0.027**			0.932
Negative/low	634(70)	243(76)	**(4.89)**	475(82)	207(82)	(0.0)
Positive	277(30)	76(24)		105(18)	45(18)	
KI67-LI			**<0.001**			**0.005**
Negative/low	289(38)	144(53)	**(16.68)**	237(50)	130(61)	**(7.90)**
High	466(62)	130(47)		242(50)	83(39)	
BCL2			**0.010**			0.836
Negative/low	305(44)	84(34)	**(6.70)**	113(25)	49(26)	(0.04)
High	396 (56)	163(66)		334(75)	139(74)	
HER1			0.480			0.117
Negative	734(79)	260(81)	(0.49)	514(89)	210(85)	(2.46)
Positive	190(21)	60(19)		66(11)	38(15)	
HER2			**0.001**	-	-	-
Negative	777(85)	299(92)	**(11.12)**			
Positive	138(15)	25(8)				
HER3			0.302			0.309
Negative	68(8)	29(10)	(1.06)	46(9)	25(11)	(1.03)
Positive	785(92)	263(90)		492(91)	205(89)	
HER4			**0.005**			**0.026**
Negative	103(11)	55(18)	**(8.01)**	77(13)	48(19)	**(4.98)**
Positive	812(89)	260(82)		500(87)	199(81)	

Table 3-19: The associations between nuclear p-C-JUN and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**0.004**	-	-	**-**
Negative	130(29)	178(22)	**(8.23)**			
Positive	321(71)	645(78)				
PgR			**<0.015**			0.512
Negative	197(46)	312(39)	**(5.88)**	60(22)	113(20)	(0.43)
Positive	231(54)	489(61)		208(78)	441(80)	
AR			**<0.001**			**0.006**
Negative	190(47)	238(33)	**(20.04)**	78(31)	107(21)	**(7.70)**
Positive	218(53)	482(67**)**		178(69)	395(79)	
CK7/8			**0.032**			0.484
Negative	12(3)	9(1)	**(4.59)**	0(0)	1(0.)	(0.48)
Positive	418(97)	790(99)		269(100)	550(100)	
CK18			**0.005**			0.085
Negative	72(18.1)	86(12)	**(7.86)**	17(7)	20(4)	(2.96)
Positive	325(81.9)	631(88)		229(93)	480(96)	
CK19			**<0.001**			**0.013**
Negative	62(15)	54(7)	**(18.32)**	20(8)	19(4)	**(6.19)**
Positive	364(85)	725(93)		245(92)	519(96)	
FOXA1			**0.001**			**0.007**
Negative	177(64)	272(51)	**(12.00)**	93(55)	148(43)	**(7.34)**
Positive	100(36)	259(49)		75(45)	199(57)	
BEX1			**<0.001**			**<0.001**
Negative	114(42)	142(28)	**(15.96)**	67(41)	87(25)	**(13.41)**
Positive	160(58)	370(72)		98(59)	264(75)	
TFF1			0.610			0.624
Negative	131(52)	227(50)	(0.26)	72(47)	149(49)	(0.24)
Positive	122(48)	230(50)		82(53)	154(51)	
TFF3			0.236			0.413
Negative	129(52)	226(7)	(1.40)	66(44)	131(40)	(0.66)
Positive	121(48)	255(53)		83(56)	194(60)	
GATA3			**<0.001**			**0.018**
Negative	191(73)	277(59)	**(12.86)**	91(59)	149(48)	**(5.60)**
Positive	72(27)	189(41)		63(41)	165(52)	
CD71			0.655			0.601
Negative	127(42)	232(43)	(0.20)	90(50)	184(52)	(0.27)
Positive	177(58)	304(57)		92(50)	171(48)	
CARM1			**<0.001**			**0.009**
Negative	97(34.0)	113(22)	**(15.89)**	66(39)	91(26)	**(9.45)**
Moderate	124(43.5)	290(56)		77(46)	196(56)	
High	64(22.5)	116(22)		26(15)	65(18)	
PELP1			0.082			**0.016**
Negative	52(17)	85(16)	(4.99)	40(21)	64(18)	**(8.29)**
Moderate	216(73)	364(68)		136(73)	245(68)	
High	30(10)	83(16)		11(6)	50(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.043**			0.513
Negative	171(40.0)	264(34)	**(4.07)**	96(36)	183(34)	(0.42)
Positive	257(60.0)	511(66)		169(64)	357(66)	
p-Cadherin			0.545			0.982
Negative	166(46.6)	317(48.5)	(0.36)	137(62)	285(62)	(0.0)
Positive	190(53.4)	336(51.5)		83(38)	172(38)	
p53			0.622			0.853
Negative/low	302(70.2)	564(71.5)	(0.24)	221(83)	454(82)	(0.03)
Positive	128(29.8)	225(28.5)		46(17)	98(18)	
KI67-LI			**0.002**			**0.003**
Negative/low	127(35.6)	304(45.8)	**(10.04)**	102(46)	262(58)	**(9.12)**
High	230(64.4)	360(54.2)		122(54)	191(42)	
BCL2			0.482			0.897
Negative/low	138(43.5%	249(41.2)	(0.49)	53(26)	108(27)	(0.01)
High	179(56.5)	355(58.8)		151(74)	300(73)	
HER1			0.899			0.117
Negative	346(80.3)	636(80.0)	(0.01)	237(89)	473(87)	(2.46)
Positive	85(19.7)	159(20.0)		28(11)	72(13)	
HER2			0.863	-	-	-
Negative	371(86.3)	687(86.5)	(0.03)			
Positive	59(13.7)	107(13.5)				
HER3			0.596			0.054
Negative	39(9.6)	63(8.7)	(0.28)	33(13)	44(9)	(3.71)
Positive	367(90.4)	665(91.3)		217(87)	462(91)	
HER4			0.383			0.713
Negative	51(11.9)	107(13.7)	(0.76)	39(15)	85(16)	(0.13)
Positive	377(88.1)	676(86.3)		226(85)	456(84)	

3.3.2.5 Overview of the expression of MAPKs in Trastuzumab treated series

Regarding nuclear p-ERK1/2, there were 152 cases valid for its assessment within this series, of which, 84 (55.3%) cases had negative/low expression while 68 (44.7%) had high expression. The cytoplasmic form had 135 (88.8%) were negative/low and 17 (11.2%) had high expression. For the total form, 108 cases have been revealed valid for the assessment, where 64 (59.3%) were negative/low but only 44 (40.7%) were high.

Regarding p-JNK1/2, 161 cases were assessed. Out of these, 85 (52.8%) cases had negative/low expression but 76 (48.2%) cases had high expression. For the total form of p-JNK1/2, 152 cases were valid for the assessment. From the total, 78 (51.3) had negative/low expression and for those had high expression were 74 (48.7%).

p-p38 had 147 cases valid, 44(29.9%) of which, had negative/low while 103 (70.1%) had high expression. Regarding the total form, 155 cases were valid for the assessment of this protein, out of them, 78 (50.3%) had negative/low while 77 (49.7%) had high expression.

For p-ATF2, 150 cases were available for the assessment for this protein, of which 87 (58%) had negative/low expression and 63 (42%) had high expression. Lastly, p-C-JUN, 149 cases were valid for its assessment and 65 (43.6%) out of them were negative/low while 84 (56.4%) had high expression. Details of these antibodies are listed in Table 3-1. The cut-off points used are the same for those in the primary series; refer to Table 2-5 in methodology.

3.3.2.5.1 The associations of MAPKs with clinicopathological variables and biological markers in Trastuzumab treated series

In This series, a positive association between high expression of nuclear p-ERK1/2 and advanced stage (p=0.012: borderline), less mitotic count (nuclear: p=0.034: borderline and cytoplasmic=0.002) and a trend for better NPI was noticed (p=0.057, Table 3-20) while no associations were found for total and phosphorylated JNK1/2 and p38 (Table 3-21 and Table 3-22). For p-ATF2, it was associated with advanced stage, lower tumour grade (both p=0.001) and low mitotic count (p=0.014: borderline) while p-C-JUN was only associated with lower NPI score (p=0.004, Table 3-23). Regarding the associations between

MAPKs themselves within this series, all the associations between MAPKs were positive; nevertheless, less associations were noticed than those observed within the primary series (Table 3-24).

For the associations of MAPKs with the biological markers, high expression of both pan ERK1/2 and its phosphorylated nuclear form were associated with upregulation of CK7/8 (p=0.006, p<0.001, respectively, Table 3-25).

High expression of pan JNK1/2 on the other hand was associated with a trend for upregulation of HER2,1 (p=0.014), HER2,3 (p=0.033) dimers and a combination of increased expression of HER2,1 and HER2,4 (p=0.030) dimers simultaneously (Table 3-26). Meanwhile, pan p38 high expression was positively associated with increased expression of all HER2 dimers: HER2-HER1, HER2-HER3 and HER2-HER4 (p=0.043: borderline, p<0.001, p=0.013: borderline) respectively and with increased expressions of dimers combinations, HER2,1 vs HER2,3, HER2,3 vs HER2,4 and HER2,3 vs HER24 (p=0.011, p=0.046, both borderline, p=0.004) respectively (Table 3-27). Meanwhile, high expression of p-ATF2 and p-C-JUN were associated with positivity of CK7/8 (p<0.001, Table 3-28).

Table 3-20: The associations between MAPKs and Clinicopathological variables in Trastuzumab treated series

	Pan ERK1/2			N-p-ERK1/2			C-p-ERK1/2		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.716			0.675			0.591
≤50	35(44)	18(41)	(0.13)	33(39)	29(43)	90.17)	56(42)	6(35)	(0.28)
>50	44(56)	26(59)		51(61)	39(57)		77(58)	11(65)	
Menopausal Status			0.493			0.345			0.475
Pre-	41(52)	20(46)	(0.46)	38(45)	36(53)	(0.89)	67(50)	7(41)	(0.51)
Post-	38(48)	24(54)		46(55)	32(47)		66(50)	10(59)	
Tumour Size (cm)			0.114			0.367			0.718
≤2.0	4(5)	0(0)	(2.28)	1(1)	0(0)	(0.81)	1(1)	0(0)	(0.13)
>2.0	74(95)	43(100)		82(99)	67(100)		130(99)	17(100)	
Stage			0.771			**0.012**			0.163
1	48(65)	29(66)	(0.51)	44(53)	45(66)	**(8.00)**	76(58)	12(70)	(3.62)
2	20(27)	10(23)		37(45)	17(25)		50(38)	3(18)	
3	6(8)	5(11)		2(2)	6(9)		6(4)	2(12)	
Grade			0.887			0.117			0.084
1	2(2)	1(2)	(0.24)	0(0)	3(4)	(4.28)	2(1)	1(6)	(4.95)
2	21(27)	10(23)		19(23)	18(27)		29(22)	7(41)	
3	56(71)	33(75)		65(77)	47(69)		102(77)	9(53)	
Tubules			0.432			0.215			0134
2	15(19)	6(14)	(61)	11(13)	14(21)	(1.35)	20(15)	5(29)	(2.24)
3	63(81)	38(86)		73(87)	54(79)		113(85)	12(71)	
Pleomorphism			0.677			0.315			0.983
2	7(9)	3(7)	(0.17)	4(5)	6(9)	(1.00)	8(6)	1(56)	(0.00)
3	71(91)	41(93)		80(95)	62(91)		125(94)	16(94)	
Mitosis			0.716			**0.034**			**0.002**
1	21(27)	11(25)	(0.13)	18(21)	16(24)	**(6.75)**	27(20)	6(35)	**(12.55)**
2	21(27)	16(36)		20(24)	28(41)		38(29)	10(59)	
3	36(46)	17(39)		46(55)	24(35)		68(51)	1(6)	
LVI			0.840			0.837			0.637
Probable/Negative	57(72)	25(57)	(2.99)	53(63)	44(65)	(0.04)	86(65)	10(59)	(0.22)
Definite	22(28)	19(43)		31(37)	24(35)		47(35)	7(41)	
NPI			0.549			0.057			0.198
GPG	3(5)	3(7)	(1.19)	4(5)	7(11)	(5.73)	9(8)	2(12)	(3.24)
MPG	37(59)	27(66)		42(56)	42(68)		70(59)	13(76)	
PPG	23(36)	11(27)		29(39)	13(21)		39(33)	2(12)	

Table 3-21: The associations between MAPKs and Clinicopathological variables in Trastuzumab treated series

	Pan JNK1/2			p-JNK1/2		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.662			0.946
≤50	31(40)	32(43)	(0.19)	34(40)	30(40)	(0.00)
>50	47(60)	42(57)		51(60)	46(60)	
Menopausal Status			0.420			0837
Pre-	36(46)	39(53)	(0.65)	40(47)	37(49)	(0.04)
Post-	42(54)	35(47)		45(53)	39(51)	
Tumour Size (cm)	-	-	-			0.343
≤2.0				1(1)	0(0)	(0.89)
>2.0				83(99)	75(100)	
Stage			0.437			0.297
1	41(53)	46(62)	(1.65)	48(56)	47(63)	(2.96)
2	32(41)	23(31)		34(40)	22(29)	
3	5(6)	5(7)		3(4)	6(8)	
Grade			0.133			0.317
1	0(0)	3(4)	(4.03)	0(0)	2(3)	(2.29)
2	17(22)	20(27)		23(27)	21(27)	
3	61(78)	51(69)		62(73)	53(70)	
Tubules			0.423			0.755
2	11(14)	14(19)	(0.64)	13(15)	13(17)	(0.09)
3	67(86)	60(81)		72(85)	63(83)	
Pleomorphism			0.840			0.510
2	7(9)	6(8)	(0.03)	8(9)	5(7)	(0.43)
3	71(91)	68(92)		77(91)	71(93)	
Mitosis			0.629			0.949
1	15(19)	19(26)	(0.92)	22(26)	18(24)	(0.10)
2	26(33)	22(30		25(29)	23(30)	
3	37(48)	33(44)		38(45)	35(46)	
LVI			0.810			0.765
Probable/Negative	51(65)	47(63)	(0.05)	54(63)	50(66)	(0.09)
Definite	27(35)	27(37)		31(37)	26(34)	
NPI			0.511			0.692
GPG	4(5)	7(11)	(1.34)	7(9)	7(10)	(0.73)
MPG	45(62)	40(60)		46(58)	43(63)	
PPG	24(33)	19(29)		26(33)	18(27)	

Table 3-22: The associations between MAPKs and Clinicopathological variables in Trastuzumab treated series

	Pan P38			p-P38		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.377			0.546
≤50	29(37)	34(44)	(0.78)	19(43)	39(38)	(0.36)
>50	49(63)	43(56)		25(57)	64(62)	
Menopausal Status			0.937			0.460
Pre-	38(49)	38(49)	(0.00)	23(52)	47(46)	(0.45)
Post-	40(51)	39(51)		21(48)	56(54)	
Tumour Size (cm)			0.319			0.508
≤2.0	1(1)	0(0)	(0.99)	0(0)	1(1)	(0.43)
>2.0	76(99)	76(100)		44(100)	100(99)	
Stage			0.356			840
1	46(59)	46(60)	(2.06)	24(56)	61(60)	(0.34)
2	29(37)	24(31)		16(37)	36(35)	
3	3(4)	7(9)		3(7)	5(5)	
Grade			0.181			0.135
1	0(0)	3(4)	(3.41)	1(2)	2(2)	(4.00)
2	20(26)	22(29)		6(14)	30(29)	
3	58(74)	52(67)		37(84)	71(69)	
Tubules			0.490			0.712
2	11(14)	14(18)	(0.47)	7(16)	19(18)	(0.13)
3	67(86)	63(82)		37(84)	84(82)	
Pleomorphism			0.980			0.697
2	7(9)	7(9)	(0.00)	3(7)	9(9)	(1.52)
3	71(91)	70(91)		41(93)	94(91)	
Mitosis			0.601			0.555
1	17(22)	22(29)	(1.01)	8(18)	26(25)	(1.17)
2	24(31)	23(30)		13(30)	32(31)	
3	37(47)	32(41)		23(52)	45(44)	
LVI			0.220			0.217
Probable/Negative	53(68)	45(58)	(1.50)	32(73)	64(62)	(1.52)
Definite	25(32)	32(42)		12(27)	39(38)	
NPI			0.942			0.262
GPG	7(10)	6(9)	(0.11)	2(5)	11(12)	(2.97)
MPG	45(62)	43(61)		22(56)	59(63)	
PPG	20(28)	21(30)		15(39)	24(25)	

Table 3-23: The associations between MAPKs and Clinicopathological variables in Trastuzumab treated series

	p-ATF2			p-C-JUN		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.423			0.692
≤50	33(38)	28(44)	(0.64)	25(39)	35(42)	(0.15)
>50	54(62)	35(56)		40(61)	49(58)	
Menopausal Status			0.657			0.845
Pre-	41(47)	32(51)	(0.19)	32(49)	40(48)	(0.03)
Post-	46(53)	31(49)		33(51)	44(52)	
Tumour Size (cm)			0.237			0.385
≤2.0	0(0)	1(2)	(1.39)	0(0)	1(1)	(0.75)
>2.0	86(100)	61(98)		63(100)	83(99)	
Stage			**0.001**			0.706
1	52(60)	38(61)	**(13.44)**	40(61)	50(60)	(0.69)
2	35(40)	16(26)		20(31)	29(35)	
3	0(0)	8(13)		5(8)	4(5)	
Grade			**0.001**			0.473
1	2(2)	2?(3)	**(13.63)**	1(2)	2(2)	(1.49)
2	12(14)	25(40)		14(21)	25(30)	
3	73(84)	36(57)		50(77)	57(68)	
Tubules			0.267			0.637
2	12(14)	13(21)	(1.23)	9(14)	14(17)	(0.22)
3	75(86)	50(79)		56(86)	70(83)	
Pleomorphism			0.585			0.614
1	6(7)	6(10)	(0.34)	4(6)	7(8)	(0.25)
2	81(93)	57(90)		61(94)	77(92)	
3						
Mitosis			**0.014**			0.373
1	13(15)	22(35)	**(8.53)**	14(21)	23(28)	(1.97)
2	28(32)	18(29)		18(28)	28(33)	
3	46(53)	23(36)		33(51)	33(39)	
LVI			0.581			0.595
Probable/Negative	59(68)	40(63)	(0.30)	44(68)	53(63)	(0.34)
Definite	28(32)	23(37)		21(32)	31(37)	
NPI8			0.837			**0.004**
GPG	7(9)	6(11)	(0.35)	0(0)	13(17)	**(11.12)**
MPG	50(62)	33(62)		41(71)	42(55)	
PPG	24(29)	14(27)		17(29)	21(28)	

Table 3-24: Associations of MAPKs used in IHC with each other in Trastuzumab treated series

		p-JNK1/2	JNK1/2	N-p-ERK1/2	C-p-ERK1/2	ERK1/2	p38	N-p-p38	p-c-jun	p-ATF2
p-JNK1/2	**Spearmann'sRank correlation**		-.113	.277**	.050	.092	.117	.218**	.071	.196*
	p-value	-	.173	**.001**	.553	.374	.163	**.007**	.405	**.020**
	Number of cases		146	145	143	95	143	151	140	141
JNK1/2	**Spearmann'sRank correlation**	-.113		.032	-.009	-.119	.085	.283**	.092	-.041
	p-value	.173	-	.701	.919	.276	.320	**.001**	.285	.635
	Number of cases	146		143	141	86	139	145	136	138
N-P-ERK1/2	**Spearmann'sRank correlation**	.277**	.032		.186*	.033	.004	.212*	.100	.382**
	p-value	**.001**	.701	-	**.022**	.757	.967	**.011**	.239	**.000**
	Number of cases	145	143		150	90	140	145	140	139
C- p-ERK1/2	**Spearmann'sRank correlation**	.050	-.009	.186*		.001	.122	.057	.264**	.155
	p-value	.553	.919	**.022**	-	.995	.153	.500	**.002**	.070
	Number of cases	143	141	150		89	138	143	138	137
ERK1/2	**Spearmann'sRank correlation**	.092	-.119	.033	.001		.231*	-.038	.087	.070
	p-value	.374	.276	.757	.995	-	**.033**	.722	.419	.522
	Number of cases	95	86	90	89		85	90	88	85
p-p38	**Spearmann'sRank correlation**	.117	.085	.004	.122	.231*	-	.149	.436**	.243**
	p-value	.163	.320	.967	.153	**.033**		.077	**.000**	**.004**
	Number of cases	143	139	140	138	85		142	138	140
Pan P38	**Spearmann'sRank correlation**	.218**	.283**	.212*	.057	-.038	.149		.057	.212*
	p-value	**.007**	**.001**	**.011**	.500	.722	.077	-	.507	**.012**
	Number of cases	151	145	145	143	90	142		138	139
p_C_JUN	**Spearmann'sRank correlation**	.071	.092	.100	.264**	.087	.436**	.057		-.004
	p-value	.405	.285	.239	**.002**	.419	**.000**	.507	-	.961
	Number of cases	140	136	140	138	88	138	138		140
p-ATF2	**Spearmann'sRank correlation**	.196*	-.041	.382**	.155	.070	.243**	.212*	-.004	-
	p-value	**.020**	.635	**.000**	.070	.522	**.004**	**.012**	.961	
	Number of cases	141	138	139	137	85	140	139	140	

(-) **Represents the analysis of each marker with itself, *1 is phosphorylated, (*2) is nuclear, (*3) is cytoplasmic and, (*)is the correlation which is significant at the 0.05 level (2-tailed), (**) is the correlation which is significant at the 0.01 level (2-tailed).**

Table 3-25: The associations between MAPKs and biological markers in Trastuzumab treated series

	Pan ERK1/2			N-p-ERK1/2			C-p-ERK1/2		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
ER			0.196			0.736			0.443
Positive	30(38)	22(50)	(1.67)	36(43)	31(46)	(0.11)	60(4)	6(35)	(0.59)
Negative	49(62)	22(50)		48(57)	37(54)		73(55)	11(65)	
PgR			0.196			0.695			0.185
Positive	39(66)	19(53)	(1.66)	45(6)	31(60)	(0.00)	70(61)	5(42)	(1.75)
Negative	20(34)	17(47)		30(40)	21(40)		44(39)	7(58)	
CK7/8			**0.006**			**<0.001**	-	-	-
Positive	16(20)	1(2)	**(7.67)**	51(61)	17(25)	**(19.38)**			
Negative	63(80)	43(98)		33(39)	51(75)				
CK18	3(7)	0(0)	0.186			0.504			0.471
Positive	40(93)	24(100)	(1.75)	2(8)	1(4)	(0.44)	3(7)	0(0)	(0.52
Negative				22(92)	25(96)		40(93)	7(100)	
BCL2			0.975			0.887			0.276
Positive	29(46)	19(46)	(0.00)	14(42)	22(44)	(0.02)	29(41)	7(58)	(1.18)
Negative	34(54)	22(54)		19(58)	28(56)		41(59)	5(42)	
P53			0.548			0.674			0.934
Positive	14(22)	7(17)	(0.36)	5(15)	9(19)	(0.017)	12(18)	2(17)	(0.00)
Negative	50(78)	34(83)		28(85)	39(81)		56(82)	10(83)	
HER2-HER1 dimer			0.365			0.150			0.735
Negative	16(28)	8(20)	(0.82)	11(36)	10(21)	(2.07)	17(25)	3(30)	(0.11)
Positive	41(72)	32(80)		20(64)	38(79)		51(75)	7(70)	
HER2-HER3 dimer			0.292			0.589			0.625
Negative	16(31)	18(42)	(1.11)	12(40)	17(34)	(0.29)	23(34)	5(42)	(0.24)
Positive	35(69)	25(58)		18(60)	33(66)		44(66)	7(58)	
HER2-HER4 dimer			0.668			0.270			0.940
Negative	21(46)	16(41)	(0.18)	14(50)	17(37)	(1.21)	26(41)	4(40)	(0.00)
Positive	25(54)	23(59)		14(50)	29(63)		37(59)	6(60)	
HER2,1 vs HER2,3			0.113			0.639			0.866
HER2,1 low-HER2,3 low	11(23)	8(20)	(5.97)	8(28)	9(20)	(1.68)	13(21)	3(30)	(0.73)
HER2,1 low-HER2,3 high	3(7)	0(0)		1(4)	1(2)		2(3)	0(0)	
HER2,1 high-HER2,3 low	3(6)	8(21)		2(7)	7(15)		8(13)	1(10)	
HER2,1 high-HER2,3 high	30(64)	23(59)		17(61)	29(63)		40(63)	6(60)	
HER2,1 vs HER2,4			0.625			0.268			0.948
HER2,1 low-HER2,4 low	10(22)	8(23)	(1.76)	8(31)	7(16)	(3.93)	12(20)	2(25)	(0.36)
HER2,3 low-HER2,4 high	1(2)	0(0)		1(4)	0(0)		1(2)	0(0)	
HER2,1 high-HER2,4 low	10(22)	5(14)		4(15)	8(19)		11(18)	1(13)	
HER2,1 high-HER2,4 high	24(54)	22(63)		13(50)	28(65)		36(60)	5(62)	
HER2,3 vs HER2,4			0.086			0.109			0.307
HER2,3 low-HER2,4 low	11(28)	12(31)	(6.59)	11(41)	9(21)	(6.06)	15(25)	4(40)	(3.61)
HER2,3 low-HER2,4 high	0(0)	5(13)		0(0)	5(11)		5(9)	0(0)	
HER2,1 high-HER2,4 low	8(20)	4(10)		3(11)	8(18)		11(18)	0(0)	
HER2,3 high-HER2,4 high	21(52)	18(46)		13(8)	22(50)		29(48)	6(60)	

Table 3-26: The associations between MAPKs and biological markers in Trastuzumab treated series

	Pan JNK/2			p-JNK1/2		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
ER			0.545			0.76
Positive	31(40)	33(45)	(0.36)	42(49)	27(36)	(3.15)
Negative	47(60)	41(55)		43(51)	49(64)	
PgR			0.450			0.300
Positive	37(56)	37(63)	(0.57)	46(64)	33(55)	(1.07)
Negative	29(44)	22(37)		26(36)	27(45)	
CK7/8	-	-	-	-	-	-
Positive						
Negative						
CK18			0.502			0.146
Positive	2(9)	1(4)	(0.45)	3(9)	0(0)	(2.11)
Negative	21(91)	24(96)		30(91)	22(100)	
BCL2			0.834			0.650
Positive	19(45)	15(43)	(0.04)	21(48)	18(43)	(0.20)
Negative	23(55)	20(57)		23(52)	24(57)	
P53			0.59			0.905
Positive	4(10)	9(26)	(3.56)	7(16)	6(15)	(0.01)
Negative	38(90)	26(74)		38(84)	35(85)	
HER2-HER1 dimer			**0.014**			0.334
Negative	15(37)	4(12)	**(6.05)**	15(33)	9(24)	(0.93)
Positive	26(63)	30(88)		30(67)	29(76)	
HER2-HER3 dimer			**0.033**			0.630
Negative	19(46)	8(23)	**(4.54)**	16(36)	17(42)	(0.23)
Positive	22(54)	27(77)		28(64)	24(58)	
HER2-HER4 dimer			0.113			0.325
Negative	19(50)	10(31)	(2.51)	22(51)	14(40)	(0.96)
Positive	19(50)	22(69)		21(49)	21(60)	
HER2,1 vs HER2,3			0.125			0.569
HER2,1 low-HER2,3 low	13(33)	3(10)	(5.73)	12(29)	8(22)	(2.01)
HER2,1 low-HER2,3 high	1(3)	1(3)		2(5)	1(3)	
HER2,1 high-HER2,3 low	5(13)	4(13)		3(7)	6(17)	
HER2,1 high-HER2,3 high	20(51)	23(74)		24(59)	21(58)	
HER2,1 vs HER2,4			**0.030**			0.354
HER2,1 low-HER2,4 low	13(36)	2(7)	**(8.91)**	13(31)	5(16)	(3.25)
HER2,3 low-HER2,4 high	0(0)	1(3)		1(2)	0(0)	
HER2,1 high-HER2,4 low	5(14)	6(20)		8(19)	6(19)	
HER2,1 high-HER2,4 high	18(50)	21(70)		20(48)	20(65)	
HER2,3 vs HER2,4			0.315			0.819
HER2,3 low-HER2,4 low	14(37)	5(17)	(3.54)	14(35)	9(26)	(0.92)
HER2,3 low-HER2,4 high	3(8)	2(7)		2(5)	3(9)	
HER2,1 high-HER2,4 low	5(13)	4(14)		6(15)	5(15)	
HER2,3 high-HER2,4 high	16(42)	18(62)		18(45)	17(50)	

Table 3-27: The associations between MAPKs and biological markers in Trastuzumab treated series

	Pan P38			p-p38		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
ER			0.816			0.844
Positive	33(42)	34(44)	(0.05)	20(46)	45(44)	(0.03)
Negative	45(58)	43(56)		24(54)	58(56)	
PgR			0.664			0.490
Positive	37(59)	40(62)	(0.18)	24(67)	51(60)	(0.47)
Negative	26(41)	24(38)		12(33.3%)	34(40)	
CK7/8	-	-	-			0.269
Positive				17(39)	50(49)	(1.22)
Negative				27(61)	53(51)	
CK18			0.415			0.783
Positive	2(9)	1(4)	(0.66)	1(8)	2(6)	(0.07)
Negative	20(91)	27(96)		12(92)	34(94)	
BCL2			0.500			0.801
Positive	18(47)	18(40)	(0.45)	12(46)	22(43)	(0.06)
Negative	20(53)	27(60)		14(54)	29(57)	
P53			0.437			0.154
Positive	5(14)	9(20)	(0.60)	6(22)	5(10)	92.03)
Negative	32(86)	36(80)		21(78)	44(90)	
HER2-HER1 dimer			**0.043**			0.748
Negative	13(36)	7(16)	**(4.07)**	6(25)	14(29)	(0.10)
Positive	23(64)	36(84)		18(75)	35(71)	
HER2-HER3 dimer			**<0.001**			0.915
Negative	23(59)	8(19)	**(13.64)**	9(36)	19(37)	(0.01)
Positive	16(41)	34(81)		16(64)	32(63)	
HER2-HER4 dimer			**0.013**			0.803
Negative	22(59)	11(31)	**(6.15)**	10(44)	21(47)	(0.06)
Positive	15(41)	25(69)		13(56)	24(53)	
HER2,1 vs HER2,3			**0.011**			0.767
HER2,1 low-HER2,3 low	13(36)	5(14)	**(11.07)**	5(23)	12(25)	(1.14)
HER2,1 low-HER2,3 high	0(0)	1(3)		0(0)	2(4)	
HER2,1 high-HER2,3 low	7(20)	2(5)		3(14)	5(11)	
HER2,1 high-HER2,3 high	16(44)	29(78)		14(63)	28(60)	
Her2,1 vs HER2,4			**0.046**			0.776
HER2,1 low-HER2,4 low	12(35)	4(12)	**(6.15)**	5(24)	10(24)	(1.10)
HER2,3 low-HER2,4 high	-	-		0(0)	1(3)	
HER2,1 high-HER2,4 low	7(21)	6(18)		3(14)	9(21)	
HER2,1 high-HER2,4 high	15(44)	24(70)		13(62)	22(52)	
Her2,3 vs HER2,4			**0.004**			0.882
HER2,3 low-HER2,4 low	17(46)	4(12)	**(13.42)**	6(27)	14(32)	(0.66)
HER2,3 low-HER2,4 high	4(11)	1(3)		2(9)	2(4)	
HER2,1 high-HER2,4 low	5(13)	6(18)		4(18)	7(16)	
HER2,3 high-HER2,4 high	11(29)	22(67)		10(46)	21(47)	

Table 3-28: The associations between MAPKs and biological markers in Trastuzumab treated series

	p-ATF2			p-C-JUN		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
ER			0.768			0.337
Positive	38(44)	26(41)	(0.08)	33(51)	36(43)	(0.92)
Negative	49(56)	37(59)		32(49)	48(57)	
PgR			0.790			0.401
Positive	42(59)	32(61)	(0.07)	39(66)	37(59)	(0.70)
Negative	29(41)	20(39)		20(34)	26(41)	
CK7/8			**<0.001**			**<0.001**
Positive	58(67)	12(19)	**(33.29)**	19(29)	50(59)	**(13.52)**
Negative	29(33)	51(81)		46(71)	34(41)	
CK18			0.218			0.762
Positive	2(12)	1(3)	(1.51)	2(7)	1(5)	(0.09)
Negative	15(88)	32(97)		26(93)	19(95)	
BCL2			0.971			0.600
Positive	13(46)	23(46)	(0.00)	20(43)	16(49)	(0.27)
Negative	15(54)	27(54)		27(57)	17(51)	
P53			0.944			0.316
Positive	4(15)	8(16)	(0.00)	10(21)	4(12)	(1.00)
Negative	22(85)	42(84)		37(79)	28(88)	
HER2-HER1 dimer			0.773			0.216
Negative	8(29)	12(26)	(0.08)	15(34)	7(21)	(1.53)
Positive	20(71)	35(74)		29(66)	26(79)	
HER2-HER3 dimer			0.520			0.212
Negative	12(43)	17(35)	(0.41)	19(43)	10(29)	(1.55)
Positive	16(57)	31(65)		25(57)	24(71)	
HER2-HER4 dimer			0.761			0.272
Negative	12(48)	19(44)	(0.09)	21(51)	11(38)	(1.20)
Positive	13(52)	24(56)		20(49)	18(62)	
HER2,1 vs HER2,3			0.690			0.127
HER2,1 low-HER2,3 low	7(26)	10(23)	(1.46)	10(25)	7(22)	(5.69)
HER2,1 low-HER2,3 high	0(0)	2(5)		3(8)	0(0)	
HER2,1 high-HER2,3 low	4(15)	5(11)		7(17)	2(6)	
HER2,1 high-HER2,3 high	16(59)	27(61)		20(50)	23(72)	
Her2,1 vs HER2,4			0.750			0.596
HER1,2 low-HER2,4 low	7(29)	8(21)	(1.21)	11(29)	5(19)	(1.88)
HER1,2 low-HER2,4 high	0(0)	1(3)		1(3)	0(0)	
HER1,2 high-HER2,4 low	4(17)	8(20)		7(18)	5(18)	
HER1,2 high-HER2,4 high	13(54)	22(56)		19(50)	17(63)	
Her2,3 vs HER2,4			0.945			0.097
HER2,3 low-HER2,4 low	8(32)	12(29)	(0.37)	14(36)	7(24)	(1.63)
HER2,3 low-HER2,4 high	2(8)	2(5)		4(10)	0(0)	
HER2,1 high-HER2,4 low	4(16)	7(17)		7(18)	4(14)	
HER2,3 high-HER2,4 high	11(44)	20(49)		14(36)	18(62)	

3.3.1 Outcome analysis

3.3.1.1 Univariate analysis in the primary series

The univariate analysis of MAPKs within the whole series of BC revealed that pan ERK and all nuclear phosphorylated forms, were associated with prolonged BCSS (pan ERK1/2: p=0.043, nuclear p-ERK1/2: p=0.004, p-JNK1/2: p=0.049, p-p38: p=0.001, p-ATF2: p<0.001 and p-C-JUN: p=0.027, Figure 3-5). In addition, the subcellular localisation of p-ERK1/2 revealed that the worst combination was encountered with the cytoplasmic form was only expressed (Figure 3-6). Additionally, only nuclear p-ERK1/2 and p-ATF2 were associated with prolonged DMFS within the whole cohort (p=0.005, p<0.001, Figure 3-7).

Within ER+ tumours, nuclear p-ERK1/2, p-p38, and p-ATF2, were associated with improved BCSS (p=0.022, p=0.003, p=0.001, Figure 3-8); however, prolonged DMFS was only attributed to nuclear p-ERK1/2 and p-ATF2 (p=0.020, p=0.002, Figure 3-9).

For those patients receiving hormonal therapy, only high expression of pan ERK1/2 revealed an association with prolonged BCSS and DMFS (p=0.009, p=0.032, respectively, Figure 3-10). Interestingly, within ER+HER2- tumours, nuclear p-ERK1/2, p-p38 and p-ATF2 were associated with prolonged BCSS (p=0.024, p=0.047, p=0.007, Figure 3-11), but only p-ATF2 was associated with longer DMFS (p=0.013, Figure 3-11). Furthermore, there was no association with DMFS in the ER+HER2+ or ER-HER2+ tumours. Of worth, in those patients with LN positive disease, p-p38 was associated with better BCSS (p=0.037) while nuclear p-ERK1/2 and p-ATF2 were associated with improved both BCSS (p=0.030, p=0.002) and DMFS (p=0.014, p=0.001, Figure 3-13).

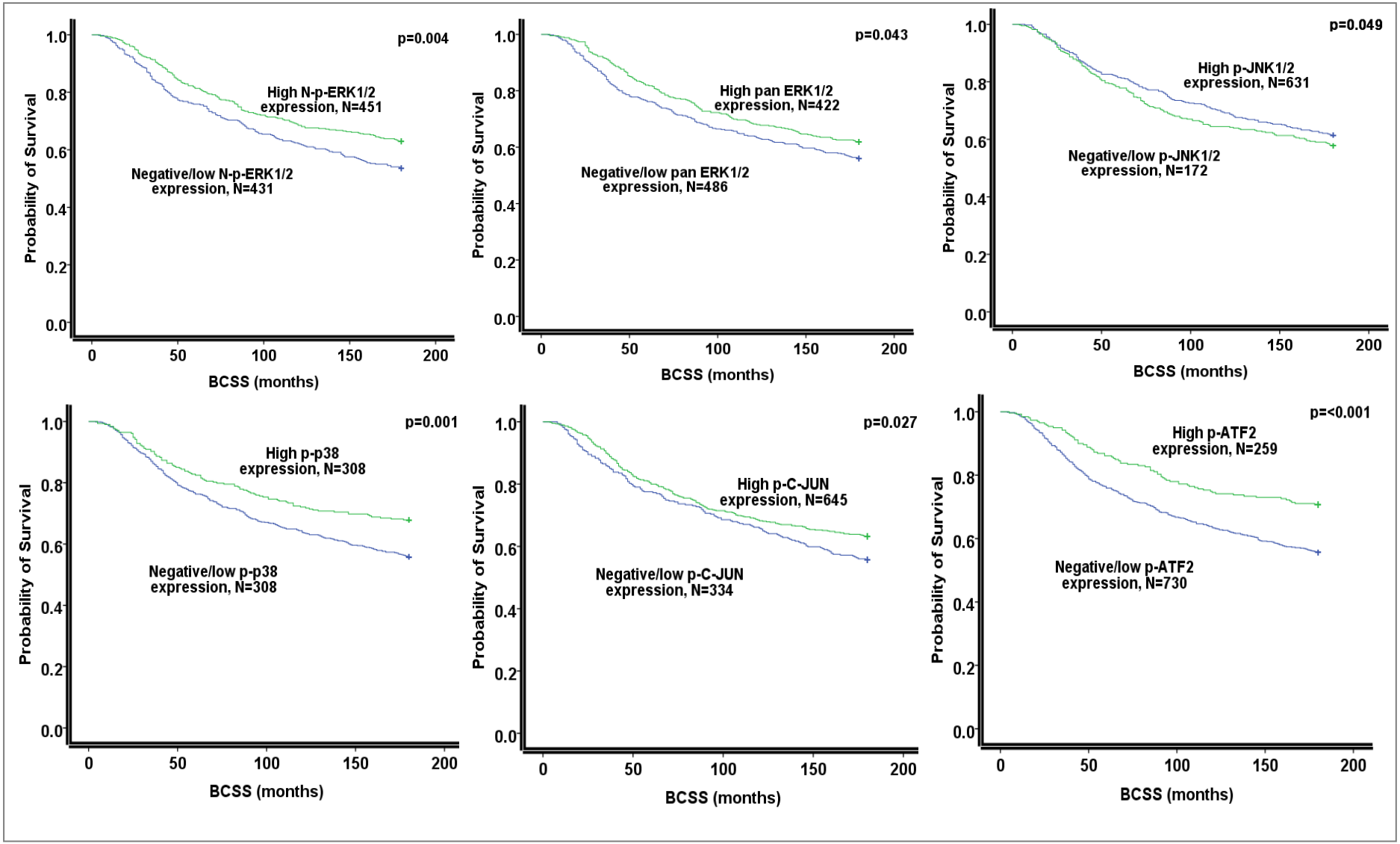


Figure 3-5: Kaplan Meier plots illustrating BCSS for MAPKs in the whole series of breast cancer

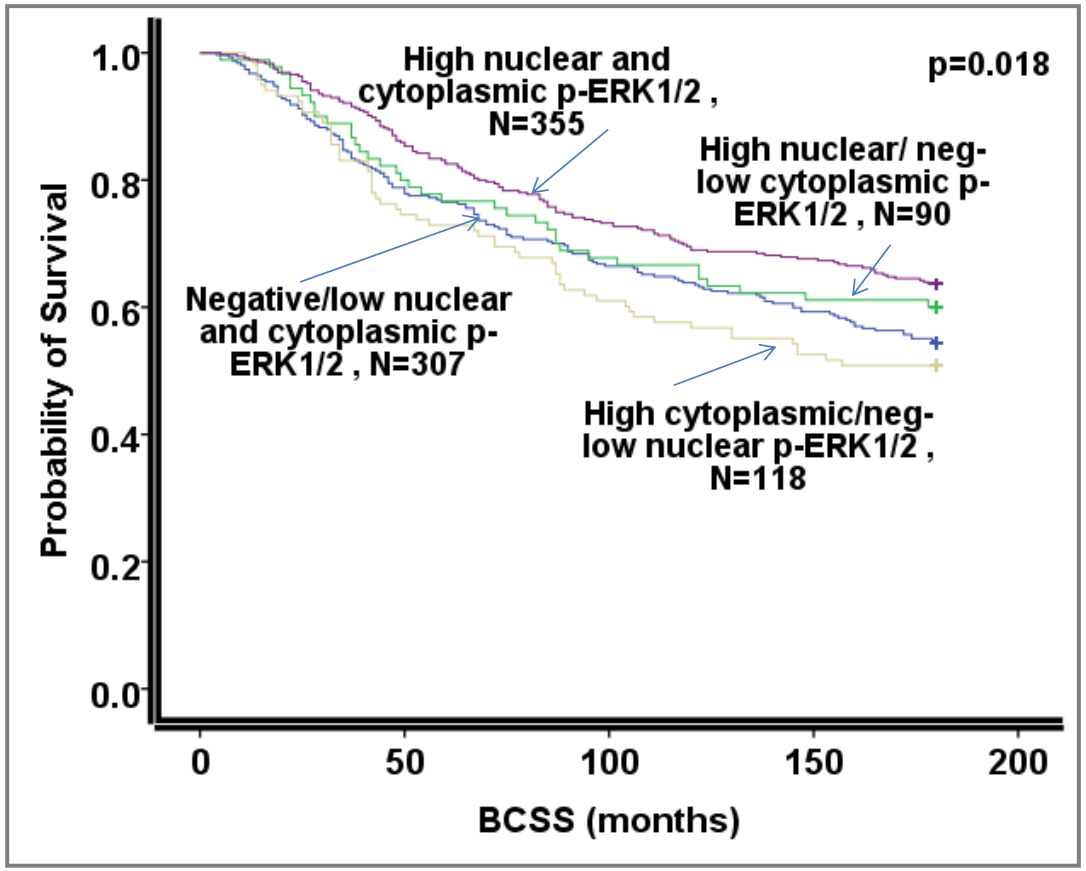


Figure 3-6: BCCS for subcellular localisation of p-ERK1/2 in the whole series of breast cancer

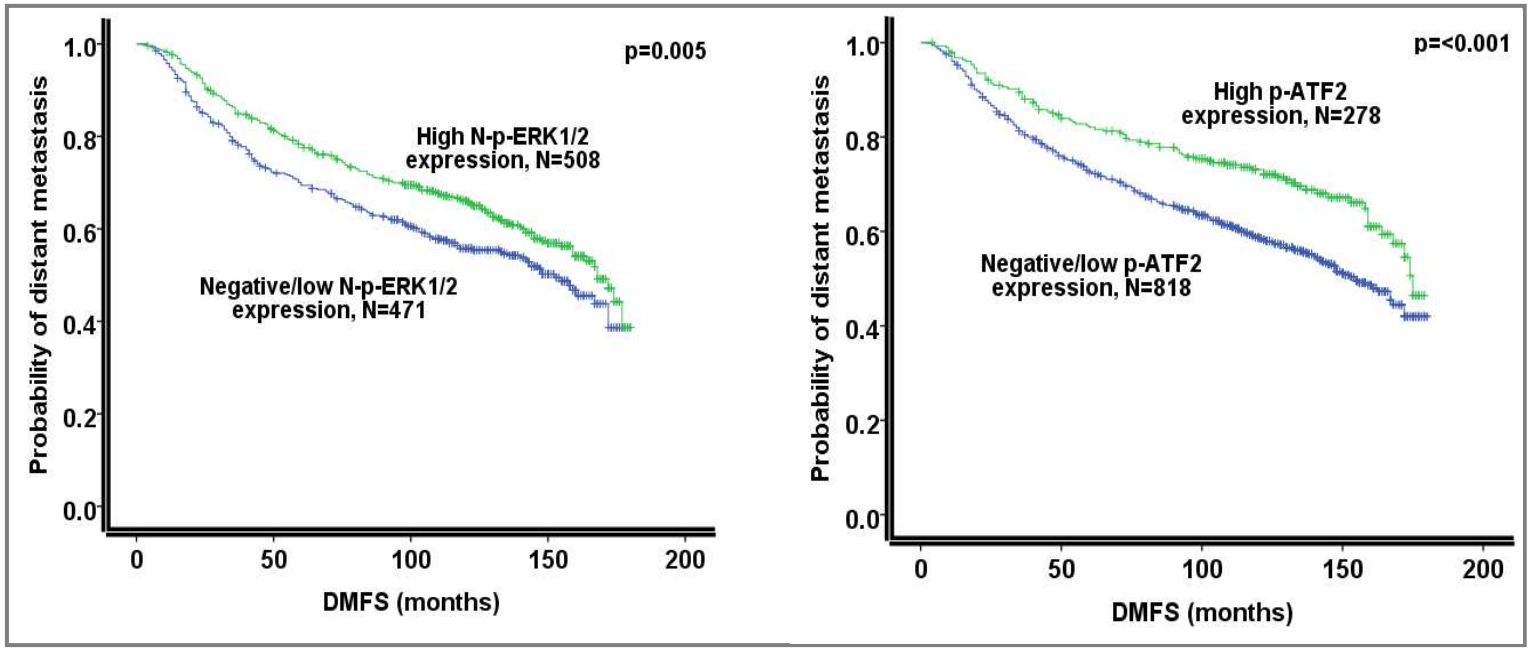


Figure 3-7: Kaplan Meier plots illustrating DMFS for MAPKs in the whole series of breast cancer

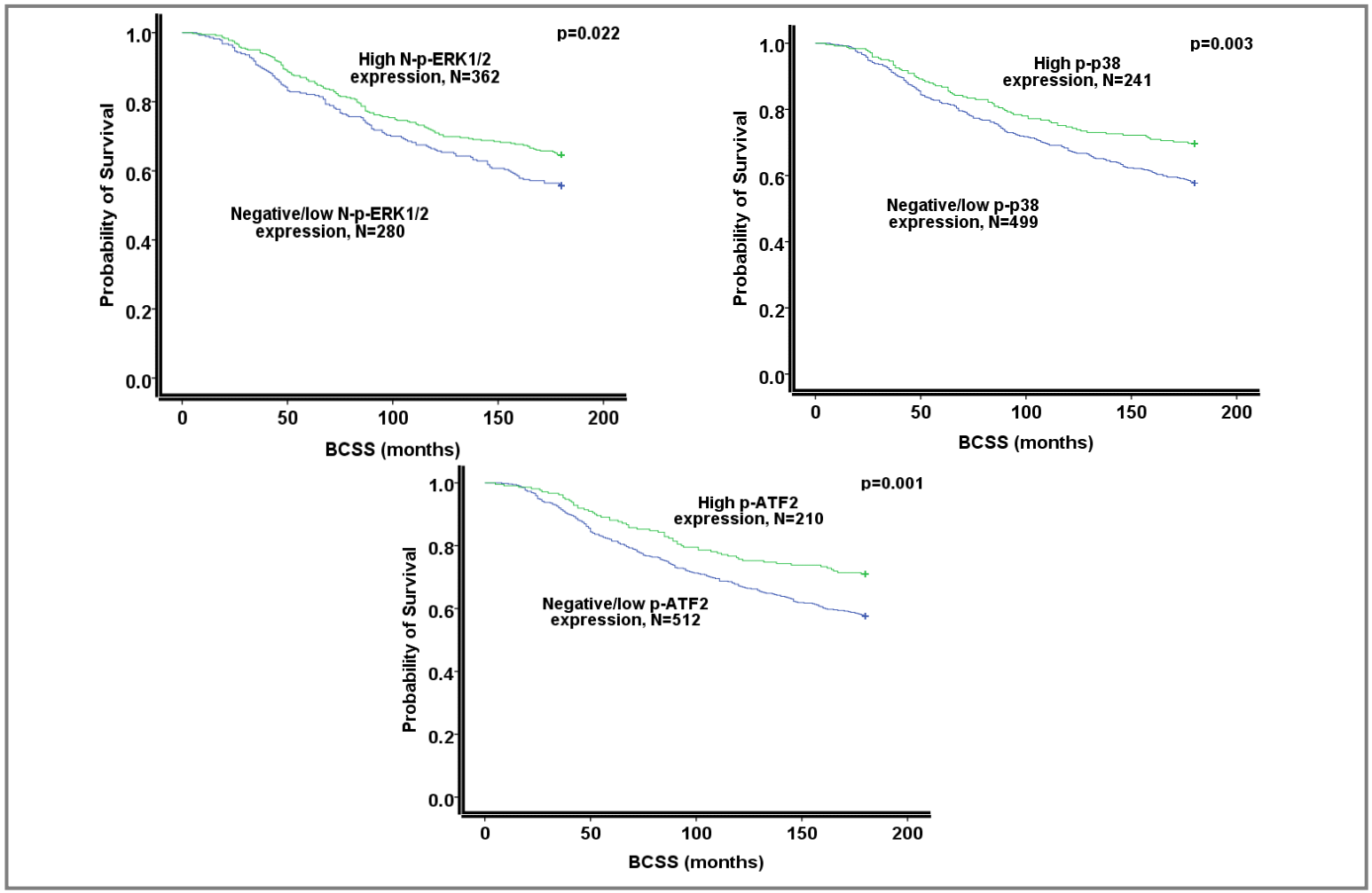


Figure 3-8: Kaplan Meier plots illustrating BCSS for MAPKs in ER+ breast cancer

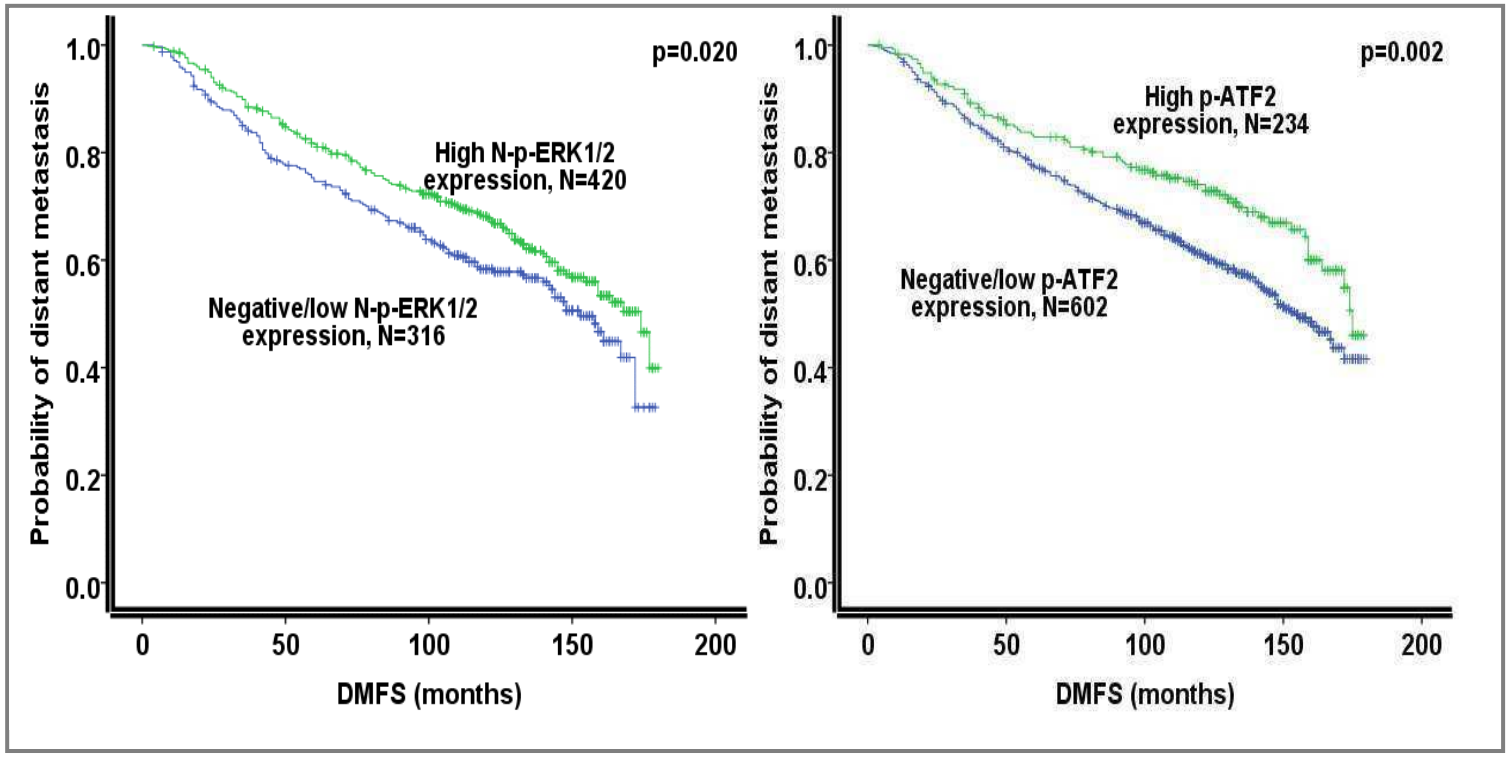


Figure 3-9: Kaplan Meier plots illustrating DMFS for MAPKs in ER+ breast cancer

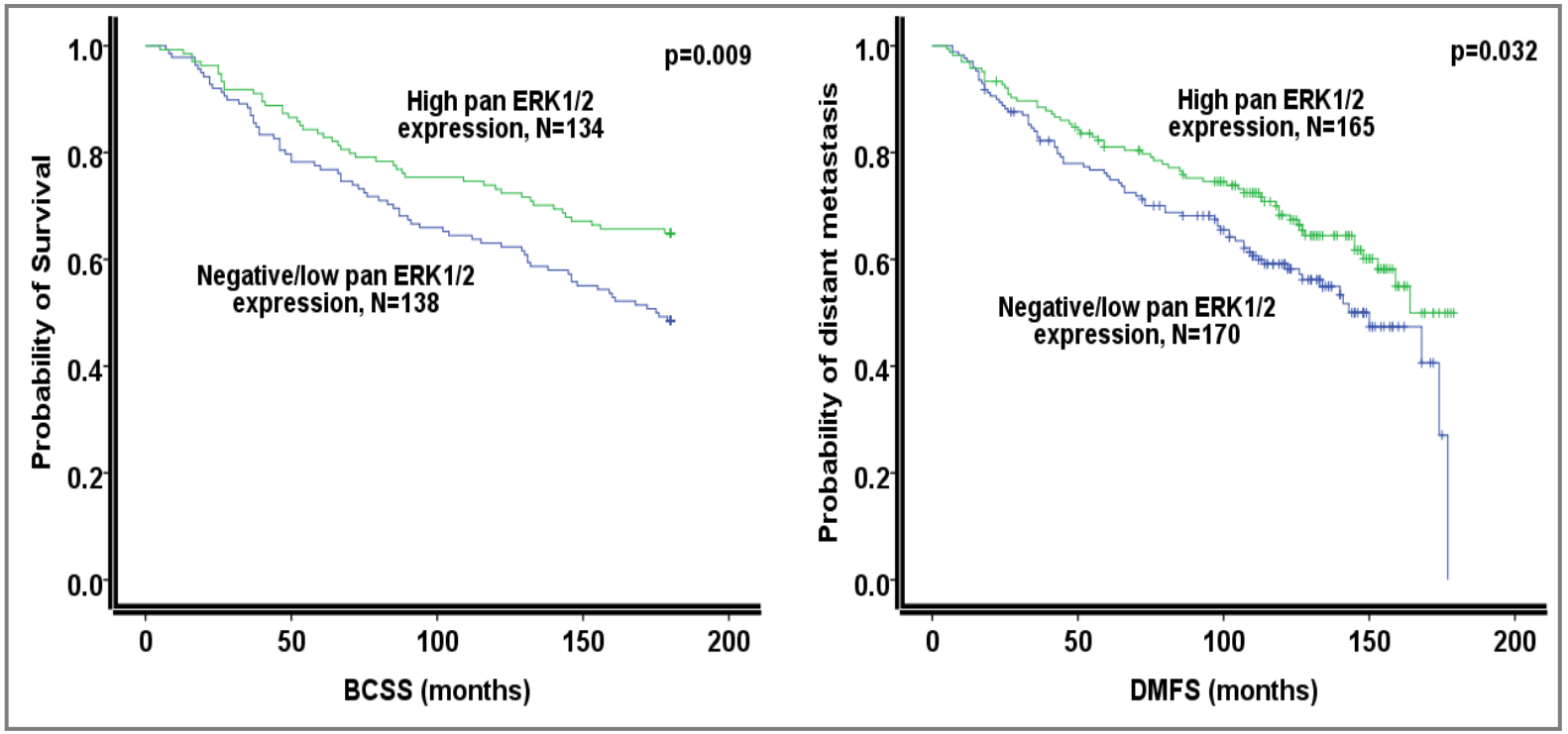


Figure 3-10: Kaplan Meier plots illustrating BCSS and DMFS for MAPKs in hormone treated patients

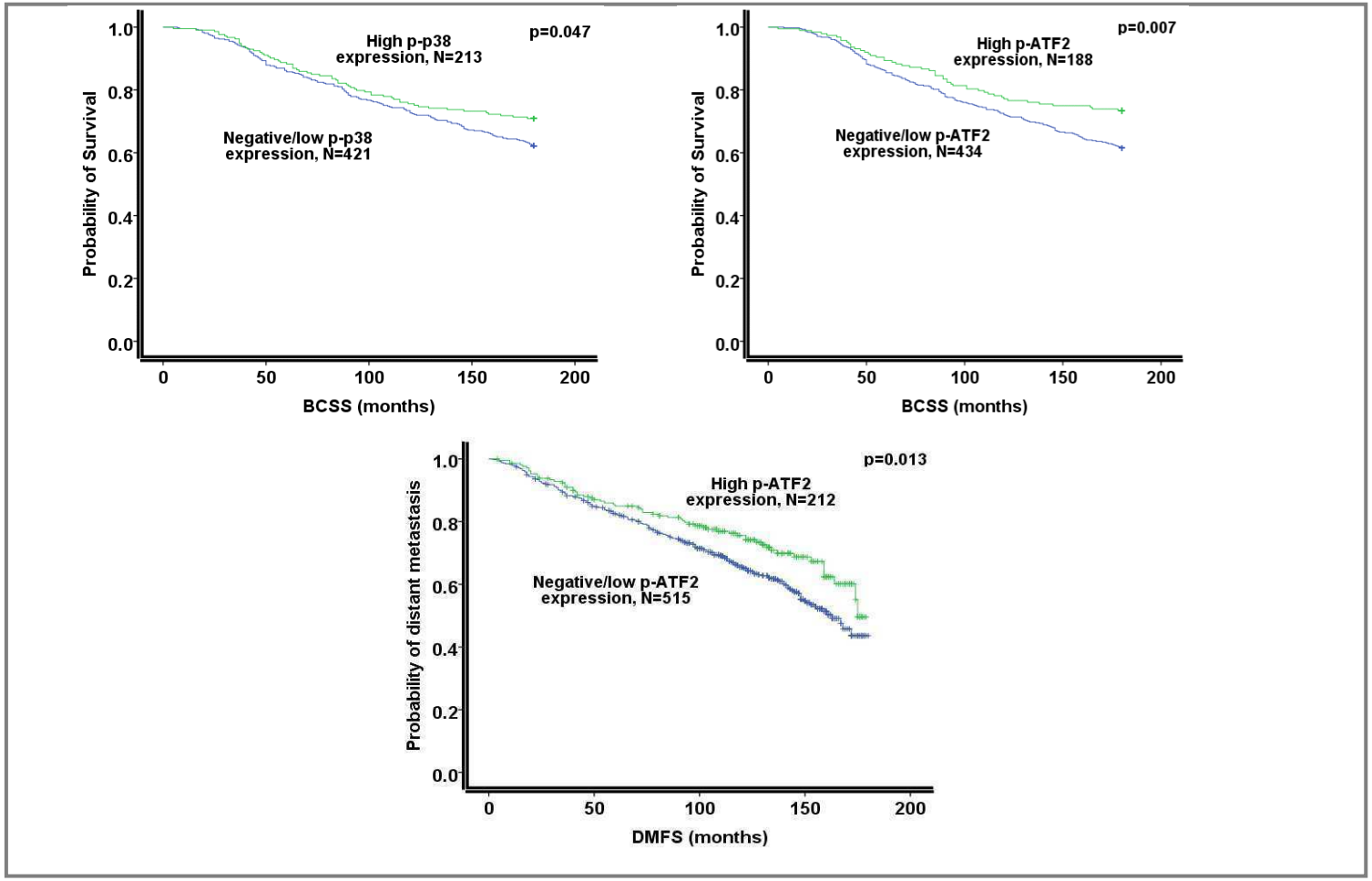


Figure 3-11: Kaplan Meier plots illustrating BCSS and DMFS for MAPKs in ER+HER2- breast cancer

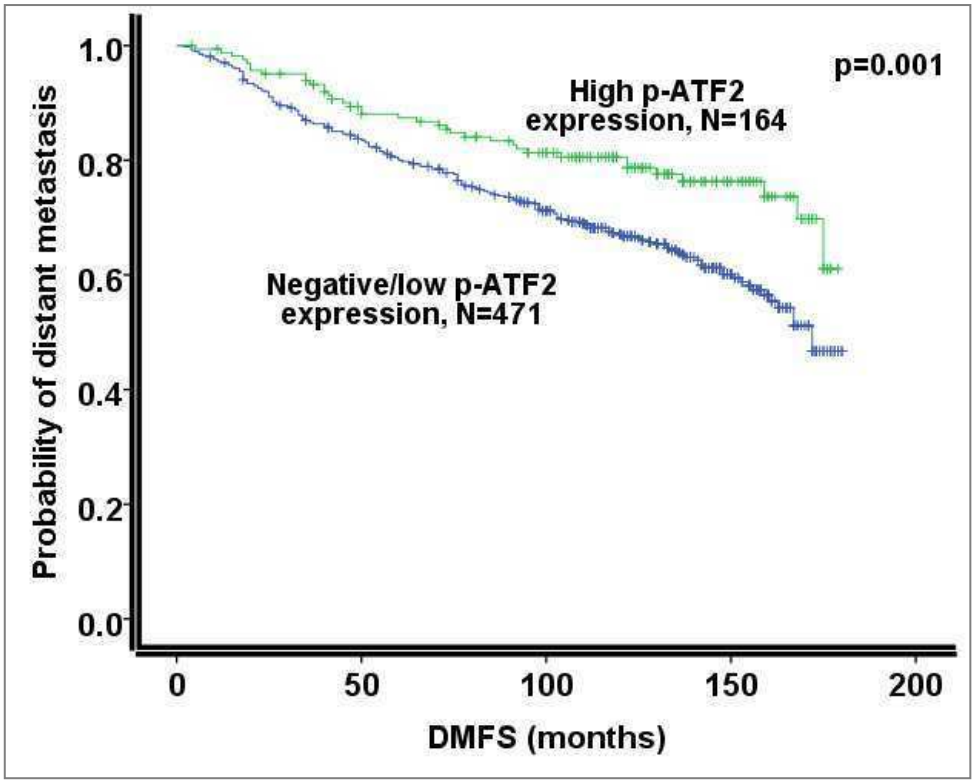


Figure 3-12: Kaplan Meier plots illustrating DMFS for p-ATF2 in ER+HER2+ breast cancer

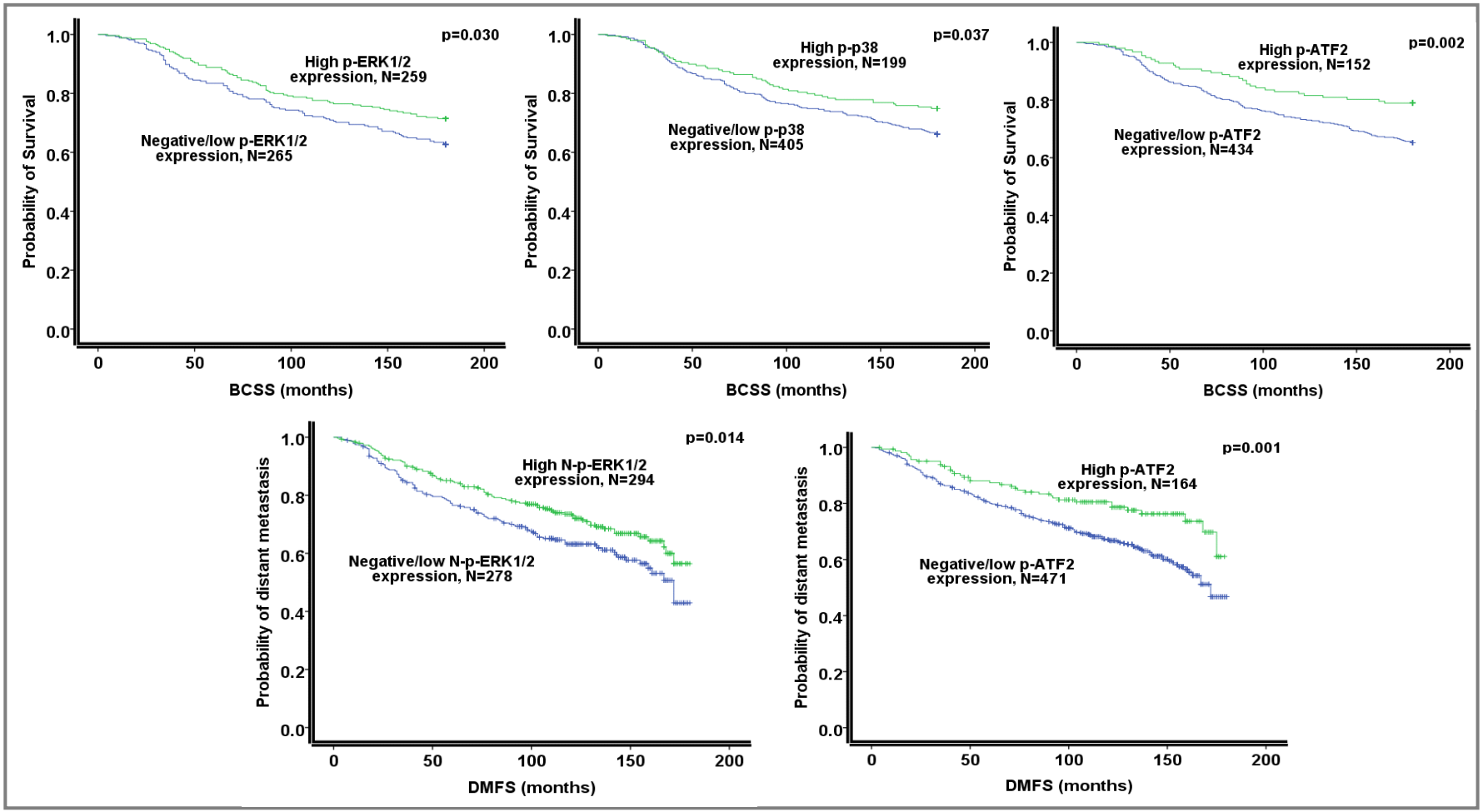


Figure 3-13: Kaplan Meier plots illustrating BCSS and DMFS for MAPKs in lymph node positive breast cancer

3.3.1.2 Univariate analysis in Trastuzumab treated Series

Regarding the association between MAPKs and outcome in Trastuzumab treated series, no significance neither for overall survival nor for DFI was determined.

3.3.1.3 Multivariate analysis (within the primary series)

Using cox regression model, MAPKs were tested against the three most powerful BC prognostic clinicopathological variables: grade, stage and size where p-p38 and p-ATF2 were both independent predictors of better BCSS (p=0.041, p=0.007) respectively (Table 3-29). p-ATF2 was also an independent predictor but of prolonged DMFS (p=0.001, Table 3-29). In ER+ tumours, only p-ATF2 was an independent predictor of both longer BCSS and DMFS (p=0.019, p=0.007) respectively (Table 3-30), but interestingly, both pan ERK1/2 and p-ATF2 were independent predictors of better BCSS and DMFS in those treated with hormonal therapy (Table 3-30).

Within ER+HER2- tumours, p-ATF2 was an independent prognostic factor which indicated better BCSS (p=0.038, Table 3-31).

Table 3-29: Cox multivariate Regression model for the predictors of survival in the whole (unselected) breast cancer series

Variable	BCSS/ unselected BC			
	***P*-value**	**Hazard ratio (HR)**	**95% CI**	
Tumour size	**0.035**	1.251	1.016	1.540
Stage	**0.000**	1.918	1.667	2.206
Grade	**0.000**	1.881	1.588	2.228
p-p38	**0.032**	.778	.619	.979
Variable	**BCSS/ unselected BC**			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.007**	1.335	1.082	1.648
Stage	**0.000**	1.947	1.686	2.249
Grade	**0.000**	1.929	1.619	2.299
p-ATF2	**0.001**	.643	.498	.830
Variable	**DMFS/ unselected BC**			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.004**	1.334	1.095	1.624
Stage	**0.000**	1.731	1.508	1.986
Grade	**0.000**	1.524	1.308	1.775
p-ATF2	**0.001**	.679	.536	.858

Table 3-30: Cox multivariate Regression model for the predictors of survival in ER+ tumours

Variable	BCSS/ ER+ tumours			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.003**	1.473	1.141	1.901
Stage	**0.000**	1.709	1.423	2.052
Grade	**0.000**	2.092	1.716	2.551
p-ATF2	**0.019**	.707	.529	.945
Variable	**DMFS/ER+ tumours**			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.000**	1.611	1.278	2.030
Stage	**0.000**	1.617	1.371	1.907
Grade	**0.000**	1.598	1.346	1.896
p-ATF2	**0.007**	.697	.536	.906
	BCSS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.014**	.629	.434	.912
Stage	0.784	.949	.652	1.381
Grade	**0.000**	2.017	1.506	2.701
Pan ERK1/2	**0.000**	2.566	1.776	3.708
	DMFS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	***P*-value**
Tumour size	0.488	1.132	.798	1.605
Stage	**0.000**	2.031	1.561	2.642
Grade	**0.000**	2.030	1.473	2.798
Pan ERK1/2	0.074	.732	.519	1.031
	BCSS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	
Tumour size	0.574	1.109	.774	1.588
Stage	**0.000**	1.988	1.514	2.610
Grade	**0.000**	2.629	1.866	3.702
p-ATF2	0.069	.687	.458	1.030
	DMFS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	
Tumour size	0.203	1.246	.888	1.749
Stage	**0.000**	1.949	1.509	2.517
Grade	**0.000**	1.997	1.473	2.708
p-ATF2	**0.043**	.673	.459	.987

Table 3-31: Cox multivariate Regression model for the predictors of survival in ER+HER2- tumours

Variable	BCSS/ ER+HER2- tumours			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.000**	1.688	1.303	2.186
Stage	**0.000**	1.581	1.312	1.906
Grade	**0.000**	1.555	1.288	1.877
p-ATF2	**0.038**	.738	.554	.984

3.3.2 RPPA results

High throughput RPPA was used to assess protein expression of a large panel of MAPKs proteins in 6 BC cell lines presenting different molecular classes including HER2 transfected cells. For each group of these MAPKs, associations for comparisons were carried out in four subsets; 1) within ER+ cell lines based on HER2 status (wild (W) ER+HER2- vs W and transfected (T) ER+HER2+ cell lines), 2) within HER2- cell lines based on ER status (wild ER+HER2- vs wild ER-HER2- cell line), 3) within HER2+ cell lines based on ER status (W and T ER+HER2+ vs W and T ER-HER2+), 4) within ER- cell lines based on HER2 status (ER-HER2- vs W and T ER-HER2+ cell lines) .

This study has highlighted that expression of MAPKs increased with HER2 negativity in ER+ cell lines but with HER2 positivity in ER- cell lines. It was observed that MAPKs are mainly related to ER and when it was lost, HER2 could enhance their expression. RPPA results for HER2- cell lines with variable ER status elucidated higher expression of MAPKs with ER+ status.

Interestingly, the expression of some MAPKs was variable within HER2+ (with variable ER expression) cell lines which could imply the biological difference in these groups.

Starting with p-C-RAF which is an upstream mediator (MAP3K) of MEK1/2 (MAP2K) which is the upstream of ERK1/2, there was not only a significant increase in the expression of p-C-RAF in ER+HER2- (MCF-7) cell line compared to T ER+HER2+ (p=0.001) but also a high expression of this protein in W ER-HER2+ cell line compared to ER-HER2- (p=0.006). In addition, this protein revealed increased expression in T ER+HER2+ cell line vs T ER-HER2+ one (p=0.022, Figure 3-14).

Importantly, For p-MEK1/2, the same association was noticed as p-C-RAF but there was an increase in its expression in T ER-HER2+ compared to T ER+HER+ and ER-HER2- cell lines (p=0.022 and p=0.024, respectively). Regarding ERK1/2 and its phosphorylated form, both showed an increase in their expression in ER+HER2- vs (W and T) ER+HER2+ (statistically significant for pan ERK1/2 only (p<0.001, both W and T). Moreover, both proteins showed high expression in W ER-HER2+ vs ER-HER2- cell line (pan ERK1/2: p>0.001, p-ERK1/2: p=0.004) but p-ERK1/2 expression was also increased in T ER-HER2+ vs the wild ER-HER2- cell line (p=0.011). Moreover, pan ERK1/2 showed increased expression

in W ER-HER2+ vs (T and W) ER+HER2+ cell line (p=0.002 and p=0.021, respectively). In addition, both proteins and their upstreams showed an increase in their expression in ER+HER2- cell line vs ER-HER2- one (Figure 3-14).

For MKK7 (upstream of JNK1/2), JNK1/2 and p-JNK1/2, all revealed, an increase in their expression in ER+HER2- vs T ER+HER2+ cell line. However, the only one which was significant is that for p-JNK1/2 (p<0.001, Figure 3-15). Furthermore, p-JNK1/2 revealed an increase in its expression in T and W ER-HER2+ vs W ER+HER2+ one. While all these three MAPKs did not show higher expression in ER+HER2- vs ER-HER2- cell lines, p-MKK7, showed an increase in its expression in ER- HER2+ vs ER-HER2- cell line (p=0.002, Figure 3-15).

Regarding MKK3/6 (upstream of p38), p38 and p-p38, an increase in the expression of these proteins was observed in ER+HER2-compared to ER+HER2+ (T) cell line and was statistically significant for p38 and its phosphorylated form (p=0.001, p=0.049, respectively); moreover, there was also an increase in the expression of pan and p-p38 in (T and W) ER-HER2+ vs ER-HER2- cell lines and this difference was significant for p38 (T, p=0.018, W, p<0.001) and for p-p38 (W, p=0.002, Figure 3-16). Moreover, these proteins showed an increase in their expression in ER+HER2- cell line vs ER-HER2- (MKK3/6, p=0.018, p38, p=0.005, p-p38, p<0.001, Figure 3-16). Regarding HER2+ BC cell lines, p38 showed increased expression in T and W ER-HER2+ cell lines vs T ER+HER2+ one (p=0.002 and p<0.001, respectively). In addition, p-p38 showed a borderline increased expression in T ER+HER2+ vs T ER-HER2+ cell line (p=0.053).

Interestingly, For the downstream transcription factors, C-JUN and its phosphorylated form, both showed an increase in their expression in ER+HER2- vs ER+HER2+ cell line and similarly, there was an increase in their expression in ER-HER2+ vs ER-HER2- cell line (not significant, Figure 3-17) but both showed increased expression in ER-HER2+ vs ER+HER2+ one (C-JUN: T and W ER+HER2- vs T ER+HER2+, both, p<0.001, p-C-JUN: T ER-HER2+ vs T ER+HER2+, p=0.018).

For p-ATF2 and p-MSK2 transcription factors, they showed an increase in their expression in ER+HER2- vs ER+HER2+ cell lines and this difference has been shown to be significant (p-ATF2: ER+HER2- vs T and W ER+HER2+, p<0.001 and p=0.049, respectively, p-MSK2: ER+HER2- vs W ER+HER2-, p=0.056,

borderline); however, only p-ATF2 showed an increased expression in ER+HER2- vs ER-HER2- cell line (p<0.001, Figure 3-17 and Figure 3-18).

Regarding other downstream transcription factors (p-ELK1, p-SMAD3 and p-STAT3), they did not show variable expression within HER2+ cell lines, instead, their expression was increased in ER+HER2+ vs ER+HER2- cell lines (p-SMAD3: T and W ER+HER2+ vs ER+HER2-, p<0.001and p=0.001, respectively, p-STAT3: T and W ER+HER2+ vs ER+HER2-, p=0.006 and p=0.022, respectively and p-ELK1: T and W ER+HER2+ vs ER+HER2-, p<0.005 and p=0.003, respectively) but their expression in T ER-HER2+ compared to ER-HER2- cell line was increased similar to other MAPKs and even other transcription factors downstream of MAPKs (p-SMAD3: T ER-HER2+ vs ER-HER2-, p=0.001, p-STAT3: T ER-HER2+ vs ER-HER2-, p=0.061). Finally, these 3 proteins did not show an increase in their expression in ER+HER2- vs ER-HER2- cell line (Figure 3-18).

Additionally, as an extra validation step for RPPA, WB has been used to check the expression of p-ERK1/2 in the six BC cell lines and it was concordant with IHC and RPPA as the expression of this protein was increased in ER+HER2- cell line (MCF-7) and in ER-HER2+ W and T cell lines (MCF-7-HER2+ and SKBR3, respectively, Figure 3-19)

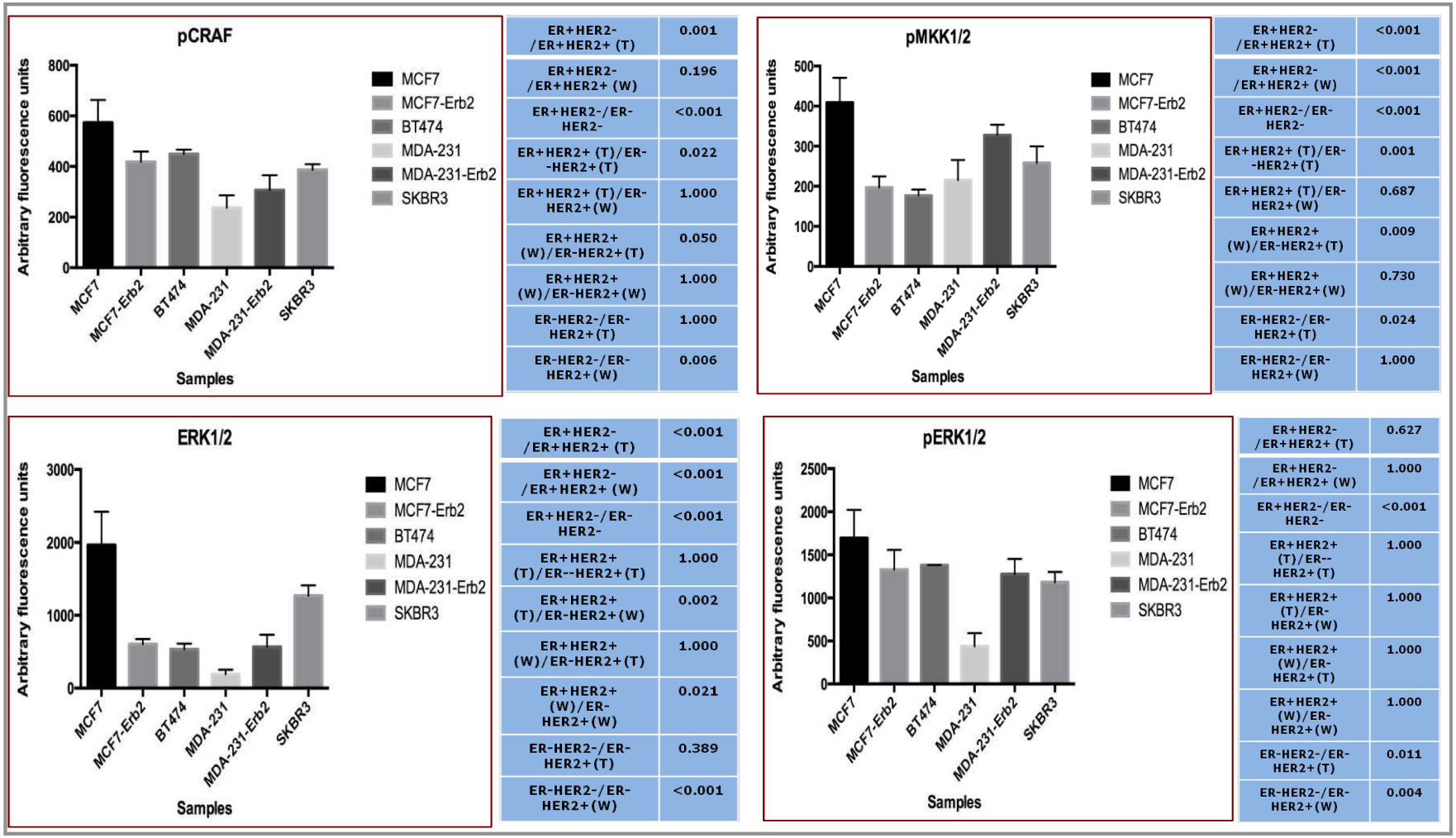
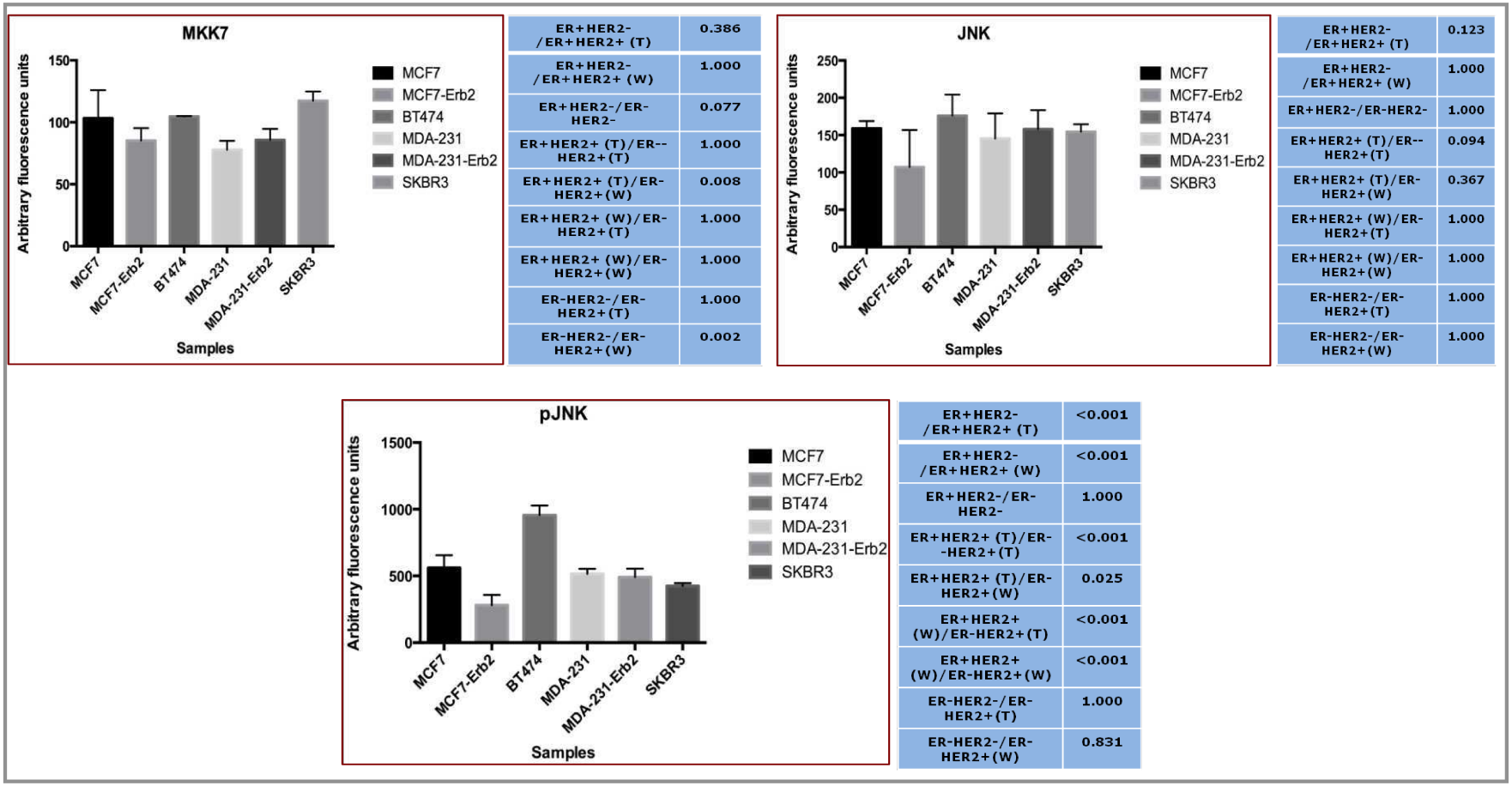


Figure 3-14: Graphical representation of the expression of p-C-RAF, p-MKK1/2, ERK1/2 and p-ERK1/2 in six BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ wild (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test).

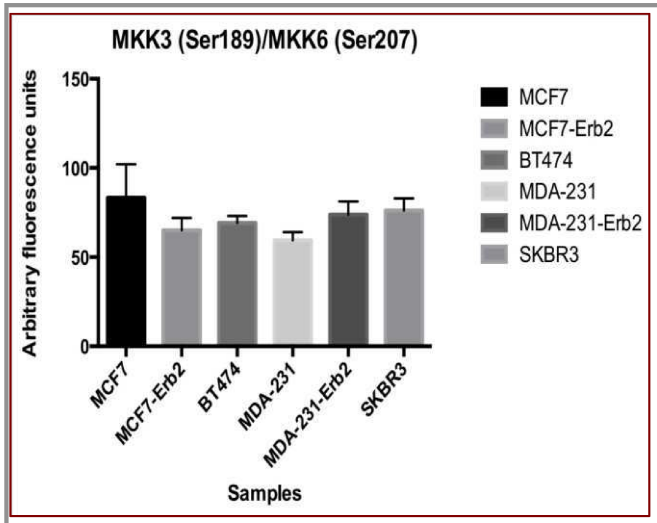


ER+HER2-/ER+HER2+ (T)	0.386
ER+HER2-/ER+HER2+ (W)	1.000
ER+HER2-/ER-HER2-	0.077
ER+HER2+ (T)/ER--HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	0.008
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	0.002

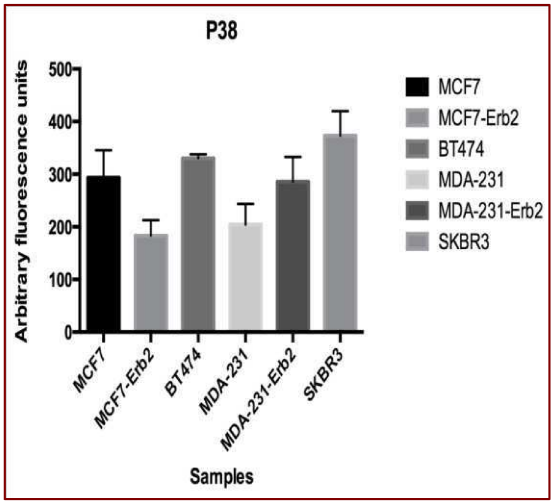
ER+HER2-/ER+HER2+ (T)	0.123
ER+HER2-/ER+HER2+ (W)	1.000
ER+HER2-/ER-HER2-	1.000
ER+HER2+ (T)/ER--HER2+(T)	0.094
ER+HER2+ (T)/ER-HER2+(W)	0.367
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	1.000

ER+HER2-/ER+HER2+ (T)	<0.001
ER+HER2-/ER+HER2+ (W)	<0.001
ER+HER2-/ER-HER2-	1.000
ER+HER2+ (T)/ER--HER2+(T)	<0.001
ER+HER2+ (T)/ER-HER2+(W)	0.025
ER+HER2+ (W)/ER-HER2+(T)	<0.001
ER+HER2+ (W)/ER-HER2+(W)	<0.001
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	0.831

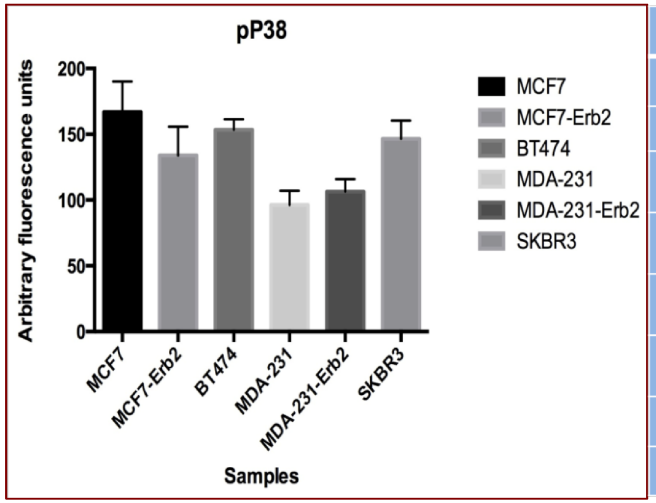
Figure 3-15: Graphical representation of the expression of p-MKK7, JNK1/2 and p-JNK1/2 in six BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test).



ER+HER2-/ER+HER2+ (T)	0.097
ER+HER2-/ER+HER2+ (W)	1.000
ER+HER2-/ER-HER2-	0.018
ER+HER2+ (T)/ER--HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	1.000
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	0.235



ER+HER2-/ER+HER2+ (T)	0.001
ER+HER2-/ER+HER2+ (W)	1.000
ER+HER2-/ER-HER2-	0.005
ER+HER2+ (T)/ER--HER2+(T)	0.006
ER+HER2+ (T)/ER-HER2+(W)	<0.001
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	0.018
ER-HER2-/ER-HER2+(W)	<0.001



ER+HER2-/ER+HER2+ (T)	0.049
ER+HER2-/ER+HER2+ (W)	1.000
ER+HER2-/ER-HER2-	<0.001
ER+HER2+ (T)/ER--HER2+(T)	0.216
ER+HER2+ (T)/ER-HER2+(W)	1.000
ER+HER2+ (W)/ER-HER2+(T)	0.053
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	0.002

Figure 3-16: Graphical representation of the expression of p-MKK3/MKK6, p38 and p-p38 in six BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test).

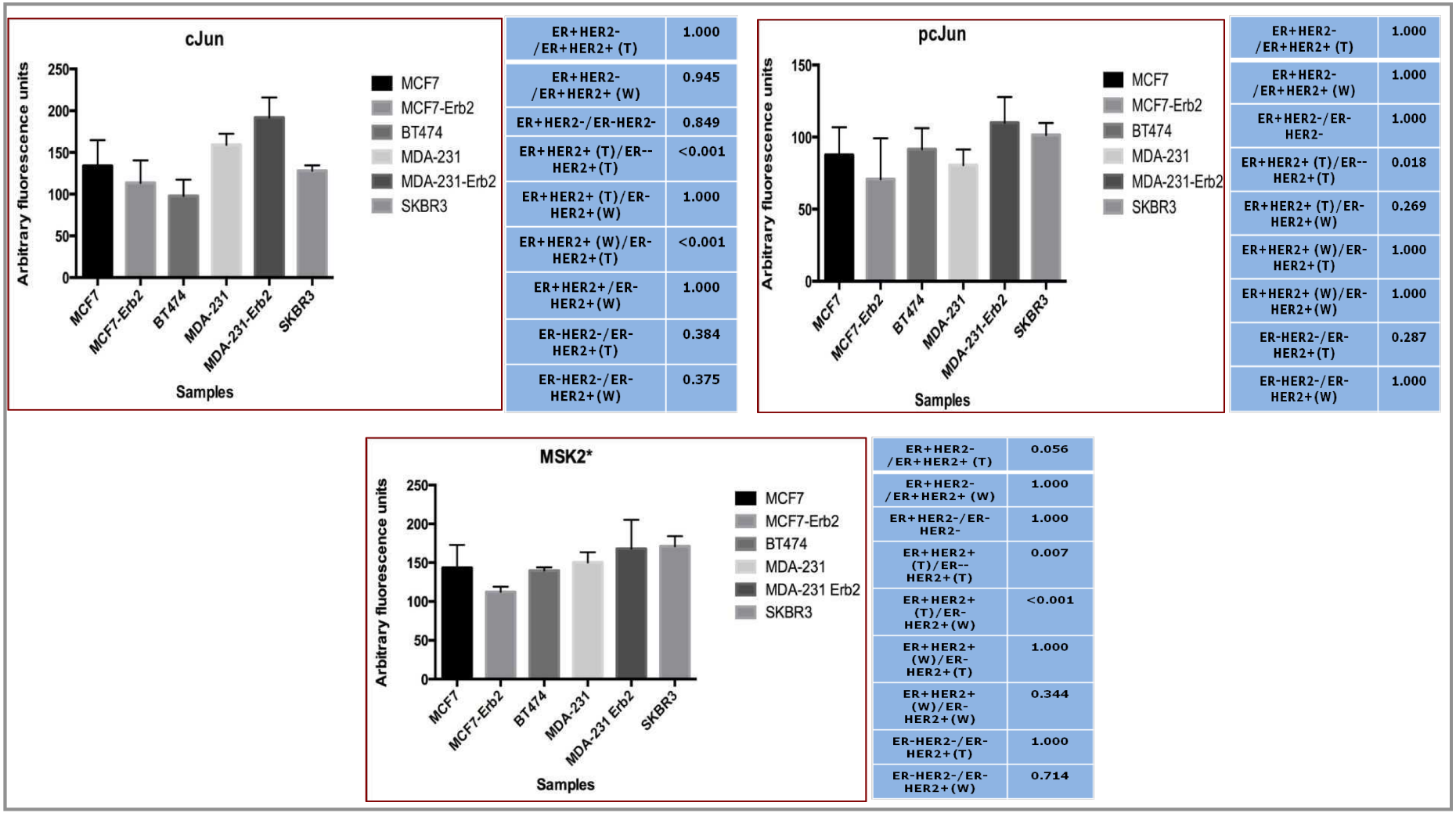
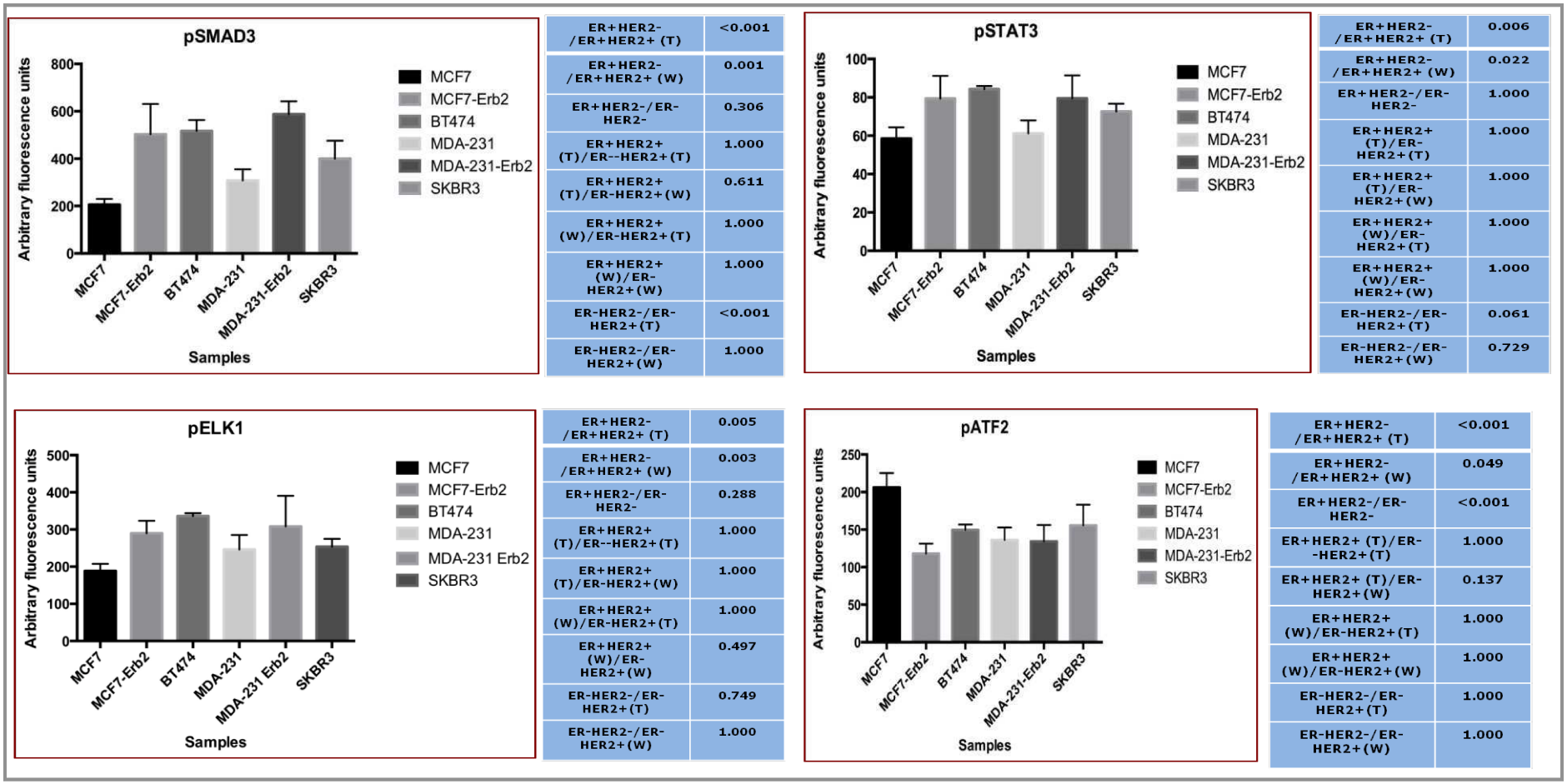


Figure 3-17: Graphical representation of the expression of C-JUN, p-C-JUN and p-MSK2 in six BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test).



ER+HER2-/ER+HER2+ (T)	<0.001
ER+HER2-/ER+HER2+ (W)	0.001
ER+HER2-/ER-HER2-	0.306
ER+HER2+ (T)/ER--HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	0.611
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	<0.001
ER-HER2-/ER-HER2+(W)	1.000

ER+HER2-/ER+HER2+ (T)	0.006
ER+HER2-/ER+HER2+ (W)	0.022
ER+HER2-/ER-HER2-	1.000
ER+HER2+ (T)/ER-HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	1.000
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	0.061
ER-HER2-/ER-HER2+(W)	0.729

ER+HER2-/ER+HER2+ (T)	0.005
ER+HER2-/ER+HER2+ (W)	0.003
ER+HER2-/ER-HER2-	0.288
ER+HER2+ (T)/ER--HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	1.000
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	0.497
ER-HER2-/ER-HER2+(T)	0.749
ER-HER2-/ER-HER2+(W)	1.000

ER+HER2-/ER+HER2+ (T)	<0.001
ER+HER2-/ER+HER2+ (W)	0.049
ER+HER2-/ER-HER2-	<0.001
ER+HER2+ (T)/ER--HER2+(T)	1.000
ER+HER2+ (T)/ER-HER2+(W)	0.137
ER+HER2+ (W)/ER-HER2+(T)	1.000
ER+HER2+ (W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	1.000

Figure 3-18: Graphical representation of the expression of p-SMAD3, p-STAT3, p-ELK1 and pATF2 in six BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test).

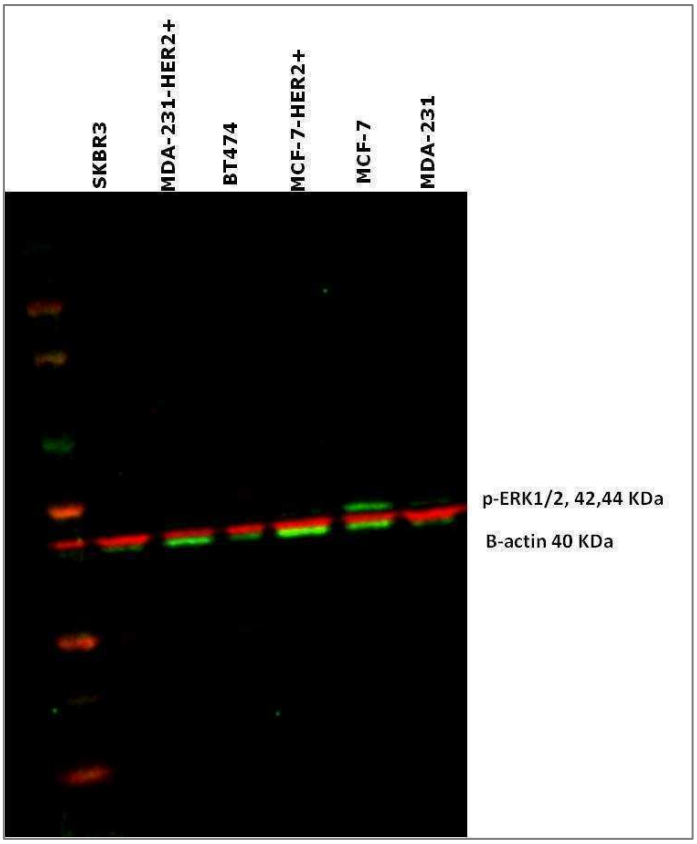


Figure 3-19: WB for p-ERK1/2 in the six BC cell lines

3.4 Discussion

Many studies have emphasised the role of MAPKs in cancer progression and different studies have been conducted using IHC or cell lines for this purpose (Reddy et al., 1999, Thrane et al., 2013, Elloumi-Mseddi et al., 2014). Nevertheless, biological and clinical significance of MAPKs expression in human BC tissue have remained largely unexplored.

In this study, the role of MAPKs was investigated in BC to determine how the expression of ER and HER2 might influence their function to indicate different biological groups. It was found that generally most of MAPKs are positively associated with good prognostic variables including lower tumour grade, an early stage and smaller tumour size in unselected and in ER+ tumours but some of these associations were lost when HER2 co-expressed with ER implying the effect of HER2. In addition, most of the associations were not encountered within ER-HER2+ BC which reflect the influence of ER loss.

There is some controversy on the role of MAPKs in influencing cellular fates and different studies have not confirmed this and importantly, the results in this chapter are supported by similar findings from other studies including Milde-Langosch et al (Milde-Langosch et al., 2005) who found that p-ERK1/2 was associated with good clinicopathological variables and Hsu et al who found that p-ERK1/2 is required for inducing apoptosis especially in MCF-7 cell line (Hsu et al., 2005). Moreover, p-JNK1/2 also has been shown to be stimulated by stress or growth factors and either one can enhance p-JNK1/2 to stimulate apoptosis (Mamay et al., 2003) and even p-JNK1/2 augments cell death signalling in slowly growing MCF-7 cells under the influence of high estradiol level(Altiok et al., 2007). Interestingly, a substance called pseudolaric acid B was used in an *in vitro* experiment and its function mimics the upstream mediators of MAPKs revealing the apoptotic function of JNK and its phosphorylated form upon activation (Yu et al., 2008).

Importantly, a plethora of studies ascertained the role of p-p38/MAPK in opposing BC progression and even more, some of these studies illustrated that this effect is mediated by Tumour Growth Factor-β (TGF-β), (Cocolakis et al., 2001, Kamaraju and Roberts, 2005, Fister et al., 2009). Importantly, a study revealed that p38 signalling inhibits BC metastasis where they indicated that stable knockdown of this protein in murine cells suppressed NF-kB p65

activation, inhibiting miR-365 expression and leading to increased IL-6 secretion. The study also reported that p38 signalling inhibits metastasis by mediated suppression of mesenchymal stem cell migration to the primary tumour and sites of metastasis, where these stem cells can differentiate into cancer-associated fibroblasts to promote tumour metastasis (Hong et al., 2015). Another study showed that p-38 can act as a tumour suppressor by regulating proliferation and transformation of tumour cells (Loesch and Chen, 2008) and probably, the bidirectional function of this protein revealed by IHC and RPPA in ER+ and ER- tumours, could imply the influence of ER expression on p38 signalling. Moreover, it has been suggested p38 has dual functions involving the regulation of survival and proliferation depending on the expression of mutant TP53 with the justification to use p38 inhibitors in this subset (Chen et al., 2009).

Moreover, apoptosis can be induced by many types of cellular stress which involves p38. These effects can be mediated by transcriptional and post-transcriptional mechanisms, influencing death receptors, survival mechanisms or pro- and anti-apoptotic BCL-2 proteins. The contribution of these different pathways to p38-induced apoptosis is presumably regulated in a stimulus- and context-dependent manner (Dolado and Nebreda, 2008). Apoptotic stimuli sometimes lead to p38 activation by alternative ways, such as the production of reactive oxygen species. This mechanism is likely to be mandatory for negatively influencing tumour initiation by p38, which enhances apoptosis in response to the expression of reactive oxygen species -inducing oncogenes in immortalised cells (Dolado et al., 2007)

p-ATF2, is thought to have dual functions independent on each other, the first is a tumour suppressor protein and the other function is related to DNA damage response pathway (Bhoumik and Ronai, 2008) and importantly, the tumour growth inhibition property of this protein has been shown by different studies (Kamaraju and Roberts, 2005, Maekawa et al., 2008, Rudraraju et al., 2014). The above results regarding the association between MAPKs and apoptosis were supported by our findings that MAPKs were positively associated with ER, BCL2 and negatively with HER2 (or no association with some of them), KI67-LI and p53. Consistent with that, is also the findings presented in this chapter regarding the positive associations between MAPKs themselves in the whole series and different BC subgroups to less extent.

It is worth mentioning that there are several observational studies which have been made in human tissue and animal models to investigate the role of MAPKs and which were in line with the results here. Firstly, some researchers based their findings on some observations that were noticed in human diploid fibroblasts and they found that RAS, which is the upstream mediator of MEK/ERK, can stop cell growth via both inducing Tp53 activity and inhibiting cyclin dependent kinases (Serrano et al., 1997) . Others attributed the growth arrest to the presence of constitutively active RAS (Zhu et al., 1998, Olsen et al., 2002). Premalignant lesions with RAS-RAF transformed tumours also have cells with senescence like growth arrest state in human cells and animal models (Braig et al., 2005, Michaloglou et al., 2005) . Furthermore, Kumagai et al (Kumagai et al., 2014) reported that high ERK activity can suppress the self-renewal of mammary cancer stem cells of mice.

In the current study, some total MAPKs were tested vs their phosphorylated forms and the latter showed some different associations rather than the total forms owing to the location of either form. For instance, pan ERK1/2 was located in the cytoplasm and p-ERK1/2 was found in the nucleus and cytoplasm but the subcellular localisation revealed distinct biological significance and in a study performed on total and phosphorylated forms of ERK 1/2, they reported that the total, unlike the other, did not show positive correlations with clinicopathological variables (ERK was assessed by WB and p-ERK was assessed by IHC), (Milde-Langosch, et al. 2005).

In our IHC data, we observed that MAPKs are related to ER and even their high expression was negatively associated with HER2 (p-ERK1/2, p-ATF2 and p-p38) in ER+ BC but with ER loss we noticed positive associations with HER2 for some MAPKs (p-p38 and pan ERK1/2). Remarkably, many studies did not indicate the differential expression of MAPKs within different biological subgroups based on ER and HER2 expressions and this perhaps could explain why different studies revealed different findings. Moreover, as our aim was to investigate the biological significance of ER+HER2+ BC compared to those with single positive expression, we found that the expression of MAPKs was related to ER and associated proteins in ER+ BC; some of these associations were still observed to less extent and others were lost with HER2 co-expression evidenced by presence of some favourable associations and loss of others.

There were some observed differences in the associations of MAPKs between HER2+ tumours in the primary and in the Trastuzumab treated series as in case of the former, the analysis was within HER2+ cohorts with either ER+ or ER- expression individually but in Trastuzumab treated series, the analysis was considered with HER2+ cohort (irrespective of ER status). In addition, some differences were encountered in the related clinicopathological variables of both cohorts as the grade and stage parameters were not similar.

In the light of some differences found with other studies, it seems that the constitution of the cohort itself could have impact on the expression and behavior of these proteins; in this context, a study conducted by Adeyinka et al (Adeyinka et al., 2002) considered a group of ER+ / LN- cases with limited number of cases compared to ours and even they used different p-ERK1/2 antibody; nevertheless, they did not confirm their results by univariate or multivariate analysis. In our study, RPPA results were considerably consistent with IHC results and it is likely that other factors that can affect the consistency of the findings did not cause conflict. For those proteins (upstream and downstream ones) that have been only tested using RPPA, their results were comparable to others tested by IHC.

By using RPPA, we indicated that the expression of some MAPKs was increased in ER+HER2- vs ER+HER2+, and ER-HER2+ vs ER-HER2- cell line. In line with the latter finding, Ostrakhovitch and Cherian showed that the expression of MAPKs is associated with loss of ER phenotype especially in those expressing HER2 (Ostrakhovitch and Cherian, 2005). Meanwhile, these findings ascertain that the role of these MAPKs is dependent on cell context and that HER2 and ER expressions are more likely to alter the expression of these proteins. In spite of the differences observed between ER+HER2+ and those with single positivity groups in RPPA with regard to the expression of MAPKs, similar to IHC, still some associations were not significant and this is expected because ER+HER2+ groups could harbour some of the features of ER+ group and other features could be due to the effect of HER2 expression.

p-c-RAF, being the upper tier of MAPK pathway was decreased in ER+ cell lines after HER2 transfection but was positively associated with HER2 in ER- cell lines which was consistent with IHC results for other MAPKs. Consistently, the expression of the middle tier MEK1/2 was also decreased after HER2 transfection in MCF-7. Furthermore, p-MEK1/2 expression was increased in ER-HER2+ vs the

double positive cell line. These findings imply the differential function of these MAPKs with HER2 in ER+ and ER- tumours. In the same scenario, ERK1/2 and p-ERK1/2 expression was decreased in the ER+ cell line after HER2 transfection; however, its expression was increased after HER2 transfection in ER- cell line and this was revealed in IHC indicating a compatible result between both techniques. P-JNK1/2 showed decreased expression in the ER+ cell line after HER2 transfection and although this result was not observed in IHC as the association between p-JNK1/2 and HER2 was not significant, still this result shown in RPPA regarding p-JNK1/2 is a supporting point for the possible actual negative association with HER2 in ER+ context.

Interestingly, although p-C-JUN expression was shown to be increased in MCF-7 vs double positive cell line the association was not significant and even p-C-JUN did not reveal a negative association with HER2 in ER+ tumours in IHC, meanwhile, it showed an increased expression in ER-HER2+ vs double positive cell lines. Taken together all these findings of p-C-JUN with the known oncogenic function of this transcription factor, it appears that it warrants further investigation. Importantly, some researchers attributed the difference in the behaviour of C-JUN protein to the availability of other JUN proteins that can have impact on C-JUN function. For instance, JUNB and JUND are often considered to be negative regulators of proliferation. Fibroblasts derived from mice with JUNB overexpression showed decreased proliferation, whereas JUND-deficient immortalized fibroblasts revealed positive association with proliferation(Passegue and Wagner, 2000, Weitzman et al., 2000). Nevertheless, reduced proliferation was also evident in primary JUND-deficient fibroblasts (Weitzman et al., 2000) implying that JUND can both positively and negatively regulate cell-cycle according to the cellular context. JUNB and JUND can modify the c-JUN-mediated activation or repression of crucial regulators of cell-cycle progression(Pfarr et al., 1994).

Taking other tissues into consideration, some studies indicated that C-JUN has dual role and the best example is its bidirectional role in neuronal cells and hepatocytes where in the former its induces the expression of proapoptotic molecules (Bim: member of BCL2 family), (Zhang et al., 2002)a(Yang et al., 1997), while its deficiency causes death of hepatocytes of mouse model due to its inhibitory effect on Tp53(Eferl et al., 1999, Eferl et al., 2003).

Importantly, the HER2 driving effect was evident on some transcription factors: p-ELK1, p-SMAD3 and p-STAT3 which were increased in T ER+HER2+ and T ER-HER2+ cell line implying the potential effect of HER2. Relevant to that, Booy et al reported that p-ELK1 is an essential element for HER2 and EGFR to enhance the action of other proteins required for promoting BC proliferation and survival (Booy et al., 2011). p-STAT3, in addition was observed to be associated with differentiation and/ or growth regulation if activated in a ligand dependent manner but it was blamed for growth derangement in case of its constitutive activation (Bromberg et al., 1996). In addition, it has been reported that many tumour derived cell lines and samples from human cancers as well, contain constitutively active STAT proteins most commonly is STAT3 (Garcia and Jove, 1998) and this has been mainly observed in SRC-transformed cell lines implying its association with HER2 (Yu et al., 1995). Regarding multiple myeloma cell lines growing independently from growth factors, they found to require STAT3 to antagonise apoptosis (Catlett-Falcone et al., 1999) and likewise a considerably high proportion of head and neck tumours had constitutively active STAT3 due to aberrant EGFR stimulation (Grandis et al., 1998). Such observations for STAT3 indicate its possible oncogenic role and its probable association with HER2.

Regarding outcome, we reported the prolonged survival of BC with high expression of MAPKs. Different studies highlighted this function. For instance, Milde-Langosch et al (Milde-Langosch et al., 2005) reported that p-ERK was associated with prolong DFI and is a predictor of prolonged survival and we reported the similar results in this regard. Moreover, Busch et al (Busch et al., 2012) found in his study regarding cancer associated fibroblasts in women with BC, that high p-ERK1/2 was an independent predictor of better outcome in tamoxifen treated patients and in line with this, we reported similar findings in our study regarding ERK1/2. Similar results were found in the current study regarding p-ATF2 and were in line with others in this regard (Rudraraju et al., 2014, Knippen et al., 2009). Despite all the results above, HER2 expression appeared to influence many of these associations as most of them were lost underscoring its potential driving effect on BC compared to ER+ group. Nevertheless, only p-ATF2 showed an association with prolonged DMFS within ER+HER2+ BC and this is probably due to the effect of ER expression.

In conclusion, MAPKs are more likely to behave in cell specific context with regard to HER2 and ER expressions. Both HER2 and ER have master role in

orchestrating different associations of MAPKs with different BC pathways and their concomitant expression revealed a distinct group with different biological features from others with a remarkable driving effect of HER2 on outcome. Furthermore, IHC is an easy, cost effective method to detect the expression of MAPKs and RPPA is a useful technique for IHC results validation. Future evaluation of MAPKs using lysate from tissue blocks might be useful in terms of comparison with IHC results.

4 mTOR Signalling in Breast Cancer as a Downstream of PI3K/Akt Pathway

4.1 Introduction

Breast cancer is a major disease that needs deciphering of its different clinical and molecular aspects to fully understand its predisposing factors, molecular background and prognosis (van 't Veer et al., 2002). It has been conceived that the activated mTORC1 is one target that has a distinct role in association with key markers of BC and plays a role in controlling cell division, motility and survival being a downstream of PI3K/Akt pathway (Nagata et al., 2004b). In addition, the PI3K-AKT-mTOR pathway can have an impact on influencing the behaviour of different cells via interactions with other key pathways including ER, HER2 and MAPK. Such interactions are thought to be responsible for the different behaviours encountered in patients and may influence certain interactions that lead to prognostic differences (Nagata et al., 2004c, Berns et al., 2007b).

The PI3K is comprised of three main classes, the most important of which is class IA, which constitutes heterodimers involving a regulatory subunit (p85a, p55a, p50a, p85b, p55g) and a catalytic subunit (p110a, p110b, p110d) that can be activated by RTKs (Engelman et al., 2006). Mutations in the PIK3CA gene encoding the p110a catalytic subunit of PI3K are a focus for therapeutic implications. This, in addition to other genetic derangements in form of: PIK3CA amplification, PTEN loss, Akt mutations and RTK amplification are able to activate the PI3K pathway (Samuels et al., 2004). mTORC1 is a downstream signalling kinase of the PI3K pathway; however, its role in BC has to date revealed conflicting results (Takei and Nawa, 2014).

Although activation of the PI3k/Akt/mTOR pathway occurs in various tumours, a paramount role in BC development was evident as such cancer is more liable for mutational activation of such pathway which can be triggered by either genomic amplification or overexpression of RTKs, such as HER2, EGFR, and IGF1R (Stephens et al., 2012).

Of notice, mutations in the catalytic PI3-kinase subunit PIK3CA occur in 36% of BC with more predominance in luminal and HER2 enriched phenotypes (29%–45%) (2012, Stephens et al., 2012, Bachman et al., 2004, Saal et al., 2005). With regard to Akt, mutations in its PH domain occur exclusively in 3% of ER+ subgroup (Lauring et al., 2010, Carpten et al., 2007), additionally, PTEN deletion or mutation primarily occurs in triple negative tumours (TNTs)(2012).

It is anticipated that inhibition of PI3K/Akt/mTOR pathway is a step towards treating HER2 overexpressing tumours; however, it has been observed that targeting this pathway in HER2+ patients, can activate feedback stimulatory loop to another compensatory mechanisms which can in turn overlaps with the function of such inhibitors (Shrivastav et al., 2014). Figure 4-1 illustrates the activators and downstreams of mTORC1 and the variable cellular fates.

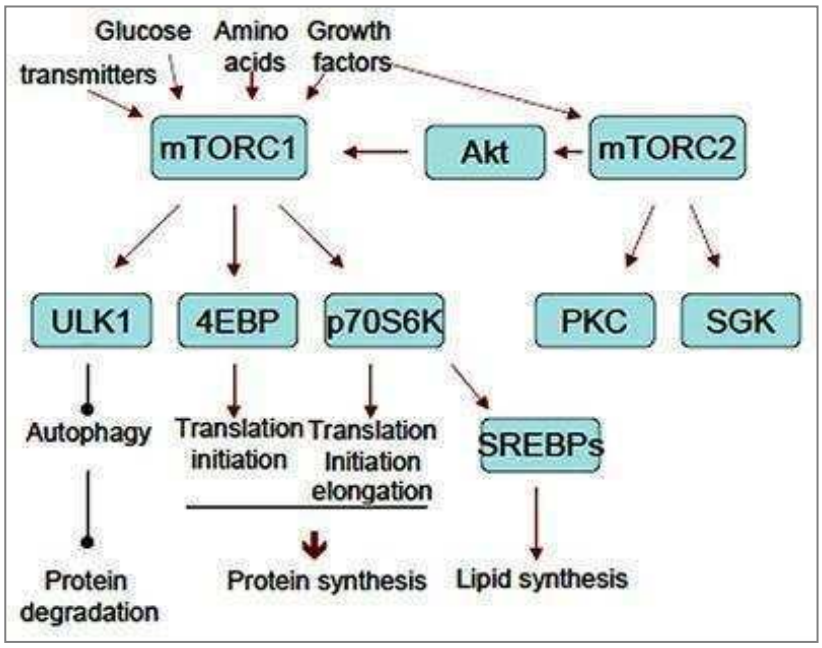


Figure 4-1: mTORC1, its upstream effectors, downstreams and different cellular effects. mTORC2 activates mTORC1 via Akt (Takei and Nawa, 2014)

4.1.1 The association between mTORC1 and hormonal therapy and the effect of mTORC1 inhibitors

It is increasingly apparent that hormone-independent BC cell growth is associated with increased PI3K/mTOR signalling and inhibition of this pathway could induce apoptosis. In addition, activation of the PI3K pathway after hormonal therapy revealed poor disease outcome (Miller et al., 2010). It has been reported that mTOR inhibition restores sensitivity to endocrine therapy in resistant BC cells expressing aberrant Akt activity (Beeram et al., 2007, deGraffenried et al., 2004). All together, these preclinical observations suggest that mTOR also plays a role in endocrine resistance.

Some investigators indicated that patients with PI3K/Akt/mTOR mutations will have more benefit from mTOR inhibitors than those with no mutations. In addition, mTOR inhibition resensitises MCF-7 constitutively expressing Akt- BC cells to tamoxifen by blocking ER and its translational effects (Janku et al., 2012). Eventually, it is thought that combining an aromatase inhibitor and an mTOR inhibitor could be beneficial to patients with HR+ tumours (deGraffenried et al., 2004). Another study combined the use of both everolimus and leterozol for those with advanced metastatic disease and has shown that this combination is safe and more useful as well than using everolimus alone (Awada et al., 2008). In line with the latter view, Baselga eta al, found that DMFS is improved in Post-menopausal HR+ BC patients after using a combination of aromatase inhibitors and everolimus and that the former is superior than tamoxifen that have been used in these patients (Baselga et al., 2012).

In spite of the above studies reports that mtor inhibitors are beneficial, it is necessary to mention that although different studies implicated the usefulness of mTORC1 inhibitors in BC management, efforts are limited in identifying the most useful biomarkers that can guide which patients can benefit the most from this proposed therapy. Interestingly, the TRAMAD trial, which is a translational study, has identified everolimus to be a useful mTORC1 inhibitor in those with low PI3K (I et al., 2013). Moreover, a retrospective study of the BOLERO-2 trial has used next generation sequencing to identify 4 common pathways which are linked with response to everolimus treatment. Furthermore, it has been revealed that everolimus is more useful in BC patients with minimal genetic changes of PI3K (GN et al., 2013).

4.1.2 Hypothesis

p-mTORC1 signalling has an important role in BC and its association with upstream PI3K/Akt proteins is evident. It is hypothesised that p-mTORC1 and other members of this pathway (upstreams and downstreams) have differential expressions in BC subtypes on the basis of ER and HER2 expressions and they have interactions with these two key proteins and their related biomarkers.

4.1.3 Aims

1- To determine the expression of p-mTORC1 in a large well characterised series of primary operable BC cases and to correlate its expression with clinicopathological variables and a panel of biomarkers related to ER and HER2 pathways, apoptosis, p53, EMT and PI3K/Akt proteins in BC and BC subgroups based on HER2 and ER expressions.
2- To assess the prognostic and predictive potential of p-mTORC1 with PI3K and Akt.
3- To determine the role of PI3K/Akt/mTORC1 in Trastuzumab treated (response and resistance) series.
4- To quantify the expression of upstream and downstream members of PI3K pathway (PI3K, AKT, p-mTORC1, PTEN, p-pTEN and p-S6K) in BC using RPPA.

4.2 Methods

Tissue microarray for breast tumours was prepared and immunohistochemical staining was performed as described (section 2.4.3., page 50) p-mTORC1 expression used in IHC was determined in TMA of the primary and Trastuzumab treated series. To quantify the expression of p-mTORC1, RPPA was used (section 2.10, page 63). For the details of concentrations used for WB and RPPA, refer to Table 2-6 and Table 2-7. In addition, a range of biological markers was used to compare with p-mTORC1 in both series (Table 2-3) and for full details of p-mTORC1 protein used, see (Table 2-5).

4.3 Results

4.3.1 Specificity of p-mTORC1 antibody

Western blot (using fluorescent method) revealed a specific band of p-mTORC1 in two BC cell lines: MCF-7 and MDA-MB-231 at the right molecular weight (289 KDa) as indicated by the supplier (Cell Signaling), (Figure 4-2).

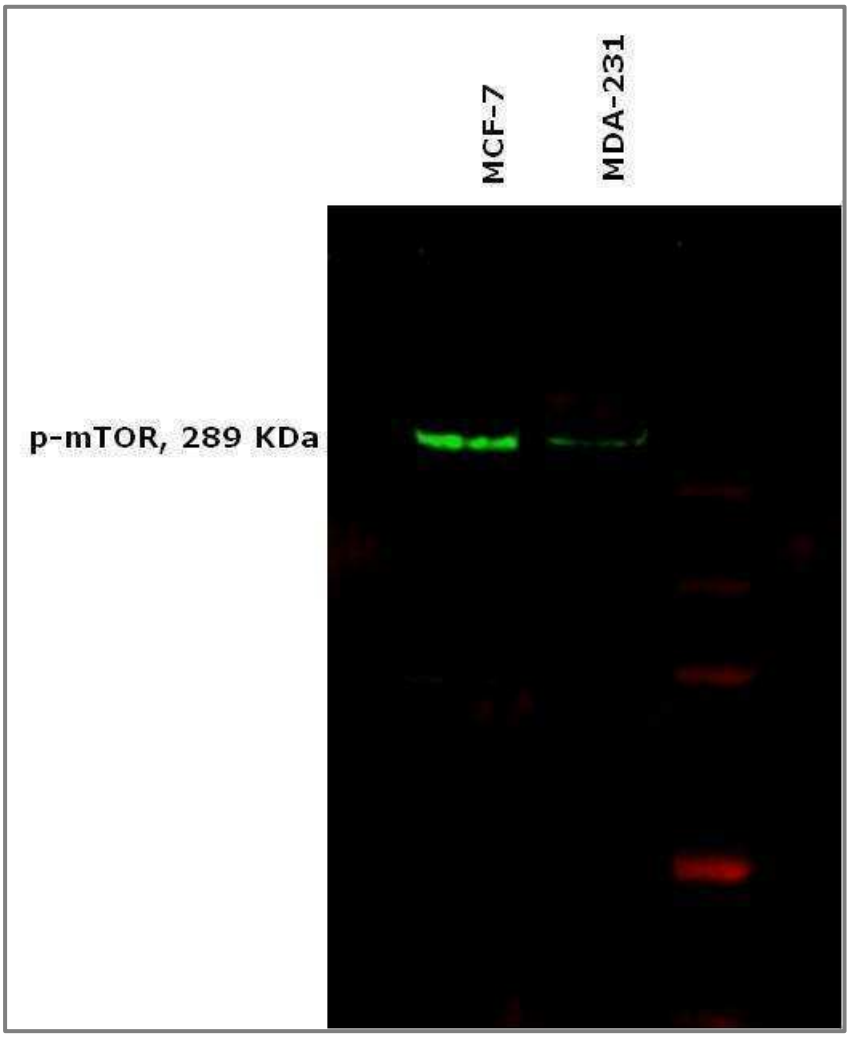


Figure 4-2: Western blot for p-mTORC1

4.3.2 Immunohistochemistry results

4.3.2.1 The expression pattern of p-mTORC1 in the primary breast cancer series

1236 cases were available for the assessment of p-mTORC1. The expression of this protein was homogenously cytoplasmic in invasive cells and in entrapped DCIS foci and normal breast tissue (Figure 4-3). The optimum cut-off point which dichotomised the cohort was set at >30 (H-score) by using X-tile software. Out of the total cases, 688 (55.7%) had negative/low expression while 548 (44.3%) cases showed high expression.

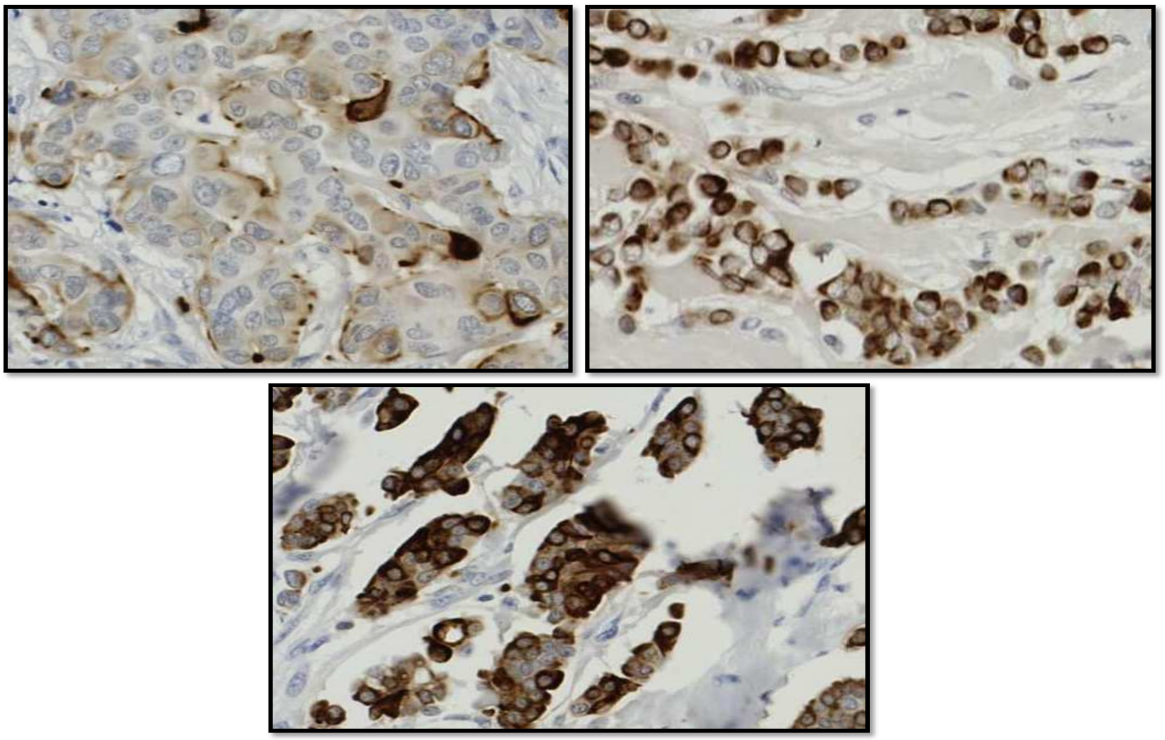


Figure 4-3: Different intensities of cytoplasmic staining of p-mTORC1, from the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20.

4.3.2.2 Associations of p-mTORC1 with clinicopathological variables in the unselected primary breast cancer series and different subgroups

High expression of p-mTORC1 was associated with good prognostic parameters: smaller tumour size (p=0.017: borderline), lobular type of BC (p=0.009), lower tumour grade and good features of all its components including extensive tubule formation, less pleomorphism and mitosis (all p=<0.001, Table 4-1). Within ER+HER2- tumours, p-mTORC1 was associated with Pre-menopausal status (p=0.023: borderline), lower tumour grade (p=0.001) and less mitotic count (p=0.016: borderline, Table 4-1). Within ER+HER2+, p-mTORC1 was associated with a trend for decreased mitosis (p=0.064) and within ER-HER2- BC, p-mTORC1 was associated with elderly patients (p=0.016: borderline, appendix table 7).

Importantly, within Akt– tumours, high expression of p-mTORC1 was associated with lobular BC type (p=0.016: borderline) and better NPI score (p<0.001). In addition, it was associated with lower tumour grade, less pleomorphism and less mitotic count (p<0.001), more tubule formation (p=0.001) and all these associations were also significant within the Akt+ cohort; however, they seem to be more significant in the former (Table 4-2).

Those BC with negative/low PI3K expression, revealed that p-mTORC1 was associated with lower tumour grade (p=0.017: borderline) and with less mitotic count (p=0.011: borderline, Table 4-3). Meanwhile, selecting the cohort to those with high PI3K expression, mTORC1 was also associated with lower tumour grade (p=0.004), more tubule formation and less pleomorphism (p=0.009, Table 4-3).

Table 4-1: The associations between p-mTORC1 and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.543			**0.023**
≤50	242(36)	186(34)	(0.370)	99(26)	140(33)	**(5.16)**
>50	435(64)	359(66)		284(74)	282(67)	
Menopausal Status			0.651			0.141
Pre-	271(40)	209(38)	(0.205)	120(31)	153(36)	(2.16)
Post-	414(60)	338(62)		262(69)	268(64)	
Tumour Size (cm)			**0.017**			0.350
≤2.0	310(46)	286(53)	**(5.59)**	198(52)	231(55)	(0.87)
>2.0	363(54)	254(47)		184(48)	188(45)	
Stage			0.140			0.187
1	420(62)	323(60)	(3.92)	246(65)	247(59)	(3.35)
2	197(29)	183(34)		110(29)	146(35)	
3	58(9)	35(6)		25(6)	25(6)	
Tumour Type			**0.009**			0.862
Ductal	566(85)	437(81)	**(11.52)**	307(81)	330(79)	(0.74)
Lobular	56(8)	9(13)		50(13)	64(15)	
Medullary-like	19(3)	6(1)		1(0)	1(0)	
Special-type	25(4)	27(5)		21(6)	24(6)	
Grade			**<0.001**			**0.001**
1	89(13)	106(20)	**(53.64)**	81(21)	94(22)	**(15.05)**
2	189(28)	232(43)		150(40)	212(51)	
3	394(59)	202(37)		149(39)	112(27)	
Tubules			**<0.001**			0.229
1	27(4)	35(7)	**(22.61)**	24(6)	32(8)	(2.94)
2	191(29)	211(40)		143(39)	176(44)	
3	434(67)	277(53)		201(55)	196(48)	
Pleomorphism			**<0.001**			0.069
1	14(2)	11(2)	**(33.01)**	13(4)	10(3)	(5.35)
2	203(31)	248(48)		174(47)	224(55)	
3	435(67)	263(50)		181(49)	170(42)	
Mitosis			**<0.001**			**0.016**
1	184(28)	225(43)	**(40.82)**	156(42)	210(52)	**(8.31)**
2	120(18)	114(22)		89(24)	92(23)	
3	348(53)	184(35)		123(34)	102(25)	
LVI			0.199			0.223
Probable/Negative	432(65)	365(68)	(1.64)	249(65)	288(69)	(1.48)
Definite	238(35)	171(32)		133(35)	128(31)	
NPI			**<0.001**			0.319
GPG	169(26)	201(39)	**(21.34)**	142(39)	179(45)	(2.28)
MPG	365(57)	253(49)		181(50)	181(45)	
PPG	112(17)	66(12)		39(11)	42(10)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 4-2: The associations between p-mTORC1 and clinicopathological variables

	Akt- tumours			Akt+ tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.110			0.972
≤50	60(46)	22(34)	(2.55)	105(33)	92(33)	(0.00)
>50	71(54)	43(66)		211(67)	186(67)	
Menopausal Status			0.075			0.360
Pre-	66(50)	24(37)	(3.16)	108(34)	105(38)	(0.83)
Post-	65(50)	41(63)		207(6)	172(62)	
Tumour Size (cm)			0.172			0.471
≤2.0	57(44)	35(54)	90.186)	147(47)	137(50)	(0.52)
>2.0	74(56)	30(46)		168(53)	139(50)	
Stage			0.073			0.924
1	80(61)	38(58)	(5.22)	193(61)	165(60)	(0.15)
2	39(30)	26(40)		98(31)	90(32)	
3	12(9)	1(2)		24(8)	21(8)	
Tumour Type			**0.016**			0.145
Ductal	113(87)	49(75)	**(10.37)**	265(85)	228(82)	(5.39)
Lobular	6(5)	8(12)		27(9)	35(13)	
Medullary-like	7(5)	1(2)		10(3)	3(1)	
Special-type	4(3)	7(11)		10(3)	10(4)	
Grade			**<0.001**			**0.001**
1	15(12)	15(23)	**(30.08)**	3912)	49(18)	**(14.16)**
2	24(18)	31(48)		99(32)	115(42)	
3	92(70)	19(29)		176(56)	112(40)	
Tubules			**0.001**			**0.020**
1	5(4)	7(11)	**(14.63)**	11(4)	15(6)	**(7.77)**
2	29(22)	28(43)		97(31)	110(41)	
3	95(74)	30(46)		199(65)	144(53)	
Pleomorphism			**<0.001**			**0.029**
1	2(2)	4(6)	**(22.39)**	3(1)	3(1)	**(7.06)**
2	31(24)	35(54)		98(32)	114(43)	
3	96(74)	26(40)		206(67)	151(56)	
Mitosis			**<0.001**			**0.007**
1	32(25)	31(48)	**(18.69)**	90(29)	112(42)	**(10.06)**
2	19(15)	16(24)		67(22)	54(20)	
3	78(60)	18(28)		15(49)	103(38)	
LVI			0.967			0.908
Probable/Negative	79(60)	39(60)	(0.00)	204(65)	180(65)	(0.01)
Definite	52(40)	26(40)		111(35)	96(35)	
NPI			**<0.001**			0.086
GPG	23(19)	25(42)	**(11.19)**	85(28)	97(36)	(4.91)
MPG	76(62)	28(46)		172(56)	130(49)	
PPG	24(19)	7(12)		49(16)	40(15)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 4-3: The associations between p-mTORC1 and clinicopathological variables

	PI3K-Negative/low			PI3K-High		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.945			0.716
≤50	28(28)	24(27)	(0.00)	95(35)	56(34)	(0.13)
>50	73(72)	64(73)		173(65)	110(66)	
Menopausal Status			0.868			0.718
Pre-	31(31)	28(32)	(0.02)	107(40)	63(38)	(0.13)
Post-	70(69)	60(68)		161(60)	102(62)	
Tumour Size (cm)			0.275			0.151
≤2.0	50(50)	51(58)	(1.19)	99(37)	72(44)	(2.06)
>2.0	50(50)	37(42)		169(63)	92(56)	
Stage			0.170			0.480
1	67(66)	51(59)	(3.54)	150(56)	83(51)	(.0146)
2	23(23)	30(34)		88(33)	63(38)	
3	11(11)	6(7)		28(11)	18(11)	
Tumour Type			0.108			0.148
Ductal	76(77)	53(60)	(6.08)	239(89)	145(88)	(5.34)
Lobular	18(18)	26(30)		9(3)	12(7)	
Medullary-like	1(1)	2(2)		12(5)	3(2)	
Special-type	4(4)	7(8)		7(3)	4(3)	
Grade			**0.017**			**0.004**
1	18(18)	22(25)	**(8.16)**	19(7)	17(11)	**(11.27)**
2	42(42)	47(54)		53(20)	53(32)	
3	40(40)	18(21)		194(73)	94(57)	
Tubules			0.866			**0.009**
1	6(6)	5(6)	(0.24**)**	4(2)	7(4)	**(9.44)**
2	32(32)	30(36)		70(27)	59(38)	
3	62(62)	49(58)		184(71)	90(58)	
Pleomorphism			0.120			**0.009**
1	4(4)	4(5)	(4.20)	3(1)	2(1)	**(9.44)**
2	49(49)	53(63)		49(19)	50(32)	
3	47(47)	27(32)		206(80)	103(67)	
Mitosis			**0.011**			0.077
1	43(43)	53(63)	**(9.04)**	43(17)	36(23)	(5.14)
2	21(21)	16(19)		41(16)	32(21)	
3	36(36)	15(18)		174(67)	88(56)	
LVI			0.911			0.768
Probable/Negative	72(72)	64(73)	(0.01)	157(59)	97(60)	(0.08)
Definite	28(28)	24(27)		110(41)	64(40)	
NPI			0.677			0.143
GPG	38(39)	38(44)	(0.78)	39(15)	36(23)	(3.89)
MPG	48(50)	41(48)		154(61)	84(53)	
PPG	11(11)	7(8)		62(24)	39(24)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

4.3.2.3 The associations of p-mTORC1 with biological markers in the unselected primary breast cancer series and different subgroups

High expression of p-mTORC1 within the whole cohort was associated with expression of ER and its associated proteins: ER, PgR, AR, CK18, CK19, BEX1, TFF1, TFF3, and GATA3 (p<0.001); in addition to CK7/8, BCL2 (both, p=0.001) and FOXA1 (p=0.002). In contrast, high expression of p-mTORC1 was associated with negative expression of P-cadherin, p53 and KI67-LI (p<0.001, Table 4-4). Within ER+HER2- tumours, p-mTORC1 was positively associated with CK18, BEX1 and GATA3 (p=0.004, p=0.032: borderline and p=0.011: borderline, respectively, Table 4-4).

High p-mTORC1 expression was associated with high Akt expression (p=0.001), low PI3K an HER1 expressions (p=0.006 and p<0.001, respectively, Table 4-5). Within ER+ tumours, when PI3K/Akt members were tested against HER2, high expression of p-mTORC1 and PTEN, displayed a negative association with HER2 expression (p=0.004and p=0.023: borderline , respectively); nevertheless, high expression of p-mTORC1, Akt, PTEN and PI3K showed a positive association with HER2 within ER- tumours (first 3: p<0.001, PI3K: p=0.002) respectively (Table 4-6). No associations were rendered within ER+HER2+ tumours but an increase in TFF1 expression (p=0.013: borderline) was revealed in association with high p-mTORC1 expression within ER-HER2+ tumours. Within ER-HER2- BC, p-mTORC1 was associated with increased expression of TFF1 but with decreased expressions of HER3 and N-Cadherin (, p=0.004, p=0.026: borderline and p=0.044: borderline, respectively, appendix table 7).

In those tumours expressing negative/low or high Akt, a direct positive association between p-mTORC1 with increased expression of HRs, CK18, CK19, GATA3 but a negative association was revealed with P-Cadherin (Table 4-7). In terms of differences between both cohorts, within Akt- BC, high expression of p-mTORC1 was associated with high expression of TFF1, E-cadherin (p=0.44: borderline, p=0.005), respectively but with decreased expression of p53 and KI67-LI (p<0.001and p=0.001, respectively, Table 4-7). On the other hand, high p-mTORC1 expression within Akt+ tumours was observed with HER1 negative expression (p<0.001) and positive BEX1, TFF3, BCL2 and HER4 expression (p=0.007, p=0.005, p=0.043 and p=0.017: borderline), respectively (Table 4-8).

High p-mTORC1 expression was associated with positive expression of ER, PgR and AR, CK18 and GATA3 in both negative and positive PI3K tumours (p<0.001, p=0.002, p=0.023: borderline, p=0.024: borderline, p=0.001) and (p<0.003, p=0.016: borderline, p=0.001, p<0.001, p=0.010: borderline), respectively, (Table 4-9). With respect to the difference between the two cohorts, within PI3K- tumours, high p-mTORC1 was associated with a trend of positive expression of CK19 and BCL2 (p=0.031, p=0.040), respectively but with a trend of negative expression of KI67-LI (p=0.033). Conversely, within PI3K+ cohort, high p-mTORC1 expression was associated with increased expression of FOXA1, BEX1 (p=0.003, p=0.007), TFF1 and TFF3 (p=0.001) but with P- Cadherin negativity (p=0.007, Table 4-9).

Regarding the association with other biomarkers, p-mTORC1 was associated with a decrease in HER1 expression within negative/low and high PI3K expressing cohort (p=0.019: borderline, p=0.008), respectively but it was associated with an increase in p-Akt expression within high PI3K expressing cohort (p=0.005, Table 4-10).

Table 4-4: The associations between p-mTORC1 and biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value**	**Neg/low N(%)**	**High N (%)**	***p*-value**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	218(32)	70(13)	**(60.70)**			
Positive	458(68)	468(87)				
PgR			**<0.001**			0.467
Negative	311(48)	157(30)	**(36.89)**	80(21)	79(19)	(0.52)
Positive	340(52)	363(70)		300(79)	337(81)	
AR			**<0.001**			0.579
Negative	257(43)	132(28)	**(24.11)**	86(24)	85(22)	(030)
Positive	346(57)	337(72)		270(76)	294(78)	
CK7/8			**0.001**			0.298
Negative	16(3)	1(0)	**(10.50)**	1(0)	0(0)	(1.08)
Positive	630(97)	520(100)		380(100)	413(100)	
CK18			**<0.001**			**0.004**
Negative	115(19)	21(5)	**(50.66)**	23(7)	8(2)	**(8.43)**
Positive	480(81)	440(95)		328(93)	362(98)	
CK19			**<0.001**			0.220
Negative	86(14)	28(6)	**(20.53)**	23(6)	17(4)	(1.50)
Positive	549(86)	482(94)		351(94)	387(96)	
FOXA1			**0.002**			0.475
Negative	254(60)	163(48)	**(9.91)**	112(48)	117(45)	(0.51)
Positive	170(40)	174(52)		122(52)	145(55)	
BEX1			**<0.001**			**0.032**
Negative	157(38)	80(24)	**(14.90)**	83(34)	66(25)	**(4.62)**
Positive	258(62)	248(76)		161(66)	195(75)	
TFF1			**<0.001**			0.115
Negative	208(56)	125(42)	**(12.37)**	109(51)	101(44)	(2.49)
Positive	166(44)	172(58)		103(49))	129(56)	
TFF3			**<0.001**			0.579
Negative	207(54.	123(41)	**(12.26)**	97(43)	97(41)	(0.30)
Positive	176(46)	180(59)		127(57)	141(59)	
GATA3			**<0.001**			**0.011**
Negative	272(72)	156(52)	**(26.28)**	117(56)	102(44)	**(6.40)**
Positive	108(28)	142(48)		91(44)	129(56)	
CD71			0.439			0.738
Negative	193(43)	156(46)	(0.60)	133(53)	136(52)	(0.11)
Positive	257(57)	185(54)		118(47)	128(48)	
CARM1			0.186			0.404
Negative	113(27)	85(25)	(3.36)	80(34)	72(28)	(1.81)
Moderate	195(47)	180(54)		116(48)	136(53)	
High	105(26)	71(21)		43(18)	50(19)	
PELP1			0.798			0.747
Negative	71(16)	61(18)	(0.45)	53(22)	51(19)	(0.58)
Moderate	291(66)	224(65)		165(64)	182(67)	
High	82(18)	60(17)		38(14)	38(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation and apoptosis						
E-Cadherin			0.416			0.722
Negative	237(37)	178(35)	(0.66)	129(35)	145(36)	(0.12)
Positive	397(63)	329(65)		244(65)	260(64)	
p-Cadherin			**<0.001**			0.614
Negative	234(43)	230(56)	**(16.11)**	197(62)	212(64)	(0.25)
Positive	316(57)	183(44)		123(38)	122(36)	
p53			**<0.001**			0.181
Negative/low	435(68)	394(77)	**(13.12)**	304(80)	343(84)	(1.78)
Positive	208(32)	115(23)		75(20)	66(16)	
KI67-LI			**<0.001**			0.259
Negative/low	195(37)	217(49)	**(14.99)**	162(51)	192(56)	(1.27)
High	335(63)	224(51)		153(49)	152(44)	
BCL2			**0.001**			0.575
Negative/low	213(45)	129(34)	**(10.21)**	66(24)	77(26)	(0.31)
High	262(55)	251(66)		213(76)	223(74)	

Table 4-5: The associations between p-mTORC1 and other biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
p-AKT			**0.001**			0.110
Negative/low	131(29)	65(19)	**(11.15)**	62(24)	51(19)	(2.55)
High	316(71)	278(81)		193(76)	223(81)	
p-PI3K			**0.006**			0.071
Negative/low	101(20)	88(22)	**(10.25)**	69(23)	78(25)	(5.28)
Moderate	136(27)	136(35)		98(33)	120(39)	
High	268(53)	166(42)		134(44)	109(36)	
PTEN			0.399			0.812
Negative	36(13)	22(11)	(0.71)	18(13)	18(12)	(0.06)
Positive	235(87)	183(89)		125(87)	136(88)	
HER1			**<0.001**			0.008
Negative	482(75)	449(86)	**(20.75)**	314(84)	370(90)	(6.94)
Positive	161(25)	72(14)		60(16)	40(10)	
HER2			0.170	-	-	-
Negative	548(86)	462(89)	(3.54)			
Positive	92(14)	57(11)				
HER3			0.976			0.772
Negative	47(8)	37(8)	(0.00)	58(156)	57(14)	(0.08)
Positive	551(92)	430(92)		314(84)	352(86)	
HER4			0.791			0.514
Negative	80(13)	62(12)	(0.07)	314(84)	370(90)	(0.42)
Positive	557(87)	452(88)		60(16)	40(10)	
HER1-HER2 dimer			0.979			0.189
Negative	279(88)	207(88)	(0.00**)**	170(99)	173(98)	(1.72)
Positive	38(12)	28(12)		1(1)	4(2)	
HER2-HER3 dimer			0.217			0.866
Negative	257(89)	183(86)	(1.52)	141(98)	162(98)	(0.02)
Positive	30(11)	30(14)		3(2)	3(2)	
HER2-HER4 dimer			0.280			0.849
Negative	297(88)	233(91)	(1.16)	165(98)	190(98)	(0.03)
Positive	41(12)	24(9)		3(2)	4(2)	
HER1,2 vs HER2,3			0.507			0.379
HER2,1 low-HER2,3 low	176(82)	129(80)	(2.33)	102(98)	105(93)	(3.08)
HER2,1 low-HER2,3 high	2(1)	4(2)		1(1)	3(2)	
HER2,1 high-HER2,3 low	9(4)	4(3)		0(0)	2(2)	
HER2,1 high-HER2,3 high	29(13)	24(15)		1(1)	2(2)	
Her1,2 vs HER2,4			0.786			0.249
HER2,1 low-HER2,4 low	163(86)	115(82)	(1.06)	94(99)	100(96)	(2.78)
HER2,3 low-HER2,4 high	3(2)	4(3)		1(1)	1(1)	
HER2,1 high-HER2,4 low	5(3)	4(3)		0(0)	3(3)	
HER2,1 high-HER2,4 high	18(9)	16(11)		-	-	
Her2,3 vs HER2,4			0.529			0.973
HER2,3 low-HER2,4 low	151(80)	107(76)	(1.21)	76(95)	92(95)	(0.22)
HER2,3 low-HER2,4 high	7(4)	3(2)		1(1)	2(2)	
HER2,1 high-HER2,4 low	8(4)	7(5)		1(1)	1(1)	
HER2,3 high-HER2,4 high	22(12)	23(17)		2(3)	2(2)	

Table 4-6: The associations of PI3K members with HER2

Biological markers	HER2 Negative N (%)	HER2 Positive N (%)	*p*-value (χ2)
Within ER+ BC			
p-AKT			0.279
Negative/low	167(88)	625(91)	(1.17)
High	22(12)	62(9)	
p-PI3K			**0.006**
Negative/low	235(25)	16(17)	**(10.34)**
Moderate	329(35)	26(27)	
High	367(40)	54(56)	
p-mTORC1			**0.004**
Negative/low	383(88)	422(94)	**(8.13)**
High	52(12)	29(6)	
PTEN			**0.023**
Negative	50(12)	0(0)	**(9.53)**
Low	85(21)	16(32)	
Moderate	124(30)	18(36)	
High	153(37)	16(32)	
Within ER- BC			
p-AKT			**<0.001**
Negative/low	86(88)	135(68)	**(12.35)**
High	12(12)	63(32)	
p-PI3K			**0.002**
Negative/low	39(16)	3(4)	**(13.27)**
Moderate	47(20)	11(13)	
High	155(64)	70(83)	
p-mTORC1			**<0.001**
Negative/low	161(80)	36(56)	**(15.05)**
High	39(20)	28(44)	
PTEN			**<0.001**
Negative	37(24)	1(2)	**(21.10)**
Low	63(41)	17(32)	
Moderate	36(24)	23(43)	
High	16(11)	12(23)	

Table 4-7: The associations between p-mTORC1 and biological markers

	Akt- tumours			Akt + tumours		
	Neg/low N (%)	**High N (%)**	***p*-value**	**Neg/low N (%)**	**High N (%)**	***p*-value**
Hormone Receptors and ER related proteins						
ER			**<0.001**			**<0.001**
Negative	56(43)	11(17)	**(12.45)**	94(30)	32(12)	**(29.88)**
Positive	75(57)	53(83)		220(70)	246(88)	
PgR			**<0.001**			**0.003**
Negative	72(55)	17(27)	**(13.979)**	138(44)	87(32)	**(8.81)**
Positive	59(45)	47(73)		175(56)	184(68)	
AR			**<0.001**			**0.003**
Negative	68(55)	14(23)	**(17.25)**	113(39)	66(27)	**(8.96)**
Positive	55(45)	47(77)		174(61)	178(73)	
CK7/8			0.059			0.141
Negative	7(5)	0(0)	(3.57)	5(2)	1(0)	(2.16)
Positive	123(95)	64(100)		307(98)	270(100)	
CK18			**0.015**			**<0.001**
Negative	33(27)	7(12)	**(5.90)**	48(16)	7(3)	**(25.86)**
Positive	88(73)	54(88)		246(84)	234(97)	
CK19			**0.022**			**0.004**
Negative	27(21)	5(8)	**(5.22)**	34(11)	12(5)	**(8.43)**
Positive	103(79)	59(92)		269(8)	252(95)	
FOXA1			0.060			0.120
Negative	61(67)	16(49)	(3.45)	133(62)	103(55)	(2.41)
Positive	30(33)	17(51)		80(38)	85(45)	
BEX1			0.185			**0.007**
Negative	42(49)	15(37)	(1.83)	78(34)	45(22)	**(7.30)**
Positive	43(51)	26(63)		150(66)	156(7)	
TFF1			**0.044**			0.051
Negative	6(67)	13(45)	**(4.06)**	106(55)	75(45)	(3.79)
Positive	23(33)	16(55)		87(45)	93(55)	
TFF3			0.227			**0.005**
Negative	56(67)	21(55)	(12.26)	107(51)	70(37)	**(8.06)**
Positive	28(33)	17(45)		101(49)	118(63)	
GATA3			**0.043**			**<0.001**
Negative	66(72)	23(55)	**(4.09)**	156(73)	107(54)	**(15.37)**
Positive	25(28)	19(45)		58(27)	90(46)	
CD71			0.583			0.168
Negative	48(50)	21(55)	(0.30)	89(41)	92(47)	(1.90)
Positive	48(50)	17(45)		131(59)	103(53)	
CARM1			0.064			0.759
Negative	33(36)	12(32)	(5.49)	55(27)	46(24)	(0.55)
Moderate	32(34)	20(54)		106(53)	105(55)	
High	28(30)	5(14)		40(20)	40(21)	
PELP1			0.415			0.834
Negative	15(16)	10(25)	(1.75)	38(16)	36(18)	(0.36)
Moderate	66(71)	24(60)		159(6)	140(69)	
High	12(13)	6(15)		35(15)	27(13)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation and apoptosis						
E-Cadherin			**0.005**			0.709
Negative	54(42)	14(22)	**(7.84)**	100(33)	91(35)	(0.13)
Positive	75(58)	51(78)		202(67)	172(65)	
p-Cadherin			**0.035**			**0.001**
Negative	49(43)	34(60)	**(4.43)**	16(43)	132(58)	**(11.27)**
Positive	66(57)	23(40)		155(57)	96(42)	
p53			**<0.001**			0.328
Negative/low	75(58)	55(85)	**(13.70)**	217(70)	196(74)	(0.95)
Positive	54(42)	10(15)		93(30)	70(26)	
KI67-LI			**0.001**			0.352
Negative/low	32(32)	32(59)	**(10.41)**	99(38)	94(42)	(0.86)
High	67(68)	22(41)		164(62)	131(58)	
BCL2			0.054			**0.043**
Negative/low	47(55)	13(36)	(3.73)	94(40)	60(31)	**(4.10)**
High	38(45)	23(64)		141(60)	136(69)	

Table 4-8: The associations between p-mTORC1 and other biological markers

	Akt- tumours			Akt+ tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
p-PI3K			0.084			0.148
Negative/low	27(26)	19(38)	(4.94)	39(15)	42(19)	(3.81)
Moderate	27(26)	16(32)		76(30)	75(34)	
High	51(48)	15(30)		143(55)	102(47)	
PTEN			0.278			0.720
Negative	11(20)	2(10)	(1.17)	15(11)	12(10)	(0.12)
Positive	44(80)	19(90)		122(89)	113(90)	
HER1			0.058			**<0.001**
Negative	102(78)	57(89)	(3.58)	234(75)	242(8)	**(15.79)**
Positive	29(22)	7(11)		79(25)	34(12)	
HER2			0.090			0.189
Negative	111(85)	60(94)	(2.87)	260(84)	237(87)	(1.72)
Positive	19(15)	4(6)		51(16)	34(13)	
HER3			0.508			0.224
Negative	7(6)	5(8)	(0.43)	18(6)	10()	(1.47)
Positive	117(94)	56(92)		272(94)	246(96)	
HER4			0.287			**0.017**
Negative	15(12)	11(17)	(1.13)	40(13)	19(7)	**(5.70)**
Positive	114(88)	53(83)		270(87)	255(93)	
HER1-HER2 dimer			0.886			0.586
Negative	65(89)	27(90)	(0.02**)**	134(86)	125(88)	(0.29)
Positive	8(11)	3(10)		22(14)	17(12)	
HER2-HER3 dimer			0.487			0.705
Negative	67(94)	18(90)	(0.48)	132(87)	116(85)	(0.14)
Positive	4(6)	2910)		20(13)	20(15)	
HER2-HER4 dimer			0.629			0.264
Negative	68(2)	34(94)	(0.23)	147(87)	131(91)	(1.24)
Positive	6(8)	2(6)		22(13)	13(9)	
HER1,2 vs HER2,3			0.923			0.400
HER2,1 low-HER2,3 low	45(83)	21(84)	(0.48)	82(79)	75(80)	(2.94)
HER2,1 low-HER2,3 high	1(2)	1(4)		0(0)	2(2)	
HER2,1 high-HER2,3 low	2(4)	1(4)		6(6)	3(3)	
HER2,1 high-HER2,3 high	6(11)	2(8)		16(15)	14(15)	
Her1,2 vs HER2,4			0.726			0.853
HER2,1 low-HER2,4 low	47(90.)	14(87)	(0.64)	82(83)	77(81)	(0.78)
HER2,3 low-HER2,4 high	-	-		2(2)	4(4)	
HER2,1 high-HER2,4 low	1(2)	0(0)		3(3)	3(3)	
HER2,1 high-HER2,4 high	4(8)	2(13)		12(12)	11(12)	
Her2,3 vs HER2,4			0.763			0.419
HER2,3 low-HER2,4 low	44(90)	15(83)	(1.15)	76(75)	67(76)	(2.82)
HER2,3 low-HER2,4 high	1(2)	1(5)		5(5)	1(1)	
HER2,1 high-HER2,4 low	1(2)	1(6)		7(7)	5()	
HER2,3 high-HER2,4 high	3(6)	1(6)		13(13)	15(17)	

Table 4-9: The associations between p-mTORC1 and biological markers

	PI3K-Negative/low tumours			PI3K-High tumours		
	Neg/low N (%)	**High N (%)**	***p*-value**	**Neg/low N (%)**	**High N (%)**	***p*-value**
Hormone Receptors and ER related proteins						
ER			**0.003**			**<0.001**
Negative	21(21)	5(6)	**(9.04)**	110(41)	38(23)	**(14.73)**
Positive	79(79)	82(94)		158(59)	127(77)	
PgR			**0.016**			**0.002**
Negative	42(42)	22(26)	**(5.77)**	146(55)	65(40)	**(9.49)**
Positive	57(58)	64(74)		117(4)	97(60)	
AR			**0.001**			**0.023**
Negative	32(34)	11(13)	**(10.35)**	119(48)	51(36)	**(5.19)**
Positive	62(66)	72(87)		130(52)	91(64)	
CK7/8			0.185			**0.065**
Negative	2(2)	0(0)	(1.75)	9(3)	1(1)	**(3.40)**
Positive	98(98)	87(100)		254(97)	160(99)	
CK18			**0.024**			**<0.001**
Negative	14(16)	4(5)	**(5.09)**	55(22)	8(6)	**(17.47)**
Positive	75(84)	76(95)		191(78)	129(94)	
CK19			**0.031**			0.086
Negative	10(10)	2(2)	**(4.66)**	36(14)	13(8)	(2.94)
Positive	88(90)	84(98)		222(86)	143(92)	
FOXA1			0.321			**0.003**
Negative	27(44)	29(53)	(0.89)	144(67)	70(51)	**(8.75)**
Positive	35(56)	26(47)		70(33)	66(49)	
BEX1			0.451			**0.007**
Negative	30(47)	22(40)	(0.56)	75(35)	28(21)	**(7.40)**
Positive	34(53)	33(60)		138(65)	103(79)	
TFF1			0.837			**0.001**
Negative	25(50)	23(48)	(0.04)	114(60)	47(41)	**(11.30)**
Positive	25(50)	25(52)		75(40)	69(59)	
TFF3			0.076			**0.001**
Negative	34(55)	19(38)	(3.14)	117(59)	53(41)	**(10.55)**
Positive	28(45)	31(62)		81(41)	77(59)	
GATA3			**0.001**			**0.010**
Negative	35(65)	15(31)	**(12.03)**	142(80)	74(67)	**(6.68)**
Positive	19(35)	34(69)		35(20)	37(33)	
CD71			0.395			0.827
Negative	45(62)	37(70)	(0.72)	71(34)	43(33)	(0.04)
Positive	27(38)	16(30)		138(66)	88(67)	
CARM1			0.162			0.424
Negative	26(42)	16(26)	(3.64)	54(26)	27(20)	(1.71)
Moderate	26(43)	33(54)		101(47)	72(54)	
High	9(15)	12(20)		58(27)	34(26)	
PELP1			0.035			0.834
Negative	14(20)	13(22)	(6.69)	31(14)	20(15)	(0.36)
Moderate	51(74)	34(58)		145(68)	93(71)	
High	4(6)	12(20)		38(18)	19(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation and apoptosis						
E-Cadherin			0.607			0.356
Negative	44(44)	35(41)	(0.26)	95(37)	50(33)	(0.85)
Positive	55(56)	51(59)		162(63)	104(67)	
p-Cadherin			0.385			**0.007**
Negative	41(53)	44(60)	(0.75)	81(36)	63(50)	**(7.39**
Positive	36(47)	29(40)		147(64)	62(50)	**)**
p53			0.966			0.089
Negative/low	87(87)	75(87)	(0.00)	162(62)	110(71)	(0.92)
Positive	13(13)	11(13)		98(38)	46(29)	
KI67-LI			**0.033**			0.352
Negative/low	38(49)	48(67)	**(4.57)**	62(29)	40(29)	(0.86)
High	39(51)	24(33)		153(71)	98(71)	
BCL2			**0.040**			0.459
Negative/low	30(44)	16(27)	**(4.21)**	101(48)	52(44)	(0.54)
High	38(56)	44(73)		108(52)	66(56)	

Table 4-10: The associations between p-mTORC1 and other biological markers

	PI3K-Negative/low tumours			PI3K-High tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
p-Akt			0.253			**0.005**
Negative	27(41)	19(31)	(1.30)	51(26)	15(13)	**(7.91)**
Positive	39(59)	42(69)		143(74)	102(87)	
PTEN			0.546			0.087
Negative	9(25)	4(18)	(0.36)	21(16)	8(8)	(2.92)
Positive	27(75)	18(82)		109(84)	87(92)	
HER1			**0.019**			**0.008**
Negative	86(85)	84(95)	**(5.52)**	181(69)	131(81)	**(7.14)**
Positive	15(15)	4(5)		81(31)	31(19)	
HER2			0.317			0.300
Negative	89(91)	83(95)	(1.00)	218(83)	128(78)	(1.07)
Positive	8(8)	4(5)		46(17)	35(22)	
HER3			0.293			0.879
Negative	6(7)	7(9)	(0.28)	18(7)	11(8)	(0.02)
Positive	84(93)	72(91)		233(93)	134(92)	
HER4			0.896			0.968
Negative	18(18)	15(17)	(0.01	21(8)	13(8)	(0.00)
Positive	81(82)	71(83)		241(92)	147(92)	
HER1-HER2 dimer			0.843			0.665
Negative	47(94)	26(93)	(0.03**)**	120(86)	82(84)	(1.87)
Positive	3(6)	2(7)		20(14)	16(16)	
HER2-HER3 dimer			0.667			0.164
Negative	31(94)	31(91)	(0.18)	132(87)	68(80)	(1.93)
Positive	2(6)	3(9)		20(13)	17(20)	
HER2-HER4 dimer			0.753			0.553
Negative	48(92)	28(90)	(0.09)	123(83)	82(85)	(0.35)
Positive	4(8)	3(10)		26(17)	14(15)	
HER1,2 vs HER2,3			0.317			0.976
HER2,1 low-HER2,3 low	30(91)	13(81)	(0.32)	74(77)	54(76)	(0.21)
HER2,1 low-HER2,3 high	0(0)	1(6)		2(2)	1(1)	
HER2,1 high-HER2,3 low				3(3)	2(3)	
HER2,1 high-HER2,3 high	3(9)	2(13)		17(18)	14(20)	
Her1,2 vs HER2,4			0.463			0.444
HER2,1 low-HER2,4 low	25(92)	13(86)	(2.576)	82(84)	49(78)	(2.67)
HER2,3 low-HER2,4 high	1(4)	1(7)		2(2)	2(3)	
HER2,1 high-HER2,4 low	0(0)	1(7)		4(4)	1(2)	
HER2,1 high-HER2,4 high	1(4)	0(0)		10(10)	11(17)	
Her2,3 vs HER2,4			0.5205			0.799
HER2,3 low-HER2,4 low	22(92)	9(69)	(4.58)	76(76)	45(70)	(1.00)
HER2,3 low-HER2,4 high	0(0)	1(8)		4(4)	2(3)	
HER2,1 high-HER2,4 low	0(0)	1(8)		5(5)	4(7)	
HER2,3 high-HER2,4 high	2(8)	2(15)		15(15)	13(20)	

4.3.2.4 The associations of p-mTORC1 with MAPKs in the primary breast cancer series and different subgroups

High p-mTORC1 expression was highly significantly associated with MAPKs proteins: p-JNK1/2 (p=0.013: borderline), nuclear p-ERK1/2 (p=0.019: borderline), cytoplasmic p-ERK1/2 (p=0.001), p38, p-p38, p-C-JUN and ATF2 (all p<0.001). Similarly, apart from p-JNK1/2, all other associations were maintained within ER+HER2- tumours (p<0.001, Table 4-11). In ER+HER2+ tumours, positive associations were observed between high mTORC1 expression and p-p38 and p-ATF2 (p=0.007). Meanwhile, there was only an association with increased pan ERK1/2 expression within ER-HER2+ cohort (p=0.044: borderline). Within ER-HER2- cohort, p-mTORC1 was positively associated with p-ATF2, p38 and p-p38 (p=0.037, p=0.010, both borderline and p<0.001 respectively, appendix table 8).

Table 4-11: The associations of p-mTORC1 with MAPKs

	Whole series			ER+HER2- tumours		
	Negative/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Negative/low N (%)**	**High N (%)**	***p*-value (χ^2)**
p-JNK1/2			**0.013**			0.186
Negative/low	114(26)	63(18)	**(6.17)**	48(21)	44(16)	(1.74)
Moderate/high	332(74)	284(82)		185(79)	230(84)	
Pan JNK1/2			0.562			0.738
Negative/low	218(56)	171(53)	(0.33)	121(55)	134(56)	(0.11)
Moderate/high	174(44)	149(47)		100(45)	104(44)	
Pan ERK1/2			0.277			0.534
Negative/low	261(54)	200(50)	(1.18)	130(47)	156(50)	(0.38)
Moderate/high	223(46)	198(50)		145(53)	157(50)	
Nuclear p-ERK1/2			**0.019**			**<0.001**
Negative/low	269(57)	161(41)	**(5.52)**	135(52)	111(36)	**(14.88)**
Moderate/high	205(43)	235(59)		126(48)	200(64)	
Cytoplasmic p-ERK1/2			**<0.001**			**<0.001**
Negative/low	250(53)	153(39)	**(17.49)**	148(57)	116(37)	**(22.51)**
Moderate/high	221(47)	241(61)		110(43)	194(63)	
Pan p38			**<0.001**			**<0.001**
Negative/low	325(68)	209(53)	**(21.49)**	171(65)	152(50)	**(12.28)**
Moderate/high	150(32)	185(47)		93(35)	151(50)	
p-p38			**<0.001**			**<0.001**
Negative/low	501(80)	329(62)	**(43.38)**	267(76)	262(64)	**(13.68)**
Moderate/high	125(20)	197(38)		83(24)	148(36)	
p-C-JUN			**<0.001**			**<0.001**
Negative/low	273(45)	119(24)	**(55.68)**	48(44)	89(23)	**(35.61)**
Moderate/high	334(55)	387(76)		191(56)	300(77)	
p-ATF2			**<0.001**			**<0.001**
Negative/low	482(79)	330(65)	**(27.70)**	264(76)	245(63)	**(15.76)**
Moderate/high	124(21)	174(35)		81(24)	144(37)	

4.3.2.5 The expression of p-mTORC1 in Trastuzumab treated series

152 cases were valid for the assessment of this protein in this studied series. 80 (52.6%) had negative/low expression while 72 (47.4%) had high expression.

4.3.2.5.1 The associations of p-mTORC1 with clinicopathological variables and biological markers in Trastuzumab treated series

High expression of p-mTORC1 was associated with an early tumour stage (p=0.005), a trend of lower tumour grade (p=0.027) and less mitotic count (p=0.047, Table 4-12). Moreover, p-mTORC1 was significantly associated with high expression pan p38 (p<0.001) and a trend of high expression of pan JNK1/2 p-p38 and p-ATF2 (p=0.012, p=0.014, Table 4-13 and Table 4-14).

Table 4-12: The associations between p-mTORC1 and clinicopathological variables

	Trastuzumab Treated BC Series		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**
Age			0.266
≤50	36(45)	26(36)	(1.24)
>50	44(55)	46(64)	
Menopausal Status			0.321
Pre-	42(52)	32(44)	(0.98)
Post-	38(48)	40(56)	
Tumour Size (cm)	-	-	-
≤2.0			
>2.0			
Stage			**0.0.005**
1	38(48)	51(72)	**(10.70)**
2	37(46)	15(21)	
3	5(6)	5(7)	
Grade			**0.0.27**
1	4(5)	0(0)	**(7.22)**
2	15(19)	24(33)	
3	61(76)	48(67)	
Tubules			0.712
2	14(18)	11(15)	(0.13)
3	66(82)	61(85)	
Pleomorphism			0.723
2	8(10)	6(8)	(0.12)
3	72(90)	66(92)	
Mitosis			**0.047**
1	18(22)	19(26)	**(6.11)**
2	19(24)	28(39)	
3	43(54)	25(35)	
LVI			0.576
Probable/Negative	51(64)	49(68)	(0.31)
Definite	29(36)	23(32)	
NPI			0.588
GPG	7(10)	6(9)	(1.06)
MPG	41(57)	43(65)	
PPG	24(33)	17(26)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 4-13: The association between p-mTORC1 with biological markers and HER2 dimers Trastuzumab Treated BC Series

	mTORC1		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**
ER			0.559
Negative	34(43)	34(47)	(0.34)
Positive	46(57)	38(53)	
PgR			0.441
Negative	38(59)	39(66)	(0.59)
Positive	26(41)	20(34)	
p-AKT			0.677
Negative/low	8(22)	6(18)	(0.17)
High	28(78)	27(82)	
p-PI3K			0.1000
Negative/low	-	-	(0.00)
Moderate	1(3)	1(3)	
High	37(97)	37((97)	
PTEN			0.352
Negative/low	0(0)	1(4)	(2.09)
Moderate	1(3)	0(0)	
Positive	34(97)	25(96)	
P53			0.591
Negative	6(15)	8(20)	(0.028)
Positive	33(85)	32(80)	
BCL2			0.252
Negative/low	15(39)	21(51)	(1.31)
Moderate/high	24(61)	20(49)	
HER1-HER2 dimer			0.494
Negative	11(31)	10(24)	(0.46**)**
Positive	24(69)	31(76)	
HER2-HER3 dimer			0.386
Negative	16(41)	13(32)	(1.52)
Positive	23(59)	28(68)	
HER2-HER4 dimer			0.295
Negative	18(53)	15(41)	(1.09)
Positive	16(47)	22(59)	
HER1,2 vs HER2,3			0.975
HER2,1 low-HER2,3 low	9(26)	9(23)	(0.21)
HER2,1 low-HER2,3 high	1(3)	1(3)	
HER2,1 high-HER2,3 low	4(12)	4(10)	
HER2,1 high-HER2,3 high	20(58)	25(64)	
Her1,2 vs HER2,4			0.616
HER2,1 low-HER2,4 low	7(23)	9(25)	(1.79)
HER2,3 low-HER2,4 high	1(4)	0(0)	
HER2,1 high-HER2,4 low	7(23)	6(17)	
HER2,1 high-HER2,4 high	15(50)	21(58)	
Her2,3 vs HER2,4			0.422
HER2,3 low-HER2,4 low	11(32)	10(27)	(2.81)
HER2,3 low-HER2,4 high	3(9)	1(3)	
HER2,1 high-HER2,4 low	7(21)	5(14)	
HER2,3 high-HER2,4 high	13(38)	20(55)	

Table 4-14: The associations between p-mTORC1 and MAPKs

	Trastuzumab Treated BC Series		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**
Pan JNK1/2			**0.012**
Negative/low	46(62)	27(50)	**(6.31)**
Moderate/high	28(38)	39(59)	
p-JNK1/2			0.166
Negative/low	45(61)	34(49)	(1.92)
Moderate/high	29(39)	35(51)	
Pan ERK1/2			0.385
Negative/low	25(58)	22(49)	(1.18)
Moderate/high	18(42)	23(51)	
Nuclear p-ERK1/2			0.110
Negative/low	47(67)	32(49)	(2.55)
Moderate/high	28(37)	33(51)	
Cytoplasmic p-ERK1/2			0.150
Negative/low	69(92)	53(84)	(2.07)
Moderate/high	6(8)	10(16)	
Pan p38			**<0.001**
Negative/low	51(70)	24(35)	**(17.51)**
Moderate/high	22(30)	45(65)	
p-p38			**0.014**
Negative/low	29(39)	13(20)	**(5.04)**
Moderate/high	46(61)	53(80)	
p-C-JUN			0.075
Negative/low	37(49)	22(34)	(3.17)
Moderate/high	39(51)	43(66)	
p-ATF2			**0.014**
Negative/low	53(68)	31(48)	**(6.00)**
Moderate/high	25(32)	34(52)	

4.3.3 Outcome analysis

4.3.3.1 Outcome of p-mTORC1 within the primary series

p-mTORC1 revealed some associations with outcome in combination with other proteins of PI3K pathway rather than alone within the whole cohort. For instance, within Akt+ cohort, p-mTORC1 was associated with better BCSS (p=0.027, Figure 4-4).

Interestingly, when the analysis was restricted to those expressing negative/low p-mTORC1, p-Akt was associated with shorter BCSS (p=0.049, Figure 4-5).

4.3.3.2 Outcome of p-mTOR1 in Trastuzumab treated series

There was no significance with respect to mTORC1 and patient outcome in HER2+ BC with regard to Trastuzumab response and resistance.

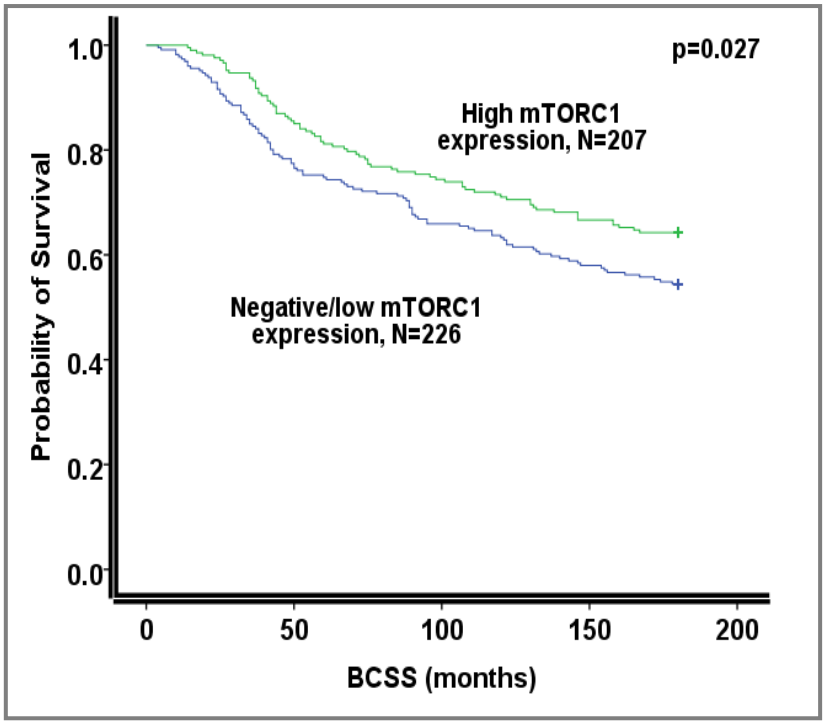


Figure 4-4: Kaplan Meier plots illustrating BCSS and DMFS for p-mTORC1 in Akt-positive tumours

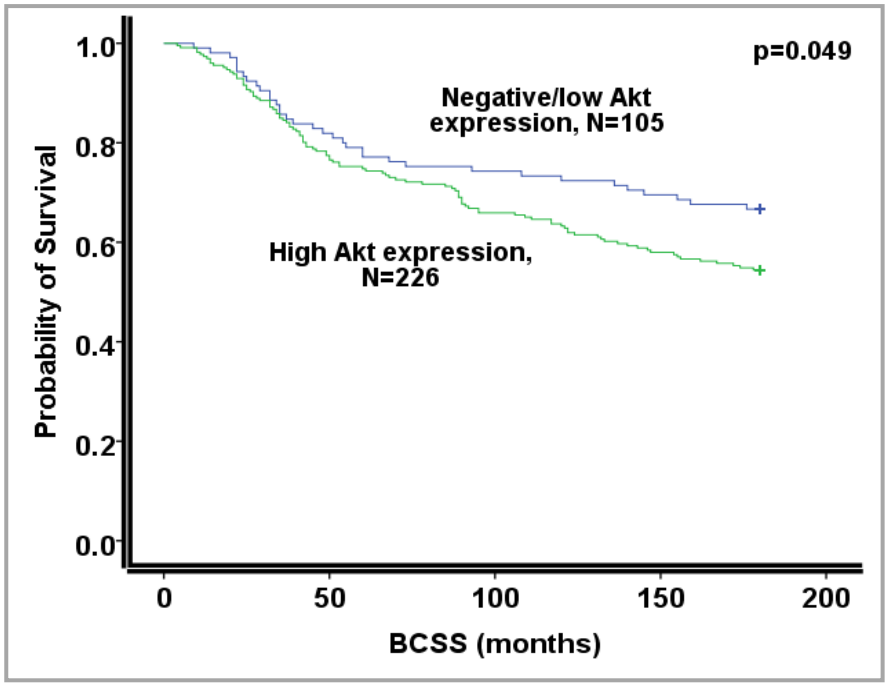


Figure 4-5: Kaplan Meier plots illustrating BCSS for Akt in Negative/low p-mTORC1 expressing tumours

4.3.3.3 Multivariate analysis for p-mTORC1 (within the primary series)

Cox proportional hazard elucidated that high p-mTORC1 expression was a predictor of prolonged survival independently from PI3K and Akt proteins (Table 4-15). In addition, when ER and HER2 were added to the model, p-mTORC1 remained a predictor of better BCSS (Table 4-15). In the same context, p-mTORC1 was not an independent predictor from grade, stage and size (data is not shown).

Table 4-15: Cox multivariate Regression models for the predictors of survival in the unselected series

Variable	BCSS/ BC			
	***P*-value**	**HR**	**95% CI**	
p-mTORC1	**0.040**	.729	.538	.986
p-Akt	0.287	1.206	.854	1.703
p-Pi3k	**0.004**	1.337	1.094	1.632

Variable	BCSS/ BC			
	***P*-value**	**HR**	**95% CI**	
p-mTORC1	0.067	.751	.552	1.020
p-Akt	0.242	1.233	.869	1.749
p-Pi3k	**0.016**	1.290	1.048	1.586
ER	0.267	.825	.588	1.158

Variable	BCSS/ BC			
	***P*-value**	**HR**	**95% CI**	
p-mTORC1	**0.031**	.714	.526	.970
p-Akt	0.401	1.160	.820	1.641
p-Pi3k	**0.017**	1.281	1.045	1.570
HER2	**0.000**	2.023	1.428	2.865

Variable	BCSS/ BC			
	***P*-value**	**HR**	**95% CI**	
p-mTORC1	**0.040**	.724	.532	.986
p-Akt	0.406	1.160	.817	1.647
p-Pi3k	**0.031**	1.261	1.021	1.558
ER	0.724	.940	.665	1.328
HER2	**0.000**	2.001	1.403	2.855

4.3.4 RPPA results

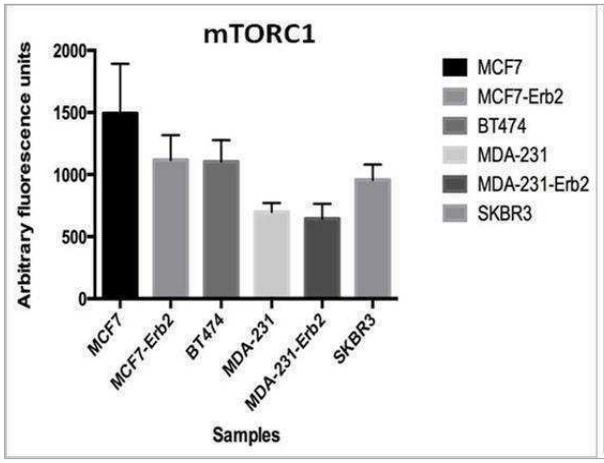
RPPA was used to assess the difference in the expression of p-mTORC1 and its related proteins Akt, PI3K, total and phosphorylated PTEN and p70S6K in BC cell lines representing different biological classes based on ER and HER2 expressions. RPPA was consistent with IHC results.

Importantly, there was an increase in p-mTORC1 expression with ER in HER2- cell lines (wild ER+HER2- vs wild ER-HER2-, $p<0.001$) and HER2+ cell lines (W and T ER+HER2+ vs W and T ER-HER2+ cell lines) where a significant increase with ER was in T ER+HER2+ cell line vs T ER-HER2+ one ($p=0.043$, Figure 4-6).

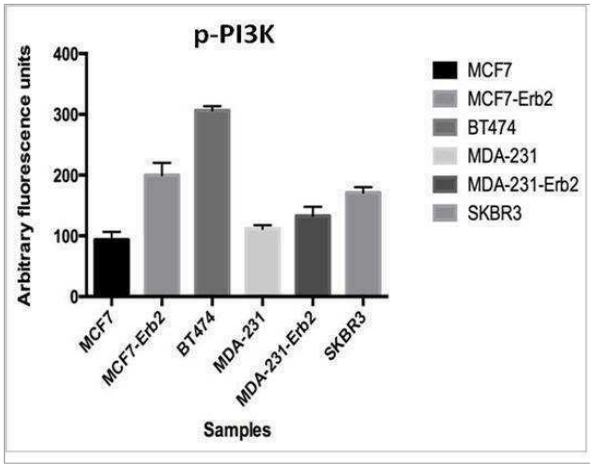
In RPPA, a significant difference in the expression of Akt and PI3K was observed based on HER2 status within ER+ cell lines where these proteins increased with HER2 expression (PI3K: T and W ER+HER2+ cell lines vs ER+HER2-, $p<0.001$, Akt: T and W ER+HER2+ vs ER+HER2-, $p=0.001$ and $p<0.001$, respectively, Figure 4-6). Within HER2- cell lines, no significant associations were deemed with variable ER expression. In contrast, within HER2+ cell lines, a significant decrease in the expression of both p-PI3K and p-Akt proteins was observed in association with ER loss if compared to ER expression (PI3K: T ER+HER2+ vs T and W ER-HER2+, $p<0.001$, $p=0.043$, respectively, W ER+HER2+ vs T and W ER-HER2+, $p<0.001$), (p-Akt: T ER+HER2+ vs T ER-HER2+, $p=0.013$, W ER+HER2+ vs T and W ER-HER2+, $p<0.001$, both, Figure 4-6).

With respect to unphosphorylated PTEN in ER+ cell lines, it was significantly negatively associated with HER2 status (ER+HER2- vs T and W ER+HER2+, $p<0.001$, both, Figure 4-7). Moreover, in HER2- cell lines, this protein revealed a direct positive association with ER expression (ER+HER2- vs ER-HER2-, $p<0.001$) but in HER2+ cell lines, this biomarker displayed negative association with ER (although this was observed in W ER-HER2+ cell line, Figure 4-7).

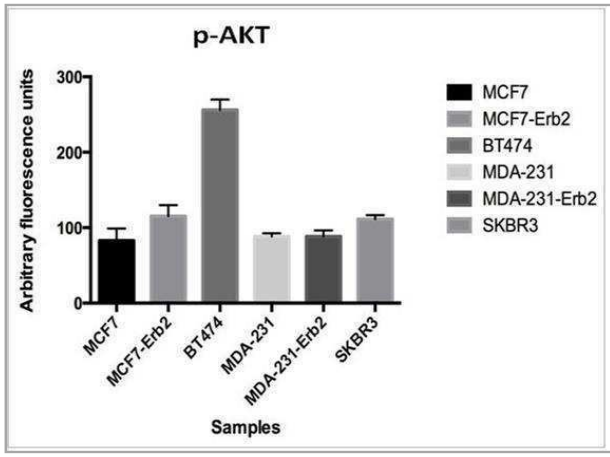
p-PTEN revealed a similar pattern of increased expression with HER2 in ER+ and ER- cell lines only (p-PTEN: T ER+HER2+ vs ER+HER2-, $p<0.004$, T ER-HER2+ vs ER-HER2-, $p=0.002$, Figure 4-7). There was no direct association between total and phosphorylated forms of PTEN ($p=0.672$, correlation coefficient= -0.087). Finally, p-S6K being a direct downstream of p-mTORC1, showed an increased expression with HER2 positivity in ER+ cell lines (T and W ER+HER2+ vs ER+HER2-, $p<0.001$, Figure 4-7).



ER+HER2-/ER+HER2+ (T)	0.128
ER+HER2-/ER+HER2+ (W)	0.695
ER+HER2-/ER-HER2-	<0.001
ER+HER2+(T)/ER--HER2+(T)	0.043
ER+HER2+(T)/ER-HER2+(W)	1.000
ER+HER2+(W)/ER-HER2+(T)	0.421
ER+HER2+(W)/ER-HER2+(W)	1.000
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	1.000



ER+HER2-/ER+HER2+ (T)	<0.001
ER+HER2-/ER+HER2+ (W)	<0.001
ER+HER2-/ER-HER2-	0.493
ER+HER2+(T)/ER--HER2+(T)	<0.001
ER+HER2+(T)/ER-HER2+(W)	0.043
ER+HER2+(W)/ER-HER2+(T)	<0.001
ER+HER2+(W)/ER-HER2+(W)	<0.001
ER-HER2-/ER-HER2+(T)	0.726
ER-HER2-/ER-HER2+(W)	<0.001



ER+HER2-/ER+HER2+ (T)	0.001
ER+HER2-/ER+HER2+ (W)	<0.001
ER+HER2-/ER-HER2-	1.000
ER+HER2+(T)/ER--HER2+(T)	0.013
ER+HER2+(T)/ER-HER2+(W)	1.000
ER+HER2+(W)/ER-HER2+(T)	<0.001
ER+HER2+(W)/ER-HER2+(W)	<0.001
ER-HER2-/ER-HER2+(T)	1.000
ER-HER2-/ER-HER2+(W)	0.107

Figure 4-6: Graphical representation of the expression of p-mTORC1, p-PI3K and p-Akt in 6 BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test)

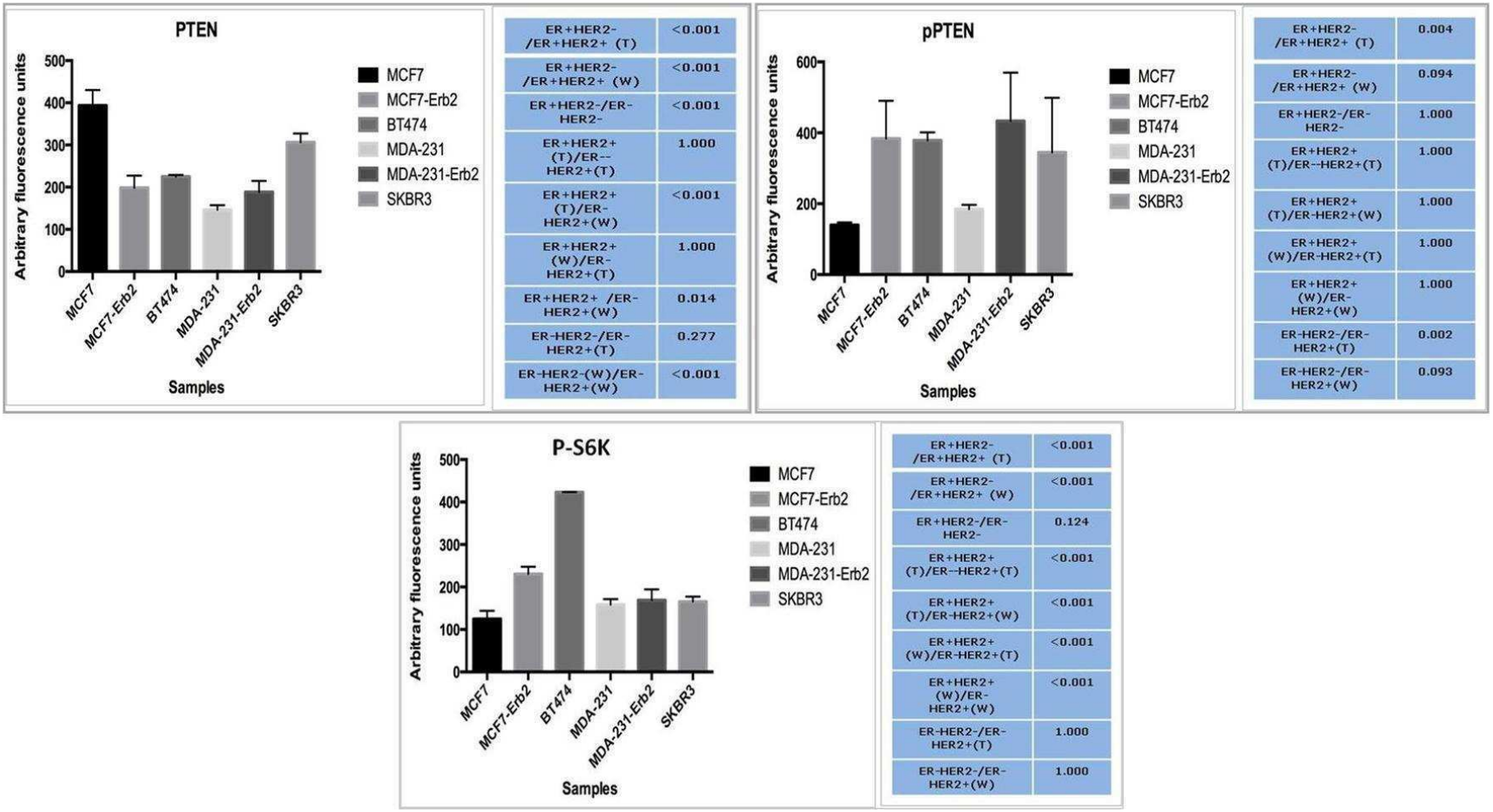


Figure 4-7: Graphical representation of the expression of p-mTORC1, p-PI3K and p-Akt in 6 BC cell lines. MCF-7: ER+HER2-, MCF-7-ERB2: ER+HER2+ transfected (T), BT474: ER+HER2+ (W), MDA-231: ER-HER2-, MDA-231-ERB2: ER-HER2+ (T), SKBR3: ER-HER2+ (W) using RPPA. The left column of the table represents certain BC cell line against another to test the association for proteins' expression, and the right column of the blue table denotes the p-value (Kruskal Wallis test)

4.4 Discussion

In spite of the development and availability of several inhibitors of mTORC1, still the role of PI3K/Akt/mTOR signalling in BC and the adverse effect it could have on outcome is to be determined. Not surprisingly, different studies have previously emphasised the role of this pathway in causing hormone therapy resistance (Rosner et al., 2012, Bakarakos et al., 2010)

The findings presented in this chapter revealed that p-mTORC1 is associated with good clinicopathological prognostic factors and strongly related to ER, ER-related proteins, luminal differentiation markers, together with high Akt and low PI3K expressions. Interestingly, some of these associations were maintained within the ER+HER2- luminal cohort. The vast majority of p-mTORC1+ tumours were HRs+ while nearly 50% of HR+ tumours showed p-mTORC1 positivity. Meanwhile, the diverse expression of p-mTORC1 within ER+ and ER- tumours with regard to HER2 merits further evaluation. Furthermore, the IHC results were also supported by RPPA data which revealed that p-mTORC1 had higher expression in ER+HER2- cell lines vs ER+HER2+ cell lines and it was increased in T ER+HER2+ vs T ER-HER2+ one. These findings could reflect the major role of ER and HER2 and how their interaction can influence the biological features of p-mTORC1.

Different views have been reported regarding mTORC1 function, one by O'Regan and Hawk (O'Regan and Hawk, 2011) who supported the role of an intact ER dependent pathway in maintaining the upregulation of p-mTORC1 in BC in cells dependent on oestrogen for their growth and survival bearing in mind that p-mTORC1 is a major regulator of cell growth and cell viability.

Moreover, to outline the possibility that the signalling of mTORC1 for BC progression can be interrupted, a study indicated that feedback inhibition of the PI3K/AKT pathway can be mediated by S6K; downstream of p-mTORC1, when there is chronic insulin stimulation, or loss of the tuberous sclerosis complex, through the phosphorylation of Insulin Receptor Substrate 1 (IRS1), leading to its degradation (Harrington et al., 2004, Shah and Hunter, 2006). Moreover, a complementary negative feedback loop has been identified in which S6K phosphorylates Rictor, leading to decrease AKT activation by p-mTORC2 (Dibble

et al., 2009, Treins et al., 2010). These studies support the findings in this chapter by underlining the role of p-mTORC1 in enhancing apoptosis.

The association between p-mTORC1 and clinicopathological variables and ER and its related proteins are in line with Shrivastav et al (Shrivastav et al., 2014), who showed that p-mTORC1 is associated with smaller tumour size and better overall survival and recurrence free survival. Those authors defined the p7-ERα score which is a combination of seven ER phosphorylated epitopes detected in any one tumour. They confirmed the inverse association between p7-ERα score and p-mTORC1. Since the low p7-ERα score represents more phosphorylation of ERα sites which are associated with good prognosis and outcome, conversely, the high p7-ERα score represent more phosphorylation of ERα sites that are associated with poor prognosis and shorter survival which indicates that p-mTORC1 is associated with good prognosis (Shrivastav et al., 2014). Moreover, the results were in line with Beca et al (Beca et al., 2014) who found that p-mTORC1 is associated with lower tumour grade and smaller tumour size.

Importantly, we are in keeping with the above studies; however, these studies did not take into consideration the different BC subgroups and how the mTORC1 could behave accordingly; therefore, we want to address the impact of interaction between ER and HER2 expressions on altering the biological significance of mTORC1. We hereby emphasise the effect of HER2 co-expression with ER which can modulate the interactions of mTORC1 with different proteins related to ER and key BC pathways. Moreover, we demonstrated that RPPA was useful tool to assess the expression of the proteins of interest in this study which showed good concordance with IHC results.

Consistent with our findings, Martina et al (Martina et al., 2012) investigated the impact of p-mTORC1 in regulating the transcription factor EB (TFEB), a member of the bHLH leucine-zipper family of transcription factors. This protein is required for the biogenesis of the lysosomes which function to clear damaged organelles and to produce autophagy enzymes when activated (Martina et al., 2012). The latter study indicated that p-mTORC1 is a kinase and is located in the lysosomes. When lysosome function is intact, p-mTORC1 phosphorylates TFEB by relating it to members of the YWHA (14-3-3) family of proteins and by this, its transcriptional activity for the autophagy enzymes can be maintained within the cytosol. In contrast, TFEB is translocated to the nucleus in case of genetic or

pharmacological inhibition of p-mTORC1 as there is lack of phosphorylation and loss of transcriptional activity.

The results of this thesis indicate the possible role of p-mTORC1 inhibitors to be applicable in ER-HER2+ early BC group as p-mTORC1 is positively associated with HER2 in this subset. Although different studies have reported the role of mTORC1 inhibitors in BC, they addressed its usefulness in HR+/HER2- advanced BC (Beck, 2015, Awada et al., 2008). The results from the present study dealt with early BC and it indicate that inhibiting mTORC1 by everolimus could be useful in ER-HER2+ BC but this needs further investigation of mTORC1 in larger data sets and with validation in different centres could be warranted.

Regarding other biomarkers in the PI3K/Akt/mTOR pathway, PI3K and p-PTEN seem to be potential therapeutic targets in ER+HER2+ and ER-HER2+ tumours as they showed increased expression with HER2 in ER+ and ER- tumours. In addition, p-S6K which is the downstream of mTORC1 could be a therapeutic target in the double positive group as it revealed positive association with HER2 in that group.
Furthermore, the associations of p-mTORC1 within AKT+/- and PI3K+/- which are main components of PI3K pathway, did not reveal a possibility of mTORC1 inhibitors to be of value but still future consideration of the role of p-mTORC1 in such subgroups is to be further explored.

With regard to outcome, mTORC1 did not reveal associations with longer survival in the whole cohort of BC or in ER+ group, instead, within the Akt+ cohort, p-mTORC1 was associated with better outcome. Furthermore, within negative/low p-mTORC1 expression, Akt was associated with shorter outcome, which could imply that p-mTORC1 might have impact on prolonged survival and this could be explained by the possible feedback effect of p-mTORC1 on Akt (Harrington et al., 2004).
In conclusion, p-mTORC1 is a downstream signalling molecule that has shown differential associations with key biomarkers including HER2 and ER in different molecular subgroups of BC using IHC and RPPA. Although p-mTORC1 was associated with good prognostic features in BC including ER+ group, these associations were decreased after HER2 expression illustrating a biological difference in ER+HER2+ BC compared to ER+HER2- and ER-HER2+ groups.

In the future, investigation of four downstream targets of p-mTORC1; 4E-BP1, eIF-4E, S6K1 (has been investigated in this study using RPPA) and S6K2, using IHC, can further explore more aspects of this pathway. For instance, 4E-BP1 is considered a factor that seems to be a channeling point at which different upstream oncogenic changes converge and transmit their stimulating signal and perhaps modulating protein translation and has been shown to associate with high grade and worst prognosis (Rojo et al., 2007). In addition, and in terms of response to therapy, S6K1 has been reported to be associated with decreased benefit from radiotherapy while S6K2 was reported to be associated with improved response to tamoxifen in ER+ patients and eIF-4E has been indicated to be associated with poor outcome (Holm et al., 2008, Perez-Tenorio et al., 2011). In this respect, IHC and RPPA or other quantitative technique can be further used to detect proteins' levels and correlate it to protein expression by other methods.

5 Oestrogen Receptor-Positive/HER2-Positive Breast Cancer is a Distinct Class: The Biological and Prognostic significance of HER2 and Proliferation in the ER-Positive Breast Cancer

5.1 Introduction

In mammary tissue, ER regulates growth and development by regulating the balance between cell proliferation and differentiation (Anderson, 2002, Clarke et al., 2004). In BC, ER expression which is observed in 80-85% of cases, provides a prognostic and predictive value; that indicating good prognosis (Mosselman et al., 1996) and response to hormone treatment (CTSU, 2005). BC has been classified into distinct molecular classes using GEP and identified ER in addition to HER2 overexpression and proliferation as key drivers regulating its molecular profile. Tumours expressing ER are clustered together in the "luminal class", which has good prognosis (Perou et al., 2000a).

Subsequent GEP investigations have sub-stratified the luminal group into at least two subsets; A and B with the latter harbours a poorer prognosis (Sorlie et al., 2001, Sorlie et al., 2003a, Parker et al., 2009). Although most immunohistochemical studies have defined luminal-A tumours as HR-positive and HER2-negative, the definition of luminal-B class remains to be determined. Some studies have defined luminal –B tumours to include HER2 positivity (Badve et al., 2007, Matos et al., 2005, Carey et al., 2006), KI67-LI (Guiu et al., 2012) or a combination of both (Hugh et al., 2009b) Others placed all HER2+ tumours, irrespective of their HRs status in the HER2+ subgroup (Bhargava and Dabbs, 2008).

Cheang et al (Cheang et al., 2009a) using qRT-PCR gene expression profiles have classified BC into luminal-A and luminal-B. Using surrogate immunohistochemical biomarkers, they also separated luminal tumours into three subgroups based on HER2 and KI67; i) Luminal-A class defined as HER2- and KI67-negative/low, ii) Luminal-B as HER2- and KI67-high and iii) luminal-HER2 positive as HER2+ regardless of KI67 expression. Survival analysis showed similar outcome for tumours classified as luminal-B and luminal-HER2 positive. Importantly, the role of proliferation in defining the luminal-B class is highlighted in recently published studies (Guiu et al., 2012, Bastien et al., 2012). In addition, Green et al, in our group, have recently carried out a study using IHC to reveal seven molecular BC classes, of which three groups were luminal and HER2 was not within the criteria to define any of these subclasses and is separated in a single main group differentially expressed by ER (Green et al., 2013).

Although HER2 is a major determinant of BC molecular profile, the interaction between HER2 and ER pathway depends on several factors including ER level, transcriptional co-repressors and co-activators (Osborne et al., 2003b) and others. Different reports highlighted the importance of proliferation in BC outcome and its association with biological, molecular and clinical features that indicates aggressive behaviour (Viale et al., 2008, Urruticoechea et al., 2005). Upregulation of proliferation-related genes are also a common theme in most prognostic gene signatures (Guiu et al., 2012). Although an inverse correlation between KI67 and HR expression has been found, correlation between KI67 and HER2 remains to be investigated and both positive and negative associations have been observed (Liu et al., 2001, Spyratos et al., 2002, Brown et al., 1996). There were some issues regarding the potential clinical utility of GEP include dealing with sample, interpretation and analysis of the data, reproducibility, validation, availability, and cost issues (Ein-Dor et al., 2006, Pusztai et al., 2006, Simon, 2006). Moreover, existing studies also have not addressed whether the proposed classification was consistent across variable cases, whether the surrogate genes involved in the cluster designation have been proven for their exact biological values, or whether the cases that cannot be classified into any of main molecular classes revealed form GEP clustering. We therefore have sought to use available data using IHC being feasible and cost effective compared to previous method.

5.1.1 Hypothesis

High proliferation and HER2 overexpression occur in ER+ BC which leads to relatively more aggressive behaviour tumours and even the response to treatment could be affected. It was hypothesised that ER+HER2+ group is a distinct class of luminal BC and there is a controversy to define luminal-B BC with relation to HER2 and proliferation.

5.1.2 Aims

This study aims to investigate the biological and clinical significance of KI67-LI, as a marker of proliferation, and HER2 overexpression in a large well-characterised series of BC to increase our understanding which of these key biomarkers merits to be considered as the main determinant of luminal B

subgroup. Such an issue appears of critical relevance considering the need to identify the molecular features of individual tumours in routine practice.

5.2 Methods

Breast cancer TMA sections of the primary series were stained. With regards to HER2, Rabbit antihuman HER2 protein (Dako, Denmark) was used as a primary antibody diluted at 1:250 with an incubation period of 45 minutes. The chromogenic substance used was 3-3' Diaminobenzidin tetrahydrochloride (Dako liquid DAB plus, K3468, Dako, Denmark). Moreover, the positive control was used in each run according to the supplier's guidelines and in such case, the kidney was used. For scoring, Hercept test was used where 0, 1+ are negative, 3+ is positive and then the equivocal +2 cases were assessed by chromogenic insitu hybridisation. The same protocol for staining HER2 was applied for KI67, but the whole sections of BC tissues were used rather than the TMA. In addition, heat induced epitope for retrieval of antigen, using microwave, was performed in citrate buffer, at pH 6.0 and the primary antibody was mouse monoclonal antibody against KI67 (clone MIB1; Dako, Denmark), diluted 1:100 in swine serum and then incubated for 60 minutes after application to each slide using full-face sections.

Regarding KI67, it was scored by assessing the hot spots and KI67-LI was calculated as the percentage from 1000 malignant cells. The cut-off point used to evaluate the KI67 labelling index (LI) was >13 as previously defined (Cheang et al., 2009b). These two key proteins; HER2, KI67-LI and a large panel of antibodies used in this chapter (including proteins related to ER, HER2, EMT, p53, BCL2 and PI3K/Akt) were all stained and scored by our group (Table 2-3). The IHC staining was performed according to standard protocol for each antibody as part of studies performed by the group. Details of IHC procedure used and the scoring method performed are the same as previously published (Abd El-Rehim et al., 2005, Rakha et al., 2009, Aleskandarany et al., 2011, Aleskandarany et al., 2012, Habashy et al., 2010a, Habashy et al., 2008a, Habashy et al., 2010c, Habashy et al., 2013).

5.3 Results

5.3.1 Immunohistochemistry results

5.3.1.1 Association of HER2 and KI67-LI with clinicopathological variables

The total number of ER+ cases ($\geq$1%) in this study was 1601, of which, 1151cases were valid for KI67-LI assessment and 1405 were valid for HER2. A total of 45.1% ER+ cases showed high KI67-LI and 7%, showed HER2 overexpression and both were positively correlated with each other (p<0.001, Table 5-1).

Within ER+ cohort, both high KI67-LI and HER2 positivity showed positive associations with younger age, higher tumour grade, invasive ductal NST and high NPI scores (all p<0.001,Table 5-2). In addition, both were associated with larger tumour size (HER2+: p=0.022: borderline, high ki67: p<0.001). High KI67-LI was, in addition, associated with advanced stage and definite vascular invasion (p<0.001) while HER2+ was associated with Pre-menopausal status (p=0.008, Table 5-2).

Of worth, comparing those ER+ tumours overexpressing HER2 and those expressing high KI67-LI, revealed that both high KI67-LI (within HER2+ =96 case) and HER2+ (within high KI67-LI=502) were associated with younger age (p=0.015, p=0.010, both borderline, respectively), Pre-menopausal status (p=0.008, p=0.006, respectively), higher tumour grade (both p<0.001) and worse NPI scores (p=0.003, p=0.004, respectively, Table 5-3).

Table 5-1: association between HER2 and KI67-LI in ER-positive/luminal tumours

	HER2-negative (%)	HER2-Positive (%)	X^2; *p-value*
Low KI67-LI	588 (58)	23 (24)	40.61, <0.001
High KI67-LI	429 (42)	73 (76)	
Total	1017 (100)	96 (100)	1113

Table 5-2: Association between HER2, KI67-LI and the clinicopathological variables in ER-positive breast cancer

	ER+ BC series			ER+ BC series		
	HER2- N (%)	**HER2+ N (%)**	***p*-value (χ^2)**	**Low KI67-LI N (%)**	**High KI67-LI N (%)**	***p*-value (χ^2)**
Age			**<0.001**			**<0.001**
≤50	370(29)	52(45)	**(12.29)**	164(27)	197(37)	**(11.51)**
>50	892(71)	64(55)		434(73)	333(63)	
Menopausal Status			**0.008**			0.060
Pre-	429(33)	53(46)	**(7.23)**	207(34)	207(39)	(2.86)
Post-	854(67)	63(54)		408(66)	325(61)	
Tumour Size (cm)			**0.022**			**<0.001**
≤2.0	708(56)	52(45)	**(5.25)**	371(62)	244(46)	**(29.85)**
>2.0	549(44)	63(55)		227(38)	284(54)	
Stage			0.051			**<0.001**
1	1831(65)	62(54)	(5.94)	422(68)	285(54)	**(25.34)**
2	357(28)	40(35)		153(25)	196(37)	
3	88(7)	13(11)		41(7)	48(9)	
Tumour Type			**<0.001**			**<0.001**
Ductal	1181(79)	212(95)	**(42.97)**	465(75)	715(91)	**(126.63)**
Lobular	190(13)	1(1)		100(16)	36(4)	
Medullary-like	30(2)	7(3)		1(0)	28(4)	
Special-type	89(6)	2(1)		56(9)	8(1)	
Grade			**<0.001**			**<0.001**
1	334(26)	4(4)	**(105.35)**	240(39)	24(5)	**(355.97)**
2	555(44)	23(20)		299(48)	176(33)	
3	387(30)	88(76)		77(13)	330(62)	
Tubules			**0.001**			**<0.001**
1	108(9)	292)	**(15.99)**	80(13)	7(1)	**(75.59)**
2	493(40)	34(30)		255(43)	184(36)	
3	628(51)	78(68)		259(44)	322(63)	
Pleomorphism			**<0.001**			**<0.001**
1	49(4)	0(0)	**(65.91)**	32(5)	2(0)	**(205.67)**
2	668(54)	22(20)		396(67)	153(30)	
3	511(42)	91(80)		165(28)	358(70)	
Mitosis			**<0.001**			**<0.001**
1	644(52)	10(9)	**(102.17)**	446(75)	72(14)	**(439.30)**
2	252(21)	25(22)		96(16)	143(28)	
3	333(27)	79(69)		52(9)	298(58)	
LVI			0.051			**<0.001**
Probable/Negative	883(70)	72(62)	(3.80)	452(76)	323(61)	**(21.68)**
Definite	370(30)	44(38)		146(24)	205(39)	
NPI			**<0.001**			**<0.001**
GPG	569(45)	12(11)	**(73.51)**	332(55)	91(12)	**(190.29)**
MPG	567(44)	71(62)		231(38)	490(65)	
PPG	142(11)	31(27)		40(7)	171(23)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 5-3: Association between KI67-LI, HER2 and the clinicopathological variables in ER+ breast cancer within HER2+ and high KI67-LI cohorts respectively

	ER+HER2+			ER+High KI67-LI		
	Low KI67-LI N (%)	**High KI67-LI N (%)**	***p*-value (χ^2)**	**HER2- N (%)**	**HER2+ N (%)**	***p*-value (χ^2)**
Age			**0.015**			**0.010**
≤50	5(22)	37(51)	**(5.95)**	149(35)	37(51)	**(6.65)**
>50	18(78)	36(49)		278(65)	36(49)	
Menopausal Status			**0.008**			**0.006**
Pre-	5(22)	39(53)	**(7.07)**	157(37)	39(53)	**(7.42)**
Post-	18(78)	34(47)		272(63)	34(47)	
Tumour Size (cm)			0.932			0.561
≤2.0	10(44)	31(43)	(0.00)	197(46)	31(43)	(0.33)
>2.0	13(5)	42(57)		230(54)	42(57)	
Stage			0.073			0.402
1	16(70)	34(46)	(5.23)	233(55)	34(46)	(1.82)
2	4(17)	32(44)		153(36)	32(44)	
3	3(13)	7(10)		40(9)	7(10)	
Tumour Type			0.166			0.122
Ductal	21(95)	71(99)	(3.59)	368(90)	71(99)	(5.78)
Lobular	0(0)	1(1)		31(7)	1(1)	
Medullary-like	-	-		3(1)	0(0)	
Special-type	1(5)	0(0)		7(2)	0(0)	
Grade			**<0.001**			**<0.001**
1	3 (13)	0(0)	**(18.59)**	23(5)	0(0)	**(19.66)**
2	9(39)	10(14)		149(35)	10(14)	
3	11(48)	63(86)		255(60)	63(86)	
Tubules			**0.036**			0.068
1	1(4)	0(0)	**(6.64)**	6(1)	0(0)	(5.37)
2	10(44)	18(25)		151(37)	18(25)	
3	12(52)	55(75)		255(62)	55(75)	
Pleomorphism			**0.001**			**0.001**
1	9(41)	8(11)	**(10.32)**	2(1)	0(0)	**(13.22)**
2	13(59)	65(89)		129(31)	8(11)	
3				281(68)	65(89)	
Mitosis			**<0.001**			**<0.001**
1	8(35)	1(1)	**(29.91)**	64(15)	1(1)	**(17.15)**
2	7(30)	14(19)		118(29)	14(19)	
3	8(35)	58(80)		230(56)	58(80)	
LVI			0.195			0.575
Probable/Negative	17(74)	43(59)	(1.68)	265(62)	43(59)	(0.31)
Definite	6(26)	30(41)		160(38)	30(41)	
NPI			**0.003**			**0.004**
GPG	7(30.4%)	3(4)	**(11.84)**	86(21)	3(4)	**(10.92)**
MPG	13(56.5%)	46(70)		244(61)	46(70)	
PPG	3(13.0%)	17(26)		73(18)	17(26)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

5.3.1.2 Association of HER2 and KI67-LI with biological markers

Within ER+ cohort, both high KI67-LI and HER2+ were associated with an increased expression of ER-co-regulators (CD-71: both $p<0.001$) and (CARM1: $p=0.019$ and $p=0.048$, PELP1: $p=0.020$ and $p=0.040$, all borderline, respectively) and poor prognostic markers (P-cadherin: $p=0.003$ and $p<0.001$, N-cadherin: $p=0.025$: borderline and $p=0.023$: borderline and p53: both $p<0.001$, respectively) and with downregulation of (AR: both $p=0.001$ and BCL2: p=0.004 and p<0.001, respectively, Table 5-4). In addition, both high KI67-LI and HER2 were associated with decreased ER levels where HER2 was associated with decreased ER mean H-score: 162 to 116 ($p<0.001$); meanwhile; high KI67-LI was significantly associated with a decrease in ER mean H-Score from 165 to 152 ($p=0.007$).

Interestingly, high KI67-LI was associated with downregulation of luminal cytokeratins (CK18: $p<0.001$, CK19: $p=0.024$: borderline) but high expression of HER family proteins (HER1: $p=0.004$, HER3: $p=0.001$ and HER4: $p<0.001$, Table 5-4). On the other hand, HER2+ was associated with high expression of differentiation-associated protein (TFF3: $p<0.001$) and with E-cadherin ($p=0.009$, Table 5-4).

To further explore the influence of HER2 and KI67-LI on the ER-pathway, the biology of HER2+/High KI67-LI cases was compared to HER2+/ low KI67-LI cases, where high KI67-LI showed positive associations with ER-related proteins: AR and a trend of TFF1 and GATA3 ($p=0.001$, $p=0.036$, $p=0.027$ respectively, Table 5-5). In addition, it was associated with downregulation of N-cadherin (EMT associated protein, $p=0.045$: borderline, Table 5-5).

In those HER2+ cases within high KI67-LI, there was low expression of PgR ($p=0.006$) but high expression of TFF1, TFF3 and E-cadherin ($p=0.008$, $p=0.004$, $p=0.018$: borderline, respectively, Table 5-5).

To determine the mere effect of HER2 and KI67-LI expressions, HER2+/low KI67-LI and HER2-/high KI67-LI were tested against biological markers, where high KI67-LI vs HER2+ was associated with increased expressions of PgR, AR, GATA3, BCL2 and P-Cadherin ($p=0.001$, $p<0.001$, $p=0.018$: borderline, $p=0.003$, $p=0.002$) but was associated with a trend of decreased expression of N-Cadherin ($p=0.030$).

Table 5-4: The associations between HER2, KI67-LI and different biological markers

	ER+ BC tumours			ER+ BC tumours		
	HER2- N (%)	**HER2+ N (%)**	***p*-value (χ^2)**	**Low KI67-LI N (%)**	**High KI67-LI N(%)**	***p*-value (χ^2)**
PgR			**<0.001**			0.205
Negative	247(20)	46(41)	**(28.13)**	113(19)	117(23)	(1.60)
Positive	1001(80)	67(59)		477(81)	402(77)	
AR			**0.001**			**0.001**
Negative	257(22)	37(36)	**(11.52)**	102(19)	136(28)	**(10.64)**
Positive	892(78)	66(64)		434(81)	352(72)	
CK7/8			0.761			0.276
Negative	1(0)	0(0)	(0.09)	0(0)	1(0)	(1.18)
Positive	1248(100)	114(100)		587(100)	522(100)	
CK18			0.954			**<0.001**
Negative	54(5)	4(4)	(0.00)	10(2)	35(7)	**(18.77)**
Positive	1088(95)	97(96)		525(98)	453(93)	
CK19			0.868			**0.024**
Negative	69(6)	6(5)	(0.02)	25(4)	39(8)	**(5.08)**
Positive	1154(94)	106(95)		552(96)	475(92)	
FOXA1			0.707			0.070
Negative	338(44)	42(46)	(0.14)	147(43)	171(49)	(3.29)
Positive	426(56)	50(54)		198(57)	179(51)	
BEX1			0.635			0.171
Negative	226(30)	29(33)	(0.22)	95(29)	108(33)	(1.87)
Positive	524(70)	58(67)		233(71)	224(67)	
TFF1			0.154			0.923
Negative	432(64)	45(55)	(2.03)	191(64)	188(61)	(0.00)
Positive	245(36)	37(45)		106(36)	119(39)	
TFF3			**<0.001**			0.318
Negative	280(41)	18(21)	**(13.09)**	118(41)	115(36)	(0.99)
Positive	401(59)	66(79)		172(59)	203(64)	
GATA3			0.071			0.569
Negative	320(49)	41(59)	(3.27)	143(49)	160(51)	(0.32)
Positive	337(51)	28(41)		147(51)	155(49)	
CD71			**<0.001**			**<0.001**
Negative	411(53)	25(31)	**(15.67)**	198(60)	152(43)	**(17.30)**
Positive	371(47)	57(69)		134(40)	201(57)	
CARM1			**0.048**			**0.019**
Negative	237(32)	19(21)	**(6.06)**	111(33)	92(27)	**(7.95)**
Moderate	388(51)	49(54)		176(53)	168(50)	
High	130(17)	23(25)		48(14)	77(23)	
PELP1			**0.040**			**0.020**
Negative	157(20)	7(9)	**(5.89)**	79(24)	63(17)	**(6.04)**
Moderate	517(65)	61(74)		212(65)	244(67)	
High	120(15)	14(17)		37(11)	60(16)	
E-Cadherin			**0.009**			0.194
Negative	461(38)	28(25)	**(6.73)**	215(37)	169(34)	(1.68)
Positive	757(62)	84(75)		366(63)	335(66)	
P-Cadherin			**<0.001**			**0.003**
Negative	642(63)	37(41)	**(17.45)**	321(65)	255(57)	**(8.54)**
Positive	378(37)	54(59)		153(32)	190(43)	
N-Cadherin			**0.023**			**0.025**
Negative	461(38)	28(25)	**(5.19)**	149(38)	123(31)	**(5.00)**
Positive	757(62)	84(75)		244(62)	276(69)	
p53			**<0.001**			**<0.001**
Negative/low	1039(83)	67(59)	**(40.06)**	507(86)	367(71)	**(38.26)**
Positive	212(17)	46(41)		81(14)	150(29)	
BCL2			**<0.001**			**0.004**
Negative/low	91(13)	25(35)	**(28.23)**	33(11)	65(20)	**(8.14)**
High	599(87)	46(65)		282(89)	259(80)	
HER1			0.082			**0.004**
Negative	1068(87)	93(82)	(3.02)	515(89)	431(83)	**(8.44)**
Positive	162(13)	21(18)		63(11)	89(17)	
HER3			0.870			**0.001**
Negative	113(10)	11(11)	(0.02)	62(12)	28(6)	**(10.83)**
Positive	1020(90)	92(89)		460(88)	453(94)	
HER4			0.147			**<0.001**
Negative	206(17)	13(12)	(2.09)	115(20)	55(11)	**(18.67)**
Positive	1020(83)	97(88)		450(80)	462(89)	

Table 5-5: The associations of KI67-LI with biological markers within HER2+ BC and association of HER2 with these markers within High KI67-LI

	ER+HER2+ Tumours			ER+High KI67-LI tumours		
	Low KI67-LI N (%)	**High KI67-LI N (%)**	***p*-value (χ^2)**	**HER2- N (%)**	**HER2+ N(%)**	***p*-value (χ^2)**
PgR	-	-	**-**			**0.006**
Negative				85(20)	25(35)	**(7.55)**
Positive				337(80)	47(65)	
AR			**0.001**	-	-	-
Negative	13(68)	19(28)	**(10.16)**			
Positive	6(32)	48(72)				
FOXA1			0.093	-	-	-
Negative	13(65)	26(43)	(2.81)			
Positive	7(35)	34(57)				
BEX1			0.081	-	-	-
Negative	10(53)	16(30)	(3.05)			
Positive	9(47)	37(70)				
TFF1			**0.036**			**0.008**
Negative	10(56)	14(28)	**(4.40)**	118(48)	14(28)	**(6.95)**
Positive	8(44)	36(72)		126(52)	36(72)	
TFF3	-	-	**-**			**0.004**
Negative				100(40)	10(19)	**(8.12)**
Positive				153(60)	43(81)	
GATA3			**0.027**	-	-	-
Negative	11(85)	21(50)	**(4.88)**			
Positive	2(15)	21(50)				
E-Cadherin			0.060			**0.018**
Negative	9(39)	14(20)	(3.54)	141(34)	14(20)	**(5.61)**
Positive	14(61)	57(80)		275(66)	57(80)	
P-Cadherin			0.050	-	-	**-**
Negative	4(22)	28(48)	(3.82)			
Positive	14(78)	30(52)				
N-Cadherin			**0.045**	-	-	**-**
Negative	1(6)	16(30)	**(4.00)**			
Positive	16(94)	38(70)				

5.3.2 Breast cancer outcome in association with HER2 and KI67-LI within ER+ tumours

HER2 negativity and low-KI67-LI were both associated with better BCSS, DFI and DMFS (p<0.001, Figure 5-1).

Interestingly, in tumours where HER2 was negative and KI67-LI was low resulted in the best outcome but HER2 positivity and low KI67-LI tumours were associated with the worst outcome (p<0.001, Figure 5-2).

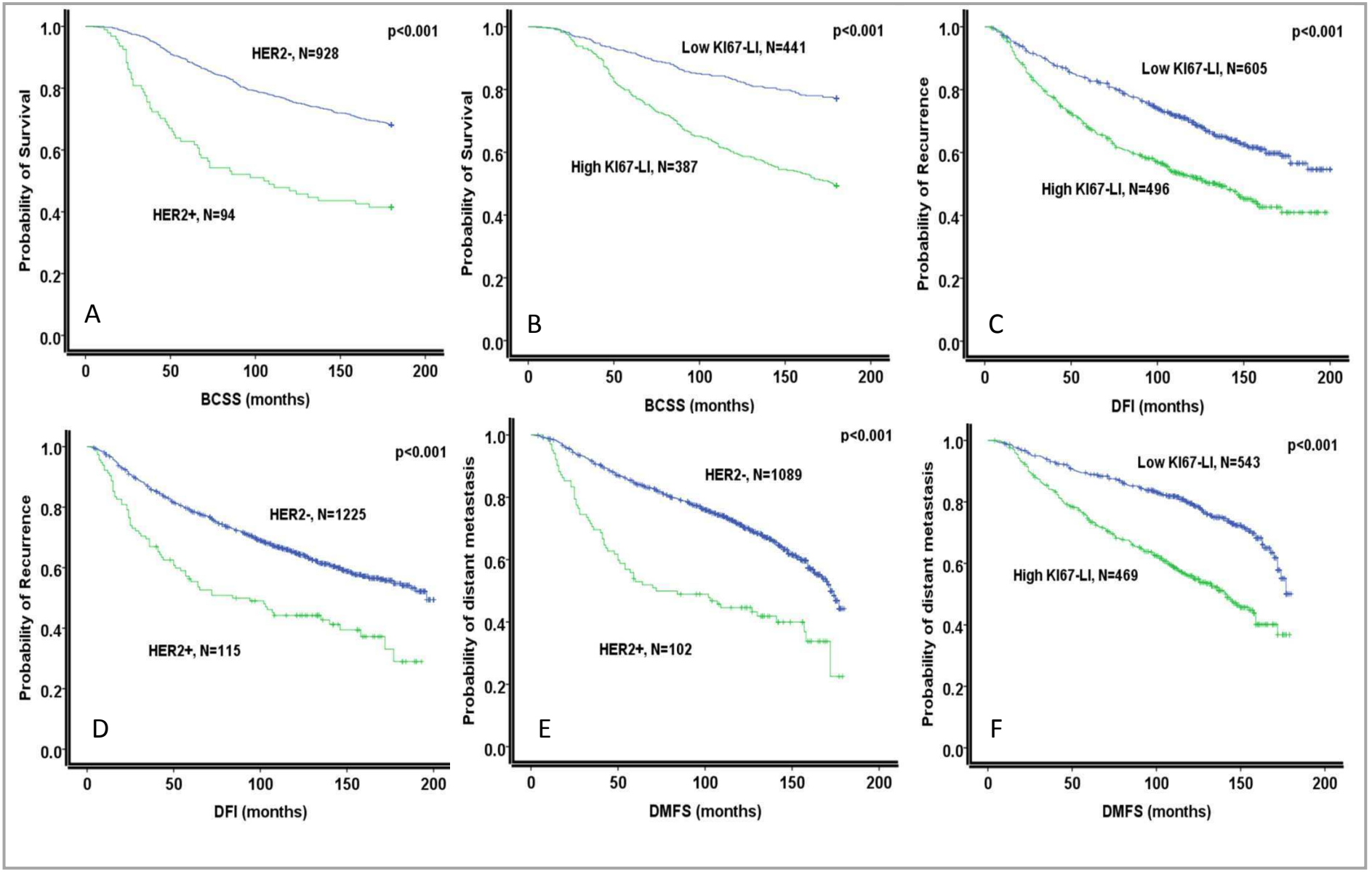


Figure 5-1: A& B: BCSS, C& D: DFI and E&F: DMFS for HER2 and KI67-LI within ER+ tumours

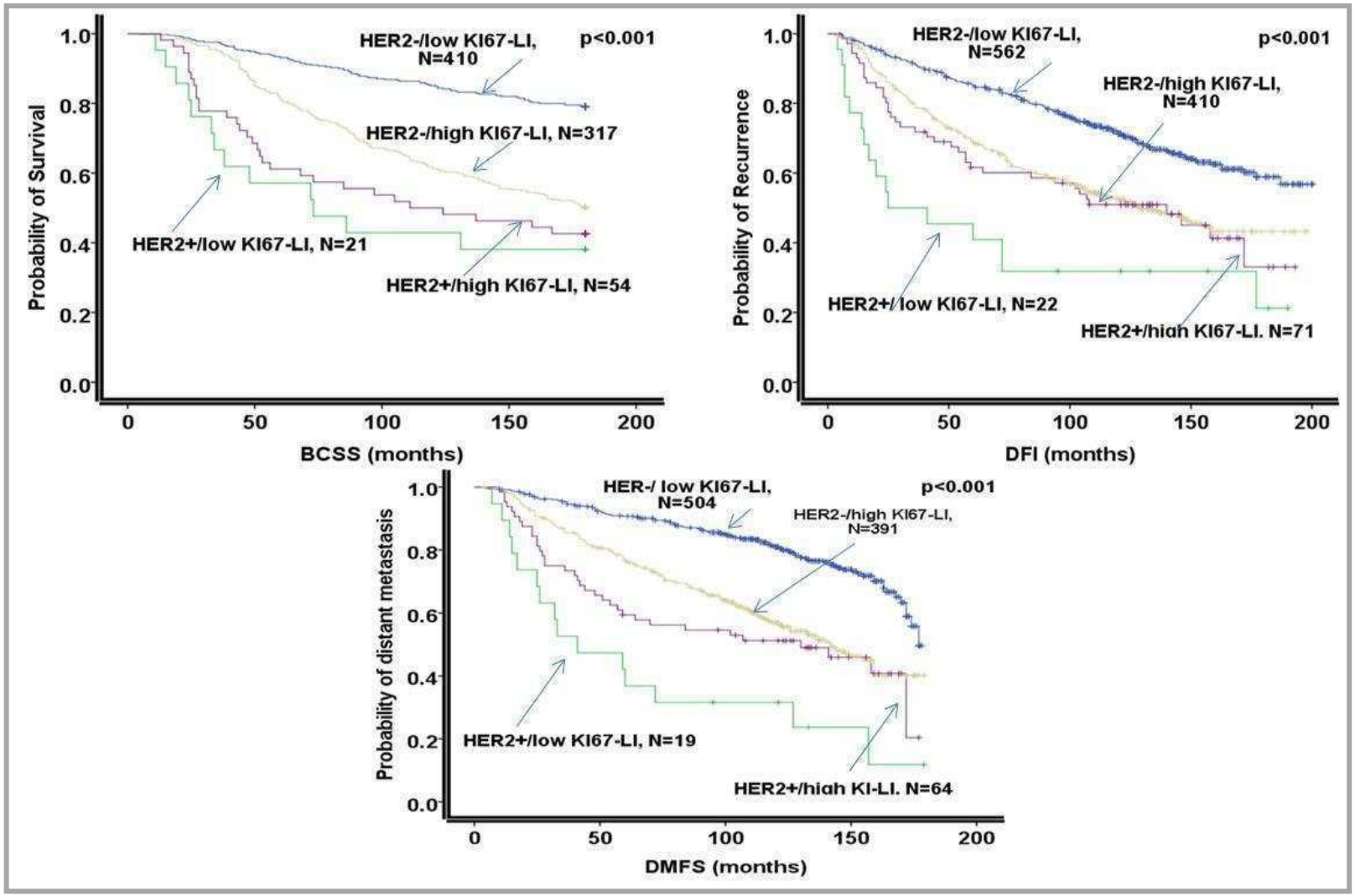


Figure 5-2: BCSS, DFI and DMFS for different combinations of HER2 and KI67-LI within ER+ cohort

5.4 Discussion

Molecular classification of BC and the concept that ER, HER2 and proliferation are key driving markers is well understood. However, the fact that luminal tumours comprise more than half of BC and HER2+ tumours, are candidates for anti-HER2 therapy regardless of the molecular class limit the clinical significance of this classification. Although high proliferation is a feature of basal and HER2+ tumours; a considerable number of ER+ tumours revealed high proliferation status. To identify whether HER2 or KI67 could identify luminal B subclass being both associated with poor outcome, GEP studies have reported a luminal-B subclass with high proliferation (Sørlie et al., 2001). However, molecular features are used to define these tumours which vary among different studies and more than one class has been identified (Sørlie et al., 2003, Draghici et al., 2006, Oh et al., 2006, Sotiriou et al., 2006b). In addition, the availability and cost associated with GEP make identification of a poor prognostic subclass of luminal tumours difficult in clinical practice.

Using surrogate immunohistochemical markers to identify a luminal-B class in routine clinical practice appears a valid and practical alternative. However, previous studies have varied in the marker(s) used to identify these tumours. Different views have been reported in this regard and whether to include KI67-LI or HER2 in the definition of luminal-B is still a matter of debate (Cheang et al., 2009c, Aleskandarany et al., 2012, Ihemelandu et al., 2007, Kurebayashi et al., 2007, Matos et al., 2005, Carey et al., 2006, Onitilo et al., 2009, Hu et al., 2006a, Hugh et al., 2009b, Cheang et al., 2009b, Bhargava and Dabbs, 2008).

Previous studies have indicated that the frequency of ER+ tumours with high proliferation status is greater than that of HER2+ tumours and of interest, this finding has been revealed by a study carried out on blocks from cell lines representing different molecular classes (Subik et al., 2010). Moreover both markers are associated with other poor prognostic features and shorter survival. However, a detailed comparative study of HER2 and KI67 in the luminal class regarding the clinical and biological molecular features is lacking. In this Chapter, HER2 appears to be associated with a worse outcome independent of proliferation and other clinicopathological features. Importantly the aggressive behaviour of HER2+ tumours is not associated with downregulation of luminal-enriched ER-related biomarkers. Compared to tumours with high KI67-LI;

HER2+ tumours retained their luminal-associated features as evidenced by positive association between HER2+ with TFF1, and TFF3and markers of good prognosis including E-Cadherin. In contrast high KI67-LI was associated with downregulation of luminal markers (CK18, CK19) and borderline significance with downregulation of FOXA1. In addition, high KI67-LI showed an association with upregulation of other HER family proteins (HER1, HER3 and HER4).

Although both HER2 and KI67 demonstrated associations with features of poor prognosis, high KI67-LI not HER2+ was significantly associated with clinical features of advanced tumours including nodal positivity, larger tumour size and definite lymphovascular invasion (LVI) which indicates that high proliferation status is a feature of biological aggressiveness rather than a unique driving genetic event. In addition, we revealed that KI67-LI is more associated with decreased luminal associated proteins than HER2 evidenced in our data by an association of KI67-LI (within HER2+ tumours) with upregulation of ER associated proteins.

With regard to outcome, although HER2 overexpression was not associated with LN stage, definite LVI or decreased expression of luminal proteins, it was associated with poorer outcome in terms of shorter BCSS, DFI and DMFS than high KI67-LI. The association with BCSS was observed in ER+ tumours as well as in the ER+/low KI67-LI subgroup reflecting the association with aggressive behaviour independent on its proliferative potential.

In conclusion, the results presented here support the hypothesis that HER2 gene amplification and protein over expression occur as a second oncogenic hit that drives the molecular portrait and clinical behaviour of ER+/HER2+ BC independent of the ER-pathway or proliferation. Current data indicates that HER2+/ER+ tumours are distinct form of luminal BC and that it provides extra justification to place them in the HER2+ BC candidate for anti-HER2 therapy.

6 Other Biomarkers Related to HER2 and ER Pathways

6.1 Introduction

In this Chapter, biomarkers related to protein homeostasis, cancer cell stemness, steroid receptors coregulators and cell cycle regulators that are related to ER and HER2 pathways were investigated. These markers (CHIP, SOX9, SRC3 and ECD) were chosen based on previous *in-vitro* observations in BC cell lines that indicated the importance of these proteins in BC and their potential interaction with HER2 and ER pathways in addition to their previously defined biological functions (Connell et al., 2001, Chakravarty et al., 2011, Ma et al., 2011, Zhao et al., 2012, Murphy et al., 2004). In addition to assessing the impact of ER phosphorylation on its function and the interaction with HER2, phosphorylated ER at SER 118 was also assessed.

1- CHIP

CHIP (35 KDa) has two important domains; one at its amino terminus end that interacts and modulates the function of Hsp-70 and another motif in its domain at the carboxy terminus end that functions to mediate ubiquitin ligase activity(Connell et al., 2001). CHIP protein has a protective role in targeting damaged proteins for degradation which eventually assists in death of those cells harbouring damaged or misfolded proteins which mainly occurs during oxidative stress (Lee et al., 2013). The significance of CHIP in enhancing the ubiquitin proteosomal degradation of ER *in vitro* after CHIP transfection has been reported (Fan et al., 2005) and this effect seems to be interrupted by a protein called Hsp-90 which function to maintain ER in a ligand dependent manner (Pratt and Toft, 1997). From the practical point of view, this appears to have implications on response to hormonal therapy (Beliakoff et al., 2003, Bagatell et al., 2001).

Similar to its role in ER stabilisation, Hsp-90 has been shown to stabilise HER2 protein preventing its degradation (Xu et al., 2002). CHIP induces Hsp-70 forming a complex with HER2 promoting its dissociation. Moreover, substances have been found to enhance the binding of CHIP to HER2 for its degradation, of which is flavenoid Quercetin (Jeong et al., 2008).

2- 1.6.3.2 SOX9

SOX9 is one of the determinants of the stem cell state of the breast tissue and it is well recognised for its nuclear function following intra-nuclear translocation as it is required for development and differentiation. Interestingly, its cytoplasmic expression was shown to be associated with higher grade tumours and poor prognosis mainly in invasive ductal carcinoma especially those having metastasis. The latter implies the benefit of SOX9 cytoplasmic expression as a predictive biomarker in predicting BC outcome (Chakravarty et al., 2011). As it is well known that the passage of neoplastic cells in EMT program renders them an aggressive phenotype which enables increased motility, invasiveness and metastasis of malignant cells (Huber et al., 2005), some studies focused on the presence of a link between EMT and MaSCs. Nevertheless, direct evidence has remained scarce thus far whether EMT can generate bona fide stem cells rather than integrating properties from actual stem cells into these cells (Mani et al., 2008, Morel et al., 2008).

3- SRC3

The importance of SRC-3/ AIB1 appears in enhancing the resistance to endocrine therapy in patients with BC if there is a concomitant HER2 over expression (He et al., 2009). The subsequent action of ER after its activation is mainly related to the levels of co-activators or co-repressors. Importantly, tamoxifen having partial agonistic activity, its action is modulated by SRC3 and will favour the transcription of genes that promote development and growth of BC (Graham et al., 2000, Smith et al., 1997). Moreover, a study revealed that in those patients, recurrence of BC is more evident in those having high SRC3 levels (Osborne et al., 2003b).

4- ECD

ECD is a cell cycle modulator that favours the progression of the cell cycle via releasing the inhibitory effect of Rb protein on E2F transcription factors enhancing gene transcription required for growth and progression of cancer. ECD expression is highly correlated to HER2 and some poor prognostic variables and is reported to be a predictive of poor outcome especially in HER2+ BC (Zhao et al., 2012).

5- SER 118 ER

ER is a transcription factor that is apart from having its specific binding site for oestrogen; it can be phosphorylated at other sites at its AF-1 domain. For instance; SER 118 ER site (Kato et al., 1995a) is perhaps required by tamoxifen to mediate a decrease in ER activation (Cheng et al., 2007). For this reason, this biomarker has been a focus of several studies, which so far yielded conflicting findings (Sarwar et al., 2006, Zoubir et al., 2008). For instance, a study reported that SER 118 ER is directly associated with ER status and low grade tumours (Murphy et al., 2004). Others highlight its role from a therapeutic point of view and reported that patients treated with tamoxifen benefit more if they have highly phosphorylated SER 118 ER compared to low levels of phosphorylation (Kok et al., 2009b). Other investigators indicate that p-SER 118 ER is available at the promoters of ER regulated genes and is unaffected by the level of HER2 (Weitsman et al., 2006). Other investigators reported that phosphorylation of ER at 167 site is a predictor of favourable outcome rather than SER 118 ER (Yamashita et al., 2008). Therefore, the role of SER 118 ER is still to be elucidated and an actual understanding for its function warrants further investigation.

6.1.1 Hypothesis

Biomarkers related to different mechanisms of the HER2 and ER pathways are directly involved with the complex interactions between HER2 and ER in BC and could act as therapeutic targets. In addition, investigation of the biological significance of a phosphorylated form of ER (SER 118 ER) rather than the total ER will help more understanding of the possible cross talk between HER2 and ER and it could have a therapeutic potential.

6.1.2 Aims

1- To study the expression of CHIP, SOX9 and SER 118 in the primary BC series prepared as TMA and the expression of CHIP, SER 118 ER, SRC3 and ECD was also assessed in Trastuzumab treated series. Association of protein expression with clinicopathological variables and a range of proteins related to

ER, HER2, and other BC related proteins will be explored in BC and different BC subgroups based on HER2 and ER expressions.

2- To assess the prognostic and predictive utility of the expression of all proteins in the BC and within different BC molecular subtypes (for those assessed in the primary series).

6.2 Method

Tissue microarrays for BC from the primary and Trastuzumab treated series were prepared and immunohistochemical staining was performed as described (section 2.4.1, page 50).

Table 6-1 shows details regarding antibodies used, manufacturer, and staining conditions.

Table 6-1: Details of the antibodies used in this chapter

Antibody	Supplier	species	Clone	Molecular weight (kDa)	Dilution
p-CHIP	Thermo scientific	Rabbit polyoclonal	PA5-29024	35	1:2000
SOX9	EMD	Rabbit polyoclonal	-	65	1:10000
ECD[1]	-	Mouse monoclonal	4A8	75	1:2000
SRC3	BD Biosciences	Mouse monoclonal	-	155	1:250
p-SER 11ER (SER 118)	Cell signalling	Mouse monoclonal	16J4	66	1:350

1: Generated by the Monoclonal Antibody Facility at the Lurie Cancer Centre, Northwestern University, Chicago. Citrate buffer antigen retrieval was used for all antibodies which were incubated Overnight at 4°C

6.3 Results

6.3.1 CHIP

6.3.1.1 The pattern of CHIP expression in breast cancer

A total of 972 cases were available for the assessment of CHIP. This biomarker was homogeneously detected in the nuclei and cytoplasm of the malignant cells, normal breast acini as well as DCIS foci entrapped within some TMA cores (Figure 6-1). The cut-off point was set at >30 (H-score) and > 140 (H-score) for the nuclear and cytoplasmic forms, respectively by using the X-tile. Out of the total, 658 (67.7%) cases had negative/low nuclear expression while 314 (32.3%) cases showed high expression. Regarding the cytoplasmic form, 542 (56.2%) cases had negative/low expression and 423 (43.8%) cases revealed high expression (Table 2-5).

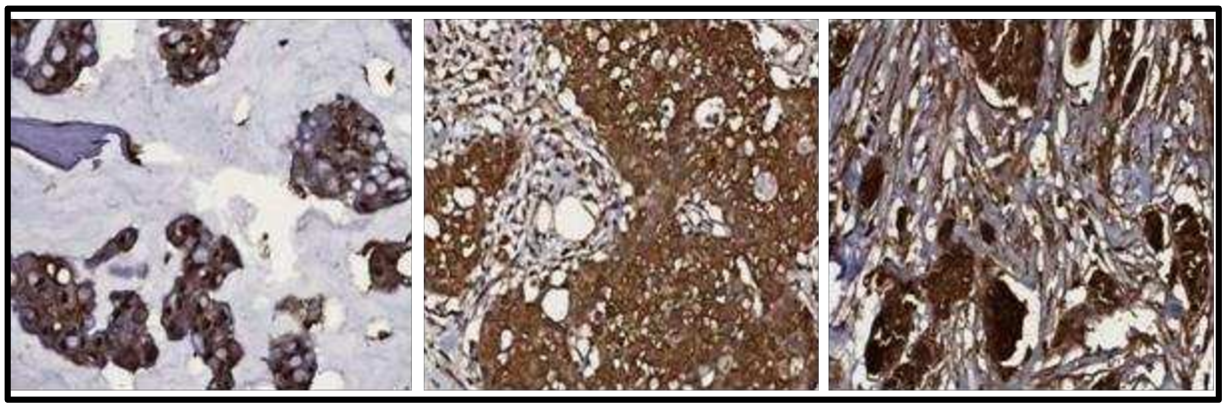


Figure 6-1: Different intensities of staining of CHIP protein. From the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20

6.3.1.2 The associations of CHIP with clinicopathological variables in the unselected primary breast cancer series and different subgroups

Within the whole BC series, nuclear CHIP was associated with smaller tumour size (p=0.008), lobular BC type, lower tumour grade, more tubule formation, less pleomorphism, lower mitotic counts and good NPI score (all p<0.001). Importantly, associations with lower tumour grade, less pleomorphism, lower

mitotic counts and lower NPI score were all maintained within ER+HER2- cohort (Table 6-2).

The cytoplasmic expression of CHIP was associated with a trend for Post-menopausal status (p=0.022), higher tumour grade and lower mitotic counts (p=0.047). In addition, when the analysis was restricted to the ER+HER2- phenotype, cytoplasmic CHIP was associated with a trend for Post-menopausal status (p=0.010), higher tumour grade (p=0.014), less tubule formation (p=0.039) and significantly with more pleomorphism (p=0.008, Table 6-2). Within ER+HER2+ cohort, high nuclear CHIP expression was associated with lower tumour grade (p=0.004), more tubule formation (p=0.005) and less frequent mitoses (p=0.009, appendix table 9). Higher cytoplasmic expression of CHIP was associated with a trend for more tubule formation (p=0.032) and less frequent mitoses (p=0.012). In addition, within ER-HER2+ borderline association for definite LVI was observed for cytoplasmic CHIP (p=0.061). No associations were observed within ER-HER2- group, (appendix table 9).

Table 6-2: The associations between nuclear CHIP and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.316			0.570
≤50	231(35)	100(32)	(1.00)	112(30)	66(28)	(0.32)
>50	427(65)	214(68)		263(70)	172(72)	
Menopausal Status			0.768			0.662
Pre-	248(38)	121(39)	(0.06)	123(33)	82(35)	(0.19)
Post-	408(62)	192(61)		251(67)	155(65)	
Tumour Size (cm)			**0.008**			0.128
≤2.0	89(45)	168(53)	**(6.94)**	186(50)	134(56)	(3.31)
>2.0	361(55)	146(47)		186(50)	104(44)	
Stage			0.558			0.377
1	398(61)	192(61)	(1.16)	237(64)	148(62)	(1.95)
2	195(30)	101(32)		108(29)	79(33)	
3	55(9)	21(7)		25(7)	11(5)	
Tumour Type			**<0.001**			0.054
Ductal	559(87)	253(81)	**(22.40)**	307(83)	186(78)	(7.62)
Lobular	43(7)	45(15)		40(11)	42(18)	
Medullary-like	20(3)	1(0)		3(1)	0(0)	
Special-type	21(3)	13(4)		19(5)	10(4)	
Grade			**<0.001**			**0.001**
1	66(10)	72(23)	**(51.78)**	61(17)	61(26)	**(12.29)**
2	194(30)	126(40)		161(43)	114(48)	
3	388(60)	116(37)		148(40)	63(26)	
Tubules			**<0.001**			0.605
1	23(4)	23(8)	**(15.29)**	23(6)	19(8)	(1.00)
2	187(30)	117(38)		139(39)	95(41)	
3	417(66)	167(54)		196(55)	120(51)	
Pleomorphism			**<0.001**			**0.015**
1	11(2)	6(2)	**(32.74)**	11(3)	4(2)	**(8.36)**
2	196(31)	155(50)		170(48)	139(59)	
3	418(67)	146(48)		177(49)	91(39)	
Mitosis			**<0.001**			**<0.001**
1	157(25)	148(48)	**(61.10)**	142(40)	134(57)	**(18.94)**
2	117(19)	63(21)		88(24)	48(21)	
3	353(56)	96(31)		128(36)	52(22)	
LVI			0.993			0.396
Probable/Negative	418(64)	202(64)	(0.00)	244(66)	164(69)	(0.72)
Definite	230(36)	111(36)		128(34)	74(31)	
NPI			**<0.001**			**0.020**
GPG	147(24)	126(42)	**(32.21)**	128(36)	109(48)	**(7.80)**
MPG	368(59)	144(48)		187(53)	103(45)	
PPG	103(17)	32(10)		38(11)	17(7)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 6-3: The associations between cytoplasmic CHIP and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.201			0.172
≤50	193(36)	134(32)	(1.63)	106(31)	70(26)	(1.86)
>50	349(64)	289(68)		234(69)	198(74)	
Menopausal Status			**0.022**			**0.010**
Pre-	222(41)	143(34)	**(5.25)**	128(38)	75(28)	**(6.55)**
Post-	318(59)	279(66)		210(62)	193(72)	
Tumour Size (cm)			0.672			0.374
≤2.0	258(48)	196(47)	(0.17)	182(54)	135(50)	(0.79)
>2.0	279(52)	224(53)		155(46)	133(50)	
Stage			0.462			0.812
1	336(63)	251(60)	(1.54)	216(64)	166(62)	(0.41)
2	162(30)	132(31)		100(30)	86(32)	
3	37(7)	37(9)		19(6)	16(6)	
Tumour Type			0.489			0.704
Ductal	455(85)	351(84)	(2.42)	277(82)	212(79)	(1.40)
Lobular	44(8)	44(11)		41(12)	41(15)	
Medullary-like	14(3)	7(2)		2(1)	1(1)	
Special-type	20(4)	14(3)		16(5)	13(5)	
Grade			**0.047**			**0.014**
1	90(17)	48(11)	**(6.12)**	82(24)	40(15)	**(8.48)**
2	169(32)	151(36)		146(44)	129(48)	
3	276(51)	221(53)		107(32)	99(37)	
Tubules			0.209			**0.039**
1	30(6)	16(4)	(3.12)	27(8)	15(6)	**(6.47)**
2	175(34)	128(31)		140(43)	93(35)	
3	311(60)	268(65)		158(49)	155(59)	
Pleomorphism			0.092			**0.008**
1	11(2)	6(2)	(4.78)	10(3)	5(2)	**(9.68)**
2	209(41)	141(34)		187(58)	121(46)	
3	295(57)	264(64)		128(39)	137(52)	
Mitosis			**0.047**			0.752
1	164(32)	141(34)	**(6.09)**	150(46)	126(48)	(0.57)
2	88(17)	91(22)		73(23)	62(24)	
3	264(51)	180(44)		102(31)	75(28)	
LVI			0.177			0.356
Probable/Negative	357(67)	262(62)	(1.81)	232(69)	175(65)	(0.85)
Definite	178(33)	157(38)		105(31)	93(35)	
NPI			0.443			0.497
GPG	153(30)	120(30)	(1.62)	138(43)	99(39)	(1.39)
MPG	293(57)	215(54)		157(49)	129(50)	
PPG	68(13)	64(16)		27(8)	27(11)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

6.3.1.3 The associations of CHIP with biological markers in the unselected primary breast cancer series and different subgroups

Nuclear CHIP revealed an association with high expression of HRs (ER, PgR and AR), GATA3 and BEX1 (all $p<0.001$). Furthermore, it was associated with an increased expression of CK18, FOXA1 (both $p=0.001$), CK19, TFF1 (both $p=0.014$) and BCL2 ($p=0.003$). In contrast, high nuclear CHIP expression was negatively associated with low CD71 ($p=0.016$: borderline), P-cadherin ($p=0.001$), p53 ($p=0.003$) and KI67-LI ($p<0.001$), HER1, HER4 (both $p=0.009$) and HER2 ($p=0.001$, Table 6-4). Of worth, some of the mentioned associations were maintained when the analysis was restricted to ER+HER2- cohort including; an association with high expression of AR, BEX1, GATA3 ($p<0.001$) but with low KI67-LI ($p<0.001$) and HER4 ($p=0.004$, Table 6-4). Unlike the nuclear form, cytoplasmic CHIP was only associated with high CK18 and TFF1 ($p=0.001$) but with low CD71 ($p=0.005$) and no association was observed within ER+HER2- cohort (Table 6-5).

Interestingly, within ER+HER2+, high cytoplasmic CHIP expression was associated with a trend for low CD71 ($p=0.010$), and high HER3 expression ($p=0.027$). In addition, within ER-HER2+ BC, high cytoplasmic CHIP expression was associated with AR ($p=0.005$) and CK18 positivity ($p=0.017$: borderline) but with low BEX1 expression ($p=0.005$). Within ER-HER2- cohort, nuclear CHIP was positively associated with GATA3 but was negatively associated with p53 ($p=0.028$ and $p=0.042$, respectively, both are trends, appendix table 10) while the cytoplasmic form was positively associated with CK18 but negatively with TFF3 and CD71 ($p=0.024$: borderline, $p=0.008$ and $p=0.039$: borderline, respectively, appendix table 10). When the analysis with HER2 dimers and combinations was considered, nuclear CHIP was associated with low HER3 ($p=0.042$: borderline) dimers and cytoplasmic CHIP was associated with high HER4 dimer expression ($p=0.008$, Table 6-6). No associations were determined between CHIP and HER2 dimers within other BC subgroups.

Table 6-4: The associations of nuclear CHIP with biological markers

	Whole series			ER+HER2- tumours		
	Neg/low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**			**-**
Negative	207(32)	44(14)	**(33.28)**			
Positive	447(68)	266(86)				
PgR			**<0.001**			0.647
Negative	296(47)	97(32)	**(18.49)**	76(21)	45(19)	(0.21)
Positive	334(53)	205(68)		293(79)	191(81)	
AR			**<0.001**			**<0.001**
Negative	272(47)	65(24)	**(40.59)**	105(31)	33(15)	**(16.31)**
Positive	313(53)	209(76)		238(69)	181(85)	
CK7/8			0.310			**-**
Negative	12(2)	3(1)	(1.03)			
Positive	621(98)	297(99)				
CK18			**0.001**			0.180
Negative	92(16)	22(8)	**(11.21)**	17(5)	6(3)	(1.79)
Positive	476(84)	258(92)		316(95)	211(97)	
CK19			**0.014**			0.187
Negative	63(10)	16(5)	**(6.03)**	17(5)	6(3)	(1.74)
Positive	554(90)	283(95)		342(95)	226(97)	
FOXA1			**0.001**			0.088
Negative	268(60)	86(47)	**(10.21)**	125(50)	57(41)	(2.90)
Positive	176(40)	99(53)		125(50)	82(59)	
BEX1			**<0.001**			**<0.001**
Negative	175(40)	48(24)	**(14.19)**	100(39)	32(21)	**(14.52)**
Positive	265(60)	149(76)		157(61)	122(79)	
TFF1			**0.014**			0.090
Negative	213(55)	69(43)	**(5.99)**	116(52)	52(42)	(2.87)
Positive	177(45)	91(57)		108(48)	71(58)	
TFF3			0.071			0.667
Negative	208(50)	74(42)	(3.26)	100(41)	52(38)	(1.86)
Positive	211(50)	104(58)		147(59)	84(62)	
GATA3			**<0.001**			**<0.001**
Negative	284(72)	85(47)	**(32.75)**	133(58)	50(37)	**(15.62)**
Positive	111(28)	95(53)		97(42)	87(63)	
CD71			**0.016**			0.476
Negative	189(40)	100(50)	**(5.80)**	141(53)	86(56)	(0.50)
Positive	279(60)	98(50)		127(47)	67(44)	
CARM1			0.118			0.108
Negative	119(28)	45(23)	(4.27)	86(35)	38(25)	(4.45)
Moderate	204(47)	110(56)		113(46)	83(56)	
High	109(25)	41(21)		48(19)	28(19)	
PELP1			0.098			0.206
Negative	62(14)	37(17)	(4.65)	46(17)	31(18)	(3.16)
Moderate	338(74)	144(66)		198(74)	115(68)	
High	55(12)	36(17)		24(9)	24(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			0.359			0376
Negative	235(38)	104(35)	(4.80)	119(33)	85(37)	(0.78)
Positive	381(62)	193(65)		239(67)	146(63)	
p-Cadherin			**0.001**			0.282
Negative	229(44)	144(57)	**(11.62)**	189(62)	131(66)	(1.16)
Positive	293(56)	109(43)		117(38)	66(34)	
p53			**0.003**			0.086
Negative/low	423(68)	228(77)	**(8.56)**	299(82)	201(87)	(2.94)
Positive	200(32)	67(23)		67(18)	30(13)	
KI67-LI			**<0.001**			**<0.001**
Negative/low	171(34)	138(55)	**(31.54)**	132(45)	121(62)	**(14.82)**
High	339(66)	114(45)		164(55)	73(38)	
BCL2			**0.003**			0.964
Negative/low	219(45)	71(33)	**(8.78)**	67(23)	39(23)	(0.00)
High	272(55)	146(67)		221(77)	130(77)	
HER1			**0.009**			0.345
Negative	482(76)	254(84)	**(6.89)**	314(85)	206(88)	(0.89)
Positive	152(24)	50(16)		54(15)	28(12)	
HER2			**0.001**	-	-	-
Negative	521(83)	273(91)	**(10.67)**			
Positive	110(17)	28(9)				
HER3			0.608			0.264
Negative	44(8)	30(11)	(0.26)	31(9)	26(12)	(1.24)
Positive	539(92)	248(89)		310(91)	190(88)	
HER4			**0.009**			
Negative	65(10)	50(16)	**(6.89)**	42(11)	47(20)	**0.004**
Positive	560(90)	254(84)		325(89)	188(80)	**(8.32)**

Table 6-5: The associations of cytoplasmic CHIP with biological markers

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			0.110	-	-	**-**
Negative	151(28)	98(23)	(2.55)			
Positive	388(72)	320(77)				
PgR			0.691			0.193
Negative	216(42)	175(43)	(0.15)	61(18)	60(23)	(1.69)
Positive	302(58)	232(57)		273(82)	206(77)	
AR			0.296			0.405
Negative	193(41)	142(37)	(1.09)	71(23)	66(27)	(0.69)
Positive	279(59)	238(63)		232(77)	183(73)	
CK7/8			0.176	-	-	**-**
Negative	11(2)	4(1)	(1.82)			
Positive	509(98)	402(99)				
CK18			**0.001**			0.791
Negative	78(17)	34(9)	**(11.99)**	12(4)	9(4)	(0.07)
Positive	384(83)	345(91)		284(96)	240(96)	
CK19			0.416			0.422
Negative	47(9)	31(8)	(0.66)	14(4)	8(3)	(0.64)
Positive	461(91)	370(92)		310(96)	254(97)	
FOXA1			0.608			0.511
Negative	203(55)	149(57)	(0.26)	105(46)	77(49)	(0.43)
Positive	163(45)	110(43)		125(54)	80(51)	
BEX1			0.173			0.385
Negative	121(33)	101(38)	(1.85)	74(31)	57(35)	(0.75)
Positive	247(67)	164(62)		169(69)	108(65)	
TFF1			**0.001**			**0.002**
Negative	148(46)	133(60)	**(11.06)**	87(42)	80(59)	**(9.51)**
Positive	176(54)	88(40)		121(58)	56(41)	
TFF3			0.455			0.309
Negative	157(46)	123(49)	(0.55)	84(38)	67(43)	(1.03)
Positive	185(54)	128(51)		140(62)	90(57)	
GATA3			0.345			0.148
Negative	205(63)	161(66)	(0.89)	100(47)	82(55)	(2.09)
Positive	122(37)	81(34)		113(53)	68(45)	
CD71			**0.005**			0.076
Negative	147(39)	139(50)	**(8.04)**	123(50)	103(59)	(3.15)
Positive	234(61)	141(50)		121(50)	71(41)	
CARM1			0.403			0.519
Negative	91(25)	73(28)	(1.81)	72(31)	52(33)	(1.31)
Moderate	179(49)	135(51)		114(48)	82(51)	
High	92(26)	55(21)		49(21)	26(16)	
PELP1			0.70			0.667
Negative	58(15)	41(14)	(0.60)	44(18)	33(18)	(1.80)
Moderate	273(72)	204(71)		182(72)	127(69)	
High	48(13)	42(15)		25(10)	23(13)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			0.608			0.109
Negative	186(37)	153(38)	(0.26)	104(32)	100(39)	(2.56)
Positive	321(63)	246(62)		220(68)	160(61)	
p-Cadherin			0.168			0.0.533
Negative	190(46)	182(51)	(1.89)	167(62)	152(65)	(0.39)
Positive	223(54)	175(49)		100(38)	81(35)	
p53			0.091			**0.017**
Negative/low	350(69)	297(74)	(2.85)	265(80)	231(88)	**(5.71)**
Positive	159(31)	105(26)		64(20)	32(12)	
KI67-LI			0.553			0.266
Negative/low	176(41)	130(39)	(0.35)	146(54)	104(49)	(1.23)
High	249(59)	201(61)		126(46)	110(51)	
BCL2			0.908			0.166
Negative/low	165(41)	123(41)	(0.01)	53(21)	53(27)	(1.92)
High	239(59)	175(59)		200(79)	147(73)	
HER1			0.192			0.055
Negative	419(80)	311(76)	(1.70)	297(89)	218(83)	(3.68)
Positive	105(20)	96(24)		38(11)	44(17)	
HER2			**0.016**	-	-	-
Negative	455(88)	333(82)	**(5.76)**			
Positive	64(12)	73(18)				
HER3			0.036			0.063
Negative	50(11)	24(6)	(4.42)	38(13)	19(8)	(3.45)
Positive	428(89)	352(94)		266(87)	229(92)	
HER4			0.535			0.805
Negative	67(13)	47(12)	(0.38**)**	50(15)	38(14)	(0.06)
Positive	450(87)	358(88)		282(85)	227(86)	

Table 6-6: The associations of nuclear and cytoplasmic CHIP with HER2 dimers and dimers' combinations in breast cancer

	Nuclear-CHIP			Cytoplasmic-CHIP		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
HER1- HER2 dimer			0.077			0.177
Low	292(83)	107(90)	(3.13)	239(87)	159(82)	(1.82)
High	59(17)	12(10)		36(13)	34(18)	
HER3- HER2 dimer			**0.042**			0.293
Low	288(84)	108(91)	**(4.14)**	223(87)	168(84)	(1.10)
High	55(16)	10(9)		32(13)	32(16)	
HER4 - HER2 dimer			0.578			**0.008**
Low	333(88)	124(90)	(0.30)	272(92)	183(84)	**(7.13)**
HIGH	45(12)	14(10)		24(8)	34(16)	
HER1,2 vs HER2,3			0.597			0.147
HER2,1 low-HER2,3 low	195(75)	61(82)	(1.88)	159(81)	97(72)	(5.36)
HER2,1 low-HER2,3 high	5(2)	1(1)		2(1)	4(3)	
HER2,1 high-HER2,3 low	14(6)	2(3)		10(5)	6(4)	
HER2,1 high-HER2,3 high	45(17)	10(14)		26(13)	28(21)	
HER1,2 vs HER1,4			0.655			0.216
HER2,1 low-HER2,4 low	187(8)	57(84)	(1.61)	140(84)	103(79)	(4.45)
HER2,1 low-HER2,4 high	7(3)	1(2)		6(4)	2(1)	
HER2,1 high-HER2,4 low	6(3)	3(4)		3(2)	6(5)	
HER2,1 high-HER2,4 high	32(14)	7(10)		18(11)	20(15)	
HER2,3 vs HER2,4			0.138			0.108
HER2,3 low-HER2,4 low	183(75)	55(80)	(5.51)	141(80)	95(71)	(6.07)
HER2,3 low-HER2,4 high	4(1)	4(6)		2(1)	6(5)	
HER2,1 high-HER2,4 low	14(6)	3(4)		10(6)	7(5)	
HER2,3 high-HER2,4 high	41(17)	7(10)		22(13)	25(19)	

6.3.1.4 The expression of CHIP in Trastuzumab treated series

Out of 153 cases of HER2+ Trastuzumab treated BC, 96 (62.7%) were negative/low and 57 (37.3%) had high expression. For the cytoplasmic form, 78 (52%) of which showed negative/low expression while the other 72 (48%) revealed high expression. The cut-off points were the same as the ones used for the primary series (Table 2-5).

6.3.1.5 The associations of CHIP with clinicopathological variables in Trastuzumab treated series

High nuclear CHIP was associated with a trend for lower tumour grade (p=0.036), less mitosis (p=0.027). However, the cytoplasmic form of CHIP was not associated with clinicopathological variables (Table 6-7).

Table 6-7: The associations of nuclear CHIP with clinicopathological variables

	Nuclear CHIP			Cytoplasmic CHIP		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.437			0.275
≤50	36(38)	25(44)	(0.60)	35(45)	26(36)	(1.19)
>50	60(62)	32(56)		43(55)	46(64)	
Menopausal Status			0.348			0.320
Pre-	43(45)	30(53)	(0.88)	41(53)	32(44)	(0.98)
Post-	53(55)	27(47)		37(47)	40(56)	
Tumour Size (cm)			0.064			0.969
≤2.0	0(0)	2(4)	(3.43)	1(1)	1(1)	(0.00)
>2.0	95(100)	54(96)		75(99)	71(99)	
Stage			0.369			0.279
1	54(57)	37(66)	(1.99)	45(58)	44(62)	(2.55)
2	36(38)	15(27)		25(33)	25(35)	
3	5(5)	4(7)		7(9)	2(3)	
Grade			**0.036**			0.688
1	1(1)	3(5)	**(6.63)**	2(3)	2(3)	(0.74)
2	19(20)	19(33)		17(22)	20(28)	
3	76(79)	35(62)		59(75)	50(69)	
Tubules			0.084			0.386
2	13(14)	14(25)	(0.03)	12(15)	15(21)	(0.75)
3	83(86)	43(75)		66(85)	57(79)	
Pleomorphism			0.949			0.861
2	7(7)	4(7)	(0.00)	6(8)	5(7)	(0.03)
3	89(93)	53(93)		72(92)	67(93)	
Mitosis			**0.027**			0.926
1	20(21)	17(30)	**(7.20)**	18(23)	18(25)	(0.15)
2	26(27)	23(40)		24(31)	23(32)	
3	50(52)	17(30)		36(46)	31(43)	
LVI			0.200			0.501
Probable/Negative	67(70)	34(60)	(1.64)	49(63)	49(68)	(0.45)
Definite	29(30)	23(40)		29(37)	23(32)	
NPI			0.672			0.784
GPG	7(8)	5(11)	(0.79)	6(8)	5(8)	(0.48)
MPG	57(64)	26(56)		46(65)	37(60)	
PPG	25(28)	15(33)		19(27)	20(32)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

6.3.1.6 The associations of CHIP with biological markers and HER2 dimers in Trastuzumab treated series

Both forms of CHIP did not reveal associations with biological markers, HER2 dimers and combinations (Table 6-8).

Table 6-8: The associations with biological markers, HER2 dimers and combinations

	Nuclear CHIP			Cytoplasmic CHIP		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (X^2)**
ER			0.812			0.969
Negative	44(46)	25(44)	(0.05)	36(46)	33(46)	(0.00)
Positive	52(54)	32(56)		42(54)	39(54)	
PgR			0.604			0.670
Negative	50(62)	26(58)	(0.26)	42(63)	33(59)	(0.18)
Positive	30(38)	19(42)		25(37)	23(41)	
CK7/8	-	-	-			-
Negative						
Positive						
CK18			0.851			0.185
Negative	2(6)	1(7)	(0.3)	3(10)	0(0)	(1.75)
Positive	33(94.)	13(93)		28(90)	17(100)	
P53			0.176			0.149
Negative	7(13)	6(25)	(1.83)	11(21)	2(8)	(2.08)
Positive	48(87)	18(75)		41(79)	23(92)	
BCL2			0.108			0.594
Negative/low	26(48)	10(39)	(2.59)	24(47)	11(41)	(0.28)
Moderate/high	28(52)	16(61)		27(53)	16(59)	
HER2-HER1 dimer			0.099			0.331
Negative	18(33)	3(14)	(2.72)	15(31)	5(20)	(0.94)
Positive	36(67)	18(86)		34(69)	20(80)	
HER2-HER3 dimer			0.060			0.392
Negative	24(46)	5(21)	(4.46)	20(41)	8(31)	(0.73**)**
Positive	28(54)	19(79)		29(59)	18(69)	
HER2-HER4 dimer			0.121			0.660
Negative	27(51)	5(29)	(2.40)	21(48)	11(42)	(0.19)
Positive	26(49)	12(71)		23(52)	15(58)	
HER2,1 vs HER2,3			0.125			0.528
HER2,1 low-HER2,3 low	15(31)	3(14)	(5.74)	12(27)	5(21)	(2.22)
HER2,1 low-HER2,3 high	2(4)	0(0)		2(4)	0(0)	
HER2,1 high-HER2,3 low	7(14)	1(5)		6(13)	2(8)	
HER2,1 high-HER2,3 high	25(51)	17(81)		25(56)	17(71)	
Her2,1 vs HER2,4			0.154			0.748
HER2,1 low-HER2,4 low	15(30)	1(7)	(5.26)	11(27)	5(21)	(1.22)
HER2,3 low-HER2,4 high	1(2)	0(0)		1(2)	0(0)	
HER2,1 high-HER2,4 low	10(20)	2(13)		8(20)	4(17)	
HER2,1 high-HER2,4 high	24(48)	12(80)		21(51)	15(62)	
Her2,3 vs HER2,4			0.234			0.900
HER2,3 low-HER2,4 low	19(38)	2(12)	(4.26)	14(33)	7(28)	(0.58)
HER2,3 low-HER2,4 high	3(6)	1(6)		3(7)	1(4)	
HER2,1 high-HER2,4 low	7(14)	3(17)		6(15)	4(16)	
HER2,3 high-HER2,4 high	21(42)	11(64)		19(45)	13(52)	

6.3.1.7 Outcome analysis

In the primary series, nuclear CHIP was associated with prolonged BCSS (p=0.003) and its subcellular localisation showed that the worst combination was where cytoplasmic expression was high and the nuclear expression was low (p=0.011, Figure 6-2). The same associations were observed with BCSS for nuclear and subcellular localisation of CHIP in ER+ tumours (p=0.002, p=0.010) respectively (Figure 6-2). Likewise associations with BCSS were observed within ER+HER2- tumours for nuclear CHIP only (p=0.016, Figure 6-3). Meanwhile, cytoplasmic only expression was associated with prolonged DMFS in LN+ tumours (p=0.030, Figure 6-3). No associations with outcome were revealed in other BC subgroups.

For the Trastuzumab treated series, associations were found between CHIP protein and survival but deemed non-significant (overall survival and DFI).

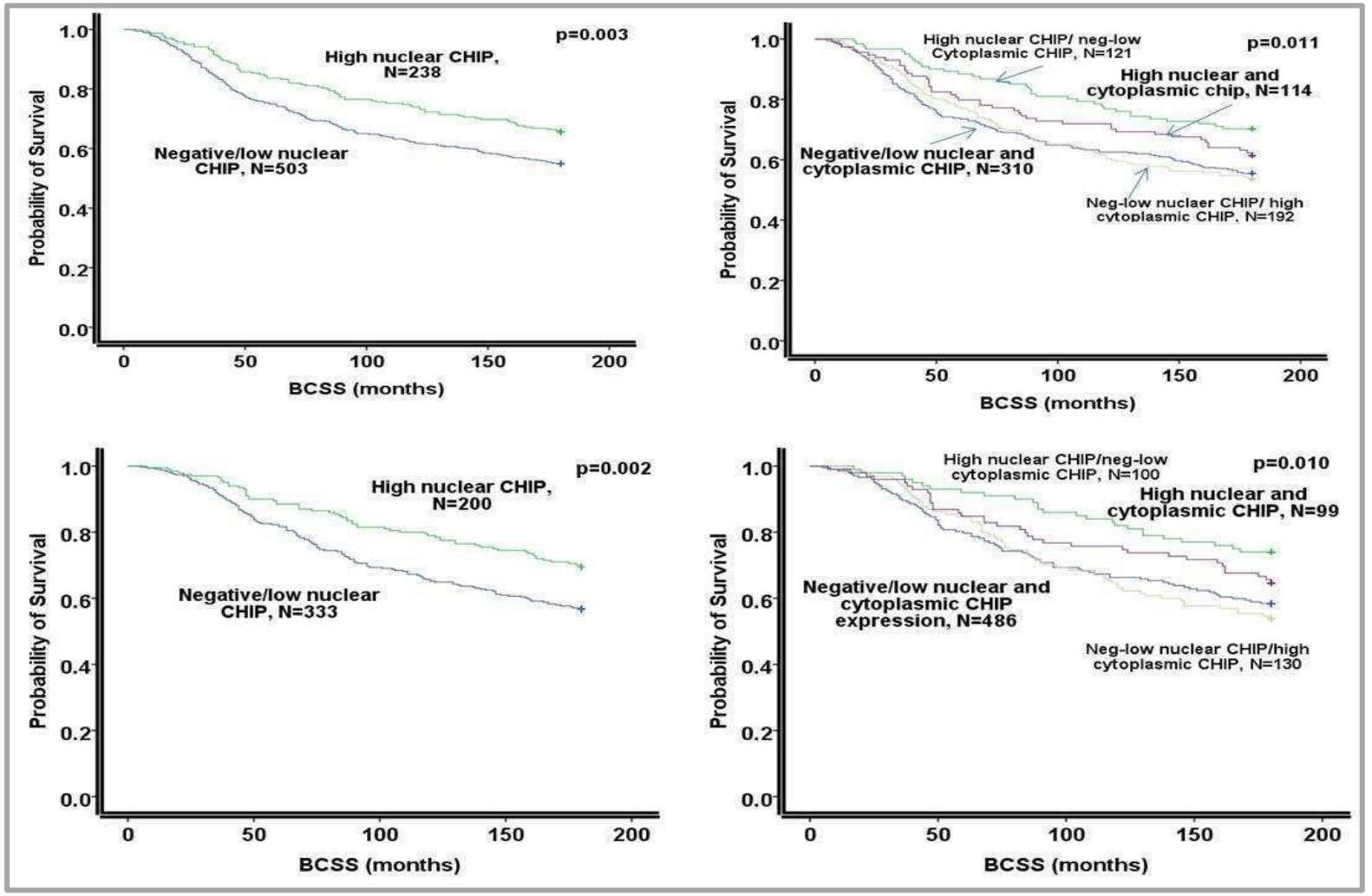


Figure 6-2: Kaplan Meier plots illustrating BCSS for nuclear CHIP and its subcellular localisation in BC and in ER+ tumours respectively

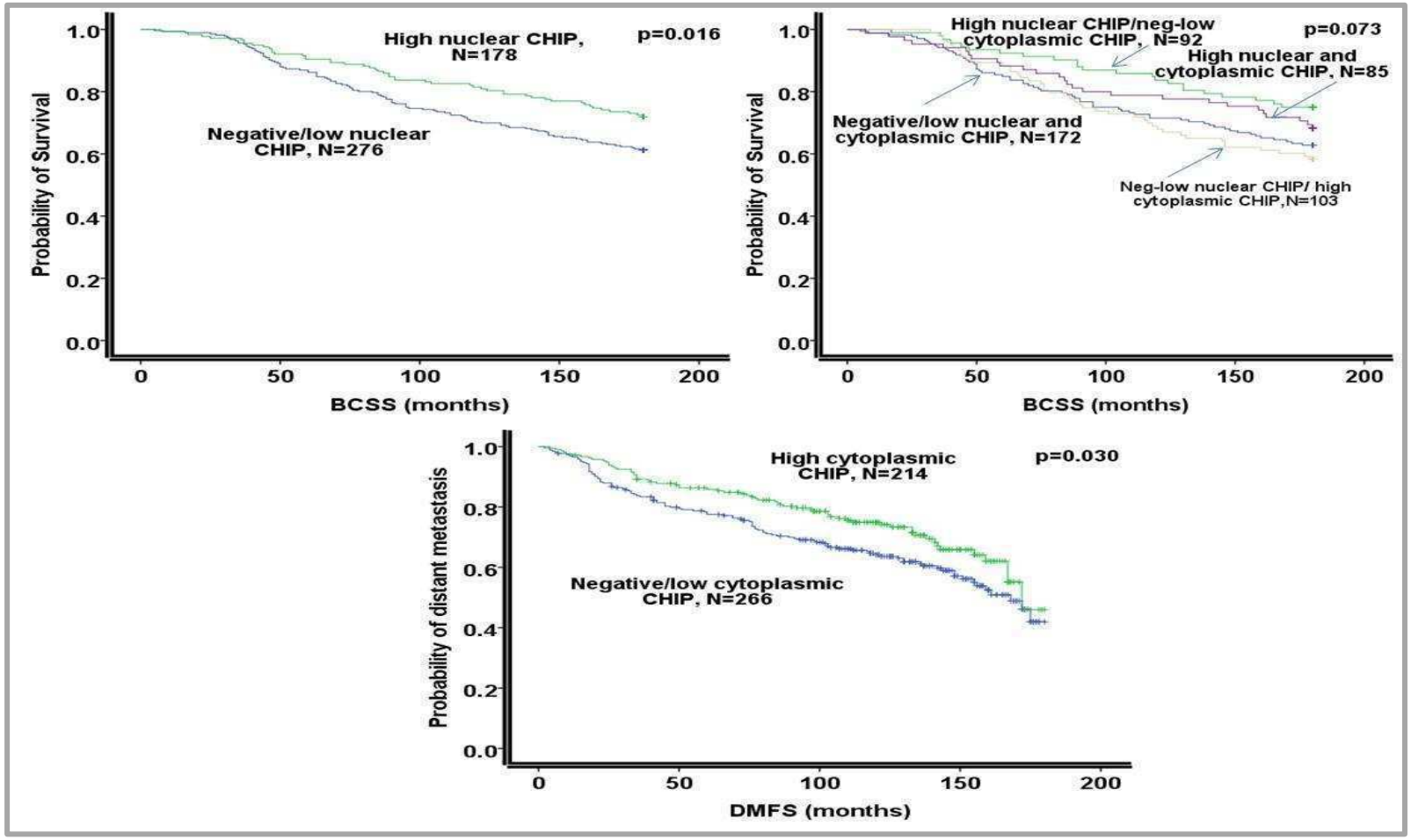


Figure 6-3: Kaplan Meier plots illustrating BCSS for nuclear CHIP and its subcellular localisation in ER+/HER2- tumours and DMFS for cytoplasmic CHIP in LN-positive tumours

6.3.2 SOX9

6.3.2.1 The pattern of expression of SOX9 in breast cancer

The analysis revealed that 1186 cases were valid for this protein. SOX9 was expressed in both the nuclei and cytoplasm of the invasive BC tissue, normal and entrapped DCIS foci within the TMA cores (Figure 6-4). The cut-off point was set at >80 (nuclear percent), by using X-tile software (Table 2-5), where 813 (68.5%) cases had negative/low nuclear expression while 373 (31.5%) had high expression.

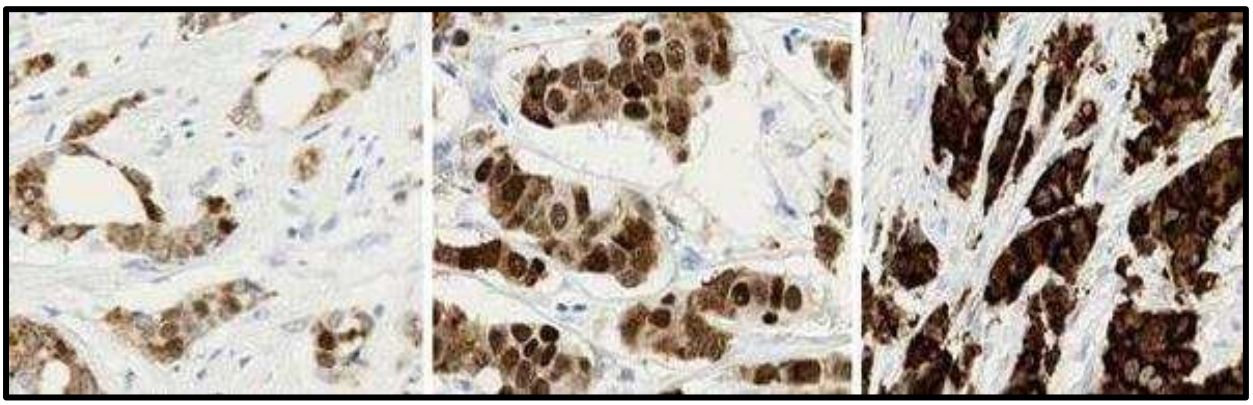


Figure 6-4: Different intensities of staining of SOX9 protein. From the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20

6.3.2.2 The associations of SOX9 with clinicopathological variables in the unselected primary breast cancer series and different subgroups

Within the whole BC series, high nuclear expression of SOX9 was associated with higher tumour grade, less tubule formation, higher mitotic count (all $p<0.001$), more pleomorphism ($p=0.002$), a trend for lobular BC type ($p=0.021$) and higher NPI score ($p=0.019$, Table 6-9). Interestingly, all these associations were lost (apart from an association with lobular BC) when the analysis was restricted to ER+HER2- subgroup (Table 6-9). Considering the cytoplasmic intensity of SOX9 it was associated with a trend for higher tumour grade ($p=0.043$), higher NPI score ($p=0.019$) but with negative LVI (p=0.027, Table 6-10). In contrast, no significant association was observed within ER+HER2- cohort apart from an association with trends for younger age and Pre-menopausal status ($p=0.049$, $p=0.046$), respectively (Table 6-10). No associations between SOX9 and other proteins were observed within other subgroups.

Table 6-9: The associations between nuclear SOX9 and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.259			**0.028**
≤50	282(35)	142(38)	(1027**)**	194(33)	46(25)	**(4.84)**
>50	531(65)	231(62)		394(67)	142(75)	
Menopausal Status			0.165			0.236
Pre-	310(38)	158(43)	(1.92)	212(36)	59(31)	(1.40)
Post-	501(62)	214(57)		375(64)	129(69)	
Tumour Size (cm)			0.176			0.245
≤2.0	402(50)	170(46)	(1.83)	305(52)	107(57)	(1.35)
>2.0	405(50)	203(54)		281(48)	81(43)	
Stage			0.064			0.430
1	484(60)	242(65)	(5.49)	364(62)	126(67)	(1.68)
2	257(32)	95(25)		181(31)	49(26)	
3	63(8)	36(10)		38(7)	13(7)	
Tumour Type			**0.021**			**0.016**
Ductal	688(86)	303(82)	**(9.68)**	490(84)	139(74)	**(10.26)**
Lobular	68(8)	39(10)		63(11)	35(19)	
Medullary-like	10(1)	14(4)		2(0)	0(0)	
Special-type	36(5)	15(4)		29(5)	13(7)	
Grade			**<0.001**			0.100
1	149(18)	43(11)	**(18.96)**	136(23)	39(21)	(4.61)
2	279(35)	107(29)		240(41)	94(50)	
3	376(47)	223(60)		207(36)	55(29)	
Tubules			**<0.001**			0.70
1	45(6)	22(6)	**(19.56)**	39(7)	20(11)	(5.31)
2	280(36)	86(23)		232(41)	62(33)	
3	450(58)	262(71)		290(52)	104(56)	
Pleomorphism			**0.002**			0.378
1	19(3)	10(3)	**(12.35)**	16(3)	8(4)	(1.94)
2	320(41)	113(30)		285(51)	101(54)	
3	434(56)	246(67)		259(46)	77(42)	
Mitosis			**<0.001**			0.120
1	278(36)	112(30)	**(18.66)**	253(45)	99(53)	(4.23)
2	171(22)	54(15)		131(23)	41(22)	
3	326(42)	204(55)		177(32)	6(25)	
LVI			0.227			0.216
Probable/Negative	15(64)	252(68)	(1.46)	384(66)	133(71)	(1.53)
Definite	288(36)	120(32)		199(34)	55(29)	
NPI			**0.0**			0.641
GPG	260(34)	92(26)	**19**	231(41)	82(45)	(0.88)
MPG	403(52)	205(57)	**(7.93)**	269(48)	81(45)	
PPG	110(14)	63(17)		59(11)	18(10)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 6-10: The associations between cytoplasmic intensity of SOX9 and clinicopathological variables

	Whole series					ER+HER2- tumours				
	0 N (%)	1 N (%)	2 N (%)	3 N (%)	p-value (χ^2)	0 N (%)	1 N (%)	2 N (%)	3 N (%)	p-value (χ^2)
Age					0.363					**0.049**
≤50	176(37)	185(35)	41(33)	22(46)	(3.18)	116(32)	97(31)	15(20)	12(46)	**(7.86)**
>50	301(63)	350(65)	84(67)	26(54)		243(68)	217(69)	62(80)	14(54)	
Menopausal Status					0.313					**0.046**
Pre-	200(42)	201(38)	45(36)	22(46)	(3.56)	133(37)	107(34)	18(23)	13(50)	**(7.99)**
Post-	275(58)	333(62)	80(64)	26(54)		225(63)	207(66)	59(77)	13(50)	
Tumour Size (cm)					0.681					0.333
≤2.0	223(47)	264(49)	59(48)	26(55)	(1.50)	178(50)	176(56)	42(54.)	16(61)	(3.40)
>2.0	251(53)	270(51)	65(52)	21(45)		179(50)	138(44)	35(46)	10(39)	
Stage					0.770					0.707
1	288(61)	340(64)	73(59)	25(53)	(3.30)	219(62)	208(66)	48(62)	15(58)	(3.77)
2	143(30)	152(28)	39(31)	18(38)		108(30)	90(29)	23(30)	9(34)	
3	41(9)	41(8)	12(10)	4(9)		28(8)	15(5)	6(8)	2(8)	
Tumour Type					0.424					0.704
Ductal	392(83)	448(85)	108(87)	42(89)	(9.14)	289(81)	253(81)	64(83)	23(88)	(1.40)
Lobular	54(12)	43(8)	7(6)	3(7)		50(14)	38(12)	7(9)	3(12)	
Medullary-like	6(1)	13(2)	4(3)	1(2)		0(0)	1(0)	1(1)	0(0)	
Special-type	20(4)	25(5)	5(4)	1(2)		17(5)	20(7)	5(7)	0(0)	
Grade					**0.043**					0.114
1	72(15)	91(17)	22(18)	7(15)	**(12.98)**	66(19)	83(26)	20(26)	6(23)	(10.25)
2	181(38)	162(30)	31(25)	12(25)		161(45)	137(44)	27(35)	9(35)	
3	219(47)	280(53)	71(57)	28(60)		128(36)	93(30)	30(39)	11(42)	
Tubules					0.851					0.180
1	29(6)	31(6)	6(5)	1(2)	(2.64)	27(8)	26(9)	6(8)	0(0)	(8.89)
2	146(32)	162(31)	43(36)	15(32)		120(35)	129(43)	33(45)	12(46)	
3	285(62)	326(63)	69(59)	31(66)		199(57)	146(48)	35(47)	14(54)	
Pleomorphism					0.282					0.486
1	13(3)	14(3)	1(1)	1(2)	(7.44)	12(4)	11(4)	1(1)	0(0)	(5.46)
2	190(41)	190(37)	38(32)	15(32)		173(50)	166(55)	34(46)	13(50)	
3	255(56)	314(60)	79(67)	31(66.)		160(46)	124(41)	39(53)	13(50)	
Mitosis					0.053					0.151
1	170(37)	176(34)	32(27)	12(26)	(12.42)	157(45)	156(52)	29(39)	10(39)	(9.42)
2	102(22)	90(17)	23(20)	9(19)		85(25)	66(22)	17(23)	4(15)	
3	188(41)	253(49)	63(53)	26(55)		104(30)	79(26)	28(38)	12(46)	
LVI					**0.027**					0.147
Probable/Negative	295(63)	356(67)	77(62)	39(83)	**(9.14)**	230(65)	216(69)	49(64)	22(85)	(5.36)
Definite	176(37)	176(33)	47(38)	8(17)		126(35)	97(31)	27(36)	4(15)	
NPI					**0.0.19**					0.121
GPG	140(31)	166(32)	33(28)	13(29)	**(7.93)**	127(37)	145(48)	30(41)	11(44)	(10.10)
MPG	249(55)	274(53)	63(53)	22(50)		173(51)	131(44)	35(47)	11(44)	
PPG	65(14)	75(15)	23(19)	9(21)		42(12)	23(8)	9(12)	3(12)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

6.3.2.3 The associations of SOX9 with biological markers in the primary breast cancer series

With regard to the nuclear expression of SOX9, high expression was associated with lower expression of HRs (ER, PgR and AR), CK18, CK19, FOXA1, TFF3, GATA3, BCL2 (all $p<0.001$), CK7/8 ($p=0.001$), and a trend for E-cadherin ($p=0.032$) and HER2 ($p=0.027$). In contrast, nuclear SOX9 was associated with high expression of P-cadherin, p53, HER1 (all $p<0.001$), HER3 ($p=0.007$) and PELP1 ($p=0.004$, Table 6-11). Within ER+HER2- tumours, high nuclear SOX9 was associated with a trend for low expression of PgR ($p=0.027$), FOXA1 ($p=0.034$), CD71 ($p=0.045$) and significantly with high KI67-LI (0.003). However, it was associated with a trend of AR ($p=0.030$) and HER3 positive expressions ($p=0.049$, Table 6-11).

The cytoplasmic intensity of SOX9 was associated with negativity of HRs (ER, PgR; $p<0.001$, $p=0.002$ respectively), BCL2 ($p=0.010$: borderline) and HER4 ($p=0.001$). Additionally, it was associated with negativity of AR ($p=0.001$) and high expression of CK18 ($p=0.036$: borderline) but with positivity of GATA3 ($p=0.005$), a trend for CARM1 and P-cadherin ($p=0.034$, $p=0.011$) and significantly with HER1 and HER2 positivity ($p<0.001$, Table 6-12). Only two associations were maintained within the ER+HER2- cohort including those of CARM1 and HER4. Additionally, there was a borderline association with high KI67-LI ($p=0.034$, Table 6-12). There were no other observations apart from a borderline inverse association between higher SOX9 cytoplasmic intensity and low CD71 ($p=0.022$) within ER-HER2+ tumours. No associations were observed with both forms of SOX9 and biological markers within other BC subgroups (ER+HER2+ and ER-HER2-).

Table 6-11: The associations of nuclear SOX9 with biological markers

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	128(16)	164(44)	**(107.84)**			
Positive	675(84)	208(56)				
PgR			**<0.001**			**0.027**
Negative	246(32)	210(58)	**(74.23)**	100(17)	45(25)	**(4.91)**
Positive	536(68)	150(42)		484(83)	139(75)	
AR			**<0.001**			**0.030**
Negative	229(32)	164(49)	**(27.25)**	140(26)	30(18)	**(4.71)**
Positive	487(68)	173(51)		400(74)	139(82)	
CK7/8			**0.001**	-	-	-
Negative	5(1)	11(3)	**(10.30)**			
Positive	776(99)	351(97)				
CK18			**<0.001**			0.080
Negative	62(9)	88(26)	**(55.07)**	30(6)	4(2)	(3.06)
Positive	647(91)	252(74)		502(94)	167(98)	
CK19			**<0.001**			0.121
Negative	49(6)	56(16)	**(25.30)**	25(4)	13(7)	(2.41)
Positive	717(94)	298(84)		546(96)	165(93)	
FOXA1			**<0.001**			**0.034**
Negative	261(49)	162(68)	**(25.63)**	169(44)	55(50)	**(4.47)**
Positive	275(51)	75(32)		212(56)	56(50)	
BEX1			0.167			0.084
Negative	162(32)	90(37)	(1.91)	108(29)	45(38)	(2.99)
Positive	343(68)	152(63)		260(71)	74(62)	
TFF1			**0.002**			0.095
Negative	222(48)	126(61)	**(9.48)**	160(48)	55(57)	(2.79)
Positive	243(52.)	82(39)		176(52)	41(43)	
TFF3			**<0.001**			0.537
Negative	209(44)	136(62)	**(21.29)**	150(43)	41(40)	(0.38)
Positive	271(56)	82(38)		197(57)	62(60)	
GATA3			**<0.001**			0.080
Negative	262(56)	167(78)	**(30.93)**	162(48)	56(58)	(3.07)
Positive	207(44)	47(22)		178(52)	41(42)	
CD71			0230			**0.045**
Negative	246(45)	98(40)	(1.44)	201(50)	69(61)	**(4.03)**
Positive	306(55)	147(60)		198(50)	44(39)	
CARM1			0.265			0.088
Negative	147(28)	61(25)	(2.65)	118(31)	47(41)	(4.87)
Moderate	268(52)	119(50)		191(51)	56(48)	
High	105(20)	61(25)		68(18)	13(11)	
PELP1			**0.004**			0.247
Negative	97(18)	37(15)	**(11.02)**	77(19)	23(19)	(2.79)
Moderate	396(72)	165(67)		292(72)	79(67)	
High	54(10)	45(18)		37(9)	17(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.032**			0.617
Negative	260(34)	144(41)	**(4.58)**	194(34)	64(36)	(0.25)
Positive	501(66)	209(59)		378(66)	114(64)	
p-Cadherin			**<0.001**			0.195
Negative	346(55)	114(36)	**(28.13)**	294(61)	103(67)	(1.67)
Positive	286(45)	199(64)		184(39)	50(33)	
p53			**<0.001**			0.603
Negative/low	579(75)	233(65)	**(31.30)**	475(82)	147(80)	(0.27)
Positive	191(25)	127(35)		104(18)	36(20)	
KI67-LI			0.278			**0.003**
Negative/low	279(43)	122(40)	(1.17)	239(50)	102(64)	**(8.82**)
High	367(57)	187(60)		237(50)	58(36)	
BCL2			**<0.001**			0.106
Negative/low	207(34)	150(58)	**(41.82)**	100(22)	37(29)	(2.61)
High	400(66)	110(42)		350(78)	90(71)	
HER1			**<0.001**			0..901
Negative	643(83)	265(73)	**(14.27)**	498(87)	162(88)	(0.01)
Positive	133(17)	97(27)		73(13)	23(12)	
HER2			**0.027**	-	-	-
Negative	661(85)	323(90)	**(4.76)**			
Positive	117(15)	37(10)				
HER3			**0.007**			**0.049**
Negative	75(11)	18(5)	**(7.19)**	59(11)	10(6)	**(3.86)**
Positive	642(89)	315(95)		473(89)	159(94)	
HER4			0.223			0.282
Negative	109(14)	41(12)	(1.48)	94(16)	24(13)	(1.15)
Positive	660(86)	315(88)		478(84)	159(87)	

Table 6-12: The associations of cytoplasmic intensity of SOX9 with biological markers

	Whole series					ER+HER2- tumours				
Intensity	**0 N (%)**	**1 N (%)**	**2 N (%)**	**3 N (%)**	**p-value (χ2)**	**0 N (%)**	**1 N (%)**	**2 N (%)**	**3 N (%)**	**p-value (χ2)**
Hormone Receptors and ER related proteins										
ER					**<0.001**	-	-	-	-	**-**
Negative	71(15)	168(32)	37(30)	16(33)	**(41.09)**					
Positive	401(85)	362(68)	87(70)	32(67)						
PgR					**0.002**					0.510
Negative	139(31)	243(47)	52(43)	22(48)	**(27.86)**	64(18)	66(21)	11(15)	4(15)	(2.31)
Positive	315(69)	278(53)	69(57)	24(52)		293(82)	245(79)	63(85)	22(85)	
AR					**0.001**					0.195
Negative	131(31)	197(41)	51(48)	14(35)	**(16.26)**	76(23)	65(23)	22(34)	7(29)	(4.70)
Positive	296(69)	283(59)	55(52)	26(65)		257(77)	223(77)	42(66)	17(71)	
CK7/8					0.404	-	-	-		0.426
Negative	4(1)	9(2)	3(3)	0(0)	2.91)					(0.63)
Positive	449(99)	513(98)	119(97)	46(100)						
CK18					**0.036**					0.495
Negative	49(12)	83(17)	16(15)	2(5)	**(8.52)**	20(6)	10(3)	3(5)	1(4)	(2.39)
Positive	370(88)	399(83)	92(85)	38(95)		307(94)	280(97)	60(95)	22(96)	
CK19					0.306					0.818
Negative	34(8)	55(11)	13(11)	3(7)	(3.61)	19(5)	16(5)	2(3)	1(4)	(0.92)
Positive	413(92)	457(89)	103(89)	42(93)		331(95)	287(95)	69(97)	24(96)	
FOXA1					0.275					0.942
Negative	154(51)	208(58)	46(53)	15(58)	(3.87)	107(46)	88(46)	22(42)	7(47)	(**0.39)**
Positive	149(49)	149(42)	41(47)	11(42)		126(54)	103(54)	31(58)	8(53)	
BEX1					0.804					0.488
Negative	94(32)	121(34)	29(38)	8(32)	(0.98)	72(33)	61(30)	18(36)	2(15)	(2.43)
Positive	199(68)	231(66)	48(62)	17(68)		148(67)	143(70)	32(64)	11(85)	
TFF1					0.606					0.823
Negative	126(49)	167(52)	42(57)	13(54)	(1.84)	102(51)	85(49)	23(52)	5(39)	(0.91)
Positive	131(51)	152(48)	31(43)	11(46)		99(49)	89(51)	21(48)	8(61)	
TFF3					0.372					0.115
Negative	131(47)	165(51)	38(57)	11(42)	(3.21)	86(41)	78(43)	24(56)	3(21)	(5.93)
Positive	150(53)	159(49)	29(43)	15(58)		125(59)	104(57)	19(44)	11(79)	
GATA3					**0.005**					0.424
Negative	152(55)	213(68)	50(69)	14(56)	**(12.83)**	104(50)	86(50)	24(57)	4(31)	(2.79)
Positive	123(45)	98(32)	22(31)	11(44)		106(50)	86(50)	18(43)	9(69)	
CD71					0.555					0.795
Negative	142(45)	154(42)	34(38)	14(50)	(2.08)	122(52)	112(55)	29(52)	7(44)	(1.02)
Positive	174(55)	209(58)	56(62)	14(50)		114(48)	92(45)	27(48)	9(56)	
CARM1					**0.034**					**0.002**
Negative	86(28)	100(29)	16(19)	6(21)	**(13.64)**	71(31)	78(40)	12(21)	4(27)	**(21.19)**
Moderate	148(49)	175(51)	54(63)	10(36)		112(50)	96(49)	35(63)	4(27)	
High	68(23)	70(20)	16(18)	12(43)		43(19)	22(11)	9(16)	7(46)	
PELP1					0.541					0.363
Negative	56(17)	61(17)	10(11)	7(23)	(5.02)	48(19)	42(21)	7(12)	3(18)	(6.56)
Moderate	231(72)	243(69)	66(73)	21(70)		178(72)	136(68)	43(73)	14(82)	
High	36(11)	47(14)	14(16)	2(7)		22(9)	23(11)	9(15)	0(0)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins										
E-Cadherin					0.156					0.561
Negative	153(35)	190(37)	50(42)	11(25)	(5.23)	120(34)	100(33)	30(42)	8(32)	(2.05)
Positive	291(65)	318(63)	68(58)	33(75)		229(66)	204(67)	42(58)	17(68)	
p-Cadherin					**0.011**					0.605
Negative	213(55)	190(44)	43(45)	14(41)	**(11.21)**	191(64)	162(64)	34(60)	10(50)	(1.84)
Positive	173(45)	240(56)	52(55)	20(59)		109(36)	92(36)	23(40)	10(50)	
p53					0.286					0.566
Negative/low	338(75)	354(69)	89(74)	31(70)	(3.78)	290(82)	249(81)	64(85)	19(73)	(2.03)
Positive	115(25)	158(31)	32(26)	13(30)		64(18)	58(19)	11(15)	7(27)	
KI67-LI					0.357					**0.034**
Negative/low	161(43)	187(43)	43(41)	10(28)	(3.23)	145(50)	159(60)	30(48)	7(37)	**(8.69**)
High	214(57)	252(57)	62(59)	26(72)		145(50)	106(40)	32(52)	12(63)	
BCL2					**0.010**					0.575
Negative/low	123(35)	181(47)	39(42)	14(47)	**(11.42)**	63(24)	59(25)	10(17)	5(28)	(1.99)
High	232(65)	207(53)	55(58)	16(53)		204(76)	174(75)	49(83)	13(72)	
HER1					**<0.001**					0.154
Negative	383(84)	385(74)	104(87)	36(80)	**(14.79)**	306(87)	265(86)	67(90)	22(88)	(5.24)
Positive	73(16)	133(26)	15(13)	9(20)		44(13)	42(14)	7(10)	3(12)	
HER2					**<0.001**	-	-	-	-	-
Negative	421(92)	433(84)	99(82)	31(67)	**(31.20)**					
Positive	36(8)	81(16)	22(18)	15(33)						
HER3					0.081					0.094
Negative	48(11)	32(7)	8(8)	5(13)	(6.73)	38(12)	20(7)	7(11)	4(17)	(2.80)
Positive	378(89)	447(93)	97(92)	35(87)		289(88)	265(93)	59(89)	19(83)	
HER4					**0.001**					**0.003**
Negative	83(18)	52(10)	9(8)	6(14)	**(17.27)**	71(20)	34(11)	7(9)	6(24)	**(13.76)**
Positive	371(82)	456(90)	110(92)	38(86)		280(80)	270(89)	68(91)	19(76)	

6.3.2.4 Outcome analysis

There were no associations between SOX9 and patient outcome (BCSS and DMFS).

6.3.3 SRC3

6.3.3.1 The pattern of expression of SRC3 in breast cancer

SRC3 expression was exclusively expressed in the nucleus (Figure 6-5) in invasive, entrapped foci of normal and DCIS tissue, where 151 cases were valid for assessment. Using the median of H-score expression [130] as a cut-off point (Table 2-5), 76 (50.3%) cases had negative/low expression and 75 (49.7%) cases had high expression.

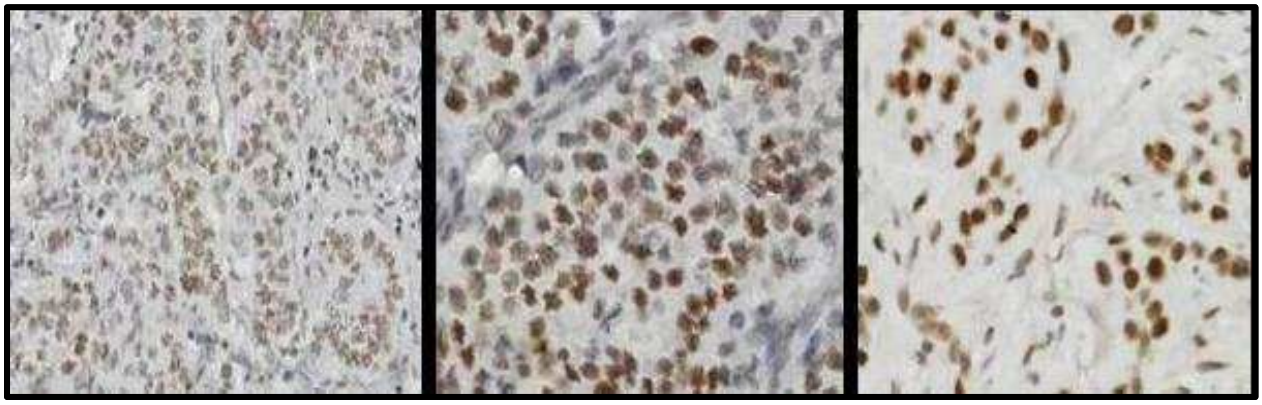


Figure 6-5: Different intensities of staining of SRC3. From the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20

6.3.3.2 The associations of SRC3 with clinicopathological variables in Trastuzumab treated series

SRC3 expression was associated with a trend for higher tumour grade (p=0.030), high pleomorphism (p=0.045) and significantly with high mitosic count (p=0.005, Table 6-13).

Table 6-13: The associations of SRC3 with clinicopathological variables

	SRC3		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**
Age			0.844
≤50	29(41)	31(43)	(0.03)
>50	41(59)	41(57)	
Menopausal Status			0.938
Pre-	36(47)	36(48)	(0.00)
Post-	40(53)	39(52)	
Tumour Size (cm)			0.319
≤2.0	1(1)	0(0)	(0.99)
>2.0	74(99)	74(100)	
Stage			0.118
1	52(68)	39(52)	(4.27)
2	21(28)	32(43)	
3	3(4)	4(5)	
Grade			**0.030**
1	2(3)	1(2)	**(7.40)**
2	27(35)	13(17)	
3	47(62)	61(81)	
Tubules			0.306
2	16(21)	11(15)	(1.04)
3	60(79)	64(85)	
Pleomorphism			**0.045**
2	10(13)	3(4)	**(4.02)**
3	66(87)	72(96)	
Mitosis			**0.005**
1	27(35)	10(13)	**(10.75)**
2	18(24)	29(39)	
3	31(41)	36(48)	
LVI			0.108
Probable/Negative	55(72)	45(60)	(2.58)
Definite	21(28)	30(40)	
NPI			0.277
GPG	7(10)	7(10)	(2.56)
MPG	46(67)	37(55)	
PPG	16(23)	24(35)	

6.3.3.3 The associations of SRC3 with biological markers, HER2 dimers and their combinations in Trastuzumab treated series

SRC3 was associated with a trend for high expression of HER2-HER1 dimer (p=0.024) and presence of HER2-HER3 dimers (p=0.017, Table 6-14). Moreover, SRC3 was associated with high expression of HER2-HER1 vs HER2-HER4 (when HER1 dimer and even when both are high, p=0.026: borderline, Table 6-14). Meanwhile, SRC3 was associated with a borderline high expression of HER2-HER3 and HER2-HER4 dimers (when either dimer is high or when both are high, (p=0.011, Table 6-14).

Table 6-14: The associations of SRC3 with biological markers and HER2 dimers

	SRC3		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**
ER			0.689
Negative	32(42)	34(45)	(0.16)
Positive	44(58)	41(55)	
PgR			0.447
Negative	36(56)	39(63)	(0.57)
Positive	28(44)	23(37)	
CK7/8	-	-	-
Negative			
Positive			
CK18			0.258
Negative	3(9)	0(0)	(1.27)
Positive	32(91)	14(100)	
P53			0.708
Negative	8(17)	4(14)	(0.14)
Positive	39(83)	25(86)	
BCL2			0.443
Negative/low	23(49)	12(40)	(0.59)
Moderate/high	24(51)	18(60)	
HER2-HER1 dimer			**0.024**
Negative	16(37)	4(13)	**(5.06)**
Positive	27(63)	26(87)	
HER2-HER3 dimer			**0.017**
Negative	21(49)	7(22)	**(5.70)**
Positive	22(51)	25(78)	
HER2-HER4 dimer			0.113
Negative	21(54)	10(35)	(2.51)
Positive	18(46)	19(65)	
HER2,1 vs HER2,3			0.075
HER2,1 low-HER2,3 low	14(36)	3(10)	(6.89)
HER2,1 low-HER2,3 high	1(3)	0(0)	
HER2,1 high-HER2,3 low	4(10)	4(14)	
HER2,1 high-HER2,3 high	20(51)	22(76)	
Her2,1 vs HER2,4			**0.026**
HER2,1 low-HER2,4 low	13(36)	2(7)	**(7.26)**
HER2,1 high-HER2,4low	5(14)	7(26)	
HER2,1 high-HER2,4 high	18(50)	18(67)	
Her2,3 vs HER2,4			**0.011**
HER2,3 low-HER2,4 low	17(47)	3(11)	**(11.12)**
HER2,3 low-HER2,4 high	1(3)	3(10)	
HER2,1 high-HER2,4 low	4(11)	7(24)	
HER2,3 high-HER2,4 high	14(39)	16(55)	

6.3.3.4 Outcome analysis

No associations were found in relation of SRC3 to survival (overall survival or DFI).

6.3.4 ECD

6.3.4.1 The pattern of expression of ECD in breast cancer

137 cases in total were valid for the assessment of ECD and its expression was detected in the cytoplasm in the TMA invasive (with more intensity), normal and DCIS entrapped foci (Figure 6-6). The cut off was chosen >80 H-score using X-tile (Table 2-5). At this cut-off, 64 (46.7%) cases had negative/low expression and 73 (53.3%) deemed high.

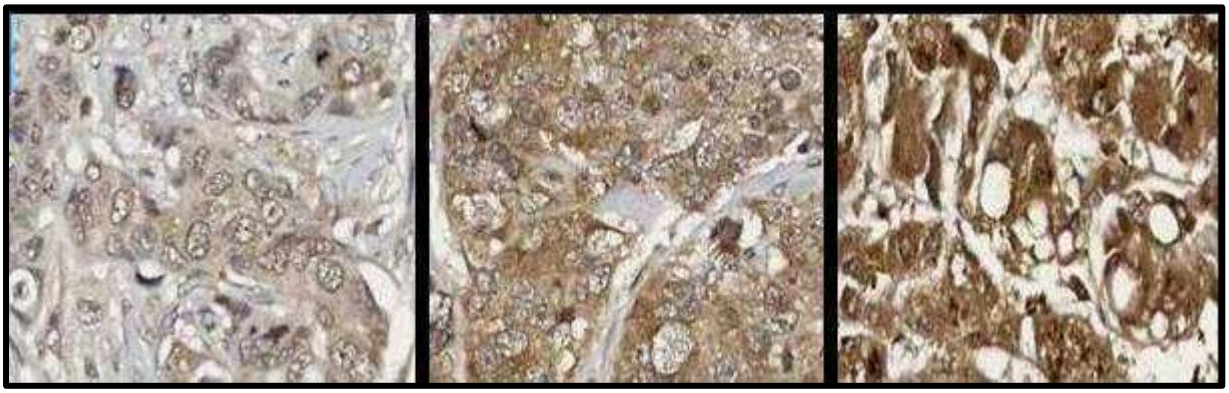


Figure 6-6: Different intensities of staining of ECD. From the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20

6.3.4.2 The associations of ECD with clinicopathological variables in Trastuzumab treated series

the cytoplasmic ECD was associated with higher tumour grade (p=0.002), less tubule formation (p=0.014: borderline), high pleomorphism (p=0.002) and more mitosis (p=0.003) in BC (Table 6-15).

Table 6-15: The associations of ECD with clinicopathological variables

	ECD		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**
Age			0.245
≤50	30(51)	28(41)	(1.34)
≥50	29(49)	41(59)	
Menopausal Status			0.258
Pre-	36(56)	34(47)	(1.27)
Post-	28(44)	39(53)	
Tumour Size (cm)	-	-	-
≤2.0			
>2.0			
Stage			0.830
1	37(59)	43(59)	(0.03)
2	23(36)	27(37)	
3	3(5)	3(4)	
Grade			**0.002**
1	2(3)	0(0)	**(12.45)**
2	22(34)	9(12)	
3	40(63)	64(88)	
Tubules			**0.014**
2	15(23)	6(8)	**(6.08)**
3	49(77)	67(92)	
Pleomorphism			**0.002**
2	8(13)	0(0)	**(9.69)**
3	56(87)	73(100)	
Mitosis			**0.003**
1	21(33)	7(10)	**(11.72)**
2	19(30)	25(34)	
3	24(37)	41(56)	
LVI			0.145
Probable/Negative	38(59)	52(71)	(2.12)
Definite	26(41)	21(29)	
NPI			0.436
GPG	6(11)	3(4)	(1.66)
MPG	35(61)	42(64)	
PPG	16(28)	21(32)	

6.3.4.3 The associations of ECD with biological markers, HER2 dimers and their combinations in Trastuzumab treated series

ECD was associated with high expression of HER2-HER4 dimer (p=0.031: borderline). Additionally, ECD was associated with a trend for high expression of HER2-HER1 and high HER2-HER3 dimer combination or when the former is only high (p=0.027, Table 6-16).

Table 6-16: The associations of ECD with biological markers

	ECD		
	Neg/Low N (%)	**High N (%)**	***p*-value (X^2)**
ER			0.228
Negative	25(39)	36(49)	(1.45)
Positive	39(61)	37(51)	
PgR			0.153
Negative	27(52)	41(65)	(2.04)
Positive	25(48)	22(35)	
CK7/8	-	-	-
Negative			
Positive			
CK18			0.137
Negative	3(14)	0(0)	(2.21)
Positive	19(86)	21(100)	
P53			0.611
Negative	5(13)	5(17)	(0.25)
Positive	34(87)	24(83)	
BCL2			0.358
Negative/low	15(37)	14(48)	(95)
Moderate/high	26(63)	15(52)	
HER2-HER1 dimer			0.077
Negative	13(33)	4(14)	(3.12)
Positive	26(67)	24(86)	
HER2-HER3 dimer			0.554
Negative	17(42)	10(35)	(0.34**)**
Positive	24(58)	19(65)	
HER2-HER4 dimer			**0.031**
Negative	20(56)	8(29)	**(4.66)**
Positive	16(44)	20(71)	
HER2,1 vs HER2,3			**0.027**
HER2,1 low-HER2,3 low	13(35)	3(10)	**(7.20)**
HER2,1 low-HER2,3 high	-	-	
HER2,1 high-HER2,3 low	2(5)	6(22)	
HER2,1 high-HER2,3 high	23(60)	18(68)	
Her2,1 vs HER2,4			0.107
HER2,1 low-HER2,4 low	11(33)	3(12)	(4.46)
HER2,1 high-HER2,4low	6(18)	4(15)	
HER2,1 high-HER2,4 high	16(49)	19(73)	
Her2,3 vs HER2,4			0.086
HER2,3 low-HER2,4 low	13(37)	6(22	(6.59)
HER2,3 low-HER2,4 high	1(3)	4(14)	
HER2,1 high-HER2,4 low	7(20)	2(7)	
HER2,3 high-HER2,4 high	14(40)	16(57)	

6.3.4.4 Outcome analysis

No associations were observed between ECD and survival (overall survival and DFI).

6.3.5 SER 118 ER

6.3.5.1 The pattern of expression of SER 118 ER in breast cancer

1160 cases were valid for the evaluation of this protein which revealed nuclear and cytoplasmic expressions not only in invasive carcinoma cores but also within normal and DCIS foci entrapped within some of the TMA cores (Figure 6-7). Out of the total, [negative/low nuclear: 630 (40.3%), high nuclear 530 (45.7)] and [negative/low cytoplasmic: 529 (45.6%), high cytoplasmic 632 (54.4%)] were revealed for nuclear and cytoplasmic forms, respectively. The optimum cut off point was set at >140 (H-score) and > 70 (percent) by using the median for the nuclear and cytoplasmic forms respectively (Table 2-5).

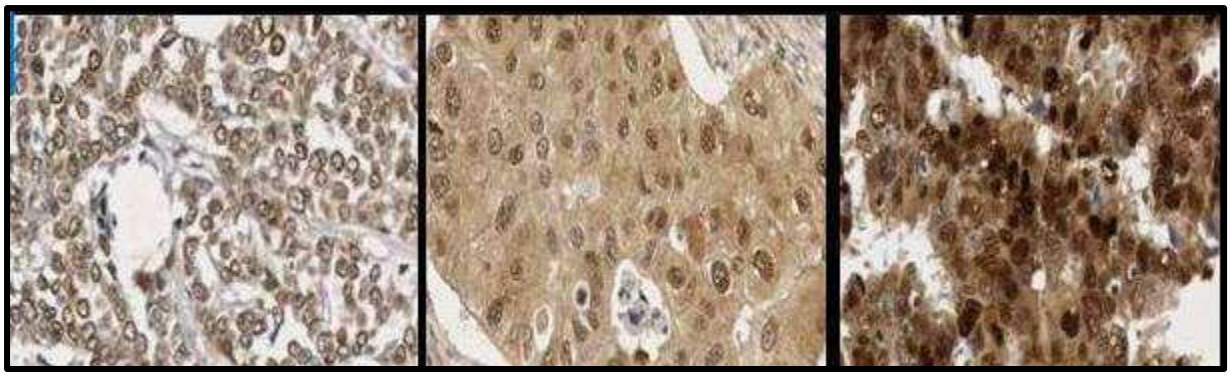


Figure 6-7: Different intensities of staining of SER 118 ER From the left to right: Weak, moderate and strong intensities. All pictures were taken using digital pathology system at x20

6.3.5.2 The associations between SER118 ER and clinicopathological variables in the unselected primary breast cancer series and different subgroups

Regarding SER 118 ER, its higher nuclear and cytoplasmic expressions were associated with smaller tumour size (p=0.009, p=0.002, respectively), lobular BC, lower tumour grade, more tubule formation, less pleomorphism, less mitotic count and lower NPI score (all, p<0.001, Table 6-17 and Table 6-18). Importantly, all these associations, except tumour size, were maintained when the analysis was restricted to ER+HER2- (Table 6-17 and Table 6-18). No associations were found within other subgroups.

Table 6-17: The associations between nuclear SER 118 ER and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.766			0.116
≤50	224(36)	184(35)	(0.08)	97(27)	131(33)	(2.47)
>50	406(64)	346(65)		257(73)	270(67)	
Menopausal Status			0.575			0.366
Pre-	252(40)	203(38)	(0.31)	116(33)	144(36)	(1.80)
Post-	377(60)	325(62)		237(67)	256(64)	
Tumour Size (cm)			**0.009**			0.186
≤2.0	278(45)	277(52)	**(6.89)**	177(50)	221(55)	(1.74)
>2.0	345(55)	252(48)		174(50)	179(45)	
Stage			0.766			0.308
1	373(60)	327(62)	(0.53)	218(62)	250(63)	(2.35)
2	198(32)	158(30)		113(32)	116(29)	
3	52(8)	43(8)		20(6)	33(8)	
Tumour Type			**<0.001**			**0.007**
Ductal	548(89)	418(79)	**(31.51)**	299(86)	308(77)	**(12.26)**
Lobular	34(5)	72(14)		34(10)	67(17)	
Medullary-like	17(3)	7(1)		2(0)	0(0)	
Special-type	17(3)	29(6)		14(4)	24(6)	
Grade			**<0.001**			**<0.001**
1	70(11)	106(20)	**(66.54)**	61(18)	97(24)	**(23.38)**
2	174(28)	227(43)		145(41)	202(51)	
3	379(61)	194(37)		145(41)	99(25)	
Tubules			**<0.001**			**0.013**
1	24(4)	31(6)	**(28.67)**	22(7)	28(7)	**(8.66)**
2	163(27)	213(41)		118(35)	180(46)	
3	408(69)	274(53)		191(58)	184(47)	
Pleomorphism			**<0.001**			**0.023**
1	9(2)	12(2)	**(31.55)**	9(3)	9(2)	**(7.55)**
2	180(30)	238(46)		151(45)	219(56)	
3	406(68)	268(52)		171(52)	164(42)	
Mitosis			**<0.001**			**<0.001**
1	146(245)	225(43)	**(63.81)**	130(39)	206(53)	**(18.65)**
2	107(18)	115(22)		79(24)	96(24)	
3	342(57)	178(35)		122(37)	90(23)	
LVI			0.657			0.981
Probable/Negative	406(65)	351(67)	(0.19)	235(67)	269(67)	(0.00)
Definite	214(35)	175(33)		114(33)	130(33)	
NPI			**<0.001**			**0.009**
GPG	137(23)	202(40)	**(39.18)**	120(36)	180(47)	**(9.33)**
MPG	356(59)	238(47)		176(53)	163(43)	
PPG	106(18)	62(13)		38(11)	39(10)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

Table 6-18: The associations between cytoplasmic SER 118 ER and clinicopathological variables

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.962			0.072
≤50	188(36)	220(35)	(0.06)	77(26)	151(33)	(3.24)
>50	341(64)	412(65)		215(74)	313(67)	
Menopausal Status			0.956			0.103
Pre-	207(39)	248(39)	(0.00)	90(31)	170(37)	(2.65)
Post-	321(61)	382(61)		201(69)	293(63)	
Tumour Size (cm)			**0.002**			0.135
≤2.0	226(43)	329(52)	**(9.64)**	143(50)	255(55)	(2.23)
>2.0	298(57)	300(48)		146(50)	208(45)	
Stage			0.480			0.081
1	312(59)	388(62)	(1.46)	175(61)	293(63)	(5.03)
2	171(33)	185(29)		99(34)	130(28)	
3	41(8)	55(9)		15(5)	39(9)	
Tumour Type			**<0.001**			**<0.001**
Ductal	464(90)	502(80)	**(39.74)**	251(87)	356(77)	**(19.41)**
Lobular	21(4)	86(14)		21(7)	81(18)	
Medullary-like	17(3)	7(1)		2(1)	0(0)	
Special-type	16(3)	30(5)		13(5)	25(5)	
Grade			**<0.001**			**<0.001**
1	66(13)	110(18)	**(49.50)**	58(20)	100(22)	**(19.86)**
2	138(26)	264(42)		110(38)	238(51)	
3	320(61)	253(40)		121(42)	123(27)	
Tubules			**0.001**			0.237
1	23(5)	32(5)	**(13.03)**	21(8)	29(7)	(2.23)
2	142(28)	234(38)		103(38)	195(43)	
3	336(67)	347(57)		149(54)	227(50)	
Pleomorphism			**<0.001**			**0.034**
1	8(2)	13(2)	**(31.55)**	8(3)	10(2)	**(6.74)**
2	150(30)	269(44)		123(45)	248(55)	
3	343(68)	331(54)		142(52)	193(43)	
Mitosis			**<0.001**			**0.001**
1	119(24)	253(41)	**(48.73)**	105(38)	232(51)	**(14.33)**
2	94(19)	128(21)		68(25)	107(24)	
3	288(57)	232(38)		100(37)	112(25)	
LVI			0.643			0.471
Probable/Negative	348(67)	410(65)	(0.21)	198(69)	307(66)	(0.58)
Definite	173(33)	216(35)		89(31)	155(34)	
NPI			**<0.001**			0.050
GPG	116(23)	223(37)	**(26.29)**	100(36)	200(45)	(5.99)
MPG	300(59)	294(49)		145(53)	194(44)	
PPG	88(18)	81(14)		30(11)	48(11)	

LVI: lymphovascular invasion, NPI: Nottingham prognostic index, GPG: good prognostic group, MPG: moderate prognostic group, PPG: poor prognostic group.

6.3.5.3 The associations between SER 118 ER and biological markers in the unselected primary breast cancer series and different subgroups

High nuclear expression of SER 118 ER revealed direct positive association with HRs (ER, PgR and AR), CK18, FOXA1, BEX1, GATA3, BCL2 (all, $p<0.001$), CK7/8 ($p=0.003$) and a trend for CK19 ($p=0.047$) and E-cadherin ($p=0.024$). However, it was associated with low CD71, P-cadherin, p53, KI67-LI ($p<0.001$), HER1 ($p=0.001$) and HER2 ($p=0.017$: borderline, Table 6-19). When the analysis was considered within ER+HER2– group, the associations with AR, FOXA1, GATA3, CD71 and p53 were all maintained (Table 6-20).

High cytoplasmic SER 118 ER similarly, was positively associated with ER, AR, CK18, FOXA1, GATA3 and BCL2 (all, $p<0.001$). Moreover, SER118 cytoplasmic expression was also positively associated with high expression of PgR ($p=0.002$), CK7/8 ($p=0.006$), CK19 ($p=0.008$), BEX1 ($p=0.001$) and E-cadherin ($p=0.025$: borderline, Table 6-20). On the other hand, the cytoplasmic expression was associated with low CD71, P-cadherin, P53, ki67-LI, HER1 (all, $p<0.001$) and HER4 ($p=0.002$, Table 6-20). Similar associations regarding PgR, AR, FOXA1, GATA3, CD71, KI67-LI, and HER4 were observed within the ER+HER2- cohort but the association with a borderline high expression of PELP1 ($p=0.046$) was an additional finding (Table 6-20).

Furthermore, Within ER+HER2+ cohort, nuclear SER118 ER was positively associated with a trend for GATA3 expression ($p=0.038$). Additionally, cytoplasmic SER118 ER showed an association with AR positivity ($p=0.042$: trend). No associations were observed within ER-HER2+ BC but when the analysis was performed within ER-HER2- cohort, nuclear SER 118 ER was associated with a trend for decreased expression of CARM1, KI67-LI and N-Cadherin ($p=0.030$, $p=0.033$ and $p=0.032$, respectively, appendix table 11) while the cytoplasmic form was associated with decreased expression of CARM1 and CD71 ($p=0.033$ and $p=0.040$, respectively, both borderline, appendix table 11). No associations were found between SER 118 ER and HER2 dimers neither in BC or other subgroups.

Table 6-19: The associations of nuclear SER 118 ER with biological markers

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
ER			**<0.001**	-	-	**-**
Negative	211(34)	71(14)	**(61.81)**			
Positive	414(66)	449(86)				
PgR			**<0.001**			0.112
Negative	309(51)	159(31)	**(43.31)**	83(24)	76(19)	(2.52)
Positive	297(49)	347(69)		263(76)	320(81)	
AR			**<0.001**			**<0.001**
Negative	277(50)	109(23)	**(78.24)**	104(33)	62(17)	**(24.49)**
Positive	276(50)	361(77)		211(67)	308(83)	
CK7/8			**0.003**			0.344
Negative	16(3)	2()	**(8.59)**	0(0)	1(0)	(0.89)
Positive	593(97)	501(100)		349(100)	390(100)	
CK18			**<0.001**			0.173
Negative	104(19)	33(7)	**(30.65)**	18(6)	13(4)	(1.85)
Positive	445(81)	435(93)		294(94)	351(96)	
CK19			**0.047**			0.677
Negative	67(11)	38(8)	**(3.95)**	17(5)	22(6)	(0.17)
Positive	529(89)	457(92)		322(95)	363(94)	
FOXA1			**<0.001**			**<0.001**
Negative	270(63)	137(43)	**(29.97)**	130(55)	88(36)	**(16.74)**
Positive	161(37)	185(57)		108(45)	156(64)	
BEX1			**<0.001**			**0.006**
Negative	161(38)	76(25)	**(12.47)**	90(36)	56(24)	**(7.68)**
Positive	266(62)	225(75)		160(64)	174(76)	
TFF1			0.510			0.990
Negative	205(53)	130(50)	(0.43)	109(50)	96(49)	(0.00)
Positive	183(47)	129(50)		111(50)	98(51)	
TFF3			0.231			0.192
Negative	204(51)	132(47)	(1.43)	88(39)	97(45)	(1.70)
Positive	195(49)	152(53)		140(61)	120(55)	
GATA3			**<0.001**			**0.001**
Negative	287(73)	145(52)	**(33.84)**	131(58)	87(42)	**(11.33)**
Positive	104(27)	136(48)		93(42)	119(58)	
CD71			**<0.001**			**0.008**
Negative	162(36)	176(53)	**(22.18)**	119(47)	145(59)	**(7.09)**
Positive	285(64)	155(47)		134(53)	101(41)	
CARM1			0.534			0.084
Negative	113(27)	79(25)	(1.25)	83(36)	65(27)	(4.94)
Moderate	208(50)	170(54)		114(49)	129(53)	
High	98(23)	67(21)		35(15)	48(20)	
PELP1			0.233			0.090
Negative	69(16)	55(17)	(2.91)	47(19)	46(18)	(4.81)
Moderate	318(72)	223(67)		185(73)	169(68)	
High	53(12)	53(16)		20(8)	35(14)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.024**			0.2.0
Negative	235(40)	163(33)	**(5.09)**	127(38)	128(33)	(1.60)
Positive	357(60)	330(67)		211(62)	259(67)	
p-Cadherin			**<0.001**			0.0977
Negative	201(42)	228(53)	**(12.57)**	170(62)	205(62)	(0.00)
Positive	283(58)	200(47)		105(38)	126(38)	
p53			**<0.001**			**0.002**
Negative/low	388(65)	394(78)	**(22.18)**	265(77)	340(86)	**(9.65)**
Positive	211(35)	113(22)		78(23)	55(14)	
KI67-LI			**<0.001**			0.001
Negative/low	165(33)	223(52)	**(32.43)**	131(46)	197(59)	(11.29)
High	330(67)	207(48)		154(54)	134(41)	
BCL2			**<0.001**			0.268
Negative/low	227(49)	125(34)	**(19.38)**	75(28)	69(24)	(1.22)
High	239(51)	247(66)		193(72)	220(76)	
HER1			**0.001**			0.757
Negative	456(76)	427(84)	**(10.29)**	300(88)	340(87)	(0.09)
Positive	146(24)	84(16)		42(12)	51(13)	
HER2			**0.017**	-	-	-
Negative	510(84)	449(89)	**(5.68)**			
Positive	96(16)	55(11)				
HER3			0.149			0.104
Negative	52(9)	32(7)	(2.08)	36(11)	28(8)	(2.64)
Positive	505(91)	435(93)		280(89)	334(92)	
HER4			0.126			0.147
Negative	68(12)	74(15)	(2.34**)**	46(14)	68(18)	(2.09)
Positive	524(88)	433(85)		293(86)	321(82)	

Table 6-20: **The associations of cytoplasmic SER 118 ER with biological markers**

	Whole series			ER+HER2- tumours		
	Neg/Low N (%)	**High N (%)**	***p*-value (x^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (x^2)**
Hormone Receptors and ER related proteins						
ER			**<0.001**	-	-	**-**
Negative	188(36)	94(15)	**(64.96)**			
Positive	338(64)	526(85)				
PgR			**0.002**			**0.043**
Negative	270(53)	198(33)	**(9.65)**	72(25)	87(19)	**(4.10)**
Positive	239(47)	406(67)		213(75)	371(81)	
AR			**<0.001**			**<0.001**
Negative	245(53)	141(25)	**(82.07)**	91(35)	75(18)	**(27.46)**
Positive	219(47)	418(75)		167(65)	352(82)	
CK7/8			**0.006**			0.426
Negative	14(3)	4(1)	**(7.43)**	0(0)	1(0)	(0.63)
Positive	498(97)	597(99)		287(100)	453(100)	
CK18			**<0.001**			0.359
Negative	94(21)	43(8)	**(36.32)**	14(6)	17(4)	(0.84)
Positive	362(79)	519(92)		239(94)	407(96)	
CK19			**0.008**			0.317
Negative	61(12)	44(7)	**(6.98)**	12(4)	27(6)	(1.00)
Positive	440(88)	547(93)		266(96)	420(94)	
FOXA1			**<0.001**			**0.034**
Negative	224(62)	183(47)	**(17.20)**	100(51)	118(41)	**(4.47)**
Positive	138(38)	208(53)		96(49)	168(59)	
BEX1			**0.001**			0.007
Negative	137(39)	100(27)	**(11.49)**	76(37)	70(26)	(7.15)
Positive	218(61)	273(73)		130(63)	204(74)	
TFF1			0.886			0.852
Negative	164(51)	171(52)	(0.02)	86(49)	119(50)	(0.03)
Positive	155(49)	158(48)		90(51)	120(50)	
TFF3			0.062			0.539
Negative	176(53)	160(46)	(3.47)	75(40)	110(43)	(0.37)
Positive	157(47)	190(54)		113(60)	147(57)	
GATA3			**<0.001**			**<0.001**
Negative	247(75)	185(54)	**(30.35)**	114(61)	104(43)	**(13.94)**
Positive	84(25)	156(46)		73(39)	139(57)	
CD71			**<0.001**			**0.028**
Negative	133(36)	205(51)	**(18.75)**	98(47)	166(57)	**(4.80)**
Positive	242(64)	198(49)		110(53)	125(43)	
CARM1			0.221			0.668
Negative	91(26)	101(26)	(3.02)	64(34)	84(30)	(0.80)
Moderate	171(49)	207(54)		94(49)	149(52)	
High	88(25)	77(20)		33(17)	50(18)	
PELP1			0.156			**0.046**
Negative	54(15)	70(17)	(3.71)	34(17)	59(20)	**(6.16)**
Moderate	268(73)	273(67)		155(76)	199(67)	
High	43(12)	63(16)		15(7)	40(13)	
Proteins of epithelial mesenchymal transition (EMT), tumour suppressor, proliferation, apoptosis and HER family proteins						
E-Cadherin			**0.025**			0.312
Negative	200(40)	199(34)	**(5.04)**	104(38)	152(34)	(1.02)
Positive	296(60)	391(66)		173(62)	297(66)	
p-Cadherin			**<0.001**			0.711
Negative	161(40)	269(53)	**(14.79)**	136(61)	240(62)	(0.13)
Positive	242(60)	241(47)		87(39)	144(38)	
p53			**<0.001**			0.088
Negative/low	325(64)	457(76)	**(16.91)**	223(79)	382(84)	(2.91)
Positive	179(36)	146(24)		60(21)	74(16)	
KI67-LI			**<0.001**			**0.001**
Negative/low	135(33)	254(49)	**(26.02)**	102(45)	227(58)	**(10.71**)
High	277(67)	260(51)		126(55)	162(42)	
BCL2			**<0.001**			0.680
Negative/low	190(49)	162(36)	**(12.63)**	60(27)	84(25)	(0.17)
High	202(51)	284(64)		164(73)	249(75)	
HER1			**<0.001**			0.693
Negative	375(74)	509(84)	**(16.49)**	248(88)	393(87)	(0.15)
Positive	132(26)	98(16)		34(12)	59(13)	
HER2			0.533	-	-	-
Negative	438(86)	522(87)	(0.38)			
Positive	73(14)	78(13)				
HER3			0.203			0.094
Negative	44(9)	40(7)	(1.61)	31(12)	33(8)	(2.80)
Positive	425(91)	516(93)		232(88)	383(92)	
HER4			**0.002**			**0.002**
Negative	48(10)	95(16)	**(9.65)**	30(11)	85(19)	**(6.32)**
Positive	454(90)	503(84)		253(89)	361(81)	

6.3.5.4 The expression of SER 118 ER in Trastuzumab treated series

SER 118 ER had 179 valid cases, for its nuclear form, 95 (53.1%) had negative/low expression while 84 (46.9%) had high expression. The cytoplasmic form of SER 118 ER had 92 (51.4%) cases which revealed low expression and 87 (48.6%) had high expression. The cut off points for both forms were the same as those in the primary series for both forms (Table 2-5).

6.3.5.5 The associations of SER 118 ER with clinicopathological variables

Nuclear SER 118 ER does not seem to be associated with clinicopathological variables but its cytoplasmic form was only associated with decreased lymphovascular invasion (p=0.002, Table 6-21).

6.3.5.6 The associations of SER 118 ER with biological markers and HER2 dimers

Nuclear and cytoplasmic forms of SER 118 ER did not reveal any association with biological markers or HER2 dimers (Table 6-22).

Table 6-21: The associations of nuclear and cytoplasmic SER 118 ER with clinicopathological variables

	Nuclear SER 118 ER			Cytoplasmic SER 118 ER		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (χ^2)**
Age			0.444			0.056
≤50	41(45)	31(39)	(0.58)	43(49)	29(35)	(3.65)
>50	50(55)	48(61)		44(51)	54(65)	
Menopausal Status			0.790			0.197
Pre-	46(48)	39(46)	(0.88)	48(52)	37(43)	(1.66)
Post-	49(52)	45(54)		44(48)	50(57)	
Tumour Size (cm)			0.942			0.955
≤2.0	1(1)	1(1)	(0.00)	1(1)	1(1)	(0.00)
>2.0	92(99)	83(99)		91(99)	84(99)	
Stage			0.286			0.074
1	61(65)	48(58)	(2.50)	61(68)	48(56)	(5.22)
2	30(32)	27(33)		27(30)	30(35)	
3	3(3)	7(9)		2(2)	8(9)	
Grade			0.386			0.688
1	1(1)	3(4)	(1.90)	1(1)	3(3)	(0.74)
2	27(28)	20(24)		27(29)	20(23)	
3	67(71)	61(72)		64(70)	64(74)	
Tubules			0.975			0.867
2	16(17)	14(17)	(0.00)	15(16)	15(17)	(0.02)
3	79(83)	70(83)		77(84)	72(83)	
Pleomorphism			0.983			0.875
2	8(8)	7(8)	(0.00)	8(9)	7(8)	(0.02)
3	87(92)	77(92)		84(91)	80(92)	
Mitosis			0.299			0.863
1	27(29)	19(22)	(2.41)	25(27)	21(24)	(0.29)
2	24(25)	30(36)		28(31)	26(30)	
3	44(46)	35(42)		39(42)	40(46)	
LVI			0.992			**0.002**
Probable/Negative	61(64)	54(64.3	(0.00)	49(5)	66(76)	**(9.94)**
Definite	34(36)	30(36)		43(47)	21(24)	
NPI			0.251			0.465
GPG	10(12)	4(5)	(2.76)	9(11)	5(6)	(1.53)
MPG	46(56)	50(65)		45(57)	51(64)	
PPG	26(32)	23(30)		25(32)	24(30)	

Table 6-22: The associations of nuclear and cytoplasmic SER 118 ER with biological markers

	Nuclear SER 118 ER			Cytoplasmic SER 118 ER		
	Neg/Low N (%)	**High N (%)**	***p*-value (χ^2)**	**Neg/Low N (%)**	**High N (%)**	***p*-value (X^2)**
ER			0.614			0.067
Negative	42(44)	34(41)	(0.25)	33(36)	43(49)	(3.36)
Positive	53(56)	50(59)		59(64)	44(51)	
PgR			0.966			0.392
Negative	46(60)	42(61)	(0.00)	43(57)	45(64)	(0.73)
Positive	30(40)	27(39)		32(43)	25(36)	
CK7/8	-	-	-			-
Negative						
Positive						
CK18			0.164			0.137
Negative	3(8)	0(0)	(1.93)	0(0)	3(8)	(2.21)
Positive	36(92)	24(100)		26(100)	34(92)	
P53			0.389			0.853
Negative	8(16)	11(22)	(0.74)	8(18)	11(20)	(0.34)
Positive	43(84)	38(78)		36(82)	45(80)	
BCL2			0.056			0.594
Negative/low	27(55)	18(36)	(3.64)	17(41)	28(49)	(0.28)
Moderate/high	22(45)	32(64)		25(59)	29(51)	
HER2-HER1 dimer			0.630			0.520
Negative	12(25)	13(29)	(0.23)	12(30)	13(24)	(0.41)
Positive	37(75)	32(71)		28(70)	41(76)	
HER2-HER3 dimer			0.209			0.154
Negative	19(43)	15(31)	(1.57**)**	16(46)	18(31)	(2.02**)**
Positive	25(57)	34(69)		19(54)	40(69)	
HER2-HER4 dimer			0.631			0.23
Negative	21(48)	17(43)	(0.23)	20(61)	18(35)	(5.18)
Positive	23(52)	23(57)		13(39)	33(65)	
HER2,1 vs HER2,3			0.165			0.686
HER2,1 low-HER2,3 low	11(26)	9(21)	(5.09)	10(30)	10(19)	(1.48)
HER2,1 low-HER2,3 high	0(0)	3(7)		1(3)	2(4)	
HER2,1 high-HER2,3 low	7(17)	3(7)		4(12)	6(12)	
HER2,1 high-HER2,3 high	24(57)	28(65)		18(55)	34(65)	
Her2,1 vs HER2,4			0.608			0.158
HER2,1 low-HER2,4 low	11(26)	7(19)	(1.83)	9(29)	9(19)	(5.19)
HER2,3 low-HER2,4 high	0(0)	1(2)		0(0)	1(2)	
HER2,1 high-HER2,4 low	9(22)	7(19)		9(29)	7(15)	
HER2,1 high-HER2,4 high	22(52)	22(59)		13(42)	31(64)	
Her2,3 vs HER2,4			0.558			0.155
HER2,3 low-HER2,4 low	14(35)	9(23)	(2.07)	12(41)	11(22)	(5.23)
HER2,3 low-HER2,4high	3(8)	2(5)		1(3)	4(8)	
HER2,1 high-HER2,4low	5(12)	8(21)		6(21)	7(14)	
HER2,3 high-HER2,4 high	18(45)	20(51)		10(35)	28(56)	

6.3.5.7 Outcome analysis

6.3.5.7.1 Univariate analysis

High nuclear and high cytoplasmic expressions were associated with prolonged BCSS and DMFS (p<0.001) and with subcellular localisation, nuclear expression alone or with cytoplasmic expression, were associated with prolonged BCSS and DMFS but cytoplasmic expression alone or absent expression of both was associated with worst survival in BC (p<0.001, Figure 6-8 and Figure 6-9). When the analysis was restricted to ER+ tumours, similar associations were noticed for nuclear, cytoplasmic and subcellular localisation of SER 118 ER with BCSS (p<0.001, p=0.001, p<0.001, respectively, Figure 6-10) and DMFS (p<0.001, Figure 6-11).

Furthermore, when the cohort analysis was restricted to ER+HER2-, nuclear, cytoplasmic and subcellular localisation of SER 118 ER, revealed similar association to that within the whole series with regard to BCSS (p<0.001, p=0.002, p=0.003) respectively (Figure 6-12) and DMFS (p<0.001, p=0.002, p=0.001) respectively (Figure 6-13). No associations with outcome of either form of SER 118 ER were observed within HER2+ groups and ER-HER2- subgroups.

Considering the analysis within a subgroup of patients taking hormonal therapy, consistent with the analysis with other subgroups, nuclear, cytoplasmic and subcellular localisation (with any nuclear expression) were all associated with prolonged BCSS (p=0.001, p=0.018, p=0.007) respectively (Figure 6-14) and likewise with prolonged DMFS (p=0.001, p=0.052, p=0.009) respectively (Figure 6-15). Meanwhile, when the analysis was considered within a subgroup of patients with LN-positive disease, the association of nuclear and cytoplasmic expressions deemed similar to others in terms of BCSS (p=0.013, p=0.020) respectively (Figure 6-16) and DMFS (p=0.001) but subcellular localisation showed that nuclear expression alone or with cytoplasmic expression was only associated with better DMFS (p<0.001, Figure 6-17).

No associations with survival (overall survival and DFI) were observed within the Trastuzumab treated series.

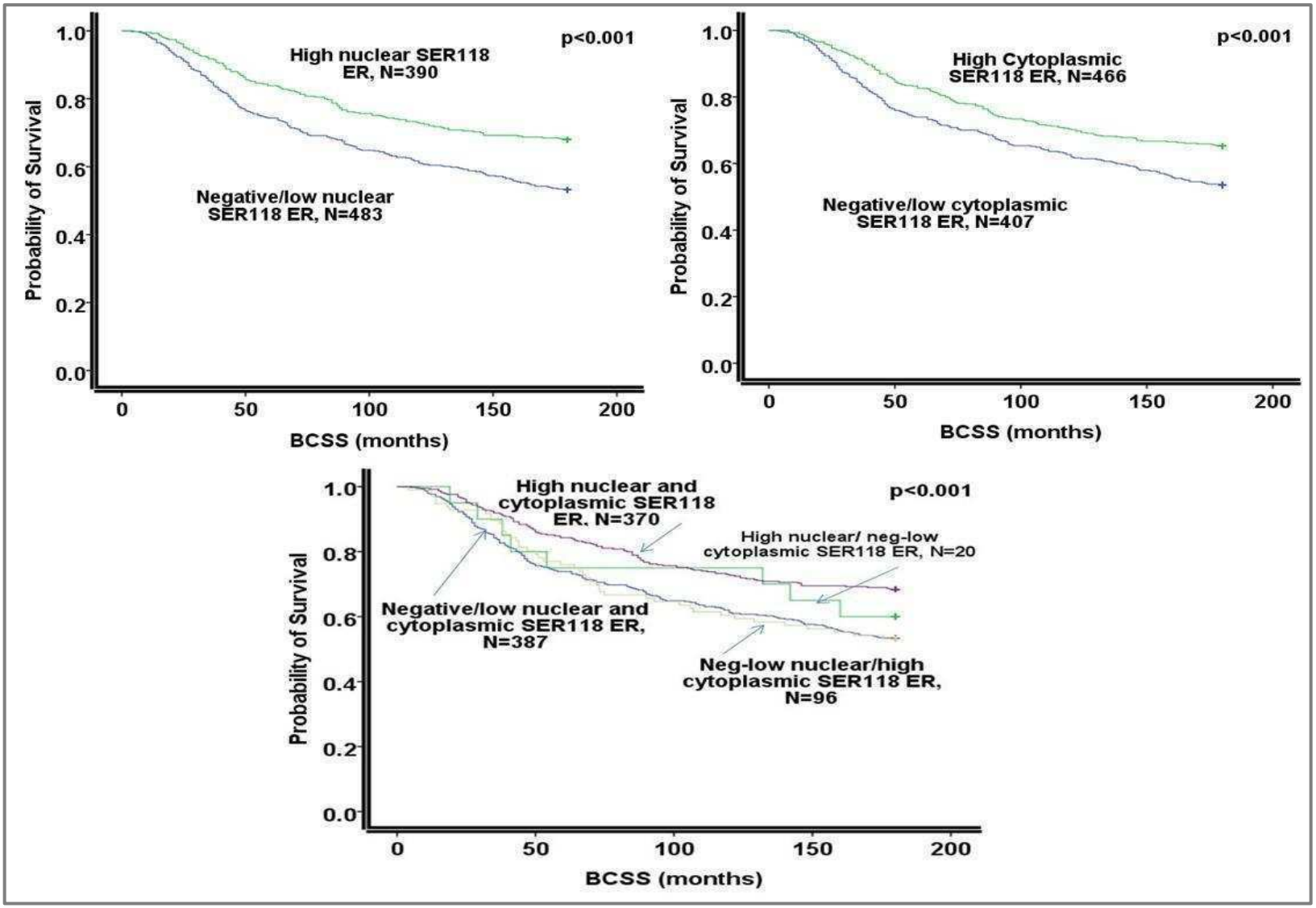


Figure 6-8: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in the unselected breast cancer

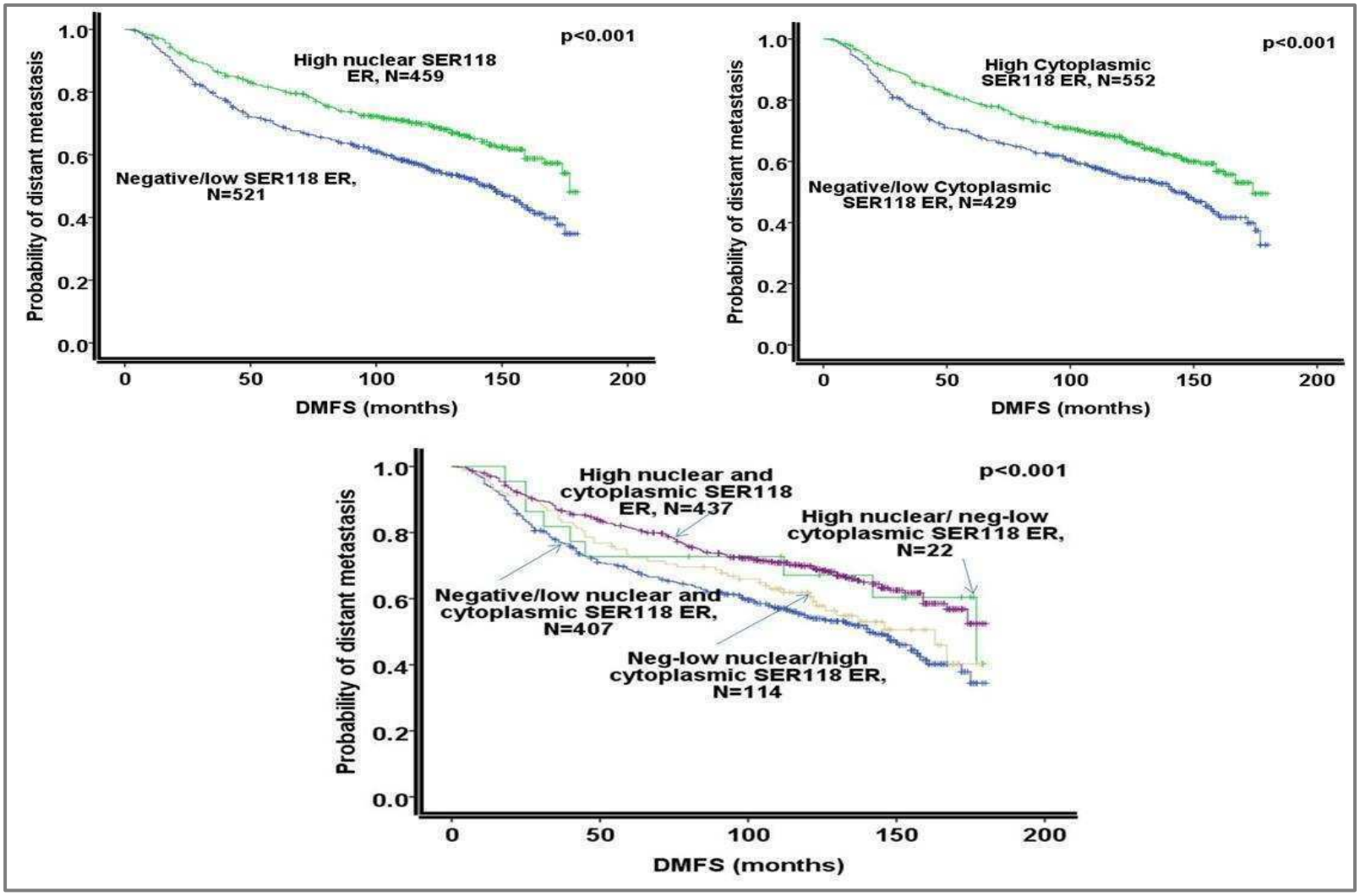


Figure 6-9: Kaplan Meier plots illustrating DMFS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in the unselected breast cancer

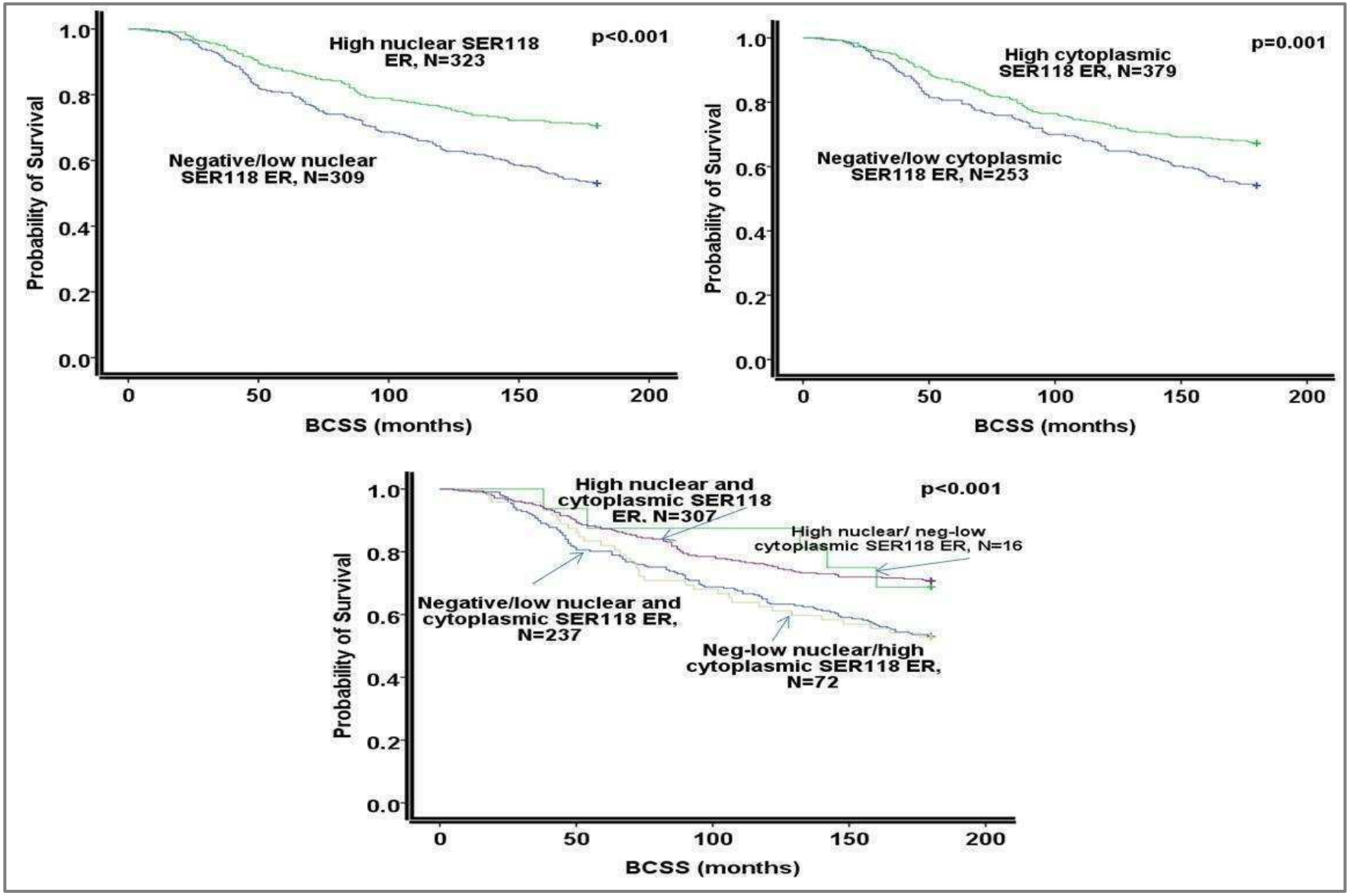


Figure 6-10: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in ER+ tumours

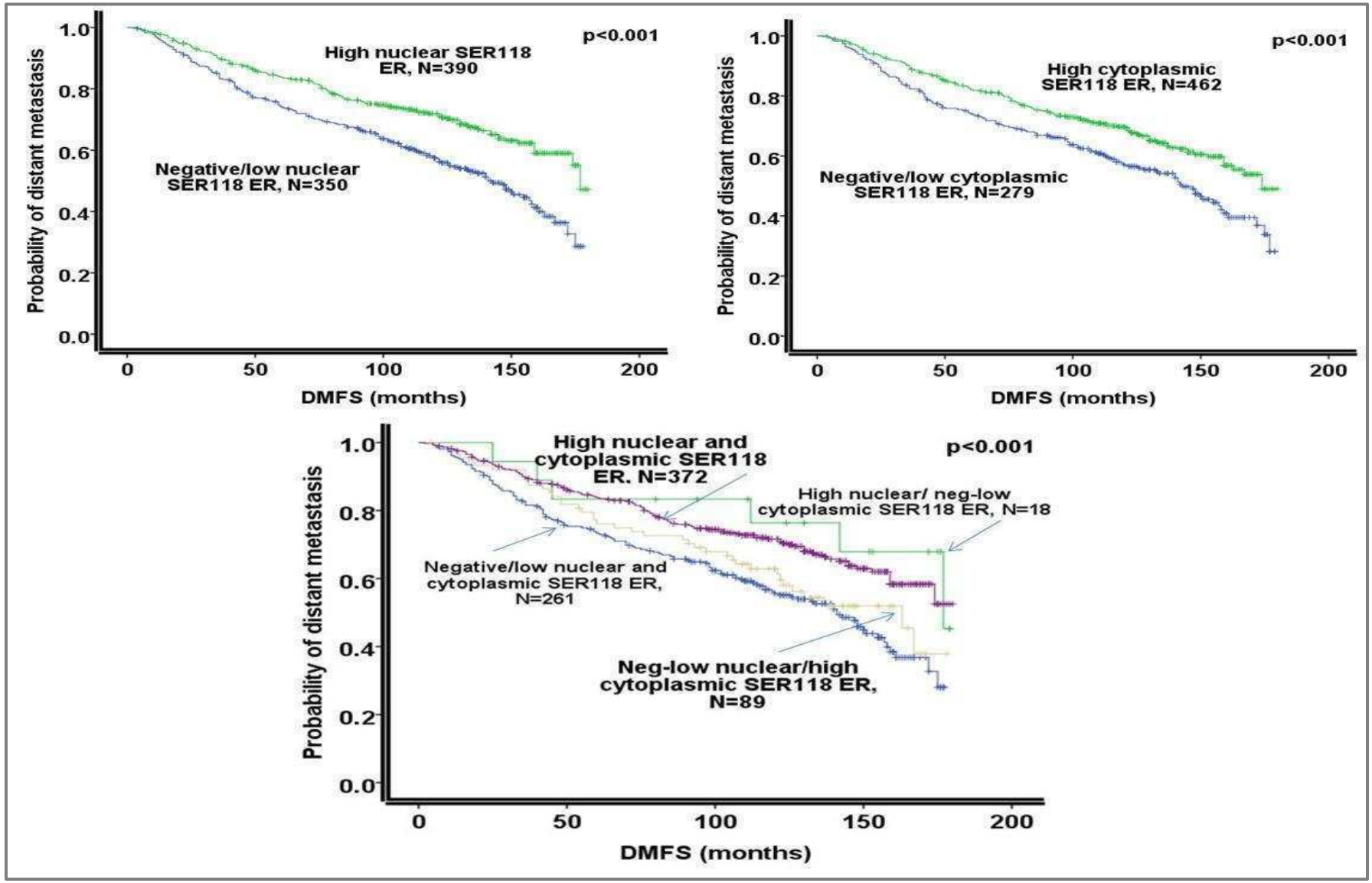


Figure 6-11: Kaplan Meier plots illustrating DMFS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in ER+ tumours

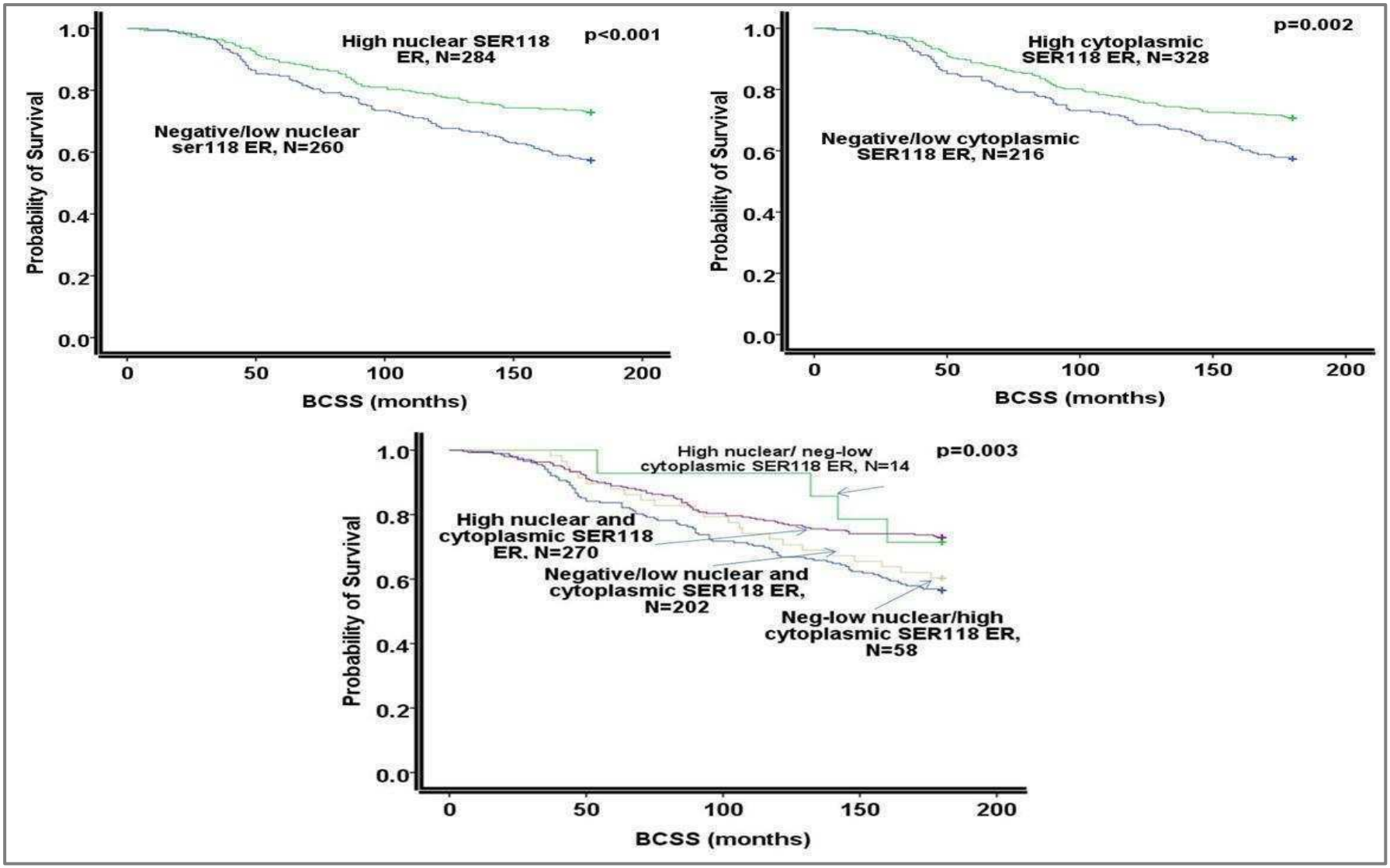


Figure 6-12: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in ER+HER2- tumours

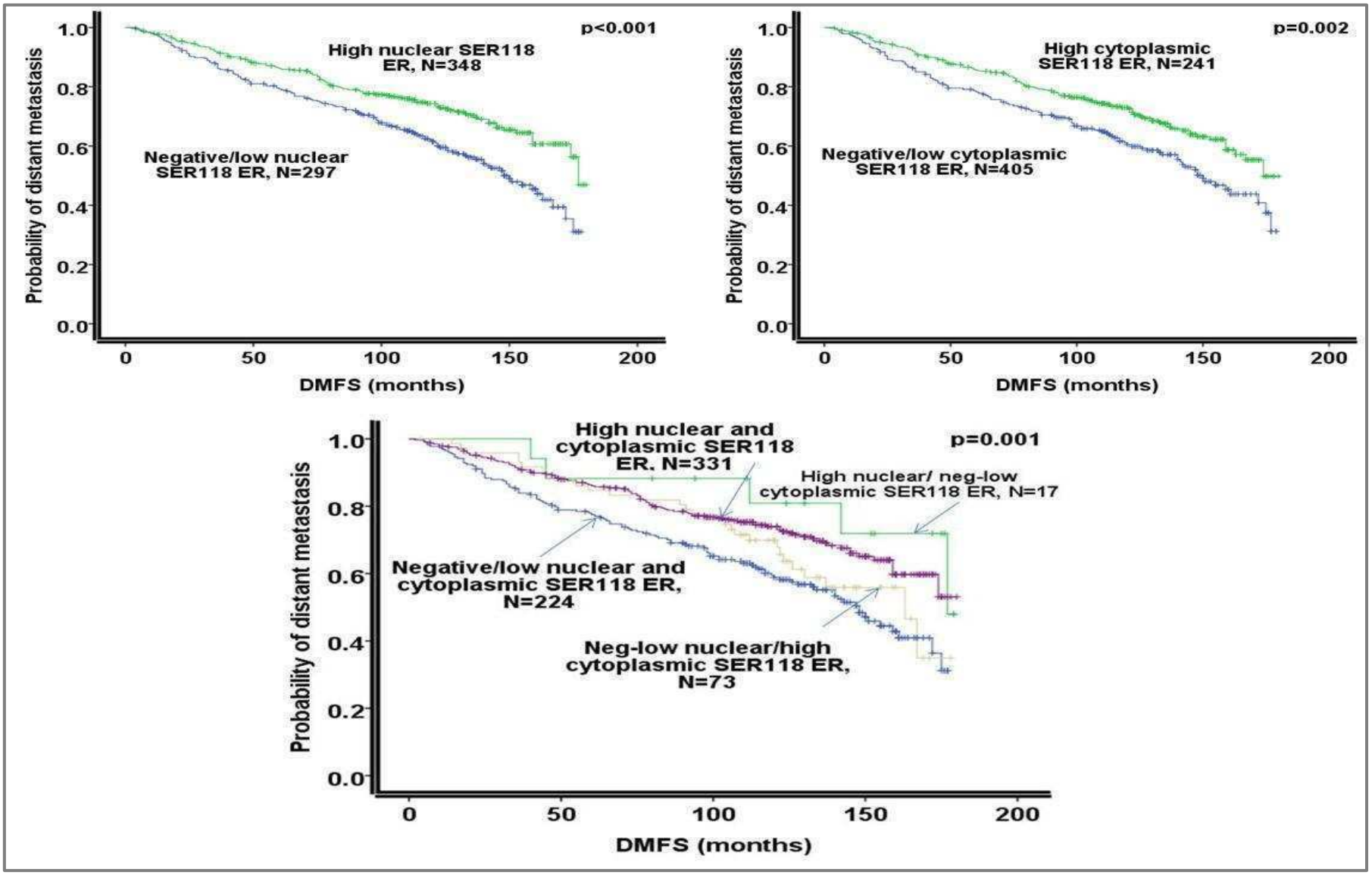


Figure 6-13: Kaplan Meier plots illustrating DMFS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in ER+HER2- tumours

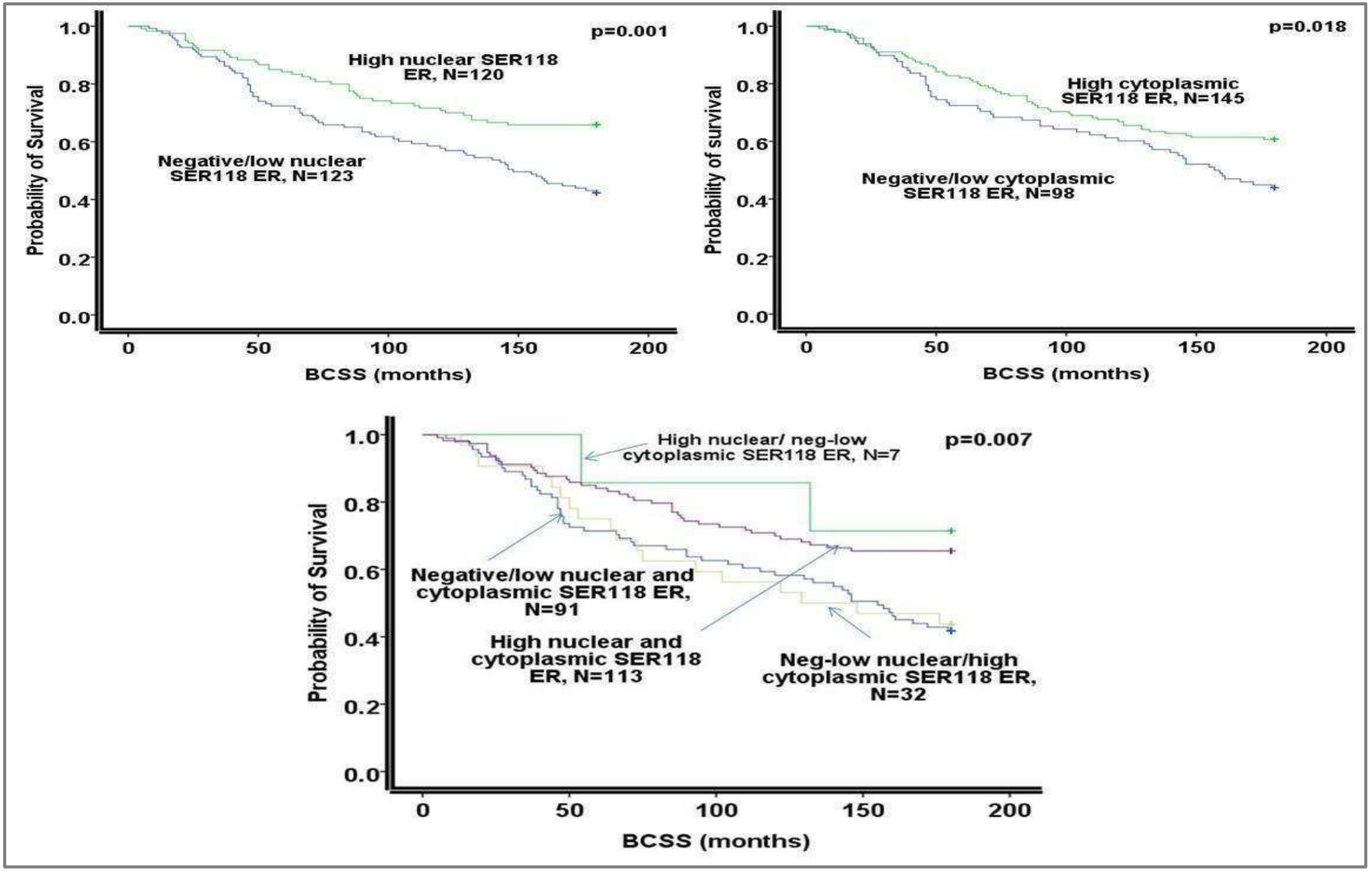


Figure 6-14: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in patients with hormonal therapy

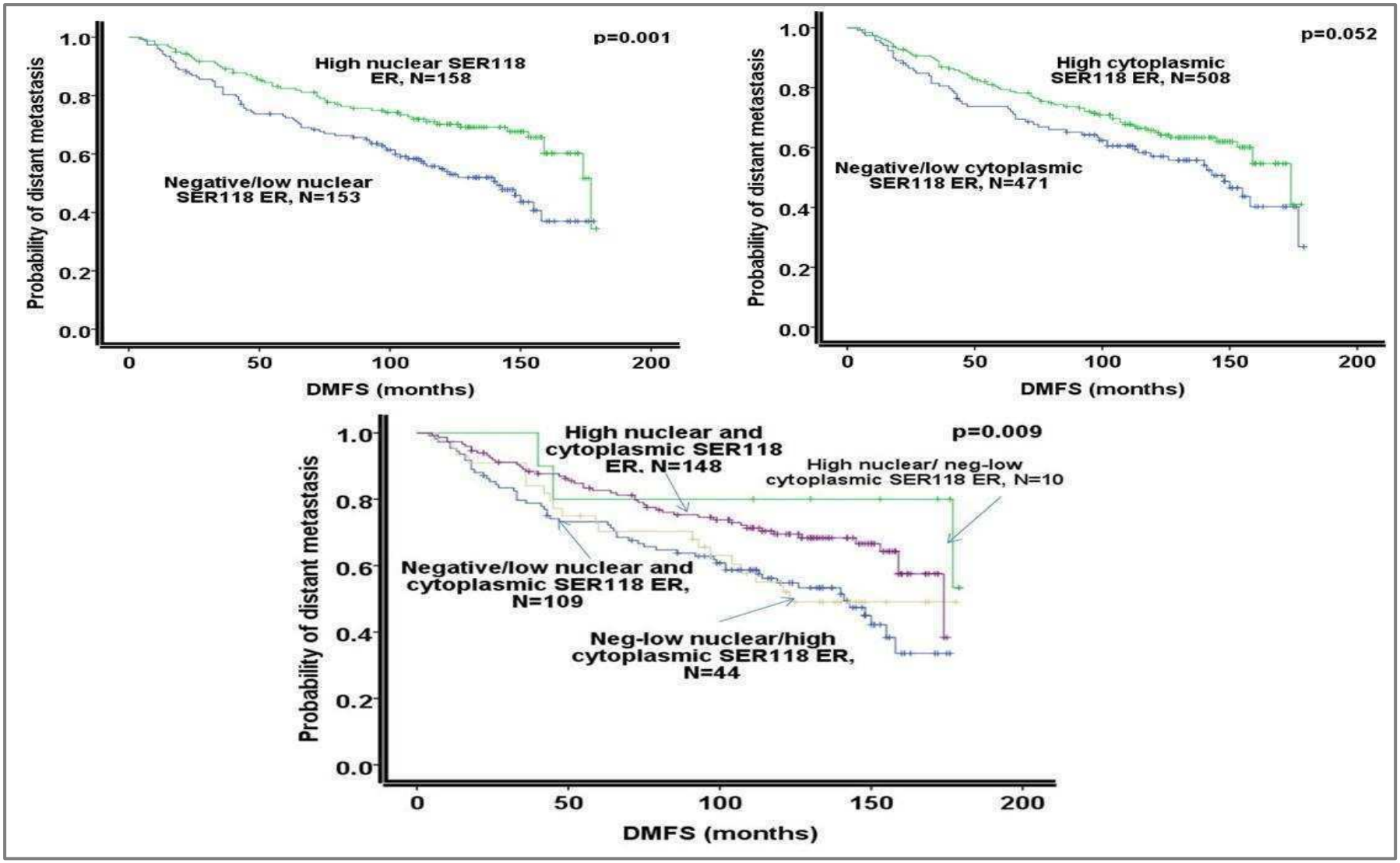


Figure 6-15: Kaplan Meier plots illustrating DMFS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in patients with hormonal therapy

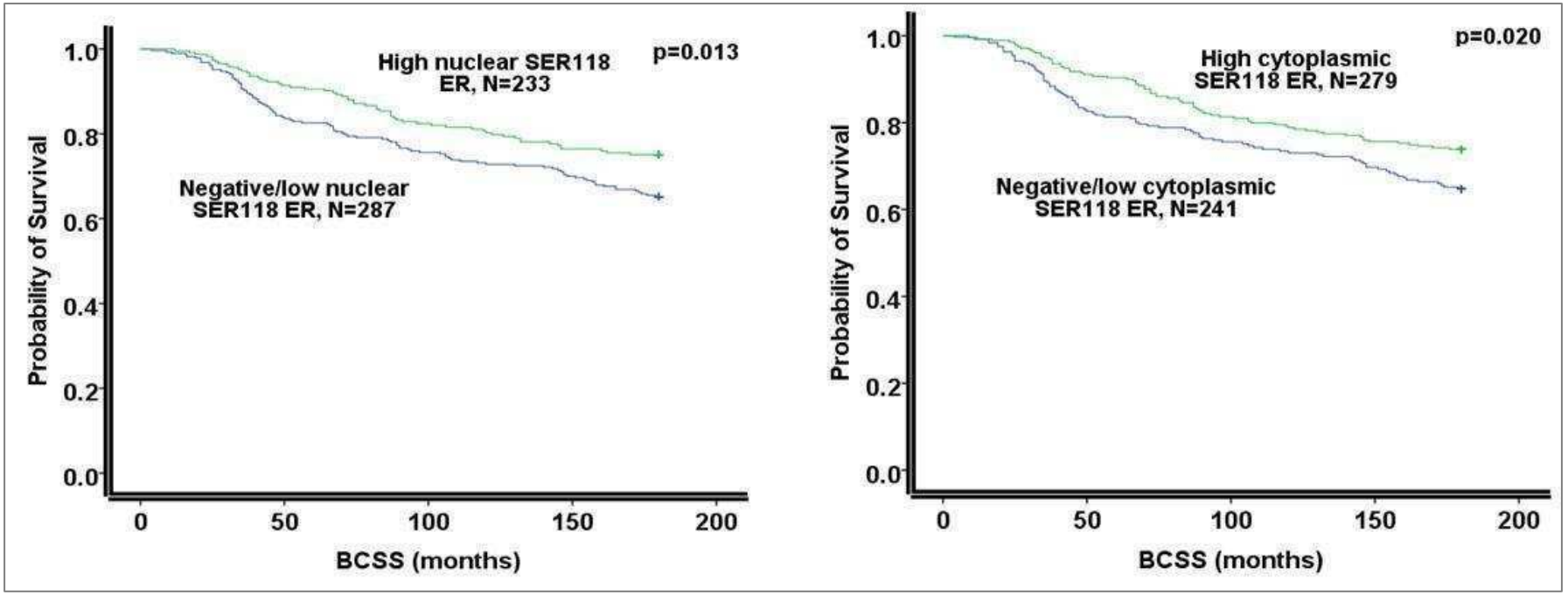


Figure 6-16: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in patients with LN positive disease

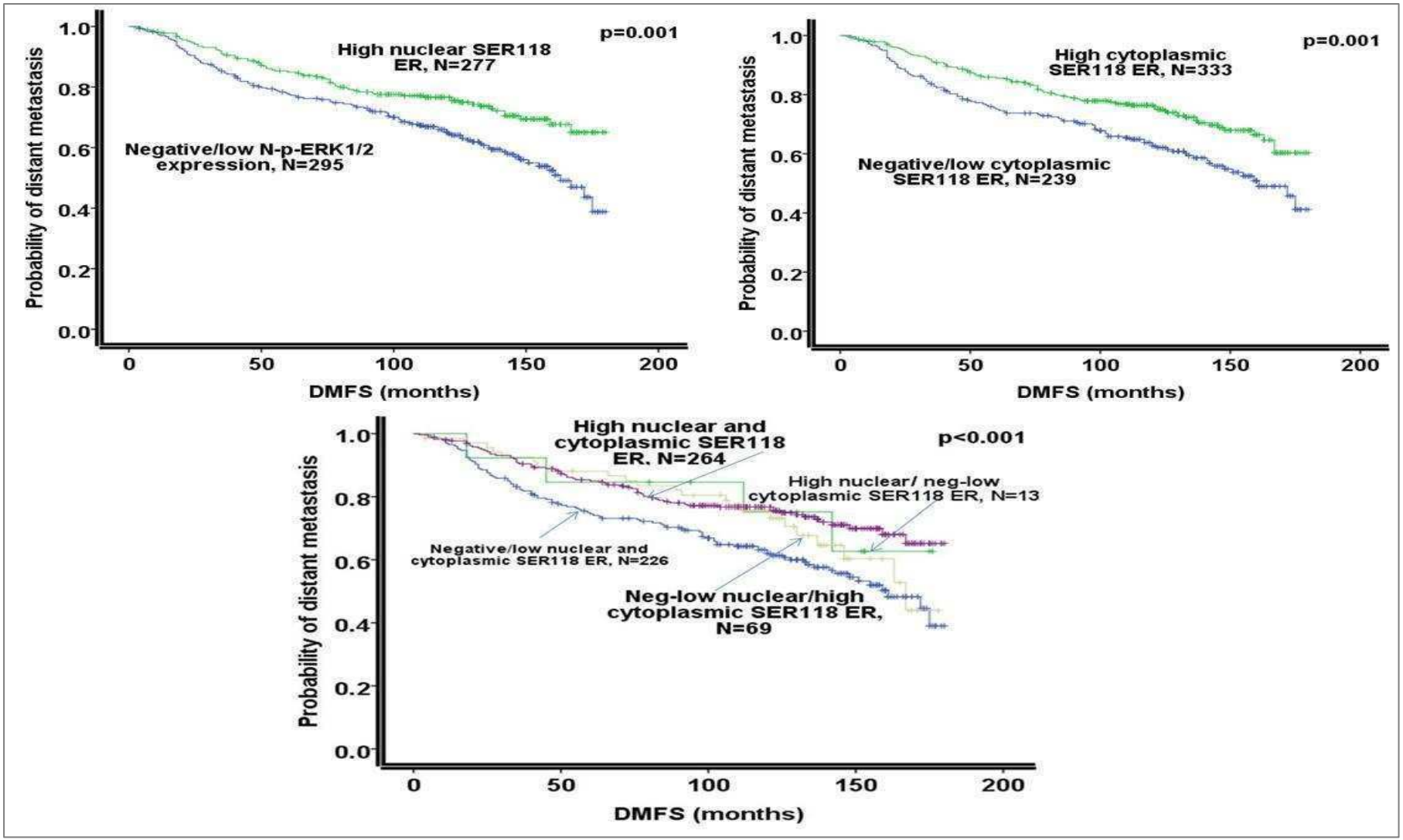


Figure 6-17: Kaplan Meier plots illustrating BCSS for nuclear, cytoplasmic and subcellular localisation of SER 118 ER in patients with LN positive disease

6.3.5.7.2 Multivariate analysis of SER 118 ER

High nuclear expression of this protein was a predictor of better BCSS (p=0.005) and DMFS (p<0.001) independent of tumour grade, size, stage, ER and HER2 (Table 6-23 and Table 6-24). Within ER+ tumours, nuclear SER 118 ER was an independent predictor for prolonged BCSS (p=0.004) and DMFS (p=0.001) independent of tumour grade, size, stage and HER2 (Table 6-23 and Table 6-24).

Moreover, within ER+HER2- tumours, nuclear SER 118 ER was a predictor of better survival for BCSS (p=0.003) and DMFS (p=0.001) independent of tumour grade, size and stage (Table 6-23 and Table 6-24). Importantly, nuclear SER 118 ER was an independent prognostic factor for better BCSS (p=0.002) and DMFS (p=0.001, Table 6-23 and Table 6-24) for patients treated with hormonal therapy in BC.

Table 6-23: Cox multivariate Regression model for the predictors of survival in breast cancer

Variable	BCSS/ whole BC			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.003**	1.429	1.125	1.814
Stage	**0.000**	2.002	1.620	2.473
Grade	**0.000**	1.856	1.585	2.175
ER	0.326	1.138	.879	1.473
HER2	**0.000**	1.739	1.333	2.270
p-Nuclear SER 118 ER	**0.005**	.715	.566	.904

Variable	BCSS/ER+ tumours			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.015**	1.420	1.071	1.881
Stage	**0.000**	2.091	1.676	2.609
Grade	**0.000**	1.713	1.410	2.081
HER2	**0.001**	1.815	1.296	2.540
p-Nuclear SER 118	**0.004**	.671	.513	.879

	BCSS/ ER+HER2- tumours			
Variable	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.027**	1.421	1.040	1.941
Stage	**0.000**	2.202	1.737	2.793
Grade	**0.000**	1.708	1.369	2.130
p-Nuclear SER 118	**0.003**	.641	.476	.863

	BCSS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	***P*-value**
Tumour size	0.839	1.042	.700	1.552
Stage	**0.000**	2.553	1.751	3.723
Grade	**0.000**	1.880	1.405	2.516
p-Nuclear SER 118	**0.002**	.529	.355	.788

Table 6-24: Cox multivariate Regression model for the predictors of survival in breast cancer

Variable	DMFS/ whole BC			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.003**	1.383	1.113	1.718
Stage	**0.000**	1.493	1.249	1.785
Grade	**0.000**	1.625	1.400	1.887
ER	0.225	1.168	.909	1.503
HER2	**0.000**	1.640	1.270	2.116
p-Nuclear SER 118 ER	**0.000**	.683	.552	.844
Variable	**DMFS /ER+ tumours**			
	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.002**	1.480	1.154	1.897
Stage	**0.000**	1.514	1.256	1.824
Grade	**0.000**	1.546	1.296	1.844
HER2	**0.000**	1.859	1.357	2.545
p-Nuclear SER 118	**0.001**	.660	.520	.838
	DMFS / ER+HER2- tumours			
Variable	***P*-value**	**HR**	**95% CI**	
Tumour size	**0.004**	1.501	1.143	1.973
Stage	**0.000**	1.567	1.281	1.916
Grade	**0.000**	1.507	1.236	1.838
p-Nuclear SER 118	**0.001**	.648	.499	.842
	DMFS/ Hormone treated patients			
Variable	***P*-value**	**HR**	**95% CI**	***P*-value**
Tumour size	0.475	1.144	.791	1.656
Stage	**0.000**	1.908	1.366	2.664
Grade	**0.000**	1.879	1.432	2.465
p-Nuclear SER 118	**0.001**	.549	.380	.793

6.4 Discussion

Over the last few decades there has been a remarkable progress in BC management resulting in earlier detection of disease and the availability of more treatment options helping in significant increase in BC survival for women living with the disease (Glass et al., 2007, Ravdin et al., 2007). Consensus regarding the best prognostic/predictive analysis has yet to be reached, but improvement continues to be made for a specific and reproducible method of identifying better treatment options based on novel biological markers. In different studies attention has been directed singularly at molecular classifications of BC (Carey et al., 2006, Liu et al., 2007, Wang et al., 2005).

Nuclear CHIP unlike the cytoplasmic form was associated with good clinicopathological variables in BC and even within ER+HER2- group. The results observed in the current study were in line with Lee et al (Lee et al., 2013) who reported that CHIP level was negatively correlated with BC progression. Parallel with these findings, outcome analysis revealed that nuclear CHIP alone or when subcellular localisation was considered, was associated with better outcome unlike the cytoplasmic form and consistently, Patani et al found that CHIP protein was associated with good prognostic features, downregulation of HER2 and better survival (Patani et al., 2010).

On the other hand, a study has shown that CHIP has an inverse association with ER and regulated genes (Fan et al., 2005) ; indeed, it has been reported that ER is held in its ligand binding status by the action of Hsp90-based chaperon and by using inhibitors to this protein, there will be interaction between ER and CHIP which will enhance ER degradation through ubiquitin proteosome pathway (Beliakoff et al., 2003, Bagatell et al., 2001). As we observed that nuclear CHIP and ER were positively correlated, it is therefore hypothesised that perhaps subcellular localisation could have a role in degradation as the cytoplasmic expression rather than the nuclear form did not reveal a positive association with ER and even more, it was associated with decreased expression of some ER related proteins. Indeed, the negative association of nuclear CHIP rather than the cytoplasmic form with HER2, HER2 dimers and with lower grade support our view for the differential function of both forms. Additionally, it has been observed that CHIP is associated with decreased proliferation but was associated

with stimulation of apoptosis in MCF-7 cell lines (Yi et al., 2008) and we think that this is probably the action of the nuclear form.

Interestingly, CHIP has demonstrated good associations with favourable prognostic parameters in ER+HER2- tumours, to less extent within ER+HER2+ with less likely associations observed within ER-HER2+ together with some preserved favourable associations within Trastuzumab treated series implies the effect of single and double expression of HER2 and ER. In fact, the difference in the associations between nuclear and cytoplasmic forms of this protein could imply a difference in their biological significance especially in their associations with ER and HER2. For instance, in the double positive group, the nuclear form was associated with lower grade, low mitotic count and more tubule formation whereas the cytoplasmic form was associated with a trend for more tubule formation and a trend for less mitotic count. In addition, the favourable association of the nuclear rather than the cytoplasmic form probably indicate a favourable action of the former. The mentioned results were supportive to the hypothesis of this study where differences were noticed by studying both forms of this protein in different subgroups based on ER and HER2. Prospectively, we believe that some functional studies could be considered in the future to explore additional function of this protein as the possibility that this biomarker could be of therapeutic value is still under investigation.

In conclusion, CHIP protein is a good example to study to reveal differential correlations with ER and HER2 and its subcellular localisation has deemed useful to illustrate the function of nuclear and cytoplasmic forms and CHIP revealed variable associations within different BC subclasses.

Regulatory networks orchestrated by necessary transcription factors have been thought to play a crucial role in the determination of stem cell states. Nevertheless, the master transcriptional regulators of adult stem cells are poorly understood (Guo et al., 2012). SOX9, its nuclear and cytoplasmic forms were associated with both poor prognostic variables and markers of adverse outcome. Importantly, SOX9 has been considered as one of the signature genes that define basal like BC (BLBC); however, direct evidence has remained scarce regarding its biological function in both normal and BC tissue (Perou et al., 2000a, Sørlie et al., 2001). Moreover, it has been reported that it had low expression in luminal and HER2+ groups (Wang et al., 2013) and we found

differential associations between HER2 and both forms although both were associated with poor prognostic features.

Recent report indicated that SOX9 marks the adult stem cell subset that can aid in regeneration of the liver cells, exocrine pancreas, and intestine (Furuyama et al., 2011). For this reason, SOX9 might also participate in normal breast homeostasis and renewal throughout puberty and pregnancy (Wang et al., 2013). Still evidence indicates that SOX9 could have a favourable role in cancer as it has been shown to be associated with decreased proliferation, cell cycle arrest and enhancement of apoptosis in prostatic carcinoma (Drivdahl et al., 2004). The results in this Chapter indicated the negative association between the nuclear form of this biomarker and ER and its associated proteins but its borderline negative association with HER2 could explain why it was not associated with poor outcome. From another point, the cytoplasmic form of SOX9 showed strong positive association with HER2 positivity and this could imply a possible difference between forms although both were strongly associated with ER negativity. Furthermore, both forms of this protein were associated with increased expression of P-Cadherin which could be a feature stem cell state. Accordingly and based on our results, the biological significance of SOX9 seems to indicate unfavourable associations with clinical variables. For this reason, further research in this field is still mandatory to investigate more aspect regarding the biological significance of this biomarker and to determine whether this protein could be a target for BC therapy and probable discrimination of the biological function of both forms of this protein could be elucidated.

For SRC3, it showed an association with poor prognostic variables and even it was associated with increased expression of HER2 dimers and combination of these adverse dimers (although were borderline associations). SRC3 was initially identified as a gene which is amplified and overexpressed in 5%-10% of ovarian cancers and 30%-60% of BCs (Anzick et al., 1997). Recently, clinical evidence indicated that high levels of this protein were associated positively with high HER2 levels, resistance to hormonal therapy and short survival (Cai et al., 2010, Dihge et al., 2008, Osborne et al., 2003a). Consistent with our findings, a study indicated that increased SRC3 level in the mammary tissue of mice is a crucial factor for enhancing HER2 signalling (Fereshteh et al., 2008). Since SRC is a co-

activator that enhances the transcriptional activity of ER for genes responsible for cancer progression and as it is a downstream target of HER2 signalling, we believe that in the future, expansion of Trastuzumab treated series will be of interest to revealing an association with outcome as this biomarker could be a therapeutic target.

Regarding ECD, in the context of its intensity, higher intensities were observed in invasive BC tissue rather than in normal and DCIS foci. This was in line with the results of other researchers who investigated the same protein using our primary series of BC (Zhao et al., 2012). With respect to the correlations, our data within Trastuzumab treated BC series showed that ECD was associated with poor prognostic variables and this was in keeping with Zhao et al (Zhao et al., 2012) who had the same observation. In addition, it was associated with increased expression of some HER2 dimers and combination of these dimers and this confirms its direct association with HER2 as indicated by the latter study. A recent study has also highlighted the role of ECD in promoting cell cycle progression where knocking down of this protein in mammary epithelial cells and in mice as well was associated with growth arrest. Consistently, whole-genome mRNA expression analysis of control vs. ECD knock down in human mammary epithelial cells revealed downregulatation of several genes which are part of the top 40 genes which were E2F targets (Bele et al., 2015).

Further assessment of this protein in a larger series with patients taking Trastuzumab treatment could be warranted to further assess the prognostic utility of this protein. In addition, as this protein was also expressed in normal tissue, future evaluation of this biomarker using other tissues could help further understanding of its biological significance and later on comparison will be of value.

Regarding SER 118 ER, it has been indicated that oestrogen binding can stimulate the phosphorylation of many ER sites and the most characterised phosphorylation site is SER 118 ER (Le Goff et al., 1994, Ali et al., 1993). SER 118 ER protein has revealed significant associations with clinicopathological variables and with biomarkers indicative of good prognosis. Meanwhile, this protein showed very significant associations with outcome in BC and within ER+ subgroup; nevertheless, its subcellular localisation has illustrated a favourable effect of the nuclear form vs its cytoplasmic one. In line with our views, a study has indicated that SER 118 ER was associated with good prognostic features.

Researchers in the latter study reported a positive association between SER 118 ER and MAPKs which indicates that the latter may have a role for direct or indirect regulation of SER 118 ER (Murphy et al., 2004). Additionally, our results were consistent with Kok et al (Kok et al., 2009a) who used IHC for detection of SER 118 ER expression and found that this protein was an independent predictor of good prognosis in patients receiving tamoxifen therapy. Furthermore, our result regarding outcome were in keeping with the findings of another study which reported that low phosphorylation of SER118 ER is associated with short outcome (Yamashita et al., 2008). For the best of our knowledge, this is the first study to show the impact of subcellular localisation of SER 118 ER and we highlighted its predictive role in tamoxifen treated and LN-positive patients and its prognostic effect in different BC subgroups in relation to HER2 and ER. Basically, the difference between the function of the nuclear and cytoplasmic forms is related to the location and targets of SER 118 ER, where the nuclear form after activation is translocated to the nucleus and probably the phosphorylation at this site will recruit co-repressors that will repress the transcription of genes involved in cancer progression and those blamed for unfavourable behaviour. Nevertheless, the activation of the cytoplasmic form will act locally in the cytoplasm and will activate certain targets like MAPKs or PI3K/Akt members that will enhance the activation of immediate early genes that will influence rapid physiological action without enhancing nuclear ER translocation to nucleus and subsequent gene transcription. Furthermore, as tumours associated with the phosphorylation of this protein were correlated with prolonged survival in those taking tamoxifen, it appears that this (the phosphorylation) together with the action of tamoxifen will both augment the recruitment of co-repressors the will subvert the transcription of unfavourable gens.

In addition, this protein also showed that despite it had positive associations with some ER-related proteins within ER+HER2+ cohort, no associations with prolonged survival (BCSS or DMFS) was revealed within this cohort implying the possible effect of HER2 as SER118 ER showed wide range of associations within ER+ subgroup.

In conclusion, SER 118 ER illustrated differential functions of its two forms in terms of its association with different biomarkers within different BC subgroups based on HER2 and ER expressions and it terms of outcome.

7 Computational Biology and Breast Cancer Classification

7.1 Introduction

Over the last few decades, identification of diseases like BC with the aid of expert system was shown beneficial over any experienced doctor (Ali et al., 2011). Expert system feed information without losing its knowledge; therefore, it is mandatory in the diagnosis of certain aspects related to health care and thus, decision tree techniques have been used in different studies due to its simple use (Kurt et al., 2008, Kupta et al., 2011, Sharma and Hota, 2013, Hota, 2012, Bendi et al., 2012, Bendi et al., 2011).

Clustering is an exploratory data analysis task that aims to find the intrinsic structure of data by organising data objects into similar clusters or groups. Clustering is often unsupervised learning as opposed to the supervised learning (e.g., classification) for which the data objects are already labelled with known classes. Decision tree algorithms are a novel clustering method, which is based on a supervised learning technique. Decision tree is a commonly used classification method being easy to understand and follow and easily trained. Decision tree is an approach used to automatically learn, through machine learning, to identify complex patterns and demonstrate relations between observed variables to reach intelligent decisions, thus, improving basic knowledge regarding cancer and its development (Cruz and Wishart, 2006). In addition, decision trees are one of the proposed means used for BC classification and this can be achieved by sorting cases according to certain feature or attribute values; e.g. tissue biomarker expression).

The structure of a decision tree is similar to the usual tree having root node, with left and right sub branches. The leaf in a tree represents a class membership. The arcs from one node to another denote the conditions on the attributes. Every instance in any set of data used by machine learning algorithms is represented using the same set of features which may be continuous, categorical or binary. If instances are given with known labels, then the learning is known as supervised; otherwise, it will be unsupervised. There are several decision tree models that can be used for classification of data including ID3, C4.5, C5, CART, CHAID, SLIQ, SPRINT, and ScalParc (Lavanya and Rani, 2012).

Perhaps the most recognised algorithm in research for building decision trees is the C4.5 designed by Ross Quinlan. C4.5 is a statistical classifier system that is

based on obtaining information to build classificatory decisions according to previously chosen target classification and each attribute can split the data into smaller subsets to make a decision (Quinlan, 1993). C4.5 decision tree is a supervised classification used to obtain a set of rules to see which are the most appropriate biomarkers involved in the classification process that will help build up a model to predict class membership of certain data. Decision tree learning is widely used approach in data mining. The goal is to create a model that predicts the value of a target variable depending on several supplied variables (Quinlan, 1993).

Importantly, BC stratification accuracy and effective decision making for the suitable treatment choice are mandatory. Many prognostic markers and models based on tissue biomarker research are still evolving; nevertheless, very few of them have fulfilled the needed criteria to be applied clinically. Unfavourable study designs and inaccurate statistical analyses have been an issue of discussion in addition to other current problems in the research field to justify the need of using other proposed models using tissue biomarkers (McShane et al., 2006)

These system outputs can be used later on as models, in the form of decision trees or sets of if-then rules, which can be used to classify other subsets bearing in mind that these should be precise and easy to understand. In general, it is often possible to prune a decision tree to obtain more desirable one (Quinlan, 1993, Kotsiantis, 2007).

7.1.1 Hypothesis

Computational biology and bioinformatics are high throughput techniques that allow classification of BC into biologically distinct subgroups based on expression of several proteins and can decipher the complex interaction of related genes. It is hypothesised that decision tree algorithms can classify BC into distinct classes based on the interaction of ER and HER2 related biomarkers and their relative contribution to driving BC molecular profile.

7.1.2 Aims

The aims of this chapter were to build decision trees algorithms for ER and HER2 related proteins, MAPKs and PI3K biomarkers to identify the specific biology of HER2 and ER in different subsets of BC and to reveal driving markers related to these two key pathways. Biomarkers' expression was assessed using IHC.

7.2 Methods

The material supplied for decision tree algorithm prediction in this chapter was obtained from a panel of biomarkers used to stain a well characterised cohort of Nottingham Tenovus primary BC series (stage I-III):

1) The biomarkers under investigation in this chapter include those directly related to ER, HER2; MAPK, mTOR pathways assessed using IHC (Table 7-1).
2) PI3K (Table 2-3)
3) HER (Table 2-3)
4) ER related proteins: CK7/8, CK18, CK19, FHIT, GCDFP, MUC1 and CD71, (Table 2-3).
5) Basal cytokeratins: CK5, CK14 and CK17 (Table 2-3).

Importantly, four class memberships were used as target classifiers in this study (have been set as an outcome variable) and each one of these classes is the final target into which some cases can assign and these classes include 1) Class one (ER+HER2-), 2) Class two (ER+HER2+), 3)Class three (ER-HER2+) and 4) Class four (ER-HER2-). Furthermore, the cut-off points for ER and HER2 to form the four classes were set by the software. In this regard, the percentage of expression was used for luminal, basal cytokeratins, HER family proteins (HER1, 3 and 4), ER related (gross cystic disease fluid protein and fragile histidine triad protein) and KI67 while for MAPKs, PI3K associated and ER and related proteins (PgR, MUC1and CD71), the H-score of expression was used (Table 7-1). Regarding HER2, a categorical data was used as the scoring of this biomarker is by Hercept test (paragraph 5.2, page 188).

To build a class membership predicting algorithm for BC patients, decision trees were computed by the expert system, the outcome variables were set (supervised) which are the four classes and then each group of biomarkers to build an algorithm from, were fed separately, then this system chose the cut-off points for each biomarker and then decided the main node (the driving

biomarker) in each decision tree and then other attributes (biomarkers' expression) are chosen by the system until at last certain number of cases are allocated in each class membership. Such step decides upon the accuracy as the minimum number of cases allowed in each class will determine the accuracy, as a result, the expert system decided the minimum number of cases under each subclass that resulted in best precision. For robustness, only cases with complete values for all biomarkers were used to compute the decision tree, for this reason, some ER related proteins were chosen to be used in the decision tree rather than others as they have less missing values compared to the rest.
To simplify how these biomarkers interact as a group and then all together, the decision trees were considered for each group separately and all together (Table 7-1).

Table 7-1: The expression of biomarkers used in decision tree

Biomarkers	The expression
ER and related proteins	
ER	H-score
PgR	H-score
AR	percent
CK7/8	percent
CK18	percent
CK19	percent
CD71	H-score
GCDFP	percent
FHIT	percent
MUC1	H-score
Basal cytokeratins	
CK5	percent
CK14	percent
CK17	percent
Proliferation	
KI67-LI	percent
MAPKs proteins	
Nuclear(N)-p-ERK1/2	H-score
Cytoplasmic (C)-p-ERK1/2	H-score
ERK1/2	H-score
p-p38	H-score
JNK1/2	H-score
p-JNK1/2	H-score
P38	H-score
p-ATF2	H-score
p-C-JUN	percent
HER family proteins	
HER1	percent
HER2	0,1
HER3	percent
HER4	percent
PI3K proteins	
PI3K	H-score
Akt	H-score
PTEN	H-score
mTORC1	H-score

7.2.1 Statistical analysis

For establishing a set of rules to determine to which group of the proposed four membership classes a patient is more likely to be assigned using values of its given variables; computing the decision tree algorithm C4.5 was performed using the WEKA software and these decision trees are designed relying on software generated cut-offs. Moreover, the boxplots were organised to visualise the differential expression of these biomarkers in four main BC subgroups (the vertical bars represent the expression whether by percentage or by H-score) and to reveal the median (represented by the bold horizontal line in the box). Furthermore, the used software decides the minimum number of cases in the last layer of each decision tree and this in turn decides upon the relevant maximum accuracy.

7.3 Results

7.3.1 Decision trees based on IHC data

7.3.1.1 MAPKs

The boxplots (Figure 7-1) show the median and range of biomarker expression in the four BC phenotypes which demonstrate variable expressions of MAPKs between the four BC subclasses.

Nuclear p-ERK1/2 was the primary distinguishing biomarker which dichotomised tumours into high or low based on 60 H-score, further markers: p-p38 (50 H-score), p-ATF2 (50 H-score) and p-JNK1/2 (200 H score) were chosen by WEKA software to split the cases into the two phenotypes including class one (ER+HER2-) and class four (ER-HER2-), (Figure 7-1B). The minimum number of cases which were considered in the last layer of the decision tree was 8 with an accuracy of 69% (Table 7-2). Class two and three (ER+HER2+ and ER-HER2+, respectively) were not encountered in this algorithm.

7.3.1.2 PI3K and associated proteins

For PI3K and associated proteins, the minimum number allowed in the last layer was 4 with a maximum accuracy of 69% (Table 7-2). Regarding prediction of membership classes, PTEN was predominant in the decision tree throughout after taking into consideration other markers. Initially, PTEN was the main node

(cut off: 60 H-score) along with p-mTOR high expression (cut off>0 H-score) which assigned the majority of cases within class one which is ER+HER2-. In addition to those cases which had PTEN >60, 0 mTOR H-score and PTEN >180 H-score (Figure 7-2 B). Furthermore, those cases having PTEN expression from 30-60 H-score with Akt >150 H-score expression and also those who had Akt expression (0-150 H-score) determined class one (ER+HER2-). In contrast, class four (ER-HER2) was assigned either to those with PTEN expression >60 H-score, p-mTORC1 0 H-score and PTEN $\leq$180 respectively. Similarly, class four was assigned to those cases whose PTEN expression was between 31-60 H-score and meanwhile to those with PTEN expression between 30-60 H-score and Akt expression from 51-150 H-score (Figure 7-2 B). Regarding class two and three, they were not revealed in this decision tree.

7.3.1.3 HER family

For HER family members, most of the cases were assigned to class membership one (those which are negative for HER2 and also low for EGFR). Furthermore, class four (ER-HER2-) was allocated to cases negative for HER2 with high EGFR (percent) and high HER3 (percent) expression. For class two, it was assigned to those cases which were positive for HER2 and with low EGFR expression but those with high EGFR expression, were put under class three (ER-HER2+). Importantly, the relevant precision was 76.2% with minimum number of cases in each class was 16 (Table 7-2), Figure 7-3 A and B; represent the boxplot and relevant decision tree.

7.3.1.4 ER, related proteins and basal cytokeratins

With relevance to ER and related proteins and basal cytokeratins, most of the cases (ER> 0 H-score) appear to be within class membership one (ER+HER2-) while those negative for ER were splitted on the basis of luminal CK18, where those with $\leq$97 H-score expression were assigned class four (ER-HER2-) in addition to those who had >97 H-score and $\geq$5 CK5. Others who had >97 CK18 H-score and $\leq$5 CK5 were assigned class three (ER-HER2+). The associated accuracy of this algorithm was 87% and similar to the previous group, 16 cases were the lowest number allowed (Table 7-2). The boxplot and related decision tree are illustrated in Figure 7-4.

7.3.1.5 All biomarkers

When all biomarkers were considered in the same algorithm, class membership one (ER+HER2-) possessed the largest number (14) of cases compared to others. Cases which were positive for HER2 and with cytoplasmic p-ERK1/2 $\leq$20 H-score were assigned class two (ER+HER2+) contrary to those with cytoplasmic p-ERK1/2 >20, they were assigned class three (ER-HER2+), (Figure 7-5). Those which were negative for HER2 and for CK5 were under the category of class one (ER+HER2-) while those positive for CK5 were categorised as class membership four (ER-HER2-). In this algorithm, the minimum number of cases was 2 in each class membership and the accuracy was 100% with no misclassified cases (Table 7-2) as they displayed the same distribution as that noticed in relevant boxplots (Figure 7-5).

Table 7-2: Accuracy and minimum number of cases in each analysed group

Biomarkers	Accuracy	Minimum number of cases in the last layer
ER and related proteins ER PgR AR CK7/8 CK18 CK19 CD71 GCDFP FHIT MUC1 **Basal cytokeratins** CK5 CK14 CK17	87%	16 cases
MAPKs proteins Nuclear(N)-p-ERK1/2 Cytoplasmic (C)-p-ERK1/2 ERK1/2 JNK1/2 p-JNK1/2 p-p38 P38 p-ATF2 P-C-JUN	69%	8 cases
HER family proteins HER1 HER2 HER3 HER4	76.2%	16 cases
PI3K proteins PI3K Akt PTEN mTORC1	69%	4 cases
All biomarkers together	100%	2 cases

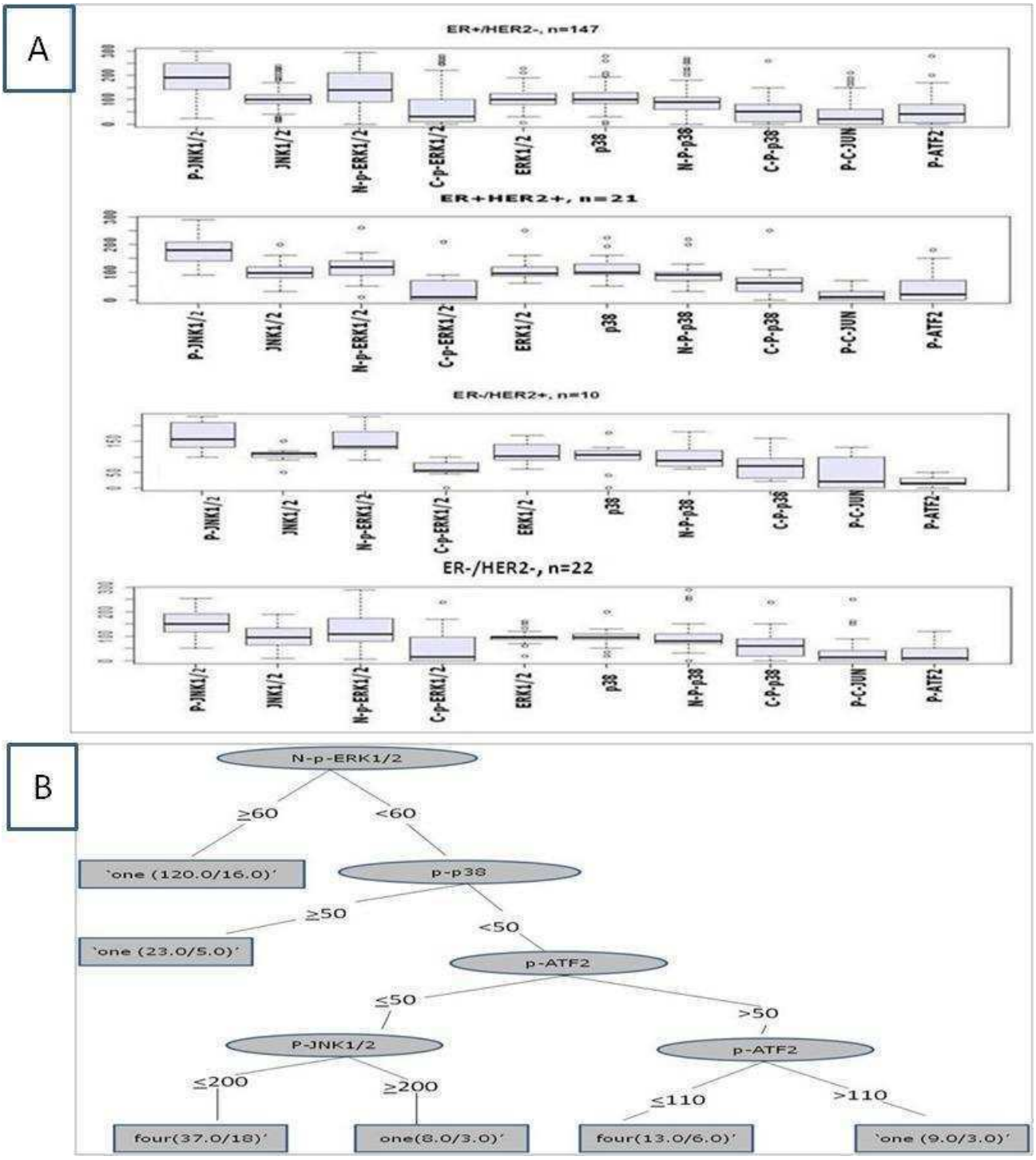


Figure 7-1: A: Box plots for MAPKs (H-score), B: Decision tree algorithm for predicting class membership in MAPKs. The circle on the top represents the main node. Other circles represent the markers in the algorithm and Rectangles represent feature value tested (class membership); and numbers between brackets in the rectangles represent subsets of patients correctly classified and misclassified, from left to right respectively. Branches emerging from each marker are levels of expression below or above which a specific case is to be classified into class one (ER+HER2-) or four (ER-HER2-) in this algorithm.

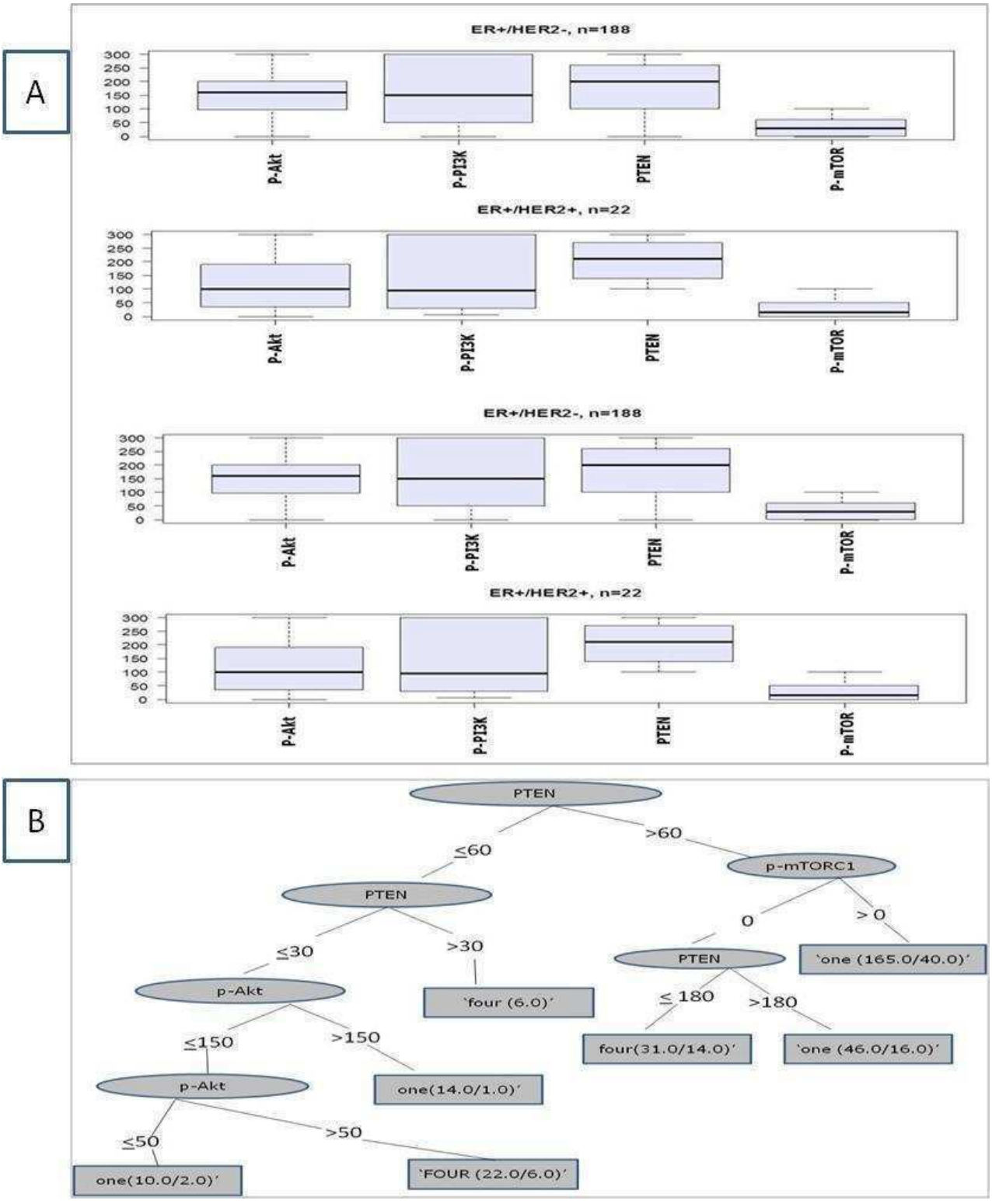


Figure 7-2: Box plots for PI3K members (H-score), B: Decision tree algorithm for predicting class membership in PI3K members. The circle on the top represents the main node. Other circles represent the markers in the algorithm and Rectangles represent feature value tested (class membership); and numbers between brackets in the rectangles represent subsets of patients correctly classified and misclassified from left to right respectively. Branches emerging from each marker are levels (H-score or percentage) of expression below or above which a specific case is to be classified into class one (ER+HER2-) or four (ER-HER2-) in this algorithm.

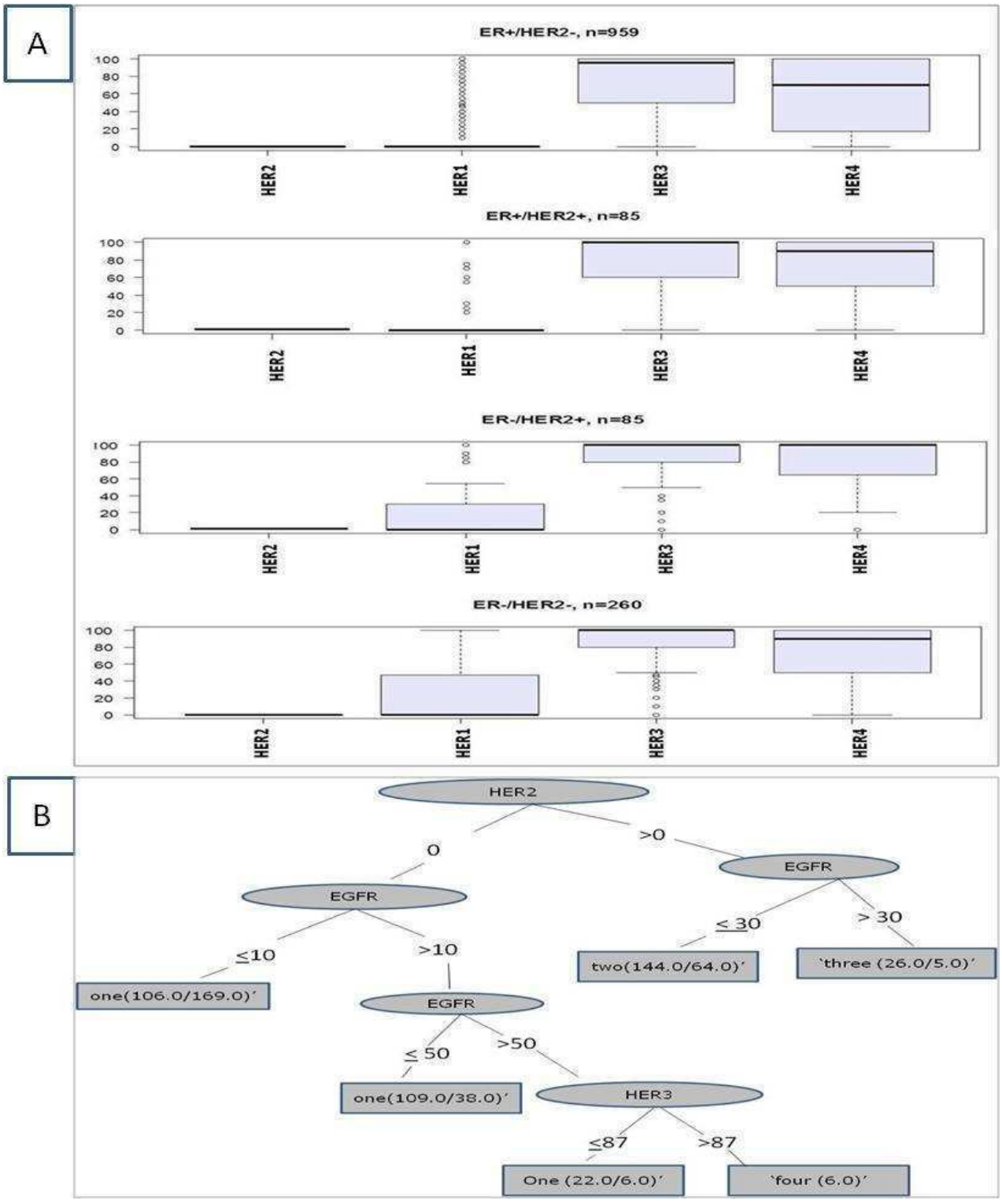


Figure 7-3: Box plots for HER family members (% for all apart from HER2: 0&1), B: Decision tree algorithm for predicting class membership in HER family members. The circle on the top represents the main node. Other circles represent the markers in the algorithm and Rectangles represent feature value tested (class membership); numbers between brackets in the rectangles represent subsets of patients correctly classified and misclassified from left to right respectively. Branches emerging from each marker are levels of expression below or above which a specific case is to be classified into class one (ER+HER2-), class two (ER+HER2+), class three (ER-HER2+) and class four (ER-HER2-) in this algorithm

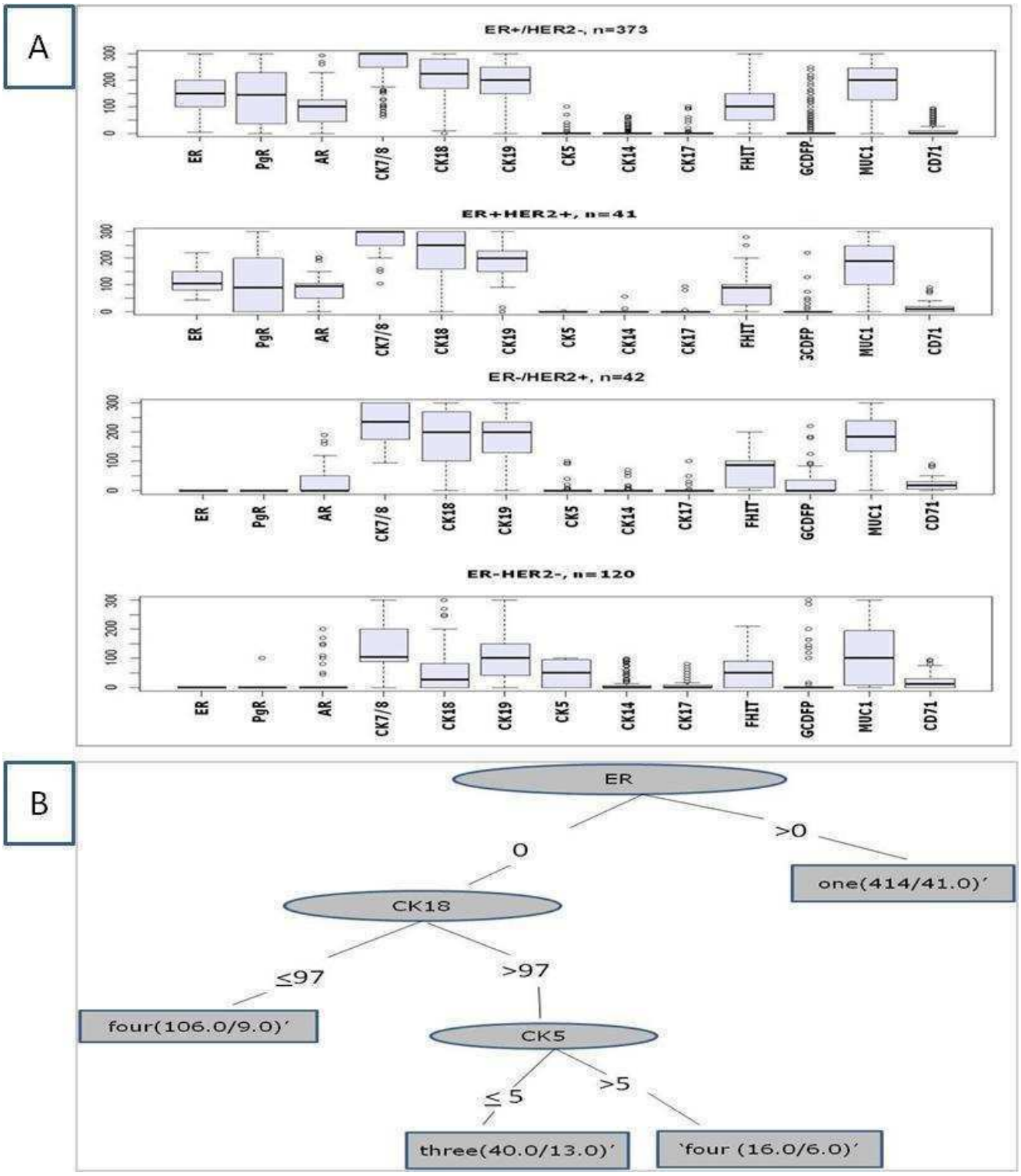


Figure 7-4: Box plots for ER, related proteins and basal cytokeratins, B: Decision tree algorithm for predicting class membership in ER and related proteins. The circle on the top represents the main node. Other circles represent the markers in the algorithm and Rectangles represent feature value tested (class membership); and numbers between brackets in the rectangles represent subsets of patients correctly classified and misclassified from left to right respectively. Branches emerging from each marker are levels of expression below or above which a specific case is to be classified into class one (ER+HER2-), class three (ER-HER2+) and class four (ER-HER2-) in this algorithm

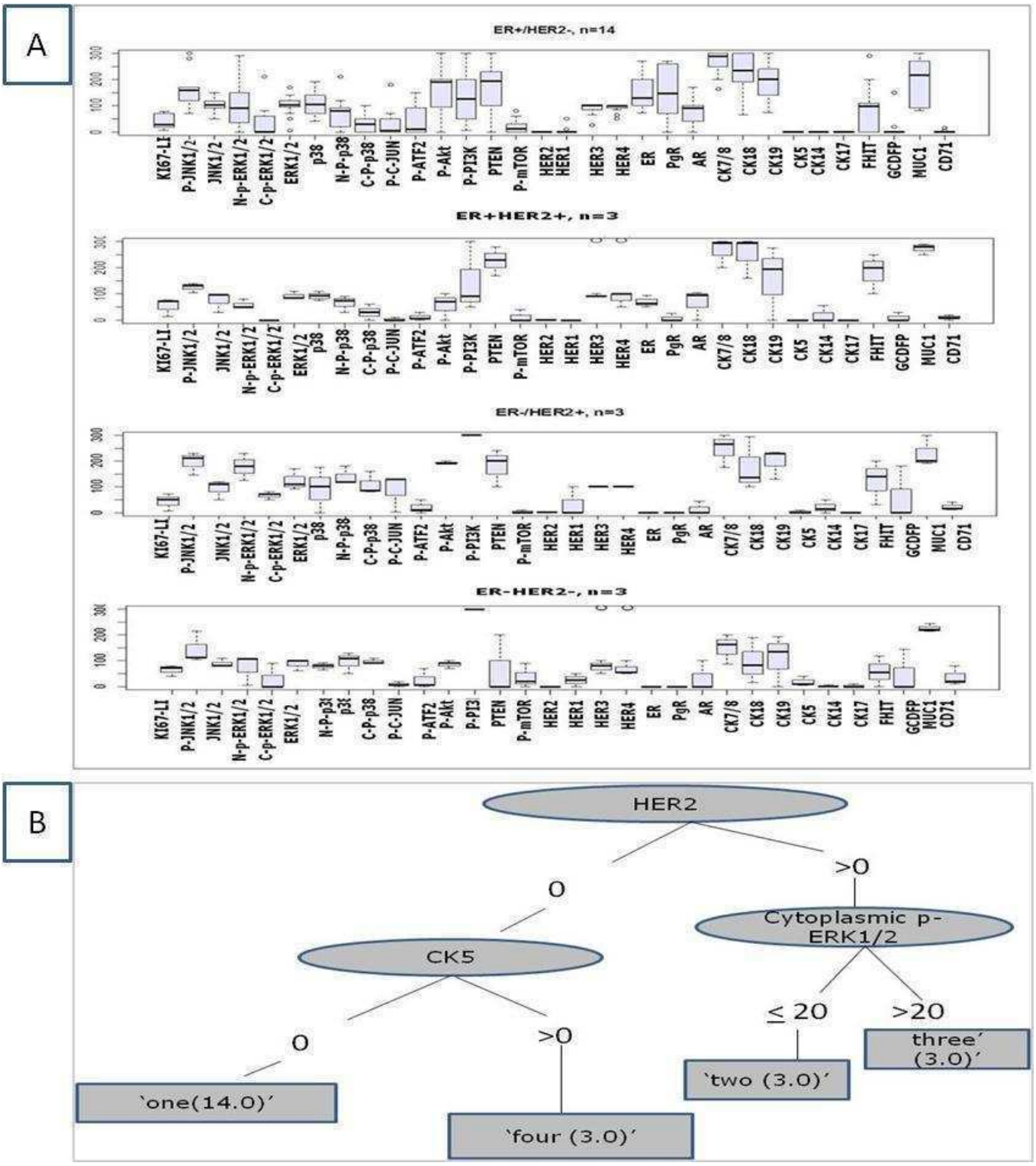


Figure 7-5: Box plots for all markers used, B: Decision tree algorithm for predicting class membership in all markers tested in IHC. The circle on the top represents the main node. Other circles represent the markers in the algorithm and Rectangles represent feature value tested (class membership); and numbers between brackets in the rectangles represent subsets of patients correctly classified and misclassified from left to right respectively. Branches emerging from each marker are levels of expression below or above which a specific case is to be classified into class one (ER+HER2-), class two (ER+HER2+), class three (ER-HER2+) and class four (ER-HER2-) in this algorithm

7.4 Discussion

Building a decision tree algorithm seems to be useful mean that can substitute some conventional statistical methods and to facilitate categorising groups of patients into their proper classes for future personalised therapy. It would be a common sense that feeding the system with data is not a heavy duty; nevertheless, sufficient knowledge on how this system is analysing the data and interpretation of them seems to be of great importance to achieve the maximum benefit. Moreover, the simplicity of interpretation of results in terms of clinical or other relevant patient characteristics makes decision tree a desired approach in clinical and epidemiologic studies (Barnholtz-Sloan et al., 2011).
Breast cancer is a heterogeneous disease and one of the challenges for the clinicians is that not all tumours behave the same, for this reason, designing these decision trees for different subgroups based on different surrogate biomarkers could help for more understanding of the complexity of this disease and perhaps aids in future treatment plans (Barnholtz-Sloan et al., 2011). In this study, the analysis of the cases within smart software to build these trees was based on IHC data. MAPKs were considered as one group and high expression of phosphorylated MAPKs allocated cases within class one and this finding coincide with our previous IHC results and RPPA in which we indicated that MAPKs are associated with ER+HER2- group (one) as also indicated by other studies that reported associations of MAPKs with ER (Milde-Langosch et al., 2005, Bhoumik and Ronai, 2008). Moreover, in this algorithm, we highlighted the importance of phosphorylated MAPKs rather than the non phosphorylated ones as p-ATF2, p-JNK1/2 and p-p38 were predominant in the tree, in particular p-ERK1/2 which constituted the main node.

Unlike IHC and RPPA, this algorithm did not predict the association of MAPKs (when were considered as a single group) within HER2+ group with or without ER expression and we think that this could be the effect of these MAPKs apart from ER or HER2 being integrated into this algorithm (apart from being part of the outcome groups). In addition, the limited number of cases could affect the prediction ability of this supervised analysis. Nevertheless, cytoplasmic form of p-ERK1/2 was part of the decision tree model when all biomarkers when considered together.

When PI3 pathway associated proteins were considered, PTEN was in the main node which went in the direction that high p-mTOR is associated with ER+HER2- group (one). This observation was one of our findings in IHC and this was consistent with our published work in this regard (Jerjees et al., 2015b) and emphasised the positive association between ER and mTORC1 (Shrivastav et al., 2014). In this decision tree and similar to MAPKs, this algorithm predict cases within groups one and four only (ER+HER2- and ER-HER2-, respectively).

With regard to ER and HER2 proteins when were analysed separately, each one was shown in the main node, however, when all biomarkers were analysed together, HER2 has been positioned at the top of the tree rather than ER. This is an important area related to our study as we are trying to figure out the biological significance of HER2 when co-expressed with ER and to determine the overwhelming driving factor. Despite of relatively small number of cases in this decision tree, HER2 appears to be the potential driving biomarker amongst all other biomarkers considered. Parallel with that, studies indicated that HER2 gene amplification and protein overexpression occurs as a second oncogenic hit that could affect the molecular behavior of BC (Malehi, 2014, Jerjees et al., 2014b). In this algorithm, HER2 could predict four classes and we did not see any influence of KI67-LI as in GEP (Hugh et al., 2009a) and in this context we expect that this sort of supervised analysis could have impact on the resultant algorithm and additionally using all biomarkers in one decision tree decreases the number of cases used for this algorithm.

Moreover, in the same decision tree, we noticed that high cytoplasmic p-ERK1/2 expression was associated with categorisation of cases within class membership number three which had worse outcome. Not surprisingly, the latter finding was noticed in the univariate survival analysis of subcellular localisation of p-ERK1/2 and this also has been shown in a our previous published work (Jerjees et al., 2014a). However, the results of this algorithm should be taken in caution since the number of cases is small.

Of worth, for the best of our knowledge we show new findings in building algorithms from some surrogate proteins mainly involving MAPKs and PI3K pathway proteins and even when ER, HER2 and some associated proteins were included together. Some models have been used by others and have been shown useful in predicting BC classes (Malehi, 2014) and in predicting survival in BC with an accuracy of the decision tree used was 90% (Chao et al., 2014). In

the same context, to emphasise the role of HER2 as a poor prognostic marker, it has been shown to be a driver of metastatic potential in BC patients on a study conducted by our group using the same cohort to predict distant metastasis decision tree algorithm (Aleskandarany et al., 2015).

Interestingly, in this study we highlighted that models adapted from this smart system could be applicable clinically in the future. The decision tree models illustrated in our study can function as a pruned biomarker panel that is useful to stratify patients according to certain criteria. For instance, to categorise patients according to ER and HER2 expressions to understand the interaction between these two key proteins as this is the aim of our study, we employed different biomarkers separately denoting the function of specific signalling pathways and only specific biomarkers of each pathway proved useful for segregation and this is useful to allocate only important biomarkers for patients assignment under certain group and ignoring others of less importance. Collectively, the surrogate biomarkers were implemented together, few of them proved essential for proper assignment of patients under one of the four groups based on ER and HER2 expressions. The final model can be used clinically as a formula to segregate patients which is cost effective where only few biomarkers will be used. Prospective validation of the current models seems beneficial as if similar results observed, this will improve the validity of such models and will probably hasten their incorporation as clinical diagnostic tools.

In conclusion, decision tree algorithm is a simple and useful model used for stratification of BC cases based on using different biomarkers' data. By applying this algorithm, we showed some concordance with our results in IHC and RPPA and this model also highlighted the role of HER2 in BC classification. In spite of that, the limitation of this approach is that in each analysed group, minimum number of missing data should be encountered and this could decrease the number of cases used in the algorithm.

In the future, selection of a panel of biomarkers with sufficient number of cases with minimum missing values will perhaps produce best models with improved precision.

8 General Discussion

8.1 Background

Despite the major recent advances in understanding the molecular diversity of BC, several issues related to the heterogeneity of BC remain to be determined including 1) the key underpinning drivers for different BC molecular subtypes, and 2) reliable surrogate biomarkers and advanced bioinformatics that can efficiently help understand the molecular portrait of BC subtypes especially with regards to HER2+ BC and how these groups with concomitant ER expression or loss can affect the signalling of different proteins related to these two key proteins (Eccles et al., 2013).

It is widely accepted that the engagement between histopathological classification, assessment of ER and HER2 status remains the mainstay of BC management. Since HER2 and ER are important pathways involved in BC development and it is well known that HER2 is an oncogenic factor that is responsible for driving adverse BC prognosis (Bjornsti and Houghton, 2004, Yarden and Sliwkowski, 2001a), it is essential to address the biological significance of ER+HER2+ as an aggressive type of luminal BC requiring both anti-HER2 and hormonal therapy (Cui et al., 2003) .

Different pathways have been recognised to have interactions with HER2 and ER including MAPKs, PI3K/Akt/mTOR pathways and proliferation (Feigin and Muthuswamy, 2009). Evidence whether HER2 or ER can overwhelm the driving effect on different proteins especially when both are co-expressed and the potential effect on therapeutic response is insufficient and needs further investigation. It is widely accepted that HER2+ BC is an aggressive subtype and it was hypothesised that ER+HER2+ BC is a distinct biological group that differs from ER+HER2-, ER-HER2+.

8.2 Hypothesis, aims and methods of the study

A set of aims were put forward to explore the hypothesis that HER2+ ER+ is a molecularly distinct entity when compared to HER+ ER- and ER+ HER2 – BC and to clarify the interactions between HER2 and ER to explore further the driving effect of HER2 and how it can influence the unfavourable behaviour of BC. For this purpose, many proteins of pathways known to be downstream effectors of HER2 or associated with it or with ER were studied. These included: 1) MAPKs,

2) PI3K/Akt/mTOR/ members, 3) Proliferation and 4) Other proteins related to ER and HER2 pathways. The selection of the panel of proteins was based on literature reviews (*in vivo* and *in vitro* findings) that reported clear associations with HER2 and ER. The expression of the investigated biomarkers' panel was studied with relevance to their biological significance in BC in association with different proteins related to ER and HER2 pathways and key BC proteins (Abd El-Rehim et al., 2005, Aleskandarany et al., 2010c) in 4 main subgroups: ER+HER2+, ER+HER2-, ER-HER2+ and double negative to assess alterations in the expression of these proteins which might help reveal distinct patterns. Moreover, the correlation with patient outcome was also considered in BC and the 4 BC subgroups.

For this purpose, integrated immunohistochemical staining was used to test the expression of the panel of proteins in two BC series: Nottingham primary BC series and a Trastuzumab treated one. RPPA was also employed to quantify the expression of these proteins in 6 BC cell lines as well. Finally, clustering analysis was performed to obtain a biomarker model to assist in BC classification.

8.3 Cardinal findings of the study

8.3.1 MAPKs expression in breast cancer: Members of this pathway act as tumour suppressor proteins or oncogenes according to cellular context

The expression of MAPKs (pan and p-ERK1/2, p-JNK1/2, pan and p-p38, p-ATF2 and p-C-JUN) revealed that these proteins are associated with good prognostic variables and longer survival in the unselected cohort and within ER+ tumours. Moreover, some MAPKs were negatively associated with HER2 in unselected BC and within ER+ tumours (p-ERK1/2, p-p38 and p-ATF2), (Jerjees et al., 2014a). The co-expression of HER2 with ER seems to influence negatively the relationships of these MAPKS with favourable prognostic variables despite of preservation of some features. Importantly, when ER was lost, HER2 was associated with higher expression of some MAPKs. From a clinical perspective, this appears to indicate that MAPKs could behave as tumour suppressors or promoters based on the surrounding context and in HER2+ tumours in particular, ER-HER2+, they could have therapeutic potential. Further

investigating of such pathways to be of value and give insight into the related upstream activators and downstream effectors some of which have been thoroughly investigated in this study. Previous studies have conflicting findings regarding MAPKs (Merlin et al., 2013, Huang et al., 2013, Kuo et al., 2013) as they could not address their actual function either due to type of cohort used, small cohort sizes, and techniques for assessment or due to other factors. Additionally, we addressed that total forms appears to be less active than the phosphorylated forms due to the location of both and that the latter are better to be considered for assessing the function of these proteins evidenced by several associations with clinical variables, outcome and even when decision tree models were built for MAPK pathway members. Indeed, a study has highlighted a difference between total and phosphorylated forms of p-ATF2, were the total was associated with short survival while the phosphorylated one revealed associations with highly differentiated tumours (Knippen et al., 2009). Actually, there were some differences observed while assessing the associations between HER2+ tumours in the primary series and Trastuzumab treated one and this is presumably due to some clinical differences between both cohorts including grade and stage variables. An interesting point regarding outcome is that although MAPKs were associated with preferable outcome in ER+ group, only p-ATF2 showed prolonged survival within double positive group, indicating the effect of ER and implying the difference between the double positive group from ER-HER2+ one supporting the aim of our hypothesis. In this thesis, the combined power of IHC with protein array analytic capability provided validation of the findings regarding the differential expressions of MAPKs with individual ER and HER2 patterns of expression or co-expression (Bertone and Snyder, 2005, MacBeath, 2002) and ER+HER2+ tumours appear to be a different entity from other subtypes of BC based on ER and HER2 expression evidenced by differential expression of MAPKs in these different BC subgroups.

8.3.2 mTORC1 and other members of PI3K/Akt pathway: Differential expression of these driving factors for progression in relation to HER2 and ER expression

It is widely accepted that PI3K/Akt/mTOR pathway is an important driving mechanism that could enhance BC develoment (Nagata et al., 2004b). Different

studies have investigated the role of this pathway in BC but there remains a lack of knowledge on how these factors can act within the HER2 and ER related subgroups. Additionally, some studies have also indicated that excessive signalling of p-mTORC1 is associated with activation of cellular senescence and apoptosis which are considered as fail safe checkpoints (Ito et al., 2009, Lee et al., 2010). This current study highlighted the bidirectional role of mTORC1 in ER+/- BC. Although mTORC1 revealed positive associations with good clinicopathological variables and markers of good prognosis and was shown to be negatively associated with HER2 in ER+ subset, loss of some of these associations with HER2 co-expression denotes a difference between these subtypes and this was also observed in RPPA where the expression of mTORC1 was decreased in ER+HER2+ vs ER+HER2-. Meanwhile, the increased expression of mTORC1 relevant to HER2 within ER- cohort (also observed in RPPA) could imply a significant biological contribution in BC development in this specific context (Jerjees et al., 2015a). Therefore, these findings imply that mTORC1 could be a therapeutic target in ER- HER2+ BC. Importantly, the favourable action of p-mTORC1 in BC could be attributed to its negative feedback on Akt. Probably, HER2 could affect this route when it is expressed especially after ER loss and could further enhance the action downstream of this pathway. The associations of p-mTORC1 with clinical variables and biological biomarkers within PI3K+/- and Akt+/- cohorts indicated favourable associations although were more significant within negative cohorts. Of worth mentioning that p-mTORC1 could reflect complexity in its action especially in relation with outcome as this protein did not reveal any association with outcome in BC subgroups based on ER and HER2 instead, it revealed prolonged association within Akt+ cohort which indicated favourable behaviour in relation to members of PI3K/Akt pathway. PTEN showed similar expression pattern to mTOR but PI3K showed positive correlations with HER2 in ER+/- cohorts. Regarding RPPA, the results indicated that PI3K, Akt and p-S6K showed increased expression in the double positive group vs ER-HER2+ one being all potential therapeutic targets that could be considered and these results can be further validated for future possible use in clinical trials and if deemed of benefit could then be used for patients' management. Importantly, the finding that the proteins in this pathway showed variable expression within HER2+/ER (+/-) groups indicates their variable biological status.

8.3.3 The biological and prognostic significance of HER2 and proliferation in ER+ breast cancer: ER+HER2+ is a distinct aggressive subset

The difference in the biological and prognostic significance of HER2 and proliferation in ER+ BC was considered in this study. Despite the fact that some studies acknowledged that HER2+ BC can be placed in the luminal category if they express ER (Badve, et al. 2007b; Matos, et al. 2005; Carey, et al. 2006), its association with worst outcome compared to high KI67-LI favours considering ER+HER2+ BC as a distinct luminal subgroup that shares some biological features with ER+ HER2- tumours (Jerjees, et al. 2014b). KI67-LI revealed features that reflected biological aggressive behaviur evidenced by its association with an advanced stage, larger tumour size and definite LVI vs HER2. Moreover, the association of KI67-LI with decreased expression of luminal cytokertains rather than HER2 implies its inverse association with differentiation. In this thesis, the data generated supports the view that HER2 overexpression is an oncogenic event that can drive the unfavourable molecular portrait of BC independently from ER underpinning the relevance of anti HER2 therapy in this group.

8.3.4 Other biomarkers related to ER and HER2 pathways:

Nuclear CHIP protein in this study was found to be associated with favourable prognostic variables in particular ER and related proteins but contrary to its cytoplasmic form, it was negatively associated with HER2. Patani et al (Patani et al., 2010) has also indicated that CHIP is associated with good prognostic parameters. Using IHC, gives an advantage that subcellular localisation assessment is feasable and regarding CHIP, it was associated with prolonged survival in the whole cohort and in ER+ rather than HER2+ group but sbubcellular localisation has indicated that cytoplasmic only expression, was associated with poor outcome. It appears that studying the biological significance of biomarkers involved in cancer process with consideration of its subcellular localisation is of value as ignoring one of them could falsly reveal diffenet associations. Some associations of CHIP with good prognostic variables were lost when the analysis was restricted to ER+HER2+ group but few associations were observed with favourable variables within ER-HER2+ groups

and double negative and no associations with prolonged survival were noticed with HER2+ BC groups. Such variable associations imply that these subgroups are not similar and each harbours its own biological characteristics reflected on the lack of similarity of associations within these subgroups. Limitted studies have addressed the biological significance of CHIP and future functional studies could explore more aspects of the function of this protein.

Stem cell status of a tumour is an evolving issue and is a matter of investigations to understand the landscape of the tumours and their heterogenity (Chakravarty et al., 2011). In this study, SOX9, a stem cell marker, exhibited both nuclear and cytoplasmic forms which displayed associations with unfavourable parameters including higher tumour grade, higher NPI scores; however, nuclear expression alone displayed negative association with HER2 positivity. Despite the latter association, this does not necessarly denote a favourable function of the nuclear form especially it was associated with a trend for decreased expression of E-Cadherin and increased expression of P-Cadherin which might imply features of stem cell state. Very few associations for both nuclear and cytoplasmic forms were observed within 4 subgroup analysis of BC. Further understanding of the function of this protein is warranted and functional studies could help reveal its relation to ER and HER2 in cell lines based on expression of these two key bimarkers. In addition, investigating other related biomarker as Slug which is has also been determined to have stem cell state features and by correlating its association with SOX9, this could help further understanding of the function of stem cell features and their impact on BC especially in determining the differences between subgroups base on ER and HER2.

With respect to bimarkers that were investigated in the Trastuzumab treated series, SRC3 and ECD, both have indicated associations with aggressive clinicopathological variables and importatnly positive associations with HER2 dimers. Although they did not reveal associations with outcome owing probably to the limitted number of cases in this cohort; nevertheless, the available findings imply their possible role in cancer progression and the potential collaboration with HER2 in enhancing their function. SRC3 is a coregulator involved directly with enhancing the transcriptional activity of ER with the fact that it is a downstream target for HER2 (Graham et al., 2000) is a step towards

giving more attension to the fuction of this protein to help reveal interaction between ER and HER2 especially if studied in more than one cohort. Additionally, ECD being a novel cell cycle regulator has been shown to be involved in mammary carcinoma using functional studies with cell lines (Bele et al., 2015). This protein merits further assessment using larger cohort of patients receiving Trastuzumab to help indicate its association with outcome and the possibility of being a therapeutic goal.

SER 118 ER is a form of ER that is activated in a ligand independent manner (Lannigan, 2003). This biomarker has been investigated with relevance to ER and HER2 to determine its biological significance with variable expression of HER2 and ER. Moreover, the associations of SER 118 ER with other biomarkers in the 4 BC subgroups indicated variability and emphasised the difference between ER+HER2+ with single expression groups with preservation of some favourable features reflected the influence of ER and HER2. The findings in this thesis have indicated that SER 118 ER is good prognostic marker in the unselected BC and in the ER+ tumours but no associations with the outcome were observed in the HER2+/ER (+/-) group. Furthermore, the subcellular localisation of SER 118 ER has added more to our understanding of the function of this protein as the cytoplasmic only expression was associated with the worst survival. In fact, there were different results reported with respect to SER 118 ER (Sarwar et al., 2006, Zoubir et al., 2008) but it seems that phosphorylation of ER at SER 118 ER is required by tamoxifen to maintain its function (Cheng et al., 2007) and this is attributed to the increased levels of co-repressors vs co-activators and consistently, we demonstrated prolonged survival with high nuclear SER 118 ER in those taking tamoxifen therapy. Within the double positive group there was only an association between high SER 118 ER and higher tumour grade and even no associations were observed within Trastuzumab treated one implying an effect of HER2 which could be due to enhancing co-activators and even affecting the function of tamoxifen which will act as an agonist when HER2 is expressed (Ellis et al., 2001). Clinically, testing this biomarkers in BC has showed that it is a predictor of prolonged survival in those under tamoxifen therapy and this was in agreement with another study that reported the same result (Kok et al., 2009b).

8.3.5 Computational biology and breast cancer classification: Building a potential future model

Supervised analysis of a panel of proteins including HER2 and related proteins, ER and related proteins, basal cytokeratins, MAPKS, PI3K/Akt/mTORC1 members and proliferation allowed designing a range of decision trees based on individual or combined consideration of these groups. Setting the outcome variable based on single positive, single negative, double positive and double negative HER2 and ER expresions has influenced the distribution of these trees. These algorithms appear potentially useful in clinical practice as they can provide simple model for disease classification for certatin disease (Bendi, Prasad, and Venkateswarlu 2011) or stratifying patients whether they are liable for distant metastasis or not (Aleskandarany et al., 2015) or for other purposes.

Further support to the main hypothesis, 3 main biomarker panel have been identified; one for ER and related proteins and the other for HER2 and other members of HER family where both HER2 and ER have been considered in the main node of related decision tree. Concomitant consideration of both pathways together showed that HER2 was present in the main node. Thus, providing further demonstration that HER2 is a strong driving factor that enhances BC progression. Interestingly and with the consideration of MAPK decision tree, it denoted the importance of the phosphorylated forms evidenced by their involvement rather than the total forms. Cost effectively, possible future consideration of the decision tree involving the whole biomarkers together could provide a simple model with the need for only few biomarkers for assigning patients into groups based on ER and HER2 with the possible improvement of this model and perhaps implementaion of different biomarkers for this purpose.

8.4 Final Conclusions

Variable pathways including HER2, ER, MAPKs, PI3K/Akt/mTOR, proliferation, proapoptotic molecules and others can have differing impacts on BC. The differential expression of MAPKs, PI3K/Akt/mTORC1 members , CHIP and SER 118 ER has indicated the biological differences of ER+HER2+ BC from other groups with variable ER and HER2 expressions. Therefore, ER+HER2+ BC is considered as a distinct biological entity which might have future therapeutic

considerations. The use of IHC was easy, cost effective and is applicable in routine clinical practice, with further validation of the results by RPPA.

8.5 Potential strength points in the study

1- Using a large cohort of BC with a lot of biomarker data available relevant to BC allowed variable detailed comparisons with the studied biomarkers.

2- Using positive and negative controls with each IHC run were useful to ensure genuine staining.

3- Using WB to detect the specificity of the investigated proteins.

4- Validation of qualitative IHC results by a high throughput RPPA which showed concordance with IHC.

8.6 Limitations of the study

1- The breast cancer series used in this study is a historic, retrospective one and no prospective cases were included.
2- The data were only assessed in one centre and no multicentre validation was considered.
3- The semiquantitative IHC method was used which can be influenced by the subjectivity of scorers despite standard approaches were followed to minimise these limitations (Oyama et al., 2007).
4- Most of the biomarkers studied showed either nuclear or cytoplasmic expression; however, this does not exclude the possibility that these biomarkers are expressed in other parts of the malignant cells. Therefore, future studies can highlight the importance of subcellular localisation of these proteins in addition to the role of the expression in the tumour surrounding areas or the tumour stromal tissues.
5- Cell lines were used in RPPA instead of the tissues used to test the biomarkers in IHC.
6- The limited number of cases available in Trastuzumab treated series compared to the primary series.
7- The effectiveness and the reliability of using TMA on a large scale are acknowledged by different studies even with high concordance rate with

full face tissue (Camp et al., 2000, Lee and Kim, 2006). However, it is worth using 3 cores per case for better assessment of these proteins.

8.7 Future work

1- Assessment of prospective cases of BC in addition to the current series would be of value.
2- Multicentre assessment of the BC cases will be more robust as this will give the opportunity to rely on the obtained results if deemed parallel with the possibility for their clinical application.
3- The results for the expression of different proteins were assessed using IHC and RPPA using different BC cell lines. Proteins could be extracted using the same tissue tested by IHC and further evaluated by RPPA. This can create a robust environment for comparison and validation of findings

4- To highlight the role of subcellular localisation, measuring different cellular components separately, known as subcellular fractionation, is warranted. For this purpose, Thermo Scientific Subcellular Protein Fractionation Kit could be used in tissue samples and later on each extract (nuclear, cytoplasmic, membranous or others) can be tested by WB.

5- Investigating other downstream signalling proteins of PI3K/Akt pathway as 4E-BP1, elF-4E and S6K (using IHC) is of worth since emerging evidence has indicated their possible role in BC development.

6- Using three cores per case in the TMA could be beneficial.

7- Expansion of the Trastuzumab treated series to support better evaluation especially in terms of treatment related outcome.

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10 Appendix

Appendix Table 1: Association of MAPKs with each other in ER+HER2-

			p-JNK1/2	JNK1/2	N-p-ERK1/2	C-p-ERK1/2	ERK1/2	p38	N-p_p38	p-c-jun	p_ATF2
Spearman's rho	***1p-JNK1/2**	Correlation Coefficient	1.000	.033	.585**	.351**	.036	.078	.420**	.246**	.360**
		Sig. (2-tailed)	.	.505	.000	.000	.421	.094	.000	.000	.000
		N	666	398	582	577	506	463	541	517	530
	JNK1/2	Correlation Coefficient	.033	1.000	.120*	.181**	.192**	.066	.083	.128**	.085
		Sig. (2-tailed)	.505	.	.012	.000	.000	.151	.063	.005	.060
		*N	398	693	440	433	468	471	505	485	487
	***2N-p-ERK1/2**	Correlation Coefficient	.585**	.120*	1.000	.681**	.183**	.216**	.527**	.396**	.472**
		Sig. (2-tailed)	.000	.012	.	.000	.000	.000	.000	.000	.000
		N	582	440	755	746	574	531	622	595	607
	***3C-p-ERK1/2**	Correlation Coefficient	.351**	.181**	.681**	1.000	.209**	.192**	.442**	.394**	.350**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000	.000	.000	.000
		N	577	433	746	746	566	523	618	590	603
	ERK1/2	Correlation Coefficient	.036	.192**	.183**	.209**	1.000	.272**	.143**	.187**	.182**
		Sig. (2-tailed)	.421	.000	.000	.000	.	.000	.000	.000	.000
		N	506	468	574	566	784	608	646	627	637
	p38	Correlation Coefficient	.078	.066	.216**	.192**	.272**	1.000	.085*	.206**	.136**
		Sig. (2-tailed)	.094	.151	.000	.000	.000	.	.037	.000	.001
		N	463	471	531	523	608	757	598	573	594
	N-p-p38	Correlation Coefficient	.420**	.083	.527**	.442**	.143**	.085*	1.000	.441**	.522**
		Sig. (2-tailed)	.000	.063	.000	.000	.000	.037	.	.000	.000
		N	541	505	622	618	646	598	874	796	804
	p-c-jun	Correlation Coefficient	.246**	.128**	.396**	.394**	.187**	.206**	.441**	1.000	.428**
		Sig. (2-tailed)	.000	.005	.000	.000	.000	.000	.000	.	.000
		N	517	485	595	590	627	573	796	834	766
	p-ATF2	Correlation Coefficient	.360**	.085	.472**	.350**	.182**	.136**	.522**	.428**	1.000
		Sig. (2-tailed)	.000	.060	.000	.000	.000	.001	.000	.000	.
		N	530	487	607	603	637	594	804	766	848

*: number of cases, (*1) is phosphorylated, (*2) is nuclear, (*3) is cytoplasmic, (*) is the correlation which is significant at the 0.05 level (2-tailed)and (**) is the correlation coefficient

Appendix Table 2: Association of MAPKs with each other in ER+HER2+

			p-JNK1/2	JNK1/2	N-p-ERK1/2	C-p-ERK1/2	ERK1/2	p38	N-p_p38	p-c-jun	p_ATF2
Spearman's rho	*1p-JNK1/2	Correlation Coefficient	1.000	.483**	.586**	.452**	.147	.219	.513**	.330*	.264*
		Sig. (2-tailed)	.	.001	.000	.000	.266	.134	.000	.010	.040
		N	74	46	69	68	59	48	63	60	61
	JNK1/2	Correlation Coefficient	.483**	1.000	.275	.211	.325*	.222	.409**	.542**	.257
		Sig. (2-tailed)	.001	.	.051	.142	.016	.126	.001	.000	.060
		N	46	69	51	50	55	49	59	58	54
	*2N-p-ERK1/2	Correlation Coefficient	.586**	.275	1.000	.691**	.060	.228	.526**	.463**	.515**
		Sig. (2-tailed)	.000	.051	.	.000	.639	.094	.000	.000	.000
		N	69	51	81	80	64	55	72	70	71
	*3C-p-ERK1/2	Correlation Coefficient	.452**	.211	.691**	1.000	-.003	.235	.313**	.338**	.324**
		Sig. (2-tailed)	.000	.142	.000	.	.984	.087	.008	.005	.006
		N	68	50	80	80	63	54	71	69	70
	ERK1/2	Correlation Coefficient	.147	.325*	.060	-.003	1.000	.472**	.266*	.306*	.192
		Sig. (2-tailed)	.266	.016	.639	.984	.	.000	.028	.011	.122
		N	59	55	64	63	83	66	68	68	66
	p38	Correlation Coefficient	.219	.222	.228	.235	.472**	1.000	.236	.318*	.311*
		Sig. (2-tailed)	.134	.126	.094	.087	.000	.	.067	.014	.018
		N	48	49	55	54	66	71	61	59	58
	N-p-p38	Correlation Coefficient	.513**	.409**	.526**	.313**	.266*	.236	1.000	.471**	.540**
		Sig. (2-tailed)	.000	.001	.000	.008	.028	.067	.	.000	.000
		N	63	59	72	71	68	61	93	87	85
	p-c-jun	Correlation Coefficient	.330*	.542**	.463**	.338**	.306*	.318*	.471**	1.000	.545**
		Sig. (2-tailed)	.010	.000	.000	.005	.011	.014	.000	.	.000
		N	60	58	70	69	68	59	87	91	80
	p-ATF2	Correlation Coefficient	.264*	.257	.515**	.324**	.192	.311*	.540**	.545**	1.000
		Sig. (2-tailed)	.040	.060	.000	.006	.122	.018	.000	.000	.
		N	61	54	71	70	66	58	85	80	88

*: number of cases, (*1) is phosphorylated, (*2) is nuclear, (*3) is cytoplasmic, (*) is the correlation which is significant at the 0.05 level (2-tailed)and (**) is the correlation coefficient

Appendix Table 3: Association of MAPKs with each other in ER-HER2+

			p-JNK1/2	JNK1/2	N-p-ERK1/2	C-p-ERK1/2	ERK1/2	p38	N-p_p38	p-c-jun	p_ATF2
Spearman's rho	*1p-JNK1/2	Correlation Coefficient	1.000	-.076	.252	.491**	.440**	.233	.208	.359*	.183
		Sig. (2-tailed)	.	.647	.069	.000	.006	.107	.192	.010	.214
		N	65	39	53	53	38	49	41	50	48
	JNK1/2	Correlation Coefficient	-.076	1.000	-.230	.109	.318*	.368*	.072	.407**	.268
		Sig. (2-tailed)	.647	.	.138	.486	.048	.012	.642	.007	.087
		N	39	67	43	43	39	46	44	42	42
	*2N-p-ERK1/2	Correlation Coefficient	.252	-.230	1.000	.435**	.293	.198	-.173	.205	.225
		Sig. (2-tailed)	.069	.138	.	.000	.054	.148	.244	.133	.099
		N	53	43	69	69	44	55	47	55	55
	*3C-p-ERK1/2	Correlation Coefficient	.491**	.109	.435**	1.000	.137	.145	.046	.375**	.115
		Sig. (2-tailed)	.000	.486	.000	.	.377	.291	.759	.005	.403
		N	53	43	69	69	44	55	47	55	55
	ERK1/2	Correlation Coefficient	.440**	.318*	.293	.137	1.000	.262	.141	.365*	-.079
		Sig. (2-tailed)	.006	.048	.054	.377	.	.075	.335	.013	.608
		N	38	39	44	44	61	47	49	46	45
	p38	Correlation Coefficient	.233	.368*	.198	.145	.262	1.000	.121	.276*	.440**
		Sig. (2-tailed)	.107	.012	.148	.291	.075	.	.417	.020	.000
		N	49	46	55	55	47	76	47	71	69
	N-p-p38	Correlation Coefficient	.208	.072	-.173	.046	.141	.121	1.000	.085	-.059
		Sig. (2-tailed)	.192	.642	.244	.759	.335	.417	.	.567	.693
		N	41	44	47	47	49	47	68	48	47
	p-c-jun	Correlation Coefficient	.359*	.407**	.205	.375**	.365*	.276*	.085	1.000	.277*
		Sig. (2-tailed)	.010	.007	.133	.005	.013	.020	.567	.	.020
		N	50	42	55	55	46	71	48	74	70
	p-ATF2	Correlation Coefficient	.183	.268	.225	.115	-.079	.440**	-.059	.277*	1.000
		Sig. (2-tailed)	.214	.087	.099	.403	.608	.000	.693	.020	.
		N	48	42	55	55	45	69	47	70	74

*: number of cases, (*1) is phosphorylated, (*2) is nuclear, (*3) is cytoplasmic, (*) is the correlation which is significant at the 0.05 level (2-tailed)and (**) is the correlation coefficient

Appendix Table 4: The associations of MAPKs with clinicopathological variables within BC subgroups

	ER+HER2+			ERHER2+			ER-HER2-		
	Neg/low , N (%)	**High,N (%)**	**p-value**	**Neg/low , N (%)**	**High, N (%)**	**p-value**	**Neg/low , N (%)**	**High, N (%)**	**p-value**
ERK1/2									
Menopausal status			**0.100**			**0.627**			**0.368**
Pre- menopause	19(41)	22(59)	**(2.70)**	11(39)	15(46)	**(0.23)**	74(55)	33(49)	**(1.80)**
Post-menopause	27(59)	15(41)		17(61)	18(54)		60(45)	35(51)	
Tumour size			**0.539**			**0.047**			**0.554**
<2	21(46)	14(39)	**(0.37)**	9(32)	19(58)	**(3.94)**	43(32)	25(36)	**(0.35)**
>2	25(54)	22(61)		19(68)	14(42)		91(68)	44(64)	
Stage			**0.603**			**0.040**			**0.911**
1	24(52)	19(53)	**(1.01)**	13(46)	20(61)	**(6.41)**	79(59)	39(56)	**(0.18)**
2	15(33)	14(39)		8(29)	12(36)		37(28)	20(29)	
3	7(15)	3(8)		7(25)	1(3)		17(13)	10(15)	
Nuclear p-ERK1/2									
Age			**0.031**			**0.28**			**0.336**
≤ 50	19(40)	22(65)	**(4.65)**	13(29)	10(42)	**(1.15)**	64(51)	31(44)	**(0.92)**
>50	28(60)	12(35)		32(71)	14(58)		62(49)	40(56)	
Menopausal status			**0.009**			**0.28**			**0.124**
Pre- menopause	18(38)	23(68)	**(6.79)**	13(29)	10(42)	**(1.15)**	72(57)	32(46)	**(2.36)**
Post-menopause	29(62)	11(32)		32(71)	14(58)		54(43)	38(54)	
Tumour size			**0.133**			**0.050**			**0.118**
≤2	17(36)	18(53)	**(2.26)**	17(38)	15(62)	**(3.84)**	36(29)	28(39)	**(2.44)**
>2	30(64)	16(47)		28(62)	9(38)		90(71)	43(61)	
Cytoplasmic p-ERK1/2									
Age			**0.370**			**0.060**			**0.161**
< 50	17(45)	23(55)	**(0.80)**	7(22)	16(43)	**(3.52)**	54(52)	39(42)	**(1.96)**
>50	21(55)	19(45)		25(78)	21(57)		49(4)	53(58)	
Mitosis			**0.038**			**4.35**			**0.065**
1	4(11)	2(5)	**(2.00)**	3(9)	1(3)	**(1.66)**	1(1)	7(8)	**(5.47)**
2	5(13)	11(26)		7(22)	6(17)		9(9)	8(9)	
3	29(76)	29(69)		22(69)	28(80)		92(90)	76(83)	
NPI			**0.274**			**0.032**			**0.026**
GPG	2(6)	7(18)	**(2.59)**	4(13)	0(0)	**(6.89)**	2(2)	7(8)	**(7.27)**
MPG	24(68)	23(59)		18(56)	29(81)		66(66)	64(74)	
PPG	9(26)	9(23)		10(31)	7(19)		32(32)	16(18)	
p-JNK1/2									
Menopausal status			**0.028**			**0.352**			**0.709**
Pre- menopause	5(29)	34(60)	**(4.80)**	4(21)	15(33)	**(0.86)**	35(51)	70(54)	**(0.140)**
Post-menopause	12(71)	23(40)		15(79)	31(67)		33(49)	59(46)	
Stage			**0.302**			**0.033**			**0.114**
1	11(65)	25(45)	**(2.39)**	9(48)	30(65)	**(6.81)**	42(62)	77(60)	**(4.34)**
2	5(29)	22(39)		5(26)	14(31)		22(32)	32(25)	
3	1(6)	9(16)		5(26)	2(4)		4(6)	20(15)	
LVI			**0.151**			**0.024**			**0.171**
Negative	12(71)	29(51)	**(2.05)**	9(47)	35'(76)	**(5.07)**	48(71)	79(61)	**(1.87)**
Definite	5(29)	28(49)		10(53)	11(24)		20(29)	51(39)	
NPI			**0.486**			**0.049**			**0.526**
GPG	1(6)	7(14)	**(1.44)**	2(10)	2(5)	**(6.01)**	1(2)	6(5)	**(1.28)**
MPG	12(75)	30(59)		10(53)	37(82)		47(72)	87(69)	
PPG	3(19)	14(27)		7(37)	6(13)		17(26)	32(26)	

Appendix Table 5: The associations of MAPKs with clinicopathological variables within BC subgroups

	ER+HER2+			ER-HER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
Pan p38									
Age			**0.040**			**0.502**			**0.299**
≤ 50	1(31)	20(56)	**(4.20)**	17(37)	6(29)	**(0.45)**	71(46)	29(54)	**(1.07)**
>50	24(69)	16(44)		29(63)	15(71)		85(54)	25(46)	
Menopausal status			**0.012**			**0.826**			**0.289**
Pre- menopause	11(31)	22(61)	**(6.28)**	18(39)	8(36)	**(0.04)**	77(49)	31(57)	**(1.12)**
Post-menopause	24(69)	14(39)		28(61)	14(64)		80(51)	23(43)	
Pleomorphism			**0.032**			**0.763**			**0.166**
1	8(24)	2(6)	**(4.61)**	3(7)	1(5)	**(0.09)**	1(1)	0(0)	**(3.59)**
2	26(76)	34(94)		42(93)	20(95)		5(3)	5(9)	
3							148(96)	48(91)	
Stage			**0.003**			**0.280**			**0.766**
1	21(60)	15(42)	**(11.34)**	27(59)	12(54)	**(2.54)**	96(62)	34(63)	**(0.53)**
2	6(17)	19(53)		12(26)	9(41)		44(28)	13(24)	
3	8(23)	2(5)		7(15)	1(5)		16(10)	7(13)	
p-p38									
Tumour size			**0.941**			**0.068**			**0.067**
≤2	32(41)	6(40)	**(0.005)**	15(31)	14(52)	**(3.32)**	56(31)	19(46)	**(3.36)**
>2	46(59)	9(60)		34(69)	13(48)		123(69)	22(54)	
Pleomorphism			**0.843**			**0.761**			**0.004**
2	15(19)	3(21)	**(0.03)**	3(6)	2(8)	**(0.09)**	0(0)	1(2)	**(11.27)**
3	63(81)	11(79)		46(94)	23(92)		3(2)	4(10)	
							172(98)	36(88)	
p-ATF2									
Grade			**0.343**			**0.023**			**0.036**
I	1(1)	1(7)	**(2.14)**	1(2)	0(0)	**(7.52)**	2(1)	3(8)	**(6.63)**
2	17(23)	2(13)		3(5)	3(30)		10(6)	3(8)	
3	55(75)	12(80)		60(94)	7(70)		164(93)	32(84)	
Tubule			**0.856**			**0.107**			**0.001**
1	1(1)	0(0)	**(0.31)**	7(11)	3(30)	**(2.60)**	0(0)	3(8)	**(13.7)**
2	21(29)	5(33)		56(89)	7(70)		20(12)	4(10)	
3	51(70)	10(67)					152(88)	31(82)	
Pleomorphism			**0.961**			**0.076**			**0.002**
1	4(19)	3(20)	**(0.002)**	3(5)	2(20)	**(3.14)**	0(0)	2(5)	**(12.7)**
2	58(81)	12(80)		60(95)	8(80)		6(4)	4(11)	
3							166(96)	32(84)	
Mitosis			**0.977**			**0.088**			**0.009**
1	6(8)	1(7)	**(0.04)**	2(3)	0(0)	**(4.85)**	5(3)	4(10)	**(9.37)**
2	15(21)	3(20)		8(13)	4(40)		10(6)	6(16)	
3	52(71)	11(73)		53(84)	6(60)		157(91)	28(74)	
p-C-JUN									
Mitosis			**0.031**			**0.509**			**0.367**
1	5(16)	2(4)	**(6.94)**	2(7)	1(2)	**(1.35)**	1(1)	5(4)	**(2.00)**
2	8(26)	9(15)		4(15)	9(20)		7(7)	8(7)	
3	18(58)	48(81)		21(78)	36(78)		86(92)	102(89)	

Appendix Table 6: The associations of MAPKs with biological marker within BC subgroups

	ER+HER2+			ERHER2+			ER-HER2-		
	Neg/low, N (%)	High,N (%)	p-value	Neg/low, N (%)	High, N (%)	p-value	Neg/low, N (%)	High, N (%)	p-value
ERK1/2									
AR			**0.001**			**0.589**			**2.52**
Negative	19(49)	4(12)	**(11.01)**	15(54)	17(61)	**(0.29)**	104(85)	49(78)	**(1.31)**
Positive	20(51)	29(88)		13(46)	11(39)		19(15)	14(22)	
BEX1			**0.335**			**0.002**			**0.421**
Negative	15(43)	8(31)	**(0.92)**	11(52)	1(6)	**(9.97)**	36(47)	16(39)	**(0.64)**
Positive	20(57)	18(69)		10(48)	17(94)		41(53)	25(61)	
Nuclear p-ERK1/2									
HER1			**0.004**			**0.464**			**0.199**
Negative	28(74)	38(90)	**(3.89)**	24(53)	15(63)	**(0.53)**	78(63)	38(53)	**(1.64)**
Positive	10(26)	4(10)		21(47)	9(37)		46(37)	33(47)	
Cytoplasmic p-ERK1/2									
BEX1			**0.009**			**0.315**			**0.218**
Negative	11(48)	4(14)	**(6.84)**	11(41)	10(2)	**(1.00)**	29(49)	24(38)	**(1.51)**
Positive	12(52)	24(86)		16(59)	25(71)		30(51)	39(62)	
CD71			**0.040**			**0.873**			**0.219**
Negative	4(20)	1(3)	**(4.19)**	8(28)	10(29)	**(0.02)**	24(33)	16(24)	**(1.51)**
Positive	16(80)	32(97)		21(72)	24(71)		48(67)	51(76)	
p-Cadherin	15(47)	11(32)	**0.228**			**0.168**			**0.043**
Negative	17(53)	23(68)	**(1.45)**	8(20)	4(14)	**(1.89)**	11(13)	3(4)	**(4.10)**
Positive				19(70)	24(86)		77(87)	76(96)	
p-JNK1/2									
BEX1			**0.009**			0.089			0.442
Negative	25(58)	28(34)	**(5.92)**	6(37.5%)	5(15.6%)	(2.88)	39(95)	73(91)	(0.59)
Positive	18(42)	55(66)		10(62.5%)	27(84.4%)		2(5)	7(9)	
Pan p38									
PgR			**0.018**	-	-	**-**	151(100)	50(98)	**0.085**
Negative	19(56)	9(27)	**(5.63)**				0(0)	1(2)	**(2.97)**
Positive	15(44)	24(73)							
KI67-LI			0.061			**0.450**			**0.786**
Negative/low	9(30)	3(10)	(3.51)	7(20)	5(29)	**(0.57)**	13(10)	5(12)	**(0.07)**
High	21(70)	26(90)		28(80)	12(71)		115(90)	38(88)	
FOXA1			**0.005**			**0.135**			**0.127**
Negative	87(92)	27(73)	**(7.82)**	23(68)	14(87)	**(2.22)**	57(68)	18(53)	**(2.32)**
Positive	8(8.4%)	10(27)		11(32)	2(13)		27(32)	16(47)	
BEX1			**0.335**			**0.624**	43(47)	7(23)	**0.031**
Negative	7(28)	11(41)	**(0.93)**	12(35)	3(27)	**(0.24)**	48(53)	23(77)	**(5.32)**
Positive	18(72)	16(59)		22(65)	8(73)				
P-P38									
PgR			**0.044**	-	-	**-**			**0.235**
Negative	27(36)	9(64)	**(4.07)**				173(99)	37(97)	**(1.41)**
Positive	49(64)	5(36)					1(1)	1(3)	
FOXA1			**0.013**			**0.940**			**0.091**
Negative	35(53)	2(15)	**(6.18)**	30(73)	13(72)	**(0.00)**	102(86)	20(71)	**(4.80)**
Positive	31(47)	11(85)		11(27)	5(28)		16(14)	8(29)	
BCL2			**0.106**			**0.028**			**0.907**
Positive	30(45)	9(69)	**(2.60)**	25(78)	19(100)	**(4.81)**	104(83)	28(82)	**(0.01)**
Negative	37(55)	4(31)		7(22)	0(0)		21(17)	6(18)	
p-ATF2									
AR			**0.035**			**0.079**			**0.974**
Negative	29(45)	2(14)	**(4.44)**	33(60)	3(30)	**(3.08)**	48(44)	8(44)	**(0.00)**
Positive	36(55)	12(86)		22(40)	7(70)		61(56)	10(5)	
PELP1			**0.039**			**0.012**			**0.916**
Negative	6(11)	0(0)	**(6.50)**	9(18)	1(17)	**(8.83)**	10(8)	2(11)	**(0.17)**
Moderate	43(77)	7(58)		37(74)	2(33)		85(70)	12(67)	
High	7(12)	5(42)		4(8)	3(50)		27(22)	4(22)	
CD71	15(27)	6(46)	**0.185**			**0.010**			**0.051**
Negative	40(73)	7(54)	**(1.75)**	5(10)	3(50)	**(6.62)**	32(25)	10(46)	**(3.80)**
Positive				43(90)	3(50)		95(75)	12(54)	
p-C-JUN									
AR			**0.148**			**0.049**			**0.503**
Negative	14(48)	17(32)	**(2.09)**	10(40)	26(65)	**(3.89)**	52(88)	69(84)	**(0.44)**
Positive	15(52)	36(68)		15(60)	14(35)		7(12)	13(16)	
P-Cadherin			**0.526**			**0.039**			**0.165**
Negative	11(41)	15(33)	**(0.40)**	7(37)	5(13)	**(4.27)**	10(12)	6(6)	**(1.92)**
Positive	16(59)	30(67)		12(63)	33(87)		71(88)	89(94)	
CARM1			**0.200**			**0.684**			**0.009**
Negative	9(33)	8(17)	**(3.22)**	3(20)	4(12)	**(0.76)**	16(24)	7(10)	**(9.67)**
Moderate	13(48)	25(52)		6(40)	17(52)		24(36)	44(60)	
High	5(19)	15(31)		6(40)	12(36)		27(40)	22(30)	

Appendix Table 7: The associations of p-mTORC1 with clinicopathological variables and biological markers within BC subgroups

	ER+HER2+			ERHER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
p-mTORC1									
Age			**0.631**			**0.077**			**0.016**
< 50	24(46)	15(52)	**(0.23)**	18(46)	7(25)	**(3.11)**	85(53)	11(31)	**(5.82)**
>50	28(54)	14(48)		21(54)	21(75)		76(47)	25(69)	
Mitosis			**0.064**			**0.975**			**0.364**
	6(12)	0(0)	**(5.49)**	1(3)	1(4)	**(0.05)**	7(4)	1(3)	**(2.02)**
1	8(16)	9(31)		7(18)	5(18)		11(7)	5(14)	
2	37(72)	20(69)		30(79)	22(79)		141(89)	30(83)	
3									
p-mTORC1									
TFF1			**0.743**			**0.013**			**0.004**
Negative	14(35)	9(39)	**(0.10)**	19(79)	7(41)	**(6.19)**	62(70)	7(35)	**(8.44)**
Positive	26(65)	14(61)		5(21)	10(59)		27(30)	13(65)	
HER3			**0.289**			**0.227**			**0.026**
Negative	5(11)	1(4)	**(1.12)**	2(6)	0(0)	**(1.46)**	5(3)	4(1)	**(4.97)**
Positive	40(89)	25(96)		32(94)	24(100)		145(97)	27(87)	
N-Cadherin			**0.053**			**0.535**			**0.044**
Negative	4(11)	8(31)	**(3.73)**	5(16)	2(10)	**(0.38)**	26(22)	10(42)	**(4.06)**
Positive	32(89)	18(69)		26(84)	18(90)		92(78)	14(58)	

Appendix Table 8: The associations of p-mTORC1 with MAPKs within BC subgroups

	ER+HER2+			ERHER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
p-mTORC1									
Pan ERK1/2			**0.063**			**0.044**			**0.361**
Neg/low	27(67)	9(43)	**(3.45)**	17(63)	5(31)	**(4.04)**	69(62)	20(71)	**(0.83)**
High	13(32)	12(57)		10(37)	11(69)		42(38)	8(29)	
Pan p38			**0.058**			**0.698**			**0.037**
Neg/low	16(47)	16(73)	**(3.59)**	22(7)	11(73)	**(0.15)**	95(82)	20(64)	**(4.33)**
High	18(53)	6(27)		6(21)	4(27)		21(18)	11(3)	
P38			**0.007**			**0.114**			**<0.001**
Neg/low	44(90)	18(64)	**(7.39)**	29(76)	15(58)	**(2.49)**	135(88)	19(58)	**(17.93)**
High	5(10)	10(36)		9(24)	11(42)		18(12)	14(42)	
p-ATF2			**0.806**			**0.963**			**0.010**
Neg/low	34(79)	22(81)	**(0.60)**	31(89)	23(85)	**(0.15)**	125(85)	21(66)	**(6.58)**
High	9(21)	5(19)		4(11)	4(15)		22(15)	11(34)	

Appendix Table 9: The associations of CHIP with clinicopathological variables within BC subgroups

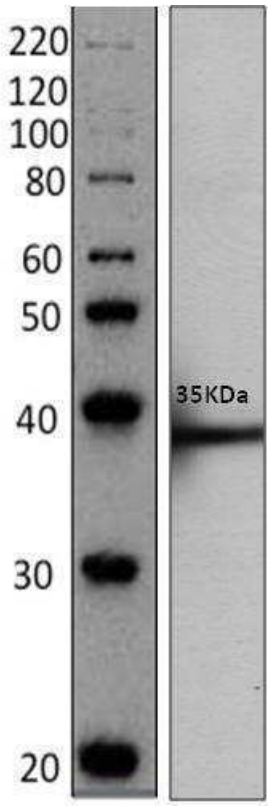
	ER+HER2+			ER-HER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
Nuclear CHIP									
Grade			**0.004**			**0.905**			**1.00**
I	0(0)	3(16)	**(11.15)**	1(2)	0(0)	**(0.20)**	2(2)	2(6)	**(4.60)**
2	10(17)	5(26)		6(12)	1(11)		9(6)	0(0)	
3	49(83)	11(58)		42(86)	8(89)		133(92)	32(94)	
Tubule			**0.005**			**0.766**			**0.040**
1	0(0)	2(11)	**(10.67)**	7(15)	1(11)	**(0.08)**	0(0)	1(3)	**(6.44)**
2	12(21)	8(42)		40(85)	8(89)		17(12)	7(21)	
3	46(79)	9(47)					125(88)	25(76)	
Mitosis			**0.009**			**0.316**			**0.101**
1	2(3)	5(26)	**(9.51)**	4(8)	0(0)	**(2.30)**	4(3)	2(6)	**(4.57)**
2	11(19)	4(21)		7(15)	3(33)		8(6)	5(15)	
3	45(78)	10(53)		36(77)	6(67)		130(91)	26(79)	
Cytoplasmic CHIP									
Tubule			**0.032**			**0.254**			**0.744**
1	2(6)	0(0)	**(6.86)**	5(20.8%)	3(10)	**(1.35)**	1(1)	0(0)	**(0.59)**
2	5(14)	15(37)		19(79.2%)	28(90)		15(13)	9(15)	
3	29(80)	26(63)					96(86)	53(85)	
Mitosis			**0.012**			**0.624**			**0.087**
1	3(8)	4(10)	**(8.81)**	2(8)	2(66)	**(0.94)**	5(4)	1(2)	**(4.88)**
2	2(6)	13(32)		3(13)	7(23)		5(5)	8(13)	
3	31(86)	24(58)		19(79)	22(71)		102(91)	53(85)	
LVI			**0.872**			**0.061**			**0.206**
Negative	22(58)	23(56)	**(0.02)**	20(77)	17(55)	**(3.02)**	76(66)	35(56)	**(1.59)**
Definite	16(42)	18(44)		6(23)	14(45)		39(34)	27(44)	

Appendix Table 10: The associations of CHIP with biological markers within BC subgroups

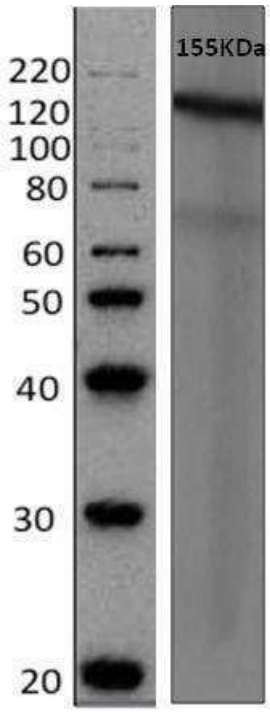
	ER+HER2+			ER-HER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
Nuclear CHIP									
GATA3			**0.694**			**0.636**			**0.028**
Negative	22(65)	7(58)	**(0.15)**	31(97)	7(100)	**(0.22)**	91(100)	18(94)	**(4.83)**
Positive	12(35)	5(42)		1(3)	0(0)		0(0)	1(5)	
P53			**0.851**			**0.514**			**0.042**
Negative	34(5)	11(61)	**(0.03)**	21(44)	5(56)	**(0.042)**	65(47)	9(27)	**(4.13)**
Positive	24(41)	7(39)		27(56)	4(44)		74(53)	24(73)	
Cytoplasmic CHIP									
AR			**0.368**			**0.008**			**0.849**
Negative	15(4)	11(32)	**(0.31)**	18(78)	12(41)	**(7.14)**	87(84)	50(83)	**(0.03)**
Positive	20(57)	23(68)		5(2)	17(5)		16(16)	10(17)	
CK18			**0.303**			**0.019**			**0.024**
Negative	3(9)	1(3)	**(1.06)**	4(18)	0(0)	**(5.53)**	59(58)	24(40)	**(5.11)**
Positive	31(91)	33(97)		18(82)	28(100)		42(42)	36(60)	
Tff3			**0.063**			**0.804**			**0.376**
Negative	3(11)	10(30)	**(3.46)**	7(39)	7(35)	**(0.06)**	63(90)	37(95)	**(0.78)**
Positive	25(89)	23(70)		11(61)	13(65)		7(10)	2(5)	
CD71			**0.010**			**0.317**			**0.039**
Negative	4(14)	14(45)	**(6.61)**	1(5)	3(14)	**(1.00)**	18(21)	17(38)	**(4.28)**
Positive	24(8)	17(55)		19(95)	18(86)		68(79)	28(62)	
BEX1			**0.973**			**0.008**			**0.776**
Negative	10(36)	12(35)	**(0.00)**	2(1)	10(50)	**(7.12)**	34(45)	19(48)	**(0.08)**
Positive	18(64)	22(65)		17(89)	10(50)		42(55)	21(52)	
HER3			**0.027**			**0.332**			**0.907**
Negative	7(19)	1(3)	**(4.88)**	0(0)	1(4)	**(0.94)**	4(4)	2(3)	**(1.00)**
Positive	29(81)	34(97)		24(100)	25(96)		101(96)	56(97)	

Appendix Table 11: The associations of p-SER 118 ER with biological markers within BC subgroups

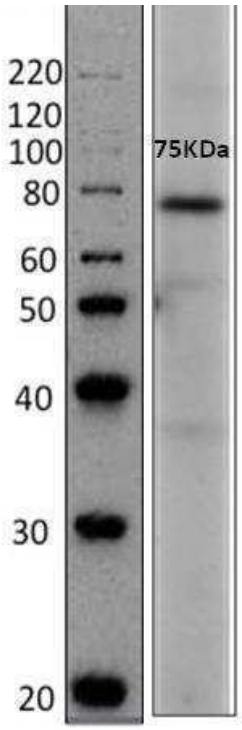
	ER+HER2+			ER-HER2+			ER-HER2-		
	Neg/low, N (%)	**High,N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**	**Neg/low, N (%)**	**High, N (%)**	**p-value**
Nuclear SER 118 ER									
GATA3			**0.038**			**0.454**			**0.313**
Negative	25(76)	9(47)	**(4.29)**	30(97)	17(100)	**(0.56)**	96(99)	25(96)	**(1.01)**
Positive	8(24)	10(53)		1(3)	0(0)		1(1)	1(4)	
CARM1			**0.816**			**0.643**			**0.030**
Negative	9(21)	4(16)	**(0.40)**	4(14)	2(11)	**(0.88)**	13(13)	6(25)	**(7.04)**
Moderate	23(52)	15(60)		16(53)	7(41)		49(47)	15(62)	
High	12(27)	6(24)		10(33)	8(47)		41(40)	3(13)	
KI67-LI			**0.495**			**0.491**			**0.033**
Low	11(28)	5(20)	**(0.46)**	9(27)	4(19)	**(0.47)**	12(10)	8(24)	**(4.54**
High	29(72)	20(80)		24(73)	17(81)		111(90)	26(76)	
N-Cadherin			**0.046**			**0.599**			**0.032**
Negative	5(12)	7(33)	**(3.97)**	2(6)	2(10)	**(0.27)**	30(24)	11(46)	**(4.59)**
Positive	36(88)	14(67)		31(94)	18(90)		93(76)	13(54)	
Cytoplasmic SER 118 ER									
AR			**0.042**			**0.253**			**0.843**
Negative	16(50)	11(27)	**(4.14)**	19(61)	13(46)	**(1.30)**	112(84)	39(83)	**(0.03)**
Positive	16(50)	30(73)		12(39)	15(54)		21(16)	8(17)	
CARM1			**0.409**			**0.109**			**0.033**
Negative	6(18)	7(19)	**(1.78)**	4(18)	2(8)	**(4.44)**	13(14)	6(19)	**(6.84)**
Moderate	16(49)	22(61)		13(59)	10(40)		43(45)	21(65)	
High	11(33)	7(20)		5(23)	13(52)		39(41)	5(16)	
CD71			**0.175**			**0.290**			**0.040**
Negative	6(21)	12(36)	**(1.84)**	2(8)	5(18)	**(1.11)**	24(23)	14(41)	**(4.20)**
Positive	23(79)	21(64)		23(92)	23(82)		80(77)	20(59)	



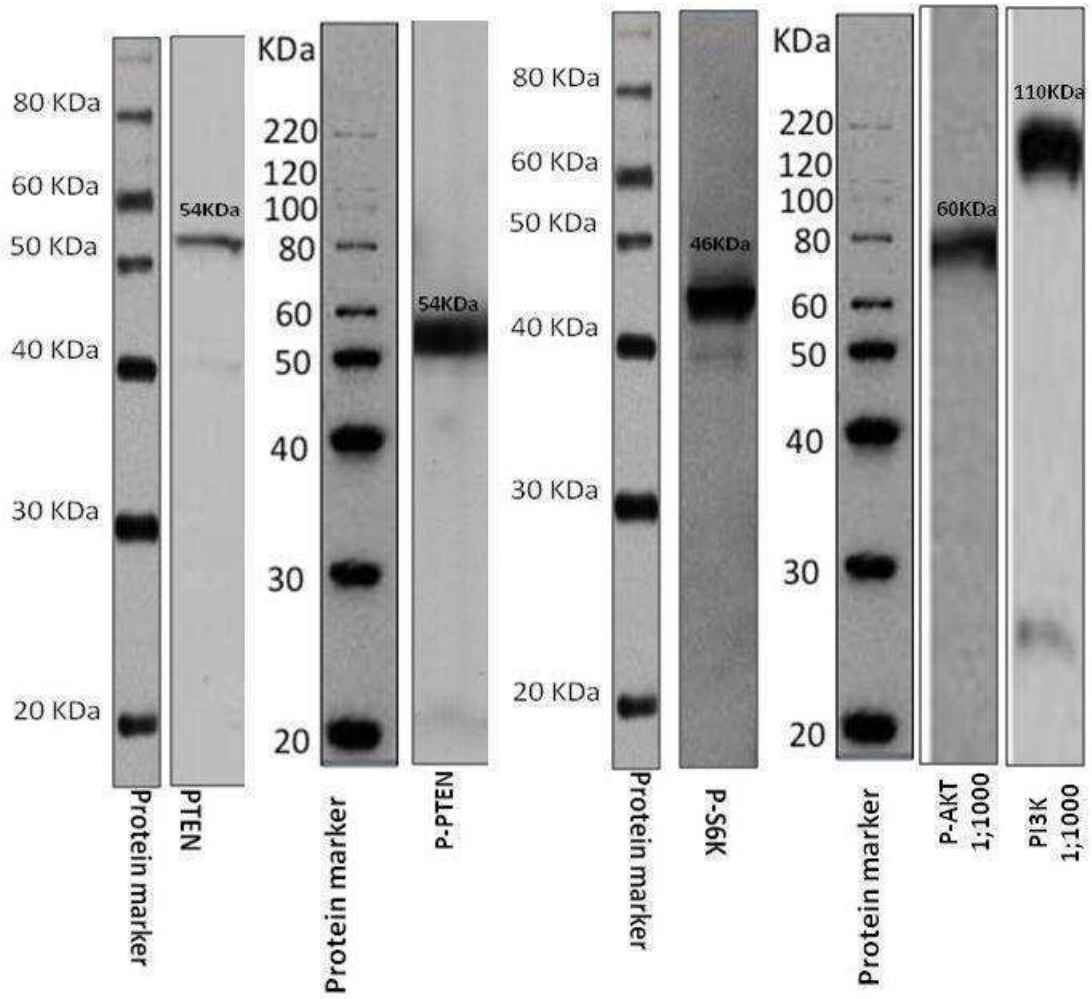
Appendix figure 1: Western blot for CHIP



Appendix figure 2: Western blot of SRC3



Appendix figure 3: Western blot for ECD



Appendix figure 4: Western blot for members of PI3K/Akt pathway