ROLE OF CALPAIN IN BREAST CANCER AND IN REGULATION OF THERAPEUTIC RESPONSE TO TARGETED TREATMENT

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Abstract

The calpain system is a group of intracellular cysteine proteases. Dysregulated calpain activity, or mutation in calpain isoforms, as well as its endogenous inhibitor calpastatin, has been found to play an important role in tumorigenesis. The main aim of the current study is to explore the differential role of calpain family members in different breast cancer molecular subtypes; validate calpain-1 as a biomarker of trastuzumab response in HER2+ breast cancer patients; and explore the mechanisms by which calpain family regulates trastuzumab response in HER2+ cells.

Three representative cell lines for different molecular subtypes were used: MDA-MB-231 (basal-like), MCF-7 (luminal) and SKBR3 (HER2+); and two additional HER2+ cell lines were used in the HER2+ study: acquired trastuzumab-resistant SKBR3 and inherent trastuzumab-resistant JIMT-1. The role of calpain in proliferation, signal transduction and apoptotic response was assessed using growth curves, phosphokinase arrays and Annexin V-FITC apoptosis assays, respectively. The effect of calpastatin knockdown, via shRNA, on cell migration was examined using Haptotaxis assay. The combined effect of calpeptin and trastuzumab on colony formation and cell cycle progression were examined using clonogenic survival and flow cytometry, respectively. Biomarker studies were conducted using standard immunohistochemistry.

The results suggested that inhibition of calpain activity showed anti-proliferative effect on breast cancer cells across different subtypes. Knockdown of calpastatin in both MDA-MB-231 and MCF-7 cells did not have significant effects on migratory ability, either with or without calcium ionophore A23187. The study also showed that combining calpeptin and trastuzumab enhanced trastuzumab-induced anti-proliferative effects on SKBR3 and SKBR3/TR cells, but not in JIMT-1 cells. Combined treatment did not further reduce clonogenic survival either in SKBR3 or SKBR3/TR cells, compared with single agent alone. In all three HER2+ cells, combined treatment had no significant effect on

trastuzumab-induced G0/G1 cell cycle arrest. Results from the immunohistochemical study suggested that high calpain-1 expression was significantly associated with adverse relapse-free survival in breast cancer patients who received adjuvant trastuzumab. Findings were validated in the expanded Nottingham and independent Newcastle patient cohort.

Based on the previous *in vitro* study, suggesting a role of calpain in regulation of phospho-MSK1/2 expression; and because there is close link between calpain and caspase family. It was decided to explore the correlation between calpain system protein expression with MSK1 and two representative caspases (caspase-3 & -8), as well as their prognostic significance, in a large cohort of invasive breast cancer patients. Results demonstrated significant correlations between calpain-1 vs MSK1, and vs caspase-3; calpastatin vs MSK1, and vs caspase-8, however with low correlation coefficients. High MSK1 expression was significantly associated with improved breast cancer-specific survival. High caspase-3, but not caspase-8, was significantly associated with adverse breast cancer-specific survival. And combinatorial calpain-1 and caspase-3 expression provided additional prognostic values, especially in basal-like subtype.

In conclusion, calpain system protein has been found to have roles in different breast cancer molecular subtypes. Calpain-1 is a potential biomarker for trastuzumab response in HER2+ breast cancer patients, and this study suggested MSK1 and caspase-3 could be potential biomarkers in breast cancer.

Publications arising from this thesis

Journal articles

Storr S. J., Thompson N., **Pu X.**, Zhang Y., Martin S. G., Calpain in Breast Cancer: Role in Disease Progression and Treatment Response. Pathobiology 2015; 82: 133-141

Pu, X., Storr, S.J., Ahmad, N.S., Chan, S.Y., Moseley, P.M., Televantou, D., Cresti, N., Boddy, A., Ellis, I.O. and Martin, S.G., Calpain-1 is associated with adverse relapse-free survival in breast cancer: a confirmatory study. Histopathology, 2016; 68: 1021-1029

Pu, X., Storr, S.J., Ahmad, N.S., Hall L., Rakha E.A., Green A.R., Ellis, I.O. and Martin, S.G., Low MSK1 is associated with adverse disease-specific survival in breast cancer patients (manuscript submitted)

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Conference presentations

Poster presentation “Calpain-1 is associated with relapse-free survival in breast cancer patients treated with adjuvant trastuzumab: a validation study.” 10th NCRI Cancer Conference, 2-5 November 2014, Liverpool, UK

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Abbreviations

2D	two dimensional
2-DE	two-dimensional electrophoresis
3D	three dimensional
5-FU	5-fluorouracil
AC	doxorubicin (Adriamycin®) and cyclophosphamide
AD	Alzheimer's disease
ADCC	antibody-dependent cell cytotoxicity
ADH	atypical ductal hyperplasia
AH	atypical hyperplasia
AI	aromatase inhibitor
AIF	apoptosis-inducing factor
ALH	atypical lobular hyperplasia
ALL	acute lymphoblastic leukaemia
AMC	7-Amino-4-methylcoumarin
AML	acute myelogenous leukaemia
ATCC	American Type Culture Collection
ATF1	activating transcription factor 1
Atg	autophagy-related gene
ATM	ataxia telangiectasia mutated
BCIS	breast carcinoma in situ
BCS	breast conserving surgery
BCSM	breast cancer specific mortality
BMI	body mass index
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BRIP1	BRCA1 interacting protein C-terminal helicase 1
Brk	breast tumour kinase
CAMK	calcium/calmodulin-dependent protein kinase
CANP	calcium-activated neutral protease
CDH1	cadherin-1
CDK	cyclin-dependent kinase
CHEK2	checkpoint kinase 2
CI	confidence interval
CK	cytokeratin
CMF	cyclophosphamide, methotrexate and 5-FU
CNS	central nervous system

CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
DAB	3, 3’-diaminobenzidine
DCIS	ductal carcinoma in situ
DFS	disease-free survival
DISC	death-inducing signalling complex
DR	death receptor
DSB	double-strand break
E2	17β-estradiol
EBRT	external beam radiotherapy
E-CMF	epirubicin, followed by CMF
EGFR	epidermal growth factor receptor
EP-HRT	oestrogen plus progesterone hormone replacement therapy
ER	oestrogen receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERT	oestrogen replacement therapy
FA	focal adhesion
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FEC	5-fluorouracil, epirubicin and cyclophosphamide
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FL	fluorescent light
GGI	gene expression grade index
H&E	haematoxylin and eosin
H_2O_2	hydrogen peroxidase
HD	huntington disease
HER	epidermal growth factor receptor
HER2	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor-1α
HPIP	hematopoietic PBX-interacting protein
HR	hazard radio
HRP	horseradish peroxidase
HRT	Hormone replacement therapy

HSP60	heat shock protein 60
HTRA2	high temperature requirement protein A2
HU	hydroxyurea
IAP	inhibitor of apoptosis protein
IBC	inflammatory breast cancer
IC50	half maximal inhibitory concentration
ICC	intraclass correlation co-efficient
ICE	interleukin-1β-converting enzyme
IDC	invasive ductal carcinoma
IGF-IR	insulin-like growth factor-I receptor
IHC	immunohistochemistry
ILC	invasive lobular carcinoma
kDa	kilo dalton
LABC	locally advanced breast cancer
LCIS	lobular carcinoma in situ
LGMD2A	limb-girdle muscular dystrophy type 2A disease
LVI	lymphovascular invasion
M	molar
MAPK	mitogen-activated protein kinase
MD	mammographic density
MIB-LI	MIB1/Ki67 labelling index
mL	milliliter
mM	millimolar
MMP2	matrix metalloproteinases 2
MSK	mitogen- and stress- activated kinase
mTOR	mammalian target of rapamycin
MUC4	membrane-bound glycoprotein mucin-4
ND	not determined
NF	nuclear factor
NGS	Nottingham Grading System
NICE	National Institute for Health and Clinical Excellence
NKs	natural killers
NP	non-proliferative disease
NPI	Nottingham Prognostic Index
OC	oral contraceptives

OR	objective response
OS	overall survival
PALB2	partner and localizer of BRCA2
PAM50	Prediction Analysis of Microarray
PARP	poly-ADP-ribose polymerase
PBS	phosphate buffered saline
pCR	pathologic complete response
PD	parkinson's disease
PDWP	proliferative disease without atypia
PE	plating efficiency
PFS	progression free survival
PI	propidium iodide
PI	phosphatidylinositol
PI3K	phosphatidylinositol kinase-3
PIP2	Phosphatidylinositol‑4, 5‑bisphosphate
PKA	phosphokinase array
PKA	protein kinase A
PKCι	protein kinase Cι
PMA	phorbol 12-myristate 13-acetate
PMD	percent mammographic density
PPase	phosphoprotein phosphatase
PR	progesterone receptor
PS	phosphatidylserine
PSS	peeling skin syndrome
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reserve transcription polymerase chain reaction
RFU	relative fluorescence units
RNAi	RNA interference
RNase A	ribonuclease A
ROR	risk of relapse
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SERMs	selective oestrogen-receptor modulators
SF	surviving fraction
shRNA	short hairpin RNA

Sig	significance
siRNA	small interference RNA
SOLH	small opticlobe-homology
Src	proto-oncogene tyrosine-protein kinase Src
SSB	single-strand break
STAT3	signal transducer and activator of transcription 3
STK11	serine/threonine kinase 11
STS	staurosporine
TAC	docetaxel (Taxotere®), doxorubicin and cyclophosphamide
TARGIT	targeted intraoperative radiotherapy
TBI	traumatic brain injury
TFF1	trefoil factor 1
TGFα	transforming growth factor α
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TNBC	triple-negative breast cancer
TNF	tumour necrosis factor
TNFRSF6	tumour necrosis factor receptor superfamily member 6
TPA	12-O-tetradecanoylphorbol-13-acetate
VEGF	vascular endothelial growth factor
WLE	wide local excision
XIAP	X-linked inhibitor of apoptosis protein
µg	microgram
µg/mL	microgram per mililiter
µL	microliter
µM	micromolar
χ^2	Pearson Chi Square test of association

Table of Contents

List of Tables

List of Figures

Chapter 1 General introduction

1.1 General introduction to breast cancer

1.1.1 Epidemiology

1.1.1.1 Incidence, mortality and survival

Worldwide there were approximately 14.1 million new cancer cases and 8.2 million cancer related deaths in 2012, these numbers increased significantly compared with 12.7 million new cancer cases and 7.6 million deaths in 2008 (Ferlay et al., 2010, Ferlay et al., 2015). Breast cancer is the most common cancer in females, in both developed and developing countries, representing 25% (1.67 million) of the total new cancer cases in female. It is the most common cause of female cancer deaths in developing countries (14.3%) and is the second most common cause in developed countries (15.4%) (Ferlay et al., 2015). The highest incidence and mortality of female breast cancer was recorded in Western Europe and Western Africa, respectively; and the lowest incidence and mortality was observed in Middle Africa and Eastern Asian, respectively. Generally, incidence and mortality rates of breast cancer vary markedly over the world; the incidence rate in most of the developed areas is much higher than developing areas, whilst the mortality rate is slightly lower in developed areas (Jemal et al., 2011, Ferlay et al., 2015). Specifically, breast cancer incidence in Africa is about the half of that in Europe (36 new cases per 100,000 women vs 71 new cases per 100,000 women); however, the mortality rate is very close between those two areas, accounting for approximately 17 per 100,000 women, due to the lack of diagnosis and treatment facilities in the poorer areas (Ferlay et al., 2015).

In the UK breast cancer is the most common cancer in females; about 1 in 8 women are at risk of developing breast cancer during their life. Similar to trends in most of the Western countries, the incidence rate of female breast cancer in the UK has experienced an increase of about 7% over the last decade; whilst the mortality rate has decreased by about 19% during the same period. In particular, significant changes in incidence and mortality have been observed in women aged 50-64 and 65-69, both age groups are currently included in the

breast screening programmes (Cancer Research UK, 2014a). It is likely that the increases in incidence may result from the introduction of screening programmes, increased longevity and changes in related lifestyle factors. The factors that led to declines in mortality may include early detection through mammography as well as improved treatment strategies (e.g. wide use of tamoxifen since late 1980s, use of radiotherapy and targeted therapies) (Youlden et al., 2012). In the UK, 87% of breast cancer patients survive for at least five years; and the ten year survival rate is about 78%. The survival rate is much lower in late stage breast cancer patients, five year survival rate for stage IV patients is only 15% (Cancer Research UK, 2014c, Cancer Research UK, 2014d).

1.1.1.2 Risk factors

Breast cancer is a heterogeneous disease; several factors can contribute to its development: for example, risk is also increased if the patient was previously diagnosed with breast cancer; at least one family member had been diagnosed with breast cancer; or mutation in breast cancer related genes (such as BRCA1). Some of the most important risk factors are discussed below.

Age and reproductive factors

The risk of developing breast cancer increases with age. In the UK, about 8 in 10 patients are diagnosed in their 50s and above; and about 1 in 4 are diagnosed in the 75's and over (Cancer Research UK, 2014b). Breast cancer incidence increases by two-fold every 10 years until menopause, after which the rate of increase begins to slow down (McPherson et al., 2000). The changes in incidence at different ages underline the correlation between breast cancer and hormonal status. Women who start menstruating at younger age and/or have menopause at older age have increased risk of breast cancer. Each 1-year delay in menarche decreases the relative risk by 5%; whilst each 1-year delay in menopause increases the relative risk by 3%. Though both early menarche and late menopause increase breast cancer risk, those effects are not equivalent. 1 year younger at menarche has a greater effect than 1 year older at menopause

(Collaborative Group on Hormonal Factors in Breast, 2012). The age at first birth is also related with breast cancer risk. The relative risk increases by 3% for each year older when women give the first birth (Collaborative Group on Hormonal Factors in Breast, 2002b). Studies have shown that the effects of early age at first birth and parity maybe limited to oestrogen receptor positive (ER+)/progesterone receptor positive (PR+) breast cancer; whilst late age at menarche has a protective effect on both ER+/PR+ and ER-/PR- breast cancer (Ma et al., 2006).

Family history and genetic factors

The risk of breast cancer is increased if women have a family history of breast cancer. The results from a meta-analysis have shown that having at least one first degree relative with breast cancer increases a woman's risk by about two-fold; having two, and three or more, relatives increases the risk by about three- and four-fold, respectively (Collaborative Group on Hormonal Factors in Breast, 2001). Hereditary breast cancer accounts for around 5-10% of the total cases, which suggests abnormal genes can be transmitted through parents to the child (American Cancer Society, 2015). Two susceptibility genes, BRCA1 and BRCA2, have been identified and are related with high risk of familial breast cancer. Women with mutations in BRCA1 have 65% chance of having breast cancer and 45% chance for women with mutation in BRCA2 (Antoniou et al., 2003). In addition, mutations in certain genes can cause familial syndromes that increase the risk of breast malignancy. For example, it has been shown that tumour suppressor gene p53 and phosphatase and tensin homolog (PTEN) gene are associated with Li-Fraumeni syndrome and Cowden syndrome, respectively; (Malkin et al., 1990, Liaw et al., 1997). Other examples are CHEK2, ATM (ataxia telangiectasia), BRIP1, PALB2, CDH1 and STK11 (Peutz-Jeghers syndrome) (reviewed in (Turnbull and Rahman, 2008)).

Clinical factors

Women who have a history of breast cancer are at high risk of developing a

second primary breast cancer (not as a recurrence); and the risk increases more markedly in women who are diagnosed before menopause (Soerjomataram et al., 2005). Increased risk has also been observed in women who have benign breast disease. Benign breast disease can be categorised as non-proliferative disease (NP), proliferative disease without atypia (PDWA) and proliferative disease with atypical hyperplasia (AH); the latter includes atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH). Basically, women with NP do not have increased risk of breast cancer. Compared with women with NP, women diagnosed with PDWA and AH have around 50% increased risk and three-fold increased risk of developing breast cancer, respectively (Zhou et al., 2011). In addition, breast carcinoma in situ (BCIS), which includes ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), has the potential to turn into invasive breast cancer. Women diagnosed with BCIS have an approximately two-fold increased risk of developing breast cancer (Robinson et al., 2008).

The breasts are made up of a combination of fibrous tissue, glandular tissue and fatty tissue; fibrous and glandular tissue show as radio-dense areas on mammograms, while fatty tissue appears transparent. Having dense breast tissue increases the risk of breast cancer. The term mammographic density (MD) refers to the percentage of different tissue in the breast, and is measured as percent mammographic density (PMD). Women who have high PMD (>75%) have a 4.6 fold higher risk compared to women with lower PMD (<5%); and the association is not affected by age or body mass index (BMI) (McCormack and dos Santos Silva, 2006, Baglietto et al., 2014).

Hormone related factors

Female sex hormones include oestrogen and progesterone, exposure to such hormones increases the risk of developing breast cancer. Women who used oral contraceptives (OC) for 10 years have a slightly increased relative risk of breast cancer compared with never users; however, there is no significant increased

risk 10 years after stopping OC use (Collaborative Group on Hormonal Factors in Breast, 1996). Hormone replacement therapy (HRT) after menopause also alters endogenous hormone levels. There are two different types of hormone replacement therapy, the use of oestrogen only (E-only HRT or ERT) for women without a uterus (hysterectomy); and combined use of oestrogen and progesterone (EP-HRT) for women who still have a uterus. The risk of breast cancer is higher in women using HRT compared with women who have never used HRT; and the effect also increases with increasing duration of use. However after stopping use of HRT for 5 years, the risk in HRT users is equivalent to that in women who have never used HRT(Collaborative Group on Hormonal Factors in Breast, 1997). Nevertheless, a history of OC use or HRT is associated with less advanced symptoms in breast cancer patients (Collaborative Group on Hormonal Factors in Breast, 1996, Collaborative Group on Hormonal Factors in Breast, 1997).

Radiation exposure

It has been estimated that previous radiation exposure attributes to the incidence of second cancers. In the UK in 2007, 0.45% of the total new cancer cases associated with radiotherapy; among those 10.6% were estimated to be breast cancer (Maddams et al., 2011). One study demonstrated that the risk of contralateral breast cancer is slightly higher in breast cancer patients who had surgery and radiotherapy, compared with patients who had surgery alone. It was estimated that radiotherapy attributes for 176 excess cases of contralateral breast cancer or 5% of the total in 1 year survivors. However, with long term follow-up, it was noticed that this effect decreases with increasing age at diagnosis. The relative risk for radiotherapy were 1.09 for contralateral breast cancer in patients received ≈ 1Gy doses (de Gonzalez et al., 2010).

Life style

Younger, obese women, smokers (15+ cigarettes per day) and alcohol drinkers (20g+ per day) have higher levels of endogenous hormones (Key et al., 2011).

Based on this, some life style related factors have been potentially linked with breast cancer. A meta-analysis suggested that obesity could increase the risk of ER+/PR+ tumours in postmenopausal women whilst it could decrease the risk in premenopausal women; and obesity did not have an effect on ER-/PR- tumours (Munsell et al., 2014). A study demonstrated that the relative risk of breast cancer increases by 7.1% for each extra unit of alcohol consumed per day (Collaborative Group on Hormonal Factors in Breast, 2002a).

Protective factors

Breast feeding is a protective factor for breast cancer; it contributes around 67% decline in breast cancer incidence and decreases the risk by 4% for every 12 months of breastfeeding (Collaborative Group on Hormonal Factors in Breast, 2002b). Such effect of breastfeeding may be more relevant for ER+/PR+ tumours, though the different effect between ER+/PR+ and ER-/PR- tumours is marginally significant (Ma et al., 2006). According to World Cancer Research Fund/American Institute for Cancer Research, diet and physical activities has been listed as protective actions against breast cancer (AICR, 2007).

Kumar et.al summarised the factors that increase the risk of breast cancer, as shown in Table 1.1.

Table 1.1 Summary of breast cancer risk factors

Well-Established Factors	Relative Risk
Geography	Varies in different areas
Age	Increases after age 30
Family history	
First-degree relative with breast cancer	1.2-3.0
Premenopausal	3.1
Premenopausal and bilateral	8.5-9.0
Postmenopausal	1.5
Postmenopausal and bilateral	4.0-5.4
Menstrual history	
Age at menarche <12 years	1.3
Age at menopause >55 years	1.5-2.0
Pregnancy	
First live birth from ages 25 to 29 years	1.5
First live birth after age 30 years	1.9
First live birth after age 35 years	2.0-3.0
Nulliparous	3
Benign breast disease	
Proliferative disease without atypia	1.6
Proliferative disease with atypical hyperplasia	>2.0
Lobular carcinoma in situ	6.9-12.0
Other Possible Factors	
Exogenous oestrogens	
Oral contraceptives	
Obesity	
High-fat diet	
Alcohol consumption	
Cigarette smoking	

Reproduced from (Kumar et al., 2007), Robbins Basic Pathology, 8th edition, 739-750;

1.1.2 Breast cancer classification

1.1.2.1 Clinicopathological classification

Generally, breast cancer can be categorised into three different types: early, locally advanced and metastatic. Early stage breast cancer contains invasive cancer, DCIS and LCIS. DCIS refers to the tumour cells that have not broken out from the boundary of the duct or spread into the surrounding normal tissues and LCIS is limited to the lobules. Invasive tumours can grow into the stroma,

and have the potential to metastasize, including invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Yarnold, 2009a). Locally advanced breast cancer (LABC) is a heterogeneous group of cancers with diverse clinical presentations and outcomes. Although there is no strict definition for this type of cancer, it is generally accepted that LABC contains stage III disease (TNM system), which means the tumour has spread from the breast to the chest wall, the skin, but it has not spread to the other organs (Sobin et al., 2010). Inflammatory breast cancer (IBC) is a very rare but aggressive form of LABC, characterised by causing erythema and oedema of the breast skin. IBC accounts for approximately 1-5% of invasive breast cancer and 8.5% of LABC, and the median survival is only 2.9 years (Hance et al., 2005, Tryfonidis et al., 2015). Breast cancer metastasis is defined by tumour cells that have spread to lymph nodes and distant organs through the blood stream and/or lymphatic system. Metastasis is a strong indicator of disease progression and the failure of primary treatments. It is estimated that 10-15% of aggressive breast cancer patients develop distant metastases within three years after diagnosis; and the most frequent metastasis sites are lung, bone, liver and pleura (Weigelt et al., 2005).

1.1.2.2 Molecular classification

Microarray technology is able to quantify the expression of thousands of genes; and has been used to define breast cancer molecular subtypes. Pioneering studies led to classify breast cancer into at least four molecular subtypes: luminal, ERBB2+/HER2+, basal-like and normal breast-like; later on, Sørlie et al. refined the luminal group into three subgroups: luminal subtype A, B and C (Perou et al., 2000, Sørlie et al., 2001, Sørlie et al., 2003). Triple-negative breast cancer (TNBC) shares high similarity with basal-like breast cancer. TNBC is characterised by the lack of ER, PR and HER2 expression. Both TNBC and basal-like account for less than 20% of all breast cancers, and have been shown to display a more aggressive behaviour and have a poorer prognosis (Badve et al., 2011). Numerous similarities existing between TNBC and basal-like breast cancer, they are not synonymous. 77% of basal-like tumours have TNBC subtype,

and only 71% of TNBC express basal-like genes from gene expression profiling (Rakha et al., 2007, Badve et al., 2011). Clinically TNBC and basal-like breast cancer are more difficult to treat and are more likely to relapse, as the tumours do not respond to hormone or targeted therapy due to the lack of ER, PR and HER2 proteins, the details will be discussed further in section 1.1.4.

A number of other classification studies have been conducted, for example a recent study has demonstrated an immunohistochemical based classification system by reducing a panel of 25 biomarkers down to 10, identifying 997 (out of 1073, 93%) breast tumours as seven subgroups: luminal A, N and B, basal-p53 altered, basal-p53 normal, HER2+/ER+ and HER2+/ER-. In this study the basal group was separated into two subgroups according to high p53 expression (p53 altered) and low p53 expression (p53 normal); and HER2+ group was further divided into two subgroups (ER+/ER-) based on differential hormone expression (Green et al., 2013). Another study examined the genomic/transcriptomic architecture of 2000 breast tumours consisted of a discovery set (997 tumours) and a validation set (995 tumours), generating a robust and tailored classification of breast cancer molecular subtypes. This method divided the population into ten integrative clusters, and each group represented distinct clinical outcomes (Curtis et al., 2012).

1.1.3 Prognostic and predictive factors

Prognostic factors are characteristics that are used to predict how the diseases will progress (e.g. recovery or relapse), irrespective of treatments. Predictive factors refer to something that can be used to predict if a patient will respond to a specific therapy. For breast cancer, prognostic factors include age, tumour size, axillary lymph node, histological type/grade, lymphatic/vascular invasion, proliferation markers and others. ER, PR and HER2 can function as both prognostic and predictive factors.

1.1.3.1 Age at diagnosis

Age is an established prognostic factor for breast cancer. Young age has been shown to be an independent factor of overall survival and disease-free interval in multivariate analysis, when considering potential confounding factors such as tumour size, lymph node status, histological grade, ER/PR status, local-regional treatment and adjuvant treatment (De la Rochefordiere et al., 1993). A study has found that in premenopausal patients, younger women have significantly lower survival rates and higher rates of local and distant recurrence compared with older women. It is worthwhile to note that molecular subtypes should be taken into account when assessing the prognostic value of age. A recent study has demonstrated that younger patients (<40 years old) have a worse prognosis compared with older patients; however when dividing patients into different subtypes (luminal A, luminal B, HER2+ and TNBC), significant association between age and event-free survival was only observed in the TNBC patients (Liedtke et al., 2015).

1.1.3.2 Tumour size and axillary lymph node

Tumour size and axillary lymph nodes are two of the most important prognostic factors for breast cancer survival (Warwick et al., 2004, Arriagada et al., 2006). Patients with a tumour size of 5cm, or more than 10 positive nodes, have an increased risk of relapse and death, compared with patients with a tumour size of 2cm, or only 1 positive node (Weiss et al., 2003). However in a large cohort study, significantly higher breast cancer-specific mortality (BCSM) was observed in patients with a tumour size <0.5cm, with four or more positive nodes, compared with those which had tumour size between 0.5-1.0cm with four or more positive nodes. This implies that the presence of very small tumours with aggressive nodal involvement can predict higher BCSM compared with larger tumours with similar nodal involvement, and may represent a more prominent invasive/metastatic potential (Wo et al., 2011). Tumour size, histological grade and nodal involvement are considered the most powerful prognostic factors for long term evaluation, though effects are time dependent (declining over time)

- the most significant effect is generally observed in the first five years (Warwick et al., 2004, Arriagada et al., 2006, Soerjomataram et al., 2008).

1.1.3.3 Histological type/grade

As mentioned in previously, breast cancer can be broadly divided into non-invasive (in situ) and invasive carcinomas, and the latter can be further refined based on the degree of differentiation. There are two ways to define the degree of differentiation: based on the morphological feature of the tumour-histological type; and based on semi-quantitative assessment of the structural characteristics-histological grade (Elston et al., 1999). Histological type has been long considered as an independent prognostic factor (Ellis et al., 1992). Generally, there are three types of invasive carcinomas: invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC) and invasive mixed ductal-lobular carcinoma (IDLC). It can be further divided into nine groups: ductal, lobular, ductal/lobular, mucinous, tubular, comedocarcinoma, inflammatory, medullary, and papillary (Li et al., 2003a, Li et al., 2003b, Li et al., 2005). The clinical outcomes vary profoundly between different histological types. Studies have found that patients with ILC have a favourable prognosis over IDC patients, regarding lower risk of mortality and improved overall survival (Ellis et al., 1992, Li et al., 2003b). Detailed prognosis of different histological types is shown in Table 1.2.

Table 1.2 Prognostic groups based on histological type

	Histological type	10-year survival
Excellent group	Tubular	>80%
	Invasive cribriform	
	Mucinous	
	Tubulo-lobular	
Good group	Tubular mixed	60%-80%
	Alveolar lobular	
	Mixed ductal (NST)/special type	
	Atypical medullary	
Moderate group	Medullary	50%-60%
	Invasive papillary	
	Classical lobular	
Poor group	Mixed lobular	<50%
	Solid lobular	
	Ductal NST	
	Mixed ductal NST/lobular	

Abbreviations: NST, "no special type". Reproduced from (Elston et al., 1999), Critical reviews in oncology/hematology, 31, 209-223; Copyright© 1999 by ELSEVIER with permission conveyed through Copyright Clearance Centre Inc.

In addition to histological type, one of the robust prognostic factors in breast cancer is histological grade. In 1991, Elston and Ellis modified the Scarff-Bloom-Richardson grading system to improve the accuracy and reproducibility of the criteria, now known as the Nottingham Grading System (NGS). NGS involves the degree of tubule formation, the degree of nuclear pleomorphism and the precise mitotic count. Each factor is assessed using a numerical scoring system and the overall grade is represented by adding three scores together. It is classified as: grade 1 (well differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated) (Elston and Ellis, 1991, Elston and Ellis, 2002). Patients with poorly differentiated tumours have significantly poorer clinical outcomes compared with those with well differentiated tumours (Elston and Ellis, 1991, Rakha et al., 2008). Despite the consistent agreement that has been achieved between grade 1 and 3 tumours; there is discordance regarding the assessment of grade 2 tumours. Therefore Sotiriou et al. developed a gene expression grade index (GGI), by using 97-gene profiling that can accurately refine grade 2 tumours into GGI1 and GGI3 and provide indication of recurrence

risk in each group (Sotiriou et al., 2006). Another example is to assess proliferation biomarker MIB1/Ki67 expression by immunohistochemistry (IHC). MIB1/Ki67 labelling index (MIB-LI) has been performed and used to divide grade 2 tumours into low and high subgroups with distinct clinical relevance (Rakha et al., 2010, Aleskandarany et al., 2011).

1.1.3.4 Lymphovascular invasion

Lymphovascular invasion (LVI) refers to the presence of tumour emboli in the lymphatic spaces and/or blood vessels, and is a critical step in cancer metastasis. LVI presents less frequently in node-negative tumours than node-positive tumours. A number of studies have been conducted to investigate the prognostic value of LVI in node-negative and node-positive disease with its presence being shown to be significantly associated with poorer survival in both groups. LVI has also been shown to be significantly associated with histological grade, tumour size and age in early invasive breast cancer (Song et al., 2011, Mohammed et al., 2011, Rakha et al., 2012). The conventional method to detect LVI in tumour cells is via routine haematoxylin and eosin (H&E) staining, however immunohistochemical staining has been shown to be able to improve sensitivity and specificity and to differentiate between lymphatic and blood vessel endothelium, providing more accurate detection of LVI. Amongst the lymphatic and blood vessel endothelial markers, podoplanin/D2-40 and factor VIII are two representative examples that can differentiate blood vessel endothelium from lymphatic at different density (Gujam et al., 2014). LVI is strongly associated with adverse prognosis; it is suggested that LVI should be incorporated into routinely breast cancer staging systems.

1.1.3.5 Grouped factors

In addition to single prognostic factors, grouped prognostic systems are also used in breast cancer, providing more precise information to assess clinical outcomes. The TNM system is used as a main method to stage different types of breast cancer. The TNM system involves the size of the primary tumour (T),

the involvement of regional lymph nodes (N) and the presence of distant metastasis (M). A number is added following the letter, indicating the size and/or extent of the tumour, and the degree of nodal involvement as well as distant spread, i.e. TX, T0, Tis, T1, T2, T3, T4; NX, N0, N1, N2, N3; MX, M0 and M1. The T, N and M values are combined together to generate an overall stage, from 0 to IV; some stages can be further subdivided using letters such as A and B. The higher the number, the tumour is more advanced (Edge et al., 2010, Kumar et al., 2015). A combination of the TNM classification system and the UICC (Union for International Cancer Control) criteria is shown in Table 1.3.

Table 1.3 TNM staging system in breast cancer

Stage	Tumour Size	Lymph Nodes	Distant Metastasis
0	DCIS or LCIS	No metastases	None
I	Invasive carcinoma ≤ 20mm	No metastases or only micrometastases	None
II	Invasive carcinoma > 20mm	1 to 3 positive LNs	None
	Invasive carcinoma > 20mm but ≤ 50mm	0 to 3 positive LNs	None
III	Invasive carcinoma > 50mm	Negative or positive LNs	None
	Invasive carcinoma (any size)	≥ 4 positive LNs	None
	Invasive carcinoma with skin or chest wall involvement or inflammatory carcinoma	Negative or positive LNs	None
IV	Invasive carcinoma(any size)	Negative or positive LNs	Present

Abbreviations: DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ. Data adapted from (Kumar et al., 2015).

Another well-established system is the Nottingham Prognostic Index (NPI). The NPI is determined based on tumour size, lymph-node stage and histological grade, and can be achieved by the formula: NPI = 0.2 x Tumour size (cm) + Lymph-node stage (1-3; 1= negative, 2= 1-3 nodes positive, 3= ≥4 nodes positive) + Histological grade (1-3). The index was stratified into three prognostic groups based on the numerical cut off points: Good, Moderate and Poor; the larger the

index the worse the prognosis (Haybittle et al., 1982, Todd et al., 1987). The clinical utility of the NPI system was subsequently confirmed in a large patient cohort of 1629 operable patients up to aged 70 (Galea et al., 1992). Blamey et al. modified the system into six prognostic groups, providing a more detailed evaluation of patients' prognosis (Blamey et al., 2007). The different prognostic systems summarised in Table 1.4.

Table 1.4 Different prognostic groups and predicted prognosis

	Group	Prognosis			
		10-year OS (%)			
TNM[1]	Stage 0	92			
	Stage I	87			
	Stage II	65			
	Stage III	40			
	Stage IV	5			
		OS (%)		Frequency (%)	
		5-year	15-year	5-year	15-year
NPI[2,3,4]	Good (≤3.4)	88	80	27	29
	Moderate (3.41<I≤5.4)	69	42	54	54
	Poor (>5.4)	22	13	19	17
		10-year BCS (%)		Frequency (%)	
		1980-1986	1990-1999	1980-1986	1990-1999
NPI[5]	EPG (2.02-2.4)	88	96	12	15
	GPG(2.41-3.4)	72	93	19	21
	MPGI(3.41-4.4)	61	81	29	28
	MPGII(4.41-5.4)	42	74	24	22
	PPG (5.41-6.4)	15	55	11	10
	VPG(6.41-6.8)	12	38	5	4

Abbreviations: NPI, Nottingham Prognosis Index; OS, overall survival; BCS, breast cancer-specific survival; EPG, Excellent prognostic group; GPG, Good prognostic group; MPG, Moderate prognostic group; PPG, Poor prognostic group; VPG, Very poor group. [1](Kumar et al., 2015), [2](Haybittle et al., 1982), [3](Todd et al., 1987), [4](Galea et al., 1992), and [5](Blamey et al., 2007).

Currently, the next generation of NPI, known as the NPI+, is under development and classifies patients tumours into seven molecular subtypes (luminal A, N, and B, basal p53 altered/normal, HER2/ER- and HER2/ER+); it is combined with several traditional clinicopathologic variables. The NPI+ contains different biological subtypes of breast cancer to give more accurate predictions (Green

et al., 2013, Rakha et al., 2014).

1.1.3.6 ER/PR status and HER2 status

ER and PR have been used to assess patients' benefit from hormone therapy such as tamoxifen. ER+ breast cancer patients given five years of adjuvant tamoxifen have an approximately 50% reduced recurrence rate and approximately 30% decrease in breast cancer mortality (EBCTCG, 2005, EBCTCG, 2011b). The prognostic values of ER and PR are limited and time-dependent. It has been shown that ER- or PR- breast cancer patients have significantly worse prognosis than ER+ or PR+ patients (10-year survival rate is 56% or 55% vs 69% or 68%, respectively). Such significant differences are only observed during the first five years of follow-up, after five years the risk for both types is almost equivalent to each other (Costa et al., 2002).

Human epidermal growth factor receptor 2 (HER-2/neu, ERBB2) belongs to the epidermal growth factor receptor (EGFR) family, amplification and/or overexpression was historically detected in approximately 25-30% of invasive breast cancer, with recent data suggesting a positivity rate closer to 15% (Slamon et al., 1987, Slamon et al., 1989, Lal et al., 2004, Wolff et al., 2013, Mustacchi et al., 2015). HER2 amplification and/or overexpression are associated with more aggressive tumorigenesis and poor prognosis. Earlier studies have shown that HER2 amplification is significantly associated with poor overall and disease-free survival in node-positive patients (Slamon et al., 1987, Slamon et al., 1989). Targeting HER2 is currently a standard treatment for HER2+ breast cancer patients providing survival benefits in both early stage and metastatic patients. Trastuzumab (Herceptin®) is a humanised recombinant anti-HER2 monoclonal antibody of proven clinical benefit in HER2+ breast cancer patients. It is generally given as a 12 month adjuvant treatment following completion of chemotherapy; which results in significant increased overall and disease-free survival (Slamon et al., 2011). There are other promising HER2 targeted agents, such as lapatinib, pertuzumab, and T-DM1, which will be

discussed in section 1.2.

1.1.3.7 Genetic profiling phenotypes

As discussed in section 1.1.2.2, gene expression microarray data has been used to identify distinct molecular subtypes of breast cancer, which yields important prognostic and predictive information. Amongst five core molecular subtypes (luminal A, luminal B, HER2+, basal-like and normal breast-like), the worst overall survival and relapse-free survival were observed in HER2+ and basal-like subtypes (Sørlie et al., 2001, Sørlie et al., 2003, Sotiriou et al., 2003). In addition to its prognostic value, genetic profiling provides some information on predicting response to specific treatments, e.g. HER2+ breast cancer responds to HER2 targeted agents; and hormone therapies are very effective in the luminal breast cancer; whereas basal-like subtype is difficult to target due to the lack of ER, PR and HER2 expression. More details will be discussed in section 1.1.4.3 and 1.2. The characteristics of each intrinsic molecular subtype of breast cancer are shown in Table 1.5.

Table 1.5 Characteristic, prognosis and treatment for five subtypes

	Characteristic[1]	5-year OS (%)[2]	DFS (%)[2]	Recommended treatment[1]
Luminal A	ER+and/or PR+, HER2-, Ki-67 low	90.3	86.8	HT
Luminal B	ER+and/or PR+, HER2-(or HER2+), Ki-67 high	88.7	83.2	HT, CT
HER2+	HER2+ (amplified and/or overexpressed), ER-	78.8	66.0	Anti-HER2, CT
Basal-like	ER-, PR-, HER2- (triple negative)	79.0	73.5	CT
Normal-like	Not relevant	-	-	Not relevant

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor; OS, overall survival; DFS, disease-free survival, HT, hormonal therapy; CT, chemotherapy. [1](Norum et al., 2014), [2](Onitilo et al., 2009).

1.1.3.8 Other factors

Ki-67 proliferation index has been used to evaluate the prognosis of breast cancer patients. Ki-67 is a nuclear protein that is expressed in the G1 through

M phases of the cell cycle, thus the percentage of Ki-67 positive cells can be used as an indicator of cell proliferation (Fitzgibbons et al., 2000). Evidence has shown that patients with *BRCA1* mutations have a significantly worse breast cancer-specific survival than those without *BRCA1* mutations; however such significant differences have not been seen in patients with *BRCA2* mutations. *BRCA1* mutations are associated with breast cancer mortality, in patients who received surgery alone (Robson et al., 2004). There are other factors such as EGFR, p53 and cathepsin-D, etc; most of the markers have shown prognostic values, with conflicting results. However the long-term prognostic role of such factors, and their independent value in multivariate analysis, have not been confirmed, thus they have not been adopted for use in routine clinical practice (reviewed in (Fitzgibbons et al., 2000)). The summary of main prognostic and predictive factors is shown in Table 1.6.

Table 1.6 Summary of main prognostic and predictive factors

	Prognostic	Predictive	Time-dependent
Age at diagnosis	√		
Tumour size	√		√
Axillary lymph node	√		√
Differentiation			
Histological type	√		
Histological grade	√		√
Lymphatic/vascular invasion	√		
ER/PR status	√	√	√
HER2 status	√	√	
Genetic profiling	√	√	

(Warwick et al., 2004, Arriagada et al., 2006, Soerjomataram et al., 2008).

1.1.3.9 Novel multi-gene assays

During recent decades, multi-gene signatures have emerged as useful alternate tools to provide prognostic and predictive information. MammaPrint® (Agendia) is a 70-gene microarray-based assay and was the first gene signature approved by the FDA (U.S. Food and Drug Administration). Currently MammaPrint® is performed, in certain countries and centres, as a prognostic tool for patients of <61 years with node-negative, stage I/II and size <5cm tumours (Weigelt et al.,

2010a). Another 50-gene profiling tool, called Prediction Analysis of Microarray (PAM50), has been recently cleared by the FDA (Toss and Cristofanilli, 2015). PAM50 generates a numerical score, the risk of relapse score (ROR), that can be used to estimate the efficacy of neoadjuvant chemotherapy in node-negative patients (Parker et al., 2009). The 21-gene Oncotype DX® (Genomic Health) was developed using quantitative reserve transcription polymerase chain reaction (qRT-PCR) assay to assess the risk of distant recurrence. It is being increasingly used to predict the outcomes of adjuvant chemotherapy in ER+ and node-negative patients (Toss and Cristofanilli, 2015). Other novel multi-gene assays includes: MapQuant Dx™ (Ipsogen), Breast Cancer Index^{SM} (BCI; BioTheranostics) and IHC4, the comparison and evaluation between such tests are ongoing in many clinical trials, aiming to provide reproducible and accurate prognostic/predictive information (reviewed in (Weigelt et al., 2010a, Toss and Cristofanilli, 2015)).

1.1.4 Treatment strategies

Optimal treatment strategies can limit recurrence, control metastasis, enhance survival status and improve patients' quality of life. Some breast cancer patients require adjuvant therapy after primary surgery to achieve better clinical outcomes. Adjuvant therapy includes radiotherapy and systematic therapy; the latter encompasses hormone therapy, chemotherapy and biological targeted therapy.

1.1.4.1 Surgery

The main aim of surgery is to completely remove the primary tumour with local excision of lymph nodes, to minimize local/distant recurrence. Surgery is the first treatment option for operable breast cancer patients, and includes mastectomy and breast conserving surgery (BCS). BCS refers to the local removal of the primary tumour and regional lymph nodes, which is more frequently applied than mastectomy (Association of Breast Surgery at BASO, 2009). It has been shown that the long term survival of patients who received

BCS is equivalent to that in patients who received mastectomy; though, BCS appeared to significantly increase the local recurrence compared to mastectomy (Veronesi et al., 2002). Breast conserving therapy (BCT) includes BCS and adjuvant radiotherapy. Radiotherapy has been shown to significantly decrease 10-year risk of recurrence and 15-year risk of breast cancer mortality from 35.0% to 19.3% and 25.2% to 21.4%, respectively (EBCTCG, 2011a). For the patients who had mastectomy as treatment, the possibility of breast reconstruction is often discussed and is performed during the primary surgery when possible (Association of Breast Surgery at BASO, 2009, Yarnold, 2009b).

1.1.4.2 Radiotherapy

Radiotherapy is given as adjuvant treatment following surgery to limit the risk of local recurrence. Internationally, radiotherapy is delivered to a total dose of 50 Gy, in 25 fractions of 2 Gy given over a 33 day period. In the UK, hypofractionated radiotherapy has been extensively used for early invasive breast cancer patients, with higher doses per fraction with the advantage of lower total doses and shorter overall treatment time -- 40 Gy in 15 fractions over 3 weeks (Yarnold, 2009c). A long term follow-up study has confirmed that hypofractionated radiotherapy can result in fewer complications (i.e. breast shrinkage, telangiectasia, and breast oedema) compared with the 50 Gy regimen; and the loco-regional recurrence rate did not differ between those two regimens (Haviland et al., 2013). As mentioned above (in section 1.1.4.1), radiotherapy after breast conserving surgery has been shown to significantly reduce the risks of recurrence and breast cancer mortality (EBCTCG, 2011a). Recently, a novel radiotherapy technique, called targeted intraoperative radiotherapy (TARGIT), has been introduced. It delivers single dose of radiation directly to the tumour bed; the process normally finishes within 20-45min during the operation. TARGIT has been shown to significantly reduce non-breast-cancer mortality; and it does not cause any difference in breast cancer mortality or overall mortality compared with external beam radiotherapy (EBRT). However, the 5-year risk of local recurrence in TARGIT group was

significantly higher than that in the whole breast radiotherapy group (3.3% vs 1.3%) (Vaidya et al., 2014). TARGIT trials have been conducted in particular subsets of patients (i.e. women aged 45 and older). It remains to be seen if it is as effective in other groups (i.e. high-risk); a TARGIT-B superiority trial (TARGIT boost vs EBRT boost; clinicaltrials.gov identifier: NCT01792726) was started in 2013 aiming to assess its efficacy in women with high risk of local recurrence. As only 1222 (35%) patients reached a median follow up of 5 years, the long term effect of TARGIT also remains to be assessed before being applied as standard procedure.

1.1.4.3 Hormone/endocrine therapy

Breast cancer patients whose tumours are ER and/or PR expression positive are considered to be potentially responsive to hormone therapy. The ways to interfere with oestrogen levels include ovarian ablation/suppression, the use of selective oestrogen-receptor modulators (SERMs) (e.g. tamoxifen) and aromatase inhibitors (AIs, e.g. letrozole) (Puhalla et al., 2012). The selection of treatment(s) is related with menopausal status. Generally, tamoxifen is recommended for premenopausal patients, whilst AIs are favoured for postmenopausal patients. Tamoxifen acts as an antagonist of oestrogen by blocking oestrogen receptors in the tumour cells (Lewis and Jordan, 2005). As mentioned in section 1.1.3.6, ER+ breast cancer patients have a reduced recurrence rate and breast cancer mortality rate after five years of adjuvant tamoxifen. AIs can prevent other hormones from transforming into oestrogens by inhibiting aromatase enzymes, leading to subsequent decreased level of oestrogen. As the ovaries are the main organ producers of oestrogen, AIs can function in women with dysfunctional ovaries (e.g. postmenopausal patients, and ovarian ablation/suppression). Evidence has demonstrated that the application of AIs either before or after using tamoxifen can cause reduction in recurrence risk (Puhalla et al., 2012). Recently hormone therapy has been used in the neoadjuvant setting for ER+ patients who have an inoperable tumour and/or are not suitable for surgery, with the aim of improving the probability

being able to conduct an operation. The objective response (OR) of neoadjuvant hormone therapy is approximately 40%, and its high tolerability makes it an attractive option for postmenopausal patients with ER+ cancers (Smith et al., 2005, Holmes et al., 2015).

1.1.4.4 Chemotherapy and targeted therapy

As one of the most chemosensitive types of solid tumour, single-agent and polychemotherapy have been applied in treating breast cancer, aiming to reduce recurrence, control micrometastases and extend patients' survival (Hassan et al., 2010). Conventional cytotoxic anticancer agents include alkylating agents, platinum compounds, antimetabolites, anthracyclines, and tubulin-binding drugs. Another type of cytotoxic agent is tyrosine kinase inhibitors; several small molecular kinase inhibitors have shown activity in different types of tumours, such as lapatinib in breast cancer and erlotinib in non-small cell lung cancer (Lind, 2011).

Generally, in conventional treatment, polychemotherapy regimens are extensively used for routine treatment of both early stage and advanced breast cancer; examples include CMF (cyclophosphamide, methotrexate and 5-FU), FEC (5-FU, epirubicin and cyclophosphamide), AC (doxorubicin (Adriamycin®) and cyclophosphamide), E-CMF (epirubicin, followed by CMF), TAC (docetaxel (Taxotere®), doxorubicin and cyclophosphamide) (reviewed in (Hassan et al., 2010)). Patients with locally advanced breast tumours, or inflammatory tumours, are also given neoadjuvant or preoperative chemotherapy. Chemotherapy is also used together with targeted therapy; details are discussed below, in section 1.2.

Although platinum agents have not traditionally been used to treat breast cancer there are recent studies suggest that they may be highly effective in particular cases i.e. *BRCA1/2* mutation associated breast tumour and TNBC. In a neoadjuvant trial, patients were received gemcitabine (G) and carboplatin (C)

plus iniparib, the overall pathologic complete response (pCR) was 39%. And patients with TNBC and *BRCA1/2* mutation achieved a higher pCR rate (56%) than patients with wild-type *BRCA1/2* (33%) (Telli et al., 2013).

Another approach, still under active investigation, is the concept of synthetic lethality –mutation in either gene alone leads to viability but simultaneous mutation in both genes causes death. Targeting the genes that are synthetic lethal to such mutations can kill only cancer cells without having effects on normal cells (with intact gene function) (reviewed in (Kaelin, 2005)). An example is the use of poly (ADP-ribose) polymerase (PARP) inhibitors in *BRCA*-mutation tumours. *BRCA*s are involved in DSB repair, and PARP1 is an enzyme involved in SSB repair (Lord et al., 2015). The use of higher dose of Olaparib (a novel PARP inhibitor) achieved an objective response rate at 41% (11 of 27) in women with *BRCA*1 or 2 mutation and advanced breast tumours, with low grades toxicities (Tutt et al., 2010).

A growing number of molecular targets have been identified in breast cancer and several targeting agents are available or currently under investigation. Examples include agents that target HER1 (EGFR), HER2, HER3, HER4 and insulin-like growth factor 1 receptor (IGF-1R); agents target intracellular signalling pathways (PI3K/AKT/mTOR); anti-angiogenic agents (vascular endothelial growth factor, VEGF) and agents that target cell cycle regulation and heat shock protein HSP90. Recently, as mentioned above, the effect of poly-ADP-ribose polymerase (PARP) inhibitors has been investigated in *BRCA1/2* mutation breast cancer and TNBC (reviewed in (Mohamed et al., 2013)). Amongst these, targeting the EGFR family has shown the greatest effect and is widely used in HER2+ breast cancer treatment.

1.2 HER2 targeted therapy in breast cancer

1.2.1 Mechanisms of action

HER2 belongs to the human epidermal growth factor receptor (EGFR) family, it

has three homologous receptors: HER1 (EGFR or *ERBB1*), HER3 and HER4, which are all tyrosine kinase (TK) receptors. Structurally, they have three domains: the intracellular TK domain, transmembrane domain and extracellular ligand-binding domain. HER1, HER3 and HER4 are activated by receptor-specific ligand binding at the cell membrane, followed by homo-/heterodimerization with other family members. HER2 does not bind with any ligands, but when it is overexpressed in breast tumour cells, it becomes a favoured co-receptor to form heterodimers with other family members, or forms homodimers with itself. Dimerization leads to further activation of TKs, facilitating phosphorylation cascades to trigger downstream signalling pathways. Consequently, activated downstream signals disrupt the expression of several genes which can accelerate tumorigenesis via promoting tumour cell proliferation, survival and invasion (Figure 1.1) (Hudis, 2007, Rimawi et al., 2015). Exceptionally, although HER3 has a defective TK, HER2/HER3 heterodimerization function can be effectively attenuated by PI3K inhibitors, indicating its involvement in the PI3K pathway (Junttila et al., 2009).

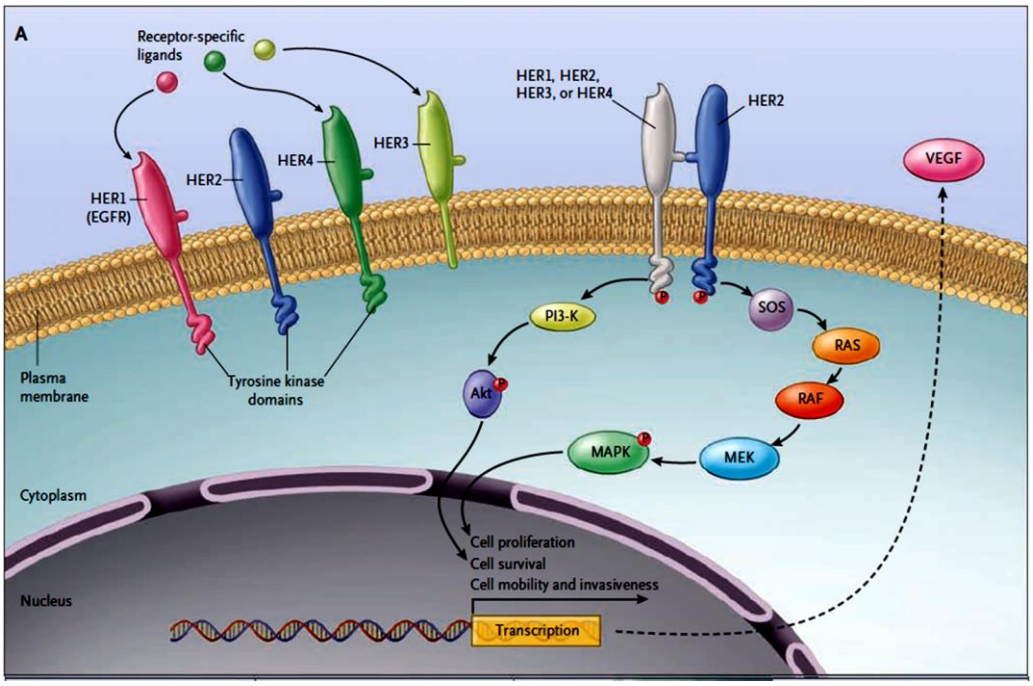


Figure 1.1 Signal transduction by the HER family and potential mechanisms of action of trastuzumab. Reproduced with permission from (Hudis, 2007) Copyright © Massachusetts Medical Society.

There are two classes of targeted drugs used in treating breast cancer: monoclonal antibodies and small molecular inhibitors. Monoclonal antibodies usually bind to the receptors to inhibit ligand-binding or dimerization; this enables recruitment of immune effectors (i.e. natural killers, NKs) or stimulates an antibody-dependent cell-mediated cytotoxicity (ADCC) effect. Small molecules can effectively supress kinase activation of the protein (reviewed in ((Pérez-Garcia et al., 2014)). So far the FDA has approved several HER2-targeted agents for HER2+ breast cancer, including monoclonal antibodies trastuzumab (Herceptin®) and pertuzumab (Perjeta®), the TK inhibitor lapatinib ditosylate (Tykerb®), and a trastuzumab drug conjugate Ado-Trastuzumab emtansine (T-DM1, Kadcyla™). Some of the drugs are combined with adjuvant or neoadjuvant chemotherapy depending on the different clinical criteria.

1.2.2 Approved HER2 targeted therapies

1.2.2.1 Trastuzumab

Although ADCC may be an important component to the efficacy of trastuzumab direct cellular effects, several mechanisms disrupting downstream signalling, are also very important (Figure 1.2).

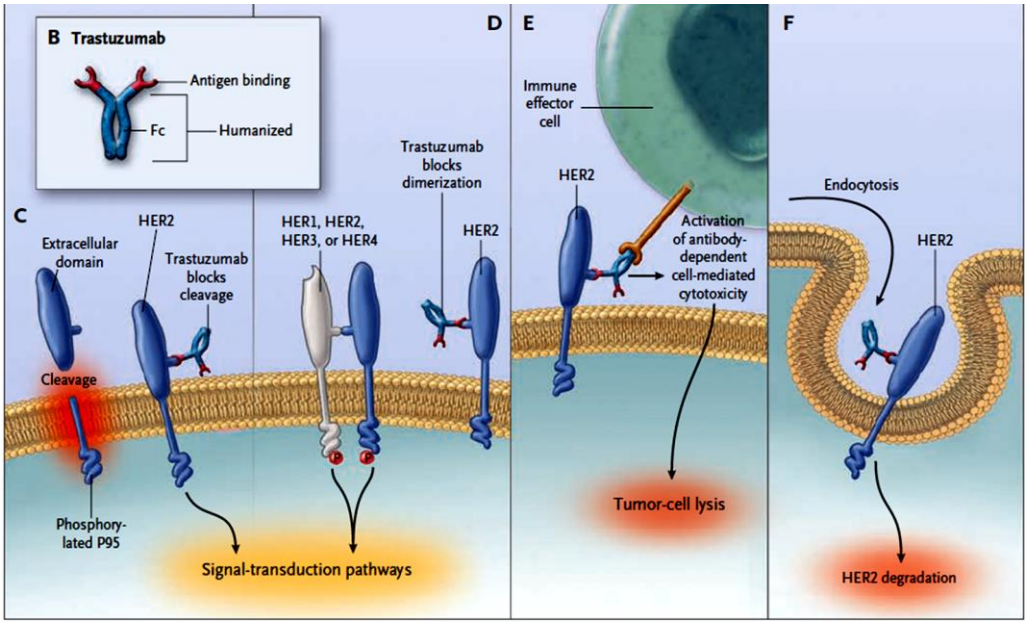


Figure 1.2 Signal transduction by the HER family and potential mechanisms of action of trastuzumab. Reproduced with permission from (Hudis, 2007) Copyright © Massachusetts Medical Society.

1) p95HER2 is a truncated active form of HER2, cleavage of the extracellular domain of HER2 leads to the exposure of p95. Binding of trastuzumab to HER2 may reduce cleavage of the extracellular domain, which blocks p95-mediated signal transduction; 2) trastuzumab may directly disrupt HER2 homo- or heterodimerization, thereby interrupting activation of downstream signalling; 3) trastuzumab may cause tumour cell death via recruiting immune effectors or initiating ADCC; 4) and trastuzumab may result in degradation of HER2 itself through endocytosis. The possible mechanisms of trastuzumab can be summarised as: reducing shedding of the extracellular domain, inhibiting receptors homo-/heterodimerization, inducing immune activity and causing endocytic degradation of the HER2 (Hudis, 2007). In addition, by using a novel quantitative assay, a study has demonstrated that the quantitative value of p95 expression is significantly associated with progression-free survival (PFS) and overall survival (OS) in HER2+ breast cancer patients treated with trastuzumab, implying p95 may be a useful biomarker for assessing trastuzumab response (Duchnowska et al., 2014). Such a conclusion may, however, be premature as certain studies suggest no prognostic/predictive significance with respect to p95 expression. In another immunohistochemical study (n=38), the p95-HER2 expression was not associated with overall survival, progression-free survival and response to trastuzumab (Kocar et al., 2014).

Trastuzumab is the first targeted agent for treating HER2+ metastatic breast cancer (MBC) and was originally approved for use in combination with first-line chemotherapy. A study of trastuzumab in early stage HER2+ breast cancer reported a significant reduction in the first disease event (disease-free survival) and risk of death in the trastuzumab group compared with the control group, which led to the FDA approval of trastuzumab in combination with adjuvant chemotherapy for treating operable HER2+ breast cancer patients (Romond et al., 2005). The efficacy of trastuzumab has also been assessed in the neoadjuvant setting. The addition of trastuzumab to anthracycline/taxane based neoadjuvant chemotherapy has shown benefit in terms of pathologic

complete response (31.7% vs 15.7%; vs control group) (Untch et al., 2010). Despite trastuzumab showing impressive effects on improving HER2+ breast cancer patient outcome in the both adjuvant and neoadjuvant settings, some patients still show resistance to therapy. One study demonstrated that the proportion of patients surviving free of disease was 93.9% at year 1, 85.8% at year 2, 79.4% at year 3, and 75.0% at year 4 (Bonifazi et al., 2014). The mechanisms of such trastuzumab resistance, inherent or acquired, remain unclear; more will be discussed in Chapter 4. Therefore, several attempts have been made to identify biomarkers to assess those patients that may not respond to trastuzumab. Previous results from our group indicate that determining the expression of the protease calpain-1 may be of benefit in this regard (Storr et al., 2011c).

1.2.2.2 Lapatinib

Lapatinib (GW2016) was designed as a dual tyrosine kinase inhibitor (TKI) of HER1 and HER2. Unlike trastuzumab, which binds to the extracellular domain of HER2, lapatinib binds to the intracellular domains of HER1 and HER2 to inhibit receptor phosphorylation and activation of downstream signalling. An earlier clinical study demonstrated the benefits of lapatinib in combination with capecitabine in HER2+ metastatic breast cancer patients whose disease progressed after adjuvant trastuzumab, which led to the FDA approval of lapatinib for HER2+ metastatic breast cancer (Geyer et al., 2006). The Adjuvant Lapatinib and/or Trastuzumab Treatment (ALTTO) study has reported that in the adjuvant setting that, using either lapatinib plus trastuzumab regimen or lapatinib followed by trastuzumab regimen, both regimens failed to show superior benefits on improving disease-free survival compared to trastuzumab monotherapy (Piccart-Gebhart et al., 2014).

1.2.2.3 Pertuzumab

Aiming to overcome trastuzumab resistance and improve survival, another monoclonal antibody, pertuzumab, has been developed and recently approved

for the treatment of HER2+ metastatic breast cancer. Pertuzumab is also directed against the extracellular domain of HER2, but an epitope of domain II, which is different from the binding of trastuzumab in domain IV. It can prevent HER2 from dimerizing with other family members, thereby blocking activation of downstream signalling (Franklin et al., 2004). Promising results from clinical trials have shown that the addition of pertuzumab to trastuzumab and docetaxel leads to significant improved progression free survival and overall survival, and similar results were observed with extended follow-up (median follow-up of 50 months) (Baselga et al., 2012b, Swain et al., 2015). Therefore, dual HER2 blockade of pertuzumab and trastuzumab can result in an enhanced anti-tumour activity that cannot be achieved by pertuzumab or trastuzumab alone.

1.2.2.4 Ado-trastuzumab emtansine

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate consisting of the humanised monoclonal antibody trastuzumab and a microtubule inhibitor emtansine (a maytansinoid derivative). Trastuzumab links with DM1 through a non-reducible linker (MCC) that allows the conjugate to be released intracellularly after binding to HER2, with increased effect and reduced toxicity. Studies have demonstrated that T-DM1 maintains all the active mechanisms of the unconjugated trastuzumab; but also provides enhanced anti-tumour activity *in vitro* and *in vivo* (Junttila et al., 2011). A clinical study of 991 HER2+ metastatic breast cancer patients has shown that the use of T-DM1 can add an extra 3.2 months to the median progression free survival and 5.8 months to the median overall survival compared with those who received lapatinib and capecitabine (Verma et al., 2012). The superiority of T-DM1 has been proved by comparing with other HER2 targeted regimens; it has been shown that T-DM1 can significantly improve progression free survival, overall survival and objective response compared with other HER2 targeted therapies (Krop et al., 2014). The promising anti-tumour effects of T-DM1, and good tolerance of the drug, led to FDA approval of T-DM1 in HER2+ metastatic breast cancer patients

who had been treated with trastuzumab and taxane to provide additional treatment options for patients.

1.2.3 Other targeted agents

Neratinib (HKI-272) is an irreversible, small molecule, orally administrated TKI with activities against HER1, HER2 and HER4. It binds to the intracellular domain of the receptors to disrupt auto-phosphorylation. A recent phase III trial of HER2+ early breast cancer patients shows that the addition of neratinib to trastuzumab can improve invasive disease-free survival (DFS, IDFS, DFS+DCIS) in patients at year 2 after the treatments. Further three year follow-up is currently underway (Chan et al., 2015). Another small molecule TKI, afatinib (BIBW-2992), is a dual irreversible blocker of HER1 and HER2, inhibiting all signalling from homo- or heterodimers formed by other family members. The efficacy of the drug has been evaluated in HER2+ metastatic breast cancer patients whose disease progressed after trastuzumab treatment. Results showed that patients given afatinib once-daily can reach a median progression-free survival at 15.1 weeks and median overall survival at 61.0 weeks (Lin et al., 2012). Clinical trial results of novel TKIs are very promising and a number of trials are underway to assess their effect in treating HER2+ patients who experienced failure to trastuzumab treatment.

Additional novel agents are under development. Amongst these, MM-111 is a novel bio-specific monoclonal antibody directed against both HER2 and HER3. It contains two different monoclonal antibody fragments thus can bind to two types of the antigen. Mammalian target of rapamycin (mTOR) plays a role in mediating cell growth and proliferation by regulating activation of the PI3K/AKT signalling pathway. Given the fact that the PI3K/AKT signalling pathway is tightly associated with HER2 family signalling, inhibitors of mTOR have been investigated as potential anti-tumour agents in HER2+ breast cancer (O'Sullivan and Smith, 2014). Heat shock protein 90 (HSP90) is a chaperone protein that interacts with over 200 proteins, the latter includes apoptotic factors, protein

kinases, transcription factors and many other proteins involved in pathways that are dysregulated in cancer cells (e.g. EGFR family). Several studies are in progress to assess the potential of HSP90 inhibitors in combination with other anti-HER2 agents and regimens (reviewed in (Lu et al., 2012)). A recent clinical trial assessed the efficacy of HSP90 inhibitor AUY922 in EGFR-mutant lung cancer patients, the partial response rate was 16% (4 of 25 patients), however this was limited by the drug toxicities—e.g causing diarrhea, hyperglycemia, and night blindness (Johnson et al., 2015). Therefore the efficacy and long term use of HSP90 inhibitors still needs to be confirmed. Examples of approved and novel HER2 targeted agents are summarised in Table 1.7.

Table 1.7 Important currently approved and novel HER2 targeted agents in HER2+ breast cancer

	Agents	Molecular targets[1]	Mechanisms of action[2]	FDA approval[1,2]
Trastuzumab	mAb	HER2 extracellular domain	Inhibits dimerization	HER2+ EBC and MBC(adjuvant)
Lapatinib	Dual TKI	HER1&HER2 intracellular TK domain	Prevents receptor phosphorylation	HER2+ MBC
Pertuzumab	mAb	HER2 extracellular domain	Inhibits dimerization	HER2+ MBC (adjuvant) HER2+ EBC (neoadjuvant)
T-DM1	Drug conjugate	HER2 extracellular domain	Delivers cytotoxic drug to HER2+cells	HER2+ MBC
Neratinib	TKI	HER1,HER2&HER4 intracellular TK domain	Prevents auto-phosphorylation	Not FDA approved
Afatinib	Dual TKI	HER1&HER2 intracellular TK domain	Inhibits kinase activity	Not FDA approved
MM-111	mAb	HER2&HER3 extracellular domain	Inhibits dimerization	Not FDA approved
AUY922	HSP90 inhibitor	HSP90	Inhibits signal transduction	Not FDA approved
Everolimus	mTOR inhibitor	PI3K/AKT/mTOR pathway	Inhibits signal transduction	Not FDA approved

Abbreviations: mAb, monoclonal antibody; HER1, human epidermal growth factor receptor-1; HER2, human epidermal growth factor receptor-2; HER3, human epidermal growth factor receptor-3; HER-4, human epidermal growth factor receptor-4; EBC, early-stage breast cancer; MBC, metastatic breast cancer; BC, breast cancer; TKI, tyrosine kinase inhibitor;; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; T-DM1, ado-trastuzumab emtansine; FDA, Food and Drug Administration. [1](Awada et al., 2012), [2](O'Sullivan and Smith, 2014)

1.3 Calpains and calpastatin

As mentioned in section 1.2.2.1, studies from our group have suggested that expression of calpain, a proteolytic enzyme, may be involved in trastuzumab response (Storr et al., 2011c). Calpains, and their endogenous inhibitor calpastatin, may, as indicated below, be involved in a number of important processes in relation to cancer progression and treatment response, with studies having been conducted in numerous tumour types including breast cancer (Zhang et al., 2013, Cai et al., 2014, Dai et al., 2014, Zheng et al., 2014).

1.3.1 Biology of the calpain system

1.3.1.1 Nomenclature and structures

The calpain family is a group of calcium-activated proteolytic intracellular cysteine proteases (EC 3.4.22.17 Clan CA, family C02), expressed in a wide range of cells and tissues (Goll et al., 2003). A neutral proteinase extracted from rat brain, in 1964, was first described by Guroff (Guroff, 1964) and was later also extracted from chicken skeletal muscle, purified to a homogenous state and named as calcium-activated neutral protease (CANP) (Ishiura et al., 1978). The cDNA for calpain was cloned by Ohno et al., in 1984, revealing the complete structure of the calpain catalytic subunit; and clarifying the functions of two important domains DII and DIV (Ohno et al., 1984). Subsequent cDNA and genomic cloning studies led to the identification of several calpain-related molecules, including its endogenous inhibitor calpastatin. Calpains can be named based on the corresponding gene products, e.g. CAPN1, CAPN2 and CAPNS1 (Goll et al., 2003, Sorimachi et al., 2010, Ono and Sorimachi, 2012). At the FASEB (Federation of American Societies for Experimental Biology) conference in 2001, a numerical calpain nomenclature was proposed: calpain-1 (large subunit encoded by CAPN1), calpain-2 (large subunit encoded by CAPN2), calpain-3 (encoded by CAPN3), calpain-4 (small subunit encoded by CAPNS1), etc. μ-calpain and m-calpain are used to denote conventional heterodimer of calpain-1 and small subunit, and heterodimer of calpain-2 and small subunit, respectively.

There are at least 15 calpain isoforms that have been identified; two of the most extensively studied, and ubiquitously expressed, isoforms are micro (μ)-calpain and milli (m)-calpain; as well as their only known endogenous inhibitor, calpastatin (Goll et al., 2003). Human μ- and m-calpain were first described according to the level of calcium ions required for their activation *in vitro* (Dayton et al., 1981). Both μ- and m-calpain are heterodimers, sharing a similar 28-kDa regulatory subunit (also called calpain-4, encoded by CAPNS1); whilst the 80-kDa catalytic subunits are distinct: calpain-1 is encoded by CAPN1 and calpain-2 is encoded by CAPN2, respectively (Goll et al., 2003, Storr et al., 2011a, Ono and Sorimachi, 2012). The catalytic subunit and regulatory subunit can be divided into four domains (DI to DIV) and two domains (DV to DVI), respectively (Figure 1.3).

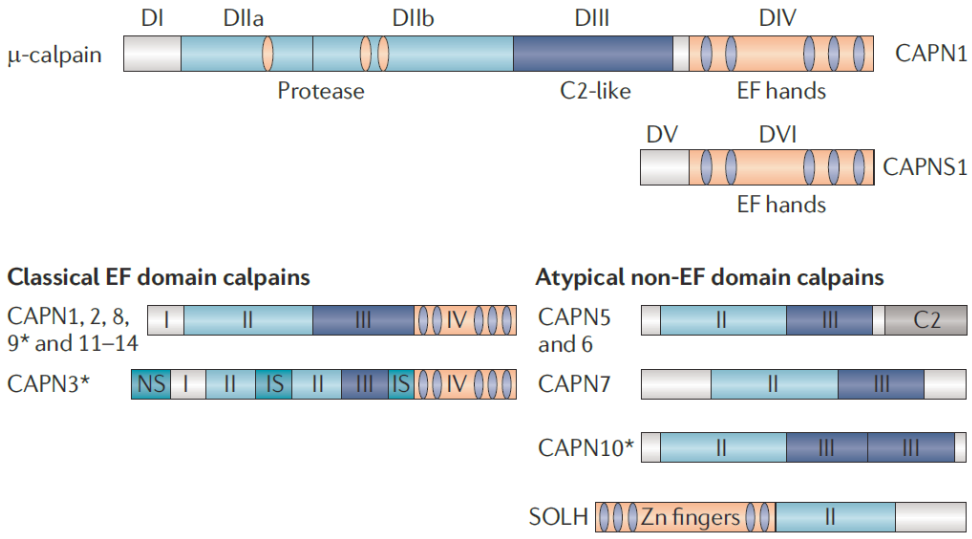


Figure 1.3 Schematic structure of μ-calpain heterodimer and calpain family members. Reproduced from (Storr et al., 2011a) , Nature Reviews Cancer, 11, 364-374; Copyright© 2011 by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

DI is autolysed when activated by Ca^{2+}, occurring far away from the catalytic site; and DII contains two core subdomains IIa and IIb, in the absence of Ca^{2+} the two subdomains can dissociate from each other. It is a highly conserved domain in the calpain family, underlying an important role in regulating protein function. DIII is characterised by a C2-like (C2L) domain and is involved in structural

conformation when interacting with calpastatin. Both DIV and the homologous DVI contains five EF-hands (penta-EF hand/PEF) - these are the domains that are involved in calcium binding; a fifth EF-hand interacts with CAPNS1 and CAPN2 and is responsible for the heterodimeric structure (Sorimachi et al., 2010, Storr et al., 2011a).

Some studies refer to μ-calpain and m-calpain as "conventional calpains", with other calpains being referred to as "unconventional calpains". Based on the structural characteristics, the calpain family can be further subdivided into two groups: typical/classical and atypical/non-classical. As shown in Figure 1.3 the "typical" calpains have DII, III and DIV, which is named by showing the similarity to the calpain catalytic subunits. Nine out of fifteen calpains are typical calpains: calpain-1 to -3,-8,-9, and -11 to-14. The atypical calpains consist of molecules that have irregular C2L domain and missing EF-hand sequences in DIV, which are considered to have mechanisms of action different from typical calpains. The atypical calpains include calpain-5 to -7 and -10, as well as SOLH (small optilobe-homology) and PalB subfamilies (Goll et al., 2003, Sorimachi et al., 2010). In addition to the structural characteristics, calpain can be grouped depending on their specific tissue expression. Of the 15 calpain members, six are expressed in a tissue-specific fashion; the remaining nine are ubiquitously expressed. The tissue specific calpains are calpain-3 (skeletal muscle), -6 (placenta and embryonic muscle), -8 and-9 (gastrointestinal system), -11 (testis) and -12 (hair follicles) (Sorimachi et al., 2010). Care should, however, be taken when considering tissue specificity as although certain calpains are preferentially expressed in certain tissues they may also be expressed in other tissues, albeit at much lower levels, or in different pathological conditions (will be discussed in section 1.3.1.3).

1.3.1.2 Activation and regulation mechanisms

The calpains can be activated by the presence of calcium and extracellular signal-regulated kinase (ERK) through growth factor signalling; whilst its activity

can be regulated by autolytic cleavage, interaction with phospholipids, phosphorylation, and more importantly, its endogenous inhibitor calpastatin. In tumour cells, calpain activity is also correlated with hypoxia and autophagy, and is discussed in subsequent sections.

The molecular mechanism of calpain activation involves two stages. Binding of Ca^{2+} leads to conformational changes in DIII, DIV and VI; followed by binding of Ca^{2+} to the protease domains (IIa and IIb), leading to rearrangement of the active site cleft (Moldoveanu et al., 2002). Evidence has shown that autolytic cleavage of the N-terminal anchor peptide, followed by dissociation of the small subunit from the large subunit, can significantly reduce Ca^{2+} requirement for activation (Nakagawa et al., 2001). In addition to Ca^{2+} regulation, calpain activity is regulated *in vivo* by the endogenous inhibitor calpastatin. Calpastatin consists of a non-inhibitory L-domain and four 15-kDa repetitive inhibitory domains. Due to its "intrinsically unstructured" nature, calpastatin can bind with up to four calpain heterodimers independently. The inhibitory domains contain three regions (A-C). In the presence of Ca^{2+}, a shift of EF-hands leads to exposure of hydrophobic areas, which allows region A and C to bind with DIV and DVI (PEF-hand) on calpains, respectively. Region B binds to DIII of calpain and blocks the active site cleft only in the presence of calcium (Wendt et al., 2004, Hanna et al., 2008). The presence of DIII to DVI is essential for the regulatory effects of calpastatin; calpain isoforms that do not have these domains can escape inhibition by calpastatin, thus calpastatin inhibits only dimeric calpains (e.g. μ- and m-calpain) (Moldoveanu et al., 2002). The structural overview of calpastatin bound to m-calpain is shown in Figure 1.4 (Moldoveanu et al., 2008).

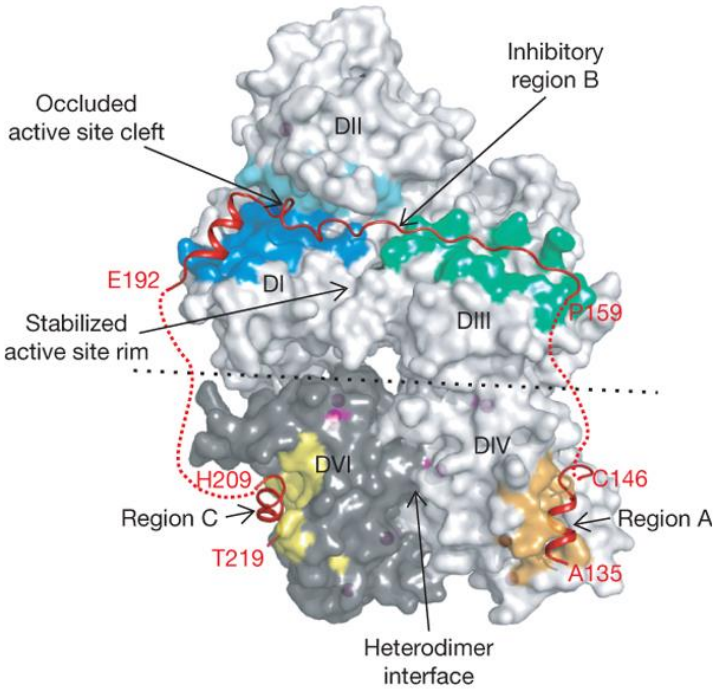


Figure 1.4 Complex between Ca^{2+}-bound m-calpain and calpastatin. Reproduced from (Moldoveanu et al., 2008), Nature, 456, 404-408; Copyright© 2008 by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

The intracellular localisation of calpastatin is altered during calpain activation, which regulates interaction between calpastatin and calpains. An early study has shown that calpastatin, unlike calpains that are dispersed in cytosol, accumulates close to nuclear invaginations, forming granule-like structures. Following an increase in Ca^{2+}, calpastatin was redistributed into the cytosol (Tullio et al., 1999). Additionally, *in vivo* data showed that the cellular localisation of calpastatin, as well as the level of soluble calpastatin protein during calpain activation, was directly regulated by protein kinase A (PKA) phosphorylation and phosphoprotein phosphatase (PPase)-dephosphorylation. *In vitro* data show that the phosphorylation of calpastatin is regulated by both PKA and protein kinase C (PKC) (Averna et al., 2001)

The calcium requirement for calpain activation can be altered through interaction with phospholipids; the latter being important components of the plasma membrane. Calpain has been shown to interact with phospholipids at the plasma membrane, which allows activation at a lower Ca^{2+} concentration.

Among three phospholipids, phosphatidylinositol-4, 5-bisphosphate (PIP_2) has the highest level of interactivity for calpains, followed by phosphatidylinositol-4-monophosphate (PIP), then phosphatidylinositol (PI) (Saido et al., 1992).

Phosphorylation is another important way to regulate calpain activity. Evidence has shown that m-calpain is directly activated and phosphorylated by ERK in response to growth factor signalling; further regulating growth factor related cell motility and adhesion (Glading et al., 2004). Moreover, m-calpain is restricted by PKA phosphorylation at a specific serine site in DIII, the phosphorylation can limit domain movement and retain m-calpain in a "frozen" state (Shiraha et al., 2002). Another study, however, suggests that phosphorylation of ERK or PKA has no direct regulatory effect on m-calpain; in fact, its activity is modulated directly by redistribution of m-calpain to the plasma membrane and binding to PIP_2. In this context, ERK phosphorylation of m-calpain promotes its binding to PIP_2 at the membrane; whilst PKA phosphorylation of m-calpain inhibits its binding to PIP_2 and keeps m-calpain away from the plasma membrane, thus leading to negative control of the activity (Leloup et al., 2010). Protein kinase Cι (PKCι) is also involved in the phosphorylation of both μ- and m-calpain, and is related to nicotine-induced cell migration and invasion in lung cancer cells (Xu and Deng, 2006).

In all, the activity of calpains in cells is strictly regulated by several mechanisms; including adjusting Ca^{2+} requirement for the activation, autolytic cleavage, phosphorylation, interaction with substrates (e.g. phospholipids, protein kinases), calpastatin interactions and both calpain and calpastatin intracellular location (as discussed above).

1.3.1.3 Calpains in health and disease

Aberrant calpain activity, or gene mutations in calpain family members, can lead to a number of diseases/pathological disorders, as well as tumorigenesis. For

example, the interruption of calcium homeostasis leads to calpain dysregulation, which results in both acute neurodegenerative disorders and chronic neurodegenerative disease (e.g. Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington disease (HD)) (reviewed in (Vosler et al., 2008)). Increased calpain-cleaved alpha-II-spectrin and its breakdown products were found in the cerebrospinal fluid (CSF) of traumatic brain injury (TBI) patients, compared to patients with other central nervous system (CNS)-disorders (Farkas et al., 2005). Recently, AD patients have shown increased level of calpain activity in CSF; and combining calpain activity in CSF with other traditional CSF biomarkers (Ab1–42, h-tau, and p-tau181) might provide prognostic values in AD patients (Laske et al., 2015). In terms of other calpain family members, mutations in CAPN3 (also called p94, encoding calpain-3) have been found to cause skeletal muscle disorder limb-girdle muscular dystrophy type 2A disease (LGMD2A), which is also the most extensively studied human calpainopathy (Richard et al., 1995, Ono et al., 2015). Studies suggested that polymorphisms in CAPN10 (encoding calpain-10) could affect susceptibility to type 2 diabetes (Horikawa et al., 2000). Calpains have also been found to be associated with a wide range of other disease states, including cataracts, multiple sclerosis, polycystic ovary syndrome, ageing, cardiac disease and pulmonary hypertension (reviewed in (Zatz and Starling, 2005, Chakraborti et al., 2012)). As discussed in section 1.3.1.2, both μ- and m-calpain are tightly regulated by their endogenous inhibitor calpastatin, thus aberrant calpain activity sometimes comes along with dysfunction of calpastatin, which will be discussed below.

Interestingly, μ- and m-calpain has been shown to play important roles in progenitor function. Results from transgenic mice have demonstrated fundamental roles for calpains during embryonic development. One early study showed that heterozygous CAPNS1 $^{+/-}$ mice are viable and fertile, but that homozygous *CAPNS1* $^{-/-}$embryos die in midgestation. Knocking out *CAPNS1* abolishes both μ- and m-calpain leading to downregulation of both heterodimers, suggesting the presence of *CAPNS1* is a prerequisite for

functional calpains in embryonic development (Arthur et al., 2000). The same group has been able to further clarify this result by showing that *CAPN2*$^{-/-}$ embryos die at the preimplantation stage, confirming that functional m-calpain is essential for embryonic development (Dutt et al., 2006). Although *CAPN1*$^{-/-}$ mice are viable and fertile, they present with impaired platelet function, suggesting normal platelet function is dependent on μ-calpain (Azam et al., 2001). Collectively, knocking out *CAPNS1* or *CAPN2* in mice can result in embryonic lethality; whilst knocking out *CAPN1* in mice causes platelet damage. Despite μ- and m-calpain sharing similarities *in vitro*, results suggest substantially different roles *in vivo*; but that m-calpain may be able to compensate for the loss of μ-calpain, at least during embryogenesis (Dutt et al., 2006). Calpastatin studies are much less common but a recent study detected CAST mutation in four individuals from three families of different ethnicities, and reported that the mutations in *CAST* gene that leads to loss of function were responsible for a rare specific type of peeling skin syndrome (PSS) (Lin et al., 2015).

Most investigations into the role of the calpain system in disease pathology have used murine models, with studies about the relationship between calpains and calpastatin in human disease being very limited. In a recent study, increased expression/activity of μ-calpain along with varying CAST gene expression was observed in high risk childhood acute lymphoblastic leukaemia (ALL) blast samples; and the abundant amount/activity of μ-calpain was associated with a lower level of spontaneous apoptosis in those patients (Mikosik et al., 2015). Another study noticed a significant negative association between calpain activity and calpastatin levels in acute myelogenous leukaemia (AML) blast samples, determining patients with lower calpain activity were more likely to have complete remission (Niapour et al., 2012). Our group initially became interested in the calpain system having shown that low levels of calpastatin mRNA and protein expression are associated with the presence of LVI in invasive breast cancer (Storr et al., 2011b, Storr et al., 2012a).

Although the number of studies is somewhat limited, aberrant calpain expression/activity has been found in numerous human cancers, though results vary between different types of tumours. Increased calpain-1 mRNA expression was observed in renal cell carcinomas, and has been shown to associate with advanced lymph node status in patients (Braun et al., 1999). Overexpression of m-calpain has been found in colorectal adenocarcinoma (Lakshmikuttyamma et al., 2004). Calpain-4 expression was elevated in nasopharyngeal carcinoma (NPC), intrahepatic cholangiocarcinoma (ICC), glioma and hepatocellular carcinoma (HCC); and was associated with aggressive tumour behaviour and poor prognosis in patients (Zhang et al., 2013, Cai et al., 2014, Dai et al., 2014, Zheng et al., 2014). Increased calpastatin protein expression was noticed in endometrial tumour specimens compared to benign samples, and no altered μ- or m-calpain protein expression was observed (Salehin et al., 2010). The roles of other calpain family members in cancer have been widely reviewed. Collectively, evidence includes reduced calpain-3 variants expression in melanoma; increased calpain-6 protein expression in uterine cervical neoplasia, uterine sarcomas/carcinosarcomas and liver cancer; reduced CAPN9 gene expression in gastric cancer (Yoshikawa et al., 2000, Storr et al., 2011a, Moretti et al., 2014, Liu et al., 2015).

A number of immunohistochemical based studies by our group have suggested that calpain-1,-2 and calpastatin are extensively expressed in breast, ovarian, gastro-oesophageal, pancreas, bile duct and ampulla tumours, and associated with patient prognosis or treatment response (Storr et al., 2011b, Storr et al., 2012a, Storr et al., 2012b, Storr et al., 2012c, Storr et al., 2013). Growing evidence, both *in vitro* and *in vivo*, has shown that the calpains are involved in the tumorigenic process, regulating processes such as invasion, migration and metastasis. For example, upregulation of calpain-6 has been suggested to promote cellular proliferation, inhibit apoptosis, and induce cell cycle arrest, which may be regulated by the PI3K-AKT signalling pathway and microRNAs (miRNA) (Liu et al., 2011, Liu et al., 2015). There is a plethora of *in vitro* data

showing a regulatory link between calpain activity and tumour cell invasion, migration and metastasis, this is described fully in section 1.3.2.

1.3.2 Calpain functions

1.3.2.1 Calpains and migration

The acquired migration ability of tumour cells is essential for their invasion into adjacent tissues, which subsequently contributes to distant metastasis. Cellular movement, in normal cells, involves multiple steps: generation of membrane protrusions at the leading edge and attachment to the new site by the cell front; connecting actin cytoskeleton to the extracellular matrix substratum; generating "pulling" force to promote detachment of the cell adhesion at the trailing edge and that allows the cell to move forward (reviewed in (Wells et al., 2005)). Calpain has been shown to regulate cell migration through targeting numerous focal adhesion related proteins, such as E-cadherin (Rios-Doria et al., 2003), β-catenin (Rios-Doria et al., 2004), talin (Franco et al., 2004), focal adhesion kinase (Chan et al., 2010), and ezrin (Hoskin et al., 2015). E-cadherin belongs to a family of transmembrane glycoproteins, and plays an important role in cell-cell adhesion, and maintaining adherence junction and tissue morphogenesis. In breast cancer cells calpain has been found to degrade E-cadherin into a truncated form, and this ionomycin-induced process can be effectively blocked by calpain inhibitors (Rios-Doria et al., 2003). Mutation in β-catenin occurs frequently in some cancers, although relatively rare in breast tumours. It has been shown that calpain-mediated proteolysis of β-catenin can disrupt stabilisation of β-catenin and lead to nuclear accumulation of protein fragments, which may lead to increased tumorigenesis in prostate and breast cancers (Rios-Doria et al., 2004). Talin is an important focal adhesion protein involved in integrin-mediated adhesion, which links the connection between integrin and the actin cytoskeleton. One study has found that calpain can directly cleave talin and regulate adhesion disassembly at the rear of the cells (Franco et al., 2004).

Furthermore, in breast cancer cells, calpain-1 has been shown to interact with ezrin; an important membrane/cytoskeleton linking protein that regulates integrin-based focal adhesion (FA) and invadopodia formation. Downregulated ezrin was accompanied by reduced cleavage of calpain substrates such as talin, FAK and cortactin, which was associated with FA and invadopodia disassembly (Hoskin et al., 2015). Silencing calpain expression by siRNA can efficiently inhibit matrix metalloproteinases (MMPs) secretion and limit the ability of cell adhesion and invasion in osteosarcoma cells (Fan et al., 2009). Calpain-4 has been shown to promote cell invasion and metastasis through upregulation of MMP2 via activating nuclear factor (NF)-κB or focal adhesion kinase (FAK)-Src signalling pathways (Dai et al., 2014, Zheng et al., 2014). Downregulation of hematopoietic PBX-interacting protein (HPIP), a microtubule-binding protein in MCF-7 cells was accompanied by decreased activation of calpain-2, resulting in reduced talin cleavage; conversely overexpression of HPIP in MDA-MB-231 cells can promote talin proteolysis. Since HPIP has been found to be involved in cell migration of breast cancer cells and was overexpressed in invasive breast tumours; the crosstalk between HPIP and calpain-2 can further regulate cell migration and adhesion in breast cancer cells (Bugide et al., 2014). In summary, growing evidence emphasises the importance of calpain in regulating tumour cell migration and invasion, this consequently contributes to distant metastasis and disease progression.

1.3.2.2 Apoptosis and survival

Apoptosis is a highly regulated and tightly controlled intracellular mechanism that leads to the death of cells. There are two cell signalling pathways involved in such regulation: the intrinsic pathway, which involves the release of proteins from the mitochondria into the cytoplasm; and the extrinsic pathway, which involves protein receptors at the plasma membrane (reviewed in (Hancock, 2010)). Conventional calpains have been shown to promote both cell survival and cell death during tumorigenesis, depending on the varying cell apoptotic stimulus, protein expression and the cellular distribution (Tan et al., 2006).

In cell survival pathways, conventional calpains cleave p53, N-Myc, c-Fos and c-Jun (Gonen et al., 1997); and treatment with calpain inhibitor I (ALLN), in various tumour cell lines, resulted in p53-dependent apoptosis, cell cycle arrest, and caspase activation (i.e. caspases-2, -3, -6, -8, and -9) (Atencio et al., 2000). In breast cancer, inhibition of calpain by calpastatin has been shown to promote TNF-α-induced NF-κB activation (Fei et al., 2013), while calpain cleavage of IκB-α encouraged HER2-mediated activation of NF-κB, further facilitating cell survival (Pianetti et al., 2001). One study has found that calpain proteolysis of IκB-α and activation of NF-κB can be attenuated by overexpression of calpastatin (Chen et al., 2000).

In addition to p53 and NF-κB, calpains can degrade the transcription factor Myc, and the latter can sensitise cells to apoptosis. Myc is estimated to be activated or dysregulated in up to 70% of human tumours (reviewed in (Dang, 2012)). A group has observed calpain-1 activity and reduced level of calpastatin protein in Eμ-Myc transgenic model of B-cell lymphoma cells; and the calpain inhibitors PD150606 or MDL28170 have been shown to induce caspase-3-dependent/caspase-9-independent apoptosis *in vitro* (Li et al., 2012). In colon cancer cells, the cleavage of Myc by calpains has been shown to promote tumour cell survival; whilst blocking cleavage of Myc with calpain inhibitors (calpeptin, calpain inhibitor XII and VI) or by overexpressing a cleavage-resistant mutant of Myc profoundly promoted cell death (Conacci-Sorrell et al., 2014).

In terms of apoptotic pathways, evidence has shown that calpain cysteine proteases are involved in the apoptotic machinery through interaction with caspase family members; calpains appear to have both positive and negative role in the induction of apoptosis. A number of caspase family members can be processed by calpains. Direct cleavage of caspase-7, -9, -10 and -12 by calpain has been demonstrated, with confounding effects on apoptosis. Amongst those, caspase-7 and -10 were activated by calpain cleavage, along with increased proteolytic activity (Gafni et al., 2009); whilst cleavage of caspase-12

was required for endoplasmic reticulum (ER) stress-induced apoptosis (Tan et al., 2006). Calpain degradation of caspase-9 produced an inactivated form of the enzyme, which was unable to activate downstream caspase-3 (Chua et al., 2000). As mentioned above, two main cysteine protease systems both participate in apoptosis. Wang et al. has summarised some characteristics of caspase-3 and calpains (Table 1.8) (Wang, 2000).

Table 1.8 Comparing and contrasting the properties of caspase-3 and calpain

	Caspase 3	Calpain
Protease class	Cysteine aspartyl proteases	Calcium dependent cysteine protease
Endogenous inhibitor (s)	IAP1, IAP2, NAIP	Calpastatin
Resting mode	Inactive pro-enzyme (32kDa)	Inactive pro-enzyme (80+29kDa)
Activation mode	Proteolytic processing (to 17+12kDa)	Ca2+, then autolytic processing (to 78+18kDa)
Preferred cleavage site (*)	AspxxAsp*x	(Leu, Val, Lle)x*x
Endogenous substrates	Subset of cytoskeletal, cytosolic and nuclear proteins or enzymes	Same
Consequence of substrate proteolysis	Produces limited fragment (s), sometimes proteolytic activation	Same
Cell-death involvement	Most forms of apoptosis	Most forms of necrosis, some forms of apoptosis
Inhibitors as neuroprotectants	Yes	Yes

Abbreviations: IAP, inhibitor of apoptosis protein; NAIP, neuronal apoptosis inhibitory protein. Reproduced from (Wang, 2000)., Trends in Neurosciences, 23, 20-26; Copyright© 2000 by Elsevier with permission conveyed through Copyright Clearance Centre Inc.

Calpastatin can be cleaved by caspase during drug-induced apoptosis. The 110 kDa high-molecular-weight (HMW) form of CAST has been found to be produced by caspase cleavage during early apoptosis in anti-FAS treated Jurkat T-cells, staurosporine treated neuroblastoma SH-SY5Y cells and TNF-α treated U937 monocytic leukemic cells (Wang et al., 1998, Pörn-Ares et al., 1998).

In addition to caspases, calpains can also facilitate apoptosis through cleavage of Bcl-2 family members. Calpain has been shown to be responsible for the cleavage of the pro-apoptotic protein Bax during drug-induced apoptosis (Wood et al., 1998). Calpain was activated during ionomycin-induced apoptosis and Bcl-2, Bid and Bcl-X_L have all been found to be truncated by calpain, which was accompanied by activation of caspase-3, -7 and -9. Calpain-mediated Bcl-2 and Bid cleavage has been shown to induce cytochrome c release from mitochondria (Gil-Parrado et al., 2002). Calpains can also cleave various apoptosis-associated proteins, further promoting apoptosis cascades, such as CDK5, JNK, JUN, FOS and APAF1 (reviewed in (Moretti et al., 2014, Storr et al., 2015)). The role of calpains in apoptotic pathways was summarised by Storr et al., as shown in Figure 1.5 (Storr et al., 2011a).

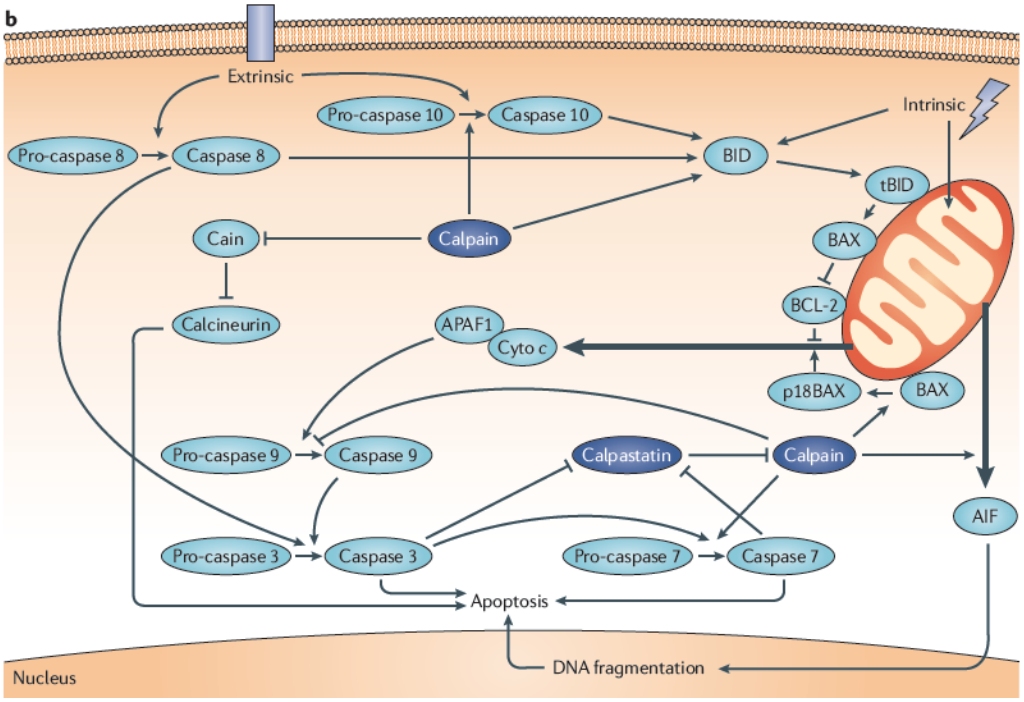


Figure 1.5 Extrinsic and intrinsic apoptotic pathways can be influenced by calpain activity. Reproduced from (Storr et al., 2011a) , Nature Reviews Cancer, 11, 364-374; Copyright© 2011 by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

A lot of conventional chemotherapy is based on the induction of apoptotic pathways, and calpain may be an important factor in chemotherapy induced cell death. The majority of studies have been carried on cisplatin induced

apoptosis. During cisplatin-induced apoptosis, calpain has been found to activate Bid and promote its translocation to mitochondria, which was accompanied with mitochondrial release of cytochrome c and apoptosis inducing factor (AIF), leading to apoptosis in human lung adenocarcinoma cells. Whereas combined inhibition of calpain activity by calpeptin and PD150606 can effectively block Bid activation, delay or reduce cytochrome c release and disrupt subsequent caspase-3/-9 activation (Liu et al., 2008, Liu et al., 2009). The treatment of calpeptin or calpain siRNA reduced cisplatin-induced apoptosis through regulating p73α and parkin-like cytoplasmic protein. The transcription factor p73α is an important member of p53 family and parkin-like cytoplasmic protein has been shown to regulate p53 distribution and function. These results suggested that inhibition of calpain attenuated the cell sensitivity to cisplatin (Al-Bahlani et al., 2011, Woo et al., 2012). During cisplatin-induced apoptosis of melanoma cells, the inhibition of calpain by combined inhibitors (i.e. MDL28170, calpeptin and PD150606) effectively protected cell apoptosis through reducing caspase-3/-7 activity or enhancing autophagic response (Del Bello et al., 2007, Del Bello et al., 2013). A limited number of immunohistochemistry based studies have suggested links between the calpain/calpastatin system and response to therapy (conventional C/T and targeted therapies– see Chapter 1) (Storr et al., 2011c, Storr et al., 2012b, Storr et al., 2013, Davis et al., 2014); but few, if any, have investigated apoptosis or caspase expression as also being involved.

1.3.2.3 Other functions

In addition to above, calpains have also been implicated in the regulation of autophagy and the response to hypoxia. Hypoxia often occurs in solid tumours, and hypoxia-inducible factor-1α (HIF-1α) is extensively overexpressed by many tumour cells; it is a proteolytic substrate of calpain (Zhou et al., 2006). Unlike other cellular functions, the relationship between autophagy, calpain and cancer is controversial. Several autophagy-related gene (Atg) proteins can be degraded by μ-calpain *in vitro*, providing links between autophagy and

apoptosis, the latter is a critical controlling process in tumorigenesis (Norman et al., 2010). Recent evidence has demonstrated that calpain inhibitors can restore calpain-dependent cleavage of p62/SQSTM1, a protein involved in assembly of organelles and degraded by autophagy, in cancer stem cells (Colunga et al., 2014).

1.3.3 Therapeutic response and calpain inhibition

The physiological functions of the calpain family have been widely studied. Several studies demonstrate that calpains are overexpressed in tumour tissues/cells compared with their normal counterparts, with variation between different types of tumours; indicating that calpains are associated with malignancy and tumour progression. Calpain overexpression is significantly associated with poor prognosis in a number of tumour types, including ICC and glioma, as well as lymphatic metastasis in renal cell carcinoma (as discussed in section 1.3.1.3). High calpain expression has been associated with adverse survival of breast (Storr et al., 2012a, Storr et al., 2011c, Pu et al., 2016) and ovarian tumours (Storr et al., 2012b); whereas high expression has been associated with improved survival of pancreatic (Storr et al., 2012c) and gastro-oesophageal tumours (Storr et al., 2013). A summary, and examples, of the involvement of calpains in cancer are listed in Table. 1.9.

Table 1.9 Examples of aberrant calpain expression/activity and their prognostic values in different types of human cancer

Calpain protein/gene	Cancer types	Aberrant expression/activity	Prognostic values	Ref
Calpain-1/CAPN1	BCC	Low expression	-	(Reichrath et al., 2003)
	SCC and BCC	mRNA overexpression	-	(Reichrath et al., 2003)
	RCC	mRNA overexpression	Associate with lymph node metastasis and histologic type	(Braun et al., 1999)
	Childhood ALL	mRNA/protein overexpression	-	(Mikosik et al., 2015)
	Gastric/GOJ adenocarcinoma	Overexpression	Associate with improved OS in patients treated with surgery + adjuvant or neoadjuvant CT	(Storr et al., 2013)
	HER2+ breast cancer	Overexpression	Associate with adverse RFS in patients treated with trastuzumab following adjuvant CT	(Storr et al., 2011c)
Calpain-2/CAPN2	Colorectal adenocarcinoma	Overexpression	-	(Lakshmikuttyamma et al., 2004)
	Basal like and TNBC	Overexpression	Associate with adverse BCSS	(Storr et al., 2012a)
	Pancreatic cancer	Low expression	Associate with adverse OS	(Storr et al., 2012c)
	Ovarian cancer	Overexpression	Associate with adverse PFS in patients treated platinum-based CT	(Storr et al., 2012b)
	Gastro-oesophageal adenocarcinoma	Low expression	Associate with adverse OS in patients treated with neoadjuvant CT	(Storr et al., 2013)

Table 1.9 Continued

Calpain protein/gene	Cancer types	Aberrant expression/activity	Prognostic values	Ref
Calpain-4/CAPN4	Glioma	Overexpression	-	(Cai et al., 2014)
	ICC	Overexpression	Correlate with adverse OS, high recurrence rate	(Zhang et al., 2013)
Calpain-9/CAPN9	Gastric cancer	Low expression	-	(Yoshikawa et al., 2000)
	Breast cancer	Low expression	Correlate with adverse DSS	(Davis et al., 2014)
Calpastatin/CAST	Endometrial cancer	Overexpression	-	(Salehin et al., 2010)
	CBD and ampulla cancer	Low expression	Correlate with adverse OS	(Storr et al., 2012c)
	Gastro-oesophageal adenocarcinoma	Low expression	Correlate with adverse OS in patients treated with neoadjuvant CT	(Storr et al., 2013)
Calpain	AML	Increased activity	-	(Niapour et al., 2012)
	Childhood ALL	Increased activity	-	

Abbreviations: SCC, Squamous cell carcinoma of skin; BCC, basal cell carcinoma of skin: RCC, Renal cell carcinoma; ALL, acute lymphoblastic leukaemia; GOJ,gastro-oesophageal junction; TNBC, triple negative breast cancer; ICC, Intrahepatic cholangiocarcinoma; AML, Acute myelogenous leukaemia; CBD, Common bile duct; CT, chemotherapy; OS, overall survival; RFS, relapse-free survival; BCSS, breast cancer-specific survival; PFS, progression free survival; DSS, disease specific survival.

The expression of calpains may be tumour type dependent with opposing effects often observed. Assumptions made from calpain expression regarding calpain activity levels in human malignancies may lead to incorrect conclusions; however expression data suggest that the use of calpain inhibitors might show potential as anti-cancer therapeutic tools in several tumour types.

Efforts have been made to develop and design calpain inhibitors that have high selectivity and potency. In addition to calpastatin, the endogenous specific inhibitor of calpain, many commercial calpain inhibitors are used such as leupeptin, calpeptin, calpain inhibitor I, E-64, PD150606 and MDL28170; however the majority of such agents also inhibit other cysteine proteases (e.g. papain and cathepsins) or have low cell permeability. Most calpain inhibitors are derived from natural compounds or from chemical synthesis, there are two main classes: non-peptide inhibitors and peptidomimetic inhibitors. Peptidomimetic calpain inhibitors were named after their mechanisms; mimicking the natural interaction between calpain and its endogenous inhibitor calpastatin can directly target the active site of calpain (Donkor, 2015). The most widely used agent is still calpeptin; a cell-permeable calpain inhibitor. Calpeptin, a synthetic peptide inhibitor, can penetrate into the cells and bind to the active site of calpain, which reversibly inhibits calpain activity. It was originally found to inhibit calpain activity in Ca^{2+} ionophore pretreated intact platelets, where leupeptin did not have any inhibitory effect. Calpeptin has shown several advantages, in that it is more specific to calpain-1 than leupeptin; furthermore it has no effect on trypsin (Tsujinaka et al., 1988).

The potential of calpain inhibitors as anti-cancer agents has been investigated using several experimental tumour models. A calpain-2 inhibitor (zLLY-CH2F) has been shown to reduce colon tumour volume *in vivo* through disruption of calpain cleavage of IκBα and NF-κB nuclear localization (Rose et al., 2015). Inhibition of calpain activity and knockdown of m-calpain in gastric cancer cells was associated with enhanced 5-FU sensitivity in resistant cells; consistently,

higher expression of calpastatin was observed in 5-FU sensitive cells compared to 5-FU resistant cells (Nabeya et al., 2011). Long-term androgen depletion is associated with more aggressive prostate cancer phenotype due to its androgen-resistance (AR) status, increased level of calpain-2 and its cleavage product filamin A (an actin-binding protein) have been observed in AR-negative, androgen-independent prostate cell lines. In this context, suppression of calpain activity with calpeptin can effectively inhibit calpain-mediated degradation of filamin A in AR-negative prostate cancer cells, which prevents the progression of cancer to an aggressive phenotype (Liu et al., 2014b).

There is growing interest in calpains involvement in tumour cell apoptosis with promoting and protective roles of calpain being demonstrated in numerous tumour types; the overall outcome may be dependent on the apoptotic stimuli, cross talk with other apoptosis family members (e.g. caspases), activity and cellular localization of the protein (reviewed in (Storr et al., 2011a)). The use of calpain inhibitor I (ALLN) and a plant extract *astragalus saponins* (AST) in combination can enhance endoplasmic reticulum (ER)-stress-induced apoptosis in colon cancer, leading to reduced tumour size *in vivo* (Wang et al., 2014b). AST was extracted from the root of a medicinal herb *Astragalus membranaceus*, which was found to have anti-cancer activity in colon cancer cells and xenografts (Tin et al., 2007). However, several studies have implied that calpain activation is required for cisplatin (cis-platinum (II) diammine dichloride, CDDP)-induced apoptosis in melanoma cells, ovarian cancer cells and lung adenocarcinoma cells. The suppression of calpain has been demonstrated to reduce the sensitivity to cisplatin in ovarian cancer, and the cell death protective effect of calpain inhibitors has been observed in melanoma cells (Del Bello et al., 2007, Liu et al., 2009, Al-Bahlani et al., 2011). In all, the role of calpain in cell survival and apoptosis is not fully understood compared with its role in cell migration and invasion, and partly depends on the cell microenvironment and tumour characteristics.

Limited studies have been carried on investigating the role of calpain in the therapeutic response in breast cancer. HER2 induced NF-κB activation can be inhibited by PTEN (tumour suppressor gene) and regulated by calpain proteolysis of IκBα (Pianetti et al., 2001). Recently, Kulkarni et al. conducted two studies to investigate the involvement of calpains in response to trastuzumab in HER2+ breast cancer. A main observation was that calpain induced cleavage of HER2 in HER2+ breast cancer cells and tumours. In addition, calpain inhibition induced downregulation of PTEN and upregulation of AKT1 in both acquired trastuzumab-resistant and parental HER2+ breast cancer cells. Finally, following trastuzumab treatment, inhibition of calpain activity by calpeptin or MDL28170 increased cell proliferation in trastuzumab-sensitive cells; but decreased cell proliferation in acquired trastuzumab-resistant cells; and knockdown of calpain-4 appeared to inhibit cell survival and enhance response to trastuzumab (Kulkarni et al., 2010, Kulkarni et al., 2012). As discussed above (section 1.3.1.3), immunohistochemical studies have shown that high calpain-1 expression is associated with worse response to adjuvant trastuzumab treatment in HER2+ breast cancer patients. Taken together, the results of such studies suggest a mechanism by which calpain activity may contribute to the sensitivity/resistance of trastuzumab. However the exact mechanism is still unclear, and may differ between acquired/inherent trastuzumab-resistant and sensitive cancer models.

1.3.4 MSKs and their role in breast cancer

Mitogen- and stress- activated kinases (MSKs), nuclear serine/threonine protein kinases, are important substrates of the mitogen-activated protein kinase (MAPK)-activated protein kinases (MAPKAPKs) family (Cargnello and Roux, 2011). Two novel protein kinases MSK1/2 were originally described by Deak et al. in 1998, based on their significant identity to the N-terminal ribosomal S6 kinases (RSKs). Human MSK1 and MSK2 are 63-75% identical to each other, and present significant homology to RSKs (approximately 40% identity). An initial study revealed that MSK1/2 are activated through MAPK/ERKs signalling in response to mitotic agents such as epidermal growth factor (EGF) and phorbol

ester (TPA, 12-O-tetradecanoylphorbol-13-acetate); or through MAPK/p38 pathway in response to stress stimulus such as UV radiation, oxidative and chemical stress (Deak et al., 1998). Consistent with this, the specific inhibitor of MAPK/ERKs (PD98059) can block growth factor-induced MSK activation, whilst the inhibitor of MAPK/p38 (SB203580) can block stress stimulus-induced signalling to MSKs (Deak et al., 1998). Because of the dual activation mechanism of MSKs, the inactivation of MSKs can only be achieved by blocking both pathways simultaneously; the activity cannot be completely inhibited if only one pathway was blocked (reviewed in (Vermeulen et al., 2009)).

Similar to other MAPKAPKs, MSK1/2 contains two distinct protein kinase domains within a single polypeptide, which is an uncommon structure in most of the protein kinases. The N-terminal domain of MSK1/2 belongs to the AGC (PKA, PKG and PKC families) protein kinase family; the C-terminal domain belongs to the calcium/calmodulin-dependent protein kinase (CAMK) family. The C-terminal domain is phosphorylated by the upstream MAPKs, which subsequently triggers the phosphorylation of N-terminal domain. Upon activation, the N-terminal domain phosphorylates other downstream substrates (reviewed in (Cargnello and Roux, 2011)). Both MSK1/2 are ubiquitously expressed, including heart, brain, placenta, lung, liver kidney and pancreas tissue, with predominant expressions observed in brain, placenta and skeletal muscle (Deak et al., 1998).

MSK1/2 accumulates in the nucleus of unstimulated and stimulated cells, and is involved in nuclear events, including transcriptional regulation and regulation of the chromatin environment. Many transcription factors have been identified as substrates of MSK1/2 and phosphorylation by MSK1/2 can increase their activity. MSK1/2 can phosphorylate cAMP response element-binding (CREB) protein and activating transcription factor 1 (ATF1) in response to mitogens and stress; the phosphorylation of CREB at Ser133 appeared to regulate transcription of several immediate early (IE) genes, including c-fos, JunB and

Egr1 (Wiggin et al., 2002). In addition to CREB and ATF1, other transcription factors have been identified as phosphorylation targets of MSK1/2, such as NF-κB, signal transducer and activator of transcription 3 (STAT3), and E26 transformation-specific [ETS]-related protein 81 (ER81) (reviewed in (Cargnello and Roux, 2011)). In terms of gene activation and chromatin regulation, MSK1/2 contributes to regulation of histone H3, a component of the nucleosome, and HMGN1 (HMG-14), a chromatin-associated protein. It has been shown that stress- and mitogen-stimulated phosphorylation of histone H3 and HMGN1 in fibroblasts is compromised in $MSK1^{-/-}MSK2^{-/-}$ mice (Soloaga et al., 2003). The physiological function(s) of MSKs remains to be completely determined, however it has been suggested that MSK1/2 may have a role in regulating the immune system and neuronal function (reviewed in (Vermeulen et al., 2009)).

In breast cancer, recent studies have suggested that MSK1 may play a role in steroid-hormone induced breast cancer cell proliferation. Depletion of MSK1 has been shown to inhibit oestrogen (E2) or progesterone-induced breast cancer cell (T-47D) proliferation and tumour growth in hormone-dependent breast cancer murine xenografts. The study also showed that MSK1 participated in G1-S phase transition and regulated cell cycle associated gene expression in response to hormones, evidenced by the recruitment of MSK1 to specific PR-binding sites in chromatin (Reyes et al., 2014). Breast tumour-associated osteoblasts (TAOB)-derived CXCL5 induced MCF-7 and MDA-MB-231 cell migration and invasion has been reported to be associated with increased MSK1 activation (Hsu et al., 2013). In T-47D cells, alcohol-exposure can cause increased expression and activation of proto-oncogene tyrosine-protein kinase (ROS1); the latter induced MSK1 activation through the MAPK/ERKs pathway. Consequently, MSK1 phosphorylated histone H3S10 coupled with increased expression of IE genes such as c-fos, resulting in enhanced breast cancer cell proliferation (Lee et al., 2013).

MSK1 and MSK2 are both nuclear serine/threonine protein kinases; although

they share similarity they are different complexes and exert differencing effects. Interestingly, a study has emphasised the differential roles of MSK1 and MSK2 in breast cancer cells following UV-radiation. UV-radiation has been shown to induce activation of the NF-κB p65 subunit in MDA-MB-231 cells, which is mainly dependent on MSK2 rather than MSK1. The depletion of MSK2 was found to reduce cell viability following UV-radiation, suggesting MSK2-mediated NF-κB activation is involved in regulating cell survival in MDA-MB-231 cells (Jacks and Koch, 2010). Recently MSK1/2 were also found to be involved in regulation of phorbol ester-induced activation of trefoil factor 1 (TFF1), a breast cancer marker, in MCF-7 cells (Khan et al., 2013). As will be described during this chapter, results from phosphokinase signalling experiments suggested that there may be a link between the calpain system and MSK1/2 – as a result of such data it was thought interesting to examine MSK expression in breast cancer samples, correlating data with calpain system protein expression.

1.4 Hypotheses and aims

1.4.1 Hypotheses

1. Calpain family involvement in proliferation, apoptosis, migration and cellular signal transduction differs in different breast cancer molecular subtypes, and contributes to the differential prognosis in patients.
2. Resistance to trastuzumab in HER2+ breast cancer cells can be modulated by agents that inhibit calpain activity or by shRNA mediated knocking down calpain/calpastatin genes. The corresponding mechanisms are different between inherent and acquired trastuzumab-resistant models.

1.4.2 Aims and objectives

1.4.2.1 Aims

1. Explore the differential role of calpain family members in regulating proliferation, migration, apoptosis and signal transduction in different breast cancer molecular subtypes.
2. Confirm the role of calpain-1 expression as a biomarker of trastuzumab

response; and explore the molecular mechanisms by which the calpain family regulates the therapeutic response to trastuzumab in HER2+ breast cancer cells.

1.4.2.2 Objectives

1. Investigate whether altering calpain activity (by using agents to inhibit activity/knocking down the gene using shRNA) has differential effects on proliferation, apoptosis, migration and signal transduction in different breast cancer molecular subtypes. Assess effects using a variety of endpoints and *in vitro* assays (i.e. cell growth curve, Annexin-V FITC assay, scratch-wound migration assay and phosphokinase arrays).
2. Based upon the previous *in vitro* study, suggesting inhibition of calpain activity may involve in the expression of phospho-MSK1/2 expression, it was decided to explore the associations between calpain and MSK1 protein expression; as well as the prognostic value of MSK1 protein in early invasive breast cancer patients.
3. There is close link between caspase family and calpain family, and regarding important of role calpain in apoptosis, it was decided to assess the associations between calpain and two representative caspases (caspase-3 & -8), as well as the prognostic value of caspase-3 & -8 in early invasive breast cancer patients.
4. Validate the prognostic role of calpain-1 in HER2+ breast cancer patients treated with adjuvant trastuzumab regimen by using expanded and independent patient cohorts.
5. Determine how manipulating calpain activity influences therapeutic response to trastuzumab in both inherent and acquired trastuzumab-resistant HER2+ breast cancer cell lines.

Chapter 2 Breast cancer calpain system protein expression: effects on phenotype and cell signalling

2.1 Introduction and aims

2.1.1 Aims

The aims and objectives of the current chapter were to:

1. Assess calpain family protein expression/activity *in vitro* across different breast cancer molecular subtypes.
2. Explore the role of calpains in regulating breast cancer cell proliferation, migration and signalling pathways.
3. Investigate the relationship between the calpain family and MSK1 protein expression in breast tumours; and evaluate the prognostic value of MSK1 in breast cancer patient samples.

2.1.2 Manipulating calpain activity

Several commercial inhibitors for calpain are currently available, such as leupeptin, calpeptin, calpain inhibitor I, E-64, PD150606 and MDL28170, etc. The majority of these are synthesised as peptide analogues that bind to the active site of the protease. However, most of the compounds lack specificity, which leads to possible cross reactivity with other cysteine proteases. This may be due to the similarity of active sites between different types of cysteine proteases (e.g. cathepsin, papain) (Wang and Po-Wai, 1994, Goll et al., 2003). It has been suggested that the use of "physiological substrates" may lower the Ca^{2+} requirement for calpain activation. However a comparison of four protein substrates (casein, casein treated with urea and EDTA, myofibrils and troponin) and three synthetic substrates (Suc-Leu-Tyr-MCA, Boc-Leu-Thr-Arg-MCA, and Suc-Leu-Leu-Val-Tyr-MCA), revealed no obvious difference in Ca^{2+} requirement for proteolytic activity of the calpains between these seven substrates (Barrett et al., 1991). In mammalian cells, RNA interference (RNAi) can be achieved by small interference RNA (siRNA) or short hairpin RNA (shRNA), resulting in silencing of sequence-specific genes. The siRNAs can be synthesised and transfected into mammalian cells, causing effective but transient suppression of specific gene expression. On the contrary, RNAi can be mediated by shRNA that results in stable suppression of gene expression over longer period

(Elbashir et al., 2001, Paddison et al., 2002). The shRNA has been used to knockdown calpain-4 in nasopharyngeal carcinoma and hepatocellular carcinoma cell lines demonstrating its validity as an approach to study the calpain system; as a result, knockdown of calpain-4 results in decreased proliferation and *in vivo* tumour growth (Dai et al., 2014, Zheng et al., 2014).

2.2 Materials and methods

2.2.1 Cell lines and growth medium

Six breast cancer cell lines including two luminal (T-47D and MCF-7), two HER2+ (SKBR3 and JIMT-1), and two basal-like (MDA-MB-468 and MDA-MB-231) were used in the current study; all cell lines were used within a 12 passage window. All cell lines were originally obtained from the American Type Culture Collection (ATCC) apart from the JIMT-1 cell line which was obtained from the Leibniz-Institute DSMZ - German Collection of Microorganisms Cell Cultures. Cells were routinely grown in T75 flasks with routine growth medium in a humidified incubator at 37^{o}C with 5% CO_2, and other sizes of tissue culture flasks and plates were used according to the requirement of specific experiments. Cell lines were cultured in defined medium listed in Table 2.1. Iron supplemented donor bovine serum (Gibco, Life Technologies) was heat-inactivated by incubating for 30 minutes in a 56^{o}C water bath before use. All tissue culture related reagents were pre-warmed to 37^{o}C in a water bath. All cell lines were regularly screened for mycoplasma infection, conducted by senior technicians every 6 months, using the MycoProbe® Mycoplasma Detection Kit (R&D Systems). Cell lines were also routinely verified for authenticity using Promega Powerplex® 16 short tandem repeat (STR) system, conducted by Dr. Sarah Storr every 6 months.

Table 2.1 The list of cell culture medium

Cell line	Reagents	Source	Volume
T-47D Luminal (ER+,PR+, *ERBB2*/HER2-)	Dulbecco's Modified Eagle's Medium (DMEM) (with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate)	D5796, Sigma, UK	500 mL
	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	56 mL
	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.6 mL
MCF-7 Luminal (ER+,PR+, *ERBB2*/HER2-)	RPMI-1640 medium	R8758, Sigma, UK	500 mL
	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	56 mL
	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.6 mL
JIMT-1 Basal-like (ER-.PR-, *ERBB2*/HER2+)	Dulbecco's Modified Eagle's Medium (DMEM) (with 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate)	D5796, Sigma, UK	500 mL
	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	56 mL
	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.6 mL
SKBR3 HER2+ (ER-,PR-, *ERBB2*/HER2+)	McCoy's 5A medium (modified, with L-glutamine and sodium bicarbonate)	M9309, Sigma, UK	500 mL
	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	56 mL
	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.6 mL

Table 2.1 Continued

Cell line	Reagents	Source	Volume
MDA-MB-231 Basal-like	Minimum Essential Medium Eagle (MEM) (with Earle's salts and sodium bicarbonate, without L-glutamine)	M2279, Sigma, UK	500 mL
(ER-,PR-,	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	57 mL
***ERBB2*/HER2-)**	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.7 mL
	L-glutamine (200 mM)	G7513, Sigma, UK	5.7 mL
	MEM Non-essential amino acids solution (100x)	M7145, Sigma, UK	5.7 mL
MDA-MB-468 Basal-like	Minimum Essential Medium Eagle (MEM) (with Earle's salts and sodium bicarbonate, without L-glutamine)	M2279, Sigma, UK	500 mL
(ER-,PR-, *ERBB2*/HER2-)	Heat Inactivated Iron Supplemented Donor Bovine Serum	Gibco, Life Technologies, UK	57 mL
	Penicillin/Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/mL)	P4333, Sigma, UK	5.7 mL
	L-glutamine (200 mM)	G7513, Sigma, UK	5.7 mL
	MEM Non-essential amino acids solution (100x)	M7145, Sigma, UK	5.7 mL

2.2.2 Subculture

Tissue culture was performed in a class II biological safety hood, which was cleaned by using 70% ethanol before and after use. Cells were cultured in flasks until reaching 70-80% confluence. To subculture, the original medium was removed and cell layer was washed with Dulbecco's phosphate buffered saline (PBS, Sigma, UK). 0.5mg/mL trypsin-EDTA (Sigma, UK) was added to the flask to ensure the monolayer was completely covered and the flask was left for 15-30s. Then trypsin-EDTA was removed and the flask was incubated at 37^{o}C for 3-5 minutes. Cells were checked under a microscope to ensure detachment. Complete medium was used to deactivate trypsin-EDTA prior to harvesting the cells. The harvested cells were split into a new tissue culture flask and fresh medium added to give an appropriate total volume (5 mL in T25 flask, 15 mL in T75 flask and 25 mL in T175 flask). The flask was then returned to the incubator at 37^{o}C with 5% CO_2.

2.2.3 Cryopreservation and recovery from cryopreservation

Cells were initially expanded then frozen in liquid nitrogen to keep them at low passage number, in order to ensure the passage number of the cells used in experiments were within the specified ranges. When reaching 70-80% confluence, cells were harvested with trypsin-EDTA and the total number of cells and percentage of viability were determined using a haemocytometer or Countess® Automated Cell Counter (Life Technologies, NY). The cell suspension was then centrifuged at 170 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in an appropriate volume of freezing medium [10% dimethyl sulfoxide (DMSO) and 90% complete medium] to achieve a final concentration of approximately 1X10^{6} cells /1 mL. The cells were stored in a labelled cryovial in a container which controlled the rate of temperature descent to 1^{o}C/minute. Cells were placed at -80^{o}C overnight before transferring into liquid nitrogen (-180^{o}C) until further use.

The cryovials containing the frozen cells were retrieved from liquid nitrogen storage and were thawed immediately in a 37^{o}C water bath. The cell suspension

was transferred dropwise into a centrifuge tube containing 9mL of complete medium, followed by centrifugation at 170 x g for 5 minutes. The supernatant was discarded and the cell pellet resuspended in fresh growth medium, put into a new tissue culture flask and returned to the incubator. Culture medium was changed the following day, and the cells cultured following routine procedures.

2.2.4 Chemicals and drug preparation

The t-Boc-Leu-Met-CMAC (Invitrogen, USA) stock solution was prepared in DMSO (Sigma, UK) at a concentration of 2 mM and stored at -20^{o}C. Calpeptin (Merck Millipore Corporation, Germany) was dissolved in DMSO at 100 mM and stored at -20^{o}C. The calcium ionophore A23187 (Sigma, UK) was stored in 10 mM stock solution in DMSO at -20^{o}C. The stock solutions were sub-diluted in complete culture medium to achieve the final concentrations for individual treatments. DMSO was added to the negative control groups at the same volume equivalent to that in the drug treatment group (the final percentage of DMSO was < 0.1% v/v, except that indicated in individual experiment).

2.2.5 Protein extraction and determination of protein concentration

Cells were harvested and pelleted by centrifugation at 170 x g for 5 minutes. The pellets were washed once in ice-cold PBS to remove residual serum proteins and pelleted by centrifuging. The pellets containing 5 x 10^6 - 1 x 10^7 cells were resuspended in 500 µL-1 mL equal volumes of RIPA buffer (Sigma, UK) or lysis buffer (as described in section 2.2.6.4 and 2.2.9) in microcentrifuge tubes, supplemented with 1X Halt Protease Inhibitor Cocktail containing protease inhibitor, phosphatase inhibitor and EDTA (ThermoFisher Scientific, USA), with gentle shaking for 30 min at 4^{o}C. The lysates were clarified by centrifugation at 14,000 x g for 5 min and the supernatants collected in fresh tubes as whole-cell protein. Lysates were aliquoted to avoid repeated freeze-thaw cycles causing protein degradation and stored immediately at -80°C for long term use. Thawed lysates were kept on ice prior to conduct Western blotting experiments.

The protein concentrations of cell lysates (used in section 2.2.6.4 and 2.2.9)

were determined using a Bio-Rad DC protein assay (Bio-Rad, USA). The DC protein assay is a colorimetric assay for detecting protein concentration following detergent solubilisation. The colour development is based on the reaction between protein with copper in alkaline and the subsequent reduction of Folin reagent by the copper-treated protein. Colour change can be detected at maximum absorbance of 750 nm and minimum absorbance of 405 nm (Lowry et al., 1951). The assay was conducted following the manufacturers' instructions, a protein standard curve was generated using bovine serum albumin (Sigma, UK) diluted in lysis buffer at different concentrations (0, 0.05, 0.1, 0.25, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 mg/mL). The absorbance values at 620 nm were plotted against corresponding protein concentrations. The average absorbance reading was carried out based on the standard curve to determine the protein concentration in each sample.

2.2.6 Detection of calpain activity

2.2.6.1 T-Boc calpain activity assay

The calpain activity in living cells was detected using a cell permeable calpain substrate t-Boc-Leu-Met-CMAC (t-Boc). Calpain cleavage of t-BOC can produce fluorescence with maximum excitation 350 nm/emission 430 nm. The treatment of cells with calpeptin, a calpain inhibitor, led to the decrease in fluorescence intensity and the calpain activity was expressed as relative fluorescence intensity. $5X10^5$ MDA-MB-231 cells were seeded into T25 tissue culture flasks. After 24 hours, the cells were treated with 0.1 µM A23187 or 100 µM calpeptin for 30 minutes; or treated with 0.1 µM A23187 for 30 minutes followed by 100 µM calpeptin for an extra 30 minutes. Alternatively, the cells were treated with increasing concentrations of calpeptin (100, 150, 200 and 250 µM) for 30 minutes. The calcium ionophore A23187 was used to transport extracellular Ca^{2+} through the plasma membrane, leading to increase of Ca^{2+} in the cytosol (Dedkova et al., 1999). DMSO was added to the negative control groups at the same volume equivalent to that in the drug treatment groups. Then the cells were harvested and diluted to $5X10^5$/mL in 500 µL serum free RPMI-1640 medium, which was aliquoted into two groups (250 µL each). 2 µL

pre-warmed t-Boc (2 mM stock) or 2 µL DMSO (final conc. 0.8%) was added to each group, respectively. The samples were added into a 96-well fluorescence plate with optical bottom (100 µl in duplicate per sample) and read using a BMG FLUOstar OPTIMA Microplate Reader (pre-heated to 37^{o}C). The fluorescence was continuously recorded on a 10 cycle kinetic window (excitation/emission 350/430, gain 2000). The values from the control group were subtracted from the total activity values to obtain pure calpain activity; and results were normalised to the baseline and medium alone. The percentage of calpain activity inhibition was calculated as: inhibition of calpain activity (%) = [1-(fluorescence of treated cells/fluorescence of untreated cells (control)] X 100%. All measurements were assessed in duplicate and data represented as the average ± standard deviation of at least two independent experiments.

2.2.6.2 AMC calpain activity assay

A second calpain activity assay was performed using a fluorogenic calpain activity assay kit (Calbiochem) following the manufacturer's instructions. The calpain substrate, Suc-LLVY-AMC (Suc Leu-Leu-Val-Tyr-AMC) was provided in the kit and the fluorescence intensity was detected at an excitation wavelength of approximately 360-380 nm and an emission wavelength of approximately 440-460 nm. The calpain activity was quantitated using an AMC (7-Amino-4-methylcoumarin) standard curve and displayed as relative fluorescence units (RFU). 2.5X10^{6} MDA-MB-231 cells were seeded into T175 tissue culture flasks. After 24 hours, cells were treated with 2 µM A23187 or 100 µM calpeptin alone for 30 minutes; or treated with 2 µM A23187 for 30 minutes followed by 100 µM calpeptin for an extra 30 minutes. The treatments were applied in complete culture medium and serum free medium. DMSO was added to the negative control groups at the same volume equivalent to that in the drug treatment groups. Cells were collected in lysis buffer provided in the kit and the protein concentration was determined using a BCA protein assay (as described in section 2.2.5). The AMC (7-Amino-4-methylcoumarin) standard curve was prepared using serial dilutions of the standard ranging from a concentration of 0.625-10 µM (i.e. 0, 0.625, 1.25, 2.5, 5, 10 µM). The substrate Suc-LLVY-AMC

was diluted 100-fold with Assay Buffer provided in the kit. 50 µL diluted standards and samples were added to wells of a 96-well plate, and 50 µL diluted substrate were added to each well. The plate was incubated at room temperature for 15 min before analysing on a BMG FLUOstar OPTIMA Microplate Reader (excitation/emission 350/430, gain 1500). The fluorescence value was assessed in duplicate and was normalised by subtracting the blank value (AMC standard curve). Data are represented as the average ± standard deviation of two independent experiments.

2.2.6.3 Detection of cleaved α-fodrin by Western blotting

Alpha-fodrin, also termed αII-spectrin, is an important component of the membrane cytoskeleton. It is a well characterised calpain substrate, when calpain is activated α-fodrin can be degraded into two specific 145-kDa and 150-kDa fragments. Thus calpain specific cleavage of α-fodrin was applied as a way to detect calpain activity by using antibodies against α-fodrin in Western blotting (Dutta et al., 2002). $1X10^6$ MDA-MB-231, MCF-7 or SKBR3 cells were seeded into T25 tissue culture flasks. After 24 hours, MDA-MB-231 and MCF-7 cells were treated with 100 µM calpeptin for 30 minutes or 6 hours; SKBR3 cells were treated with 100 µM calpeptin for 30 minutes. Alternatively, MDA-MB-231 cells were treated with 2 µM A23187 or 200 µM calpeptin in serum free and complete medium (as described in section 2.2.6.2). DMSO was added to the negative control groups at the same volume equivalent to that in the drug treatment groups. The cells were collected in the same lysis buffer prepared for casein zymography (described below) and following the procedures described in section 2.2.5.

The total cell lysates were mixed with 1 x NuPAGE™ LDS Sample Buffer (Invitrogen, USA) and 1 x NuPAGE™ Reducing Agent (Invitrogen, USA) according to manufacturer's instructions. The samples were heated at 100°C for 5 minutes. 20 µL of sample was loaded into each well and separated on a Novex™ 4-12% Bis-Tris Protein Gel (Invitrogen, USA) by electrophoresis at 125V for 90 minutes. Then the protein was transferred from the gel onto a nitrocellulose membrane

by electrophoresis at 25V for 90 minutes. The blot was blocked with 3% milk in 0.1% PBS-Tween 20 at room temperature for 1 hour and then incubated in primary antibody solution anti-α-fodrin (1:1000, Enzo Life Sciences, UK) overnight at 4°C. After 3 x 5 minutes washes with 0.1% PBS-Tween20, the blot was incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 diluted in blocking buffer), based on the host species of the primary antibody, at room temperature for 1 hour. Before detection, the blot was washed again as described above. The bands were visualised with 1:1 mixture of Amersham enhanced chemiluminescence reagents (GE healthcare, UK) and developed using Amersham hyperfilm ECL (GE healthcare, UK) or scanned using Odyssey® Fc (LI-COR Biosciences, UK). If the blot needed to be re-probed, it was washed with dH_2O for 15 minutes and incubated in stripping buffer (Abcam, UK) at room temperature for 15 minutes. The blot was blocked again as described above, followed by re-probing with anti-β-actin 1:1000 dilution (Abcam, UK) at room temperature for 1 hour. Then the blot was developed again as described above.

2.2.6.4 Casein zymography

Casein zymography, a method for detecting calpain activity after electrophoresis in casein-containing acrylamide gels, was conducted as described previously with some modifications (Arthur and Mykles, 2000). After electrophoresis, in the presence of Ca^{2+} and reducing agents, casein can be digested by active calpain generating a clear band on stained gels, and reflect calpain activity. $1X10^6$ MDA-MB-231, MCF-7 and SKBR3 cells were seeded into T25 tissue culture flasks and treated with 1 µM A23187 or 100 µM calpeptin alone for 30 minutes at 37°C. Alternatively, MDA-MB-231 cells were treated with calpeptin (100 or 200 µM) at different time points (30 minutes, 6 hours and 24 hours). DMSO was added to the negative control group at the same volume equivalent to that in the drug treatment group. The cells were then collected in lysis buffer [50 mM HEPES (pH 7.6), 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM EDTA, 10 mM 2- mercaptoethanol] and the procedures described in section 2.2.5 followed. Casein (10 mg/mL) was

copolymerised in a 10% (w/v) acrylamide resolving gel and a 4.8% (w/v) acrylamide gel (without casein) was used as the stacking gel. The preparation of the gels is listed in Table 2.2 (Arthur and Mykles, 2000).

Table 2.2 Resolving and stacking gels

Solution	Resolving gel (mL)	Stacking gel (mL)
Acrylamide (30%)	3.3	0.8
Buffer A, 1.5 *M* Tris-HCl, pH 8.8	1.5	
Buffer A, 0.5 *M* Tris-HCl, pH 6.8		1.25
Casein, 10 mg/mL	2	
dH_2O	3.1	2.95
	Mix and degas briefly	
TEMED	10 µL	5 µL
10% ammonium persulphate	50 µL	25 µL

Abbreviations: TEMED, Tetramethylethylenediamine. Reproduced from (Arthur and Mykles, 2000), Methods in Molecular Biology, 144, 109-116; Copyright© 2000 by Springer with permission conveyed through Copyright Clearance Centre Inc.

Cell lysates were mixed with an equal volume of 1 x NuPAGE™ LDS Sample Buffer (Invitrogen, USA) then loaded into each well. The casein gel was electrophoresed at 125 V for 2 hours at 4°C in a pre-cooled electrophoresis buffer [25 mM Tris base, 125 mM glycine, 1 mM EDTA, and 10 mM 2-mercaptoethanol, pH 8]. The gel was then rinsed in 30-50 mL Ca^{2+} incubation buffer [50 mM Tris-HCl (pH 7), 5 mM $CaCl_2$, 10 mM 2- mercaptoethanol] twice at room temperature, 20-30 minutes each time; then incubated in fresh Ca^{2+} incubation buffer at 4°C with shaking overnight (to activate calpain). After incubation with calpain activation buffer, the gel was washed three times in dH_2O, and then microwaved at 450W for 1 minute. The gel was then stained in GelCode™ Blue Stain Reagent (ThermoFisher, USA) for 30 minutes, followed by washing in dH_2O for 30-60 min. Calpain activity was visualised as a clear band in the dark blue gel. Gel scanning was conducted using Odyssey® Fc (LI-COR Biosciences, UK).

2.2.7 Cell growth curves

Growth curves were initially conducted over a 96 hour time course to determine

control cell characteristics; they were subsequently used to determine the role of calpain in cell proliferation by applying the cell permeable inhibitor calpeptin. $1x10^5$ MDA-MB-231, $1x10^5$ MCF-7 and $6x10^5$ SKBR3 cells were seeded into each well of a 6-well tissue culture plate. After 24 hours cells were treated, in triplicate, with calpeptin at different concentrations (0, 25, 50 and 100 μM). DMSO was added to the negative control groups at the same volume equivalent to that in the drug treatment groups. At 24, 48, 72 hours after drug treatment, cells were trypsinised and the total cell number counted by haemocytometer. Growth inhibition was expressed as the percentage of total cells in drug treatment cultures compared with untreated cultures. Data represent the average ± standard deviation (SD) of at least two independent experiments.

2.2.8 Haptotaxis (scratch wound) migration assay

Haptotaxis (scratch wound) migration assays were performed as described previously (Safuan et al., 2012). MDA-MB-231 and MCF-7 cells were non-transfected, had calpastatin knockdown by shRNA, or were transfected with non-targeted/scrambled shRNA – transfection and clonal selection was conducted by Dr. Sarah Storr (Table 2.3).

Table 2.3 Cell lines transfected with shRNA used in migration assay

Cells	Transfected	Seeding density	Abbreviations
MDA-MB-231 wild-type	Wild-type cells	$4X10^5$ cells/well	231-WT
MDA-MB-231 negative ctrl	Transfected with vector only	$4X10^5$ cells/well	231-Neg
MDA-MB-231 scrambled ctrl	Transfected with non-target shRNA	$5X10^5$ cells/well	231-Scr
MDA-MB-231 CAST knockdown	Transfected with calpastatin shRNA	$4X10^5$ cells/well	231-calpastatin KD
MCF-7 wild-type	Wild-type cells	$5X10^5$ cells/well	MCF7-WT
MCF-7 negative ctrl	Transfected with vector only	$5X10^5$ cells/well	MCF7-Neg
MCF-7 scrambled ctrl	Transfected with non-target shRNA	$5X10^5$ cells/well	MCF7-Scr
MCF-7 CAST knockdown	Transfected with calpastatin shRNA	$5X10^5$ cells/well	MCF7-calpastatin KD

In the current study transfected cells were pre-incubated with A23187 at a non-toxic concentration of 2 µM for 24 hours, to induce Ca^{2+} afflux into the cells. Our group's previous data showed that the 24 hours treatment of 2 µM A23187 in complete culture medium caused only 5.1% cell apoptosis and necrosis in MDA-MB-231 cells, but in serum free conditions the percentage increased to 95.6% (conducted by Dr. Yimin Zhang). In the same manner, A23187 in complete culture medium resulted in 5-10% cell apoptosis in MCF-7 (Chen et al., 2004). A range between 0.1 µM to 100 µM of A23187 has been shown to be able to cause a large Ca^{2+} influx in MCF-7 cells (Journé et al., 2004); and addition of 3 µM A23187 can rapidly lead to activation of heterodimeric calpains in erythrocytes (Molinari et al., 1994). For the migration assay, $4X10^5$ or $5X10^5$ cells/well (as indicated above) was seeded into a 12-well tissue culture plate. After 24 hours, a confluent tumour cell monolayer had medium replaced with complete medium with or without 2 µM A23187 and incubated at 37°C for 24 hours. After treatment, confluent cell monolayers were scratched with a pipette tip to create a wound. The monolayer was rinsed twice with 500 µL of PBS to remove any cellular debris and smooth the edge of the wound. The medium was replaced with complete medium containing 10 µg/mL mitomycin-C (Sigma, UK) for the duration of the assay, to inhibit cellular proliferation. Wound closure was monitored by photomicrographs taken at 0, 2, 4, 6 and 24 hours following the scratch. The percentage reduction of the scratch area at different time points represented the rate of tumour cell migration and was acquired using Image J 1.43u software (National Institute Health, USA). All of the experiments were performed in triplicate and repeated at least three times. Data represent the average ± standard deviation (SD) of three independent experiments.

2.2.9 Human phosphokinase signalling array

The role of calpain in regulating signal transduction was assessed using a Human Phosphokinase Array Kit (R&D Systems, USA). The kit can simultaneously detect the relative level of phosphorylation of 43 kinases at phosphorylation sites and 2 related total proteins. Phosphokinase array experiments for MDA-MB-231 and MCF-7 cells were conducted previously by Dr. Sarah Storr. Here $5x10^6$ SKBR3

cells were seeded into T175 flasks and treated with or without 100 µM calpeptin for 30 minutes. DMSO was added to a negative control group at the same volume equivalent to that in the drug treatment group. The cells were harvested and collected in Lysis Buffer (provided in the kit) and the concentrations of proteins determined by the Bio-Rad protein assay following the process described in section 2.2.5. 600 µg of total protein was mixed with Lysis Buffer and Array Buffer 1 following manufacturers' instructions to reach a final volume of 2 mL. The arrays were blocked with Array Buffer 1 for one hour in advance followed by incubation with the protein samples overnight at 4^{o}C. The array was then washed with 1x Wash Buffer for 3X10 minutes to remove unbound proteins and incubated with corresponding Detection Antibody Cocktails for 2 hours at room temperature. Streptavidin-HRP and ECL reagents were applied to the array sequentially with extensive washes following each step. The arrays were visualised using X-ray films with multiple exposure time points. A signal was produced at each spot on the array corresponding to the amount of phosphorylated protein bound. The positive signals (reference spots) were included to ensure that the arrays have been incubated with Streptavidin-HRP during the assay procedure. The relative change in phosphorylated kinase proteins between treated/untreated samples were determined by comparing corresponding signals on different arrays.

2.2.10 Western Blotting

The specificity of antibodies to be used for immunohistochemical studies (in the current chapter) was determined by Western blotting, as described in section 2.2.6.3. Blots were then blocked and probed with various primary antibodies, followed by the corresponding conjugated secondary antibodies (Table 2.4).

Table 2.4 List of antibodies used in Western blotting

Antibodies	Source	Host Type	Clone	Dilution	Size (kDa)
Anti-α-fodrin	Enzo Life Sciences	Mouse	AA6	1:1000	240
Anti-Calpain-1	Santa Cruz	Mouse	P-6	1:1000	80
Anti-Calpain-2	Abcam	Mouse	3G1	1:2000	80
Anti-Calpain II	Merck Millipore	Rabbit	-	1:1000	80
Anti-Calpain small subunit	Merck Millipore	Mouse	P-1	1:1000	30
Anti-Calpastatin	Merck Millipore	Mouse	PI-11	1:1000	70-150
Anti-MSK1	Bethyl Laboratories	Rabbit	9252	1:2000	90
Phospho-MSK1(S376)/MSK2(S360)	R&D	Rabbit	1013D	1:1000	90
Anti-β-actin	Abcam	Mouse	8226	1:1000	42
Anti-β-actin	Abcam	Rabbit	8227	1:1000	42
Anti-mouse secondary	Dako	Mouse-HRP	-	1:1000	-
Anti-rabbit secondary	Dako	Rabbit-HRP	-	1:1000	-

2.2.11 Clinical samples

The immunohistochemical study was performed using a cohort of 1902 early stage breast cancer patients treated at Nottingham University Hospitals between 1986 and 1998 with long term follow-up. Information on clinical history and outcome was maintained on a prospective basis and patients' clinical history and tumour characteristics were assessed in a standardised manner. The median age of the patients was 55 years (ranging from 18 to 72) and median follow-up time was 177 months (ranging from 1 to 308 months). 63.2% (1203 of 1902) of the patients had stage I disease.

Patients were managed under a uniform protocol, where all underwent mastectomy (n=1067, 56.1%) or wide local excision (n=819, 43.1%) and approximately half of the patients received radiotherapy (n=1025, 53.9%).

Systemic adjuvant treatment was given dependent upon NPI values, ER and menopausal status. Patients with an NPI value less than 3.4 did not receive adjuvant chemotherapy, whereas patients with an NPI value of 3.4 or above were chosen for CMF chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil, n=320, 16.8%) if they were ER negative or premenopausal; patients with ER positive disease were candidates for hormone therapy (n=674, 35.4%).

Breast cancer-specific survival was defined as the time interval (in months) between the start of primary surgery to death resultant from breast cancer. ER, PR and HER2 status were available for this cohort and have been described previously (Abdel-Fatah et al., 2010). The positivity of ER and PR were based upon and determined if staining was above 1%. HER2 expression was determined by immunohistochemistry with chromogenic in situ hybridisation (CISH) used as the arbiter in cases with an immunohistochemistry score of 2. Basal-like molecular subtypes was defined as the detection of cytokeratin (CK)-5/6 and/or CK-14 expression in 10% or more of invasive breast tumour cells, irrespective of ER, PR or HER2 status (Rakha et al., 2006). This study was reported in accordance with REMARK criteria (McShane et al., 2005). The Nottingham cohort was approved by Nottingham Research Ethics Committee 2 under the project “Development of a molecular genetic classification of breast cancer R&D “(No. 03HI01 REC Ref.C202313)”. The clinicopathological variables of the cohort are shown in Table 2.5.

Table 2.5 Clinicopathologic variables of patient cohort

Variables	No. (%)	Variables	No. (%)
Age (mean ± SD, years)	54.25 (±9.77)	**ER status**	
≤40 years	165 (8.7%)	Positive	1370 (72.0%)
>40 years	1736 (91.3%)	Negative	476 (25.0%)
ND	1 (0.1%)	ND	57 (3.0%)
Tumour size (mm)	2.06±1.14	**PR status**	
≤20 mm	1185 (62.3%)	Positive	1035 (54.4%)
>20 mm	708 (37.2%)	Negative	739 (38.9%)
ND	9 (0.5%)	ND	128 (6.7%)
Tumour stage		**HER2 status**	
I	1203 (63.2%)	Positive	243 (12.8%)
II	531 (27.9%)	Negative	1602 (84.2%)
III	160 (8.4%)	ND	57 (3.0%)
ND	8 (0.4%)	**Basal status**	
Tumour grade		Positive	368 (19.3%)
I	346 (18.2%)	Negative	1390 (73.1%)
II	632 (33.2%)	ND	144 (7.6%)
III	915 (48.1%)	**Triple-negative status**	
ND	9 (0.5%)	Positive	315 (16.6%)
NPI	4.16±1.18	Negative	1516 (79.7%)
≤3.4	619 (32.5%)	ND	71 (3.7%)
3.41-5.4	948 (49.8%)	**Breast cancer-specific survival**	
>5.4	324 (17.0%)	Alive	1064 (55.9%)
ND	11 (0.6%)	Dead	505 (26.6%)
Lymphovascular invasion		ND	333 (17.5%)
Positive	492 (25.9%)	**Recurrence**	
Negative	1070 (56.3%)	Present	752 (39.5%)
ND	340 (17.9%)	Not present	1103 (58.0%)
Operation type		ND	47 (2.5%)
Mastectomy	1067 (56.1%)	**Distant metastasis**	
WLE lumpectomy	819 (43.1%)	Present	579 (30.4%)
ND	16 (0.8%)	Not present	1310 (68.9%)

Continuous data are shown as mean ± standard deviation (SD). Abbreviations: NPI, Nottingham Prognostic value; WLE, wide local excision; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; ND, not determined.

2.2.12 Tissue microarray construction and immunohistochemistry

Protein expression was investigated in a tissue microarray (TMA) by using immunohistochemistry. The TMA was constructed by the Breast Pathology Group at the University of Nottingham. A single 0.6 mm tissue core was used

for each patient with the core being taken from a representative tumour area as assessed by a specialist breast cancer histopathologist. Freshly cut 4 µm thick TMA sections, mounted onto X-tra adhesive slides (Leica Biosystems), were used and immunohistochemistry was performed (Storr et al., 2011c). The specificity of antibodies was confirmed by Western blotting, as described in section 2.2.10, prior to use on the tumour specimens. Antibodies generating expected single bands without non-specific bands were applicable for TMA staining. The optimal concentrations of antibodies to be used for immunohistochemistry were initially determined using breast tumour composite sections which consisted of six stage I breast tumours (grade I to III), following the procedures described below.

Briefly, slides were heated at 60^{o}C for 10 minutes then dewaxed in two xylene baths (5 minutes each) and sequentially rehydrated in three industrial methylated spirit (IMS) baths (2 minutes each), followed by a 5 minute wash in running tap water. The whole procedure was conducted automatically using an Autostainer XL staining System ST5010 (Leica, USA). Antigen retrieval was performed in 0.01 mol/L sodium citrate buffer (pH 6) in a microwave, 750W for 10 minutes followed by 450W for 10 minutes. The slides were then loaded into a sequenza slide rack (ThermoFisher Scientific, USA) and staining was achieved using a Novolink Polymer Detection System (Leica, Denmark) following the manufacturers' instructions. Briefly, endogenous peroxidase activity was neutralised with Peroxidase Block reagent for 5 minutes at room temperature; followed by application of Protein Block reagent for 5 minutes at room temperature, to minimise non-specific interactions of the subsequent detection reagents. Primary antibody (anti-MSK1, 1: 200, Bethyl Laboratories, USA) was diluted in Bond Primary Antibody Diluent (Leica, Denmark) and applied to the tissue for 1 hour at room temperature. Post Primary reagent, a polymer penetration enhancer, was applied on the slides for 30 minutes; followed by NovoLink Polymer (anti-mouse/rabbit IgG-Poly-HRP) for another 30 minutes. Immunohistochemical reactions were visualised with 3, 3'-diaminobenzidine (DAB, Dako) and counterstained with haematoxylin. 2 x 5 minutes wash with

Tris buffered saline (TBS) was applied following each step during the procedure. Then the slides were loaded into the Autostainer for dehydration: 5 minutes wash with running tap water followed by 3 x 2 minutes IMS baths, and then 2 x 5 minutes xylene baths. The tissues were mounted with DPX (Leica, Germany) and left to dry overnight. Breast tumour composite sections were used as positive and negative controls; negative controls had primary antibody omitted.

Staining was assessed at 20X magnification following high-resolution scanning (Nanozoomer Digital Pathology Scanner, Hamamatsu Photonics). Protein expression was assessed using a semi-quantitative immunohistochemistry H-scoring as previously described (Storr et al., 2011c). The staining intensity was assessed as: none (0), weak (1), medium (2) and strong (3), and H-scores were calculated by multiplying the percentage of positive areas by the staining intensity, giving rise to a score ranging between 0 and 300. 30% of scores were examined by a second independent assessor, blinded to clinicopathological and survival endpoints. Single measure intraclass correlation coefficients (ICC) analysis was conducted to determine the level of agreement between independent scorers. For MSK1, ICC was 0.881 (conducted by Dr. Narmeen Ahmad), showing good concordance between both scorers. The cut point of immunohistochemical scores was determined in a non-biased fashion using X-tile software. The X-tile software provides a non-biased cut-point by describing the presence of substantial tumour subpopulations and showing the strong associations between tumour biomarker expression and patient outcomes (Camp et al., 2004).

2.2.13 Statistical analysis

Results from *in vitro* experiments were represented as average ± standard deviation (SD) of at least two independent experiments with each performed in duplicate or triplicate. The Student t-test (comparing two groups) and ANOVA one-way test (comparing >2 groups) were used to evaluate the variation between control and drug treatment groups. For the immunohistochemistry study, spearman's rank test was performed to assess correlation between the

expression levels of different proteins. The relationship between categorised protein expression and clinicopathologic factors were examined using Pearson's chi-square test of association (χ^2) or Fisher's exact test if a cell count was less than 5 in a 2 x 2 table. Survival curve analysis was conducted using the Kaplan-Meier method and significance determined using the Log-rank test. Multivariate survival analysis used the Cox proportional hazards regression model. All differences were considered statistically significant at the level of $P<0.05$. Statistical analysis was performed using SPSS 22.0 software or GraphPad Prism 6 software (GraphPad Software Inc.).

2.3 Results

2.3.1 Detection of calpain activity

The analytical techniques for detecting calpain activity in living cells, including immunofluorescence assays, Western blotting and zymography, were optimised in the current study prior to studying the functional roles of calpain. Calpains have abundant substrates that exist in a wide range of tissues and cells, such as cytoskeletal proteins MAP-2 and tau. Among these, casein and α-fodrin are two well characterised calpain substrates and are widely used for detecting calpain activity. Several synthetic fluorogenic substrates such as Suc-Leu-Tyr-AMC and t-Boc-Leu-Met-CMAC are available and were used as calpain substrates in the assays (Goll et al., 2003).

2.3.1.1 T-Boc calpain activity assay

The calpain cleavage of the fluorogenic substrate t-Boc was used to measure the calpain activity in breast cancer cells. MDA-MB-231 cells were treated with 100 µM calpeptin alone; or 0.1 µM A23187 for 30 minutes followed by 100 µM calpeptin for 30 minutes; alternatively, increasing concentrations of calpeptin (100, 150, 200 and 250 µM) were added to the culture medium and incubated for 30 minutes. The results from many independent experiments (n=26) were pooled together and, as shown in Figure 2.1-A, treatment with 100 µM calpeptin for 30 minutes resulted in an average of 26% inhibition (the 10^{th} cycle), with considerable variation between experiments.

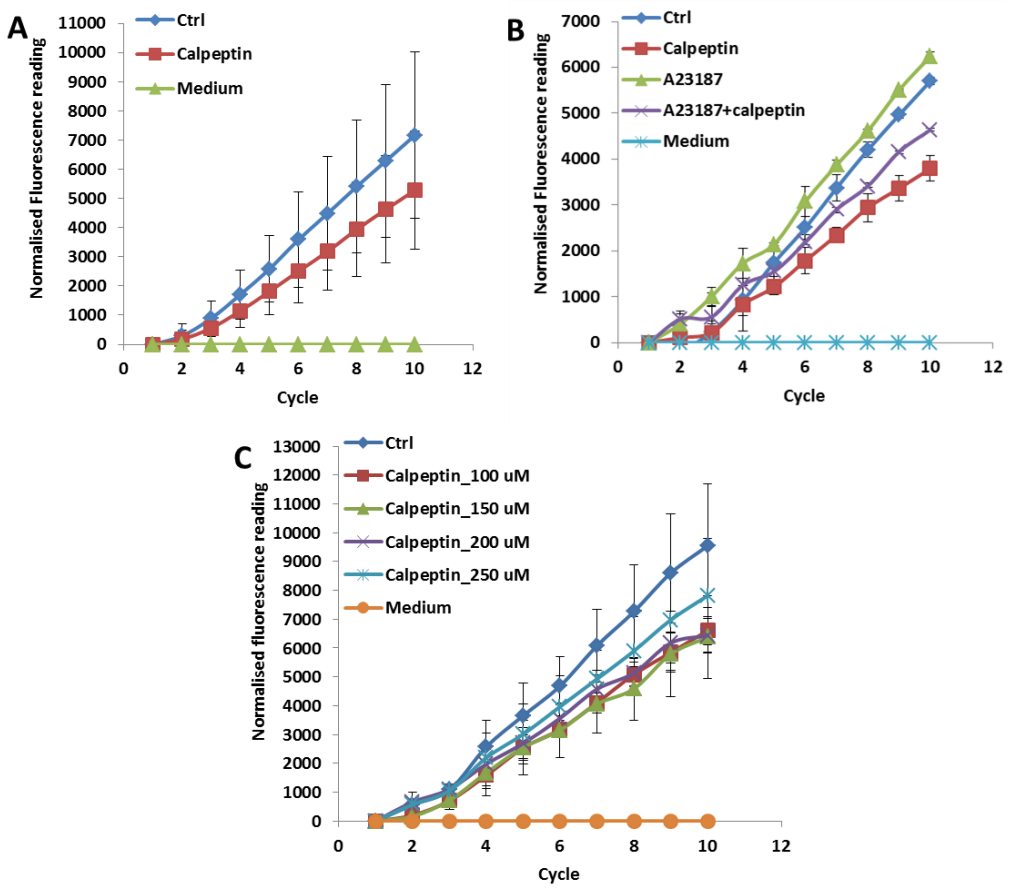


Figure 2.1 Detection of calpain activity was optimised by using t-Boc activity assay. (A) MDA-MB-231 cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes (n=26); (B) MDA-MB-231 cells were treated with 100 µM calpeptin or 0.1 µM A23187 alone for 30 minutes; or treated with A23187 for 30 minutes followed by calpeptin for 30 minutes (n=2); (C) MDA-MB-231 cells were treated with calpeptin for 30 minutes at various concentrations (n=2). Data represent the average ± standard deviation (SD) of at least two independent experiments, with each experiment performed in duplicate.

As shown in Figure 2.1-B (n=2), 100 µM calpeptin alone caused an average of 34% inhibition (the 10th cycle); while 0.1 µM A23187 increased calpain activity by 10% compared to the control. When adding A23187 followed by calpeptin, the calpain activity was decreased by 19%, suggesting calpeptin can attenuate activation effects of A23187. Increasing concentrations of calpeptin did not show dose-dependent inhibitory effects, causing 18-33% inhibition of calpain activity (Figure 2.1-C, n=2). Inconsistent results were observed over experiments, the maximal inhibition effects of calpain activity was ~53% (the 10th cycle); however eight of them showed no detectable alteration after treatment with the inhibitor (the fluorescence of calpeptin treated cells was nearly equivalent to untreated cells). Although the IC50 of calpeptin in this

assay can be achieved at a concentration of 100 µM for 30 minutes, the instability and reproducibility difficulty of the assay has been noted when interpreting the data.

2.3.1.2 AMC calpain activity assay

Calpain activity was also detected using the Suc-LLVY-AMC substrate in a fluorometric assay. MDA-MB-231 cells were treated with 2 µM A23187 or 100 µM calpeptin alone for 30 minutes; or treated with A23187 for 30 minutes followed by calpeptin for an extra 30 minutes. As shown in Figure 2.2-A, in serum free medium, the treatment of 100 µM calpeptin alone inhibited calpain activity by 32%; however, the treatment of 2 µM A23187 also appeared to inhibit calpain activity by 61%. When the combinational treatment applied, the calpain activity was still decreased by 69%, compared with the control.

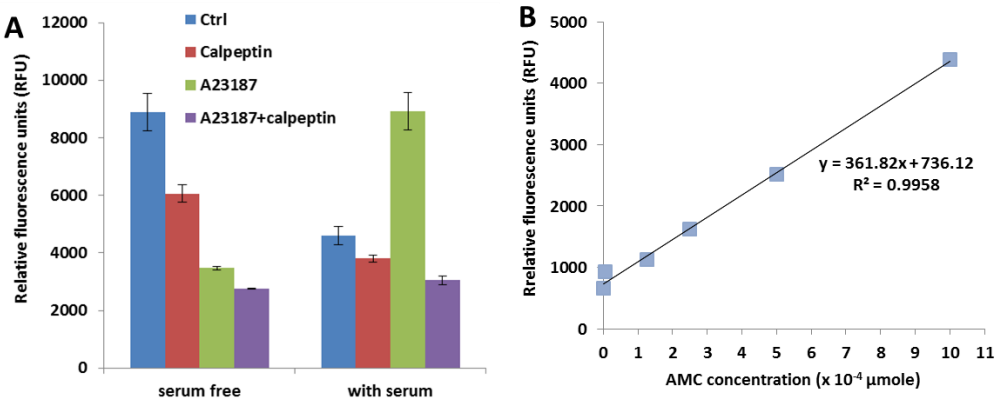


Figure 2.2 Detection of calpain activity was optimised by using AMC activity assay. (A) MDA-MB-231 cells were treated with 2 µM A23187 or 100 µM calpeptin alone for 30 minutes; or treated with A23187 for 30 minutes followed by calpeptin for 30 minutes. (B) The AMC standard curve was prepared following manufacturer's instruction. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in duplicate.

In complete culture medium, A23187 markedly increased calpain activity by 94%, compared with control; and A23187 treatment followed by calpeptin effectively inhibited calpain activity. However, the treatment of calpeptin alone had no obvious inhibitory effect on calpain activity (decreased by 17%). The calpain activity was quantitated using an AMC standard curve (Figure 2.2-B) and

displayed as relative fluorescence units (RFU). In all, calpeptin did not show marked inhibitory activity in this assay either in the serum free or complete medium. Thus it is difficult to obtain accurate assessment of calpain activity by using this assay.

2.3.1.3 Detection of cleaved α-fodrin in Western blotting

As described above, calpain degradation of α-fodrin can generate two proteolytic breakdown products (145-&150-kDa). The distinct fragmentation pattern of α-fodrin has been widely used as an assay for calpain activity in skeletal muscle and neuronal cells (Dutta et al., 2002, Takamure et al., 2005). Treatment with 10 µM and 20 µM A23187 have been shown to stimulate cleavage and generate 145- and 150-kDa fragments in rat myoblasts within 1 hour (Huang and Forsberg, 1998). As shown in Figure 2.3-A, the full length 240-kDa protein of α-fodrin can be seen from the immunoblot analysis; however the cleavage products of α-fodrin could not be detected either in 100 µM calpeptin treated or untreated cells.

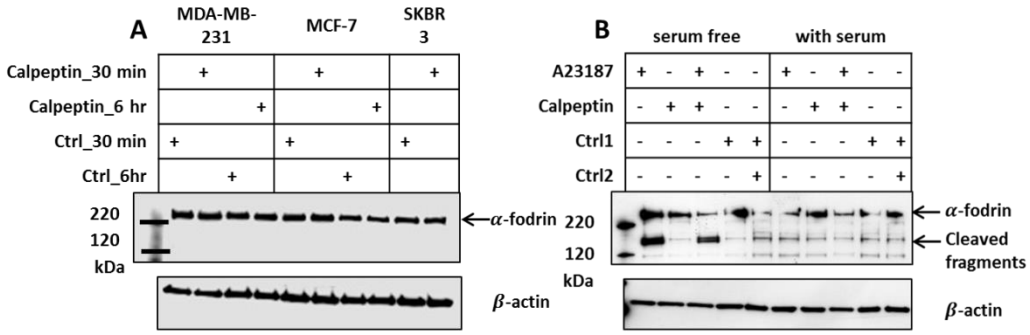


Figure 2.3 Detection of calpain activity by assessing α-fodrin degradation was optimised by using Western blotting. (A) Cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes and 6 hours; (B) MDA-MB-231 cells were treated with 2 µM A23187 or 200 µM calpeptin alone for 30 minutes; or treated with A23187 for 30 minutes followed by calpeptin for 30 minutes. Each experiment was conducted once. DMSO was added to Ctrl1 and Ctrl2, with the same volume corresponding to A23187+calpeptin, calpeptin only.

After treatment of 2 µM A23187 for 30 minutes followed by 200 µM calpeptin for 30 minutes, the immunoblot showed a 240-kDa band, in addition to a ~150-kDa fragment (Figure 2.3-B). In serum free condition, α-fodrin was protected

from calpain-mediated cleavage following calpeptin treatment. A marked increase of 150-kDa α-fodrin fragment induced by A23187 was detected; the formation of 150-kDa fragments was partially attenuated by the addition of calpeptin to A23187. In complete medium, relatively weak but detectable signals were seen at size 150- and 240 -kDa, but there was no obvious difference between A23187-treated, calpeptin-treated or combined treated-samples.

2.3.1.4 Casein zymography

A zymographic assay for calpain activity, using a casein-containing acrylamide gel, was also optimised. In the current assay, the gel was pre-incubated with Ca^{2+} buffer overnight to initiate calpain activity. As shown in Figure 2.4-A, for the cell lysates from SKBR3 and MDA-MB-231, casein zymography detected no obvious differences in casein degradation between 100 µM calpeptin-treated and untreated cells; while the bands were barely visible in MCF-7 samples.

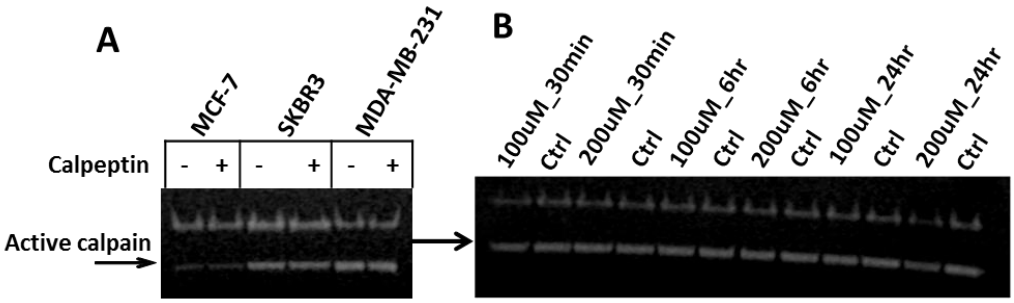


Figure 2.4 Detection of calpain activity by assessing casein degradation was optimised in casein zymography. (A)Cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes; (B) MDA-MB-231 cells were treated with 100 or 200 µM calpeptin for 30 minutes, 6 or 24 hours. Each experiment was conducted once.

There was no apparent change in casein degradation in MDA-MB-231 cells treated with 100 or 200µM calpeptin for 30 minutes, 6 or 24 hours, in comparison with controls (Figure 2.4-B). The major difficulty of casein zymography is to obtain precise quantified results. The non-standardised staining/washing steps, as well as scanning might also affect sensitivity and reproducibility of the assay (Arthur and Mykles, 2000). One study has demonstrated that in casein zymography, µ-calpain produced the most prominent activity at pH7.5 (ranging from 7.2-7.8), while m-calpain was at

pH7.3 (ranging 7.2-7.4); indicating analysis of calpain activity by casein zymography is highly dependent on pH level (Zhao et al., 1998). Therefore a strict pH control and standardised protocol should be considered for further investigation if using casein zymography.

Collectively, amongst four different assays, fluorometric assays (i.e. t-Boc and AMC) can generate quantitative measurements of calpain activity; as for detection of calpain activity via substrate degradation (α-fodrin and casein), the results were difficult to quantify. Inconsistent results were obtained from the t-Boc assay, and it could not detect a dose-dependent inhibitory effect of calpeptin. However it appeared that t-Boc showed favourable sensitivity over other assays, as it can detect up to approximately 50% of calpain inhibition in the cells. Based on such data, it was decided to use the t-Boc activity assay for future experiments, to ensure and check that calpain was active.

2.3.2 Calpain/calpastatin protein expression

In order to study the functional role of calpain and calpastatin, protein expression across six breast cancer cell lines were first examined by Western blotting. As shown in Figure 2.5, apart for MDA-MB-231 cells, that expressed slightly lower level of calpain-1, the remaining five cell lines showed similar calpain-1 expression levels; which was consistent with quantitative Western blotting results conducted by another group member (Ms Bhudsaban Sukkarn).

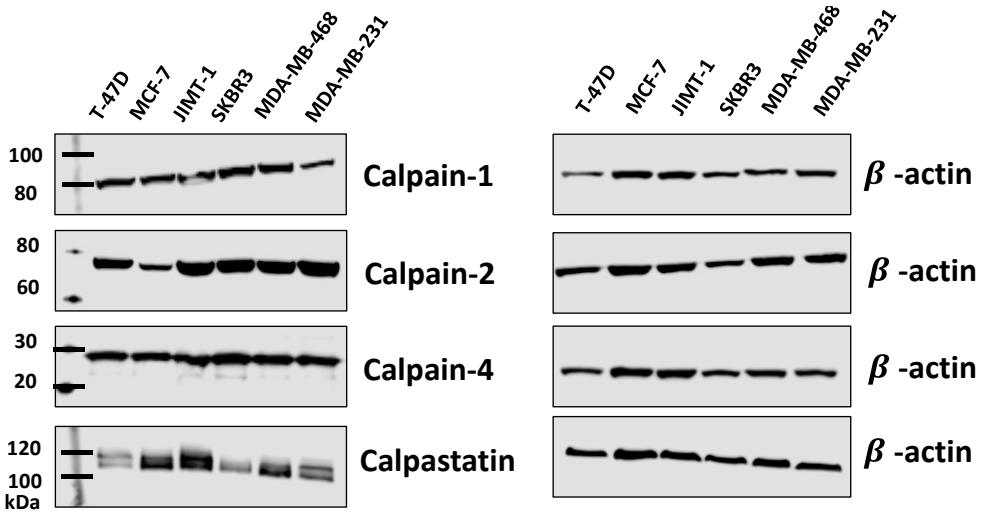


Figure 2.5 Calpain family and calpastatin protein expression across different molecular subtypes of breast cancer cells. Primary antibodies against calpain-1, calpain-2, calpain-4 and calpastatin were used. Experiments were repeated twice with separate lysates from different passage numbers and the representative blots are shown above.

MCF-7 cells expressed an observable lower level of calpain-2 compared with the other breast cancer cell lines, which was consistent with a previous report from Wu et al. that calpain-2 was not expressed in MCF-7 cells (Wu et al., 2006). There was no obvious difference in calpain-4 expression between six breast cancer samples. Calpastatin protein was expressed across six breast cancer cells with two fragments being detected in all of the samples. MCF-7, JIMT-1 and MDA-MB-468 cells expressed relatively high levels of calpastatin, while relatively low but detectable amounts of calpastatin protein were observed in T-47D, SKBR3 and MDA-MB-231 cells. Calpastatin protein expression was quantified using two colour fluorescence Western blotting. As with ECL Western blotting, high levels of calpastatin were seen in MCF-7, JIMT-1 and MDA-MB-468 cells, while lower amounts were observed in T-47D, SKBR3 and MDA-MB-231 cells (Figure 2.6).

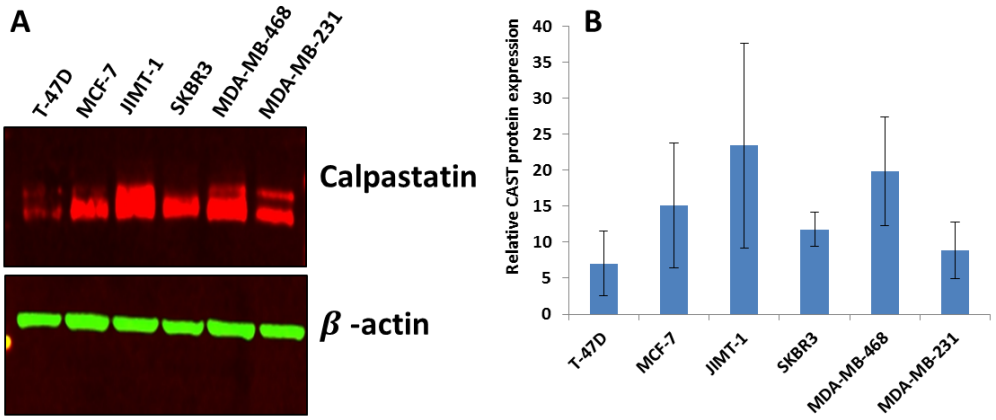


Figure 2.6 Quantification of calpastatin protein expression across different molecular subtypes of breast cancer cells. Primary antibody against calpastatin was used. Experiments were repeated three times with separate lysates from different passage numbers of cells. The representative blots as above. (A) Representative blots of three independent experiments; (B) data are presented as the average ± standard deviation (SD) of three independent experiments.

There is a trend showed that the sequence of endogenous calpastatin expression level, from high to low, is JIMT-1 > MDA-MB-468 > MCF-7 > SKBR3 > MDA-MB-231 > T-47D. However, the expression levels between all cell lines were not significantly different, and varying amounts of protein expression can be seen between experiments.

2.3.3 Calpain activity across different breast cancer cell molecular subtypes

The inhibitory effects of calpeptin on calpain activity at different incubation time points (30 minutes, 6 and 24 hours) were assessed in three different breast cancer cells: MDA-MB-231, MCF-7 and SKBR3. They are representative cells lines for basal-like, luminal and HER2+ subtypes, respectively. As expected, the treatment of the three cell lines with calpain inhibitor calpeptin resulted in a decrease in calpain activity (Figure 2.7-A, B&C).

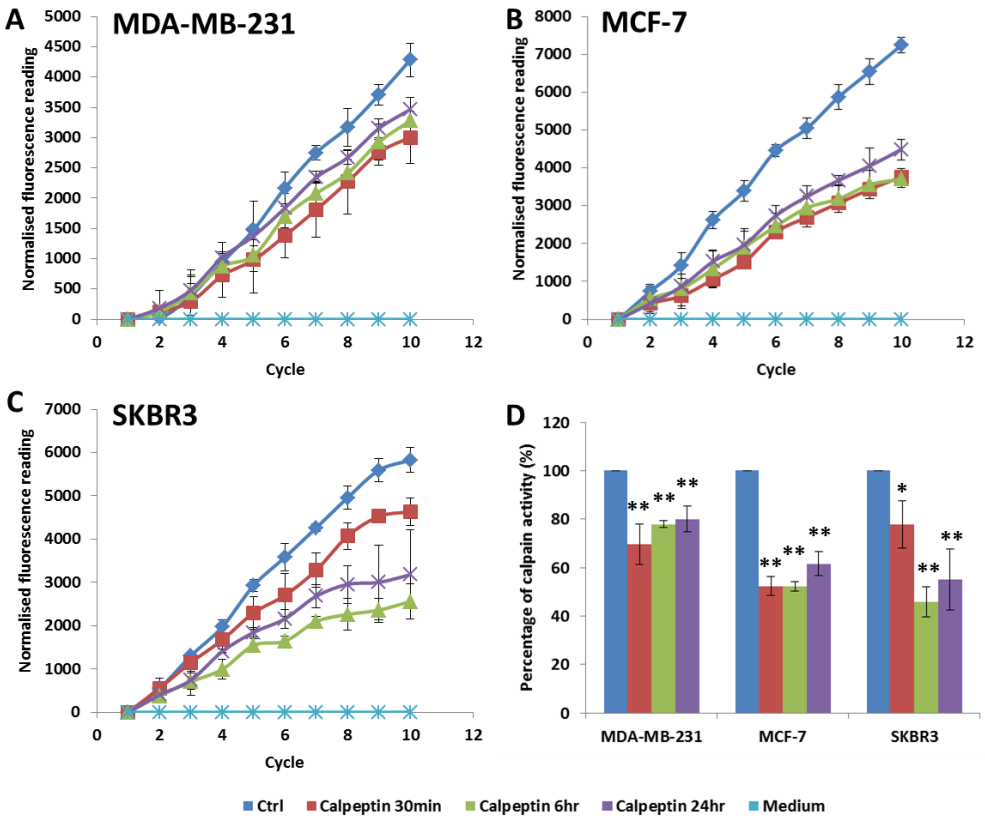


Figure 2.7 Inhibition of calpain activity across different molecular subtypes of breast cancer cells. MDA-MB-231 (A), MCF-7 (B) and SKBR3 (C) cells were treated with 100µM calpeptin for 30 minutes, 6 and 24 hours. (D) The fluorescence readings at the 10th cycle in the three cells. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in duplicate. Statistical significance determined by one-way ANOVA test compared to control is indicated by asterisk. **P*<0.05, ***P*<0.01 vs control.

However, in the three cell lines, the inhibition effects of calpeptin did not show a time-dependent pattern; even with prolonged exposure up to 24 hours, calpeptin did not completely block calpain activity. In MDA-MB-231 cells, calpeptin at a concentration of 100 µM led to approximately 20-30% inhibition of calpain activity (the 10th cycle) as shown in Figure 2.7-D, with significant difference seen at all time points. In MCF-7 cells, calpeptin resulted in significant reduction (approximately 38-48%) in calpain activity at all three time points. In SKBR3 cells, 20% calpain inhibition was observed at 30 minutes; larger significant inhibition effects (approximately 45-54%) were observed at 6 and 24 hour time points. The relatively low level of inhibition effect was observed at 30 minutes only for SKBR3 cells, but not in other two cell lines, which might be a reflection of the phenotypic differences. MDA-MB-231 and MCF-7 cells are non-

HER2 overexpressing cell lines, whereas in SKBR3 cells HER2 is overexpressed. The incubation of purified human calpain-1 has been shown to cleave HER2 and phospho-HER2 in SKBR3 cells, and such effect can be inhibited by 100 μM of calpeptin for 6 hours (Kulkarni et al., 2010). In contrast, HER2 can inhibit calpain-1 activity by upregulating calpastatin (Ai et al., 2013). These studies suggested the potential link between HER2 and calpain-1, which might provide a possible explanation for the low level of inhibitory effect of calpeptin in SKBR3 cells at 30 minutes.

It is difficult to compare activity across the three cell lines; it appeared that MCF-7 cells had the highest level of active calpain (fluorescence reading between 7000-8000), followed by SKBR3 cells (between 5000-6000), and MDA-MB-231 cells presented the lowest calpain activity (between 4000-5000). Unexpectedly, MDA-MB-231 cells had the most aggressive tumorigenesis and migratory ability, but with the lowest level of calpain activity in the current study. However, it is difficult to draw a direct link between calpain activity and migratory ability; as several other factors are attributable for cell migration, i.e. cell focal adhesion and interaction with extracellular matrix (as introduced in Chapter 1). More details will be discussed in section 2.4.

2.3.4 Role of calpain in functional effects

2.3.4.1 Proliferation

The anti-proliferative effects of calpeptin on MDA-MB-231, MCF-7 and SKBR3 cells, were assessed by conducting growth curves following treatment of the cells with increasing concentrations of calpeptin (0, 25, 50 and 100 μM) at 24, 48 and 72 hours. For the three cell lines the growth curves showed a dramatically inhibition at every time point following 100 μM calpeptin treatment (Figure 2.8-A, B&C, right). After 24 and 48 hours treatment with concentrations lower than 50 μM, calpeptin did not show obvious anti-proliferative effects on the three cell lines. Calpeptin caused a maximal dose-dependent reduction at the highest concentration after 72 hours, reaching the lowest percentage of cell number at 29, 14 and 32% in MDA-MB-231, MCF-7

and SKBR3 cells, respectively (Figure 2.8-A, B&C, left).

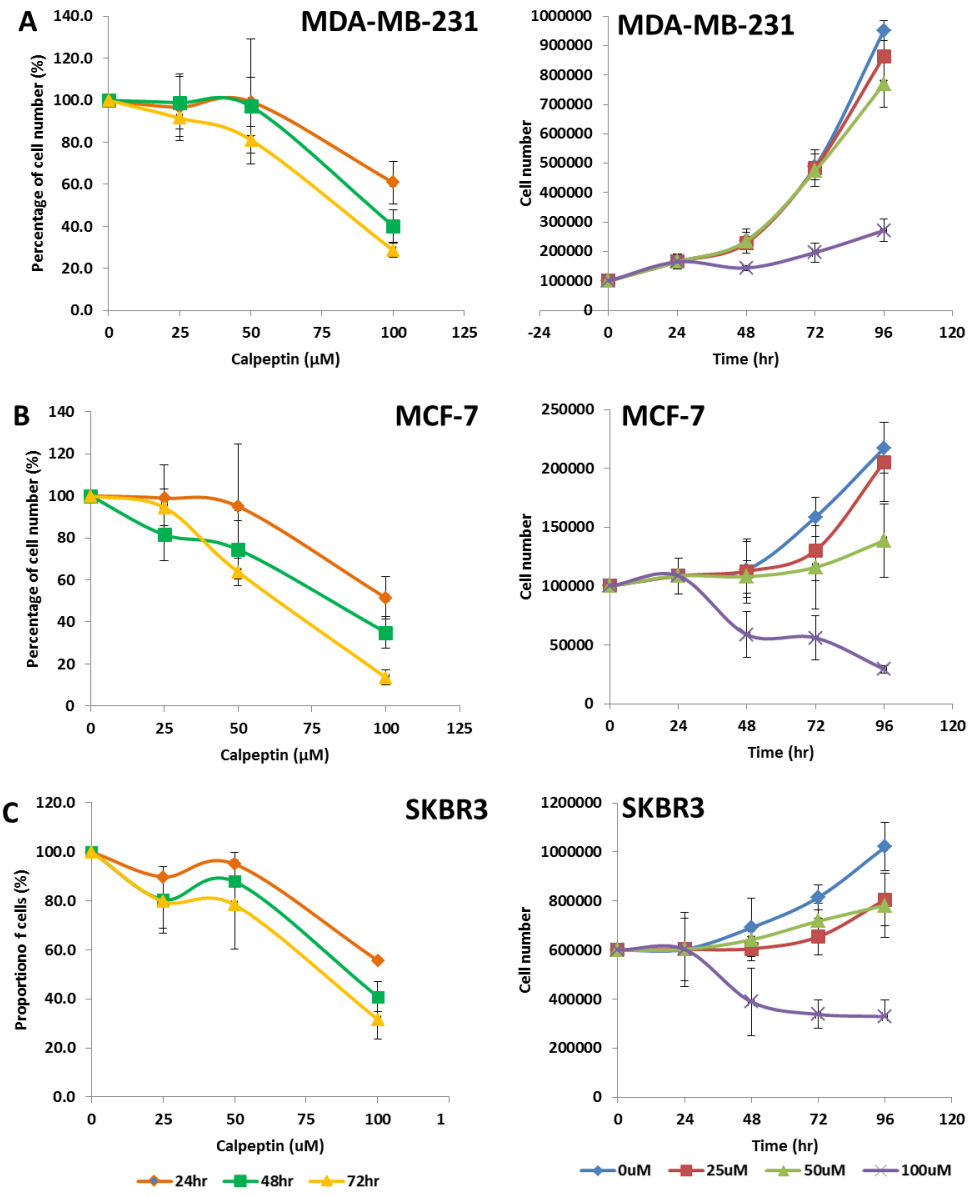


Figure 2.8 Effect of calpeptin on proliferation of breast cancer cells and corresponding growth curves. MDA-MB-231 (panel A), MCF-7 (panel B) and SKBR3 (panel C) were treated with various doses of calpeptin for 24, 48 and 72 hours. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in triplicate.

When comparing between the three cell lines, 50% inhibition was seen in MCF-7 and SKBR3 cells as early as 24 hours at a dose of 100 µM; whilst at 48 hours' treatment, the three cell lines demonstrated very similar sensitivity to calpeptin, estimated IC50 values were approximately equal, ranging between 80-90 µM. When the incubation time increased to 72 hours, MCF-7 cells had an estimated

IC50 value of between 60-65 µM; MDA-MB-231 and SKBR3 cells both having estimated IC50 values of between 80-85 µM.

2.3.4.2 Migration

To examine the function of calpastatin in regulating breast cancer cell migratory ability, MDA-MB-231 and MCF-7 cells were stably transfected with pRS plasmid vectors encoding calpastatin shRNA (conducted by Dr. Sarah Storr), with successful knockdown of calpastatin verified by Western blotting. As demonstrated in Figure 2.9-A, for MDA-MB-231 cells, calpastatin was partially knockdown by shRNA (construct 1), compared with wild-type, negative and scrambled control. For MCF-7 cells, calpastatin shRNA construct 2 was selected to use in the migration assay, as it showed complete depletion of calpastatin compared to construct 1 (Figure 2.9-B).

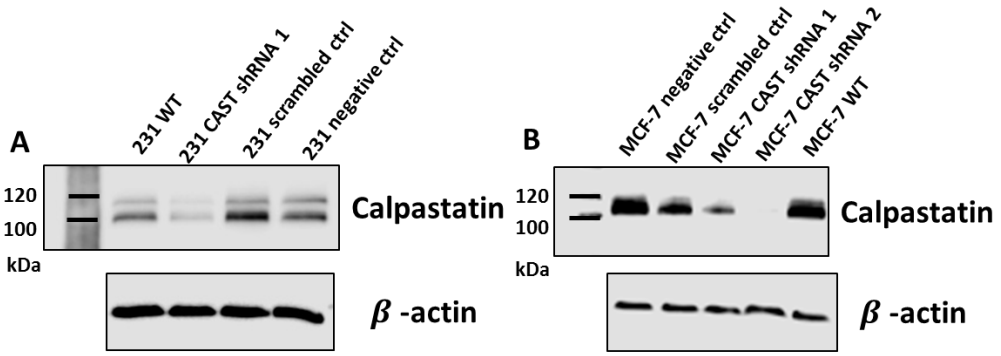


Figure 2.9 Knockdown of calpastatin in MDA-MB-231 and MCF-7 cells was tested by Western blotting. MDA-MB-231 (A) and MCF-7 (B) cells: calpastatin knockdown, scrambled control, negative control and wild-type cells. Primary antibody against calpastatin was used and β-actin expression was detected to serve as loading controls. Each experiment was conducted once.

The primary antibody detected duplicate bands in MDA-MB-231 cells, while only one band was detected in MCF-7 cells. The reason for this was unclear; the difference may be dependent on the different breast cancer subtypes. By using Western blotting, anti-calpastatin antibody can detect single band in protein extracted from skeletal muscle tissues; and it can detect duplicate bands in protein from cardiac tissues (Parr et al., 2001).

As shown in Figure 2.10-A&B, knockdown of calpastatin had no significant effect on MDA-MB-231 cell migration either with or without A23187. Although the difference was not statistically significant, the addition of A23187 to the culture medium seemed to slightly decrease migration of MDA-MB-231 calpastatin knockdown cells, compared with wild-type cells.

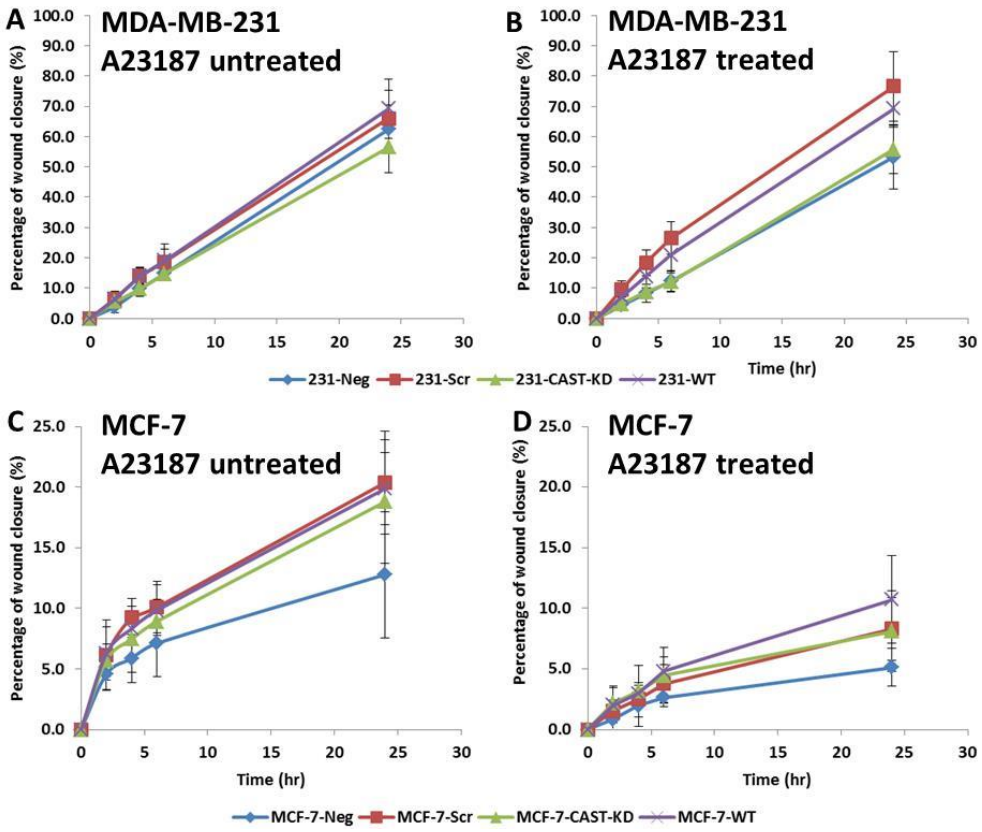


Figure 2.10 Effect of calpastatin knockdown on migration of breast cancer cells. MDA-231 (A&B) and MCF-7 (C&D) cells, calpastatin knockdown (CAST-KD), negative control (Neg), scrambled control (Scr) and wild-type (WT) cells were included. Data represent the average ± standard deviation (SD) of three independent experiments, with each experiment performed in triplicate.

Knockdown of calpastatin in MCF-7 cells did not alter cells migratory ability either in the presence of A23187 or the absence of A23187, observed in Figure 2.10-C&D. It was also noted that A23187 supplemented medium caused a reduction in migration of MCF-7 wild-type cells; the percentage of wound closure at 24 hours decreased from 19.9% to 10.7%. MDA-MB-231 (basal-like) exhibited a more aggressive migratory ability than MCF-7 (luminal). When comparing the average speed of migration within 24 hours, the speed of

percentage wound closure between two cell lines is 2.9% per hour vs 0.8% per hour (P<0.01). Untreated MDA-MB-231 cells started to migrate within the first 6 hours and almost closed the wound within 24 hours (approximately 70% wound closure); whereas MCF-7 cells remained at the initial wound margin after 6 hours (approximately 10% wound closure) and wound closure was approximately 20% after 24 hours.

2.3.4.3 Signalling pathways

The regulatory role of calpain on cell signalling pathways was analysed using a phosphokinase array that assessed the phosphorylation levels of 43 human phosphokinases and 2 total proteins. MDA-MB-231, MCF-7 and SKBR3 cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes - calpain activity under these conditions was previously described in section 2.3.3. The arrays for MDA-MB-231 and MCF-7 cells were performed by Dr. Sarah Storr.

Results show that the phosphorylation levels of MSK1/2 (S376/S360) were increased in MDA-MB-231 cells after treatment with calpeptin, but with no detectable MSK1/2 phosphorylation changes in MCF-7 or SKBR3 cells after the treatment (Figure 2.11, indicated by arrows).

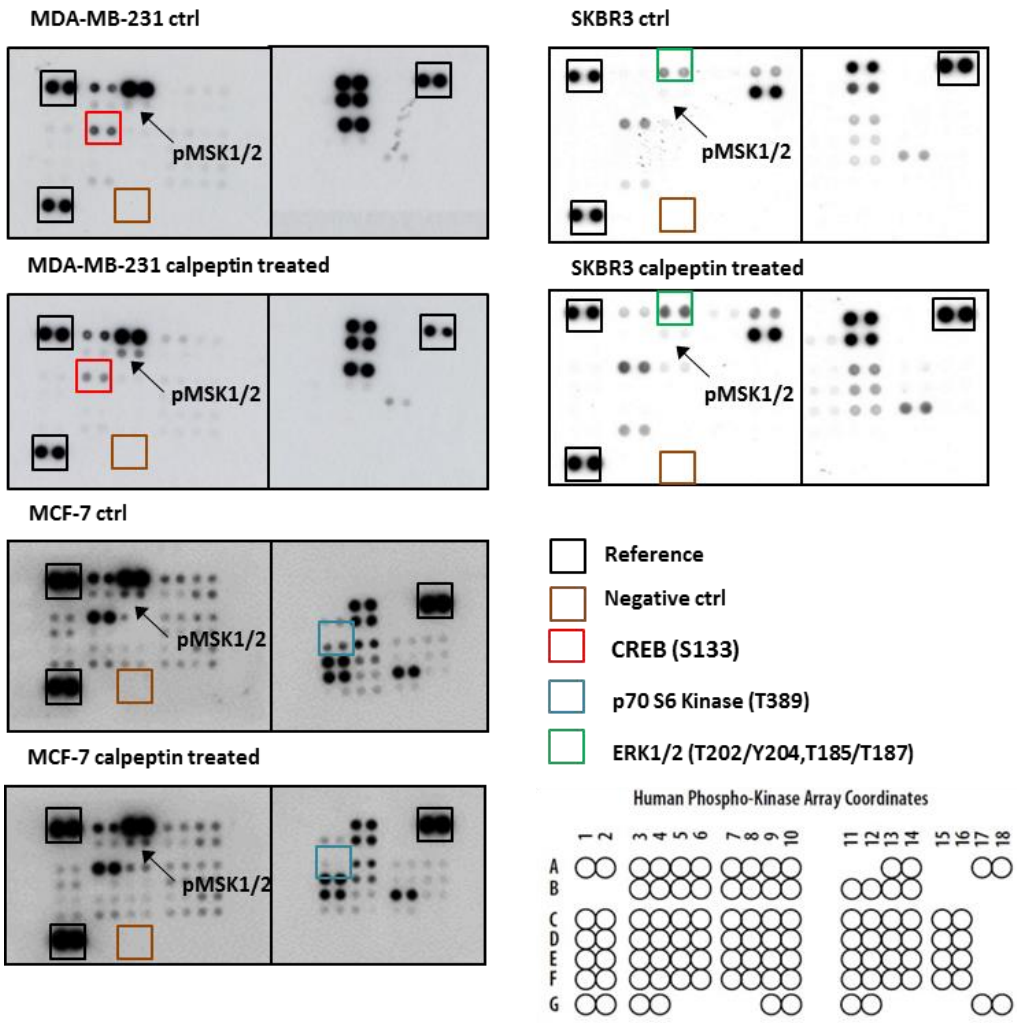


Figure 2.11 Effect of calpeptin on phosphorylation of 23 human phosphokinases in breast cancer cells. MDA-MB-231, MCF-7 and SKBR3 cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes. The experiment for each cell line was conducted once.

The phosphorylation of CREB (S133) and p70 S6 kinase (T389) was markedly downregulated by calpeptin in MDA-MB-231 and MCF-7 cells, respectively (Figure 2.11, red and blue squares). ERK1/2 phosphorylation (T202/Y204, T185/T187) was activated in all three cell lines, with the lowest level in SKBR3 cells. Calpain inhibition by calpeptin slightly enhanced ERK1/2 phosphorylation in SKBR3 cells (Figure 2.11, green square). The three cell lines expressed high endogenous levels of phosphorylated p53 (S392/S46/S15); while SKBR3 cells expressed slightly lower level of p53 at S15 compared with other two cell lines. In addition to the proteins mentioned above, several other phosphokinases were differentially expressed in the three cell lines, and seem to be regulated by calpain/calpeptin – these are listed in Table 2.6.

Table 2.6 The phosphokinases expression in breast cancer cells and changes in phosphorylation induced by calpeptin.

		Changes in phosphorylation			Phosphokinase expression					Changes in phosphorylation			Phosphokinase expression		
Coordinate	**Target**	**231**	**MCF-7**	**SKBR3**	**231**	**MCF-7**	**SKBR3**	**Coordinate**	**Target**	**231**	**MCF-7**	**SKBR3**	**231**	**MCF-7**	**SKBR3**
A3, A4	p38α (T180/Y182)	O	O		M	H		D9, D10	STAT5a (Y694)						
A5, A6	**ERK1/2 (T202/Y204, T185/Y187)**	O	O	+	H	H	M	D11, D12	p70 S6 Kinase (T421/S424)		O			H	
A7, A8	JNK 1/2/3 (T183/Y185, T221/Y223)	+	O			M		D13, D14	RSK1/2/3 (S380/S386/S377)		O	O		M	L
A9, A10	GSK-3α/β (S21/S9)		O	O		M	M	D15, D16	eNOS (S1177)						
A13, A14	p53 (S392)	O	O	O	H	H	H	E1, E2	Fyn (Y420)						
B3, B4	EGFR (Y1086)	O	O		L			E3, E4	Yes (Y426)						
B5, B6	**MSK1/2 (S376/S360)**	+	O		L	L		E5, E6	Fgr (Y412)						
B7, B8	AMPKα1 (T183)					M		E7, E8	STAT6 (Y641)		O				
B9, B10	Akt 1/2/3 (S473)		O	O		L	H	E9, E10	STAT5b (Y699)		O			M	
B11, B12	Akt 1/2/3 (T308)					M		E11, E12	STAT3 (Y705)		O			H	
B13, B14	p53 (S46)	O	O	O	H	H	H	E13, E14	p27 (T198)			O		L	L
C1, C2	TOR (S2448)		-			L		E15, E16	PLC-γ1 (Y783)	O	O	+	L	H	M
C3, C4	**CREB (S133)**	-	O	O	M	H	M	F1, F2	Hck (Y411)		O				
C5, C6	HSP27 (S78/S82)		O			L		F3, F4	Chk-2 (T68)	O	O	+	L		L
C7, C8	AMPKα2 (T172)					L		F5, F6	FAK (Y397)						
C9, C10	β-Catenin		O			L		F7, F8	PDGF Rβ (Y751)						
C11, C12	**p70 S6 Kinase (T389)**		-			L		F9, F10	STAT5a/b (Y694/Y699)		O				
C13, C14	p53 (S15)	O	O	O	H	H	L	F11, F12	STAT3 (S727)						
C15, C16	c-Jun (S63)					L		F13, F14	WNK1 (T60)					L	
D1, D2	Src (Y419)					L		F15, F16	PYK2 (Y402)						
D3, D4	Lyn (Y397)							G3, G4	PRAS40 (T246)						
D5, D6	Lck (Y394)							G11, G12	HSP60						
D7, D8	STAT2 (Y689)														

Abbreviations: "H", high phosphorylation level; "M", moderate phosphorylation level; "L", low phosphorylation level; "+", phosphorylation level increased after calpeptin treatment; "-", phosphorylation level decreased after calpeptin treatment; "O", phosphorylation level did not change after calpeptin treatment; blank, phosphorylation level was too low or cannot be detected.

As MSK1/2 (S376/S360) phosphorylation differences were particularly evident across the three lines, and showed differential effects with calpain activity, we decided to study its expression in breast cancer cell lines, by Western blotting, and tumour specimens, by immunohistochemistry.

In order to generate firm conclusions, as arrays were conducted only once for each cell line, the array results were tested in Western blotting by using the primary antibody against phospho-MSK1/2 (S376/S360) (R&D, USA). MDA-MB-231, MCF-7 and SKBR3 cells were treated under the same conditions as used in the phosphokinase array experiments. Unexpectedly, phospho-MSK1/2 (S376/S360) protein expression was not detectable, either before or after calpeptin treatment (data not shown; conducted with separate lysates from two independent experiments; without inclusion of the positive control lysates). The reason for such lack of detection is unclear; it may be that phospho-MSK1/2 is expressed at relatively low levels, as suggested by the phosphokinase array data with MDA-MB-231 and MCF-7 cells, any slight variation in signal may be due to alterations from external stimuli, leading to inconsistencies between experiments and making reproducibility difficult. If further investigation is required, Hela cells treated with phorbol 12-myristate 13-acetate (PMA) or EGF can be considered to include as positive control.

2.3.5 Immunohistochemistry on patient samples

2.3.5.1 Antibody specificity and immunohistochemistry optimisation

Based on the phosphokinase array results, the prognostic value of MSK1/2 in breast tumours were further examined by using immunohistochemistry. Before conducting the immunohistochemistry, the specificity of MSK1/2 antibodies were assessed by Western blotting. Several antibodies from different sources were screened by Western blotting by using various tumour and normal cell lysates. The anti-MSK1 antibody (Sigma, UK) only detected a weak signal (band at size 90-kDa) in MDA-MB-231 cells, along with other unspecific bands at different sizes (Figure 2.12-A).

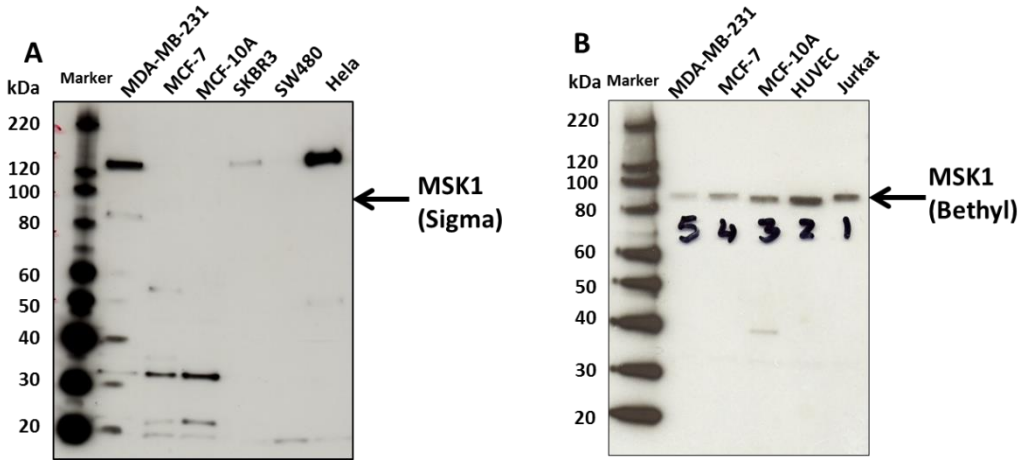


Figure 2.12 Specificity of anti-MSK1 antibodies were determined in Western blotting by using various cell lines. Primary antibodies against (A) MSK1 (Sigma), (B) MSK1 (Bethyl) were used. Each experiment was conducted once.

As shown in Figure 2.12-B, the anti-MSK1 antibody from Bethyl Laboratories can detect a single specific band of the expected size at 90-kDa over multiple human cell lines - a degraded protein band between 30-40 kDa can be seen in MCF-10A normal breast cells (conducted by Mr. Laurence Hall). Therefore this antibody was deemed suitable for immunohistochemical staining on breast tumour specimens and the antibody concentration was optimised using breast composite sections prior to staining on the full tissue microarray (conducted by Mr. Laurence Hall). The specificity of MSK2 antibodies was also tested. As shown in Figure 2.13-A, the anti-MSK2 antibody (NOVUS Biologicals, USA) detected a weak band at the expected size in MDA-MB-231, MCF-7, SKBR3 and MCF-10A cells, but two non-specific bands can be seen between 30-40 kDa and 100-120 kDa, respectively.

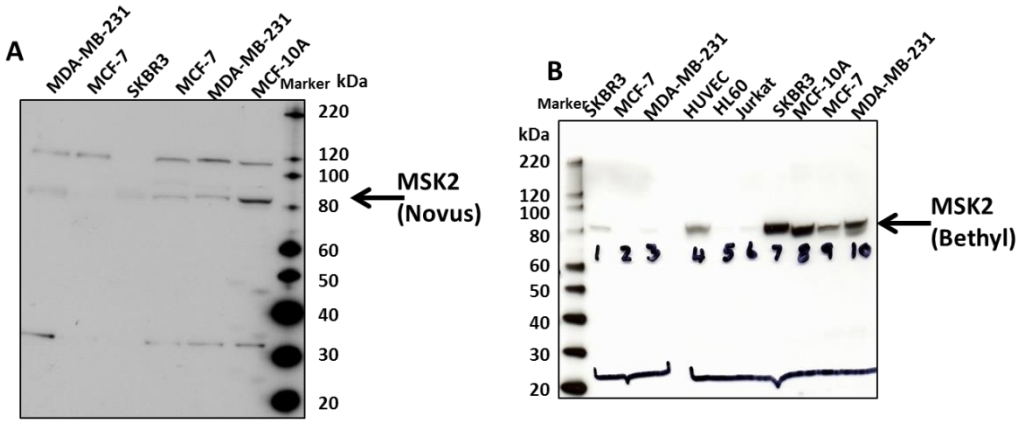


Figure 2.13 Specificity of anti-MSK2 antibodies were determined in Western blotting by using various cell lines. Primary antibodies against (A) MSK2 (Novus), (B) MSK2 (Bethyl) were used. Each experiment was conducted once.

Another anti-MSK2 antibody (Bethyl Laboratories, UK) was subjected to Western blotting, and the specificity was confirmed as shown in Figure 2.13-B (conducted by Mr. Laurence Hall). MSK1/2 are active in the nucleus, so MSK1/2 is expected to show nuclear staining in the tumour cells. However, when the concentrations of anti-MSK2 antibody (Bethyl Laboratories, UK) were optimised in the immunohistochemistry, unexpected predominant cytoplasmic staining was observed (data not shown). Therefore, this antibody was not eligible for immunohistochemistry. The specificity of phospho-MSK1/2 (S376/S360) were also assessed in Western blotting, after trying three different antibodies (including another pMSK1(S376) antibody that could not detect any signal, data not shown), none of them could detect a specific single band of the expected size (Figure 2.14-A&B).

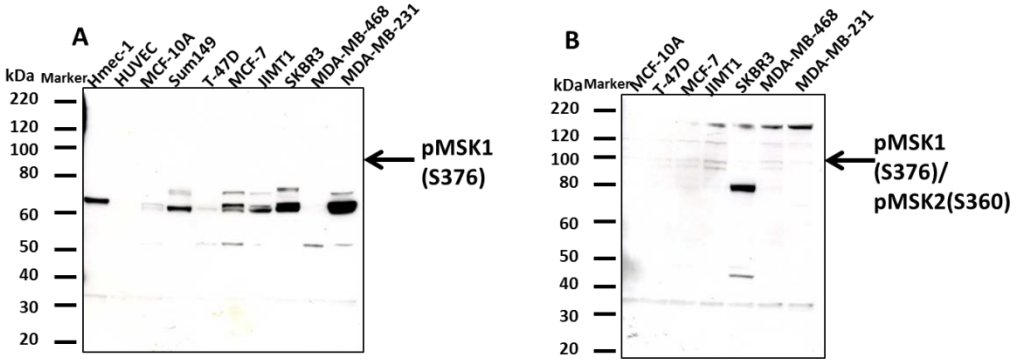


Figure 2.14 Specificity of phospho-MSK1/2 antibodies were determined in Western blotting by using various cell lines. Primary antibodies against (A) phospho-MSK1 (Abcam) and (B) phospho-MSK1/MSK2 (R&D) were used. Each experiment was conducted once.

Collectively, after testing the specificity of several total and phospho-MSK1/2 antibodies (i.e. two antibodies for MSK1, two for MSK2, two for phospho-MSK1 (376), and one for phospho-MSK1/2 (S376/S360)), the anti-MSK1 antibody (from Bethyl Laboratories) was selected for use.

2.3.5.2 Staining pattern and statistical analysis

Following confirmation of anti-MSK1 antibody specificity, MSK1 protein expression was detected by using immunohistochemistry in a large cohort of breast cancer patients (conducted by Mr. Laurence Hall). MSK1 demonstrated diffuse nuclear staining with some heterogeneity between adjacent tumour cells, varying form weak to intense staining. No cytoplasmic staining was observed. DCIS cells showed variable intensity staining in some cases (4 out of 13 TMA slides were independently scored by Dr. Narmeen Ahmad, with descriptive pathology was provided by pathologist Dr. Mohammed A Aleskandarany, with all being assessed by the current author). Some TMA cores were not scored due to insufficient tumour or the core being missing, a total number of 1270 cases were assessed. Typical staining patterns are shown in Figure 2.15.

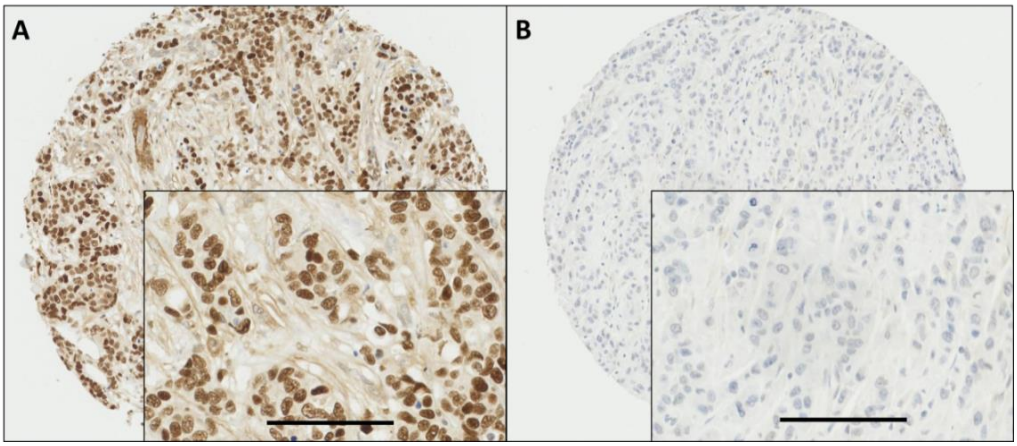


Figure 2.15 Representative photomicrographs of high and low MSK1 protein expression. Panel A (high expression) and panel B (low expression) at X10 magnification with X20 magnification inset panel and scale bar representing 100 µm.

MSK1 staining had a median H-score of 190±83 and ranged from 0 to 300. X-tile was used to generate an unbiased cut point of 115, with 364 cases (28.7%)

having low expression and 906 cases (71.3%) having high expression. Spearman's rank order correlation demonstrated significant, albeit weak, biological correlation between MSK1 and calpain-1 (r=0.117, *P*<0.001), and calpastatin expression (r=0.134, *P*<0.001); though the associations were marginally relevant. No significant correlation was found between MSK1 and calpain-2 (r=0.07, *P*=0.061) or calpain-9 expression (r=0.004, *P*=0.92). Further significant, weak, correlations were found between MSK1 and calpastatin (r=0.169, *P*=0.021) in the basal-like subgroup; between calpain-2 (r=0.182, *P*=0.026) and calpastatin (r=0.173, *P*=0.031) in the triple-negative subgroup (Table 2.7). Due to correlation coefficient values, the associations were not deemed to be biologically relevant.

Table 2.7 Correlation between calpain family, calpastatin and MSK1 protein expressions in different subgroups

	HER2+		Basal-like		Triple-negative	
	Correlation Coefficient	Sig.	Correlation Coefficient	Sig.	Correlation Coefficient	Sig.
Calpain-1	-0.077	0.385	0.091	0.213	0.099	0.222
Calpain-2	0.009	0.923	0.115	0.118	0.182	**0.026**
Calpain-9	-0.123	0.26	0.149	0.11	0.009	0.929
Calpastatin	0.565	0.052	0.169	**0.021**	0.173	**0.031**

Abbreviations: Sig, significance.

Relationship with clinicopathologic variables

Pearson's Chi-squared test was performed to examine for association between MSK1 expression with clinicopathologic criteria. High MSK1 expression was significantly associated with patients over 40 years (χ^2=8.283, d.f.=1, *P*=0.004), lower tumour grade (χ^2=34.505, d.f.=2, *P*<0.001), lower NPI value (χ^2=9.804, d.f.=2, *P*=0.007), ER positive tumours (χ^2=50.186, d.f.=1, *P*<0.001) and PR positive tumours (χ^2=41.147, d.f.=1, *P*<0.001). While low MSK1 expression was significantly associated with the presence of triple-negative tumours (χ^2=56.406, d.f.=1, *P*<0.001) and basal-like tumours (χ^2=13.850, d.f.=1, *P*<0.001). Low MSK1 expression was also significantly associated with the presence of distant metastasis (χ^2=13.850, d.f.=1, *P*<0.001), recurrence (χ^2=6.220, d.f.=1, *P*=0.013)

and occurrence of death due to breast cancer (χ^2=6.711, d.f.=1, *P*=0.01). The associations between protein expression and clinicopathologic variables are shown in Table 2.8.

Table 2.8 Associations between MSK1 protein expression and various clinicopathologic variables

	MSK1 (n=1270)		
Variables	**Low expression**	**High expression**	***P*-value**
Age (years)			
≤40 years	49 (3.9%)	74 (5.8%)	**0.004**
>40 years	315 (24.8%)	831 (65.5%)	
Tumour size (mm)			
≤20mm	207 (16.4%)	545 (43.0%)	0.275
>20mm	156 (12.3%)	358 (28.3%)	
Tumour stage			
I	227 (17.9%)	540 (42.7%)	0.544
II	105 (8.3%)	275 (21.7%)	
III	30 (2.4%)	89 (7.0%)	
Tumour grade			
I	38 (3.0%)	169 (13.3%)	**<0.001**
II	96 (7.6%)	326 (25.8%)	
III	229 (18.1%)	408 (32.2%)	
Nottingham Prognostic Index			
⩽3.4	85 (6.7%)	290 (22.9%)	**0.007**
3.41-5.4	198 (15.7%)	451 (35.7%)	
>5.4	79 (6.3%)	161 (12.7%)	
Lymphovascular invasion			
Positive	107 (10.1%)	248 (23.4%)	0.263
Negative	190 (17.9%)	517 (48.7%)	
Operation type			
Mastectomy	213 (16.9%)	520 (41.3%)	0.682
WLE Lumpectomy	147 (11.7%)	378 (30.0%)	
ER status			
Positive	205 (16.7%)	704 (57.2%)	**<0.001**
Negative	139 (11.3%)	183 (14.9%)	
PR status			
Positive	147 (12.3%)	538 (45.0%)	**<0.001**
Negative	196 (16.4%)	314 (26.3%)	

Table 2.8 Continued.

	MSK-1 (n=1270)		
Variables	**Low expression**	**High expression**	***P*-value**
HER2 status			
Positive	49 (3.9%)	125 (10.1%)	0.894
Negative	306 (24.6%)	762 (61.4%)	
Basal-like status			
Positive	96 (8.1%)	153 (13.0%)	**<0.001**
Negative	247 (20.9%)	685 (58.0%)	
Triple-negative status			
Positive	104 (8.5%)	109 (8.9%)	**<0.001**
Negative	237 (19.4%)	774 (63.2%)	
Breast cancer-specific survival			
Alive	577 (41.7%)	342 (24.7%)	**0.01**
Dead	259 (18.7%)	207 (14.9%)	
Recurrence			
Present	167 (13.5%)	362 (29.3%)	**0.013**
Not present	178 (14.4%)	530 (42.8%)	
Distant metastasis			
Present	138 (11.0%)	267 (21.2%)	**0.002**
Not present	220 (17.5%)	635 (50.4%)	

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Correlations between MSK1 protein expression and clinicopathologic variables was assessed using Pearson's Chi-square test of association (χ^2) or Fisher's exact test if in a 2X2 tables and cell count was less than 5. Significant *P*-values are indicated by bold font.

Relationship with clinical outcomes

Low MSK1 expression was, in the total patient cohort, significantly associated with adverse breast cancer-specific survival (*P*=0.009) as shown by Kaplan-Meier survival analysis (Figure 2.16).

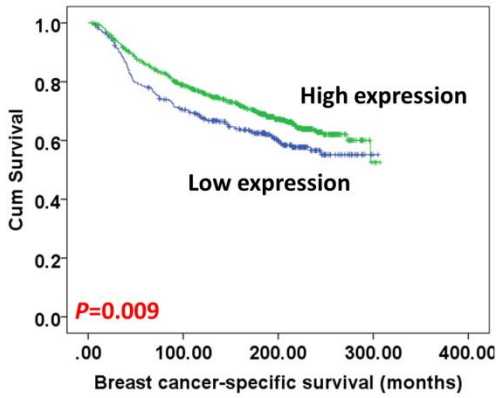


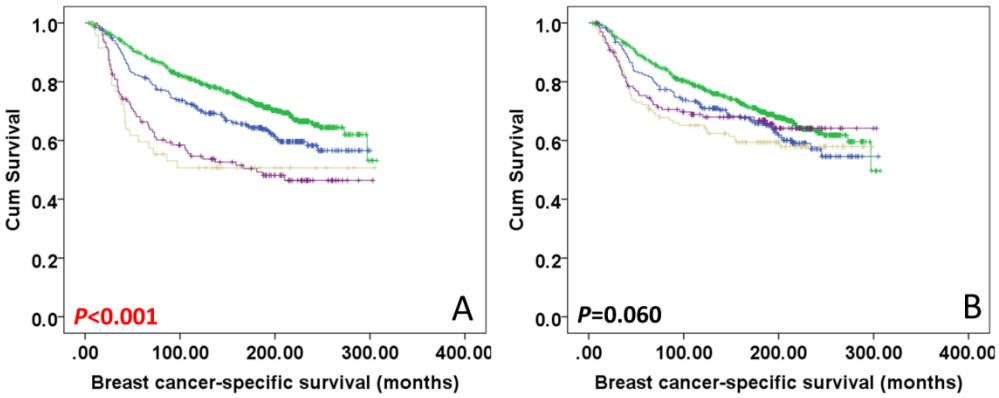
Figure 2.16 Kaplan-Meier survival curves for breast cancer-specific survival based upon MSK1 expression (total patient cohort). Curves show low (blue line) and high protein expression (green line) with significance determined using the log-rank test. 364 (28.7%) cases had low expression and 906 (71.3%) cases had high expression.

For multivariate analysis, potential confounding factors including age, tumour size, tumour stage, tumour grade, NPI value, lymphovascular invasion, ER, PR and HER2 status (with individual Kaplan-Meier statistics of $P<0.05$ for all variables) were included. MSK1 expression was not an independently associated with breast cancer-specific survival (hazard radio (HR) =0.899, 95% confidence interval (CI) 0.696–1.161; $P=0.415$).

Subgroup and combined biomarker analysis

As mentioned previously, MSK1 showed differential phosphorylation levels in different subtypes of breast cancer cells, as well as being differentially regulated by calpeptin in MDA-MB-231 cells (basal-like). The prognostic significance of MSK1 protein was therefore further assessed in different subtypes of breast tumours. As shown in Table 2.5, 243 (12.8%) patients had HER2+ tumours, 315 (16.6%) patients had triple-negative tumours, and 368 (19.3%) patients had basal-like tumours.

MSK1 expression was investigated in, HER2, triple-negative and basal-like diseases and showed interest in both HER2 and basal-like groups ($P<0.001$ and $P=0.022$, respectively; Figure 2.17-A&C). This was further investigated in the individual subgroups.



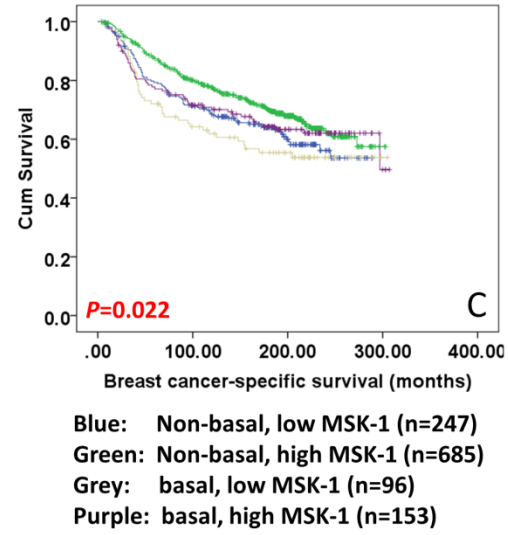


Figure 2.17 Kaplan-Meier survival curves for breast cancer-specific survival based upon MSK1 expression shown as subgroups. (A) HER2 positive and negative tumours with high and low MSK1 expression; (B) triple-negative and non-triple negative tumours with high and low MSK1 expression; (C) basal-like and non-basal like tumours with high and low MSK1 expression.

When additional analyses were performed on only HER2+ and basal-like subgroups, there were no significant associations (Figure 2.18-A&E); significant associations were only observed in HER2 negative and non-basal like subgroups (*P*=0.006 and *P*=0.024, respectively, Figure 2.18-B&F).

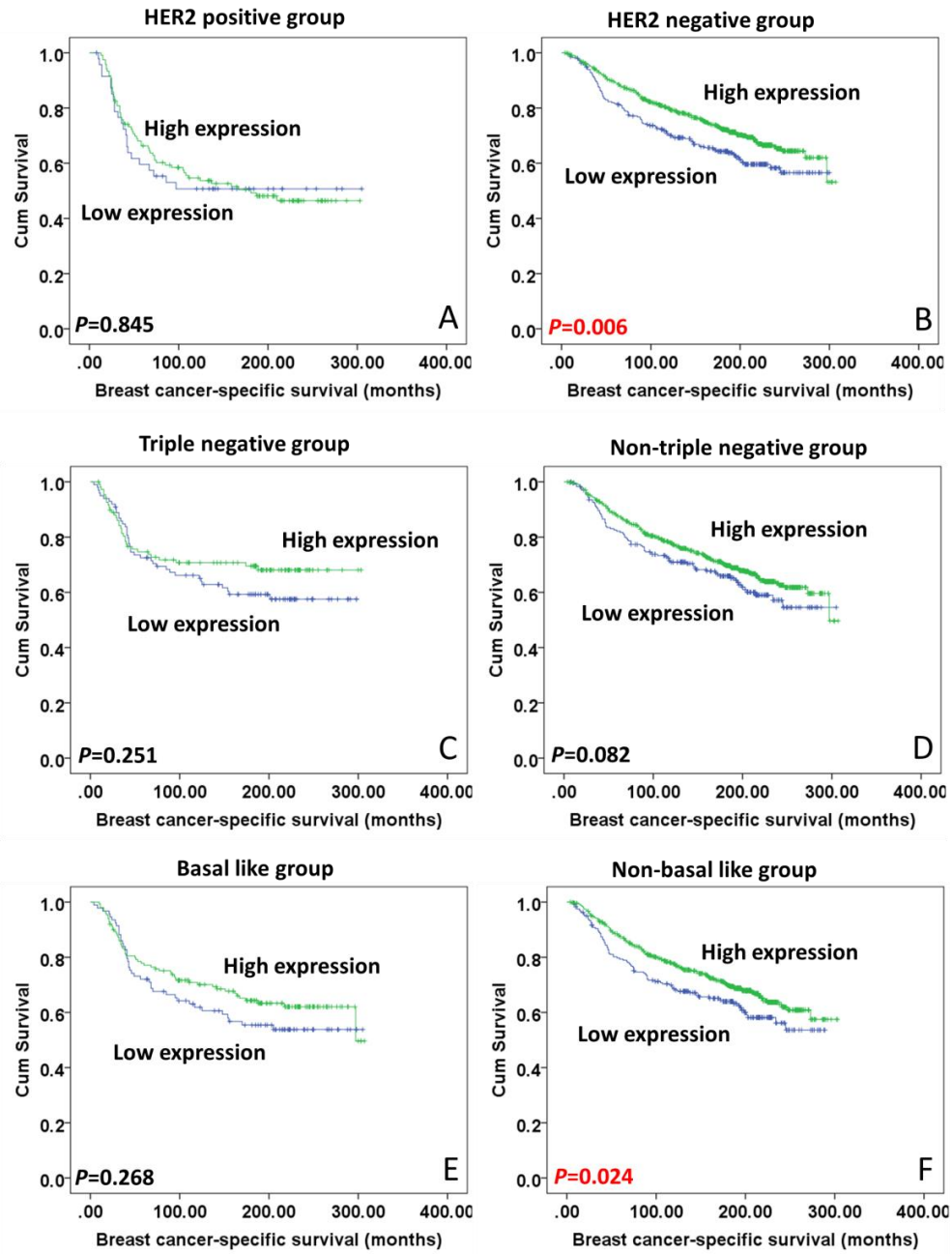


Figure 2.18 Kaplan-Meier survival curves for breast cancer-specific survival based upon MSK1 expression (subgroups). (A) HER2 positive; (B) HER2 negative; (C) triple-negative; (D) non triple-negative; (E) basal-like and (F) non-basal like.

Additional combinational analyses were conducted to examine prognostic significance of MSK1 plus calpain-1, -2, -9 or calpastatin: high MSK1/high calpain, low MSK1/low calpain, high MSK1/low calpain and low MSK1/high calpain. None of the groupings were associated with breast cancer-specific survival in either the total group or any of subgroups (Appendix A -Table A 1).

2.4 Discussion

Impaired calpain activity or genetic mutation in calpain family members has been described in several pathological conditions and human carcinogenesis. The calpain family has been implicated in tumour progression through the proteolysis of numerous specific key substrates including focal adhesion kinase, talin, inhibitor of NF-kB and several oncogenes (discussed in Chapter 1). Despite solid evidence supporting the critical role of calpains in tumorigenesis, few studies have provided direct evidence of calpain activity in breast tumours. No studies of the calpain family have systematically compared their differential roles in different subtypes of breast tumours.

In order to investigate if calpain demonstrated altered expression patterns across different subtypes, six breast cancer cell lines from luminal (T-47D and MCF-7), HER2+ (JIMT-1 and SKBR3) and basal-like (MDA-MB-468 and MDA-MB-231) were screened; with three of them being selected to assess calpain activity and inhibition by calpeptin. Data show that the calpain family members (calpain-1, -2 and -4) and calpastatin are expressed across all six breast cancer cell lines. Semi-quantitative Western blotting results showed that for calpain-1 and -2 was similar across cell lines with MDA-MB-231 cells expressing a relatively low level of calpain-1, with MCF-7 cells expressing a very low level of calpain-2. Such results are consistent with quantitative Western blotting results conducted by another group member (Ms Bhudsaban Sukkarn), showing that calpain-1 and -2 are expressed lowest in MDA-MB-231 and MCF-7 cells, respectively. Another study also measured calpain protein expression in tumour cells, including MCF-7, T-47D and MDA-MB-231, and demonstrated that MCF-7 cells specifically express high levels of calpain-1 but not calpain-2 (Wu et al., 2006). The small subunit shared by calpain-1 and -2, calpain-4 protein expression was similar among the six cell lines but calpastatin protein expression varied; JIMT-1 had the relatively higher calpastatin level, while T-47D had the relatively low level, however the difference was not significant. The antibody used to assess calpastatin expression detected duplicate bands

between 100-120 kDa. Calpastatin has four distinct isoforms with different molecular weights that are differentially expressed in different tissues. By Western blotting, a single band at 135-kDa was detected in proteins extracted from skeletal muscle tissues, duplicate bands at 135/145-kDa were seen in cardiac tissues, and anti-calpastatin anti-serum detected three 135, 145 and 172 sizes bands in skeletal whole muscle homogenates (Parr et al., 2001).

From the present study MCF-7 cells had the highest level of active calpain activity followed by SKBR3 cells then MDA-MB-231 cells. MCF-7 cells expressed lowest calpain-2 protein but had the highest calpain activity compared with other breast cancer cells. It is difficult to make direct comparisons between calpain protein expression and activity; especially calpain-1 and -2 has differential roles in tumorigenesis. ERK can directly activate and phosphorylate m-calpain (calpain-2) rather than μ-calpain (calpain-1) through growth factor receptor signalling, which is an important signalling pathway in tumorigenesis (as discussed in Chapter 1). Additionally, by using a large breast cancer patients cohort (n=1371) and a verification cohort (n=387), previous study from our group indicated that high calpain-2 expression, but not calpain-1, was associated with survival in patients with basal-like and triple-negative disease. Calpain-2 expression was also associated with presence of peri-tumoural lymphovascular invasion (Storr et al., 2012a). Our group also got information on calpain-9 being important (Davis et al., 2014) and unpublished information that, in locally advanced primary breast cancer patients with non-inflammatory diseases, high calpastatin expression was significantly associated with adverse breast cancer-specific survival, as was low calpain-2 expression. In inflammatory cases, high calpain-1 and high calpastatin expression is associated with improved breast cancer-specific survival (Storr et al., 2016). Those results emphasised that calpain-1 and -2 has differential role in different types of breast cancer.

Several commercial inhibitors of calpain are available, such as leupeptin,

calpeptin, calpain inhibitor I, E-64, PD150606 and MDL28170, etc. Although each of these has shown effective inhibition of calpain activity, none of them are particularly specific. The majority also have inhibitory effects on other cysteine proteases (e.g. papain and cathepsin) (as introduced in section 2.1.2). Calpeptin, a widely used synthetic calpain inhibitor, was used to assess calpain inhibition in breast cancer cells. Based on preliminary studies in our group (conducted by Dr. Sarah Storr), the sensitivity of calpeptin was superior to another inhibitor, PD150606. From current results, calpain activity was partially attenuated by calpeptin at a concentration of 100 μM for 30 minutes to 24 hours treatment (approximately 20-54% inhibition); however the inhibition did not show a time-dependent relationship and calpeptin could not completely abolish activity even with longer incubation times. Calpeptin seemed to have the most effective inhibition on MCF-7 cells compared with MDA-MB-231 and SKBR3 cells. In MCF-7 cells, the maximal inhibition of calpain activity by calpeptin was seen as early as 30 minutes treatment; while in SKBR3 cells the most significant inhibition was only observed after 6 and 24 hours treatment; and calpeptin only resulted in 20-30% inhibition in MDA-MD-231 cells even after 24 hours.

To determine if calpain modulates breast cancer cell proliferation, and compare effects between breast cancer subtypes, the cells were pre-treated with different concentrations of calpeptin for different incubation times. The results demonstrated that inhibition of calpain activity by calpeptin inhibited proliferation in MDA-MB-231, MCF-7 and SKBR3 cells. More specifically, calpeptin did not show any obvious anti-proliferative effect during the first 24 hours if the concentration was lower than 50 μM. At 48 hours treatment, the comparison of estimated IC50 values showed that calpeptin presented similar effects across three cell lines. While after 72 hours, an estimated IC50 value between 80-85 μM was observed in SKBR3 and MDA-MB-231 cells, and between 60-65 μM for MCF-7 cells. Another study found that calpeptin treatment for 96 hours caused 40-50% and 10% decrease in cell proliferation in

MCF-7 (10 μg/mL) and MDA-MB-231 cells (7.5 μg/mL), respectively (Mataga et al., 2012). Although the dose of calpeptin in our study is nearly 4 to 5-fold of that in their study (100 μM is equivalent to ~36.25 μg/mL), taken together it may indicate that the growth inhibition of calpeptin is more effective in the luminal subtype breast cancer cells (i.e. MCF-7 cells) and calpeptin may preferentially affect oestrogen receptor positive cells. E2 (17β-estradiol) is an oestrogen hormone that tightly correlates with biological functions of oestrogen. It has been reported that E2-induced proliferation of MCF-7 cells can be inhibited by pre-treatment with 100 μM calpeptin for 30 minutes (Wang et al., 2014a). Four aldehyde calpain inhibitor analogues have shown differential anti-proliferative effects on one prostate cancer, one cervical cancer and two leukaemia tumour cell lines, with IC50 values ranging between 2.2-48.9 μM (Guan et al., 2006).

To determine whether calpastatin plays a role in cell migration, cell haptotaxis assays were performed on calpastatin knockdown cells. MDA-MB-231 (basal-like) and MCF-7 (luminal) cells were stably transfected using calpastatin shRNA (conducted by Dr. Sarah Storr); and the knockdown of calpastatin confirmed by Western blotting. Calpastatin negatively regulates calpain activity. It has been shown that calpastatin expression level has a direct opposite effect on calpain activation and proteolytic activity in a transgenic murine model of Alzheimer's disease (Vaisid et al., 2007). Previous data from our group found that knockdown of calpastatin in MDA-MB-231 cells did not elevate calpain activity compared to the controls or wild-type counterparts (Dr. Sarah Storr, unpublished data). Results from the current migration assays showed that there is no statistical difference between the migration of wild-type and calpastatin knockdown cells either in MDA-MB-231 or MCF-7 cells. There was an indication that in the presence of calcium ionophore A23187, calpastatin knockdown MDA-MB-231 cells had reduced migratory ability; however, this was not statistically significant in comparison with controls. Such a change was not found in the absence of A23187. Treatment with A23187 has been shown to

cause subsequent activation of calpain activity in platelets, fibroblasts and erythrocytes (Molinari et al., 1994, Small et al., 2002, Nayak et al., 2011). In the current study, calpastatin was not completely inhibited, therefore it is unknown if any residual calpastatin activity would have an effect on cell migration. Additionally, the concentration of A23187 and the incubation time may not be sufficient to activate calpain activity in breast cancer cells. In retrospect it would be ideal to try different concentrations and incubation time with A23187 and detect the calpain activity level in the cells. Also it should be considered to include positive controls. Further investigation is required to provide a firm conclusion and detailed explanation. It was also noted that incubation with A23187 slightly decreased migration of wild-type MCF-7 cells. Earlier evidence have suggested that A23187 can downregulate ER protein/mRNA expression and regulate its transcriptional activity in MCF-7 cells (Ree et al., 1991, Journé et al., 2004). Such alterations may have potential effect on cells migratory ability.

Basal-like breast tumours have more aggressive clinical behaviour and poor prognosis compared with luminal subtype. In the current results, wild-type MDA-MB-231 cells displayed a markedly increased migratory ability than wild-type MCF-7 cells. Together with the Western blotting results, as MCF-7 expressed lower calpain-2 protein levels, it may suggest that calpain-1, rather than calpain-2, has a dominant role in regulating migration in MCF-7 cells. This assumption can be evidenced by Wu et al. 's findings that showed that calpain-1 is exclusively responsible for degradation of filamin and talin (two important adhesion components) in MCF-7 cells, and that restored expression of calpain-1 but not calpain-2 could enhance the cellular migration rate (Wu et al., 2006). In comparison, MDA-MB-231 cells expressing high level of calpain-2 with increased migratory ability. As mentioned previously, high calpain-2 expression was associated with poor prognosis in basal-like breast cancer patients. It may suggest that calpain-2 has a dominant role in regulating migration in MDA-MB-231 cells. It would be interesting to investigate if knockout of calpain-1 and calpain-2 separately, rather than knockdown calpastatin in cells, would show

differential effects on MDA-MB-231 and MCF-7 cells.

Calpastatin is the only known endogenous inhibitor of calpain; several studies have reported that calpains increase tumour invasion and metastasis. Previous studies from our group have shown that lower expression of calpastatin mRNA/protein was linked with increased lymphovascular invasion in breast cancer (Storr et al., 2011b, Storr et al., 2012a). Lymph node metastasis is associated with upregulation of calpain-1 mRNA in renal cell carcinomas (Braun et al., 1999). In contrast, inhibition of calpain activity has led to a decrease in cell migration via enhancing integrin-cytoskeletal linkage and reducing the speed of cell detachment (Huttenlocher et al., 1997). Reduced cellular migration has been shown in MCF-7 cells transfected with calpain-1 siRNA (Wu et al., 2006).

Although calpain has been shown to increase tumour cell migration and invasion, the regulatory mechanisms remain unclear. One study showed that overexpression of calpastatin led to calpain inhibition in melanoma transgenic mice, and actually increased metastatic dissemination to regional lymph nodes by promoting tumour cell migration (Raimbourg et al., 2013). Predominant calpain-1 activity has been detected in resting neutrophils; specific inhibition of calpain-1 in neutrophils promoted cellular polarisation, chemokinesis and migration speed (Lokuta et al., 2003). Interestingly, calpain-mediated cleavage of paxillin was reported to impair focal adhesion disassembly and cell migration in Hela cells (Cortesio et al., 2011).

The role of calpain on signal transduction was examined using phosphokinase arrays. The current study focused mainly on MSK1/2, as inhibition of calpain activity by calpeptin upregulated MSK1/2 phosphorylation in MDA-MB-231 cells; while in MCF-7 cells the phosphorylation was not affected. Compared to other two cell lines, MSK1/2 phosphorylation was not detectable in SKBR3 cells either before or after calpeptin treatment. To date it is the first evidence that

links calpains and MSKs in breast cancer cells, especially in basal-like subtype. MSK1/2 and calpains share several key substrates and signalling pathways in tumorigenesis. As discussed in previously, firstly, MSK1/2 can be activated through MAPK/ERKs signalling in response to EGF, whilst conventional m-calpain can be activated by ERK through growth factor receptor signalling. In addition, both MSK1/2 and calpains have been shown to promote breast cancer cell survival via transcription factor NF-κB. Lastly, CREB has been confirmed as a phosphorylation target of MSK1/2 (as introducd before). Our results showed that suppressed calpain activity in MDA-MB-231 cells upregulated MSK1/2 phosphorylation, in parallel with decreased CREB phosphorylation, which may suggest a potential connection between calpain, MSK1/2 and CREB signal transduction in basal-like subtype.

Although the above data from phosphokinase arrays may be interesting and may have added information to the known regulatory functions of calpains in signal transduction, the results must be viewed with caution, as MSK1/2 results could not be validated in Western blotting. In addition, 100 μM calpeptin for 30 minutes caused approximately 50% inhibition of calpain activity; it remains to be seen if the results would be different if the residual calpain activity were completely inactivated. Also cross reactivity between antibodies, the specificity of the antibody, and distinct lysis buffer used in phosphokinase arrays and Western blotting may also contribute to the discrepancies between phosphokinase array and Western blotting data. Thus the information that comes from the arrays is limited, and further investigation and evaluation should be made in order to draw any firm conclusions.

The initial array data, showing differential levels of MSK1/2 phosphorylation, was interesting and it was thought worthwhile to further explore the importance of MSK1/2 in breast tumours, particularly as total MSK1/2 expression had not previously been assessed in breast tumour specimens. After trying several antibodies from different sources, a suitable anti-MSK2 antibody

that presented nuclear staining in immunohistochemistry could not be found; equally, using antibodies directed against phospho-MSK1/2, no MSK1/2 phosphorylation could be reliably detected in cell samples. Future work might consider using such stimuli to activate MSK1/2 phosphorylation. Ultimately, it was decided to focus on expression and prognostic significance of MSK1.

In the current study, by using a large invasive breast cancer patient cohort, we found a significantly positive correlation between MSK1 and calpain-1, and calpastatin expression, respectively. Significant coefficients suggest that such associations may not be biologically relevant. Kaplan-Meier survival analysis showed a significant association between high MSK1 expression and improved breast cancer-specific survival, but this did not remain as an independent factor in multivariate analysis. High MSK1 expression was significantly associated with older age, lower tumour grade, lower NPI values, ER positive and PR positive tumours, all of which are indicative of a better prognosis. In agreement with this low MSK1 expression was also found to be associated with the presence of distant metastasis, recurrence and death due to breast cancer, as well as the presence of triple-negative and basal-like disease. Collectively, the association of MSK1 expression with ER, PR status, triple-negative and basal-like disease is of particular interest as it potentially links with the *in vitro* phosphokinase array results.

The importance of MSK1 in predicting clinical outcomes in different subtypes of breast cancer was also examined. The significant association was seen in the HER2 negative and non-basal like subtypes; high MSK1 expression was significantly associated with improved breast cancer-specific survival. This study is the first to examine the total MSK1 protein expression in breast tumour tissue and evaluate its prognostic significance in breast cancer patients.

Combinational analyses were performed to determine the prognostic significance of MSK1 plus calpain-1, calpain-2, calpain-9 and calpastatin (i.e.

high MSK1/high calpains, low MSK1/low calpain, high MSK1/low calpains and low MSK1/high calpains), in the total patient group and each subgroup; however, no association with survival was observed. The current study did not compare MSK1 protein expression in breast tumours with that found in normal breast tissues. Several lines of evidence suggest that phosphorylated MSK1 is significantly overexpressed in chronic solar UV-exposed mice skin, human squamous cell carcinoma (SCC) samples and poorly differentiated nasopharyngeal carcinoma tissues compared with their normal tissue counterparts (Yao et al., 2014, Li et al., 2015). More recently, a novel compound was identified as a MSK1 inhibitor, and has shown effective anti-cancer effects on skin cancer by disrupting MSK1 phosphorylation and MSK1-mediated downstream signalling (Liu et al., 2014a). It might be interesting to examine whether combined use of this MSK1 inhibitor and calpain inhibitor would have further effect on regulation of calpain activity. The role of MSK1 in tumorigenesis may be varied in different tumour types because of its wide range phosphorylation substrates and the involvements in transcription, chromatin transformation, inflammation and cell apoptosis (Vermeulen et al., 2009).

2.5 Summary

1. Calpain/calpastatin protein family expression was expressed in breast cancer cells with different molecular subtypes.
2. Inhibition of calpain activity had anti-proliferative effects on breast cancer cells.
3. Knockdown of calpastatin by shRNA had no significant effect on cellular migratory ability in MDA-231 and MCF-7 cells, with or without calcium ionophore stimulation.
4. High MSK1 expression was significantly associated with improved breast cancer-specific survival, but was not an independent factor when considering potential confounding factors; the association between MSK1 expression and survival was also observed in HER2 negative patients and patients without basal-like diseases.

Chapter 3 Calpain expression and apoptosis

3.1 Introduction and aims

3.1.1 Aims

The aims and objectives of the current chapter were to:

1. Explore the effect of manipulating calpain activity on breast cancer cell apoptosis and apoptotic related signalling pathways.
2. Evaluate the prognostic value of caspase-3/-8 expression in breast cancer patients, determining if there were any relationship(s) between calpain family and caspase-3/-8 protein expression.

3.1.2 Apoptosis and cancer

Apoptosis is a highly conserved and tightly regulated intracellular mechanism that leads to the cell death. The term was first described by Kerr et. al., which highlighted the significance of dying cells by distinct biochemical and morphological changes, including chromatin condensation, DNA fragmentation, cytoplasmic shrinkage and membrane blebbing (Kerr et al., 1972). There are two major pathways that initiate apoptosis: the activation of death receptors at the plasma membrane (extrinsic or death receptor pathway); and the release of proteins from mitochondria into the cytoplasm (intrinsic or mitochondrial pathway). Generally, the extrinsic pathway is initiated in response to ligand binding of death receptors (DRs) superfamily (such as CD95 and tumour necrosis factor (TNF)) at the cell surface, resulting in activation of capase-8 followed by caspase-3. The intrinsic pathway is triggered by mitochondrial release of cytochrome c, leading to formation of Apaf-1 and cytochrome c complex (apoptosomes) with the assistance of ATP, that subsequently activates caspase-9 followed by caspase-3 (Hengartner, 2000, Hancock, 2010).

The pro-apoptotic Bcl-2 family member Bid connects mitochondrial and death receptor pathways. Caspase-8 cleavage of Bid generates a truncated Bid fragment (tBid) and causes its translocation to mitochondria, resulting in the release of cytochrome c and amplifying the apoptotic signal (Li et al., 1998). In both pathways, central players of apoptosis are caspases - a group of cysteine

aspartyl proteases, which function through proteolytic cleavage of various substrates.

Dysfunction in apoptosis has been implicated in numerous pathological disorders, such as autoimmune disease, inflammation, neurodegenerative disease, ischaemia/reperfusion, heart failure, and most importantly, cancer (reviewed in (Fadeel and Orrenius, 2005)). Resistance to apoptosis is considered as a hallmark of cancer; it contributes not only to tumorigenesis, but also underlies cancer cell resistance to a variety of treatments. Cancer cells can acquire resistance to apoptosis by overexpression of anti-apoptotic proteins such as Bcl-2, Bcl-X_L, Bcl-W and Mcl-1 or by the downregulation or mutation of pro-apoptotic proteins such as Bax, Bak, Bid and Bad (reviewed in (Igney and Krammer, 2002)). Early studies of B-cell follicular lymphoma found that the chromosomal translocation t(14;18) coupled with enhanced Bcl-2 activation, could promote lymphoma cell survival; whilst mutation in the Bax gene has been observed in 21% (6 of 28) of hematopoietic malignant cell lines (McDonnell et al., 1989, Meijerink et al., 1998). *In vivo* data has provided evidence that Bax can act as tumour suppressor. Expression of Bax has been shown to be induced by p53 in transgenic murine brain tumours, leading to slow tumour growth and a high level of apoptotic cells; whereas in Bax-deficient mice, tumour growth was accelerated and apoptosis was significantly decreased, suggesting that Bax is essential for the functional p53-dependent response (Yin et al., 1997).

A number of anti-cancer chemotherapeutic agents and ionising radiation can target cancer cells by induction of apoptosis, failure to activate apoptotic programme has been implicated in drug and radioresistance. A recent study has shown that high Bcl-2 expression is associated with poor disease-free survival and overall survival in patients with ER/PR negative and triple-negative tumours who received no adjuvant treatment (Honma et al., 2015). On the contrary, high expression of the pro-apoptotic Bak can predict a favourable overall survival rate in breast cancer patients treated with Taxol, especially in luminal and

HER2+ subtypes; and increased Bak expression sensitised breast cancer cells response to Taxol treatment (Luo et al., 2015). In addition to the above, there are other mechanisms that can affect the sensitivity of tumour cells to apoptosis, such as alteration of p53 pathways, disruption of PI3K/AKT pathways, impaired activity of transcription factor NF-κB, and aberrant microRNAs expression (reviewed in (Igney and Krammer, 2002, Ouyang et al., 2012)).

3.1.3 Caspase-3/-8 in cancer

The caspase family belongs to a group of cysteine proteases, and 14 family members have been identified (Pop and Salvesen, 2009). Caspases are, based on their function, location and structural characteristics, generally classified as apoptotic caspases and pro-inflammatory caspases. Although the first member of the caspase family, interleukin-1β-converting enzyme (ICE; also known as caspase-1), was identified as having a distinct role in inflammatory response (Thornberry et al., 1992), at least 8 of 14 caspases are important regulators of apoptosis (Shi, 2002). Typical apoptotic caspases can be subdivided into two groups: the initiator caspases (i.e. caspase-2,-8,-9 and -10); and effector caspases (i.e. caspase-3,-6 and -7). All caspases exist as inactive zymogens and their activation requires proteolytic activity during apoptosis. An effector caspase (e.g. caspase-3) can be activated by an initiator caspase (e.g. caspase-8) through cleavage at the internal Asp residue, leading to disassembly of the large and small subunit; the inhibitor caspases, however, are activated by dimerization via the signal obtained from death receptors (such as TNF and TNF ligand, Fas and Fas ligand, TRAIL and its ligand). The downstream caspase cascade ultimately leads to cell death (Shi, 2002, Riedl and Shi, 2004).

As mentioned above, caspases are central players in the apoptotic machinery and aberrant caspase expression and/or activation has been implicated in tumorigenesis. Amongst the 14 caspase family members, caspase-3 is a key downstream effector, which is activated by initiator caspase-9 or -8, proceeding apoptotic signalling by cleavage of several downstream proteins. Aberrant caspase-3/-8 expression has been implicated in various types of cancer. For

example, caspase-3 protein expression was found to be significantly reduced in moderately and poorly differentiated prostate cancer specimens compared with well-differentiated prostate tumours and normal tissues (Winter et al., 2001) and high caspase-3 expression was observed in oral squamous cell carcinomas and acute myelogenous leukaemia compared with normal samples (Estrov et al., 1998, Hague et al., 2004). In addition, cleaved active caspase-3 can predict a favourable overall and disease-free survival in a distinct type of T-cell non-Hodgkin lymphoma and intracranial meningioma (Konstantinidou et al., 2007, Ling et al., 2011), and high caspase-3 expression was significantly associated with improved prognosis in patients with hepatocellular carcinomas that had complete resection (Huang et al., 2010a). On the other hand, loss of caspase-8 mRNA/protein expression has been noticed in high grade small cell lung cancers, neuroendocrine lung cancers and childhood neuroblastoma. Both studies suggested that caspase-8 might act as a tumour suppressor in certain types of lung cancer and neuroblastoma (Teitz et al., 2000, Shivapurkar et al., 2002). Studies have shown that CASP8 (encoding caspase-8) gene polymorphism may contribute to the increased risk of oesophageal squamous cell carcinoma and advanced gastric cancer (Soung et al., 2005, Umar et al., 2011, Yin et al., 2014).

Thus far there have been limited studies carried out investigating the role of caspase-3/-8 protein expression, as well as their prognostic potential, in breast cancer. Immunohistochemical based studies have reported that the levels of caspase-3 (uncleaved and cleaved forms), -6, and -8 protein expression were increased in breast carcinomas (patients numbers ranging from 81 to 210); with reports from Vakkala et al. and Blazques et al. showing that there was no significant association between caspase-3 expression and patient overall survival (Vakkala et al., 1999, Hadjiloucas et al., 2001, O'Donovan et al., 2003, Blazquez et al., 2006). Interestingly, one study explored caspase-3 expression in a relatively small breast cancer patient cohort (n=137), showing that high caspase-3 expression had a negative influence on overall survival in breast cancer patients (Nakopoulou et al., 2001).

3.2 Materials and methods

3.2.1 Cell lines and culture

Subculture, cell line verifications and mycoplasma screening were described in section 2.2.1 and 2.2.2.

3.2.2 Chemicals and drug preparation

Staurosporine (Sigma, UK) was dissolved in DMSO (Sigma, UK) as stock solution of 1 mM and stored at -20^{o}C. Calpeptin was prepared and stored as described in section 2.2.4. Dilution and the negative controls were prepared the same as described in section 2.2.4.

3.2.3 Annexin V-FITC assay

As discussed in section 3.1.2, apoptotic cells undergo a series of characteristic morphological and biochemical changes. Under physiological conditions phosphatidylserine (PS) is normally confined to the internal side of the plasma membrane. In the early stage of apoptosis, cells expose PS from the inner plasma membrane to the outside of the cell, while the plasma membrane remains intact. Externalisation of PS on the cell surface makes such apoptotic cells amenable to detection by Annexin V, a recombinant protein that strongly binds with PS residues in the presence of calcium ions, and can be applied for the detection of early apoptosis. Externalisation of PS is not, however, exclusive to apoptosis, but also occurs during cell necrosis. The latter is characterised by loss of cell membrane integrity, followed by leakage of cellular components (i.e. nucleic acids), that can be assessed via propidium iodide (PI) staining (Vermes et al., 1995, Van Engeland et al., 1998). Bearing this in mind, cells were stained with fluorescein isothiocyanatc (FITC)-labelled Annexin V, with dye exclusion of PI, and measured by flow cytometry (i.e. fluorescence-activated cell sorting, FACS). The assay was used to determine viable (Annexin^{-}/PI^{-}), early stage of apoptosis (Annexin^{+}/PI^{-}), and late stage of apoptosis & dead/necrotic cells (PI^{+}).

MDA-MB-231, MCF-7 and SKBR3 cells were treated with 100 µM calpeptin for

30 minutes, 6 and 24 hours. DMSO was added to the negative control group at the same volume equivalent to that in the drug treatment group. MDA-MB-231 and MCF-7 cells were treated with 1 μM of staurosporine for 24 hours (optimisation was conducted by Dr. Yimin Zhang) and SKBR3 cells were treated with 1 μM of staurosporine for 6 hours, were set as positive controls (Kruman et al., 1998, Belmokhtar et al., 2001). Apoptosis of cells were measured using an Annexin V-FITC apoptosis detection kit (Sigma, UK) following the manufacturer's instructions. Briefly, after treatment floating cells in the culture medium and cells attached on the flask were collected following trypsinsation. And all the cells were combined into the same tube. Cells were pelleted by centrifugation at 170 g for 5 minutes at room temperature, followed by washing twice with ice-cold PBS. The supernatant was removed and the cell pellet was resuspended in 1x binding buffer (provided in the kit) at a concentration of $1X10^6$ cells /mL. Then 500 μL of cell suspension was transferred into a plastic 12 x 75 mm test tube, and incubated with 5 μL of 50 μg/mL Annexin V-FITC conjugate and 10 μL of 100 μg/mL PI solutions (both provided in the kit). The mixture was incubated in the dark at room temperature for 10 minutes. The fluorescence of the cells were immediately analysed using a Beckman Coulter FC500 MCL flow cytometer system (Beckman Coulter, USA) by reading on multichannel of FL1 (525 nm for FITC) and FL3 (620 nm for PI). Data generated from the flow cytometer were analysed using FlowJo 7.6.5 software (Tree Star). The percentage of cells in each population (i.e. viable, apoptotic and dead/necrotic cells) was plotted. Data represent the average percentage ± standard deviation (SD) of three independent experiments.

3.2.4 Human apoptosis array

The role of calpain in regulating apoptosis-related signal transduction was assessed by using a Human Apoptosis Array Kit (R&D systems, USA). The kit can simultaneously detect the relative expression levels of 35 apoptosis-related proteins (in duplicate). MDA-MB-231 and MCF-7 cells were treated with or without 100 μM calpeptin for 30 minutes. The cells were harvested and collected in Lysis Buffer (provided in the kit) and the concentrations of proteins

were determined by Bio-Rad protein assay following the process described in section 2.2.5. 300 µg of total protein was mixed with Lysis Buffer and Array Buffer 1 following manufacturers' instructions to reach a final volume of 1.5 mL. The arrays were blocked with Array Buffer 1 for 1 hour in advance followed by incubation with the protein samples overnight at 4^{o}C. The array was then washed with 1X Wash Buffer for 3X10 minutes to remove unbound proteins and incubated with corresponding Detection Antibody Cocktails for 1 hour at room temperature. The arrays were visualised as described in section 2.2.9.

3.2.5 Western Blotting

The specificity of caspase-3 and -8 antibodies was determined using Western blotting, prior to immunohistochemical staining of patient specimens, following the steps in section 2.2.10. A number of antibodies were checked for specificity and those used in the current chapter are listed in Table 3.1.

Table 3.1 List of antibodies used in Western blotting

Antibodies	Source	Host Type	Clone	Dilution	Size (kDa)
Anti-caspase-3	Cell Signalling Technology	Rabbit	9662	1:1000	35
Anti-caspase-8	Thermo Scientifi	Rabbit	PA1-29159	1:500	62
Anti-HSP60	R&D Systems	Mouse	264233	1:1000	62
Anti-β-actin	Abcam	Mouse	8226	1:1000	42
Anti-mouse secondary	Dako	Mouse-HRP	-	1:1000	-
Anti-rabbit secondary	Dako	Rabbit-HRP	-	1:1000	-

3.2.6 Clinical samples

The immunohistochemical study was performed using a cohort of 1902 early stage breast cancer patients. The Information on clinicopathologic variables were described in section 2.2.11.

3.2.7 Tissue microarray construction and immunohistochemistry

The TMA was constructed by the Breast Pathology Group at the University of

Nottingham and cut sections supplied for immunohistochemistry use, and as described in section 2.2.12. The concentrations of antibodies were optimised following the procedures described in section 2.2.12. Caspase-3 staining was achieved using a Vectastain® Universal Elite® ABC Kit (Vector Laboratories, USA); caspase-8 staining was achieved using or a Novolink Polymer Detection System (Leica, Denmark) (as described in section 2.2.12). For the Vectastain® Universal Elite® ABC Kit, the majority of the procedures were the same as a Novolink Polymer Detection System except for the following modifications. Endogenous peroxidase activity was blocked by using 0.3% solution of hydrogen peroxide (H_2O_2) in methanol with incubation for 10 minutes. The blocking solution was replaced with 2% serum solution (provided in the ABC kit) with incubation for 30 minutes at room temperature. The primary antibody was diluted in blocking solution in an appropriate ratio decided according to the optimisation (described in section 3.3.3.1). The post primary block solution was replaced with 2% biotinylated secondary antibody (provided in the kit) with 1 hour incubation at room temperature. The Novolink polymer was replaced with avidin coupled to biotinylated horseradish peroxidase reagents (ABC reagents), which was 2% of reagent A and B diluted in PBS, and incubated for 30 minutes at room temperature.

Staining was assessed at X20 magnification following high-resolution scanning as described in section 2.2.12. ICCs between scorers were 0.898 and 0.732 for caspase-3 and caspase-8, respectively (conducted by Dr. Yimin Zhang), showing good concordance between both scorers. The cut points of immunohistochemical scores were determined in a non-biased fashion using X-tile software as described in section 2.2.12.

3.2.8 Statistical analysis

The same as described in section 2.2.13.

3.3 Results

3.3.1 FACS analysis of apoptosis

The role of calpain in breast cancer cells apoptosis was assessed by using flow cytometry. 1 µM Staurosporine (STS) was used as the positive control, (Mooney et al., 2002, Xue et al., 2003). The treatment time of STS for MDA-MB-231 and MCF-7 cells (24 hours) was optimised previously (conducted by Dr. Yimin Zhang, data not shown), and the treatment time of STS for SKBR3 cells was optimised. As shown in Figure 3.2, 1 µM STS induced apoptosis in SKBR3 cells, with maximal increase being observed at 6 hours, the percentage of apoptotic cells in the STS treatment group was 1.69 – fold of control group. 24 hour treatment with STS produced inconsistent results, which was excluded in the optimisation (data not shown). Therefore, treatment with 1 µM of STS for 6 hours was set as the positive control for SKBR3 apoptosis analysis.

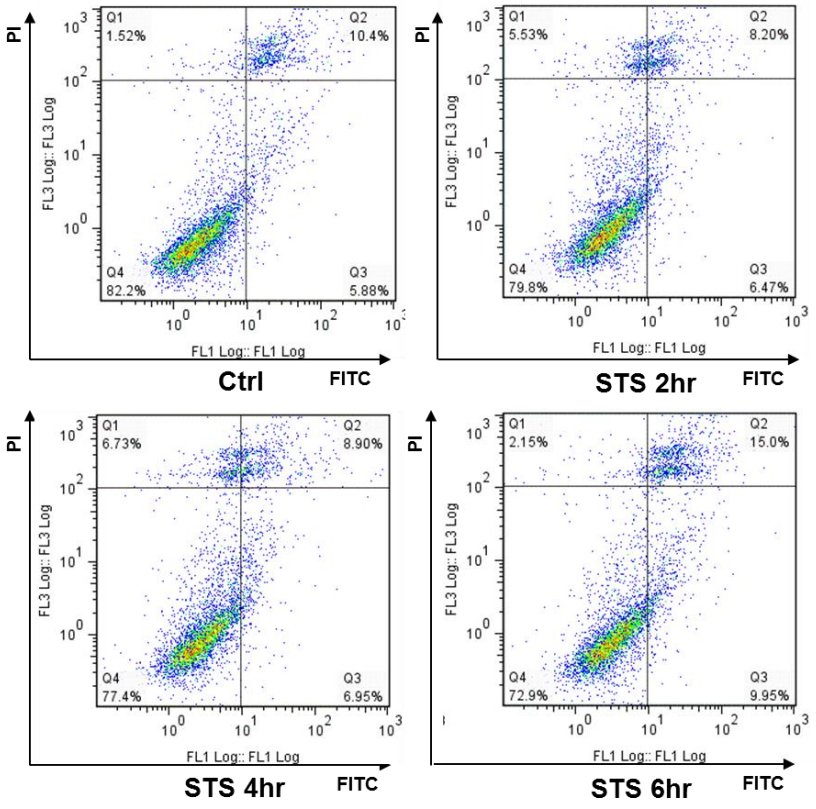


Figure 3.1 Optimisation of staurosporine (STS) treatment time in Annexin V-FITC apoptosis assay. Results are displayed as dot plots of FL-1 (Annexin V-FITC) vs. FL-3 (PI) florescence. The figure shows representative results from two independent experiments.

The role of calpain in breast cancer cells apoptosis was assessed using MDA-MB-231, MCF-7 and SKBR3 cells. As shown in Figure 3.3, treatment with 1 μM of STS for 24 hours induced significant increase in both apoptotic and necrotic cells in MDA-MB-231 cells; and in MCF-7 cells, STS induced significant increase in necrotic cells but not in apoptotic cells. Treatment with 1 μM of STS for 6 hours induced significant increase in apoptotic cells in SKBR3 cells, but had no significant effect on necrotic cells. The results also reassured STS as being a suitable positive control for the Annexin V-FITC apoptosis assay.

MCF-7 cells are caspase-3 deficient (Devarajan et al., 2002); one study found that MCF-7 wild-type cells are less sensitive to STS than MCF-7 cells expressing caspase-3, suggesting STS-induced apoptosis is caspase-3 dependent (Xue et al., 2003). This may provide a possible explanation for the unchanged apoptosis in STS treated MCF-7 cells. When comparing three cell lines, the endogenous apoptosis level in MDA-MB-231 cells (0.9%) was lower than MCF-7 (12.9%) or SKBR3 (12.0%) cells. A similar pattern was also observed in the Yuan et al. study, showing lower endogenous apoptotic death in MDA-MB-231 cells compared with MCF-7 and SKBR3 cells (Yuan et al., 2012).

As mentioned previously, treatment with 100 μM calpeptin for 30 minutes, 6 or 24 hours inhibited 20-30% of calpain activity in MDA-MB-231 cells. As shown in Figure 3.2-A, in MDA-MB-231 cells, although 100 μM calpeptin for 30 minutes or 6 hours caused no significant change on the percentage of viable, apoptotic or dead/necrotic cells, treatment for 24 hours significantly reduced the percentage of viable cells from 94.8% to 89.7% (vs control, $P<0.05$), and increased the percentage of apoptotic cells by 2.17-fold of control ($P<0.05$). Data showed that calpeptin-induced apoptosis increased with duration of drug treatment with minimal apoptosis observed at the shortest exposure time: 0.8% at 30 minutes, 1.3% at 6 hours, and 1.9% at 24 hours.

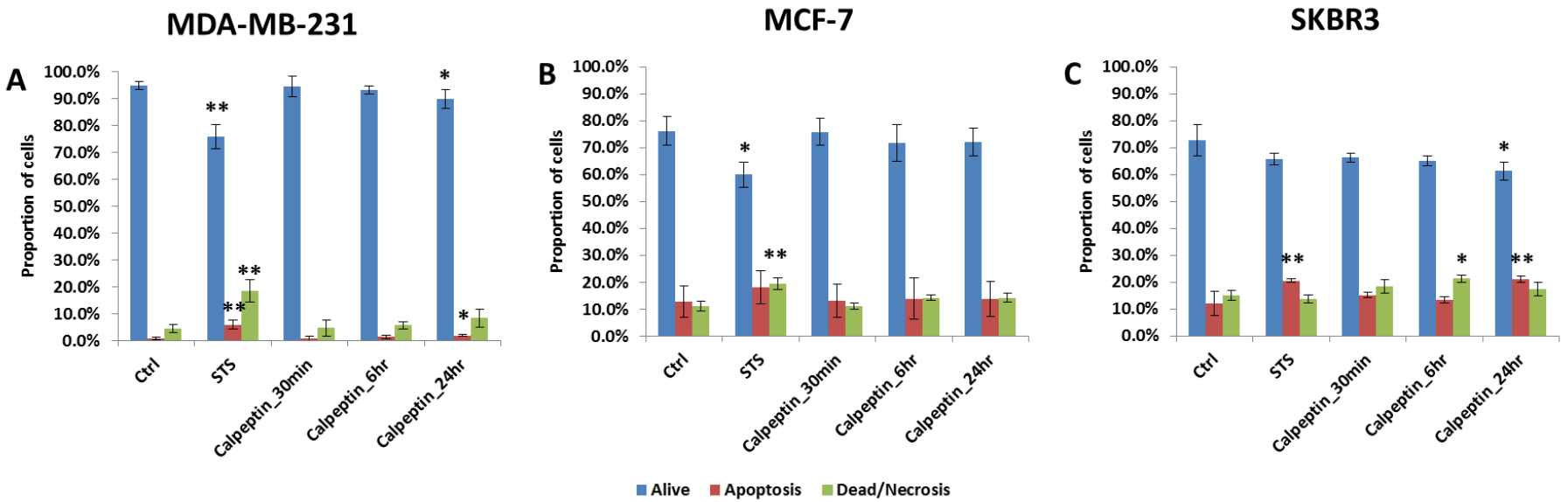


Figure 3.2 Effect of calpeptin on apoptosis of breast cancer cells. MDA-MB-231 (A), MCF-7 (B) and SKBR3 (C) cells were treated with 100µM calpeptin for 30 minutes, 6 and 24 hours. MDA-MB-231 and MCF-7 cells were treated with 1 µM STS for 24 hours, and SKBR3 cells were treated with 1 µM STS for 6 hours, as positive controls. The percentage of cells in each population was plotted as: dead/necrotic (Q1+Q2), apoptotic (Q3), and viable (Q4). Data represent the average percentage ± standard deviation (SD) of three independent experiments. Statistical significance was determined by one-way ANOVA compared to control and is indicated by asterisk: **P*<0.05, ***P*<0.01 vs control.

For MCF-7 cells, 100 µM of calpeptin at three time points resulted in significant reduction of calpain activity (38-48%); however calpeptin treatment had no effect on apoptosis at any time point assessed in the present study (Figure 3.2-B). For SKBR3 cells, 100 µM calpeptin inhibited 45-54% of calpain activity at 6 and 24 hours' time points. As shown in Figure 3.2-C, when exposed to calpeptin, a significant increase in the percentage of dead/necrotic cells was observed at 6 hours (1.14-fold of control, $P<0.05$). After 24-hour, the percentage of viable cells decreased from 72.8 to 61.4% ($P<0.05$); and the percentage of apoptotic cells increased by 1.76-fold of control ($P<0.01$). Representative dot plots from flow cytometry analysis are shown in Appendix A-
Figure A **1**&Figure A 2&Figure A 3.

3.3.2 Apoptosis array and validation

Human apoptosis arrays were used to simultaneously assess the expression of 35 apoptosis-related proteins in order to determine whether inhibition of calpain activity exerts regulatory effects on apoptosis-related signal transduction in MDA-MB-231 and MCF-7 breast cancer cells. MDA-MB-231 and MCF-7 cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes. As described before, the treatment of 100 µM calpeptin for 30 minutes can inhibit 20-30% and 38-48% of calpain activity in MDA-MB-231 and MCF-7 cells, respectively. As shown in Figure 3.4 (red squares), in both treated and untreated MDA-MB-231 cells, pro-caspase-3 was highly expressed; however cleaved-caspase-3 (active form) was not expressed. As expected, MCF-7 cells did not express either pro-caspase-3 or cleaved caspase-3.

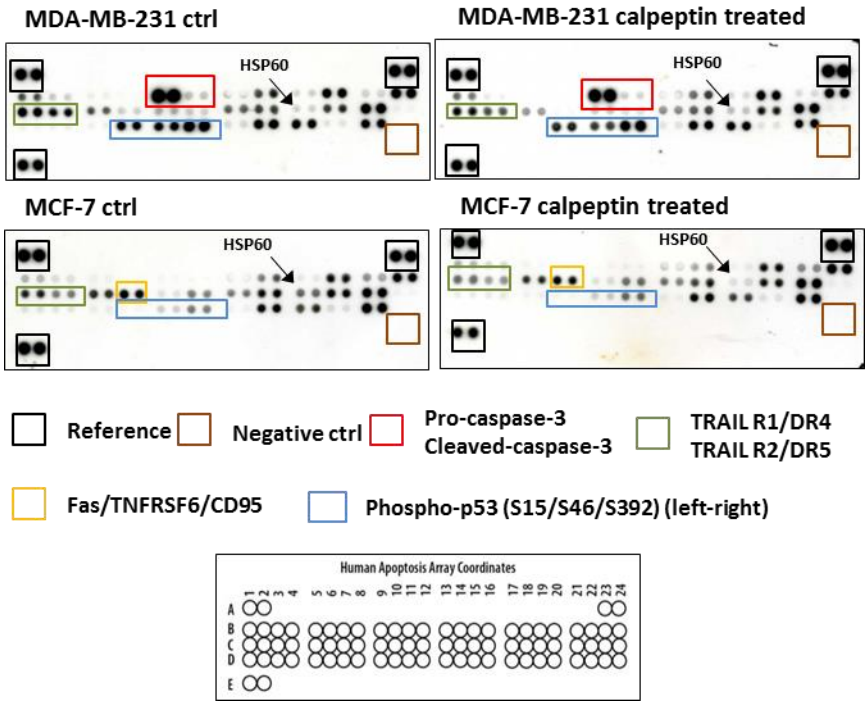


Figure 3.3 Effect of calpeptin on expression of 35 apoptosis-related proteins in breast cancer cells. MDA-MB-231 and MCF-7 cells were treated in the presence or absence of 100 µM calpeptin for 30 minutes. The experiment for each cell line was conducted once.

In MCF-7 cells, HSP60 was markedly suppressed in the presence of calpeptin, whereas such change was not detected in MDA-MB-231 cells (Figure 3.3, indicated by arrows). In addition, both cells expressed tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1/DR4 and -R2/DR5, with MDA-MB-231 cells expressing a higher level of TRAIL proteins than MCF-7 cells. Calpeptin treatment reduced the expression of TRAIL-R1 and R2 in both cell lines (Figure 3.3, green squares). Fas, also known as tumour necrosis factor receptor superfamily member 6 (TNFRSF6, or CD95), was expressed to a higher level in MCF-7 cells, with a nearly undetectable signal in MDA-MB-231 cells (Figure 3.3, yellow squares). On the contrary, phospho-p53 (S15/S46/S392) was overexpressed in MDA-MB-231 cells but with markedly lower expression in MCF-7 cells (Figure 3.3, blue squares). The signal of phospho-p53 at S392 was stronger than at other two serine sites in both cell lines. In addition to the proteins mentioned above, a number of other apoptosis-associated protein expressions are listed in Table 3.2.

Table 3.2 The apoptosis-related proteins expression in breast cancer cells and changes induced by calpeptin

		Calpain-induced changes		Differential protein expression	
Coordinate	**Target**	**231**	**MCF-7**	**231**	**MCF-7**
B1, B2	Bad	O	O	M	L
B3, B4	Bax			L	L
B5, B6	Bcl-2				
B7, B8	Bcl-x				
B9, B10	Pro-Caspase-3	O		H	
B11, B12	Cleaved Caspase-3			L	
B13, B14	Catalase	O	O	L	
B15, B16	cIAP-1	O	O	M	M
B17, B18	cIAP-2			L	
B19, B20	Claspin	O	O	M	M
B21, B22	Clusterin		O	L	L
B23, B24	Cytochrome c	O	O	H	H
C1, C2	TRAIL R1/DR4	-	-	H	M
C3, C4	TRAIL R2/DR5	-	-	M	L
C5, C6	FADD	O	O	M	M
C7, C8	Fas/TNFRSF6/CD95		O		M
C9, C10	HIF-1α			L	
C11, C12	HO-1/HMOX1/HSP32			L	M
C13, C14	HO-2/HMOX2	O	O	M	M
C15, C16	HSP27	O	O	M	M
C17, C18	**HSP60**	O	-	**L**	**M**
C19, C20	HSP70	O	O	M	M
C21, C22	HTRA2/Omi	O	O	H	H
C23, C24	Livin				
D1, D2	PON2				
D3, D4	p21/CIP1/CDKN1A				
D5, D6	p27/Kip1				
D7, D8	Phospho-p53 (S15)	O		M	
D9, D10	Phospho-p53 (S46)	O		M	L
D11, D12	Phospho-p53 (S392)	O	O	H	M
D13, D14	Phospho-Rad17 (S635)			L	
D15, D16	SMAC/Diablo	O	O	M	M
D17, D18	Survivin	O	O	M	M
D19, D20	TNF RI/TNFRSF1A				
D21, D22	XIAP	O	O	M	M

Abbreviations: 231, MDA-MB-231; "H", high expression level; "M", moderate expression level; "L", low expression level; "+", expression level increased after calpeptin treatment; "-", expression level decreased after calpeptin treatment; "O", expression level did not change after calpeptin treatment; blank, expression level was too low or could not be detected.

As shown in Table 3.2, survivin, cytochrome-c, X-linked inhibitor of apoptosis protein (XIAP) and high temperature requirement protein A2 (HTRA2/Omi) were overexpressed in both cell lines. However such results, generated from a single experiment, needs further investigation to provide firm conclusions with current data supplying 'clues' as to potential areas of interest.

As mentioned above, HSP60 protein expression appeared to be differentially regulated by calpain in MDA-MB-231 and MCF-7 cells; as its expression was shown to be downregulated by calpeptin in MCF-7 cells rather than in MDA-MB-231 cells. The results obtained from such array data were further investigated via Western blotting using an anti-HSP60 antibody (R&D Systems, USA) on a panel of 6 breast cancer lines representing two basal/TN, two luminal, and two HER2+. As shown in Figure 3.4, HSP60 expression could be detected in both MDA-MB-231 and MCF-7 cells, however, no obvious change was observed, in any of the lines, following calpeptin treatment, even with a longer incubation times (6 and 24 hours). It appears, from such data, that HSP60 apoptosis array results cannot be verified by Western blotting and such reproducibility difficulties with array results should be noted when interpreting the data.

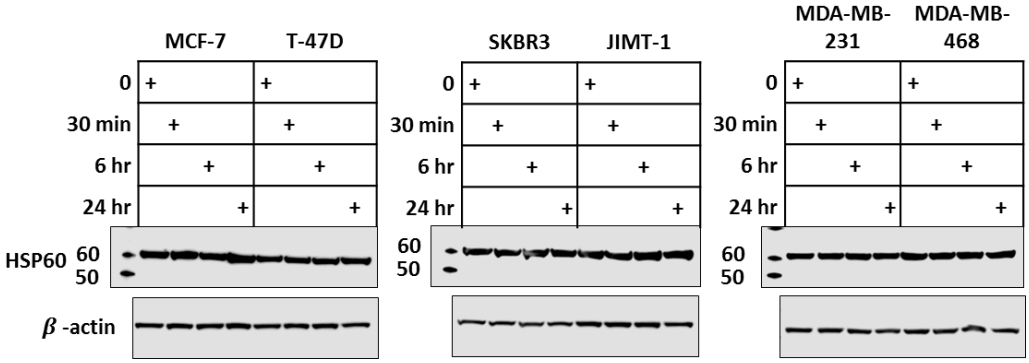


Figure 3.4 Effect of calpeptin on HSP60 protein expression. Cells were treated in the presence or absence of 100 µM calpeptin for 30min, 6 and 24 hours. Primary antibody against HSP60 was used. The cell samples for 30 minutes treatment were collected and repeated in two independent experiments; the samples for 6 and 24 hours treatment were collected in one independent experiment. Representative blots are shown above.

3.3.3 Immunohistochemistry studies on caspase-3/-8 with calpains

As introduced in sections 3.1.3, caspase-3 and -8 are key executioners in apoptotic pathways. Impaired caspase-3/-8 expression and/or activity can lead

to dysfunctions in apoptosis, and have been implicated in the carcinogenic process. There are close links, and direct interactions, between the two cysteine protease families; calpains and caspases. Based upon this, and the important role of calpain in apoptosis cascades, it was thought interesting to investigate whether caspase-3/-8 protein expression is associated with calpain family protein expression, and if caspase-3/-8 expression was of prognostic significance in a large cohort of early invasive breast cancer patients.

3.3.3.1 Antibody specificity and immunohistochemistry optimisation

The expression and prognostic value of caspase-3/-8 were investigated in breast tumour tissues using standard immunohistochemistry. Before conducting immunohistochemistry, the specificity of anti-caspase-3/-8 antibodies was assessed by Western blotting. As shown in Figure 3.5 (left), anti-caspase-3 antibody (Cell Signalling, USA) detected a single band of the expected size, 35-kDa, with no signal in MCF-7 cells. For caspase-8, the first antibody from Abcam did not detect any signal in multiple human cell lines (data not shown). A second anti-caspase-8 antibody (Thermo Scientific, USA), detected a single specific band of the expected size, at 62-kDa, across multiple human cell lines (Figure 3.5-right).

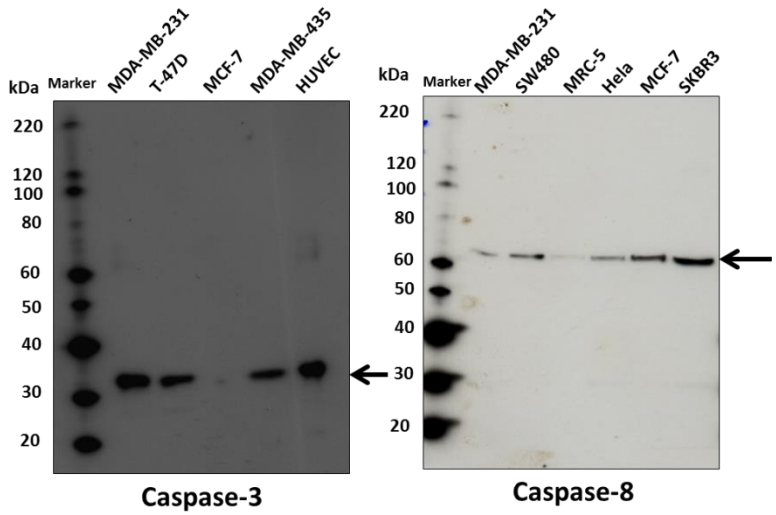


Figure 3.5 Specificity of anti-caspase-3/-8 antibodies were determined in Western blotting by using various cell lines. Primary antibodies against caspase-3/-8 were used. Each experiment was conducted once.

Once specificity was proven, the optimal concentration of each antibody to be

used in immunohistochemistry was determined, as described below. For anti-caspase-3 antibody, dilutions of 1:400, 1:200 and 1:100 incubated for 1 hour were assessed. For anti-caspase-8 antibody, a dilution of 1:250, 1:100 and 1:50 incubated for 1 hour showed no staining on freshly cut sections from the composite block breast tissue sections (data not shown). Therefore dilutions of 1:50 and 1:25, incubated overnight, were then assessed. Based on results, a dilution of 1:100 (1 hour) for anti-caspase-3 and 1:25 (overnight) for anti-caspase-8 antibodies were used experimentally to give optimal staining and were used on tissue microarrays. Representative staining patterns are shown in Figures 3.6 & 3.7.

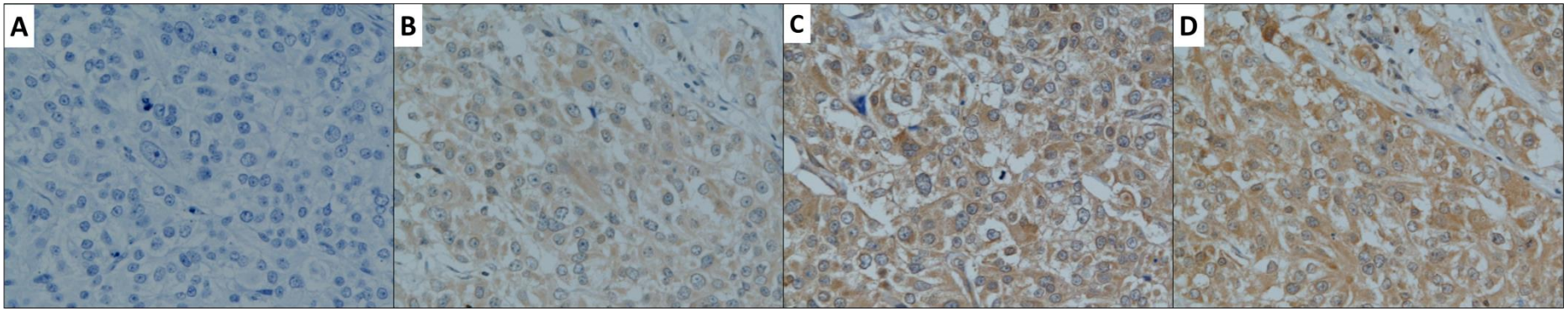


Figure 3.6 Optimisation of anti-caspase-3 antibody on breast tumours. Breast tumour composite sections were subjected to immunohistochemistry. (A) No staining in negative control; (B) 1:400 dilution of primary antibody; (C) 1:200 dilution; (D) 1:100 dilution. Photomicrographs are at X20 magnification.

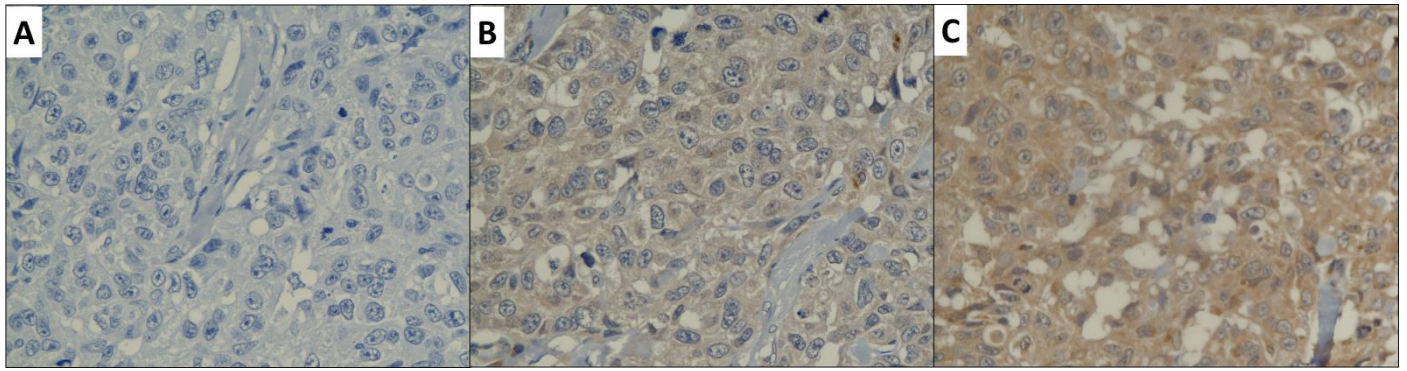


Figure 3.7 Optimisation of anti-caspase-8 antibody on breast tumours. Breast tumour composite sections were subjected to immunohistochemistry. (A) No staining in negative control; (B) 1:50 dilution of primary antibody; (C) 1:25 dilution of primary antibody. Photomicrographs are at X20 magnification.

3.3.3.2 Staining pattern and statistical analysis

After confirming the specificity of caspse-3/-8 antibodies by Western blotting, caspase-3/-8 protein expression was determined by using immunohistochemistry in a cohort of breast cancer patients (clinicopathologic variables of the patient cohort were described in section 2.2.11). As shown in Figure 3.8, caspase-3/-8 demonstrated mainly granular/diffuse cytoplasmic staining with some heterogeneity between adjacent tumour cells, varying from weak to intense staining. A few cores showed nuclear staining in some cases. Some inflammatory cells showed positive signals in some cases (4 out of 13 TMA slides were independently scored by Dr. Yimin Zhang, with descriptive pathology was provided by pathologist Dr. Mohammed A Aleskandarany, with all being assessed by the current author).

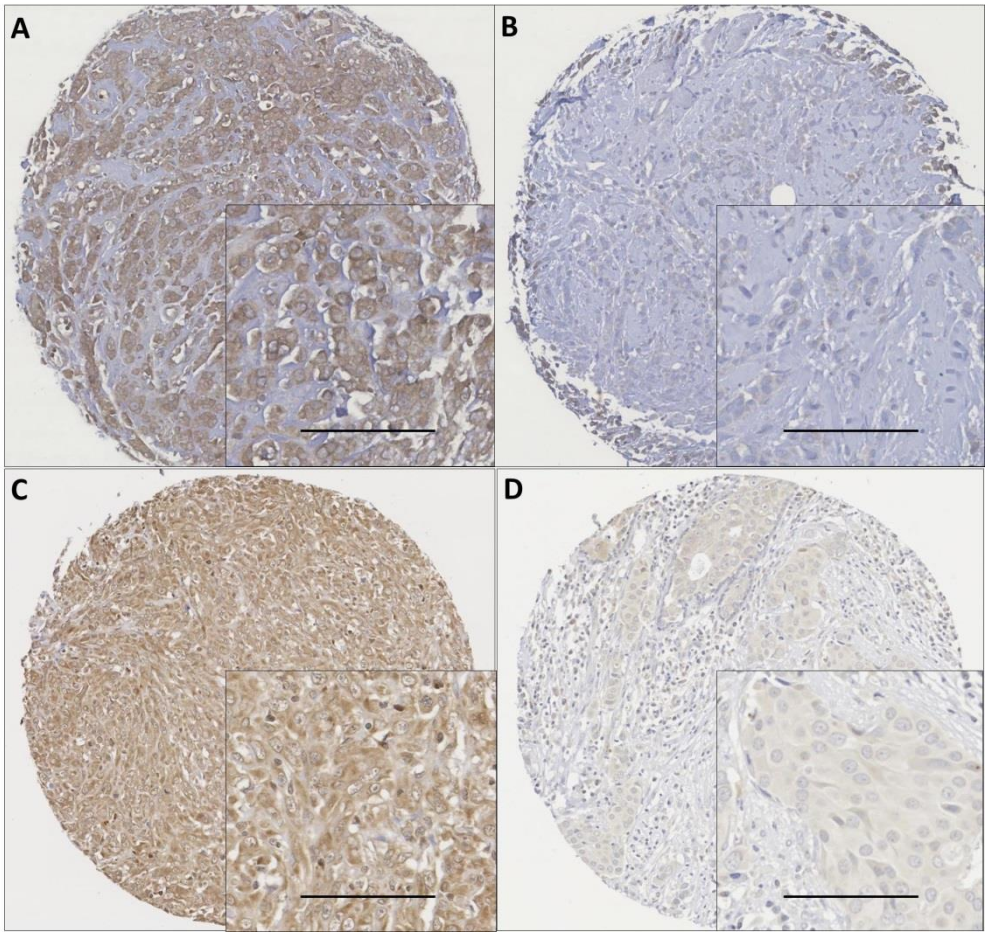


Figure 3.8 Representative photomicrographs of high and low caspase-3/-8 protein expression. High expression (panel A) and low expression (panel B) of caspase-3 protein; high expression (panel C) and low expression (panel D) of caspase-8 protein. Photomicrographs are at X10 magnification with inset panel at X20 magnification. Scale bar represents 100 µm.

A few TMA cores were not assessed due to insufficient tumour or the core being missing, a total number of 1421 cases for caspase-3, and 1402 cases for caspase-8 were assessed. Caspase-3 staining had a median H-score of 80±74 and ranged from 0 to 260; caspase-8 had a median H-score of 185±43 and ranged from 0 to 275. The X-tile cut point for caspase-3 was 128, with 556 (39.8%) cases having high expression and 855 (60.2%) cases having low expression. The X-tile cut point for caspase-8 was 183, with 736 (52.5%) cases having high expression and 666 (47.5%) cases having low expression. Spearman's rank order correlation was used to assess the correlation between caspase-3/-8, calpains, and calpastatin protein expression (Table 3.3). Although significant correlation was found between calpain-1 and caspase-3 (r=0.062, *P*=0.047), calpastatin and caspase-8 (r=0.086, *P*=0.008), the low correlation coefficient suggested the correlation was not biologically relevant.

Table 3.3 Correlation between calpain family, calpastatin and caspase-3/-8 protein expressions

	Caspase-3		Caspase-8	
	Correlation Coefficient	Sig.	Correlation Coefficient	Sig.
Calpain-1	0.062	**0.047**	-0.053	0.092
Calpain-2	0.062	0.051	0.017	0.606
Calpain-9	0.031	0.443	0.047	0.251
Calpastatin	-0.053	0.099	0.086	**0.008**

Significant *P*-values are indicated by bold font.

As shown in Table 3.4, a significant correlation was also found between calpain-2 and caspase-3 expression in the basal-like subgroup (r=0.143, *P*=0.045), calpain-2 and caspase-3 expression in the triple-negative subgroups (r=0.194, *P*=0.01), again, due to the low correlation coefficients, the correlation were unlikely to be biologically relevant. Calpastatin and caspase-8 (r=0.22, *P*=0.009), and calpain-9 and caspase-8 expression (r=0.25, *P*=0.019) in the HER2+ subgroup was also significantly correlated. Again, all the correlations were unlikely to be biologically significant, as also with the correlation between caspase-3 and -8 expression (r=-0.069, *P*=0.011).

Table 3.4 Correlation between calpain family, calpastatin and caspase-3/-8 protein expressions in different subgroups

	HER2+		Basal-like		Triple-negative	
	Correlation Coefficient	Sig.	Correlation Coefficient	Sig.	Correlation Coefficient	Sig.
Caspase-3						
Calpain-1	0.126	0.122	0.047	0.506	0.076	0.308
Calpain-2	-0.057	0.49	0.143	**0.045**	0.194	**0.01**
Calpastatin	-0.028	0.745	0.058	0.418	0.009	0.908
Calpain-9	0.088	0.408	-0.067	0.467	-0.056	0.557
Caspase-8						
Calpain-1	-0.067	0.402	0.05	0.485	-0.003	0.966
Calpain-2	0.062	0.461	0.088	0.228	0.013	0.868
Calpastatin	0.22	**0.009**	0.047	0.515	-0.009	0.904
Calpain-9	0.25	**0.019**	-0.005	0.957	-0.089	0.358

Significant *P*-values are indicated by bold font.

Relationships with clinicopathologic variables

Pearson's Chi-squared test was performed to assess the association between caspase-3/-8 protein expression with various clinicopathologic criteria (Table 3.5). High caspase-3 expression was significantly associated with HER2 positivity (χ^2=6.624; d.f.=1; *P*=0.01). High caspase-3 expression was also significantly associated with death due to breast cancer (χ^2=6.711; d.f.=1; *P*=0.01), recurrence (χ^2=8.111; d.f.=1; *P*=0.004) and distant metastasis (χ^2=5.724; d.f.=1; *P*=0.017). No significant association was found between caspase-8 expression and clinicopathologic variables or clinical outcomes.

Table 3.5 The associations between caspase-3/-8 protein expressions and clinicopathologic variables.

Variables	Caspase-3 (N=1421)			Caspase-8 (N=1402)		
	Low	High	*P*	Low	High	*P*
Age (years)						
≤40 years	91(6.4%)	45(3.2%)	0.09	66(4.7%)	65(4.6%)	0.494
>40 years	763(53.7%)	521(36.7%)		600(42.8%)	670(47.8%)	
Tumour size (mm)						
≤20 mm	512(36.2%)	341(24.1%)	0.943	410(29.4%)	432(30.9%)	0.241
>20 mm	339(23.9%)	224(15.8%)		252(18.1%)	302(21.6%)	
Tumour stage						
I	517(36.5%)	353(24.9%)	0.227	419(30.0%)	453(32.4%)	0.578
II	267(18.9%)	156(11.0%)		191(13.7%)	212(15.2%)	
III	68(4.8%)	55(3.9%)		52(3.7%)	69(4.9%)	
Tumour grade						
I	148(10.5%)	97(6.9%)	0.979	122(8.7%)	114(8.2%)	0.219
II	274(19.4%)	180(12.7%)		219(15.7%)	234(16.8%)	
III	429(30.3%)	288(20.3%)		321(23.0%)	386(27.7%)	
NPI						
≤3.4	251(17.7%)	175(12.4%)	0.416	203(14.6%)	215(15.4%)	0.516
3.41-5.4	452(31.9%)	280(19.8%)		348(24.9%)	378(27.1%)	
>5.4	148(10.5%)	109(7.7%)		111(8.0%)	140(10.0%)	
LVI						
Positive	238(20.1%)	162(13.7%)	0.456	117(15.3%)	207(17.9%)	0.747
Negative	484(40.9%)	300(25.3%)		349(30.1%)	425(36.7%)	
Operation type						
Mastectomy	493(35.0%)	325(23.1%)	0.99	378(27.2%)	429(30.9%)	0.511
WLE Lumpectomy	356(25.3%)	235(16.7%)		283(20.4%)	299(21.5%)	
ER status						
Positive	614(44.5%)	394(28.6%)	0.526	463(34.1%)	532(39.1%)	0.439
Negative	219(15.9%)	152(11.0%)		178(13.1%)	186(13.7%)	
PR status						
Positive	467(35.1%)	293(22.0%)	0.35	349(26.4%)	400(30.3%)	0.469
Negative	337(25.3%)	235(17.6%)		278(21.0%)	294(22.3%)	
HER2 status						
Positive	100(7.2%)	94(6.8%)	**0.01**	84(6.2%)	110(8.1%)	0.201
Negative	729(52.7%)	460(33.3%)		565(41.4%)	606(44.4%)	

Table 3.5 Continued.

Variables	Caspase-3(N=1421)			Caspase-8(N=1402)		
	Low	High	*P*	Low	High	*P*
Basal like status						
Positive	164(12.4%)	110(8.3%)	0.938	140(10.7%)	128(9.8%)	0.098
Negative	630(47.7%)	418(31.6%)		482(37.0%)	553(42.4%)	
Triple Negative status						
Positive	154(11.2%)	96(7.0%)	0.604	124(9.2%)	122(9.0%)	0.27
Negative	673(48.9%)	452(32.9%)		516(38.1%)	593(43.8%)	
Breast cancer specific survival						
Alive	577(41.7%)	342(24.7%)	**0.01**	444(32.6%)	464(34.0%)	0.157
Dead	259(18.7%)	207(14.9%)		204(15.0%)	251(18.4%)	
Recurrence						
Present	318(23.0%)	255(18.5%)	**0.004**	263(19.3%)	305(22.4%)	0.496
Not present	510(36.9%)	298(21.6%)		382(28.1%)	411(30.2%)	
Distant metastasis						
Present	250(17.7%)	201(14.2%)	**0.017**	204(14.7%)	240(17.2%)	0.431
Not present	597(42.3%)	364(25.8%)		457(32.8%)	491(35.3%)	

Abbreviations: NPI, Nottingham Prognostic Index; LVI, lymphovascular invasion (determined using IHC); WLE, wide local excision; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Significant P-values (≤0.01) indicated by bold font.

Relationships with clinical outcomes

In terms of Kaplan-Meier survival analysis, high caspase-3, but not caspase-8, expression was significantly associated with adverse breast cancer-specific survival in the total patient cohort (*P*=0.008 and *P*=0.056, respectively, Figure 3.9-A&B). The expression of high caspase-3/high caspase-8 was combined and was associated with adverse breast cancer-specific survival in the total patient cohort (*P*=0.021, Figure 3.9-C). No further significant association was found between combinations of caspase-3/caspase-8 protein expression and prognosis in any patient subgroup (HER2+, *P*=0.152; triple-negative, *P*=0.992; and basal-like, *P*=0.647).

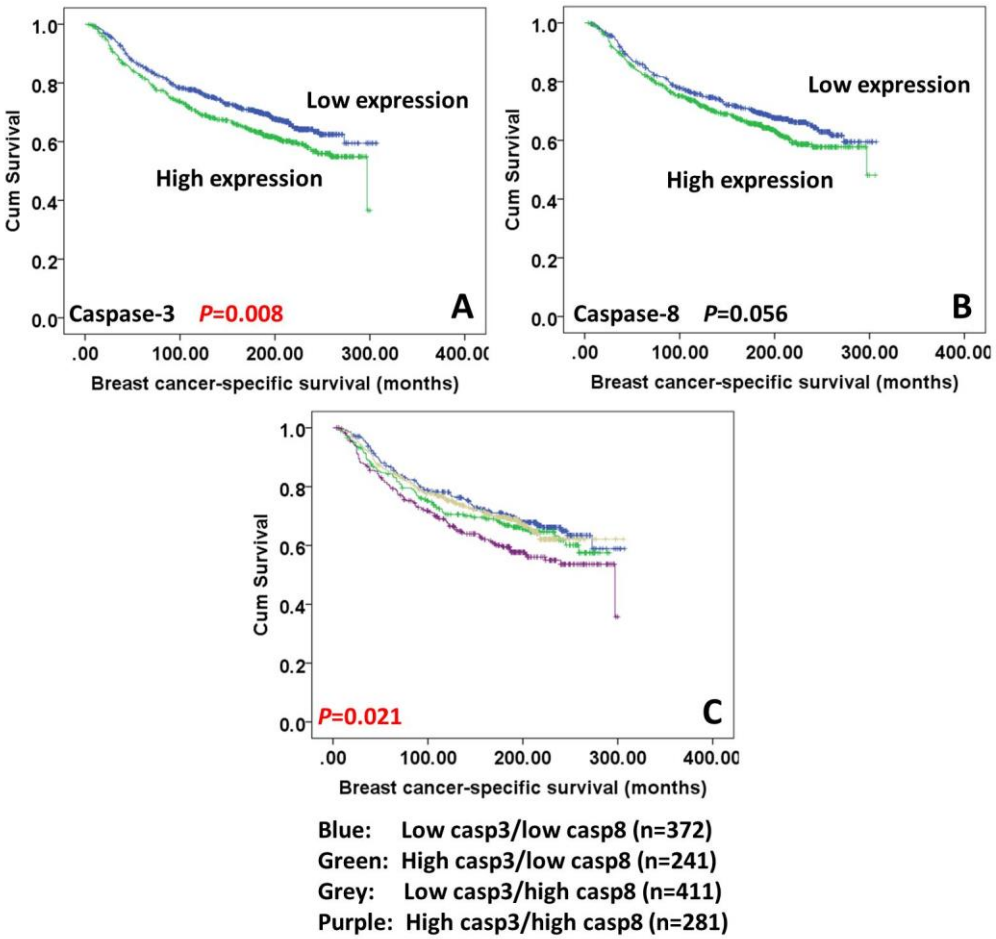


Figure 3.9 Kaplan-Meier survival curves for breast cancer-specific survival based upon caspase-3/-8 protein expressions (total patient cohort). Curves show low (blue line) and high protein expression (green line) with significance determined using the log-rank test. (A) 556 (39.8%) cases had high caspase-3 expression and 855 (60.2%) cases had low expression; (B) 736 (52.5%) cases had high caspase-8 expression and 666 (47.5%) cases had low expression (C) combinational caspase-3 and caspase-8 expression.

In multivariate analysis, including the potentially confounding factors age, tumour size, tumour stage, tumour grade, NPI value, lymphovascular invasion, ER, PR and HER2 status (with individual Kaplan-Meier statistics of $P<0.05$ for all variables), caspase-3 remained significant for breast cancer-specific survival (HR=1.347, 95% CI 1.086-1.670; $P=0.007$; Table 3.6).

Table 3.6 Cox proportional hazards analysis for breast cancer-specific survival

	Sig.	Exp(B)	95.0% CI for Exp(B)	
			Lower	Upper
Age	0.939	1.013	0.724	1.418
Tumour size	**0.003**	1.539	1.163	2.037
Tumour stage	**<0.001**	1.779	1.375	2.302
Tumour grade	**0.001**	1.595	1.197	2.125
Nottingham Prognosis Index	0.782	0.944	0.629	1.418
Lymphovascular invasion	**<0.001**	1.595	1.276	1.993
ER status	**0.009**	1.528	1.109	2.104
PR status	**0.001**	0.624	0.469	0.831
HER2 status	**0.035**	1.362	1.022	1.816
Caspase-3	**0.007**	1.347	1.086	1.670

Abbreviations: Exp (B), hazard ratio; 95% CI, 95% confidence interval. ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Significant P values are indicated by bold font.

Subgroup and combined biomarker analysis

Caspase-3 and -8 expression was investigated in HER2, triple negative and basal-like diseases and showed interest in HER2, triple-negative and basal-like diseases (P<0.001, P=0.004 and P=0.009, respectively; Figure 3.10 A-C). This was further investigated in the individual subgroups.

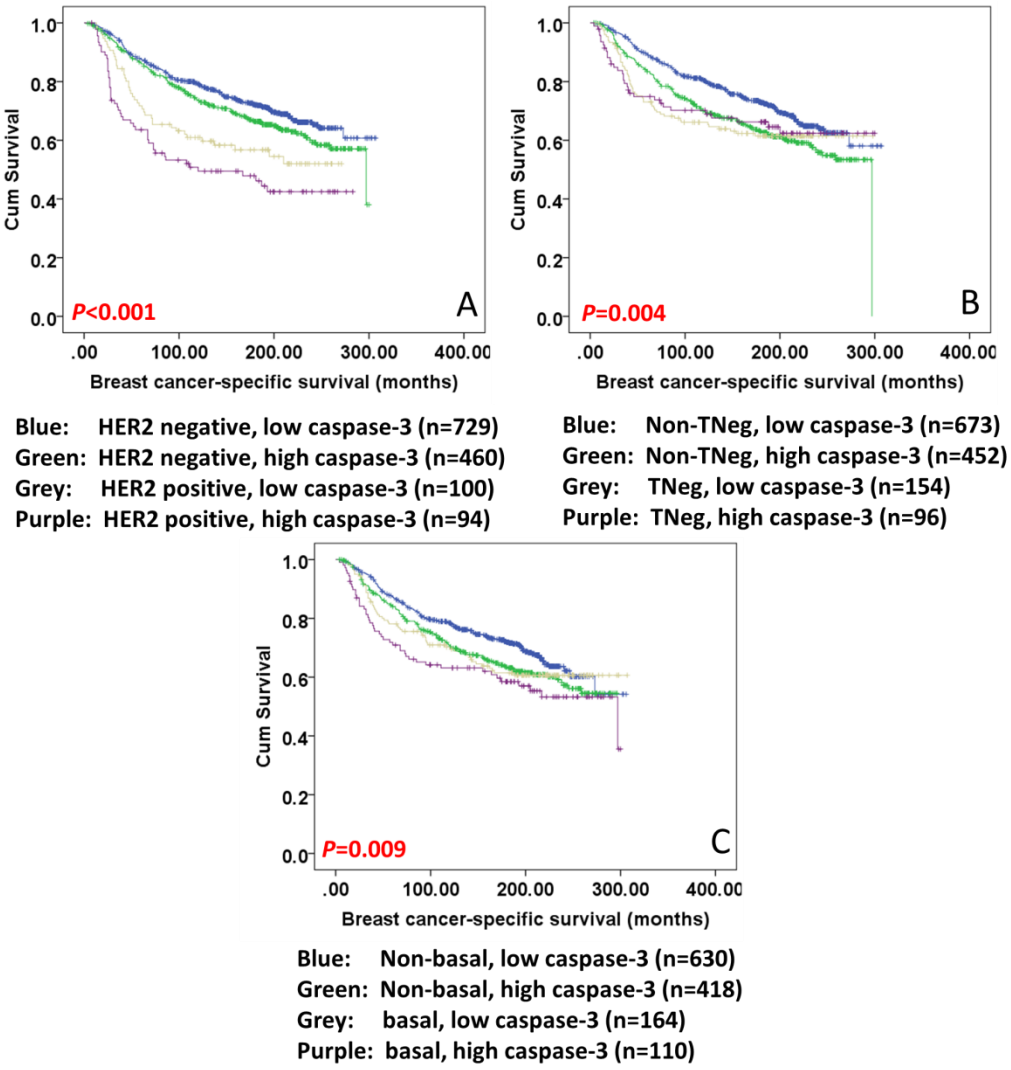


Figure 3.10 Kaplan-Meier survival curves for breast cancer-specific survival based upon caspase-3 expression shown as subgroups. (A) HER2 positive and negative tumours with high and low caspase-3 expression; (B) Triple-negative and non-triple negative tumours with high and low caspase-3 expression; (C) Basal-like and non-basal like tumours with high and low caspase-3 expression.

Analyses were further performed within the specific subgroups (HER2+, triple-negative, and basal-like) and caspase-3 protein expression was not significantly associated with breast cancer-specific survival in any of the groups (Figure 3.11-A, C&E).

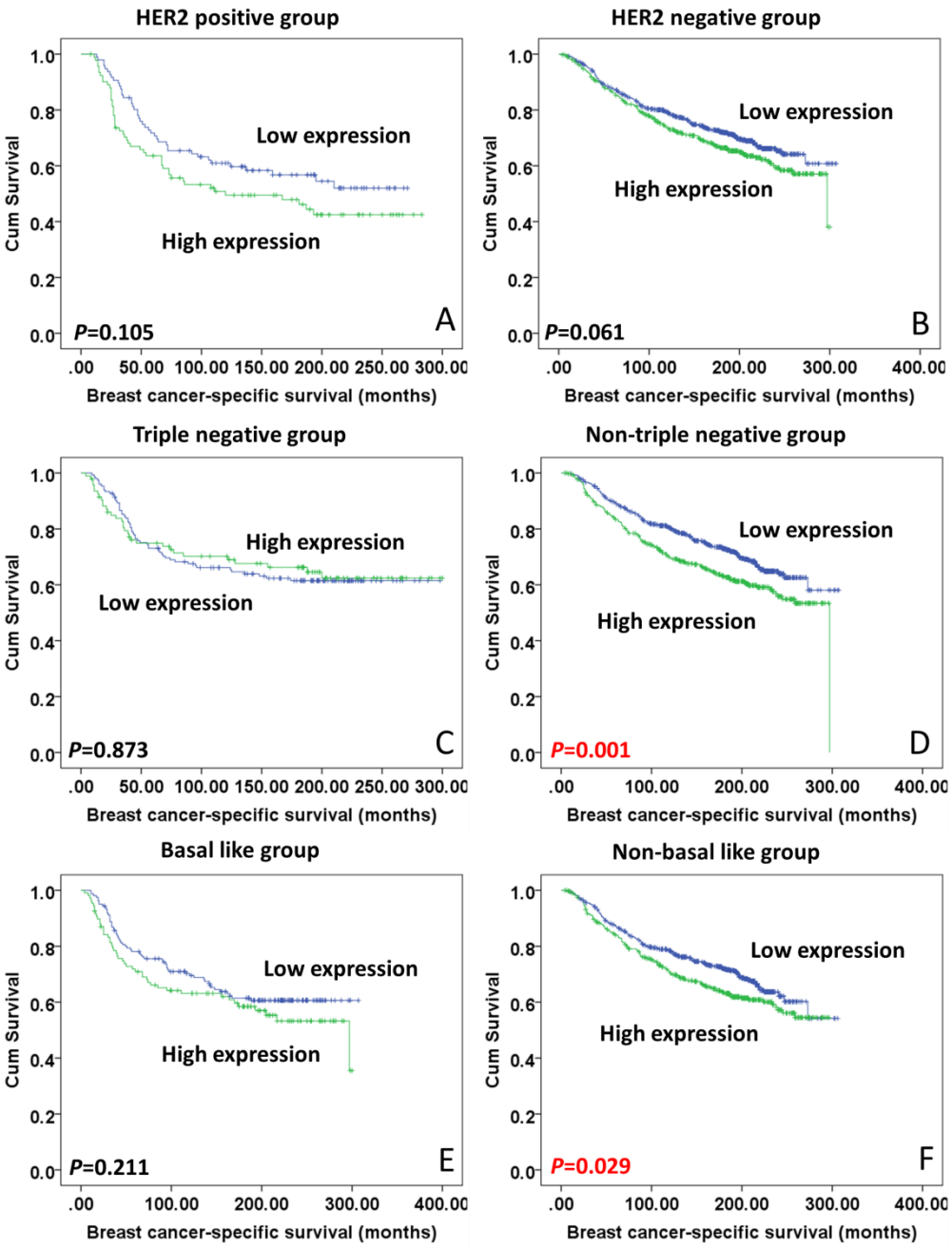


Figure 3.11 Kaplan-Meier survival curves for breast cancer-specific survival based upon caspase-3 expression (subgroups).(A) HER2 positive; (B) HER2 negative; (C) triple negative; (D) non triple negative; (E) basal like and (F) non-basal like.

Caspase-3 expression was associated with survival in receptor positive (ER, PR or HER2) patients (*P*=0.001) and in non-basal like subgroup (*P*=0.029) (Figure 3.11-D&F). In comparison, caspase-8 expression also showed interest in HER2+ and basal-like patients (*P*<0.001 and *P*=0.039, respectively; Figure 3.12-A), which was further investigated in the individual patient diseases.

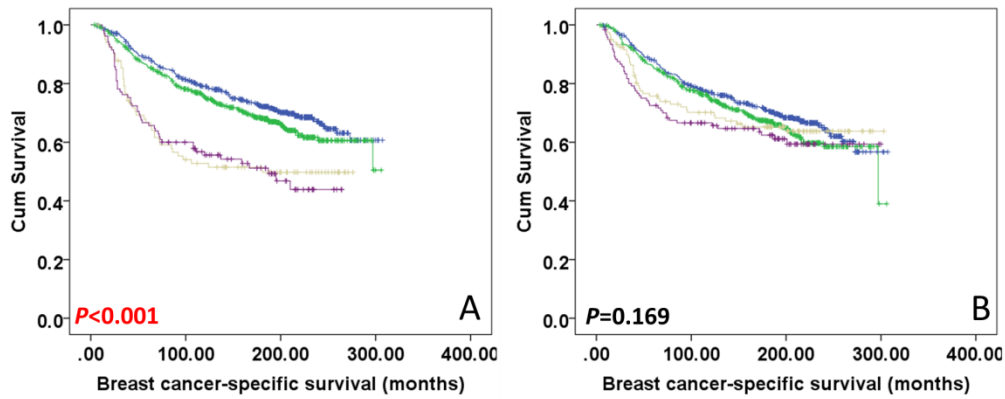


Figure 3.12 Kaplan-Meier survival curves for breast cancer-specific survival based upon caspase-8 expression shown as subgroups. (A) HER2 positive and negative tumours with high and low caspase-8 expression; (B) Triple-negative and non-triple negative tumours with high and low caspase-8 expression; (C) Basal-like and non-basal like tumours with high and low caspase-8 expression.

When analysis was further conducted in individual subgroups, no additional significant associations were observed (Figure 3.13.A-F).

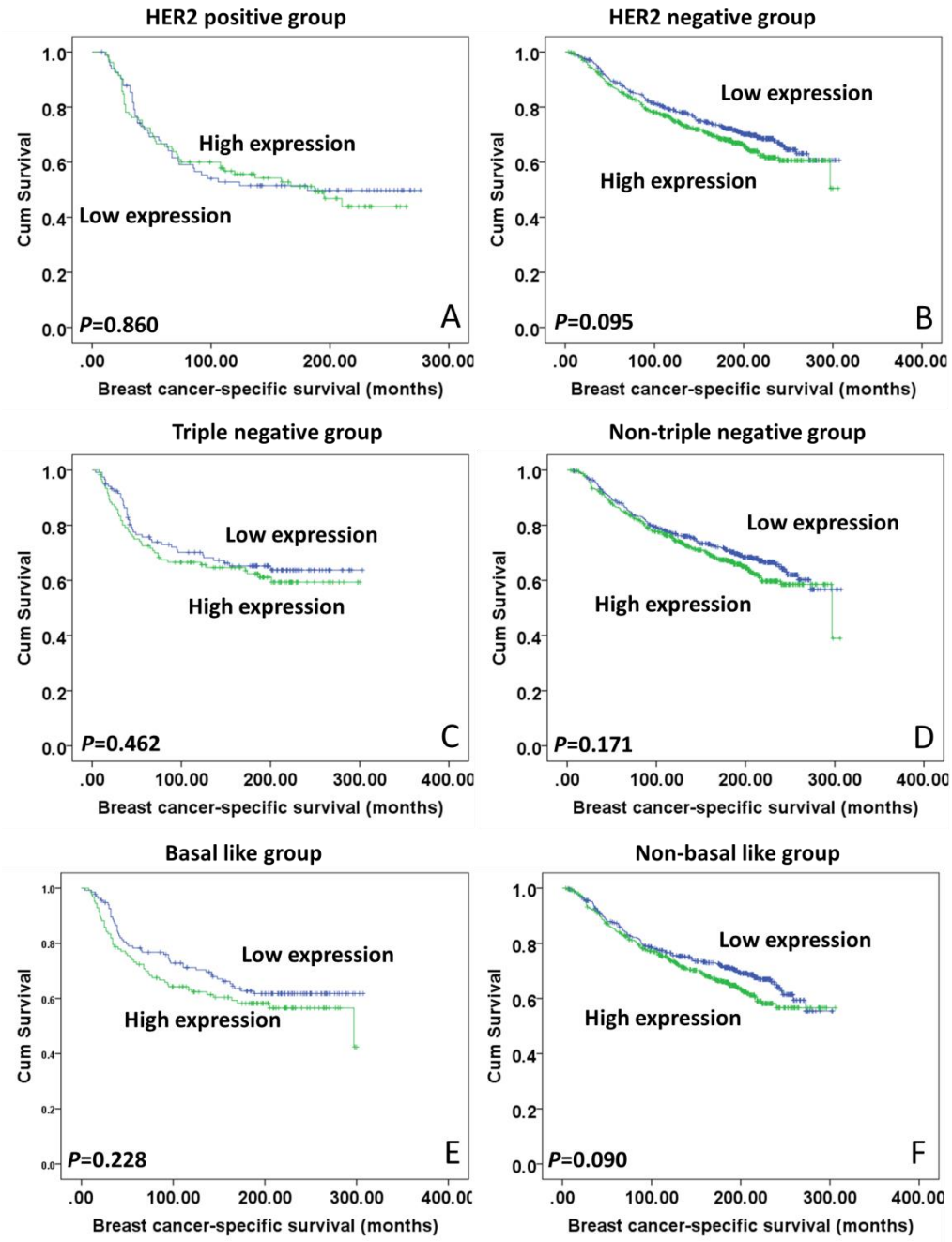


Figure 3.13 Kaplan-Meier survival curves for breast cancer-specific survival based upon caspase-8 expression (subgroups). Significance was determined using the log-rank test. (A) HER2 positive; (B) HER2 negative; (C) triple negative; (D) non-triple negative; (E) basal-like and (F) non-basal like.

To determine whether the combination of caspase-3/-8 and calpain expression had any additional prognostic value, in the total and different breast cancer subtype cohorts, patients were grouped based upon the expression of both proteins (e.g. high caspase/high calpain, low caspase/low calpain, high caspase/low calpain, and low caspase/high calpain). As shown in Figure 3.14,

patients had significantly adverse breast cancer-specific survival if tumours had high caspase-3/high calpain-1 expression (*P*=0.005), high caspase-3/high calpain-2 expression (*P*=0.049), high caspase-3/low calpastatin expression (*P*=0.02), or high caspase-8/low calpain-1 expression (*P*=0.02).

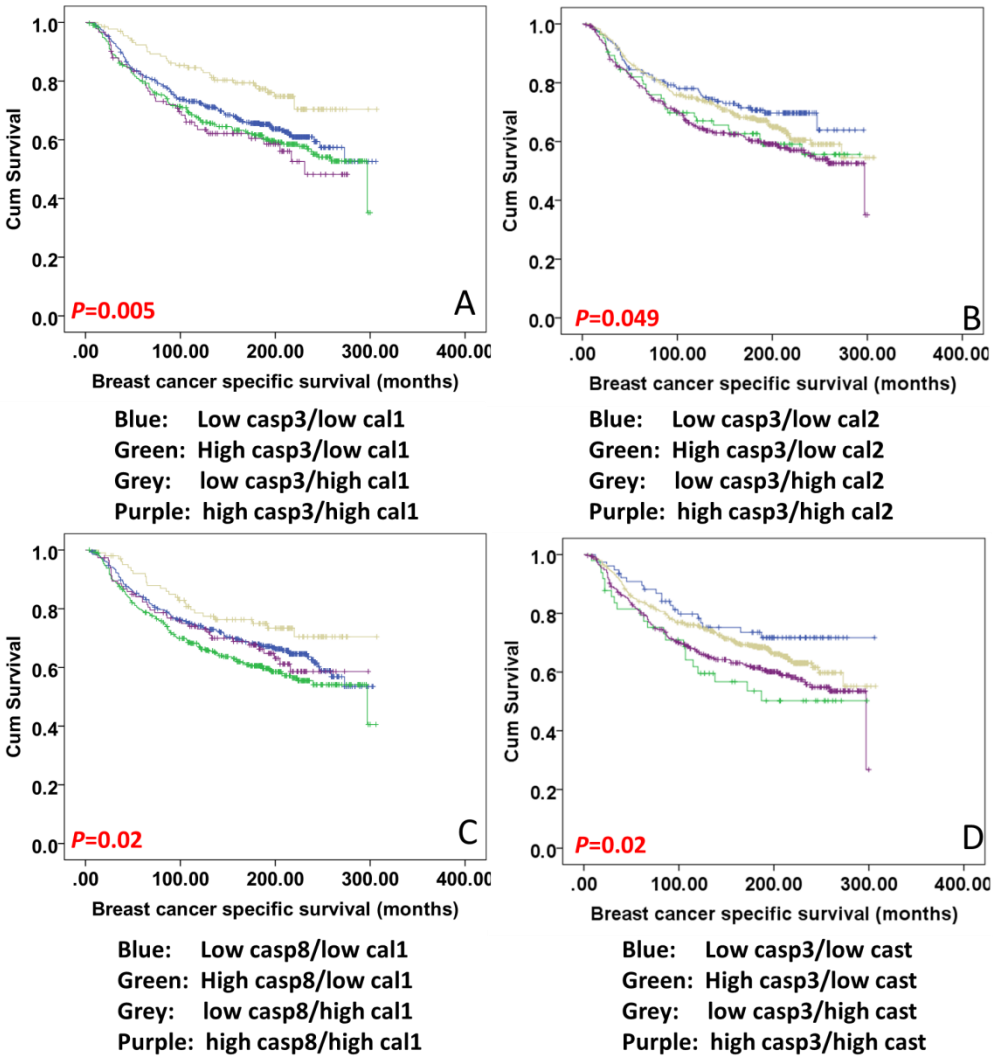


Figure 3.14 Kaplan-Meier survival curves for breast cancer-specific survival based upon combinational protein expression (total patient cohort). (A) Combinational caspase-3 and calpain-1 expression; (B) combinational caspase-3 and calpain-2 expression; (C) combinational caspase-8 and calpain-1 expression; (D) combinational caspase-3 and calpastatin expression.

As shown in Figure 3.14-A, B&C, patients with tumours that had high caspase-3/high calpains and high caspase-3/low calpastatin expression, both had adverse prognosis compared with other categories. This may suggest that high caspase-3 level and high calpain level are associated with a poor prognosis in breast cancer patients; however, it is difficult to confirm the caspase-3 and calpain activities by using such antibodies in the immunohistochemistry based study. Our group previously applied the antibody against calpain specific cleaved α-fodrin to detect calpain activity on frozen tissue sections; however it was difficult to generate consistent and reproducible results. Still, it would be of interest to determine caspase-3 and calpain activity simultaneously, by possibly applying the antibodies against the proteolytic products of calpain and caspases. In comparison, patients that had tumours with high caspase-8/low calpain-1 expression also had worse prognosis compared with other categories (Figure 3.14-D), which might suggest the differential interaction between caspase-3 and -8 with calpain-1. In subgroup analyses, significant associations were found between caspase-3/calpain-1 and breast cancer-specific survival in the basal-like subgroup (P=0.034), as in the same pattern showing in the total group, high caspase-3/high calpain-1 was associated with poor prognosis. No other significant association was found between any combinational protein expressions and breast cancer-specific survival (data shown in Appendix A - Table A 1).

3.4 Discussion

In order to examine the role of calpain in breast cancer cell apoptosis, calpeptin induced apoptosis was assessed using flow cytometry. As described in Chapter 2, 100 μM calpeptin treatment resulted in approximately 20 to 30% inhibition of calpain activity in MDA-MB-231 cells; however, such inhibition did not have significant effect on the induction of apoptosis following 30 minutes or 6 hours treatment. A significant change was observed at 24 hours, indicated by the increased percentage of apoptotic cells (2.17-fold of control). In MCF-7 cells, calpain inhibition had no effect on apoptosis at any time point; suggesting that calpeptin induced apoptosis may depend on capase-3 activity. However, in SKBR3 cells approximately 20% of calpain activity inhibition with calpeptin (30 minutes), the same level as in MDA-MB-231 cells, showed no significant effect on the induction of apoptosis. Significant induction of apoptosis was detected at 24 hours; with approximately 45-54% calpain activity inhibition.

When comparing the three cell lines, under the same conditions, MDA-MB-231 cells appear to be more sensitive to calpeptin induced apoptosis than SKBR3 cells, as revealed by the 2.17-fold increase in the percentage of apoptotic cells vs 1.76-fold increase. Although MDA-MB-231 cells were resistant to calpeptin inhibition (the maximal inhibition was approximately 20% at 24 hours), such inhibition was sufficient to induce apoptosis. Notably, the percentage of apoptotic cells increased with longer exposure time in MDA-MB-231 cells, the percentage was less than 2%; while greater apoptosis was observed in MCF-7 and SKBR3 cells, ranging from 13.1% to 21.1%. Calpain expression may also be associated with calpeptin induced apoptosis in these three breast cancer cell lines. As discussed in Chapter 2, Western blotting results showed that MCF-7 cells expressed a markedly low level of calpain-2, whilst MDA-MB-231 cells expressed a slightly low level of calpain-1. In this context, calpeptin did not induce apoptosis in MCF-7 cells, which might be linked with low calpain-2 expression (or them being caspase-3 deficient as mentioned above); and MDA-MB-231 cells were more sensitive to apoptosis in response to calpain inhibition,

corresponding to the low calpain-1 expression.

Calpains have positive and negative roles in apoptotic pathways, as a result, the inhibition of calpain, by either pharmacological inhibitors or shRNA; have generated some opposing results. In one study, treatment with calpeptin for 96 hours induced apoptosis in both MDA-MB-231 and MCF-7 cells (Mataga et al., 2012), which is consistent with our finding in MDA-MB-231 cells. The effect of calpeptin on MCF-7 cells observed in our study contradicts such data. Differences in experimental conditions including the concentration and exposure time applied (i.e. 100 μM for 24 hours vs ~36 μM for 96 hours) may contribute to such conflicting results; or it may be the inherent characteristic of the cells themselves. The conflicting results may also suggest that during a short exposure time, calpeptin induces caspase-3 dependent apoptosis; but when exposure time is prolonged calpeptin triggers apoptosis through alternative caspase-3 independent pathways. In a study of Burkitt lymphoma cells, μ-calpain, caspase-1, -3, -6 and -7 were activated during radiation-induced apoptosis, within which μ-calpain was activated 15 minutes after radiation and caspase-3 activation occurring after 2 hours. Calpain was, from such work, thought to play a role in the early stages of the apoptotic pathway and act upstream of caspase-3 (Waterhouse et al., 1998). Another study also indicated that a caspase-9-dependent but caspase-3-independent apoptotic pathway existed in oridonin (a herbal medicine, *rabdosia rubescens*)-induced apoptosis in MCF-7 cells (Cui et al., 2007).

In the current study, a significant increase in the percentage of dead/necrotic cells 6 hours after calpeptin treatment was detected in SKBR3 cells (1.14 fold of control), whilst 24-hour treatment induced apoptosis but had no effect on dead/necrosis. The reason for this result is unclear; it may be partially due to the fact that – the relatively longer cell cycle duration of SKBR3 cells (doubling time is approximately 72hours), which may be a reflection of a late apoptotic response. The inhibition of calpain activity by calpain inhibitor I (ALLN)

protected Ca^{2+}-induced caspase-independent necrosis in neurons; meanwhile the calpain inhibitor preserved caspase-3-like protease activity in neurons after glutamate receptor activation (Lankiewicz et al., 2000). The selection of the apoptotic or necrotic pathway is complicated, and depends on cell type, stimulus and cellular circumstance (reviewed in (Festjens et al., 2006)). The current study requires further investigation to provide more detailed explanations.

The effects of calpain activity on apoptosis related protein expression was assessed by use of apoptosis arrays. Results showed that inhibition of calpain activity, by calpeptin, could downregulate HSP60 expression in MCF-7 cells (luminal), but not in MDA-MB-231 cells (triple-negative).

The lack of induction of apoptosis in MCF-7 cells, following calpeptin treatment (indicated by Annexin V-FITC assay), suggests that such calpain-mediated HSP60 expression might not be involved in apoptotic responses. HSP60 belongs to the heat shock protein family, a family of molecular chaperones, whose major functions are to ensure the correct assembly of other polypeptides. HSPs are involved in the synthesis and folding of proteins and are expressed in cells in response to stress (Hendrick and Hartl, 1993). It has been reported that HSP60 can promote pro-caspase-3 activation in the presence of ATP, in both Hela and Jurkat cells (Xanthoudakis et al., 1999). In the current array results, high pro-caspase-3 expression and nearly undetectable caspase-3, along with low HSP60 expression, has also been shown in MDA-MB-231 cells. This was similar with Xanthoudakis et al. study, as pro-caspase-3 was unable to be activated in the absence of HSP60.

In terms of the interaction between calpains and HSP60, a recent study investigated synovial membrane samples from the knees of six osteoarthritic patients, demonstrating that after irradiation mitochondrial HSP60 expression was significantly increased, while calpain-4 expression was significantly

decreased in at least three patients' samples (Barabás et al., 2014). Studies have shown that 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT) triggers mitochondrial apoptosis and DNA fragmentation in leukaemia cells, accompanied with increased HSP60 expression; however, inhibition of m-calpain did not prevent DNA fragmentation (Grebeňová et al., 2003). Although limited studies have investigated the correlation between calpains and HSP60; with numerous lines of evidence suggesting that HSP60 plays an important role in breast cancer. The report from two-dimensional electrophoresis (2-DE) analyses suggested that HSP60 expression was higher in breast carcinomas than in fibroadenoma (Franzén et al., 1997). Another study used serological proteomics-based approach (SERPA) to identify proteins in the sera from infiltrating ductal breast cancer patients (n=40); among which antigens against HSP60 has been found to have a significant higher frequency compared with other proteins (Hamrita et al., 2008). In 80 breast cancer patient samples, a significant association was found between HSP60 expression and lymph node metastasis by using immunohistochemistry (Li et al., 2006).

The data generated from the apoptosis arrays is limited, and should be viewed with caution, particularly as they were conducted only once and the fact that HSP60 results were not confirmed in independent Western blotting experiments. It also remains to be seen if results would be different if calpain activity was blocked completely by other pharmacological agents and/or an shRNA mediated gene knockdown approach; this could be carried out through future work/investigations by others continuing with these studies.

Calpains and caspases are both proteolytic cysteine proteases, with research showing that the calpain family is involved in apoptotic pathways through interactions with caspases. In the current study, by using the same breast cancer patient cohort (as in Chapter 2), we assessed the correlation between caspase-3/-8, calpain family and calpastatin protein expression; as well as the prognostic significance of caspase-3/-8 protein expression in early invasive breast cancer

patients. With respect to the correlation between calpains and caspases, significant correlations were observed although unlikely to be biologically relevant. It has shown that cleaved caspase-3 can translocate into the nucleus, where it interacts with other nuclear substrates to mediate apoptosis (Krajewska et al., 1997). In the current study, the antibody epitope is in a part of the full length protein that is also retained in the cleaved caspase-3. In the Western blotting no cleaved caspase-3 was detected; however these cells were healthy and viable prior to lysis. Anti-caspase-3 antibody staining showed predominantly cytoplasmic localisation from immunohistochemistry, very few nuclear staining was observed in some cases, which may suggest a low level of apoptotic response in the tissue samples. It would be interesting to further assess the pro- and total caspase-3 separately in breast cancer.

Kaplan-Meier survival data suggests that caspase-3 expression is significantly associated with adverse breast cancer-specific survival, and remains as an independent factor from multivariate analysis. Although no significant association was found between caspase-8 and breast cancer-specific survival, a similar trend was observed (patients with high caspase-8 expression was associated with adverse survival). The current study did not compare caspase protein expression in breast tumours against that in normal breast tissues. Several studies agree that caspase-3 (both active and inactive forms) overexpression and enhanced apoptotic activity are found in breast carcinomas compared with controls. However none show significant associations between protein expression and patient prognosis (Vakkala et al., 1999, Blazquez et al., 2006) (as introduced in section 3.1.3). One study suggested that 75% (35 out of 46) of breast tumours lacked the caspase-3 transcript and protein expression, and the remaining samples had substantially decreased expression (Devarajan et al., 2002). This finding was in contrast with previous reports by Nakopoulou et al., in which high caspase-3 protein expression presented in 75.2% (103 out of 137) of invasive breast tumour samples, in comparison with non-neoplastic breast tissues (Nakopoulou et al., 2001). Results from the current study are in

agreement with the Nakopoulou et al. work, suggesting high caspase-3 expression is associated with worse prognosis in breast cancer patients. Many factors might be responsible for such discrepancies, for example the difference in demographics including patient ages and clinicopathological criteria, treatment strategies and tissue fixation procedures, etc.

As mentioned previously, an increased caspase-3 expression level does not directly correspond to high levels of caspase activity or apoptotic potential in breast cancer cells, as programed cell death in cancer cells is also determined and modulated by a number of other apoptosis related factors (e.g. Bcl-2 family, cytochrome-c and death receptor family, etc). Another study demonstrated that overexpression of caspase-3 splicing variant (caspase-3s) in caspase-3 proficient cells can effectively inhibit drug-induced apoptosis. The study found that locally advanced breast cancer patients with a high ratio of caspase3/caspase-3s expression was associated with a better response to neoadjuvant cyclophosphamide-based chemotherapy; consistently, increased caspase-3s/caspase-3 expression had no response to chemotherapy in the same cohort (Végran et al., 2006). Therefore, an impaired caspase-3/caspase-3s balance may present a possible mechanism for dysregulation of apoptosis in cancer cells, and subsequently result in resistance to treatment and poor prognosis in breast cancer patients.

Interestingly, the prognostic significance of caspase-3 appears to be linked with the balance between active and inactive forms of the enzyme, and differs across various tumour types. Our present study showed that high caspase-3 expression was associated with recurrence. Another study found that a high level of caspase-3 (active and inactive forms) is significantly associated with worse event free survival in AML patients. Whereas a high level of cleaved caspase-3 appeared to be linked with improved survival (marginally significant) (Estrov et al., 1998). In non-small cell lung cancer, inactive caspase-3 expression was associated with worse five year survival (Takata et al., 2001); while in

another study caspase-3 (active and inactive forms) expression was associated with improved prognosis (young Yoo et al., 2004). In colon cancer patients who received 5-fluoracil adjuvant chemotherapy, increased caspase-3 activity was associated with a decreased recurrence rate and better disease-free survival (de Oca et al., 2008).

In the current study casapase-3 expression was associated with HER2 status. HER2 belongs to the human epidermal growth factor receptor family, its dimerization follows ligand binding and results in the activation of tyrosine kinases, facilitating a phosphorylation cascade to trigger downstream signalling pathways, including PI3K/AKT pathways, which can further regulate cell survival (Rimawi et al., 2015). AKT has been found to directly phosphorylate caspase-9 at serine 196 and reduce its activity, resulting in inhibition of apoptosis (Cardone et al., 1998). An immunohistochemical based study described a significant correlation between phosphorylated AKT (S473) and caspase-9 (S196) expression in gastric and colorectal cancer (Sangawa et al., 2014). It may be possible that caspase-3 may interact with other substrates and potentially regulate apoptosis through the HER2 signalling pathway.

The prognostic significance of caspase-3 expression in different breast cancer molecular subtypes was also examined. The Kaplan-Meier subgroup analyses demonstrated a significant association in receptor positive (ER, PR or HER2) and non-basal like subgroups. In the case of caspase-8, no significant association was observed in any individual subgroup. The results further emphasised the prognostic importance of caspase-3 in receptor positive patients.

The combinational analysis of caspase-3/-8 and calpain expression was further evaluated in the total patient cohort and individual subgroups. Results indicated that high caspase-3/high calpain-1 and high caspase-3/high calpain-2 was significantly associated with adverse breast cancer-specific survival; and high caspase-3/low calpastatin expression was associated with adverse breast

cancer-specific survival. High caspase-3/high calpain-1 was also associated with survival in the basal-like subgroup, which further emphasised the prognostic potential of combinational caspase-3/calpain-1, especially in the basal-like subtype. In comparison, patients that had tumours with high caspase-8/low calpain-1 appeared to have worse prognosis in the total patient cohort. It would be difficult to make direct comparison between the functional role of caspase-3 and -8 during apoptosis, as caspase-8 is an initiator caspase in the apoptosis cascade, while caspase-3 acts as a downstream effector caspase requiring activation by other initiator caspases (as described in section 3.1.2). Equally, although calpain protein expression was assessed by immunohistochemistry this does not equal to protein activity.

3.5 Summary

1. The calpain inhibitor calpeptin exerted differential effects, in terms of apoptosis, in different breast cancer molecular subtypes.
2. High caspase-3, but not caspase-8, expression was associated with worse prognosis in breast cancer patients, especially in patients without basal-like diseases & with receptor (ER, PR or HER2) positive tumours.
3. High calpain-1/high caspase-3 protein expression was associated with a worse prognosis in breast cancer patients, especially in the basal-like subtype.

Chapter 4 Calpain system effects on trastuzumab response

4.1 Introduction and aims

4.1.1 Aims

The aims and objectives of the current chapter were to:

1. Validate the role of calpain-1 protein as a biomarker of trastuzumab response in HER2+ breast cancer patients.
2. Investigate the biological mechanisms by which the calpain family may regulate the therapeutic response to trastuzumab in parental, acquired and inherent trastuzumab-resistant HER2+ breast cancer cells (i.e. proliferation, clonogenic survival, cell cycle and signal transduction).

4.1.2 Mechanism of resistance to trastuzumab

As introduced in Chapter 1, several potential mechanisms of action of trastuzumab have been proposed, for example, induction of antibody-dependent cell-mediated cytotoxicity (ADCC), reduction in cleavage of the extracellular domain of HER2 (i.e. inhibiting generation of active truncated form of HER2), inhibition of HER2 dimerization with other EGFR family members, inhibition of downstream signalling (e.g. PI3K/AKT pathway and IGF-IR pathway), leading to inhibition of tumour angiogenesis and DNA damage repair, induction of cell cycle arrest and apoptosis (reviewed in (Spector and Blackwell, 2009, Arteaga et al., 2012)). Some of these mechanisms are further described below.

Resistance to HER2 targeted agents might arise through impaired trastuzumab binding to extracellular domain of HER2. The truncated active form of HER2, p95HER2, lacks the trastuzumab-binding extracellular domain, but sensitivity to tyrosine kinase inhibitors (i.e. lapatinib) is maintained as they bind intracellularly. p95HER2 expression has, albeit in a small study, been associated with trastuzumab resistance, only 11.1% (1 out of 9) of patients with their tumour expressing p95HER2 had a response to trastuzumab; whereas 51.4% (19 out of 37) of patients with tumours expressing full-length HER2 achieved a complete or partial response (Scaltriti et al., 2007). Recent immunohistochemical studies have suggested limited prognostic/predictive

significance with respect to p95 expression – there was no correlation between p95 expression and overall survival, progression free survival or trastuzumab response (Kocar et al., 2014). Resistance has also been shown to relate with impaired expression of HER2 and other EGFR family members. Increased, and loss of, HER2 expression has been shown to be involved in acquired and inherent resistance to trastuzumab, respectively. Increased HER2 expression has been observed in acquired trastuzumab-resistant SKBR3 cells; whilst relatively lower expression of HER2 protein was found in inherent trastuzumab-resistant JIMT-1 cells (Vazquez-Martin et al., 2007, Köninki et al., 2010). *In vivo* xenograft models have shown increased expression and phosphorylation of HER1 in trastuzumab resistant tumours, suggesting overexpression of HER1 is also a possible mechanism of acquired resistance to trastuzumab (Ritter et al., 2007).

The interaction between HER2 and other receptor tyrosine kinases (RTKs) can protect cells against trastuzumab. In acquired trastuzumab-resistant HER2+ breast cancer cells, insulin-like growth factor-I receptor (IGF-IR) has been shown to form heterodimers with HER2 (Nahta et al., 2005) or heterotrimers with HER2 and HER3 (Huang et al., 2010b), which maintained HER2 signalling even in the presence of trastuzumab. Another receptor tyrosine kinase Met also contributes to trastuzumab resistance. Met inhibition has been shown to sensitise cells to trastuzumab-induced growth inhibitory effects, and conversely ligand-induced Met activation inhibited trastuzumab-induced growth inhibition by abolishing cell cycle inhibitor p27 induction (Shattuck et al., 2008). Increased expression of membrane-bound glycoprotein mucin-4 (MUC4) was detected in inherent trastuzumab-resistant HER2+ breast cancer cells (JIMT-1), masking HER2 from binding to trastuzumab; and its level was inversely associated with trastuzumab binding ability. While downregulation of MUC4 can sensitise the cell response to trastuzumab (Nagy et al., 2005). The level of transforming growth factor α (TGFα) expression was dramatically increased after trastuzumab treatment, compared to pre-treatment level; expression of TGFα

has been shown to correlate with trastuzumab-induced HER2 internalisation (Valabrega et al., 2005). In this context, cross talk between HER2 and TGFα might contribute to resistance to trastuzumab.

Activation of alternative compensatory downstream signalling pathways is thought to be another major mechanism of trastuzumab resistance. Studies have revealed that activation of the PI3K/AKT pathway, through loss of PTEN expression or by mutations in oncogenic PIK3CA, contributes to resistance to trastuzumab (Nagata et al., 2004, Berns et al., 2007, O'Brien et al., 2010). Nagata et al. have also demonstrated that patients with PTEN deficient tumours exhibit worse response to trastuzumab than patients with PTEN expressing tumours; and PI3K inhibitors restored trastuzumab sensitivity in PTEN-deficient breast cancer cells. Src, a nonreceptor tyrosine kinase, has been found to be highly activated in acquired and inherent trastuzumab-resistant breast cancer cells. Src activation was significantly associated with lower clinical response rate and poorer overall survival in patients treated with trastuzumab and the use of a Src inhibitor can sensitise cells to trastuzumab *in vitro* and *in vivo* (Zhang et al., 2011).

A dysregulated cell cycle has also been linked to trastuzumab resistance. It has been shown that long term exposure of HER2+ breast cancer cells to trastuzumab leads to downregulation of cyclin-dependent kinase inhibitor $p27^{kip1}$ and amplification of cyclin E. The subsequent increased cyclin-dependent kinase 2 (CDK2) activities and lack of antibody induced G1 cell cycle arrest are responsible for the unresponsiveness to trastuzumab (Nahta et al., 2004, Scaltriti et al., 2011). In addition to the mechanisms described above, there are other possible mechanisms that contribute to trastuzumab resistance, Chung et al. has summarised some examples of the proposed mechanisms, as listed in Table 4.1.

Table 4.1 Proposed mechanisms of trastuzumab resistance

Methods of resistance	Protein	Mechanism
Epitope masking	MUC4	Disrupts binding of trastuzumab to HER2
	CD44	Binding to hyaluronan activates PI3K/AKT
	p95HER2 (truncated form of HER2)	Dimerizes with HER3 to interfere with receptor-antibody binding
Upregulation of HER2 downstream signals	PTEN mutation	Loss of PTEN allows PI3K activation
	PI3K mutation	Disrupts binding of trastuzumab with HER1/HER3/PI3K allowing AKT activation
	PDK1	AKT signalling
Overexpression of HER2 family members	HER1,HER3,eGFR, HER2	Increased MAPK, PI3K signalling
Overexpression of HER ligands	TGFα, EGF, hereguilin	Activation of PI3K
Alteration of ADCC	Impaired immune-mediated mechanism	Interferes with trastuzumab mediated ADCC

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; EGF, epidermal growth factor; eGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; MUC4, mucin-4; PDK1, phosphoinotiside-1; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; PTEN, phosphatase and tensin homolog; TGF, transforming growth factor. Reproduced from (Chung et al., 2013), Clinical breast cancer, 13, 223-232; Copyright© 2013 by Elsevier with permission conveyed through Copyright Clearance Centre Inc.

4.1.3 Calpain and HER2 signalling

A number of experimental studies have demonstrated the involvement of calpain in HER2-related signalling pathways. Calpain-2 has been shown to be activated by EGFR through ERK/MAPK pathway-mediated phosphorylation (Glading et al., 2000, Glading et al., 2004). HER2 can induce NF-κB activation through PI3K/AKT pathway, which is regulated by PTEN and calpain proteolysis of IκBα (Pianetti et al., 2001). Calpain also negatively modulates PI3K/AKT signalling through regulating expression of phospho-AKT and ribosomal S6. It

has also been shown that calpain-4 interacts with the PI3K protein in response to serum starvation, whilst calpain-1 and -2 can cleave PI3K protein, resulting in reduced PI3K kinase activity (Beltran et al., 2011). Knockdown of CAPN2 in mouse mammary carcinoma cells was associated with impaired PI3K/AKT signalling (Ho et al., 2012). Studies have shown that HER2 can inhibit calpain-1 activity through upregulation of calpastatin, resulting in increased protein kinase-6, Src and FAK expression (Ai et al., 2013). Protein/mRNA expression of calpain-6 from, an atypical member of calpain family (as introduced previously), has been shown to be increased by the PI3K/AKT pathway; and calpain-6 has been shown to promote cancer proliferation and survival, as well as inducing cell cycle dysregulation and apoptosis resistance through regulating the PI3K/AKT pathway (Liu et al., 2011).

In terms of trastuzumab response, Kulkarni et al. have investigated the role of calpain in HER2+ breast cancer cells, with differing results. They suggest that calpain is activated following trastuzumab treatment and directly cleaves the HER2 protein and that inhibition of calpain activity causes downregulation of PTEN and upregulation of AKT phosphorylation. Knockdown of calpain-4 by shRNA inhibited HER2+ breast cancer cell survival following trastuzumab treatment, thus enhancing cell response to trastuzumab. However, their data also showed that, compared with trastuzumab treatment alone, inhibition of calpain activity by pharmacological inhibitors (i.e. calpeptin or MDL28170) increased survival of trastuzumab-sensitive cells, but decreased survival of acquired trastuzumab-resistant cells. Expression of catalytically inactive mutant calpain-1 also encouraged survival of trastuzumab sensitive cells after treatment (Kulkarni et al., 2010, Kulkarni et al., 2012). A previous study from our group suggested that high calpain-1 expression is associated with poor relapse-free survival in HER2+ breast cancer patients that received trastuzumab treatment following chemotherapy (Storr et al., 2011c). A recent study, using rat *in vivo* models, has demonstrated a further role for increased calpain-2 expression in the protective effect of dexrazoxane on the cardiotoxicity induced

by trastuzumab and chemotherapy combination therapy (Zhang et al., 2015).

Taken together, the crosstalk between calpain and HER2 related pathways, as well as results from our previous studies, suggest that calpain may be involved in trastuzumab response in HER2+ breast cancer cells. The underlying mechanism remains, however, to be fully understood; with little known regarding the role of calpain in inherent versus acquired trastuzumab-resistant cells.

4.2 Materials and methods

4.2.1 Clinical samples

The first patient cohort consisted of 194 primary breast cancer patients treated at Nottingham University Hospitals between July 2004 and February 2012. The second cohort consisted of 87 primary breast cancer patients treated at Newcastle General Hospital and the Freeman Hospital, Newcastle between September 2002 and April 2013.

The 194 patients treated at Nottingham University Hospitals had a median follow up time of 49 months (range 6-100 months). The majority of patients (95%) were treated with trastuzumab following surgery and adjuvant therapy, the remainder received neo-adjuvant therapy followed by surgery and trastuzumab; according to local guidelines. Trastuzumab treatment was given on a 3 weekly regimen for 52 weeks. Most (42%) patients received trastuzumab following six cycles of 3 weekly FEC combination therapies (fluorouracil, epirubicin and cyclophosphamide). A further group (37%) received a regime of three cycles of FEC followed by three cycles of taxane (FEC-T), to which trastuzumab was frequently added from the second cycle of taxane onwards. The remainder received a selection of trial treatments using taxane alone (2%), in or combination (4%), as a result of poor response following neoadjuvant treatment (3%). A few (5%) cases received neo-adjuvant therapy only. Adjuvant hormonal therapy was received by 56% of patients (109/194), and the majority of patients (79%) also received adjuvant radiotherapy (153/194). Relapse-free survival (RFS) was defined as the date of surgery to relapse (includes local relapse and regional relapse) with a median relapse-free survival of 45 months. HER2 expression was determined by immunohistochemistry with fluorescence in situ hybridization (FISH) used as the arbiter in cases with an immunohistochemistry score of 2. During the follow up period, there were 26 patients that suffered a relapse and 18 patients who died from their disease.

The Newcastle cohort of 87 patients had a median follow up time of 52 months

(range 10-117 months). All received 3 weekly trastuzumab treatments for 52 weeks following adjuvant therapy. All received AC (doxorubicin and cyclophosphamide) or FEC, alone or in combination with a taxane. The majority received adjuvant hormonal (51/87) and adjuvant radiotherapy (69/87). RFS was defined using the same criteria as the Nottingham cohort; with a median relapse-free survival of 48 months. HER2 expression was determined by immunohistochemistry with chromogenic in situ hybridization (CISH) if the patient had an immunohistochemistry score of 2. During the follow-up period, 12 patients suffered a relapse and 6 died from their disease.

This study was reported in accordance with REMARK criteria (McShane et al., 2005). The Nottingham cohort was approved by Nottingham Research Ethics Committee 2 under the project "Development of a molecular genetic classification of breast cancer R&D "(No. 03HI01 REC Ref.C202313)". The Newcastle cohort was approved by Newcastle & North Tyneside 2 Research Ethics Committee. The clinicopathologic variables of two patient cohorts are shown in Table 4.2.

Table 4.2 Clinicopathologic variables of the two patient cohorts

Variables	Nottingham cohort	Newcastle cohort
	***N* (%)**	***N* (%)**
Age at surgery (mean± SD) , years	52±10	54±10
≤40 years	25(13.0)	10(11.5)
>40 years	168(87.0)	77(88.5)
ND	1	0
Tumour size (mm)		
≤20mm	120(62.5)	30(34.9)
>20mm	72(37.5)	56(65.1)
ND	2	1
Nottingham Prognostic Index		
Good	15(7.7)	11(12.8)
Moderate	103(53.1)	37(43)
Poor	50(25.8)	38(44.2)
ND	26(13.4)	1
Grade classification		
I	4(2.1)	1(1.2)
II	56(28.9)	24(27.9)
III	134(69.1)	61(70.9)
ND	0	1
Lymph node status		
Positive	124(63.9)	47(54.7)
Negative	70(36.1)	39(45.3)
ND	0	1
ER status		
Positive	112(57.7)	53(61.0)
Negative	82(42.3)	31(35.6)
ND	0	3(3.4)
PR status		
Positive	61(31.4)	30(34.5)
Negative	96(49.5)	43(49.4)
ND	37(19.1)	14(16.1)

Abbreviations: ER, oestrogen receptor; PR, progestogen receptor; ND, not determined. Nottingham cohort: ER and PR status was assessed from tumour biopsy material by H-score (0-300), a value above 10 was positive; Newcastle cohort: ER and PR status in biopsy material were assessed by Quick Score (0-8), a value above 3 was positive.

4.2.2 Tissue microarray construction and immunohistochemistry

TMA's were constructed and sections cut by Paul Mosley & Chris Nolan for the Nottingham cohort and by Nicola Cresti & Despina Thomaido for the Newcastle cohort. For construction of the Nottingham TMA a single 0.6 mm tissue core

was used for each patient with the core being taken from a representative area as assessed by a specialist breast cancer histopathologist. For the Newcastle cohort TMA, two 1 mm tissue cores, from the central tumour areas, were used with areas being identified by a specialist breast cancer histopathologist. Freshly cut 4 µm thick TMA sections were used and immunohistochemistry performed as described previously (Storr et al., 2011c). The primary antibodies were: murine anti-calpain-1 (1:2500, clone P-6), rabbit anti-calpain-2 (1:2500, polyclonal AB1625), murine anti-calpain small subunit (1:100,000, clone P-1) and murine anti-calpastatin (1:40,000, clone PI-11) (all from Merk Millipore, USA). The specificity of the antibodies was confirmed by Western blotting previously (Storr et al., 2011c), and as also shown in section 2.3.2. Staining was achieved using a Novolink Polymer Detection System following the steps described in section 2.2.12.

Staining was assessed at X20 magnification following high-resolution scanning (Nanozoomer Digital Pathology Scanner, Hamamatsu Photonics). Protein expression was assessed by immunohistochemistry H-scoring (range 0-300) as described in Chapter 2 (Storr et al., 2011c). 50% of slides for the Nottingham cohort and 40% of slides for the Newcastle cohort were assessed by a second independent scorer, blind to clinicopathological and survival endpoints. In the Nottingham cohort, the single measure intraclass correlation coefficients (ICC) between the scorers were 0.876, 0.779, 0.711 and 0.930 for calpain-1,-2,-4 and calpastatin, respectively (conducted by Dr. Sarah Storr). In the Newcastle cohort, ICCs were 0.784 and 0.809 for calpain-1 and -2, respectively (conducted by Dr. Narmeen Ahmad). The stratification cut point for immunohistochemical scores was determined in a non-biased fashion using X-tile software, as described in Chapter 2 (Camp et al., 2004).

4.2.3 Cell lines and culture

The SKBR3 cell line was obtained from the American Type Culture Collection (ATCC); the JIMT-1 cell line (inherent trastuzumab-resistant) was obtained from

Leibniz-Institut DSMZ - German Collection of Microorganisms Cell Cultures (Tanner et al., 2004). The acquired trastuzumab-resistant SKBR3 (SKBR3/TR) cell line was obtained from Dr. Anthony Kong's laboratory (Oxford University, UK) and maintained in 40 µg/mL (270 nM) trastuzumab in complete culture medium and the medium was refreshed every 3-4 days. Subculture, cell line verifications and mycoplasma screening were described in section 2.2.1 and 2.2.2.

4.2.4 Chemicals and drug preparation

Trastuzumab (Herceptin®; kindly provided by Nottingham University Hospitals NHS Trust Pharmacy) was predissolved in Bacteriostatic Water for Injection (BWFI) as a stock solution at 20.83 mg/mL and stored at 4°C for long term use. Calpeptin was prepared and stored as described in section 2.2.4. Hydroxyurea (Hu) (Sigma, UK) was dissolved in sterile water as a stock solution at 250 mM and stored at -20°C. Dilution and the negative controls were prepared the same as described in section 2.2.4.

4.2.5 T-boc calpain activity assay

The methodology was described in section 2.2.6.1.

4.2.6 Cell growth curves

Cell growth curves were conducted to determine the combined effects of calpeptin and trastuzumab on proliferation of HER2+ breast cancer cells. For single agent experiments, $3X10^5$ SKBR3, $2X10^5$ SKBR3/TR and $2X10^5$ JIMT-1 cells were seeded into each well of 6-well tissue culture plates. After 24 hours, the cells were treated, in triplicate, with calpeptin at different concentrations (0, 25, 50 and 100 µM) or with trastuzumab at different concentrations (0, 20, 40, 80, and 160 µg/mL) (equivalent to 0, 135, 270, 540 and 1080 nM) and incubated for the indicated time points for individual experiments. For combined treatment experiments, $3X10^5$ SKBR3, $3X10^5$ SKBR3/TR and $3X10^5$ JIMT-1 cells were seeded into each well of the 6-well tissue culture plates. After 24 hours, the cells were pretreated with 50 µM of calpeptin for 2 hours prior to applying

trastuzumab. Culture medium was then replaced with 50 µM calpeptin alone, 40 µg/mL (270 nM) trastuzumab alone, or 50 µM calpeptin plus 40 µg/mL (270 nM) trastuzumab, and incubated for 72 hours (for SKBR3 and SKBR3/TR cells) or 48 hours (for JIMT-1 cells). Different incubation times were chosen for SKBR3 and JIMT-1 cells, because of the different doubling time for the two cell lines (approximately 72 and approximately 40 hours, respectively). Following drug exposure, cells were trypsinised and the total cell number was counted by haemocytometer. Growth inhibition was expressed as the percentage of total cells in drug treatment groups compared with untreated controls. Data represent the average ± standard deviation (SD) of two independent experiments.

4.2.7 Clonogenic survival assays

Cells were treated with calpeptin alone, trastuzumab alone or calpeptin plus trastuzumab as described in section 4.2.6. Following drug exposure cells were trypsinised, counted (total cells counts rather than only viable) and plated into T25 tissue culture flasks then incubated for appropriate time periods (3.5 weeks for SKBR3 cell line) at 37°C, 5% CO_2 to allow colony formation. Following incubation flasks were washed once with PBS and colonies fixed with 50% methanol in 0.9% saline solution for 15 minutes, and then with methanol for another 15 minutes, followed by staining with 0.5% crystal violet. Colonies containing more than 50 cells were scored as survivors. Cell lines were initially assessed for their ability to form colonies and the plating efficiency (PE) for each cell line was used to determine optimal plating densities for drug treatment experiments. The PE was generated from untreated controls and calculated as:

$$\frac{Number\ of\ colonies\ formed}{Number\ of\ colonies\ plated} \times 100\%$$

The surviving fraction (SF) of cells following treatment was calculated as:

$$\frac{Number\ of\ colonies\ formed\ after\ treatment}{Number\ of\ colonies\ plated \times PE}$$

(Woolston and Martin, 2011). All individual experiments were performed in triplicate. Data represent the average SF ± standard deviation (SD) of at least

two independent experiments.

4.2.8 Cell cycle analysis

The effect of combined calpeptin and trastuzumab treatment on regulation of progression through the cell cycle was investigated via propidium iodide (PI) staining and analyses by flow cytometry. The proportion of cells in each cell cycle phase (G1 vs S vs G2/M) was reflected by the cellular DNA content, determined by PI intensity and represented by the histograms produced from cell cycle analysis software (Crissman and Hirons, 1994). Treatment with 2 mM hydroxyurea (Sigma, UK) for 48 hours was used as a positive control (Pfeiffer and Tolmach, 1967, Ashihara and Baserga, 1979). 48-hour incubation time was used for SKBR3 cells, as the 72 hours treatment had been shown to cause too much cell death. Cells were treated with calpeptin alone, trastuzumab alone or calpeptin plus trastuzumab (as described in section 4.2.6). After treatment cells were harvested by trypsinisation and at least 1 x 10^6 cells pelleted by centrifugation at 170 g for 5 minutes at room temperature, followed by washing once with ice-cold PBS. The supernatant was removed and the cell pellet resuspended and fixed in 5 mL of ice cold 70% ethanol in dH_2O, then immediately stored at 4^oC for at least 24 hours. Samples could be stored in this way for up to 7 days prior to analysis. When samples were analysed, the cells were pelleted again by centrifugation at 170 g for 5 minutes. The ethanol was removed and each sample resuspended in a mixture of 400 µL of PBS and 100 µL of 1 mg/mL ribonuclease A (RNase A) (Sigma, UK) in dH_2O. As PI can also bind to double-stranded RNA, it is essential to digest RNA with RNase for optimal results (Burrell, 1993). 500 µL of cell suspension was transferred into a plastic 12X75 mm test tube, and incubated with 5 µL of PI (Sigma, UK) solution at 0.25 mg/mL in dH_2O. The mixture was incubated in the dark at 37^oC for 10 minutes. Samples were analysed using a Beckman Coulter FC500 MCL flow cytometer system (Beckman Coulter, USA) by reading with FL3 (620 nm for PI). Gating was set to include single cells and exclude doublets and debris. Analysis of FL3-A histograms was conducted using FlowJo 7.6.5 software (Tree Star). The

percentage of cells in each phase of cell cycle (G0/G1, S and G2/M) was plotted. Data represent the average ± standard deviation (SD) of three independent experiments.

4.2.9 Human phosphokinase array

The combined effect of calpeptin and trastuzumab on signal transduction was detected and analysed using a human phosphokinase array (R&D Systems, USA). SKBR3 cells were treated with 5 µg/mL (33.75 nM) trastuzumab alone for 1 hour, or 100 µM calpeptin for 30 minutes followed by 5 µg/mL trastuzumab for an extra 1 hour. Cells were collected and following the steps described in section 2.2.9.

4.2.10 Western Blotting

The validation of phosphokinase array results was conducted, via Western blotting, using phospho-AKT (S473) antibody (R&D Systems, USA, 1:2500). The knockdown of calpastatin and calpain-4 in SKBR3 cells was assessed using antibodies against calpastatin and calpain-4. The antibody details were shown in section 2.2.10. The methodology for Western blotting followed the same steps as described in section 2.2.10.

4.2.11 Plasmid transfection using shRNA

Knockdown of calpastatin and calpain-4 was performed by transfection of short hairpin RNA into the SKBR3 cell line. The shRNA plasmid were delivered using FuGENE® HD transfection reagent (Promega, USA) and diluted in Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, USA). Four different HuSH 29mer shRNA constructs (OriGene Technologies, USA) against calpain-4 or calpastatin; along with respective scrambled non-effective shRNA's and negative control shRNA's (with vector only) in pRS plasmid were purchased (only scrambled non-effective shRNA was provided in the calpain-4 kit). To reach approximately 80% confluency at 24 hours post-plating, prior to conducting transfection, $8X10^6$ SKBR3 cells were seeded into 6-well tissue culture plates.

After 24 hours the cell medium was replaced with 500 µL of antibiotic free medium (each well). Then 5 µL of 0.1 µg/uL plasmid was added into a mixture of 25 µL Opti-MEM I Reduced Serum Medium and 3 µL FuGENE® HD transfection reagent, and incubated at room temperature for 10 minutes. The mixture was added dropwise to the corresponding wells. 48 hours post-transfection, drug selection was initiated by applying 1 µg/mL of puromycin (Thermo Fisher Scientific, USA) and incubated for 2-4 weeks (depending on the confluency); the selective medium was refreshed every 2-3 days. The transfection efficiency was determined by comparison with protein levels from the transfected cells, scrambled control, negative control and wild-type in Western blotting. The construct with highest transfection efficiency was selected and the cells being able to form distinct single colony was expanded and stored.

4.2.12 Statistical analysis

The same as described in section 2.2.13.

4.3 Results

4.3.1 Immunohistochemistry validation studies

4.3.1.1 Staining patterns

Calpain-1,-2,-4 and calpastatin protein expression showed cytoplasmic staining with some heterogeneity between adjacent tumour cells, varying from weak to intense staining. No nuclear staining was observed for the four proteins assessed in the current study. For calpain-4, some ductal carcinoma in situ cells showed staining. For calpastatin, some peri-nuclear staining was observed and low grade (grade 1) tumours showed negative staining in some cases (40-50% of slides were assessed by Dr. Sarah Storr and Dr. Narmeen Ahmad, with descriptive pathology provided by pathologist Dr. Mohammed A Aleskandarany, with all being assessed by the current author). Two tumour cores were scored from the Newcastle cohort, and an average H-score used for analysis. A few TMA cores were not scored due to insufficient tumour or the core being missing. Medians and X-tile generated cut points were as follows: in the Nottingham cohort, calpain-1 had a median score of 210±31 and an H-score cut point of 235 with 20.2% (36 out of 178) of cases having a high score; calpain-2 had a median score of 175±27 and an H-score cut point of 180 with 25.7% (46 out of 179) of cases having a high score; calpain-4 had a median score of 190±16 and an H-score cut point of 195 with 21.7% (39 out of 180) of cases having a high score; and calpastatin had a median score of 195±21 and an H-score cut point of 195 with 38.4% (68 out of 177) of cases having a high score. In the Newcastle cohort, calpain-1 had a median score of 185±42 and an H-score cut point of 190 with 24.4% (21 out of 86) of cases having a high score; and calpain-2 had a median score of 178±42 and an H-score cut point of 170 with 65.1% (54 out of 83) of cases having a high score. As cut off points were not uniform between the two cohorts, reproducibility studies using additional cohorts to standardise cut points are warranted, to increase applicability for clinical practice. In addition to analysing cohorts separately, data for calpain-1 from each cohort were combined, to yield a large combined dataset that was analysed as an individual cohort.

The previous study (n=93 patients from Nottingham) had a median score of 155±44; with an H-score cut point of 215 which resulted in 10 (10.8%) cases with high expression of calpain-1 (Storr et al., 2011c). This original cohort differed from the current cohort as two cores (both from intratumoural areas) were available from each patient; the expression of calpain-1 in these areas was significantly correlated with each other, however to a relatively low extent (r=0.39, *P*<0.001). Average calpain-1 expression from the previous study was compared with calpain-1 expression in matched cases from the current Nottingham cohort and demonstrated a significant correlation between scores (r=0.61, *P*<0.001). Of the 194 patients, 72 had been previously analysed. Ideally duplicate or triplicate cores from representative tumour areas would have been analysed. In the Newcastle cohort, a significant correlation also was observed between the two cores (r=0.641, *P*<0.001). The patient cohorts from Nottingham and Newcastle were similar in most clinicopathological criteria; except the Newcastle cohort had more patients with tumours larger than 20 mm (65.1% vs 37.5%, as shown in Table 4.2). Typical staining patterns of calpain-1, -2, -4 and calpastatin expression in the two patient cohorts are shown in Figures 4.1 & 4.2.

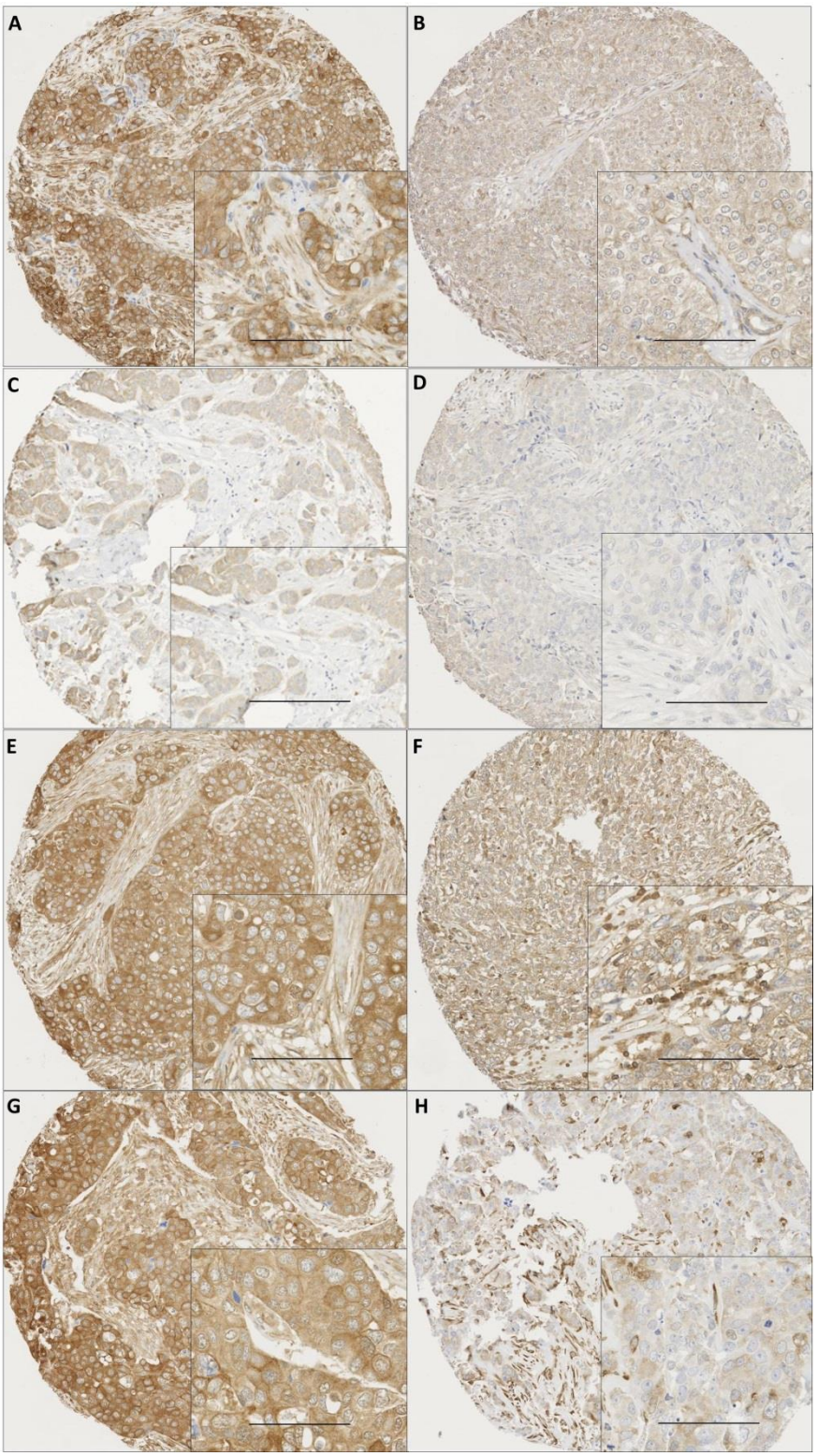


Figure 4.1 Representative photomicrographs of high and low calpain and calpastatin protein expression (Nottingham patient cohort). High (panel A) and low expression (panel B) of calpain-1; high (panel C) and low expression (panel D) of calpain-2; high (panel E) and low expression (panel F) of calpain-4; high (panel G) and low expression (panel H) of calpastatin expression. Photomicrographs were at X10 magnification with X20 magnification inset panel and scale bar representing 100 μm.

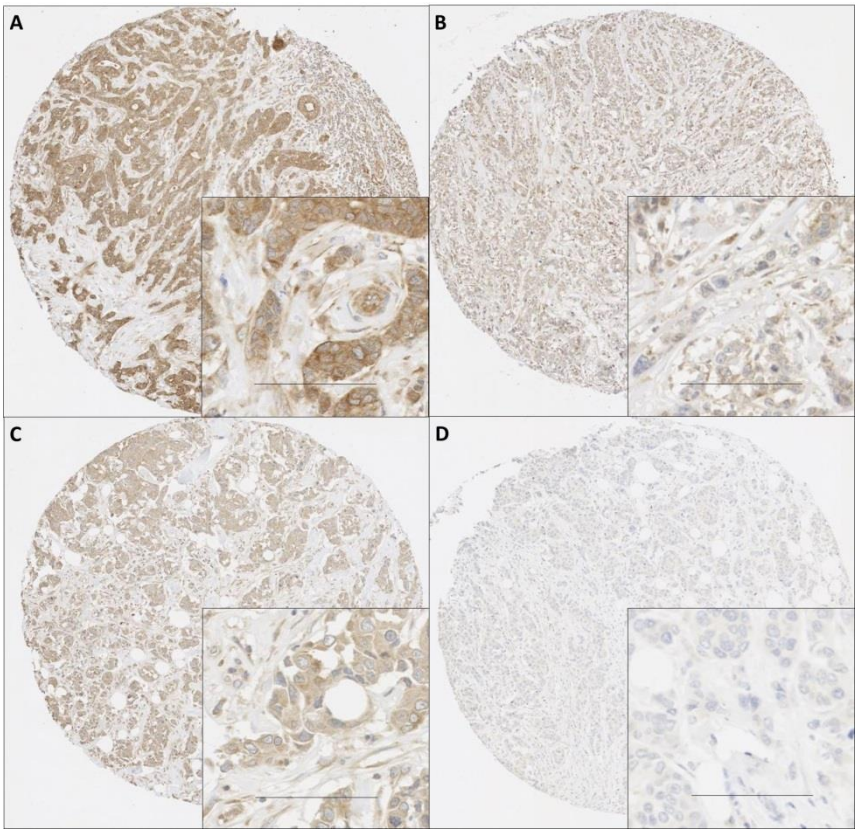


Figure 4.2 Representative photomicrographs of high and low calpain protein expression (Newcastle patient cohort). High (panel A) and low expression (panel B) of calpain-1; high (panel C) and low expression (panel D) of calpain-2. Photomicrographs were at X5 magnification with X20 magnification inset panel and scale bar representing 100 µm.

4.3.1.2 Statistical analysis

Relationship with clinicopathologic variables

Pearson's Chi-squared test was performed to assess for association between protein expression and clinicopathologic criteria, as shown in Table 4.3.

Table 4.3 Associations between calpain family, calpastatin protein expression and various clinicopathologic variables

		Nottingham cohort				Newcastle cohort		
		Calpain-1 (*N* =178)	Calpain-2 (*N* =179)	Calpain-4 (*N* =180)	Calpastatin (*N* =177)		Calpain-1 (*N* =86)	Calpain-2 (*N* =83)
Variables	***N* (%)**	***P* -value**	***P* -value**	***P* -value**	***P* -value**	***N* (%)**	***P* -value**	***P* -value**
Age								
≤40 years	25(13.0)	0.421*	0.294	0.584	0.483	10(11.5)	0.701*	0.308*
>40 years	168(87.0)					77(88.5)		
Tumour size								
≤20mm	120(62.5)	0.264	0.196	0.057	0.276	30(34.9)	0.828	0.66
>20mm	72(37.5)					56(65.1)		
NPI								
Good	15(7.7)	0.537	0.184	0.375	0.560	11(12.8)	0.547	0.148
Moderate	103(53.1)					37(43)		
Poor	50(25.8)					38(44.2)		
ND	26(13.4)					1(1)		
Grade								
I	4(2.1)	**0.001**	**0.026**	**0.032**	0.546	1(1.2)	0.839	**0.015**
II	56(28.9)					24(27.9)		
III	134(69.1)					61(70.9)		
Lymph node status								
Positive	124(63.9)	**0.021**	0.916	**0.047**	0.742	47(54.7)	0.184	0.356
Negative	70(36.1)					39(45.3)		
ER status								
Positive	112(57.7)	**0.041**	0.540	**0.046**	**0.002**	53(61.0)	0.803	0.917
Negative	82(42.3)					31(35.6)		
ND	0					3(3.4)		
PR status								
Positive	61(31.4)	**0.008**	0.685	0.404	**0.014**	30(34.5)	0.441	0.943
Negative	96(49.5)					43(49.4)		
ND	37(19.1)					14(16.1)		
Relapse status								
Not present	168(86.6)	**0.034**	0.834	0.177	0.536	75(86.2)	0.063*	0.100*
Present	26(13.4)					12(13.8)		
Survival status								
Living	176(90.7)	1.000*	1.000*	0.548*	0.965	81(93.1)	**0.029***	0.417*
Dead	18(9.3)					6(6.9)		

Abbreviations: NPI, Nottingham Prognostic Index; ER, oestrogen receptor; PR, progesterone receptor; ND, not determined. Correlation between calpain-1 protein expression and clinicopathologic variables using Pearson's Chi-square test of association (χ^2) or Fisher's exact test if in a 2X2 tables and cell count was less than 5 (indicated by *). Significant *P*-values are indicated by bold font.

In the Nottingham patient cohort, high calpain-1 expression was significantly associated with lower tumour grade, lymph node positivity, and the presence of ER+ and PR+ tumours [(χ^2=14.030; d.f.=2; *P*=0.001), (χ^2=5.342; df=2; *P*=0.021), (χ^2=4.171; d.f.=1; *P*=0.041) and (χ^2=7.127; d.f.=1; *P*=0.008), respectively]. High calpain-2 expression was significantly associated with lower tumour grade (χ^2=7.326; d.f.=2; *P*=0.026). High calpain-4 expression was significantly associated with lower tumour grade, lymph node positivity and the absence of

ER positive tumours [(χ^2=6.901; d.f.=2; *P*=0.032), (χ^2=3.959; d.f.=1; *P*=0.047) and (χ^2=3.977; d.f.=1; *P*=0.046), respectively]. Low calpastatin expression was significantly associated with the presence of ER+ and PR+ tumours [(χ^2=9.398; d.f.=1; *P*=0.002) and (χ^2=6.002; d.f.=1; *P*=0.014), respectively]. In the Newcastle cohort, only high calpain-2 expression was significantly associated with low tumour grade (χ^2=8.432; d.f.=2; *P*=0.015). However the small number of cases (less than 3) available in the low tumour grade subset may limit interpretation.

Pearson's Chi-squared and Fisher's exact tests were also performed to assess association between protein expression with relapse status and survival status (Table 4.3). High calpain-1 expression was significantly associated with relapse status (χ^2=4.486; d.f.=1; *P*=0.034) and overall survival status (χ^2=6.238; d.f.=1; *P*=0.029) in the Nottingham and Newcastle patient cohort, respectively. There were no significant associations observed between calpain-2,-4 or calpastatin with relapse status or overall survival status in either patient cohort.

Relationship with clinical outcomes

Kaplan-Meier analyses showed that calpain-1 expression was significantly associated with relapse-free survival in both Nottingham (*P*=0.01) and Newcastle patient cohorts (*P*=0.019), with high expression associated with poor relapse-free survival (Figure 4.3-A&E), confirming the previous findings from our group (Storr et al., 2011c). No significant association was found between calpain-2, -4 or calpastatin and relapse-free survival in either patient cohort.

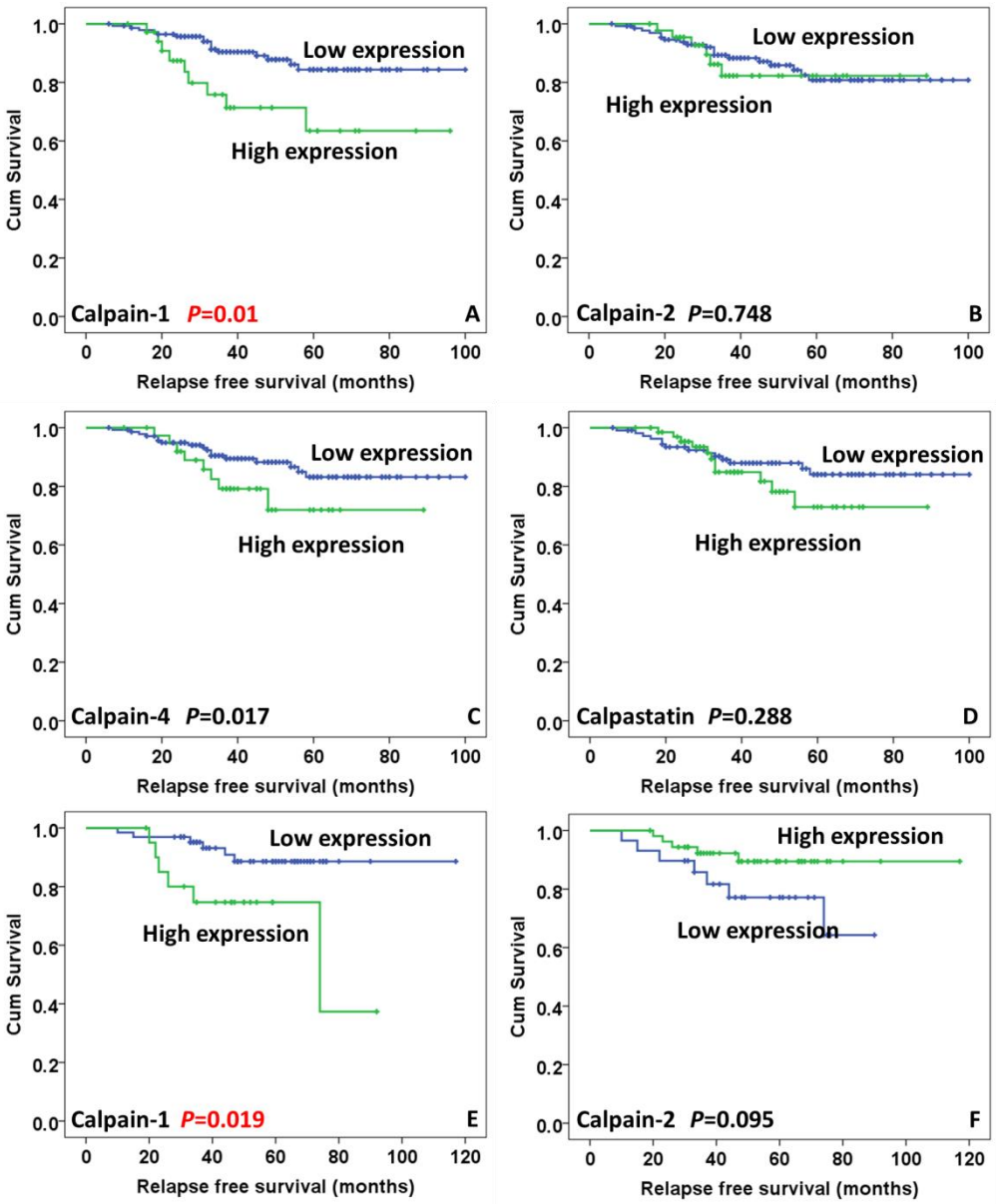


Figure 4.3 Kaplan-Meier survival curves for relapse-free survival based upon calpains and calpastatin protein expression in the Nottingham (A-D) and Newcastle patient cohort (E&F). The curve showing the impact of low (blue line) and high protein expression (green line) with significance determined using the log-rank test. (A) calpain-1 (36 patients with high expression); (B) calpain-2 (46 patients with high expression); (C) calpain-4 (39 patients with high expression); (D) calpastatin (68 patients with high expression); (E) calpain-1 (21 patients with high expression); (F) calpain-2 (54 patients with high expression).

When the extended patient follow-up period was used in the original 72 cases from the previous study, calpain-1 expression was still associated with relapse-free survival (*P*=0.029).

In multivariate analysis for the Nottingham cohort, tumour size, Nottingham

Prognostic Index and lymph node status were included in Cox regression, with individual Kaplan-Meier statistics of *P*=0.012, *P*=0.008 and *P*=0.019, respectively. Unlike the previous study, calpain-1 expression was not an independent factor for the relapse-free survival (hazard radio (HR) =1.594, 95% confidence interval (CI) 0.505–5.028; *P*=0.427). In the Newcastle cohort, tumour size, NPI and PR status were included in the multivariate analysis, with individual Kaplan-Meier statistics of *P*=0.046, *P*=0.041 and *P*=0.033, respectively. Calpain-1 expression remained an independent factor for relapse-free survival (HR=5.169, 95% CI 1.468–18.200; *P*=0.011). The multivariate analysis for both patient cohorts is shown in Table 4.4.

Table 4.4 Cox proportional hazards analysis for relapse-free survival

Nottingham cohort	**Sig.**	**Exp(B)**	**95.0% CI for Exp(B)**	
			Lower	**Upper**
Calpain-1 expression	0.427	1.594	0.505	5.028
Tumour size	0.563	1.400	0.447	4.382
Lymph node status	0.914	1.090	0.228	5.202
Nottingham Prognosis Index	0.181	2.572	0.645	10.254
Newcastle cohort				
Calpain-1 expression	**0.011**	5.169	1.468	18.200
Tumour size	0.252	3.974	0.374	42.178
Nottingham Prognostic Index	0.347	2.049	0.460	9.129
Progesterone receptor status	0.057	0.197	0.037	1.052

Abbreviations: Sig., significance; Exp (B), hazard ratio; 95.0% CI, 95% confidence interval.

Analysis of the combined cohort

The Nottingham and Newcastle patient cohorts were combined together to further analyse the prognostic significance of calpain-1 expression for relapse-free survival. A new X-tile cut point was generated for the combined patient cohort (n=281) resulting in 14% (n=37) of cases having a high calpain-1 expression. Kaplan-Meier analysis demonstrated that high calpain-1 expression was significantly associated with poor relapse-free survival in the combined patient cohort (*P*=0.01, Figure 4.4).

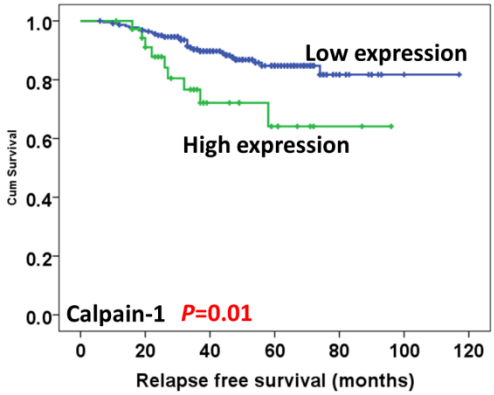


Figure 4.4 Kaplan-Meier survival curves for relapse-free survival based upon calpain-1 protein expressions in the combined patient cohort. The curve showing the impact of low (blue line) and high (green line) protein expression with significance determined using the log-rank test. 37 patients with high expression.

Tumour size, NPI and lymph node status were included in the multivariate analysis, with individual Kaplan-Meier statistics of P=0.003, P=0.004 and P<0.001, respectively. However calpain-1 expression was not an independent factor for relapse-free survival in the combined patient cohort (HR=1.631, CI 0.554–4.798; P=0.375).

4.3.2 Cell growth curves

The immunohistochemical study suggested that calpain-1 has a role in trastuzumab response in HER2+ breast cancer patients; therefore, we thought to investigate if calpain can regulate trastuzumab response in HER2+ breast cancer cells. In order to address this, the effects of the calpain inhibitor calpeptin and trastuzumab on cell proliferation was assessed in HER2+ breast cancer cells, SKBR3 parental cells, acquired trastuzumab-resistant SKBR3 cells and inherent trastuzumab-resistant JIMT-1 cells. Initially the effect of calpeptin on SKBR3/TR and JIMT-1 cells (effects on SKBR3 cells as shown in section 2.3.4.1) were assessed by treating cells with increasing concentrations of calpeptin (0, 25, 50 and 100 µM) at 24, 48 and 72 hours, and the corresponding growth curves plotted. As shown in Figure 4.5-panel A&B, both cell lines showed a decrease in cell proliferation at the highest concentration of calpeptin (100 µM), which was similar to the SKBR3 trastuzumab-sensitive parental cell line (as shown in section 2.3.4.1).

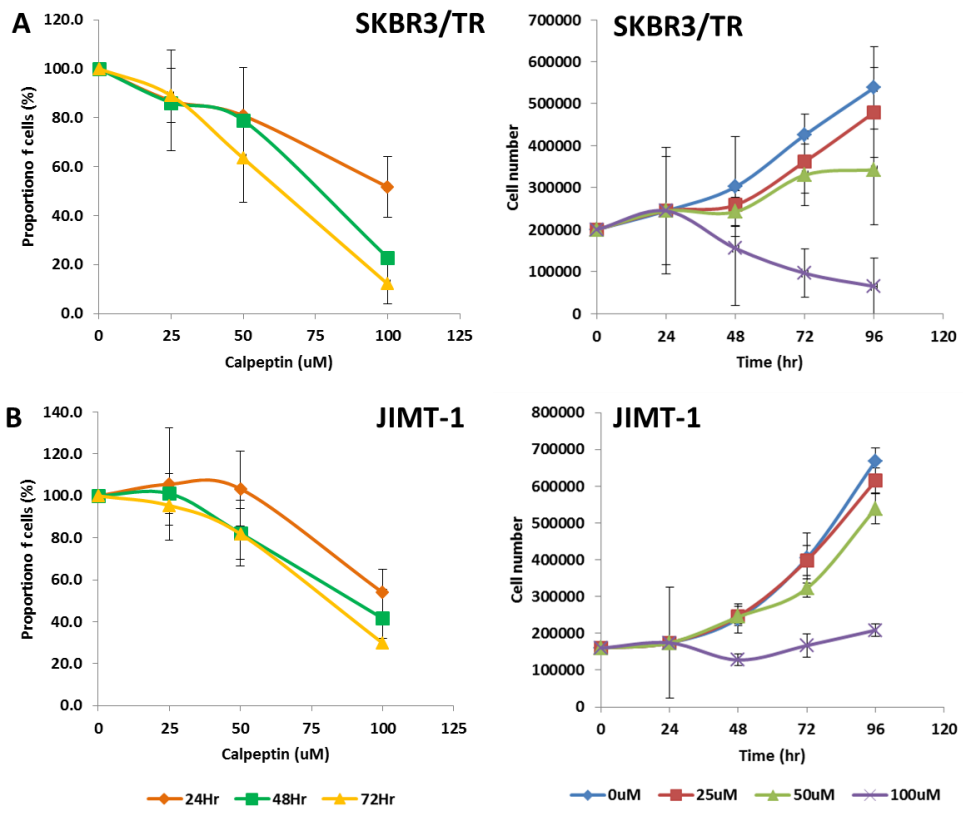


Figure 4.5 Effect of calpeptin on cell proliferation of HER2+ breast cancer cells and the corresponding growth curves. SKBR3 trastuzumab-resistant (panel A) and JIMT-1 (panel B) cells were treated with various doses of calpeptin for 24, 48 and 72 hours. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in triplicate.

Calpeptin caused maximal dose-dependent anti-proliferative effects after 72 hours, resulting in 32%, 12% and 30% decrease in SKBR3, SKBR3/TR and JIMT-1 cells proliferation, respectively. The estimated IC50 values for the three cell lines were very close at 24 hours, showing 50% inhibition at a dose of 100 µM. SKBR3 and JIMT-1 cells showed similar sensitivity to calpeptin, with estimated IC50 values ranging between 80-90 µM after either 48 or 72 hours treatment. SKBR3/TR cells had an estimated IC50 value between 75-80 µM at 48 hours and 60-65 µM at 72 hours. Additionally, growth curves demonstrated that the doubling time for SKBR3/TR cells was shorter than SKBR3 cells (approximately 60 vs 72 hr). Nahta et al. also found a shorter doubling time in SKBR3/TR cells than that in parental SKBR3 cells (24-36 vs 72 hr); but they developed the resistant pools by maintaining the cells in 4 or 8 µg/mL trastuzumab (Nahta et al., 2004) whereas in the present study SKBR3/TR cells were maintained in a

much higher concentration of trastuzumab (40 µg/mL). In comparison, JIMT-1 cells had a doubling time of approximately 40 hours. In all lines, 50 µM calpeptin had minimal (<50%) effects on cell proliferation. A treatment time of 72 hours for SKBR3 and SKBR3/TR cells, and 48 hours for JIMT-1 cells, and so it was decided to use such conditions in subsequent experiments.

The effect of trastuzumab on the cell proliferation of the three HER2+ breast cancer cells was also assessed. As shown in Figure 4.6-A, MDA-MB-231 (non HER2 overexpression/amplification) cells were resistant to trastuzumab treatment, even after 6 days; while trastuzumab showed significant effect on SKBR3 cells, decreased cell proliferation by approximately 60% at a low dose of 5 µg/mL (33.75 nM), which confirmed the specific action of the agent.

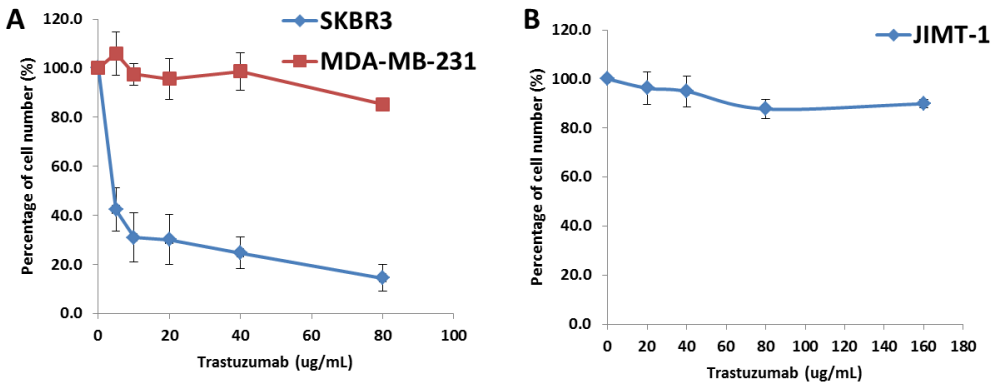


Figure 4.6 Effect of trastuzumab on cell proliferation of HER2+ breast cancer cell lines. (A) Cells were treated for 6 days; (B) Cells were treated for 48 hours. Data represent the average ± standard deviation (SD) of three independent experiments, with each experiment performed in duplicate (A); or two independent experiments, with each experiment performed in triplicate (B).

In contrast, trastuzumab for 48 hours had no effect on JIMT-1 cell proliferation (Figure 4.6-B). The response of SKBR3 cells to trastuzumab appears to vary between different studies. In the current study, the estimated IC50 value for trastuzumab in a 6-day treatment in SKBR3 cells was between 4-4.5 µg/mL. However in other studies the IC50 value of trastuzumab at 6 days was below 1 µg/mL (Junttila et al., 2009, Cheyne et al., 2011); and another group demonstrated a dose of 4 µg/mL trastuzumab can kill SKBR3 cells after 5 days

(Nahta et al., 2004). Several other studies also demonstrated that after treatment of 10 or 100 µg/mL of trastuzumab for 72 hours more than 50% of SKBR3 cells survive (Tseng et al., 2006, Barok et al., 2007, Köninki et al., 2010). Whilst the reports from Nahta et al. and Huang et al., showing that 6.25 or 20 µg/mL of trastuzumab for 72 hours caused a decrease of 50% in cell proliferation (Nahta et al., 2005, Huang et al., 2010b). The reasons for such discrepancies are unclear; it may due to different methods applied in individual studies. A concentration of 40 µg/mL trastuzumab, based on the current results, was used in subsequent studies as SKBR3/TR was maintained under such concentrations, and also because trastuzumab is administrated as 4 mg/kg in the clinical setting, which corresponds to a detectable serum concentration at 40 µg/mL (Tokuda et al., 1999).

4.3.3 Calpain activity across HER2+ breast cancer cells

As described above 50 µM calpeptin was used in the present study, therefore, the inhibitory effects of calpeptin was assessed, at this concentration, in the three HER2+ breast cancer cells: SKBR3, SKBR3/TR (for 72 hours), and JIMT-1 (for 48 hours). As expected, calpeptin caused inhibition of calpain activity in the three cells lines, as shown in Figure 4.7 A-C.

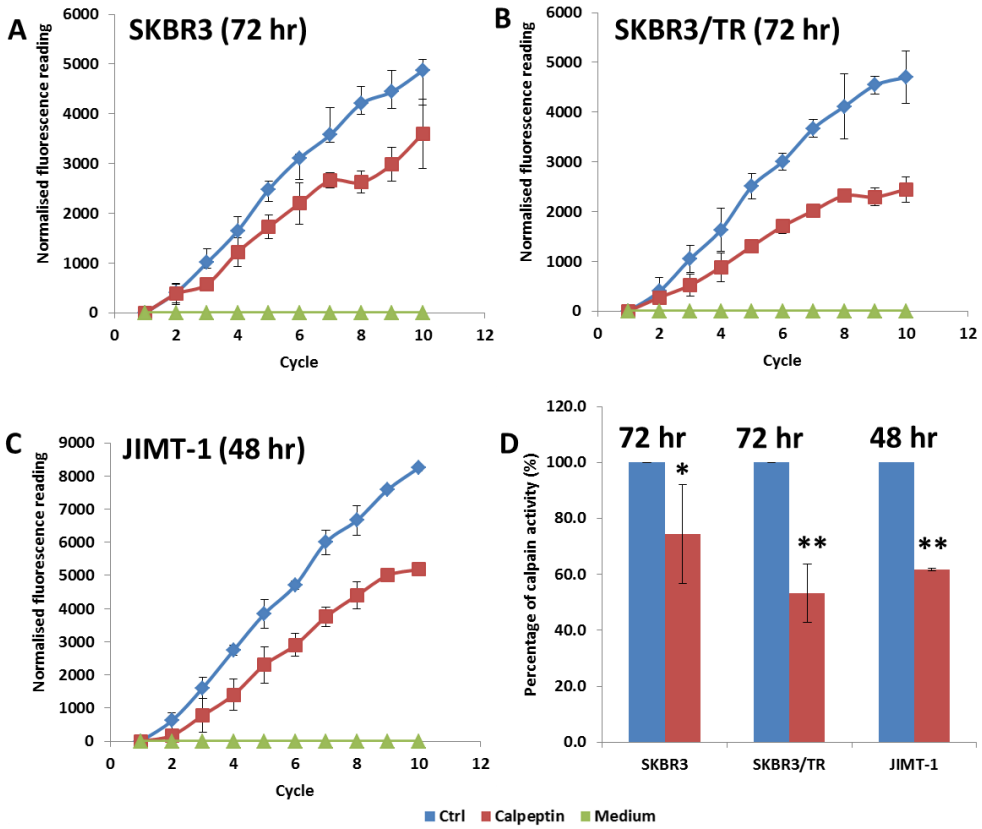


Figure 4.7 The inhibition of calpain activity across different HER2+ breast cancer cells. (A) SKBR3 and (B) SKBR3/TR cells were treated with 50µM calpeptin for 72 hours, and (C) JIMT-1 cells for 48 hours. (D) The fluorescence readings at the 10th cycle. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in duplicate. Statistical significance determined by student T-test compared to control is indicated by asterisk. **P*<0.05, ***P*<0.01 vs single agent.

In SKBR3 and SKBR3/TR cells, calpeptin led to significant inhibition of calpain activity, with 26% and 47% inhibition at the 10th cycle, respectively (*P*<0.05 and *P*<0.01, respectively). Calpeptin also caused significant reduction in calpain activity (38% of control) in JIMT-1 cells (*P*<0.01, Figure 4.7-D). SKBR3 and SKBR3/TR cells had similar level of active calpain activity (fluorescence reading between 4000-5000), whilst JIMT-1 showed slightly higher calpain activity level (fluorescence reading between 8000-9000); suggesting differential endogenous calpain level between trastuzumab-sensitive and inherent trastuzumab-resistant HER2+ cell lines. Therefore, treatment with 50 µM calpeptin led to approximately 30-50% inhibition of calpain activity in the three cell lines, which was applied in the present study.

4.3.4 Combined calpeptin and trastuzumab effects

4.3.4.1 Proliferation

The effects of combined calpeptin and trastuzumab on SKBR3, SKBR3/TR and JIMT-1 cells proliferation were assessed. Cells were pretreated with calpeptin at 50 µM for 2 hours; at which point the cells were supplemented with 50 µM calpeptin and 40 µg/mL trastuzumab, and exposed for 72 hours (SKBR3 cells) or 48 hours (JIMT-1 cells). As shown in Figure 4.8, treatment with calpeptin alone had greater effect on SKBR3 and SKBR/TR cell proliferation (approximately 40% inhibition) than JIMT-1 cells (approximately 20% inhibition).

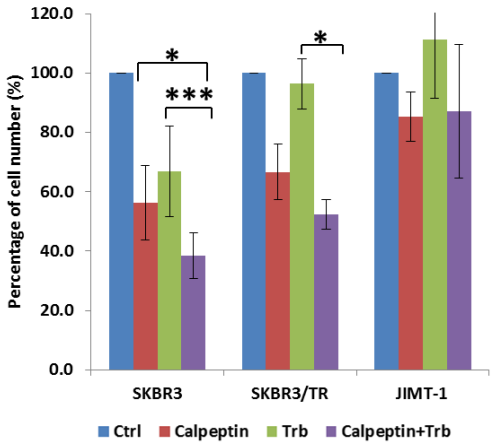


Figure 4.8 Effect of drug combinations (calpeptin and trastuzumab) on cell proliferation. Abbreviations: Ctrl – control, Trb – trastuzumab. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in triplicate. Statistical significance determined by one-way ANOVA test is indicated by asterisk. *P<0.05 and *** P<0.001 vs control.

In SKBR3 cells, the addition of calpeptin to trastuzumab resulted in a 62% decrease in proliferation compared to calpeptin alone and trastuzumab alone (P<0.05 and P<0.001, respectively, Figure 4.8). In SKBR3/TR cells, the addition of calpeptin resulted in greater inhibition of proliferation (51%) over trastuzumab alone (P<0.05, Figure 4.8). Whereas the combined treatment of calpeptin and trastuzumab for 48 hours caused approximately 15% growth inhibition of JIMT-1 cells and did not show significant reduction compared to calpeptin or trastuzumab alone. In all, the combination of calpeptin and trastuzumab produced significantly greater anti-proliferative effect on

trastuzumab-sensitive and acquired trastuzumab-resistant SKBR3 cells, but had no effect on inherent trastuzumab-resistant JIMT-1 cells.

4.3.4.2 Clonogenic survival

Prior to conducting further studies, the plating efficiency (PE) of each cell line, an inherent characteristic of the cells, was determined using the clonogenic survival assay. Briefly, subconfluent cells were plated, at a series of cell densities (i.e. 100, 200, 400 and 800 cells per flask) and incubated for different time periods (depending on the cell type) without disturbance. The cell density and the incubation time that generated the highest plating efficiency and appropriate colony size was applied in the following studies. The cells were incubated for 3 weeks (JIMT-1 cells) and 3.5 week (SKBR3 cells). The plating efficiency of SKBR3 and SKBR3/TR cells reached to 12.4% and 11.4%, respectively. The plating efficiency values of SKBR3 cells generated from our studies was consistent with others', showing plating efficiency values of 9% and 15.0%±6.6 (Ekerljung et al., 2012, Karasawa et al., 2014). In attempts to improve plating efficiency flasks were also pre-coated with 0.2% gelatin, 1% collagen, as well as using flasks from different suppliers (i.e. Falcon™), however there was no significant change or improvements (data not shown). For JIMT-1 cells, in addition to pre-coating with 0.2% gelatin, or 1% collagen, diluted BD Matrigel™ was also used (2.5%, 1.25% and 0.83%; diluted as 1:40, 1:80 and 1:120); colonies were not obtained either with or without coating and so this cell line could not be used in experiments with clonogenic survival as an end-point.

The effect of trastuzumab on clonogenic survival of SKBR3 and SKBR3/TR cells was assessed. As shown in Figure 4.9, 40 and 160 µg/mL (270 and 540 nM) trastuzumab for 72 hours markedly decreased clonogenic survival of SKBR3 cells compared with control, with corresponding survival fractions of 0.3 and 0.2, respectively. And 40 µg/mL (270 nM) of trastuzumab resulted in a survival fraction of 0.8 in SKBR3/TR cells, whilst 160 µg/mL (540 nM) of trastuzumab had no effect on clonogenic survival (data was obtained from one experiment,

as the number of colonies were too high, therefore accurate colony counting was not achieved). It should be noted, when plating at higher density, SKBR3 and SKBR3/TR cells showed increased resistance to trastuzumab. Trastuzumab had greater effect on clonogenic survival of SKBR3 cells, when cells were plated at low density (1500-2500 cells per flask); however it had no effect on clonogenic survival with high seeding density (3000-5000 cells per flask). The results reassured the effect of trastuzumab on clonogenic survival of parental SKBR3 cells and resistance response in SKBR3/TR cells. Others using trastuzumab treatment, with varying concentrations and exposure times, have shown decreased colony formation in SKBR3 cells, ranging between 10-50% (Cheyne et al., 2011, Kulkarni et al., 2012, Ahmad et al., 2013).

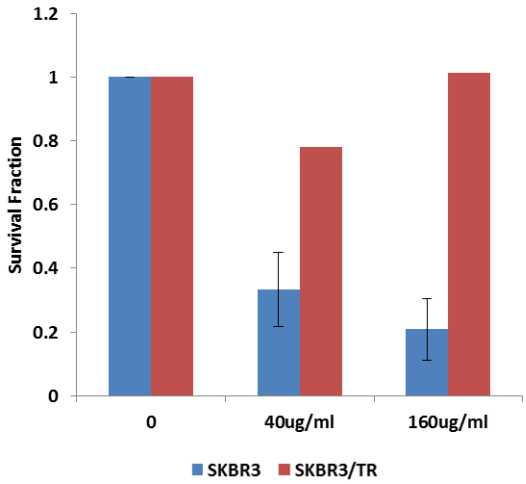


Figure 4.9 Effect of trastuzumab on clonogenic survival. PEs for SKBR3 and SKBR3/TR cells were 4.4% and 7.8%, respectively. Data represent the average ± standard deviation (SD) of two independent experiments (SKBR3), with each experiment performed in triplicate. Data for SKBR3/TR was acquired from one experiment, due to the number of colonies were too high (>250), in which case the accurate colony counting is not achieved.

The combined effect of calpeptin and trastuzumab treatment on colony formation was assessed by clonogenic survival assay. The cells were treated the same as described in section 4.2.6, and plated for clonogenic survival. As shown in Figure 4.10, calpeptin alone inhibited clonogenic survival in SKBR3 cells but not in SKBR3/TR cells; the survival fractions from 50 µM calpeptin treatment alone in SKBR3 and SKBR3/TR cells were 0.4 and 0.9, respectively. Survival fractions for 40 µg/mL (270 nM) trastuzumab alone in SKBR3 and SKBR3/TR cells were 0.5 and 1.0, respectively.

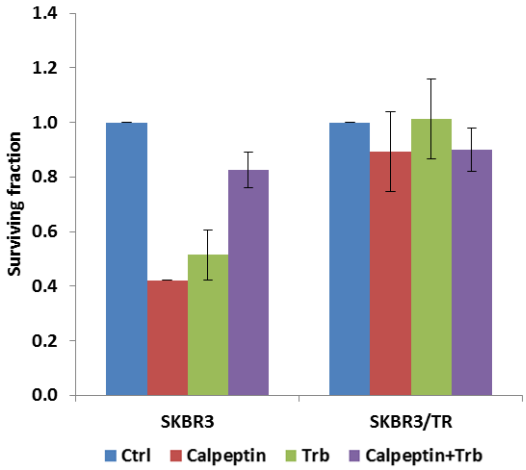


Figure 4.10 Effect of drug combinations (calpeptin and trastuzumab) on cell clonogenic survival. Abbreviations: Ctrl – control, Trb – trastuzumab. PEs for SKBR3 and SKBR3/TR cells were 10.0%and 11.6%, respectively. Data represent the average ± standard deviation (SD) of two independent experiments, with each experiment performed in triplicate. Calpeptin treatment for SKBR3 cells was generated from one experiment.

Treatment with calpeptin and trastuzumab for 72 hours yielding survival fractions of 0.8 and 0.9 for SKBR3 and SKBRR/TR cells, respectively. In SKBR3 cells, combined treatment seemed to increase clonogenic survival compared with single agent alone, but such an effect was not seen in SKBR3/TR cells. Such results contradict those of Kulkarni et al.'s, in which knockdown of calpain-4 inhibited clonogenic survival following trastuzumab treatment (Kulkarni et al., 2012). The different methodologies used to inhibit calpain (pharmacological inhibitors vs knockdown of calpain-4) may be a contributing factor to such a discrepancy. Also in the current study, the plating efficiency of SKBR3 cells was too low to produce representative data by clonogenic survival assays; therefore, it was difficult to generate firm conclusions. The relatively low and unstable plating efficiency of SKBR3 cells was also mentioned in a study by Karasawa et al. (Karasawa et al., 2014).

4.3.4.3 Cell cycle

The combined effects of calpeptin and trastuzumab on cell cycle progression were assessed by PI staining and analysed by flow cytometry. Hydroxyurea (Hu) was applied as the positive control for cell cycle analysis (Peasland et al., 2011, Lee et al., 2014). The concentration and exposure time of Hu used in the current

study was optimised. After the treatment with 2 mM of Hu for 48 hours approximately 50% of cells remained viable in both SKBR3 and JIMT-1 cells; and 2 mM of Hu for 48 hours resulted in 1.50-fold and 1.23-fold increase in G0/G1 population in SKBR3 and JIMT-1 cells, respectively (as shown in Figure 4.11-A&B). 2 mM of Hu for 48 hours was set as positive control for cell cycle analysis in experiments using SKBR3 and JIMT-1 cells.

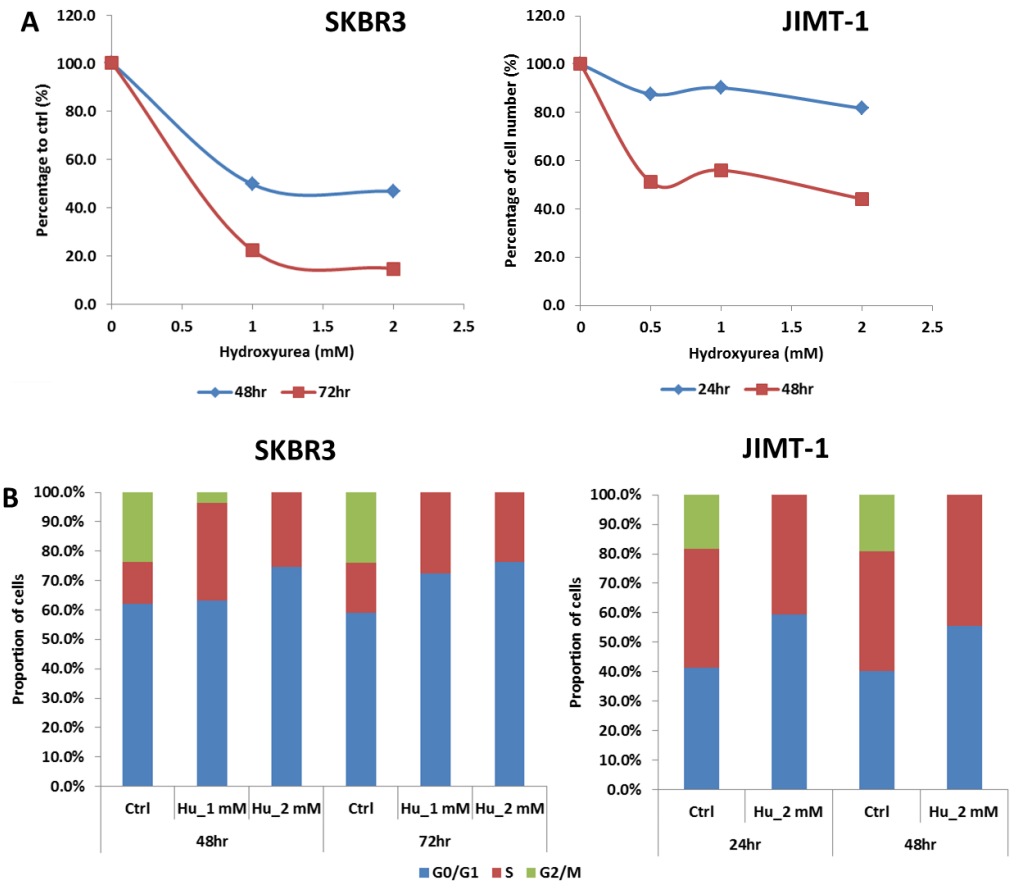


Figure 4.11 Optimisation of hydroxyurea (Hu) as positive control in cell cycle analysis. (A) The cell counting of two cell lines was plotted as percentage of control. (B) The proportion of cells in each phase of cell cycle was plotted. Data was derived from one experiment. Representative histograms are shown in Appendix B-Figure B 1.

To investigate whether calpeptin and trastuzumab treatment affected cell cycle progression, cells were treated as described in section 4.2.6 and cell cycle parameters assessed. As shown in Figure 4.12-A&C, the treatment of Hu, as expected, effectively caused G0/G1 arrest in both SKBR3 and JIMT-1 cells compared to the control (P<0.01 and P<0.05, respectively).

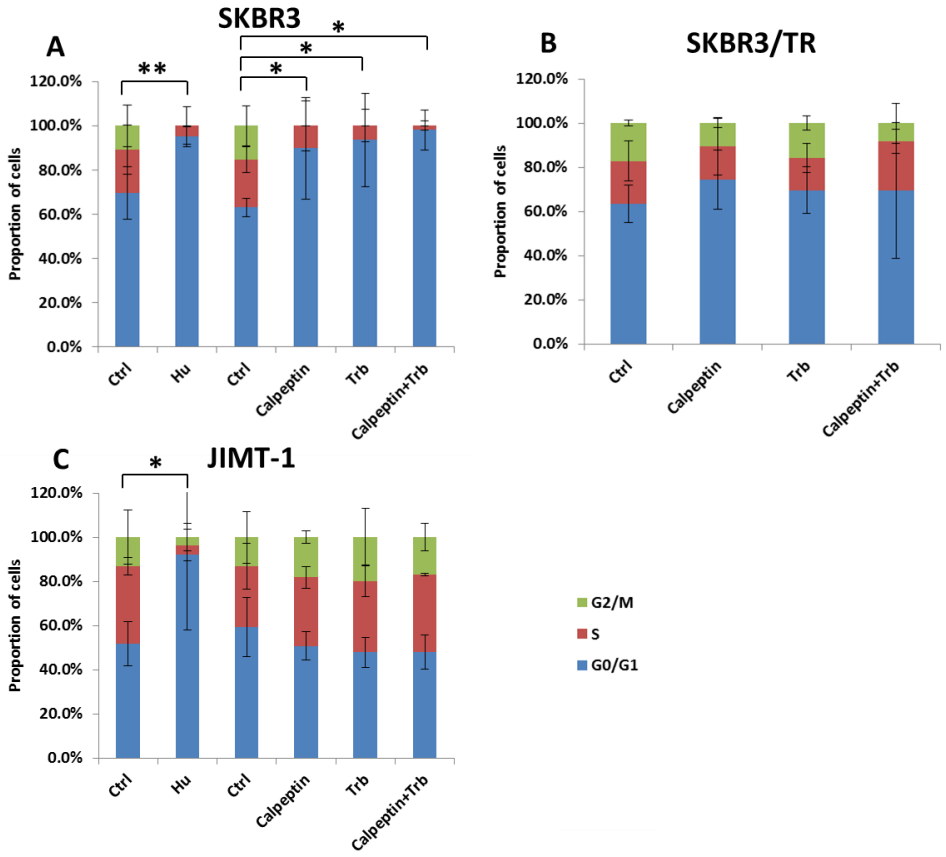


Figure 4.12 Effect of drug combinations (calpeptin and trastuzumab) on cell cycle progression. SKBR3 (A), SKBR3/TR (B) and JIMT-1 (C) cells. 2 mM of Hu for 48 hours were set as positive control. Data represent the average ± standard deviation (SD) of three independent experiments. Statistical significance determined by one-way ANOVA test or student T-test compared to control is indicated by asterisk. **P*<0.05, ***P*<0.01 vs control in respect to the percentage of cells in G0/G1 phase.

Treatment with calpeptin or trastuzumab alone caused significant G0/G1 cell cycle arrest in trastuzumab-sensitive SKBR3 cells, compared with control (all *P*<0.05), but not in the other two trastuzumab-resistant cell lines. As shown in Figure 4.12-A, in SKBR3 cells, compared with control, treatment with 50 µM calpeptin for 72 hours led to accumulation of cells in the G0/G1 phase (1.96-fold vs control), coupled with a reduction of cells in S phase (from 21.5% to 13.7%). Results indicate that calpain inhibition affects the cell cycle distribution of SKBR3 cells; leads to G0/G1 cell cycle arrest, and that 40 µg/mL (270 nM) of trastuzumab for 72 hours induced an increase in the fraction of cells in G0/G1 phase (1.97-fold vs control) with a decrease of cells in S phase (from 21.5% to 8.4%). Consistent with such results, trastuzumab treatment has been

demonstrated to cause cell cycle arrest in several studies, with various concentrations and treatment times (Lewis et al., 1996, Le et al., 2003, Nahta et al., 2004). Combination treatment of calpeptin and trastuzumab also produced a higher proportion of cells in G0/G1 phase, though the difference was not significant when compared with either single agent alone (Figure 4.12-A). The cell cycle progression of SKBR3/TR and JIMT-1 cells was not affected by either single agent alone or combined treatments (Figure 4.12-B&C). Representative histograms from flow cytometry analysis are shown in Appendix B-Figure B 2.

It is interesting to note that the combination of calpeptin and trastuzumab induced a G0/G1 arrest that was more pronounced in SKBR3 cells compared to the two trastuzumab-resistant cell lines, which is consistent with the enhanced anti-proliferative effects in SKBR3 cells, with limited action in SKBR3/TR and JIMT-1 cells.

When control groups are compared across the three cell lines, the proportion of G0/G1 cells was equal in SKBR3 and SKBR3/TR cells (62.3% and 62.2%, respectively), with a slightly lower proportion of G0/G1 cells in JIMT-1 (54.8%). Another study demonstrated similar proportion of G1 cells between parental and trastuzumab-resistant SKBR3 cells, which is concordance with our results (Nahta et al., 2004). However, in their study the proportion of S phase cells in SKBR3/TR cells was 3-fold higher than that in SKBR3 parental cells, which was not observed in the current study (21.5% in SKBR3 and 18.9% in SKBR3/TR). This may due to differences in experimental conditions, as they exposed SKBR3/TR cells to 4 µg/mL trastuzumab, while in the current study trastuzumab resistance was developed by exposing the cells to 40 µg/mL (270 nM) trastuzumab.

4.3.4.4 Signalling pathways

The effect of calpeptin and trastuzumab on phosphorylation of 43 human phosphokinases and 2 total proteins was assessed using phosphokinase arrays. SKBR3 cells were treated with 5 µg/mL (33.75 nM) trastuzumab alone for 1 hour;

alternatively the cells were pretreated with 100 µM calpeptin for 30 minutes prior to adding trastuzumab. As shown in Figure 4.13-A&D (indicated by arrows) results show that AKT1/2/3 phosphorylation was reduced by the trastuzumab treatment, which is consistent with another study showing a rapid reduction in phospho-AKT after 1 hour treatment of trastuzumab at 2 µg/mL (Nagata et al., 2004).

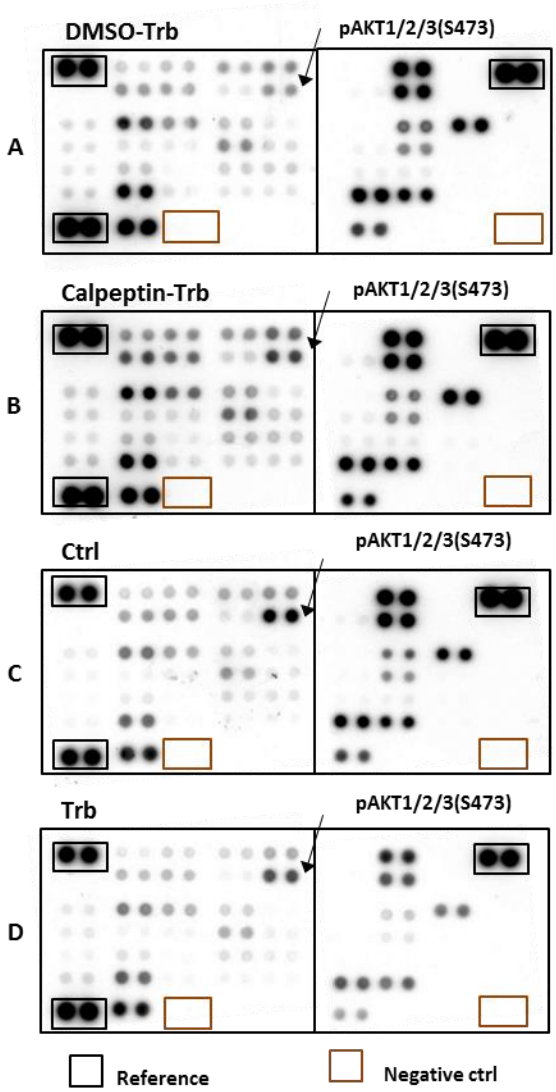


Figure 4.13 Effect of calpeptin and trastuzumab on phosphorylation of 23 human phosphokinases in HER2+ breast cancer cells. Abbreviations: ctrl, control; Trb, trastuzumab.(A) Trastuzumab alone; (B) calpeptin followed by trastuzumab; (C) control; (D) trastuzumab alone. The experiment was conducted once.

Similar effects on AKT phosphorylation have also been observed by other studies (Dubská et al., 2005, Junttila et al., 2009). Pretreatment with calpeptin seemed to restore AKT1/2/3 phosphorylation status, compared to the cells

treated with trastuzumab alone (Figure 4.13-B, indicated by arrows). Data imply that calpain activity is involved in trastuzumab anti-cancer activity through interaction with AKT signalling.

Based on the present findings, the regulation of phospho-AKT (S473) by calpeptin was further examined by Western blotting using an antibody against phospho-AKT (S473), in both trastuzumab-sensitive SKBR3 and inherent trastuzumab-resistant JIMT-1 cells. JIMT-1 and SKBR3 cells were treated using the same protocols as used in phosphokinase array experiments. As shown in Figure 4.14, phospho-AKT (S473) was detected in both JIMT-1 and SKBR3 cells at the expected size (57kDa); however, no obvious alterations in phospho-AKT expression was observed after trastuzumab treatment, compared with untreated cells.

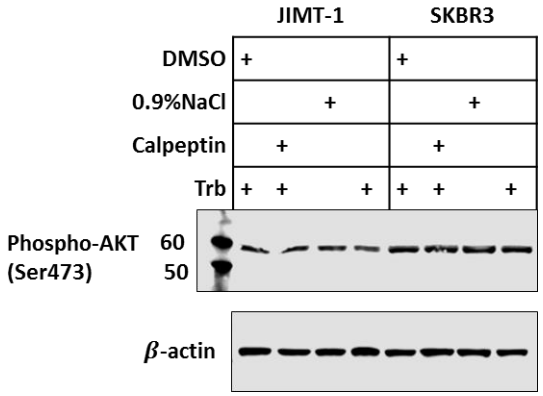


Figure 4.14 Effect of calpeptin and trastuzumab on phospho-AKT (S473) expression in HER2+ breast cancer cells. Primary antibody against phospho-AKT (S473) was used. The experiment was conducted twice from separate lysates of different passage numbers of cells, representative blot is shown as above.

Pretreatment with calpeptin followed by trastuzumab had no effect on regulation of phospho-AKT expression in either cell line, which is inconsistent with observations from the phosphokinase arrays. Again, as discussed previously, as phosphokinase array data was not verified by Western blotting, the information obtained from the arrays has to be viewed with caution. In order to generate firm conclusion, further investigation and validation are required.

4.3.5 Knockdown of calpastatin and calpain-4

To gain stable calpastatin and calpain-4 transfected cell lines, shRNA mediated knockdown of calpastatin and calpain-4 was initially attempted with SKBR3 cells. As shown in Figure 4.15-A, using four constructs, construct 2 markedly reduced calpastatin expression, compared with the other three constructs. As shown in Figure 4.15-B, expression of calpastatin protein was inhibited by approximately 80%, compared with wild-type, scrambled and negative controls.

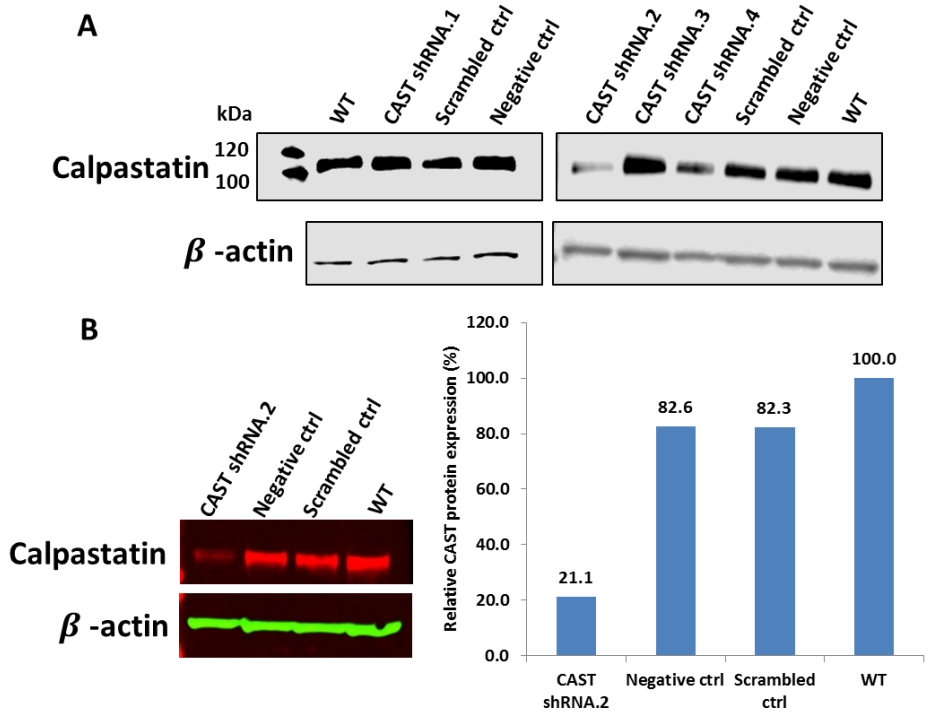


Figure 4.15 Stable shRNA-mediated knockdown of calpastatin in SKBR3 cells. Primary antibody against calpastatin was used. The bar charts showed relative expression level of the proteins.

Calpain-4 is the small subunit shared by both heterodimeric calpain-1 and -2, knockdown calpain-4 can effectively supress calpain-1 and -2 activities. Knockdown of calpain-4 was also attempted, in SKBR3, with four constructs. Following transfection two constructs were unable to survive after drug selection and no cells survived the procedure – the levels of expression in the remaining two were assessed by Western blotting (Figure 4.16). The results showed that expression of construct 1 led to suppression of calpain-4 expression by approximately 60%, versus wild-type and scrambled control.

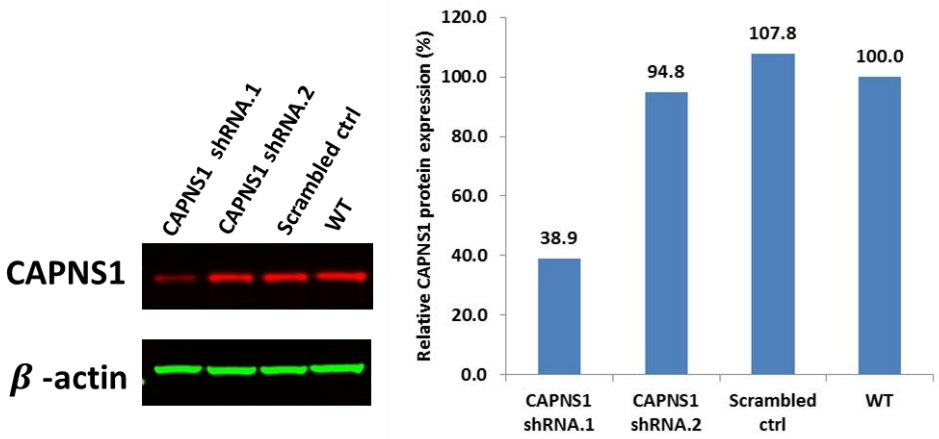


Figure 4.16 Stable shRNA-mediated knockdown of calpain-4 in SKBR3 cells. Primary antibody against calpain-4 was used. The bar charts showed relative expression level of the proteins.

As mentioned above, SKBR3 cells stably transfected with calpastatin and calpain-4 shRNA showed 80% and 60% decrease in calpastatin and calpain-4 expression, respectively. In order to gain complete knockout cell lines, single colony selection was conducted using the construct with highest transfection efficiency, along with scrambled and negative controls. However, single colony expansion was unsuccessful, perhaps due to the low plating efficiency characteristic of SKBR3 cells. Due to time limitations, further investigation and optimisation was not possible. It would be interesting to explore the role of calpain or calpastatin involved in trastuzumab response by using such stable transfected cell lines, which can be included in the future work by other researchers in our group. If single colony selection is not possible then the total cell population could be used. Knockdown of calpain-4 using SKBR3/TR cells was also attempted, but cells did not survive drug selection, so optimisation is required for future studies.

4.4 Discussion

As described previously, HER2+ breast cancer is associated with an aggressive clinical behaviour and poor prognosis; it is estimated that up to 15% of breast cancer patients are diagnosed with this subtype. Trastuzumab has been shown to effectively improve patients' survival when combined with chemotherapy in both the adjuvant and neoadjuvant setting. Despite the survival benefit some patients do not respond to the therapy (inherent resistance), while some patients develop resistance during or after treatment (acquired resistance); unfortunately there are no robust means to identify the HER2+ individuals that may not benefit from this targeted agent. A wide variety of potential mechanisms have been proposed for inherent and acquired resistance to trastuzumab; nevertheless, the underlying mechanisms still need to be fully understood; there is a need to identify biomarkers that are associated with trastuzumab response and a need to means to combat inherent and acquired resistance.

A previous study from our group found that high calpain-1 expression significantly associated with poor relapse-free survival in HER2+ breast cancer patients treated with adjuvant trastuzumab (Storr et al., 2011c). In the current study, we successfully validated this finding in an expanded patient cohort from Nottingham and an independent patient cohort from Newcastle; confirming that calpain-1 expression is significantly associated with adverse relapse-free survival in HER2+ breast cancer patients (Pu et al., 2016). When the two patient cohorts were combined together, analyses continued to show that calpain-1 expression was significantly associated with adverse relapse-free survival. Consistent with the findings from Kaplan-Meier analyses, Pearson's Chi square tests and Fisher exact tests also revealed that high calpain-1 expression was significantly associated with relapse and survival status in the Nottingham and Newcastle patient cohort, respectively. Calpain-1 remained an independent factor for relapse-free survival in the Newcastle patient cohort; however, it was not significant in either Nottingham or combined patient cohort; which

contradicts the finding from the initial Nottingham study. Such discrepancies may be potentially due to the differences existing between the current and the previous study. In the previous study, protein expression was generated from two representative intratumoural cores, whereas in the current study only one core was assessed. Nevertheless, when comparing matched 72 patients cases between the previous and the current Nottingham TMAs, there was a good correlation between H-scores (r=0.61, P<0.001). In addition, the previous study showed that calpain-1 expression was associated with relapse status, which was also found in the current expanded patient cohort.

In the Nottingham patient cohort, high calpain-1 and low calpastatin expression were found to be significantly associated with ER+ and PR+ tumours; interestingly low calpain-4 expression was associated with ER+ tumours. Calpain-4 is the small subunit shared by calpain-1 and -2, the reasons for such conflicting results are unclear; however a previous *in vitro* study has shown that treatment of breast cancer cells with E2 (estradiol) can increase calpain-4 expression, implying a potential link between calpain-4 and oestrogen receptor status (Wang et al., 2014a). In the Nottingham patient cohort, high calpain-1, -2 and -4 expression was associated with lower tumour grade, with significant association seen for calpain-2 in the Newcastle cohort. The small number of patient cases (less than 3) in the high calpain/low tumour grade subgroup will limit interpretation. Significant associations were also found between high calpain-1 expression and low tumour grade in a large cohort of breast cancer patients (including all subtypes of breast cancer) (Storr et al., 2012a).

In addition to calpain-1 being associated with relapse-free survival in HER2+ breast cancer patients receiving trastuzumab, other calpain family members (i.e. calpain-2, -9 and calpastatin) have been implicated in therapeutic response in different types of cancers, including breast cancer patients treated with endocrine therapy (Davis et al., 2014), ovarian cancer patients treated with platinum based chemotherapy (Storr et al., 2012b) and gastro-oesophageal

cancer patients treated with platinum neoadjuvant chemotherapy (Storr et al., 2013). Results from current, and previous, immunohistochemical studies suggest that calpain may play a role in trastuzumab response – as a result of such data it was decided to explore whether manipulating calpain activity could regulate trastuzumab sensitivity in HER2+ breast cancer cells; and also to compare acquired and inherent trastuzumab-resistant cell lines.

In order to investigate combined effects of calpeptin and trastuzumab on cell proliferation, cell growth curves were conducted by treating the cells with calpeptin in combination with trastuzumab, using three cell lines: SKBR3, SKBR3/TR and JIMT-1 cells. In general, results demonstrated that the combination of calpeptin with trastuzumab resulted in a significant greater anti-proliferative effect in both SKBR3 and SKBR3/TR cells, compared with trastuzumab alone, but not in JIMT-1 cells. Calpeptin alone decreased the proliferation of SKBR3 and SKBR3/TR cells by approximately 40% when used in the absence of trastuzumab; whilst trastuzumab alone inhibited approximately 40% and 20% proliferation in SKBR3 and SKBR3/TR cells, respectively. When used in combination, calpeptin and trastuzumab led to a further reduction in cell proliferation in both cells (62% and 51%, respectively). Combination response was different in JIMT-1 cells, as no further reduction in cell proliferation seen. The combined calpeptin and trastuzumab had equal effect on the proliferation of JIMT-1 cells, compared with calpeptin alone.

The effect of combined calpeptin and trastuzumab on SKBR3 cells observed in our study contradicts one report from Kulkarni et al. In their study, the treatment with calpeptin or MDL28170 both significantly enhanced cell proliferation of SKBR3 cells. Compared with trastuzumab alone, they showed that the cell proliferation of parental SKBR3 cells was increased by approximately 20% when 50 μM calpeptin used in combination with 10 μg/mL (67.5 nM) trastuzumab for 72 hours; in contrast, under the same condition, the cell proliferation of trastuzumab-resistant SKBR3 cells was reduced by

approximately 30% (Kulkarni et al., 2010). Current data is, however, consistent with another report from Kulkarni et al., showing that suppression of calpain-4 by shRNA resulted in greater anti-proliferative effect in SKBR3 cells, BT474 (HER2+) cells and acquired trastuzumab-resistant BT474 cells, compared to trastuzumab alone (Kulkarni et al., 2012). There are probably many factors responsible for such conflicting results, including the concentration of trastuzumab used to develop trastuzumab resistance (i.e. 4 μg/mL vs 40 μg/mL). As mentioned previously, 40 μg/mL, used in the current study, is the concentration that can be detected clinically in patient serum, when patients are treated with 4 mg/kg trastuzumab (Tokuda et al., 1999). It may also be due to, cells being plated on fibronectin-coated dishes in their study, which may have potential effects on cell behaviour and drug response. Altogether, current results suggest that the inhibition of calpain activity can enhance anti-proliferative effect of trastuzumab on SKBR3 and SKBR3/TR cells, but not on inherent trastuzumab-resistant JIMT-1 cells, which implies that calpain has differential roles in the mechanisms of acquired and inherently resistance to trastuzumab.

Given that addition of calpeptin to the treatment of trastuzumab enhanced short-term growth inhibitory effects; it was decided to further assess the combined effect on long-term clonogenic survival in HER2+ breast cancer cells. Combined treatment had no significant effect on clonogenic survival of the three HER2+ cell lines, compared with single agent. Treatment with trastuzumab alone appeared to inhibit clonogenic survival of SKBR3 cells, but not in SKBR3/TR cells. The other study using BT-474 trastuzumab-resistant cell model, in which the colony formation of parental BT-474 cells was profoundly decreased after trastuzumab treatment, whereas the colony forming ability of trastuzumab-resistant cells did not change (Ritter et al., 2007). The treatment of calpeptin alone also seemed to reduce clonogenic survival of SKBR3 cells, rather than SKBR3/TR cells. Our data was similar with Liu et al. and Ho et al. reports, showing that depletion of calpain-2 in murine mammary carcinoma

cells and knockdown of calpain-6 in Hela cells exhibited reduced colony forming ability compared with the parental cells (Liu et al., 2011, Ho et al., 2012). There was indication that SKBR3 cells had slightly increased clonogenic survival after the treatment of combined calpeptin and trastuzumab treatment; as the experiments was only repeated twice, it was difficult to draw firm conclusion based on the current results. It is unfortunate that little information on clonogenicity was obtained in the current study. There is, however, evidence in the literature that, trastuzumab decreased colony formation in calpain-4 knockdown SKBR3 cells, compared with control cells (Kulkarni et al., 2012). The different methodologies used to inhibit calpain activity (pharmacological inhibitors vs gene knockdown) may potentially contribute to such discrepancy. Also the plating efficiency of SKBR3 cells used in the present study was too low to give confidence that data would be representative of the behaviour and characteristics of the majority of the population of the cell line. It would be ideal to obtain data from more than one representative cell line for HER2+ subtype. Further investigations, using other HER2+ breast cancer cell lines (e.g. BT474 and HCC1954), to generate firm results, should be considered for inclusion in future studies.

Cell cycle progression was also assessed following the combined treatment. Results from cell cycle analysis showed combined treatment of calpeptin and trastuzumab has no significant effect on G0/G1 phase arrest when compared with single agent alone, in all three HER2+ breast cancer cells. It is interesting that significant effect of calpeptin alone on cell cycle progression was only seen in trastuzumab-sensitive HER2+ breast cancer cells, but not in trastuzumab-resistant HER2+ cells. Calpain directly interacts with HER2 signalling pathways (as introduced in section 4.1.3), and long term exposure (five months) to trastuzumab leads to significant increased HER2/mRNA expression, compared with parental SKBR3 cells (Vazquez-Martin et al., 2007); which may help to explain such effect was only observed in SKBR3 parental cells.

In other studies, enhanced expression of G1 cyclin-dependent inhibitor $p27^{kip1}$ and reduced cyclin D1 expression after trastuzumab treatment has been shown to associate with inactivation of AKT (Yakes et al., 2002); and trastuzumab resistance has also been shown to associate with downregulation of $p27^{kip1}$ and amplification/overexpression of cyclin E (Nahta et al., 2004, Scaltriti et al., 2011). Depletion of calpain-4 in chondrocytes caused cell accumulation in G1 phase, reduced cyclin D gene transcription, and enhanced expression of cyclin D, E and $p27^{kip1}$ (Kashiwagi et al., 2010); whilst depletion of calpain-2 in mouse mammary carcinoma cell line led to increased $p27^{kip1}$ expression and reduced expression of AKT (Ho et al., 2012). These studies suggested that trastuzumab and calpain have been involved in shared cell cycle related pathways. Although current data did not provide direct evidence to support the involvement of calpain in trastuzumab-induced cell cycle arrest, it is possible that calpain might play a role in trastuzumab response in HER2+ breast cancer cell lines - through regulating cell cycle related signalling pathways, such as cyclin D and $p27^{kip1}$. Further investigation is needed to determine the direct interactions between calpains and other factors regulating trastuzumab response.

The effect of calpeptin and trastuzumab on regulation of signalling was assessed using phosphokinase arrays. Results showed that treatment with trastuzumab induced a decrease in phospho-AKT expression in SKBR3 cells, which was restored by pretreatment with calpeptin. When calpeptin was used alone, the phosphokinase array data for expression of phospho-AKT (described in section 2.3.4.3) was consistent with reports from the another study, showing that expression of phospho-AKT was unchanged in SKBR3 parental cells following calpeptin treatment (Kulkarni et al., 2010). They also showed that in acquired trastuzumab-resistant SKBR3 cells, AKT phosphorylation was increased, and the addition of calpeptin or MDL28170 attenuated the increase effects. This study suggested that AKT signalling in acquired trastuzumab-resistant SKBR3 cells is regulated by calpain activity; and inhibition of calpain activity reduces AKT activation, enhancing trastuzumab sensitivity. The role of calpain on AKT

signalling varies considerably across different studies. Mouse embryonic fibroblasts from calpain-4 knockout mice show decreased AKT phosphorylation (Tan et al., 2006), however, by using an established mouse embryonic fibroblast cells NIH 3T3, another study demonstrated that inhibition of calpain activity by calpain inhibitor I (ALLN) can enhance AKT phosphorylation at Ser473 and Thr308 sites after serum starvation (Beltran et al., 2011). The reason for such conflicting effects of calpain on AKT phosphorylation is unknown; it may be associated with different methods used to manipulate calpain activity such as by gene knockdown or pharmacologic inhibitors. Together with our results, studies suggest that the interaction between calpain and AKT signalling might have an important role in regulating trastuzumab response. As we could not validate phosphokinase array results in Western blotting, and only one HER2+ breast cancer cell line was assessed in the array; the results must be interpreted carefully.

In the present study, calpeptin treatment resulted in maximal 50% inhibition of calpain activity; it would be of interest to see if the effects would be different if the residual active calpain was completely blocked. We developed calpastatin and calpain-4 knockdown SKBR3 cells, knocking down 80% and 60% calpastatin and calpain-4 expression, respectively. To obtain complete knockout of the genes, several single colony selections were attempted; however, the cells were not viable when grown from a single colony (perhaps due to the low plating efficiency of these cell lines). Due to time limitations further optimisation and investigations were not conducted; this could be included in future work to be conducted by other researchers in the group.

JIMT-1 cells originated from an invasive ductal carcinoma pleural metastasis of a 62 years old patient diagnosed with HER2 gene amplification, who did not respond to trastuzumab treatment (Tanner et al., 2004). From this study, two key proteins in HER2 related signalling pathways, AKT and ERK, were differentially regulated following trastuzumab treatment in JIMT-1 cells. The

fact that AKT phosphorylation was unchanged, while ERK phosphorylation was still markedly decreased, indicating that unresponsiveness to trastuzumab-induced anti-cancer effects in JIMT-1 cells is associated with AKT phosphorylation (Tanner et al., 2004). On the other hand, as cytokeratins 5/14 and 8/18 are expressed in JIMT-1 cells. Its basal-like phenotypic character was also associated with inherent resistance to trastuzumab (Jönsson et al., 2007, Oliveras-Ferraros et al., 2010, Oliveras-Ferraros et al., 2012). Therefore basal-like phenotypic background should potentially be taken into account when studying basal/HER2+ breast cancer cells, such as JIMT-1 cells.

Caution should be used when interpreting data, as current studies were conducted using two-dimensional (2D) monolayer cultures, rather than three-dimensional (3D) cell culture models. After screening 102 chemical compounds using JIMT-1 cells, substantial variations in drug sensitivity were observed between 2D and 3D models (i.e. Matrigel and poly 2-hydroxyethyl methacrylate-induced anchorage-independent growth): 63 were profoundly more potent in 3D assays (Hongisto et al., 2013). In this context, as we are unable to get JIMT-1 cells forming colony in traditional 2D cultures, it is difficult to make direct comparison between inherent and acquired trastuzumab response based in the present study. Another study also demonstrated that trastuzumab caused significantly greater growth inhibition in 3D (Matrigel®) than in a 2D culture model, in SKBR3 cells. Phosphorylation of AKT and MEK1/2 was detected in SKBR3 cells following trastuzumab treatment in 2D cultures, but not in 3D cultures; and phosphorylation of HER2 was significantly reduced in the 3D model. The study suggested that HER2 signalling was mediated by distinct pathways in 2D and 3D cultures in the SKBR3 cell line – through MAPK and AKT pathways in 2D cultures, but through other alternative pathways in 3D cultures (Weigelt et al., 2010b). Therefore, traditional 2D cultures may not be representative to mimic the tumour microenvironment; if possible, the use of alternative culture models (i.e. 3D, *in vivo*) need to be taken into account in future work testing trastuzumab response.

4.5 Summary

1. High calpain-1 expression was associated with adverse relapse-free survival in HER2+ breast cancer patients treated with adjuvant trastuzumab (validation study).
2. Inhibition of calpain activity significantly enhanced the anti-proliferative effect of trastuzumab in parental and acquired trastuzumab-resistant SKBR3 cells; but has no effect in inherent trastuzumab-resistant JIMT-1 cells.
3. Inhibition of calpain activity had significant effect on cell cycle arrest in parental SKBR3 cells, but not in acquired or inherent trastuzumab-resistant JIMT-1 cells.

Chapter 5 General discussion

5.1 General discussion

As mentioned in earlier chapters the calpain system, a group of proteolytic cysteine proteases and their endogenous inhibitor calpastatin, is expressed ubiquitously or tissue-specifically in a wide range of cells and tissues and plays an important role in a variety of physiological cellular events. Dysregulated activation/expression or gene mutation in calpain family members has been implicated in several pathological conditions, such as neurodegenerative disorders (Laske et al., 2015), skeletal muscle disorder (limb-girdle muscular dystrophy type 2A disease) (Richard et al., 1995), cardiovascular disease (myocardial infarction) (Mani et al., 2009), type 2 diabetes (Horikawa et al., 2000); and cancer.

Since the first description of calpain in 1964 (Guroff, 1964), there has been growing interest in calpain's involvement in tumour development with numerous proteolytic substrates of calpain being demonstrated to be involved with tumour progression - such as NF-κB, regulating cell survival (Pianetti et al., 2001); talin, regulating cell migration and adhesion (Franco et al., 2004); and caspase-3, regulating cell apoptosis (Chua et al., 2000). On the other hand, pharmacological inhibitors or activators of calpain, as well as targeting specific gene expression, have been applied in several studies to explore their potential anti-tumour effects or to regulate treatment response. For example, the use of calpain-2 inhibitor (zLLY-CH2F) has been shown to disrupt calpain cleavage of IκBα and NF-κB nuclear localisation, resulting in reduced colon tumour volume (Rose et al., 2015). Knockdown of m-calpain in gastric cancer cells enhanced 5-FU sensitivity in 5-FU resistant cells (Nabeya et al., 2011). Additionally, impaired calpain expression has been demonstrated in a number of different tumour types, along with their correlations with clinicopathological variables, providing important prognostic value. For example, increased calpain-4 has been found in nasopharyngeal carcinoma (Zheng et al., 2014), intrahepatic cholangiocarcinoma (Zhang et al., 2013), hepatocellular carcinoma (Dai et al., 2014) and glioma (Cai et al., 2014); and also contributed to poor clinical

prognosis.

As for breast cancer, the most common cancer in the UK (as introduced in Chapter 1), the calpain family, and its endogenous inhibitor calpastatin, has also been shown to be of importance. Breast cancer is a heterogeneous disease, by using gene expression microarrays, it can be classified into at least five distinct molecular subtypes: luminal A, luminal B, HER2+, basal-like and normal breast-like, within which HER2+ and basal-like subtypes present with the worse prognosis compared with other subtypes (Sørlie et al., 2001, Sørlie et al., 2003, Sotiriou et al., 2003). Our group has previously shown that the expression of calpain family members is associated with patient survival and therapeutic response in specific breast cancer subtypes, i.e. basal-like, triple-negative and HER2+ (Storr et al., 2011c, Storr et al., 2012a, Davis et al., 2014), and that decreased calpastatin mRNA and protein expression associated with the presence of lymphovascular invasion (Storr et al., 2011b, Storr et al., 2012a). It was therefore thought of interest to examine the role of calpain in different breast cancer subtypes, and whether manipulating calpain activity could have differential effects on different phenotype of breast cancer cells; which might provide useful information on improving patient prognosis and clinical treatment response.

In order to explore the role of calpain in breast cancer, the present study first screened calpain protein expression in six representative breast cancer cell lines from different subtypes (two luminal, two HER2+ and two basal-like), with three of them being selected to examine calpain activity level. Results showed that calpain-1, -2, -4 and calpastatin are expressed across all six breast cancer cell lines; with a relatively lower level of calpain-1 in basal-like breast cancer cells MDA-MB-231 and lower level of calpain-2 in luminal breast cancer cells MCF-7; a similar phenomenon was previously found by Wu et al. showing lower calpain-2 expression in MCF-7 cells compared with other breast cell lines (Wu et al., 2006).

Such differences between basal-like and luminal subtypes further raised our interest to investigate calpain activity levels across different breast cancer molecular subtypes. By using the T-boc calpain activity assay, MCF-7 cells were found to have the highest level of calpain activity, followed by SKBR3 cells then MDA-MB-231 cells. However, such *in vitro* results contradict clinical information relating to the cancer type and also well characterised cellular behaviours; i.e. basal-like breast cancer exhibit a more aggressive clinical behaviour compared with luminal, which would be expected to have higher levels of calpain activity. Calpain-1 and -2 have differential activation and functional mechanisms, both of them can be detected in the T-boc assay; together with the protein expression detected in Western blotting, it may be that calpain-2 is dominant in MDA-MB-231 cells, contributing to a relatively lower level of calpain activity but an invasive phenotype; while calpain-1 is dominant in MCF-7 cells, correlating with higher level of calpain activity but a less invasive phenotype. This could also be reflected by immunohistochemical data showing that high calpain-2 expression is associated with poor survival in basal-like breast cancer patients (Storr et al., 2012a). Another potential explanation, for low calpain expression in cells with high level of activity, might be the autolytic activity of calpain. Another study also observed decreased calpain activity but increased calpain-1 and -2 protein expression; suggesting the accumulation of protein due to decreased activity in the cells, and decreased autolysis (Niapour et al., 2008).

The current study went on to examine the role of calpain in regulating breast cancer cell proliferation, migration and apoptosis. Results show that inhibition of calpain activity by calpeptin (up to 50% inhibition) resulted in anti-proliferative effects in basal-like, luminal and HER2+ breast cancer cells, with similar estimated IC50 values seen across the three cell lines used. Calpeptin is not a specific calpain inhibitor and it also inhibits other proteases, albeit to a lower level i.e. cathepsin (Wang and Po-Wai, 1994). Although the estimated IC50 values were close across the three cell lines; taken together, the most rapid and significant inhibition of calpain activity, as well as the highest calpain

activity level, was observed in MCF-7 cells. One possible explanation for such a phenomenon could be the interaction between ER and calpain in oestrogen-dependent breast cancer. This can be supported by evidence showing that pre-treatment with calpeptin can reduce E2-induced proliferation effects in MCF-7 cells (Wang et al., 2014a).

With respect to cell migration, although results were not statistically significant, data showed that under the stimulation of calcium ionophore, calpastatin knockdown MDA-MB-231 cells had slightly reduced migratory ability, but it was not statistically significant in comparison with controls. Initial studies conducted by Dr. Sarah Storr, also showed that knockdown of calpastatin in MDA-MB-231 cells did not show increased calpain activity. Calpastatin, in this situation, was not completely knocked-down and it is unknown if any residual calpastatin level would be sufficient to exert an effect on cell migration. It may also because the concentration of calcium ionophore was not sufficient to activate calpain activity. In comparison, the migratory ability of MCF-7 cells was unaffected by knockdown of calpastatin (with or without calcium ionophore stimulation); wild-type MCF-7 cells showed slightly reduced migration followed by incubation with calcium ionophore. The reason for this is unclear, but calcium ionophore A23187 has been shown to decrease ER protein/mRNA expression and further regulate ER transcriptional activity in MCF-7 cells (Ree et al., 1991, Journé et al., 2004). The effect of A23187 on ER protein may have potential effects on MCF-7 cells migration.

Consistent with clinical observations, of basal-like breast cancer having a more aggressive and invasive behaviour than luminal subtype; current data show MDA-MB-231 cells having a markedly enhanced migratory ability compared with MCF-7 cells. As MCF-7 cells expressed the lowest calpain-2 level, it might imply that calpain-1, rather than calpain-2, plays a dominant role in determining migratory ability of luminal breast cancer cells. This can be supported by others' studies showing that knockdown of calpains in MCF-7 cells reduced migration

activity, and that restoring expression of calpain-1, rather than calpain-2, increased the migration rate. It is of interest to note that calpain-1 is exclusively involved in the cleavage of two important adhesion components, filamin and talin (Wu et al., 2006).

Calpain has been shown to regulate both cell survival and apoptotic pathways, but its exact role in breast cancer cell apoptosis is not fully understood. The role of calpain in breast cancer cell apoptosis was therefore studied. Treatment with calpeptin caused a significant induction of apoptosis in basal-like breast cancer cells MDA-MB-231 and HER2+ cells SKBR3, but not in luminal MCF-7 cells. Such results are in accordance with Mataga et al.'s finding in MDA-MB-231 cells, but contradict their finding with MCF-7 cells as calpeptin induced apoptosis in both MDA-MB-231 and MCF-7 cells (Mataga et al., 2012). MCF-7 cells are a caspase-3 deficient line (Devarajan et al., 2002), In the Magataga et al.'s study the treatment of calpeptin was for 96 hours, while in the current study it was 72 hours. The reason for such discrepancies may be that, calpeptin induced caspase-3 dependent apoptosis during short time, while it triggered compensatory caspase-3 independent apoptosis during longer incubation. Also, calpain has been reported to be involved in the early stage of apoptosis through interaction with caspase-3 (Waterhouse et al., 1998). Additionally, calpain protein expression level might also reflect the different effect of calpeptin on apoptotic response. Lower calpain-1 expression in MDA-MB-231 cells may reflect higher sensitivity to calpeptin induced apoptosis; whereas lower calpain-2 expression in MCF-7 cells may reflect higher resistance to calpeptin induced apoptosis. This might imply that apoptotic activity in luminal breast cancer cells requires sufficient calpain-2 expression level.

The role of calpain in signal transduction was studied in the current work and used both phosphokinase arrays and apoptosis arrays, which allow evaluation of 43 phosphokinases, 2 total proteins and 35 apoptosis related proteins simultaneously. There is an indication showed that inhibition of calpain activity

by calpeptin upregulated MSK1/2 (S376/S360) phosphorylation in MDA-MB-231 cells, but not in MCF-7 or SKBR3 cells; whilst HSP60 expression was decreased in MCF-7 cells rather than MDA-MB-231 cells. However, Western blotting failed to confirm array data either for phoshpo-MSK1/2 or HSP60. As no phospho-MSK1/2 signal was detected across various cell samples, which may due to the lack of external stimuli to activate MSK1/2 in cells *in vitro*. It may well be that as calpain activity was not completely blocked in the cells; any residual activity may have potential effects on signal transduction, which might affect data interpretation. In retrospect it may have been wise to run a number of independent arrays but costs would have been prohibitive.

The initial results from arrays suggested a potential interaction between calpain and MSK1/2 – as a result of such data it was decided to assess MSK1/2 expression in breast tumours, and examine for correlations with calpain family protein expression. After trying several antibodies from different sources, a specific MSK2 antibody that presented nuclear staining in immunohistochemistry could not be found; also phospho-MSK1/2 phosphorylation could not be detected using various cell lysates (as described in Chapter 2). Consequently, it was decided to assess MSK1 expression in breast tumour specimens and examine its prognostic significance. In addition to assessing MSK1 protein expression in a large early stage invasive breast cancer patient cohort there are numerous reports in the literature suggesting a close link between the caspase and calpain family, it was therefore thought worthwhile to also examine two representative upstream and downstream caspases, caspase-3 and -8, and assess their correlation with calpain family expression. Calpain can directly cleave caspase-9, leading to impaired activation of downstream caspase-3 (Chua et al., 2000); and calpastatin can be degraded by caspases during drug-induced apoptosis (Wang et al., 1998, Pörn-Ares et al., 1998).

Immunohistochemical results showed significant correlations between MSK1 vs calpain-1, MSK1 vs calpastatin and caspase-8 vs calpastatin, though all were of marginally relevance due to their low correlation coefficients. It should be noted, however, that it is difficult to make a direct comparison between protein expression level and calpain activity in the cells. It would be ideal to assess calpain activity and caspase activity in breast cancer tissues, by detecting proteolytic products of calpain and caspase. Some reagents have been tested, calpain specific cleaved α-fodrin antibody was used to detect calpain activity on frozen tissue sections, but consistent and reproducible results were not obtained.

In terms of prognostic significance, high MSK-1 expression was significantly associated with improved breast cancer-specific survival but the result was not significant in multivariate analysis. From subgroup analysis, high MSK1 expression seems to play important role in patients with HER2 negative and non-basal like breast cancer. This is the first report to reveal potential prognostic significance of MSK1 expression in breast cancer patients; further validation in independent patient cohorts is required. In comparison, high caspase-3, rather than caspase-8 expression, was significantly associated with adverse breast cancer-specific survival, particularly in patients with receptor positive (ER, PR or HER2) and non-basal like tumours; and remained an independent factor in multivariate analysis. Three previous, albeit small, studies showed high caspase-3 and enhanced apoptotic activity in breast carcinomas compared with normal tissues (Vakkala et al., 1999, Nakopoulou et al., 2002, Blazquez et al., 2006); with only one study showing reduced caspase-3 transcript and protein expression in breast tumours (Devarajan et al., 2002). Amongst these four studies, only one has showed a significant association between caspase expression and patients' prognosis. Current results are consistent with this finding from Nakopoulou et al. Several reasons may contribute to discrepancies between these studies. High caspase-3 expression does not necessarily correspond to a high level of apoptotic response or caspase activity in cells. It

might also relate to the impaired caspase-3/caspase-3 splicing variant level as a study suggested that overexpression of caspase-3s can interrupt drug-induced apoptosis and predict a poor response to neoadjuvant chemotherapy in breast cancer patients (Végran et al., 2006).

Calpain-1 was previously suggested to be involved in trastuzumab response in HER2+ breast cancer patients (Storr et al., 2011c). An important component of the present study was to validate this result in expanded and independent patient cohorts; and also to explore how calpain regulates trastuzumab response in HER2+ breast cancer cells. From the immunohistochemical study, we successfully validated previous findings, showing that high calpain-1 expression was significantly associated with adverse relapse-free survival in HER2+ breast cancer patients treated with trastuzumab (P=0.01 and P=0.019, in Nottingham and Newcastle cohort, respectively) (Pu et al., 2016). This led us to investigate if manipulating calpain activity would sensitise cells to trastuzumab, with a particular focus on sensitising resistant cells (inherent and acquired).

The combined effects of a calpain inhibitor, calpi-peptin, and trastuzumab were assessed in three HER2+ cells (parental SKBR3, acquired-resistant SKBR3, and inherent-resistant JIMT-1), with respect to proliferation, clonogenic survival, cell cycle and signal transduction. Generally, results suggested that a combination of calpeptin and trastuzumab may have a role in trastuzumab-sensitive HER2+ breast cancer cells, with limited action in acquired and inherent trastuzumab-resistant HER2+ breast cancer cells. The combined treatment produced a greater anti-proliferative effect than trastuzumab alone in sensitive and acquired resistant cells, but had no effect in inherent resistant cells. The combined treatment did not have significant effect on clonogenic survival in either trastuzumab-sensitive or-resistant cells. It should be noted that, due to the low plating efficiency of SKBR3 cells (10-15%), substantial differences were observed between independent experiments – a similar phenomenon was also

noticed in other study (Karasawa et al., 2014); therefore the low plating efficiency of SKBR3 cells and variable results, make it difficult to make any firm conclusions from this assay. It would be ideal to include other HER2+ cell lines, such as BT474 and HCC1954, to hopefully generate more robust data.

The combined treatment had no significant effect on cell cycle arrest in all three cell lines, compared with single agent alone. Although there was no significant effect, it is interesting to note that combined treatment appeared to cause G0/G1 cell cycle arrest in SKBR3 cells, rather than in SKBR3/TR or JIMT-1 cells. Also, calpeptin alone caused a significant G0/G1 cell cycle arrest only in trastuzumab-sensitive cells; with no such effect seen in the other two trastuzumab-resistant cell lines. This may suggest that calpain play a role in cell cycle regulation in trastuzumab-sensitive cells, but not in trastuzumab-resistant cells. As discussed in Chapter 4, the function mechanisms of trastuzumab and calpain are reported to be quite similar - both involves altered cyclin, $p27^{kip1}$ and AKT expression (Yakes et al., 2002, Kashiwagi et al., 2010, Ho et al., 2012). Further experiments are required to fully explore the role of calpain in trastuzumab-induced cell cycle arrest, for example it would be of interest to examine the effect of combined treatment on the expression of related cell cycle proteins (e.g. $p27^{kip1}$ and cyclins), and their crosstalk with HER2 signalling.

Although the array data from the current study has to be viewed with caution, having been conducted once, pretreatment with calpeptin seemed to restore trastuzumab-induced downregulation of phospho-AKT (S473) expression in SKBR3 cells. Kulkarni et al's study showed that AKT phosphorylation was increased in acquired-resistant SKBR3 cells, and that treatment of calpeptin or MDL28170 attenuated the increase effect (Kulkarni et al., 2010). Together with current data, this might imply that calpain regulates AKT signalling differently in trastuzumab-sensitive and -resistant HER2+ breast cancer cells; as in sensitive cells calpeptin restored AKT phosphorylation, and in resistant cells, calpeptin attenuated AKT phosphorylation.

It is unlikely that AKT is the only protein interacting with calpain. Nagata et al. indicated that following trastuzumab treatment there was a rapid increase in PTEN phosphatase activity and this was responsible for the rapid dephosphorylation of AKT, which happened before downregulation of HER2 and PI3K, providing another novel mechanism for anti-cancer action of trastuzumab (Nagata et al., 2004). PTEN functions as a negative regulator of AKT and is involved in phospholipid activity, i.e. phosphatidylinositol-3,4,5-triphosphate (PIP3) (Myers et al., 1998). Another phospholipid, phosphatidylinositol-4, 5-bisphosphate (PIP2) is also known as a direct substrate of calpain (as introduced in Chapter 1), further emphasising the potential link between calpain, AKT and PTEN. A separate study demonstrated that inhibition of ERK phosphorylation, but not AKT phosphorylation, increased calpain-1 activity in MCF-7 cells expressing HER2, suggesting that HER2 can regulate calpain activity via ERK/MAPK signalling pathway (Ai et al., 2013).

The results of the current study are summarised in Table 5.1 & 5.2.

Table 5.1 Summary of the study (different molecular subtypes)

	Calpain inhibition		**Basal-like (MDA-MB-231)**	**Luminal (MCF-7)**	**HER2+ (SKBR3)**
In vitro	Proliferation		↓	↓	↓
	Apoptosis*		↑	**O**	↑
	Calpastatin knockdown				
	Migration		↓	**O**	
IHC (BCSS)	**Biomarkers**	**Total**	**Triple-negative**	**Basal-like**	**HER2+**
	MSK1	+	**O**	**O**	**O**
	Caspase-3	-	**O**	**O**	**O**
	Caspase-8	**O**	**O**	**O**	**O**
	Combinational biomarkers				
	High casp-3/high casp-8	-	**O**	**O**	**O**
	High casp-3/high cal-1	-	**O**	-	**O**
	High casp-3/high cal-2	-	**O**	**O**	**O**
	High casp-3/low cast	-	**O**	**O**	**O**
	High casp-8/low cal-1	-	**O**	**O**	**O**

Abbreviations: IHC, immunohistochemistry; BCSS, breast cancer-specific survival; ↑, increased after treatment; ↓, decreased after treatment; **O**, did not change after treatment/not statistically associated; **+**, high expression was associated with improved prognosis; **-**, high expression was associated with adverse prognosis; blank, did not apply; * statistically significant (*P*<0.05 vs control).

Table 5.2 Summary of the study (trastuzumab response)

IHC (RFS)		**Nottingham cohort**	**Newcastle cohort**	
	Calpain-1	-	-	
	Combined calpeptin and Trb	**Trb-sensitive (SKBR3)**	**Acquired Trb-resistant (SKBR3/TR)**	**Inherent Trb-resistant (JIMT-1)**
In vitro	Proliferation*	↓	↓	**O**
	Clonogenic survival	↑	**O**	
	Cell cycle	↓	**O**	**O**

Abbreviations: RFS, relapse-free survival; Trb, trastuzumab; ↑, increased after treatment; ↓, decreased after treatment; **O**, did not change after treatment/not statistically associated; **-**, high expression was associated with adverse prognosis; blank, did not apply; * statistically significant (*P*<0.05 vs single agent).

5.2 Limitations and suggestions for future work

In the present study, calpeptin was used as the calpain inhibitor to explore calpain regulation in breast cancer cells. Several inhibitors are commercially available, such as leupeptin, calpain inhibitor I and II, E-64, PD150606 and MDL28170. Initial work showed that calpeptin had advantage on sensitivity over another inhibitor, PD150606. Throughout the literature, calpeptin is one of the most widely used cell permeable calpain inhibitors. It was first synthesised as a peptide inhibitor, and when compared with leupeptin its inhibitory efficacy was 4-fold higher against calpain-1, 28-fold higher against calpain-2, and 45-fold higher against platelet calpain-1; and was 2.6 times less potent against papain (Tsujinaka et al., 1988). Even though calpeptin showed superiority over leupeptin and PD150606, many inhibitors lack specificity - leading to possible cross reactivity with other cysteine proteases (cathepsin) (Wang and Po-Wai, 1994).

Calpain activity could not, in the present study, be completely blocked; and so results should be reviewed with caution as residual calpain activity may mask effects. As calpeptin blocks both heterodimeric calpain-1 and also -2, the exact roles of calpain-1 and -2 in regulating breast cancer progression cannot be clarified by using this inhibitor. It would, therefore, be interesting to use gene knockdown to examine the potential differential roles of calpain-1 and -2. Knockdown of calpains or calpastatin by using shRNA, to inhibit or activate calpains respectively, could be used to observe the role of calpain in regulating breast cancer cell proliferation, clonogenic survival, migration, invasion, cell cycle, apoptosis, as well as therapeutic response - this could be carried out through future work/investigations by others continuing the study. Caution should, however, be applied when trying to compare pharmacologic inhibition with knockdown approaches as complete removal of a protein may often result in different effects from inhibiting enzymatic activity but with the protein still being present. It should also be noted, as experienced in the current study, that complete knockdown is often difficult to achieve by shRNA methodologies with

some residual protein, and therefore enzymatic activity, potentially remaining – to obtain entire removal of the protein CRISPR-Cas9 technology may have to be considered (a time consuming, laborious and expensive technique, particularly when applied to cancer cells that usually have an abnormal number of chromosomes).

Most data in the current study was generated from a limited number of cell lines - only one cell line for each molecular subtype was used, which may not be sufficiently representative. In order to strengthen conclusions additional cell lines from each molecular subtype should be included in future work.

A recent study, using four representative breast cancer cell lines MCF-7 (luminal A), SKBR3 (luminal B/HER2+), MDA-MB-231 (basal-like), JIMT-1 (basal like/HER2+), assessed mRNA and protein expression correlation, and compared the proteomes of clinical tumour samples and corresponding tumour cells. The quantitative results revealed a low correlation between protein and relative mRNA expression in those five cell lines. There is a statistically significant enrichment in 3681 proteins (from tumour samples) related with haemostasis, response to wounding, immune response, and intracellular trafficking and signalling; while in 3417 proteins identified from the cell lines, enrichment for proteins were observed to relate to metabolic processes, intracellular transport and signalling, and cell duplication – no shared enrichment was observed between cell lines and tumour samples. Therefore, the study provided evidence to that, proteomes of cell lines are not truly representative of that in clinical samples (Cifani et al., 2015). Additional studies may wish to consider using tumour xenografts models; further linking experimental studies with the clinical setting.

Current experiments with HER2+ cell lines were carried out using traditional 2D culture methods. As discussed in Chapter 4, JIMT-1 cells cultured in 3D models show improved sensitivity to the 102 chemical compounds than when cultured

in 2D (Hongisto et al., 2013). There is also evidence suggesting that in SKBR3 cells HER2 signalling pathway is mediated through distinct pathways in 2D versus 3D cultures (Weigelt et al., 2010b). Considering such differences observed between 2D and 3D culture models, further investigations could be carried on 3D models (e.g. Matrigel®), as the latter is more representative to mimic the tumour microenvironment *in vivo* and may be a more appropriate choice to study drug response in cells. The expense associated with use of Matrigel® may be prohibitive and, if so, then other 3D matrices may have to be considered e.g. hydrogels.

High-resolution genomic profiles have indicated that the BT474 cell line is both ER and HER2 positive, and has a strong correlation to luminal A subtype; that the JIMT-1 cell line is ER negative and HER2 positive, having a strong correlation to the basal and HER2+ subtype; and that SKBR3 cells show no significant correlation to any subtype, but is closest to luminal B followed by HER2+ (Jönsson et al., 2007). The data suggested that despite HER2 amplification and/or overexpression in these cell lines they still have distinct phenotypic characteristics. Although the current work used the ER negative SKBR3 model it is possible that oestrogen receptor signalling might contribute to the calpain regulation of trastuzumab response. Combined treatment of insulin-like growth factor I receptor (IGF1R) and anti-oestrogens induced a significant apoptotic response in HER2+/ER+ BT474 cells and HER2-/ER+ MCF-7 cells; with either single agent not inducing apoptosis; and combined treatment also enhancing growth inhibition in both cell lines (Chakraborty et al., 2010). Such studies suggest that there may be a potential crosstalk between HER2 and oestrogen signalling pathways. It would, therefore, be of interest to investigate how calpain regulates trastuzumab response in HER2+ breast cancer cells with distinct phenotypic features. Future work could be done with trastuzumab-sensitive BT474 versus acquired trastuzumab-resistant BT-474 cell lines; and also with other inherent trastuzumab-resistant cell lines such as HCC1569 and

HCC1954. HCC1569 is a PTEN deficient cell line and HCC1954 has mutant activation of PIK3CA (Weigelt et al., 2011, Chakrabarty et al., 2013).

It might also be interesting to extend the work to include other HER2+ targeting agents, such as pertuzumab or lapatinib, since the mechanisms of action of pertuzumab and lapatinib are different from that of trastuzumab (as discussed in Chapter 1). The current study did not assess the combined effect of drugs on apoptosis or cell migration; so by applying a pharmacological inhibitor or specific gene knockdown, it would be interesting to examine if calpain could regulate effects of HER2 targeted agent (trastuzumab, pertuzumab, lapatinib) response, with respect to cell cycle progression, apoptotic response and migratory ability.

The present study validated calpain-1 as a potential biomarker for relapse-free survival in HER2+ breast cancer patients treated with trastuzumab. As discussed in Chapter 1, there are other HER2 targeted agents currently used clinically, such as lapatinib, pertuzumab and T-DM1. Those drugs were designed to overcome trastuzumab resistance in patients pretreated with trastuzumab; it would worthwhile to further explore the role of calpain in regulating therapeutic response of such HER2 targeted agents, including immunohistochemical based studies with patient samples. Taking lapatinib as an example, although combination of lapatinib with trastuzumab has shown superior efficacy than trastuzumab alone in neoadjuvant setting (Baselga et al., 2012a), a recent report from the ALLTO study suggested that the combined regimen failed to show superior benefits on improving disease-free survival compared to trastuzumab alone (Piccart-Gebhart et al., 2014). Experimental studies have also shown that the mechanisms contributing to trastuzumab and lapatinib resistance are different (Köninki et al., 2010, O'Brien et al., 2010).

In conclusion, the current study suggests that 1) calpain has a differential role in different breast cancer molecular subtypes; and 2) calpain-1 is a potential

biomarker for trastuzumab response in HER2+ breast cancer patients; and it might play a more important role in trastuzumab-sensitive HER2+ breast cancer cells, with limited action in trastuzumab-resistant HER2+ breast cancer cells. Inhibition of calpain activity by calpeptin reduces proliferation in basal-like, luminal and HER2+ breast cancer cells; induces apoptosis in basal-like and HER2+ cell lines, but not in luminal type. Immunohistochemical results in patient samples suggested that high MSK1 expression is associated with improved breast cancer-specific survival, especially in HER2 negative and non-basal like subtypes; while high caspase-3, but not caspase-8, expression is associated with adverse breast cancer-specific survival, especially in receptor positive (ER, PR or HER2) and non-basal like patients. The immunohistochemical based validation study showed that calpain-1 is a potential useful biomarker for trastuzumab response in HER2+ breast cancer patients. Current *in vitro* results may suggest that inhibition of calpain activity enhances the anti-proliferative effect of trastuzumab in sensitive cells, which might be associated with regulation of cell cycle progression.

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Appendix A. Supplementary information for Chapter 2&3

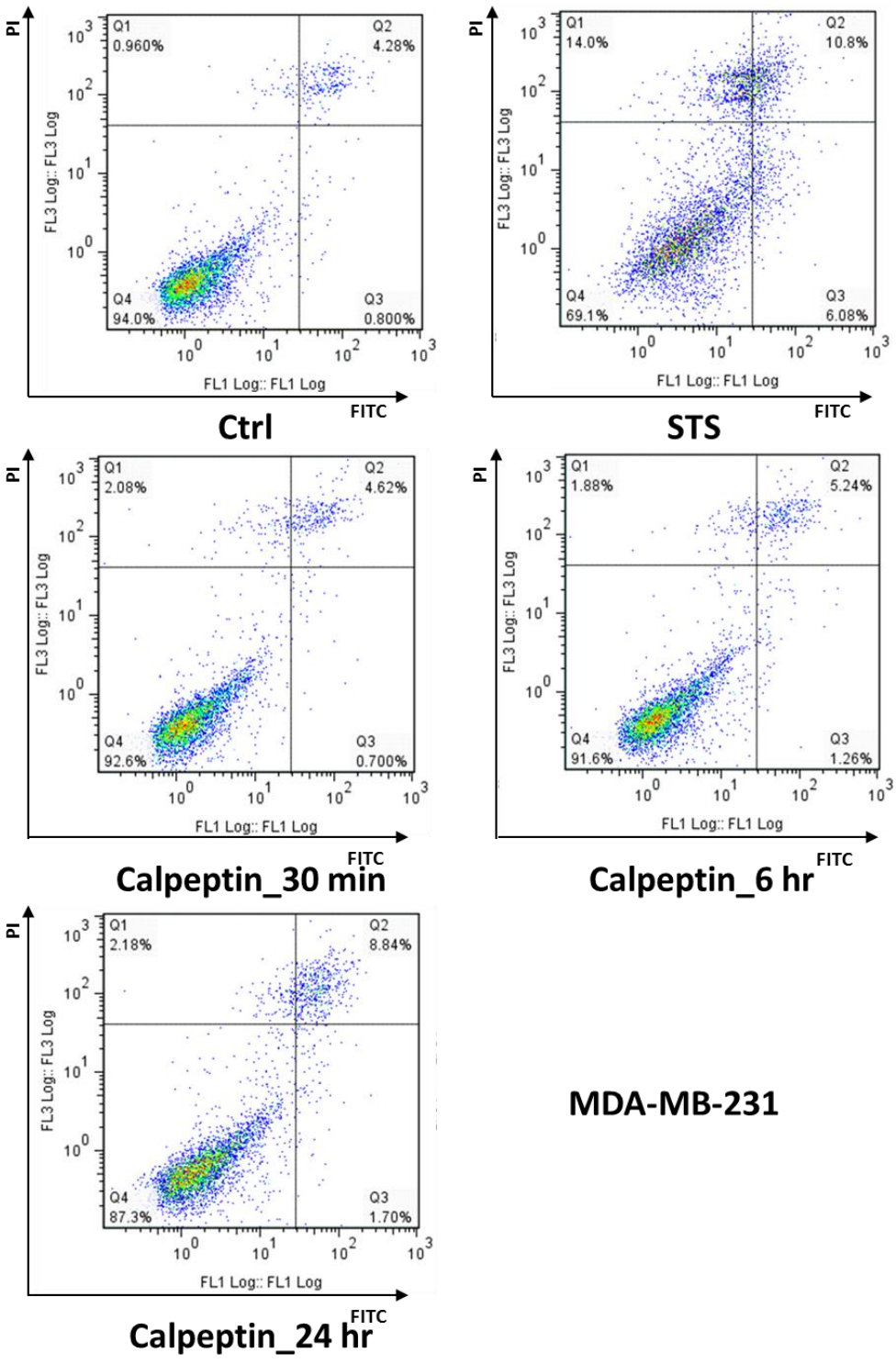

Figure A 1 Effect of calpeptin on apoptosis of MDA-MB-231 cells at different time points. Cells were treated with 100 µM calpeptin for 30 minutes, 6 and 24 hours. 1 µM STS for 24 hours, were set as positive controls. The representative dot plots are shown above.

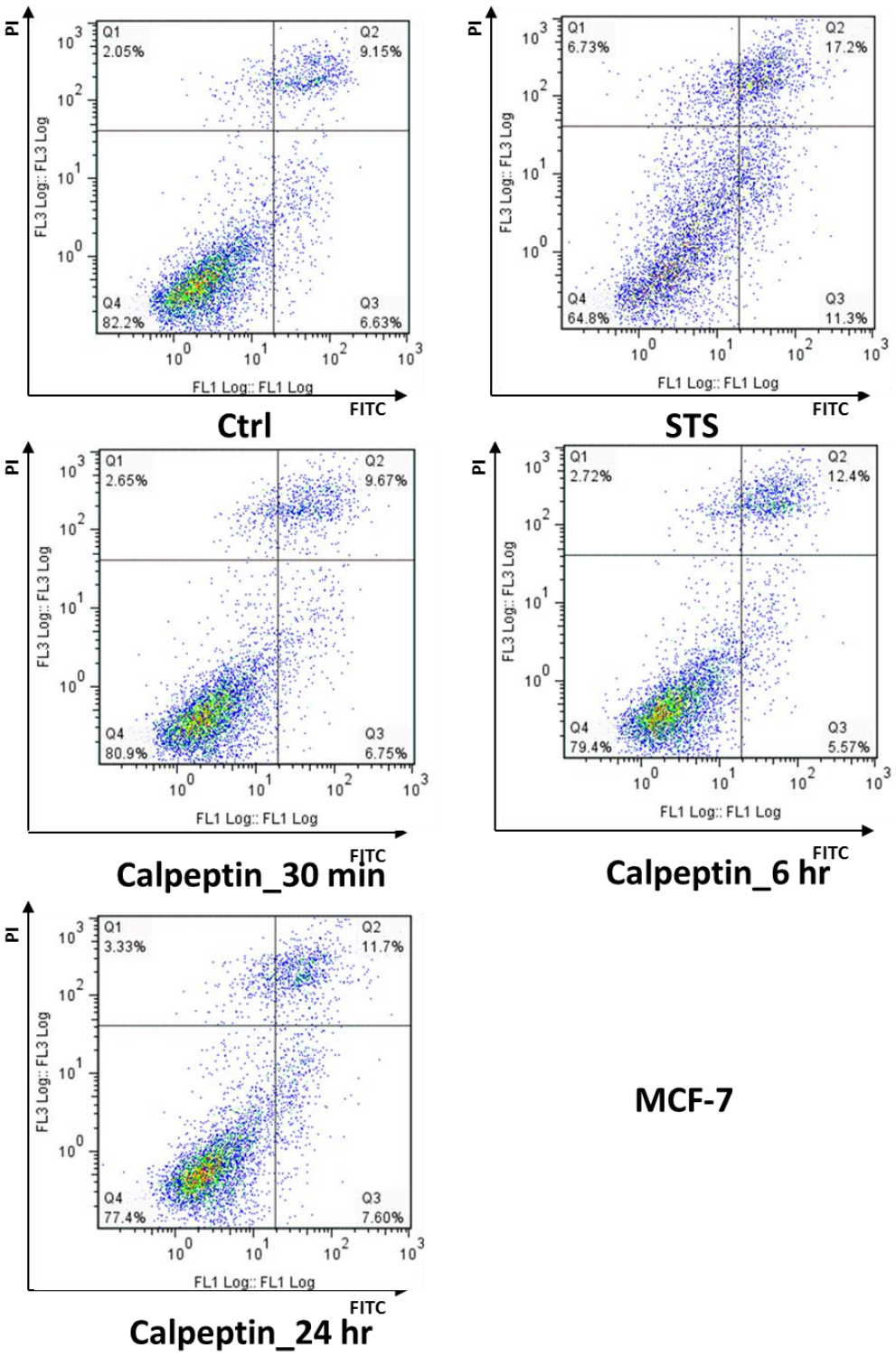

Figure A 2 Effect of calpeptin on apoptosis of MCF-7 cells at different time points. Cells were treated with 100 µM calpeptin for 30 minutes, 6 and 24 hours. 1 µM STS for 24 hours, were set as positive controls. The representative dot plots are shown above.

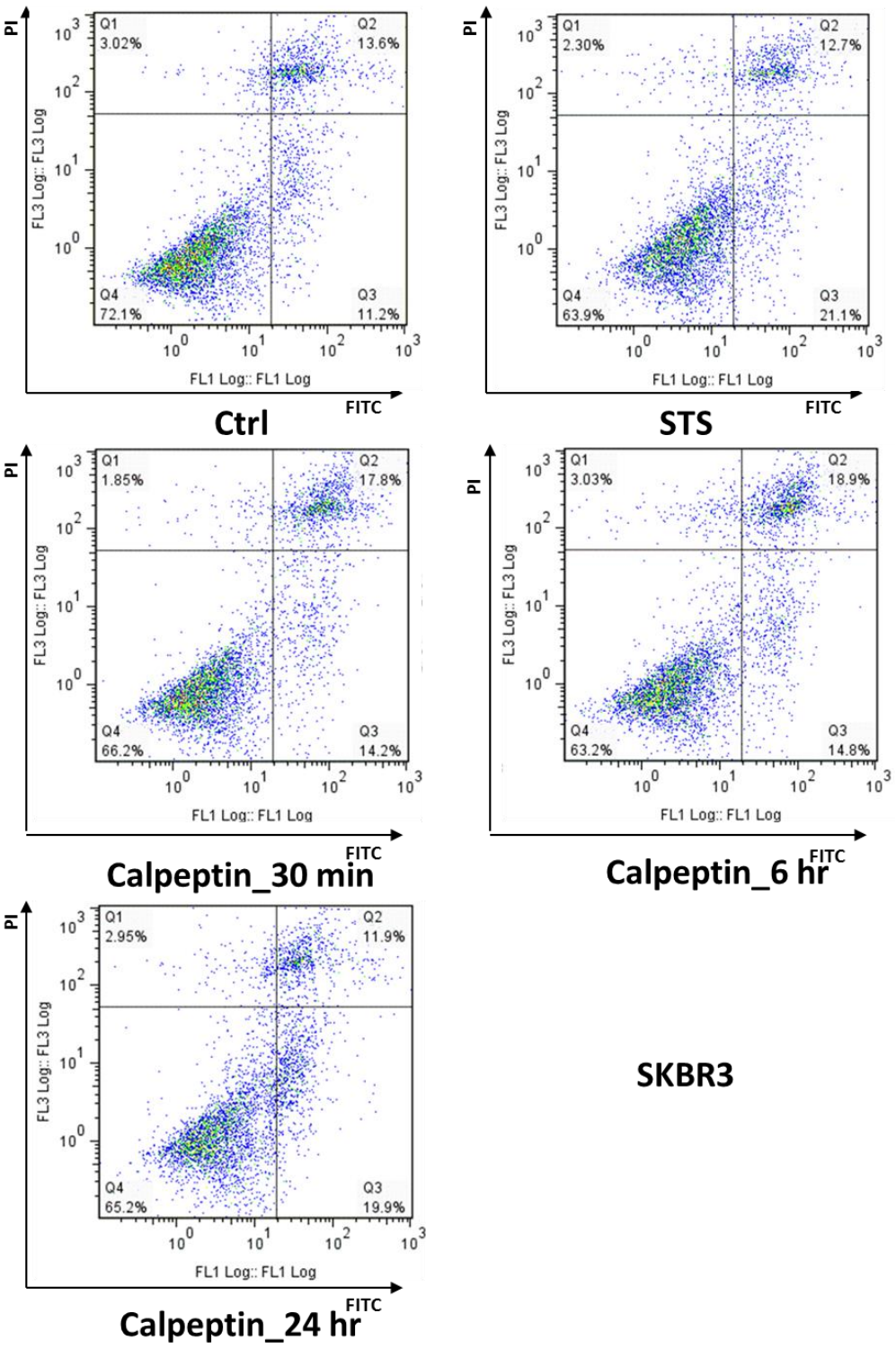

Figure A 3 Effect of calpeptin on apoptosis of SKBR3 cells at different time points. Cells were treated with 100 µM calpeptin for 30 minutes, 6 and 24 hours. 1 µM STS for 6 hours, were set as positive controls. The representative dot plots are shown above.

Table A 1 Kaplan Meier survival analyses for breast cancer-specific survival based upon combinational protein expression

	Calpain-1		Calpain-2		Calpain-9		Calpastatin	
	***P*-value**	**Patients (N)**	***P*-value**	**Patients (N)**	***P*-value**	**Patients (N)**	***P*-value**	**Patients (N)**
Total								
MSK1	0.116	885	0.105	864	0.255	543	0.273	854
Caspase-3	**0.005**	994	**0.049**	998	0.139	589	**0.02**	951
Caspase-8	**0.02**	971	0.056	943	0.072	570	0.085	930
HER2+								
MSK1	0.607	122	0.352	119	0.783	84	0.616	118
Caspase-3	0.575	145	0.855	141	0.248	89	0.244	135
Caspase-8	0.382	142	0.706	138	0.779	86	0.49	133
Triple-negative								
MSK1	0.681	149	0.317	146	0.096	97	0.825	152
Caspase-3	0.109	175	0.292	172	0.205	108	0.654	175
Caspase-8	0.579	166	0.235	163	0.071	103	0.291	166
Basal-like								
MSK1	0.516	184	0.223	181	0.452	114	0.557	182
Caspase-3	**0.034**	197	0.059	193	0.253	118	0.228	194
Caspase-8	0.25	190	0.054	185	0.308	114	0.322	187

Kaplan-Meier survival analyses for breast cancer-specific survival based upon combined protein expression in the total patient cohort and different breast tumour phenotypes. Significance was determined using the log-rank test. Significant *P* values are indicated by bold font.

Appendix B. Supplementary information for Chapter 4

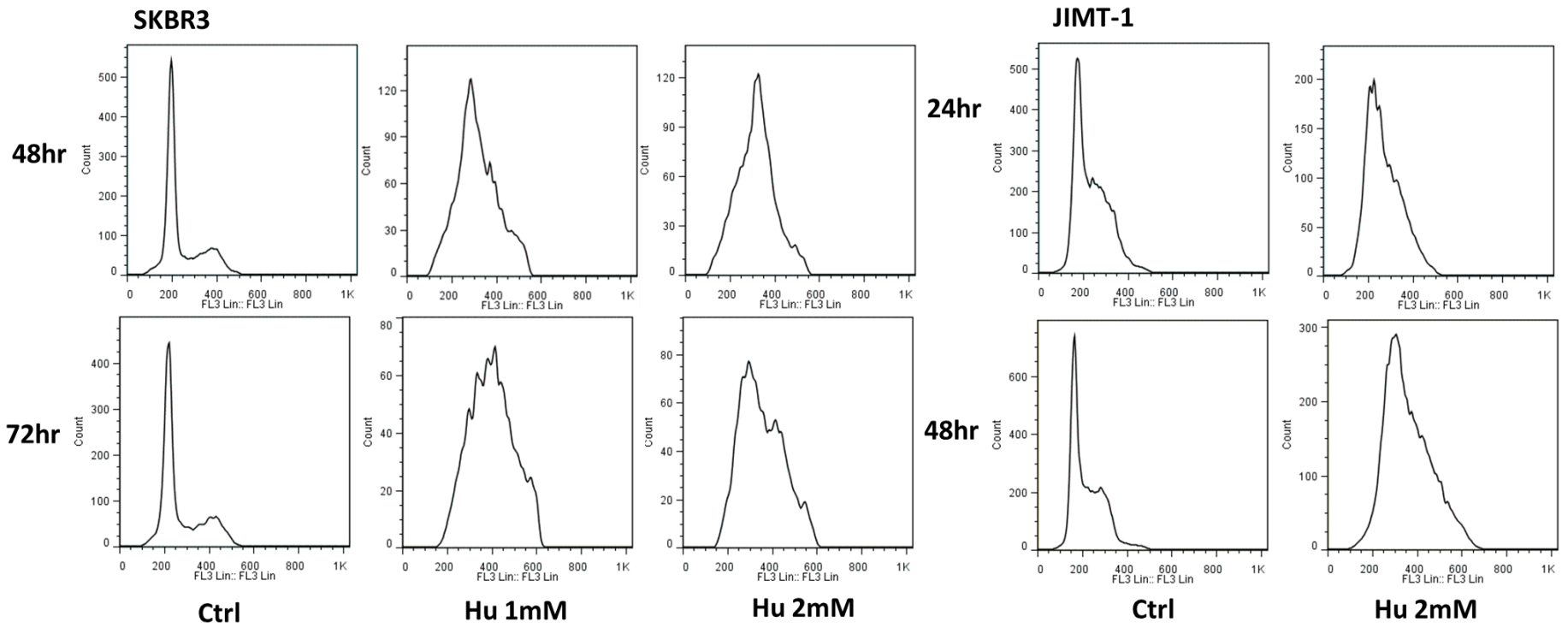

Figure B 1 Optimisation of hydroxyurea as positive control in cell cycle analysis (histograms). SKBR3 were treated with Hu (1 and 2 mM) for 48 and 72 hours. JIMT-1 cells were treated with Hu (2 mM) for 24 and 48 hours. Data was derived from one experiment.

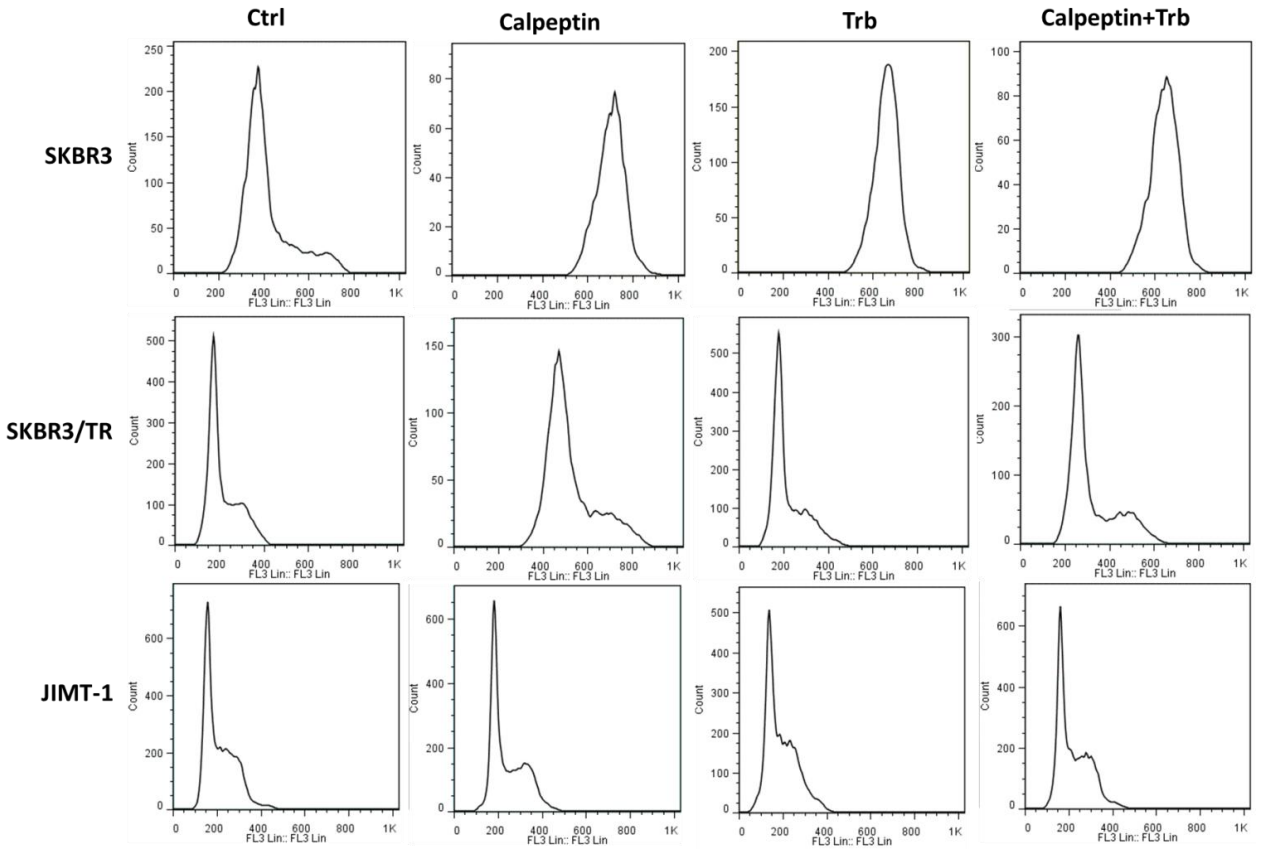

Figure B 2 Effect of drug combinations (calpeptin and trastuzumab) on cell cycle progression (histograms).

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