CALPAIN IN OVARIAN CANCER PROGRESSION AND CHEMOTHERAPEUTIC RESPONSE

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Abstract

The calpain system is associated with cancer chemotherapeutic response in both *in vivo* and *in vitro* studies. Previous immunohistochemistry (IHC) data conducted in our group indicated that high calpain-2 expression was associated with both the resistance to platinum-based adjuvant chemotherapy and worse patient outcome; moreover calpain-2 appeared as an independent prognostic factor in multivariate analysis.

To test the hypothesis that conventional calpain subunits, especially calpain-2, are associated with the chemo-resistance of ovarian cancer cells to platinum-based chemotherapy (cisplatin and/or carboplatin), five ovarian cancer cell lines, with varying platinum-based chemotherapy sensitivities, were chosen as *in vitro* models: the platinum-sensitive A2780 cells and its resistant counterpart A2780-cis cells; the platinum-resistant SKOV3 cells; and the platinum-sensitive PEO1 cells and its platinum-resistant counterpart PEO4 cells. Western blotting was used to assess the expression of the conventional calpain subunits (i.e. calpain-1, -2 and -4) and calpastatin in this panel of cell lines. Calpain activity was regulated by inhibitor calpeptin and calpain-2 short hairpin RNA (shRNA) was used in attempt to specifically downregulate calpain-2 expression. Calpain activity was assessed by an activity assay using fluorogenic peptidase substrate t-BOC. The role of calpain in proliferation and resistance to platinum-based chemotherapy were examined *in vitro* using growth curves and colony formation. Moreover the study of calpain-4 expression was added into the current project, in addition to verifying the association between the expression of calpain-1, -2 and calpastatin and clinicopathologic variables (e.g. chemo-resistance and patient outcome) by standard immunohistochemistry (IHC) with a larger patient cohort (n=575). To test the hypothesis that conventional calpain subunits and calpastatin are associated with ovarian tumour metastasis, the effect of calpain inhibition (by

calpain inhibitors) and activation (by calcium ionophore) on ovarian cancer cell migration was examined using haptotaxis (scratch wound) migration assay. Based on information from 2016 FASEB calpain conference, microtubule-associated protein 4 (MAP4) and spleen tyrosine kinase (Syk) appeared as potential calpain-related proteins associated with angiogenesis and epithelial-mesenchymal transition. Using IHC, their protein expression (i.e. MAP4 and Syk) was assessed and their associations with clinicopathological variables in ovarian cancer patient samples were studied; besides, their correlations with conventional calpain subunits and calpastatin, together with EMT (epithelial-to-mesenchymal transition)-associated proteins and angiogenesis-associated proteins (n=87, data provided by Dr S. Deen) were analysed.

Significant variations of calpain system protein expression levels were observed between the different cell lines. Among the 5 cell lines, A2780 and A2780-cis cells (likely to be endometrioid carcinoma cell lines) expressed very low levels of the conventional calpain subunits and calpastatin; whilst PEO1 and PEO4 cells (high-grade serous carcinoma cell lines) expressed comparatively higher level of these proteins. Thus, different expression of the calpain system seemed to be associated with ovarian cancer histological subtypes, which was supported by the IHC study. No significant difference of the calpain system expression was detected and calpeptin caused a similar inhibition of cell proliferation between chemo-sensitive ovarian cancer cells and their resistant counterparts. Because SKOV3 and PEO4 cells expressed the highest levels of calpain-2, shRNA was used for specific knockdown of calpain 2 in these two cell lines, unfortunately numerous attempts proved unsuccessful. Although with the optimised concentration and treatment duration, calpeptin could inhibit approximately 30% of calpain activity, down-regulation of calpain activity via calpeptin could not sensitise ovarian cancer SKOV3, PEO1 and PEO4 cells to cisplatin and carboplatin. Hence, to revisit the question as to whether conventional calpains and calpastatin are associated with chemoresponse and patient survival, a larger cohort was used

to validate the previous study. In the current study, the expression of the conventional calpain subunits and calpastatin were positively associated with each other. Calpain-2, -4 and calpastatin expression were associated with overall survival (OS) but none of them was an independent marker of OS in multivariate analysis. Neither the conventional calpain subunits nor calpastatin expression was found associated with resistance to platinum-based adjuvant chemotherapy. Since low calpain-1 expression was associated with low tumour stage, cellular processes that involved in cancer spread were studied. Again calpeptin was used for the inhibition of calpain activity, but no significant inhibition was observed on ovarian cancer cell migration. In contrast, upregulating calpain activity by A23187 using optimised concentration was found able to significantly inhibit the migration of SKOV3 cells. The recently found calpain-related proteins MAP4 and Syk were then included into the current study. Like calpain-1, neither MAP4 nor Syk expression was associated with patient outcome. Next, cases were grouped by clinicopathological variables for the examination of the association between the protein expression and survival; in such a case only high nuclear Syk expression was significantly associated with better patient outcome in certain subgroups. Low calpain-1 expression was associated with low tumour stage, so were MAP4 and Syk expression. MAP4, Syk and calpain-1 expression were significantly associated with tumour histological subtypes and their expression were significantly correlated with each other. Integrin α2β3 was moderately correlated with calpain-1, MAP4 and cytoplasmic DARC expression.

In conclusion, although in both the previous and the current study, calpain-2 expression was adversely associated with OS of ovarian cancer patients, the current results did not support the initial hypothesis that calpain can sensitise ovarian cancer cells to cisplatin/carboplatin. The roles that calpain system played in cancer cell haptotactic migration appeared to vary with cell context. Calpain-1, MAP4 and Syk expression were significantly correlated with each other and were closely related to ovarian cancer spread.

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Abbreviations

3D	three dimensional
AIF	apoptosis-inducing factor
AKT2	RAC-beta serine/threonine-protein kinase
ALLM	N-Acetyl-Lue-Lue-Norleu-al
ALLN	N-Acetyl-Leu-Leu-Met-al
ANXA	annexin A-encoding gene
ARID1A	AT-rich interactive domain-containing protein 1A-encoding gene
ATCC	American Type Culture Collection
ATM	ATM Serine/Threonine Kinase
BID	BH3 interacting-domain death agonist
BRAF	human gene that encodes B-Raf protein
BRCA	breast cancer gene
BRIP1	BRCA1-interacting protein 1
CA-125	cancer antigen 125, carcinoma antigen 125, or carbohydrate antigen 125, also known as mucin 16
Calpain	short for calcium-dependent papain-like enzyme
CCC	clear cell carcinoma
CCNE1	endcoding cyclin E1
CDH1	gene that encode cadherin-1 or epithelial cadherin
CHEK2	human gene checkpoint kinase 2
CHD5	chromodomain-helicase-DNA-binding protein 5-encoding gene
CHEK2	checkpoint kinase 2
CI	confidence interval
CMAC	fluorophore 7-amino-4-chloromethylcoumarin
CTNNB1	β-catenin-encoding gene
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DAB	3, 3’-diaminobenzidine
DARC	duffy antigen receptor for chemokines
DBS	donor bovine serum
DDP	cisplatin or cis-diamminedichloroplatinum (II)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECACC	European Collection of Authenticated Cell Cultures
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum

ERK	extracellular signal-regulated kinase
ESMO	European Society for Medical Oncology
FAK	focal adhesion kinase
FASEB	Federation of American Societies for Experimental Biology
FFPE	formalin-fixed paraffin-embedded
FIGO	the International Federation of Gynecology and Obstetrics
FRET	fluorescence resonance energy transfer)-based
GCIG	The Gynecologic Cancer InterGroup
GOG	Gynecologic Oncology Group
HE4	human epididymis protein 4
HER2	human epidermal growth factor receptor 2
HLA-G	human leukocyte antigen G
hMLH1	human mutL homolog 1
hMSH2	human mutS homolog 2
HNF-1β	hepatocyte nuclear factor-1 homeobox β
HR	hazard radio
HRP	horseradish peroxidase
HUVEC	human Umbilical Vein Endothelial Cells
IC50	half maximal inhibitory concentration
ICC	intraclass correlation co-efficient
IGF	insulin-like growth factor
IHC	immunohistochemistry
IκBα	inhibitor of nuclear factor kappa-B kinase subunit alpha
IP	Intraperitoneal chemotherapy
IP3	inositol 1,4,5- trisphosphate
IV	Intravenous chemotherapy
kDa	kilo dalton
KRAS	oncogene first identified in Kirsten rat sarcoma virus
LGALS4	galectin-4-encoding gene
M	molar
MAP	microtubule-associated protein
MARK	microtubule affinity regulating kinase
mL	milliliter
mM	millimolar
MMP	matrix metalloproteinases
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MOPS	3-(N-morpholino)propanesulfonic acid buffer

NF1	neurofibromin 1
NF-κB	nuclear factor-kappa B
OS	overall survival
OGG1	human 8-oxoguanine-DNA glycosylase 1
p21/WAF1	cyclin-dependent kinase inhibitor 1
PALB2	partner and localizer of BRCA2
PARC	p53-associated parkin-like cytoplasmic protein
PARP	poly-ADP-ribose polymerase
PBS	phosphate buffered saline
PE	plating efficiency
PFS	progression free survival
p53	tumour protein p53 (TP53), also known as cellular tumour antigen p53 (UniProt name), phosphoprotein p53, tumour suppressor p53, antigen NY-CO-13, or transformation-related protein 53 (TRP53)
PFI	platinum-free interval
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha- encoding gene
PKA	protein kinase A
PKCiota	protein kinase Ciota
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reserve transcription polymerase chain reaction
REMARK	REporting recommendations for tumour MARKer prognostic studies
Rb	retinoblastoma protein
Rs	Spearman rho
RSF1	Remodeling and spacing factor 1
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SF	surviving fraction
shRNA	short hairpin RNA
Sig	significance
siRNA	small interference RNA
Src	proto-oncogene tyrosine-protein kinase Src
SRB	sulphorhodamine-B
STAT3	signal transducer and activator of transcription 3
STK11	serine/threonine kinase 11
STR	short tandem repeating
Syk	spleen tyrosine kinase

t-BOC	fluorescent substrate CMAC, t-BOC-Leu-Met, 7-Amino-4-Chloromethylcoumarin, t-BOC-L-Leucyl-L-Methionine amide
TMA	tissue microarray
TNF	tumour necrosis factor
UGT1A1	UDP-glucuronosyltransferase 1-1-encoding gene
VEGF	vascular endothelial growth factor
WHO	World Health Organization
µg	microgram
µg/mL	microgram per mililiter
µL	microliter
µM	micromolar
χ2	Pearson Chi Square test of association

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1. Ovarian cancer

1.1.1 Epidemiology and mortality

Worldwide, ovarian cancer is the seventh most common cancer in females and the 17th most common cancer overall with over 238,000 newly diagnosed cases and around 152,000 women died of the disease, as estimated by the GLOBOCAN project in 2012 (Ferlay *et al*., 2013). In the general population, around 1.3% will be diagnosed with ovarian cancer during their lifetime (Howlader *et al*., 2016). The association with high mortality is due to around 75% of patients having progressed into advanced stage (presence of metastatic spread beyond the ovaries, FIGO (the International Federation of Gynecology and Obstetrics) stages III or IV) when they are diagnosed (Dinh *et al*., 2008; Vergote *et al*., 2011). Bloating, pelvic or abdominal pain, difficulty eating/fullness and urinary symptoms, have been listed in the Ovarian Cancer Symptom Index (Brustmann & Naudé, 2002). Difficulties in diagnosis lie in the lack of disease-specific symptoms and the lack of robust biomarkers (Della Pepa *et al*., 2015).

1.1.2 Risk factors

Family history is one of the most significant risk factors for ovarian cancer (Permuth-Wey and Sellers, 2009), with an estimation that around 7% of patients have a positive family history of this disease (Nguyen *et al*., 1994). Prat, Ribe & Gallardo, (2005) reviewed three main groups of family history of hereditary ovarian cancer patients. The first group, breast and ovarian cancer syndrome, can be caused by germline pathogenic variants in BRCA1/2 (breast cancer gene). The second group is hereditary site-specific ovarian cancer syndrome, caused by gene(s) yet to be identified (Woodruff, 2010). The third is lynch syndrome, also known as hereditary nonpolyposis colorectal cancer which is mostly due to germline mutations of the DNA mismatch repair genes

human mutL homolog 1 (hMLH1), human mutS homolog 2 (hMSH2), MSH6 and PMS1 homolog 2 (PMS2) (Nakamura K *et al*. 2014; Weissman *et al*., 2011; Prat, Ribe & Gallardo, 2005). Of all the hereditary epithelial ovarian cancer, around 90% harbour germline mutations in the BRCA1 and BRCA2 genes with most of the remaining cases belonging to the third group (Narod *et al*., 1995; Frank *et al*., 1998; Lee *et al*, 2013). Graffeo *et al*. (2016) reviewed some less known high-penetrance cancer genes carrying mutations (e.g. PTEN, TP53, STK11, CDH1) and moderate-penetrance genes which carry mutations (e.g. PALB2, CHEK2, ATM, NF1, RAD51C, RAD51D, BRIP1) that have the potential to be used as multigene panels to assess cancer risk.

Hormones (e.g. estrogen and progesterone) are considered as risk factors in promoting ovarian carcinogenesis which can be either endogenous or exogenous (e.g. from oral contraceptives or hormone replacement therapy) (Jeon, Hwang & Choi, 2016). Comparing women that have never used oral contraceptives with women who have used them less than 10 years ago, the risk reduction was 29% (95% CI 23-34%); for those who had ceased 10-19 years the risk reduction was 19% (14-24%); and those that ceased 20-29 years the risk reduction was 15% (9-21%) (Beral *et al*., 2008).

Gynaecologic procedures such as prophylactic bilateral oophorectomy, hysterectomy and tubal ligation are also risk factors of ovarian cancer. Elective bilateral oophorectomy can be used for preventing ovarian cancer or for the treatment of benign conditions such as pelvic pain, ovarian cysts or endometriosis (Jacoby *et al*., 2009). It is well established that among high-risk women, bilateral prophylactic oophorectomy reduces their risk of developing ovarian cancer by at least 80-95% (Domchek and Rebbeck, 2007; Chan *et al*., 2014). Numerous research data suggests that either hysterectomy or tubal ligation (without oophorectomy) can reduce the risk of ovarian cancer (reviewed in Permuth-Wey and Sellers, 2009). The protective effect against ovarian cancer seems to last for at least 10–15 years (Permuth-Wey and Sellers, 2009). The protective effect of tubal ligation is around 15% to 25% risk reduction of ovarian cancer (Sieh *et al*., 2013). Bilateral salpingectomy (BS) is

supported by many gynecologic oncologists as an effective way to reduce the risk of ovarian cancer after the understanding of the potential origin of high-grade serous ovarian cancer (HGSC) changing from ovarian capsule to fallopian tube (**Figure 1.1**) (Suh *et al*., 2015). Although lack of direct support from long-term follow-up trial, a large-scale population-based cohort study on women with previous surgery on benign indication suggested that salpingectomy with or without other benign surgery is effective in reducing ovarian cancer risk in the general population (Falconer *et al*., 2015).

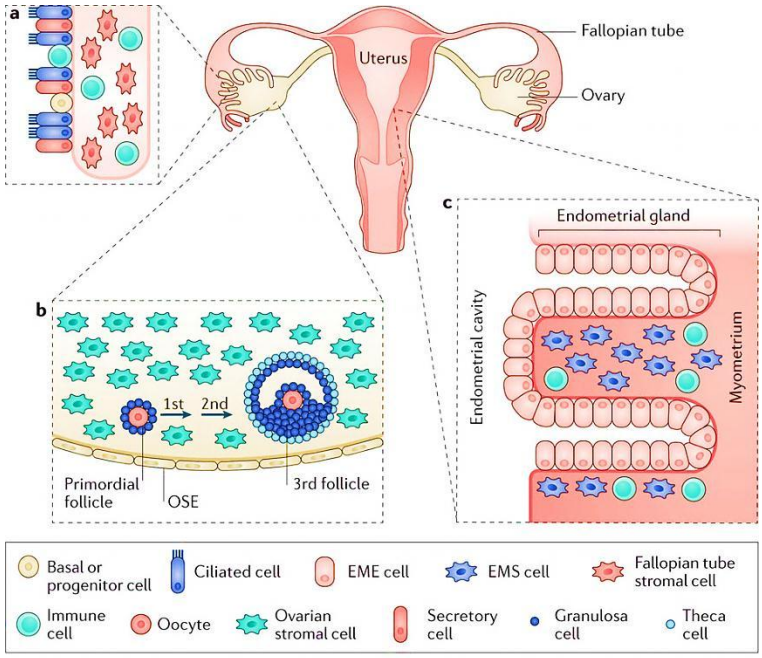


Figure 1.1 Anatomy and biology of the ovary, fallopian tube and uterus.
Reproduced from (Karnezis *et al*., 2017), Nature Reviews Cancer, 17 (1), 65-74; Copyright © 2016, Rights Managed by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

One of the most consistent findings is the effect of full-term pregnancies (parity) on reducing ovarian cancer risk (Sopik *et al*., 2015; Permuth-Wey and Sellers, 2009) knowing that pregnancy can cause the anovulation and suppression of pituitary gonadotropins secretion. Parous women had lower risk of all ovarian cancer subtypes compared with nulliparous women-with the strongest risk reduction observed in clear cell carcinomas (CCCs) and the least risk reduction in the serous cancers (Wentzensen *et al*., 2016). However, it is uncertain whether the late age at first birth would increase or reduce ovarian

cancer risk (Permuth-Wey and Sellers, 2009). The associations between ovarian cancer risk and infertility, breastfeeding and incomplete pregnancies are inconsistent and indeterminate (Permuth-Wey and Sellers, 2009; Webb and Jordan, 2016).

Lifestyle Factors such as cigarette smoking, alcohol consumption, sedentary behaviour (Shen *et al*., 2014; Hildebrand *et al*., 2015), diet, talc fiber (Gertig *et al*., 2000; Webb and Jordan, 2016; Terry *et al*., 2013), coffee and tea in relation to ovarian cancer risk are inconsistent and possibly limited to particular histologic subtypes (Permuth-Wey and Sellers, 2009).

1.1.3 Ovarian cancer biology

1.1.3.1 Histological subtypes

Normal ovary consists of germ cells (i.e. oocytes), epithelial cells, endocrine and interstitial cells (produce oestrogen and progesterone) (Romero & Bast, 2012). The endocrine cells mentioned here are steroidogenic cells such as granulosa cells and theca cells (Havelock *et al*., 2004). The cortex of normal ovary mainly consists of ovarian stromal cells and ovarian follicles (**Figure 1.1**). Ovarian follicles have granulosa and theca cells surrounding the oocytes (Karnezis, *et al*., 2017). The ovarian surface epithelium, a specialised coelomic epithelium, covers the cortex (Karnezis, *et al*., 2017). Ovarian tumours may derive from any of these cell types; specifically, they are germ cell tumours, surface epithelial-stromal tumours and sex-cord-stromal tumours (Romero & Bast, 2012). In addition to germ cell tumours, adult granulosa cell tumour and Sertoli-Leydig cell tumour are of real ovary origin based on the following criterias: anatomically, the existence of solitary tumour in ovary; phenotypically, the histological and immunophenotypic resemblances between tumour and normal ovarian cell type; biologically, via certain mutations a normal ovarian cell type gains the hallmarks of cancer which resemble to a certain tumour (Karnezis *et al*., 2017). Among the primary malignant ovarian tumours, around 90% are epithelial ovarian cancers (carcinomas) (Scully *et al*., 1998; Cho & Shih, 2009). Around 10% of all

ovarian cancers are non-epithelial ovarian cancer (Smith *et al*., 2006) and are reviewed by Colombo N. *et al*. & ESMO Guidelines Working Group (2009; 2012) and Reed N *et al*. & ESMO Guidelines Working Group (2010). In developed countries more than 90% of ovarian cancers originate from epithelium, 2–3% from germ cells and 5–6% from sex cord/stromal cells (Sankaranarayanan and Ferlay, 2006). Undifferentiated or unclassifiable ovarian cancers and borderline tumours each take up about 10% of ovarian carcinoma (Cancer Research UK, 2016a).

Ovarian carcinoma is not a single disease; as a heterogeneous group of neoplasms, ovarian carcinomas are further grouped into the following histological subtypes according to the 2003 World Health Organization (WHO) criteria of ovarian carcinoma (Clarke & Gilks, 2011) which is entirely based on tumour cell morphology (Cho & Shih, 2009). The WHO classification systems have been updated and the 2014 version was reviewed by Meinhold-Heerlein *et al*. (2015), with ovarian, fallopian tube and primary peritoneal cancer being classified uniformly. In addition, subclassification of stage 3 and 4 has been changed and histological subtypes have been simplified as high-grade serous, low-grade serous, mucinous, seromucinous, endometrioid, clear cell and Brenner tumours. Tumours of uncertain malignant potential are known as borderline tumours which contain microscopic features of malignancy but have no tumours appear in surrounding stroma yet (Sankaranarayanan and Ferlay, 2006). The new classification attached more importance to borderline tumors and its role in malignant progression (Meinhold-Heerlein *et al*., 2015). **Table 1.1** listed the description of 2003 WHO critetia of ovarian carcinoma and updates from 2014 classification on malignant type of serous, mucinous, endometrioid and clear cell carcinomas without further address the benign type and borderline tumours. Brenner tumours and newly added seromucinous tumours from 2014 classification were also listed in the table.

Table 1.1 WHO criteria classification

Histological subtypes	description of histological subtypes from 2003 WHO criteria and updates from 2014 WHO classification systems
Serous carcinoma	"composed of cells ranging in appearance from those resembling fallopian tube epithelium in well-differentiated tumours to anaplastic epithelial cells with severe nuclear atypia in poorly differentiated tumours" (Clarke & Gilks, 2011). Tumours with invasive implants are now classified as LGSC. Tumour with negative p53 immunohistochemical staining is classified LGSC whilst tumour with positive p53 staining is classified HGSC (Meinhold-Heerlein et al. 2015).
Mucinous carcinoma	"in its better differentiated areas resembles intestinal or endocervical epithelium" (Clarke & Gilks, 2011). With the presence of mucinous tumours, the importance of considering metastasis was highlighted in the 2014 classification, "even if no primary (extragenital) tumour has been identified". (Meinhold-Heerlein et al. 2015).
Endometrioid carcinoma	"closely resembles the common variant of endometrioid carcinoma of the uterine corpus" (Clarke & Gilks, 2011). "It is important to consider the possibility of metastasis when evaluating lesions presenting as endometrioid tumours" (Meinhold-Heerlein et al. 2015).
Clear cell carcinoma	"composed of glycogen-containing clear cells and hobnail cells and occasionally other histological types" (Clarke & Gilks, 2011). "CCCs are irregular, with papillary structures, solid parts with desmoplastic hyalinized stroma and high-grade nuclear atypia" (Meinhold-Heerlein et al. 2015).
Brenner tumours	previously entitled as transitional cell tumours which is defined as "composed of epithelial elements histologically resembling malignant urothelial neoplasms and does not have a component of benign or borderline Brenner tumour" (Clarke & Gilks, 2011). Intraepithelial carcinoma is also included in this category now (Meinhold-Heerlein et al. 2016). Brenner tumours are "characterized by transitional cell differentiation and display focal, high-grade nuclear atypia" whilst its invasive malignant type is "characterized by destructive stromal invasion of unspecified morphology." (Meinhold-Heerlein et al. 2015).
Seromucinous tumours	they are characterized by "an admixture of various cell types including endocervical-type mucinous, endometrioid and squamous type epithelium, an immunophenotype which is "müllerian" and clinical and molecular features demonstrating a close relationship with endometriosis similar to that of endometrioid and clear cell tumours."(Kurman & Shih, 2016)

Shih and Kurman (2004) proposed a simplified two-type model for epithelial ovarian cancer (Kohn & Hurteau, 2013) (**Table 1.2**). At clinicopathological, cellular, and molecular levels, this model classified ovarian carcinomas into two broad groups as type I and type II cancers, with histological grade, molecular phenotype and genotype as important distinguishing factors (Landen, Birrer and Sood, 2008; Shih & Kurman, 2004;

Cho & Shih, 2009; Bast, Hennessy &Mills, 2009). Type I cancers include the histotype of low-grade serous carcinoma (LGSC), low-grade endometrioid carcinoma, mucinous carcinoma and clear-cell carcinoma (CCC); whilst type II cancers include HGSC, high-grade endometrioid carcinoma and undifferentiated carcinoma (Shih & Kurman, 2004). Type II cancers are generally classified as HGSC by most pathologists, moreover, since malignant mixed mesodermal tumours (carcinosarcomas) have epithelial components which are identical to the pure type II carcinomas, they are also grouped into type II tumours (Kurman & Shih, 2010). Approximately 75% of all epithelial ovarian carcinomas are type II cancers (Kurman & Shih, 2010). The morphologic differences among type I tumours are more clear-cut and distinctive, unlike type II tumours which are ambiguous (Kurman & Shih, 2010).

Mostly, type I cancers are diagnosed during stage I to II with low-grade, slow growing (although large, tumours usually are confined to the ovary at diagnosis), low mitotic index, and resist platinum-based chemotherapy but may respond to hormonal treatment; in contrast, type II cancers are diagnosed during stage III to IV with high-grade, growing aggressively and responding to conventional chemotherapy but less often to hormonal manipulation (Romero & Bast, 2012; Cho & Shih, 2009; Kohn & Hurteau, 2013). Type II cancers have uniformly poor outcomes (Matulonis *et al*., 2011; Merritt & Cramer, 2010; Vang, Shih & Kurman 2009).

Table 1.2 Histology, precursors and distinctive molecular features of epithelial ovarian cancer

Histology (Type)	Possible Precursors	Molecular alterations that were observed
Low-grade serous carcinoma (I)	Appear to grow from serous borderline tumours which derived from endosalpingiosis or papillary tubal hyperplasia (Karnezis *et al*., 2017). With same origin as HGSC which is fallopian tube secretory epithelial cell or progenitor cell (Karnezis *et al*., 2017)	▲Often have a normal karyotype and wild-type *TP53* and *BRCA1/2*. They are genetically stable (Bell 2005); ▲Approximately two thirds have mutations in *KRAS*, *BRAF*, and *HER2* genes; ▲May have the expression of IGF receptor; ▲May have high level of *p21/WAF1* gene; ▲Frequent deletion of sub-chromosomal regions harboured genes *CHD5* and *miR-34* (genes of potential tumour suppressors).
Low-grade endometrioid carcinoma (I)	Appear to grow from serous borderline tumours which derived from endometriosis (Nezhat *et al*. 2008)	▲Mutations that deregulate the Wnt signalling pathway (i.e. Wnt/β-catenin and/ or PI3K/Pten signalling) involving 1) somatic activating mutations of *CTNNB1*, which encoding β-catenin and *CTNNB1* mutations was found in around one-third of the cases (Moreno-Bueno *et al*. 2001; Francis-Thickpenny *et al*., 2001), 2) inactivating mutations and epigenetic silencing of *PTEN* and 3) activating mutations of *PIK3CA* that up-regulate PI3K signalling. ▲Lack TP53 mutations; ▲Microsatellite instability; ▲Loss of heterozygosity; ▲The expression of *ANXA4* and *UGT1A1*genes which are associated with chemo-resistance; ▲Inactivating mutations of *ARID1A*, a chromatin-remodelling gene.
Mucinous carcinoma (I)	Some may come from gastrointestinal tract (i.e. colon, appendix, stomach) and metastasis to the ovary (Lee & Young 2003, Kelemen & Kobel 2011, Zaino *et al*. 2011); others appear to grow from Brenner tumour, teratoma, endometriosis (Karnezis *et al*. , 2017) or mucinous borderline Tumours which derived from mucinous cystadenoma of ovary	▲KRAS is mutated in 60% of specimens (Shih & Kurman, 2004); ▲Without the involvement of BRCA mutations in mucinous carcinoma development (Palmirotta *et al*., 2017); ▲Expression of mucin 2, mucin 3 and mucin 17; ▲Expression of genes that encode caudal type homeobox transcription factors CDX1 and CDX2; ▲Expression of gene LGALS4; ▲KRAS and LGALS4 also expressed in the possible processors of mucinous carcinoma.

Data adapted from (Shih & Kurman, 2004; Cho & Shih, 2009; Romero & Bast, 2012; Kuo *et al*., 2009)

Table 1.2 Continued.

Histology (Type)	Possible Precursors	Molecular alterations that were observed
Clear-cell carcinoma (I)	Appear to grow from borderline tumours which derived from endometriosis (Nezhat *et al*. 2008)	▲ Inactivating mutations of ARID1A, a chromatin-remodelling gene; ▲5-16% occurrence of *KRAS* mutations (Shih & Kurman, 2004), low occurrence of *BRAF* and *TP53* mutations; ▲Often have high percentage of *PIK3CA* activating mutations and *PTEN* mutations/loss that deregulate PI3K/Pten signalling (Palmirotta *et al*., 2017); ▲Some genes that are preferentially expressed, especially HNF-1β; ▲The expression of ANXA4 and UGT1A1 genes; ▲About 66% occurrence of TGF-β mutations (Moreno-Bueno *et al*. 2001; Francis-Thickpenny *et al*., 2001); ▲Microsatellite instability; ▲Loss of heterozygosity; ▲The gene expression patterns are similar between clear-cell and low-grade endometrioid carcinomas, which are also shared with their common origin, endometriosis.
HGSC (II)	Appear to grow from cortical inclusion cysts, the ovarian surface epithelium (Bell 2005, Crum *et al*., 2007) and/or from pre-invasive lesions in the distal fallopian tube (i.e. fimbrial end of the fallopian tubes) designated "serous tubal intraepithelial carcinoma (STIC)" rather than having an "ovarian" origin, based on both pathological and gene expression study (Kurman RJ., 2013; Erickson, Conner & Landen, 2013)	▲As the prototypic type II tumour, *TP53* mutations occurred in 50- 80% of all cases (Chan *et al*., 2000; Kohler *et al*., 1993; Kupryjanczyk *et al*., 1993; Berchuck & Carney, 1997; Wen *et al*., 1999); ▲BRCA1/2 (germline or somatic) mutations and epigenetic inactivation of BRCA1; ▲BRAF and KRAS gene mutations rarely occur; ▲Frequent amplification of sub-chromosomal regions harbour gene CCNE1 (endcoding cyclin E1), NOTCH3, RSF1, AKT2 and PIK3CA (these genes associate with cell growth, proliferation, survival, motility, genetic instability and drug resistance); ▲The overexpression of HLA-G gene; ▲Dysfunction of the Rb pathway; ▲Around 20-67% amplification of HER2/neu (Ross *et al*.,1999); ▲Around 12-18% mutation of AKT2 (Cheng *et al*., 1992; Bellacosa *et al*., 1995).
High grade of endometrioid carcinoma (II)	Appear to grow from epithelial inclusion cysts or endometriotic lesions (Nezhat *et al*. 2008).	▲Often have TP53 mutations, but lack Wnt/β-cat and/ or PI3K/Pten signalling defects; ▲ BRCA1/2 (germline or somatic) mutations and BRCA1 methylation; ▲Activating mutation of CTNNB1.
undifferentiated carcinoscoma (II)	\	undifferentiated carcinoscoma /malignant mixed mesodermal tumour often have TP53 mutations

1.1.3.2 Grading and staging systems

Grading systems divide ovarian carcinomas based on the degree of differentiation. Current grading systems include histotype-specific systems and universal grading systems (Malpica, 2008). Grading systems that have commonly been applied historically include the World Health Organization (WHO) system (Rosen DG *et al*., 2010) and the Gynecologic Oncology Group (GOG) system (Silverberg, 2000). Most of the grading systems are universal such as the Shimizu-Silverberg and the International Federation of Gynecology and Obstetrics (FIGO) that can be applied to all histological types of ovarian carcinomas (McCluggage, 2011). The Shimizu-Silverberg system is based on the Nottingham grading system for breast carcinoma and assesses three parameters: 1) the degree of nuclear atypia, 2) the mitotic count and 3) the architectural features including the amount of glandular, papillary or solid growth (Silverberg, 2000). A score of 1–3 is given to each parameter and the grade is the summation of each score (Silverberg, 2000). The FIGO 3-tier grading system is mainly categorised according to tumour architectural features (Clarke & Gilks, 2011); grades 1, 2, and 3 correspond to percentages <5%, 5–50%, and >50% solid tumour growth which represent the areas of glandular or papillary structures as the proportion of the areas of solid tumour growth (Cho & Shih, 2009). Although many pathologists chose to apply one of the universal grading systems, increasing tendency points to the application of the histotype-specific grading systems (Wilkinson & McCluggage, 2008). The grading systems for ovarian carcinoma were previously thought to have prognostic significance (Clarke & Gilks, 2011), but there are limitations. For example, the grading scale is different for each tumour type, and the scoring is subjective without guaranteed reproducibility (Connolly JL *et al*., 2003; McCluggage, 2011). It is therefore still necessary to discover additional histopathological features or biomarkers (Clarke & Gilks, 2011).

Ovarian cancer stage is assessed to understand the extent of cancer and the prognosis of the patient, based on which a treatment decision is made.

The International Federation of Gynaecology and Obstetrics (FIGO) ovarian cancer staging system (**Table 1.3**) considers the following factors: primary tumour size, localised invasion, the presence of cancer cells in lymph nodes and distant metastasis. Non-epithelial ovarian cancers generally share the FIGO staging system with epithelial ovarian cancer (Colombo *et al*., 2012). Similarly, the TNM system stages ovarian cancer according to primary tumour size (T), the number of lymph nodes spread with tumour cells (N), and the presence of metastasis (M) or secondary tumours formed by cancer cells distributed to distant area of the body (Tavassoli & Devilee, 2003; Prat & FIGO Committee on Gynecologic Oncology., 2014).

Table 1.3 FIGO staging and corresponding TNM system

Stage	Definition	TNM
Stage I	The tumour is confined to the ovaries or fallopian tube(s)	T1-N0-M0
Stage II	The tumour involves one or both ovaries or fallopian tube(s) and the pelvic organs or primary peritoneal carcinoma	T2-N0-M0
Stage III	The tumour involves one or both ovaries or fallopian tube(s) or primary peritoneal carcinoma and has macroscopic tumour growth within and beyond the pelvis to the peritoneum and/or retroperitoneal lymph node metastases (verified cytologically or histologically).	T1/2-N1-M0
Stage IV	The tumour involves one or both ovaries accompanied by distant metastasis excluding peritoneal or retroperitoneal nodal metastases below the diaphragm.	Any T-Any N-M1

Changes between FIGO 2003 and FIGO 2014 are marked in red. Reproduced from (Mutch & Prat 2014), Gynecologic Oncology, 133(3):401-404; Copyright© 2014 Elsevier Inc. with permission conveyed through Copyright Clearance Centre Inc.

1.2. Treatment of ovarian cancer

1.2.1 Surgery and chemotherapy

1.2.1.1 Conventional therapies

Debulking surgery and platinum-based chemotherapy are the standard treatment of ovarian cancer (Griffiths and Fuller, 1978). The integration of surgical cytoreduction and intravenous (IV) platinum-based (platinum and platinum–taxane) chemotherapy is the cornerstone of ovarian cancer treatment (Kwa & Muggia, 2014). Surgery always plays an important role in initial therapy (Ozols RF., 2006). The use of ascitic fluid, serum CA125 levels

and imaging before chemotherapy cannot replace tissue biopsy which can provide information for tumour typing, excluding a metastasis from other sites and for future studies and research (McCluggage, 2011). For early disease, diagnosis and staging of ovarian cancer require surgical resection of the tumour and the organs affected by the tumour; for advanced disease, surgery is essential for staging and cytoreduction (Kwa & Muggia, 2014). Before anti-cancer treatment, the following factors of ovarian cancer are important in deciding the therapeutic strategy: histological subtype, grade of differentiation, age, residual disease after primary debulking surgery and cancer stage which is often assessed at primary surgery following the FIGO classification (Pignata S. *et al*., 2011). The aim of primary debulking surgery is to achieve the absence of residual disease. As for chemotherapy, it is used as adjuvant treatment or neoadjuvant therapy for patients with advanced disease, with further chemotherapy being required for those patients that encounter a relapse after front-line treatment (Pignata S. *et al*., 2011).

Cisplatin was introduced into ovarian cancer treatment in the early 1980s. In the early 1990s, with the discovery of a component extract from the bark of the Pacific Yew tree, Taxus brevifolia, paclitaxel was added to ovarian cancer treatment (Pignata S. *et al*., 2011). Carboplatin and cisplatin improved the overall survival (OS) and disease free survival of ovarian cancer patients to a better extent than any other chemotherapeutic drug (Neijt J.P. *et al*., 1991). These platinum containing compounds are alkylating agents that disrupt the DNA structure by forming intra- and inter-strand cross-links (Wong & Giandomenico 1999; Wang & Lippard, 2005). Whilst such DNA damage is recognised as the main mechanism of action, additional mechanisms have also been recognised such as the generation of free radicals and oxidative stress (Masuda, Tanaka & Takahama, 1994; Woolston *et al*., 2010). However, while killing the proliferating cancer cells, cisplatin also brings severe damage to normal cells and causes a generalised toxicity (Ganta *et al*., 2014). Due to the significant toxicity (mainly peripheral neurotoxicity, hematological and renal toxicity, Piccart *et al*., 2000; Muggia *et al*., 2000), cisplatin-paclitaxel

combination has been replaced by the carboplatin-paclitaxel combination which can achieve similar curative effects as cisplatin-paclitaxel (Ozols RF., 2006) and is comparatively less toxic and easier to administer (Pignata S. *et al*., 2011). Currently, the conventional first-line chemotherapy for ovarian cancer is the combination of carboplatin-paclitaxel. The Gynecologic Cancer InterGroup (GCIG) recommended to administer paclitaxel (175 mg/m²) intravenously (i.v.) for over 3 hours, followed by intravenously infusing carboplatin over 30–60 min at an adjusted dosage to ensure an area under the plasma concentration–time curve (AUC) of 5–6 mg/mL per minute and then repeat the above process every 3 weeks for six cycles (Thigpen *et al*., 2011).

Advanced ovarian cancer tends to recur within 3 years and relapsed ovarian cancer is rarely curable which leads to low 5-year survival rates (Chien *et al*., 2013). Chemotherapy resistance which can be either intrinsic (primary) or acquired (secondary) is a major factor in influencing the observed cancer-associated mortality. To help clinical management, ovarian cancer patients can be broadly divided into three groups, mainly according to the clinical evidence of their initial response to platinum-based chemotherapy (Pfisterer & Ledermann, 2006). The time course between the last round of chemotherapy and the relapse is termed the platinum-free interval (PFI) or treatment-free interval. The three groups are platinum-refractory, platinum-resistant, and platinum-responsive patients. 1) Platinum-refractory patients show no sign of response to platinum-based therapy and show progression during the course of therapy (Chien *et al*., 2013). 2) From empirical evidence, if patients show an initial response to chemotherapy but their PFI is less than 6 months, then they are defined as platinum-resistant (Banerjee, Bookman & Gore, 2011). 3) If the PFI is above 6 months, the patients are classified as platinum-responsive. The platinum-responsive groups are further subdivided into platinum-sensitive patients (PFI≥12 months) and partially sensitive patients (6 months≤PFI≤12 months) (Pignata er al., 2011). In some studies, group 1 and 2 are counted as platinum resistant (PFI≥6 months) whilst group 3 as platinum sensitive (PFI≥6 months) (Luvero, Milani &

Ledermann, 2014). Platinum-sensitive recurrent ovarian cancer patients account for the majority of ovarian cancer patients (Chien *et al*., 2013).

For the improvement of treatment responses, and to overcome chemo-resistance to first-line paclitaxel–platinum treatment, different adjustments are applied using various combinations, dosages and schedules of chemotherapy agents (Yap, Carden & Kaye, 2009). For years, different drugs (e.g. topotecan, pegylated liposomal doxorubicin and epirubicin) and their different combination with platinum-based chemotherapy in doublet or triplet have been studied to improve the prognosis of ovarian cancer (Pignata S. *et al*., 2011). But clinical responses remain poor and currently no agreement has been reached on the maintenance therapy after the initial platinum–taxane doublet chemotherapy treatment (Yap, Carden & Kaye, 2009).

1.2.1.2 Neoadjuvant chemotherapy followed by interval debulking and primary debulking surgery followed by chemotherapy.

Primary cytoreductive (or debulking) surgery is defined as an operation that intended to remove the primary tumour and its metastases as much as possible before adjuvant treatment (Berek *et al*., 1999). Adjuvant treatment is any treatment performed subsequent to surgical removal of all macroscopic residual tumours to eradicate any microscopic residual tumour that may remain after surgery in order to minimise the risk of recurrence (Winter-Roach, Kitchener & Lawrie 2012). Neoadjuvant treatment is the treatment adopted as the initial attempt to reduce the extent of disease or improve patient performance status before the initial surgical cytoreduction (Pecorelli *et al*. 2002). Interval cytoreductive surgery (or interval debulking surgery) is defined as an operation performed after a short course (usually three cycles) of neoadjuvant chemotherapy (Berek *et al*., 1999). Although clinical trials have not offered enough evidence to show improvement of survival outcome, interval cytoreduction has commonly been adopted into treatment practice (Pecorelli *et al*. 2002).

Aure, Hoeg & Kolstad (1971), Griffiths (1975) and colleges, and Fuller

(1978) showed that the extent of cytoreductive surgery and the volume of residual tumour after primary surgery are among the most important prognostic factors of advanced ovarian cancer treatment(Chi *et al*., 2001; Della Pepa C. *et al*., 2015). Therefore, aiming for leaving no residual tumour, the following questions need to be answered; when is the best time for surgery (before or after chemotherapy) and how are patients selected for either primary debulking or interval debulking surgery? The standard care for advanced ovarian cancer has been primary debulking surgery. Neoadjuvant chemotherapy following interval debulking surgery was applied in two clinical trials (EORTC55971 trial and CHORUS trial), both included stage IIIC or IV epithelial ovarian cancer patients, which suggest that neoadjuvant chemotherapy is not inferior to primary debulking surgery (Vergote *et al*., 2010; Kehoe *et al*., 2013). Neoadjuvant chemotherapy has been recommended by some researchers as a better alternative treatment option for patients with poor general condition, advanced disease or cancer with a very high metastatic tumour load, because beginning with surgical cytoreduction will be too aggressive and cause unnecessary post-operative morbidity and mortality (Vergote *et al*., 2011). It has also been suggested that gynaecologic oncologists should estimate the extent of disease (with consideration of predictive factors including co-morbidities, age, tumour burden and stage, location of metastases, performance status etc.) before deciding on primary debulking or neoadjuvant chemotherapy; currently, chemotherapy following primary debulking is still the standard treatment for stage IIIb and below (Vergote *et al*., 2011; Della Pepa C. *et al*., 2015).

1.2.1.3 Intravenous (IV) and Intraperitoneal chemotherapy (IP)

About 70–80% of women with advanced ovarian cancer develop subsequent relapse or further progression, despite their initial response to platinum-based first-line therapy (Kwa & Muggia, 2014). Conventionally intravenous (IV) chemotherapy is applied for the treatment of ovarian cancer, however, in more than 85% of ovarian cancer cases, the dissemination of cancer remains restricted to the peritoneal cavity throughout its course, so

delivery of cytotoxic agents in a locoregional way, via intraperitoneal (IP) chemotherapy, is considered a promising therapeutic option for advanced ovarian cancer and has been assessed in clinical trials (Zeimet AG *et al*., 2009). Completed and on-going clinical trials have investigated IP, such as GOG114 (Alberts *et al*., 2002), GOG172 (Tewari *et al*., 2015), GOG252, iPocc Trial (GOTIC-001/JGOG-3019) and OV-21/GCIG which are reviewed by Della Pepa C. *et al*. (2015) and Fujiwara (2012). Another novel loco-regional treatment approach for treating peritoneal surface malignancies is "optimal cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC)" (Kwa & Muggia, 2014). Use of IP is limited by the toxicity (side-effects and quality of life), cost effectiveness, technical difficulties associated with implementation (e.g. insertion and management of IP catheter) and the weaknesses of data from clinical trials, (Zeimet AG *et al*., 2009) - as such IV is still currently preferred in most countries (Pignata S. *et al*., 2011; Palmirotta *et al*., 2017).

1.2.1.4 Dose-dense chemotherapy

Alternative schedules derived from the "dose-density" concept have been explored without changing the doublet used in the conventional tri-weekly regimen (**section 1.2.1.1**). The concept is based on the Norton-Simon regression hypothesis (proposed in the 1970s) which suggests the cytoreduction/regrowth rate between treatments is proportional to the tumour growth rate at the time of treatment. So to reduce tumour burden and improve cytotoxic therapeutic effect the interval between treatment cycles should be shortened to minimise the opportunity for tumour regrowth (Katsumata N., 2011; 2015). Increased dose density is achieved by reducing the interval between each dose of chemotherapy to retain the constancy of cumulative drug dose (Katsumata N., 2011). This concept has been translated into the 'dose-dense therapy' strategy and applied in the administration of chemotherapy (Katsumata N., 2011; 2015). Unfortunately dose-dense therapy has not significantly prolonged progression-free survival (PFS) compared with tri-weekly regimens among patients treated with bevacizumab based on

several trials, such as JGOG 3016 trial (Chan *et al*., 2016; Tanday, 2016), GOG 262 (Suh *et al*., 2014), MITO-7 (Della Pepa C. *et al*., 2015). Hope is placed on future studies and ongoing trails (e.g. ICON8 and ICON8b) to obtain explicit "dose, schedule, and route of administration" (Katsumata, 2015).

1.2.2 Chemo-resistance and resistance mechanisms

Platinum drugs are, in addition to ovarian cancers, applied to a variety of cancer types including testicular carcinomas, melanoma, lung cancer, lymphomas and myelomas (Apps *et al*., 2015). In spite of their success, there are two main limitations: 1) severe side effects caused by indiscriminately attacking all rapidly dividing cells; 2) the development of drug resistance which is partially due to the sub-lethal doses of platinum drug applied for patients to limit the side effect whilst leaving the cancers a chance to develop resistance (Apps *et al*., 2015). The complicated side effects include nephrotoxicity and ototoxicity while the therapy-limiting side effects include the high occurrence of nausea and vomiting (Eckstein, 2011). Other common side effects are diarrhoea, myelosuppression, neuropathy and hepatotoxicity (Apps *et al*., 2015).

Ovarian cancer chemoresponse changes with different histological subtypes: HGSC patients have a better response rate to first-line chemotherapy comparing to CCCs, mucinous and LGSCs. Overall, the response rates in ovarian cancer to paclitaxel plus platinum-based chemotherapy varies from approximately 70% to 80% (Bookman *et al*., 1996; McGuire *et al*., 1996; du Bois *et al*., 1997; Ozols *et al*., 2003). Studies conducted by Pectasides *et al*. (2005, 2006) and Sugiyama *et al*. (2000) yielded similar response rates of 70%, 81%, and 72.5% respectively in patients with serous carcinomas (100%, 100% and 78% at an advanced stage) to primary platinum-based chemotherapy. Divided by tumour grade, the response rate was 52% in low grade serous ovarian carcinoma patients (Gershenson *et al*., 2006) and 85% in high-grade serous ovarian carcinoma patients (Cooke and Brenton, 2011). In comparison, the response rate of ovarian clear-cell carcinoma (CCC) patients to

platinum-based chemotherapy was 11.1% (Sugiyama *et al*. 2000). Specific to advanced CCC, data from Goff *et al*. (1996) and Pectasides *et al*. (2006) showed that 48% and 45% of the patients responded to primary chemotherapy respectively. The overall response rate for advanced stage mucinous carcinoma was 38.5% (Pectasides *et al*., 2005) and 42% (Pisano *et al*. 2005). In a retrospective analysis conducted by Hess *et al*. (2004), the response rate to platinum-based therapy was 26% and 65% in patients with advanced stage mucinous ovarian cancer and matched non-mucinous carcinoma respectively. The comparatively higher response rate in HGSC patients to chemotherapy may be due to BRCA-1/-2 mutations that are harboured in the HGSC patients bringing a significant increase in response rate and a longer survival (Zhong *et al*., 2015).

The identified possible molecular mechanisms behind platinum-based chemo-resistance include: 1) the reduction of intracellular drug accumulation and diminished formation of drug-DNA adducts caused by decreasing drug influx and/or increasing efflux from cells via transmembrane transport alterations and/or reduced drug endocytosis; 2) cell growth-promotion, epithelial-to-mesenchymal transition (EMT) or the secretion of protective cytokines and soluble factors which facilitate survival and growth; 3) increased tolerance to DNA damage and enhanced DNA damage repair pathways (nucleotide excision repair and mismatch repair) by altered gene (e.g. transcription factors, tumour suppressor gene, oncogenes) expression levels via abnormal genetic or epigenetic changes (e.g. chromatin, DNA-methylation, histones) (e.g. BRCA secondary mutations (Swisher *et al*., 2008) and loss of BRCA promoter methylation (Dhillon *et al*., 2011) were found in association with platinum-resistance); 4) Increased degradation and detoxification of the drugs inside the cells by altering redox homeostasis antioxidants and radical scavengers such as glutathione (Balch *et al*., 2004; Galluzzi *et al*., 2012, 2014; Shen *et al*., 2012; Alkema *et al*., 2016; Patch *et al*., 2015; Castells *et al*., 2013; Woolston *et al*., 2010; Marengo *et al*., 2016). However, none of the above has lead to any clinical application.

Numerous studies have focused on adding other therapeutic agents to the conventional regimen. Improved understanding of the underlying mechanisms of ovarian cancer has set the direction for the development of molecular targeted therapies. Many investigations are focusing on small-molecule inhibitors and monoclonal antibodies which target crucial molecular pathways that drive carcinogenesis and cancer progression via angiogenesis (e.g. Pazopanib, Cediranib and Bevacizumab, reviewed in Han *et al*., 2013; Aravantinos & Pectasides, 2014; Conteduca *et al*., 2014), cell cycle progression and proliferation, survival and apoptosis (e.g. DNA repair inhibitors, Brown *et al*., 2016; anti-hormone drugs, Modugno F *et al*., 2012), protein synthesis driving cell growth and metastases (Yap, Carden & Kaye, 2009; Syrios, Banerjee & Kaye. 2014).

Ovarian cancer is often classified as an immunogenic disease with almost half of advanced ovarian cancer patients identified with spontaneous anti-tumour immune response (Vaughan *et al*., 2011). In certain solid tumours (e.g. melanoma), cancer immunotherapy has been suggested as a novel effective tool for clinical treatments (Rosenberg and Restifo, 2015). Possible immunotherapeutic strategies for ovarian cancer can be summarised into the following four groups: antibodies, immune checkpoint inhibitors, vaccines, and adoptive cell therapy (Schwab *et al*., 2014; Chester *et al*., 2015). Clinical research is at an early stage and need further studies for a better understanding of the immunotherapy efficiency, tolerability, and safety (Mandai *et al*., 2016).

1.3. Biomarkers for ovarian cancer

For ovarian cancer patients with late-stage diagnosis and treated by conventional regimen, the five-year survival rates are under 20% while the survival rates of women with the early-stage diagnosis are around 90% (De Angelis R *et al*., 2014; Siegel, Miller & Jemal, 2016). As mentioned above, only 25% ovarian cancer can be detected at an early stage (Badgwell D and Bast RC Jr., 2007) which is partly due to current insufficient screening and diagnostic

methods for early detection. Cancer Research UK (2016b) indicated that the lifetime risk for women to develop ovarian cancer is 1 in 52 in the UK in 2012. Due to the low prevalence of ovarian cancer, an effective screening strategy is required with both high sensitivity for early stage disease (>75%) and extremely high specificity (99.6%) to avoid unnecessary surgery-related complications and gain a 10% positive predictive value (i.e. for each actual case of ovarian cancer diagnosed there were less than ten exploratory operations) (Zhang *et al*., 2010; Kobayashi *et al*., 2012). Currently, no screening strategy achieves this specificity (Zhang *et al*., 2010; Menon, Griffin & Gentry-Maharaj, 2014). Therefore, searching for specific molecular biomarkers/panels is of high importance for early detection of ovarian cancer and also for the direction of ovarian cancer treatment. Candidate tumour biomarkers might be extracellular matrix or cell adhesion molecules, receptors, growth factors, cytokines, or products of abnormal metabolism. On occasion, the markers can be molecules released by other tissues and organs reacting to the tumour signals or autoantibodies from the immune system against tumour antigens (Kobayashi *et al*., 2012); besides which a number of directions have been followed in search of novel biomarkers such as circulating tumour cells, peritoneal ascitic fluids and epigenetic modifications (El Bairi *et al*., 2016).

1.3.1 Gene-based biomarkers

Due to the close link between genetic mutations and ovarian tumourigenesis, reviewed by Lee *et al*. (2013) gene-based biomarkers have been studied according to the following aspects: inherited gene mutations (mentioned in **section 1.1.2**), epigenetic modifications (e.g. small non-coding RNAs (miRNAs), DNA/histone methylation, acetylation or phosphorylation (El Bairi *et al*., 2016)), gene expression (studied via DNA-microarray technology), and whole genomic sequencing (e.g. genic amplification and chromosomal deletions; using second-generation sequencing). They identified genes (e.g. tumour suppressor genes and oncogenes) associated with ovarian cancer development and offered potential markers for further studies and validations (Longuespée *et al*., 2012).

1.3.2 Protein biomarkers

Compared to the genome, the proteome of a sample is able to reveal a subset of all possible products from the genome including the protein products after various modifications such as translational, post-translational, regulatory, and degradative processes; protein biomarkers have an advantage over gene-based biomarkers with respect to a more specific detection of cancer type and status (Zhang *et al*., 2010). With the improvement of techniques, various proteomics-based molecular biomarkers have been identified to screen early stages of ovarian cancer using the serum, ascites, and tissue samples, although validations are still required. The proteins that have been identified by proteomic studies are involved in a number of endpoints, i.e. cell proliferation, cytoskeletal signalling, metastasis, immune response modulation (Longuespée *et al*., 2012).

Longuespée *et al*. (2012) reviewed the signalling pathways that are associated with ovarian cancer pathogenesis – these include: the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, the activator of transcription 3 (Jak-STAT 3) pathway, the Interleukin-6/Interleukin-6 receptor (IL-6/IL-6R) or the Janus Kinase 2 signal transducer pathways (Toss *et al*., 2013), the mitogen-activated protein kinase (MAPK) pathway, the proto-oncogene tyrosine protein kinase Src pathway, the ErbB activation pathway, the lysophosphatidic acid (LPA) pathway, the phosphatidylinositol 3-kinases (PI3K) pathway, the Müllerian inhibitory substance receptor pathway, the epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) pathways and the estrogen receptor beta pathway.

El Bairi *et al*., (2016) reviewed currently investigated ovarian cancer biomarker panels which combine old and novel proteomic biomarkers (reviewed by Nolen & Lokshin in 2012) for early stage ovarian cancer detection. Only serum tumour markers CA-125 and HE4 have been approved by the US Food and Drug Administration for the purpose of disease recurrence and

therapeutic response monitoring but not for screening (Lee *et al*., 2013). In addition to tumour markers, serum CA-125 values have cooperated with ultrasound-detected ovarian morphology (by two-dimensional sonography or three-dimensional ultrasonography) and menopausal status in the 'Risk of Malignancy Index' which has been widely applied in clinical practice to predict whether the adnexal mass of an individual is malignant or not (Gentry-Maharaj A & Menon U., 2012). So far there have insufficient evidence to support that ovarian cancer screening could lead to a stage shift to an earlier stage or reduce morbidity or mortality (Sölétormos *et al*., 2016).

1.4. Calpain and calpastatin in cancer biology

Although numerous studies have been conducted there is still a lack of an effective, robust, prognostic biomarker. Moreover, ovarian cancers are known to consist of heterogeneous populations, yet are often treated with the same standard regimen whose effectiveness remains subject to chemo-resistance and has severe side effects. Accordingly, more effort should be made to improve the sensitisation of ovarian cancer to conventional chemotherapy and to personalise precision treatment. The expression of the calpain system may be useful in this area.

Calpains (short for calcium-dependent papain-like enzyme) are intracellular cytoplasmic neutral cysteine proteases operating their regulatory function by cleavage of certain substrates (Benyamin Y., 2006; Campbell & Davies, 2012). Their activation is regulated by intracellular Ca^{2+} at neutral pH (Storr *et al*., 2011a). Although a lot is known, the physiological functions of calpain are not yet fully elucidated. Up to now, studies indicate that they are involved in various cellular processes including signal transduction, cell proliferation, gene expression, cell cycle progression, differentiation, apoptosis, membrane fusion, cytoskeletal organisation and platelet activation (Glading, Lauffenburger & Wells, 2002; Franco & Huttenlocher, 2005; Abe, & Takeichi, 2007; Suzuki K *et al*., 2004; Saez *et al*., 2006). During growth and tissue regeneration, calpains are involved in protein renewal (Benyamin Y., 2006).

The functions of calpains cover a wide span of physiological processes, which link calpains with various pathological conditions when their activity is deregulated. Such pathological conditions include neurological disorders, muscular dystrophies, ophthalmic diseases (e.g. cataracts), brain injury, cancer, cardiovascular disorders, diabetes and parasitic, fungal, viral and bacterial infections (Saez *et al*., 2006; Kumar & Ali, 2017).

1.4.1 Family members and structure of calpains

Numerous calpain family members have been identified: with 14 isoforms of the 80K family and two genes of 30K subunits in the human genome (Baudry, Chou & Bi, 2013). Based on protein domain-structure calpains are classified into classical EF domain calpains (including conventional and unconventional EF-calpain subunits) and atypical non-EF domain calpains (i.e. non-classical calpains). **Figure 1.2** shows the domain structures of calpains. The members of calpain system are listed in **Table 1.4**. Calpain-1, calpain-2 and calpain-9 form heterodimers with small subunit while calpain-3 and calpain-13 form homodimers (Ravulapalli *et al*., 2009).

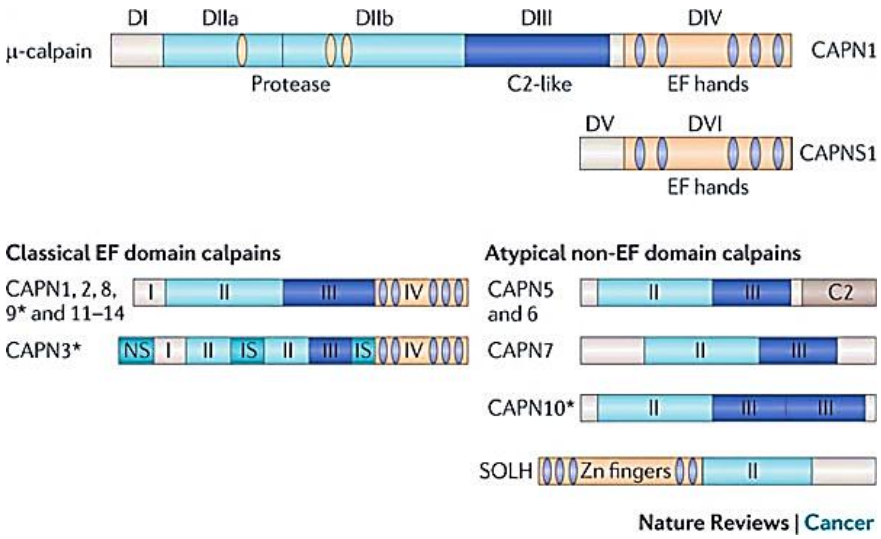


Figure 1.2 Schematic structure of μ calpain heterodimer and calpain family members. Reproduced from (Storr *et al*., 2011a), Nature Reviews Cancer, 11, 364-374; Copyright© 2011 by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

Table 1.4 The calpain family

Calpain protein	Gene	Other names	Isoforms	Expression	Implications or alterations of calpains in diseases
Calpain-1	CAPN1	μ-calpain large subunit	--	Ubiquitous	Breast cancer, bladder carcinoma
Calpain-2	CAPN2	m-calpain large subunit	--	Ubiquitous, except erythrocytes	Colorectal, lung, brain, breast, prostate, renal, skin cancer, melanoma, meningioma, neuroblastoma, osteosarcoma, rhabdomyosarcoma.
Calpain-3	CAPN3	p94, nCL-1	Yes	Skeletal muscle, lens, retina, testis	Urothelial cancer, melanoma.
Calpain-4	CAPNS1	Calpain small subunit (CSS)1, 30K	--	Ubiquitous	Breast cancer, hepatocellular carcinoma, large granular lymphocytes (LGL) Leukaemia
Calpain-5	CAPN5	hTRA-3, nCL-3	--	Central nervous system, colon, small intestine and testis	Huntington's disease polycystic ovarian syndrome, metabolic syndrome
Calpain-6	CAPN6	CAPNX	--	Placenta	Uterine cancer
Calpain-7	CAPN7	PalBH	--	Ubiquitous	
Calpain-8	CAPN8	nCL-2	Yes	Stomach mucosa	
Calpain-9	CAPN9	nCL-4	Yes	Digestive track	Gastric cancer, breast cancer
Calpain-10	CAPN10	CAPN8	Yes	Ubiquitous	Laryngeal cancer
Calpain-11	CAPN11	\	--	Testis	
Calpain-12	CAPN12	\	Yes	high in hair follicle	
Calpain-13	CAPN13	\	--	Testis and lung	
Calpain-14	CAPN14	\	Yes	Ubiquitous	
Calpain-15	CAPN15	small optic lobe homology (SOLH)	--	Ubiquitous	
Calpain-16	CAPN16	\	\	Ubiquitous	
CAPNS2	CAPNS2	CAPN14, CSS2	--	Lens	

Data adapted from (Saez *et al*., 2006; Suzuki K *et al*., 2004; Leloup & Wells, 2011, Sorimachi *et al*., 2011, Ravulapalli *et al*., 2009, Farkas *et al*., 2003, Singh *et al*., 2014)

Classical calpains (80kDa) are composed of four domains (I–IV). Non-classical calpains have certain domains of the classical calpains deleted or replaced. The classical calpains include calpain-1 and calpain-2, which are calpain large subunits of micro-calpain (μ-calpain) and milli-calpain (m-calpain) respectively – they form heterodimers separately and both need a common small subunit of 28 kDa (calpain-4) to form functional heterodimeric calpain (Benyamin Y., 2006; Saez *et al*., 2006). μ-calpain and m-calpain, also known as conventional calpains (Shinkai-Ouchi *et al*., 2016; Ono *et al*., 2016), are named based on *in vitro* studies showing the Ca^{2+} concentrations they require for optimal activity - in the micromolar range (3-50μM) and in the millimolar range (0.2-1.0mM) respectively (Benyamin Y., 2006; Baudry, Chou & Bi, 2013). The small subunit consists of domain V and VI; domain V is an N-terminal glycine-rich hydrophobic domain and domain VI a calmodulin-like domain homologous with domain IV of the large subunit (Zimmerman *et al*., 2000). Other EF-hand calpains include calpain-3, -8, -9, -11, -12, -13 and -14, which have similar domain-structure to the classical calpains; aside from calpain-9 (Ravulapalli R. *et al*. 2009), the others have not been found to form heterodimers with a calpain small subunit (Saez *et al*., 2006; Ono *et al*., 2016).

The non-classical calpains, calpain-5, -6, -7, -8b, -10a and -15 (i.e. SOLH), do not have penta-EF-hand motifs (Suzuki K *et al*., 2004). Zhao and collogues (2012) reported that a massive expansion of the calpain family may have occurred in unicellular eukaryotes and calpain gene variants may have been formed in the early evolution of eukaryotes through assembling ancient domains that were already in existence in prokaryotes. Calpains are well-conserved in evolution and ubiquitously expressed in organisms ranging from protists and plants to humans (Benyamin Y., 2006, Friedrich & Bozóky, 2005; Rawlings, 2015).

Domain I is the N-terminal anchor helix region, and autolysis occurs in this region during Ca^{2+}-induced calpain activation, which sometimes further

involves in the dissociation of the large and small subunits (Nakagawa K. *et al*., 2001). Domain II (catalytic domain), as a conserved proteolytic domain, has been considered as the structural and functional element of calpains (Saez. *et al*., 2006). It harbours the catalytic triad sequence Cys-His-Asn (Goll *et al.*, 2003) and the location of these amino acids suggest that calcium ions are needed to form an active catalytic pocket (Saez *et al*., 2006). Domain II of calpain, as with other cysteine proteases, also consists of two protease core subdomains assigned as IIa and IIb, linked by a substrate binding cleft (Moldoveanu, Jia & Davies, 2004; Suzuiki *et al*., 2004). The active site Cys-105 located on IIa and His-262 and Asn-286 on IIb of calpain-2 (Cys-115, His-272, Asn-296 in calpain-1, Goll *et al.*, 2003), due to their distance (> 10 angstrom), cannot form a functional catalytic triad (Hosfield C.M. *et al*., 1999; Strobl S. *et al*., 2000); stabilisation of this structurally inactive conformation of domain II is achieved by the interaction between domain I and VI on one side and strong electrostatic bonds between domain II and III on the other side (Suzuki K *et al*., 2004; Benyamin, 2006). Upon Ca^{2+} binding to each core domain, the two subdomains form an active cysteine catalytic site and interact with substrates or calpastatin, the endogenous calpain inhibitor (Moldoveanu, Jia & Davies, 2004). Domain III has a spatial structure that resembles C2 domains found in proteins such as phospholipase C, protein kinase C, synaptotagmin, etc., which bind calcium ions and phospholipids (Tompa *et al*., 2001a; Suzuki K *et al*., 2004). Calcium ions bind to DII and disrupt the salt bridges that keep DII in an open conformation, followed by reducing the distance between DIIa and DIIb and activating DII. This activation leads to “proteolysis activity, intermolecular cleavage in DI then DV with possible subunit dissociation, substrate proteolysis” and ends up with the “loss of calpain activity via increased autolysis of the large subunit (Benyamin Y., 2006)”.

Domain IV is presumably able to bind calcium ions since it has 5 sets of EF-hand motifs and resembles the structure of calmodulin (Hosfield *et al*.,

1999; Suzuiki *et al*., 2004), yet the presence of Ca^{2+} does not lead to obvious structural changes in domain IV of calcium-dependent calpains (Saez *et al*., 2006). Calpain-8b, an alternative splicing product of CAPN8, does not have domain IV and most of domain III is missing but it still needs calcium ions for activation, suggesting the existence of other calcium-binding sites in domain II (Hata *et al*., 2001; Moldveanu *et al*., 2002). The extreme COOH-terminal fifth EF-hand motif in domain VI of the small subunit and in domain IV of large subunit cannot bind calcium; they interact with each other and bridge the two subunits to form μ- or m-calpains (Suzuiki *et al*., 2004; Benyamin Y., 2006). Transgenic mice studies have indicated that inhibition of the large subunit stopped cell division, growth, and proliferation; and deletion of the small subunit gene resulted in early embryonic lethality (Zimmerman *et al*., 2000; Arthur, J. S. *et al*., 2000; Takano J. *et al*., 2011). Current understanding interprets the large subunits function as catalytic and the small subunit as regulatory, regulating the stability of the large subunits (Benyamin Y., 2006). New functions of conventional calpains, μ- and m-calpains, are still being discovered; whilst they are considered to be of fundamental importance in regulating various biological phenomena (Sorimachi *et al*., 2011).

1.4.2 Calpastatin

Both μ-calpain and m-calpain can be regulated by their endogenous specific inhibitor calpastatin which, at physiological Ca^{2+} concentrations, blocks the mobile structure of calpains to form a long-lived complex (Benyamin Y., 2006). With the influx of calcium ions, calpastatin is released from intracellular storage into the cytosol to interact with calpain (Averna *et al*., 2003). Calpastatin is expressed ubiquitously in all human tissues and tissue specific isoforms have been found, generated by alternative splicing and proteolytic processing (Carragher & Frame, 2002). The conventional calpains and their inhibitor calpastatin can stay relatively stable with a half-life of up to 5 days under normal physiological conditions (Carragher *et al*., 2002). Under

certain stimuli, such as elevated intracellular calcium or activation of v-Src oncoprotein, rather than inhibit calpain, calpastatin can be proteolyzed by calpain and then enhance calpain activity (Carragher *et al*., 2002).

Calpastatin is a heat-stable unstructured protein (Murachi *et al*., 1989; Tompa, 2002a) with large molecular size (Murachi *et al*., 1980; Takano er al., 1984). There are two types of calpastatin identified in mammals which are tissue type (100~120kDa) and erythrocyte type (70 kDa) (Takano er al., 1993). In most tissues and cells of mammals, the tissue type exists predominantly except for erythrocytes (Inomata *et al*., 1993). A number of types and isoforms exist through alternative promoter use and/or exon skipping and splicing. The domain structure of calpastatin is as follow: a single polypeptide chain harbours N-terminal L-domain which presumably enhances calpastatin binding to calpains (Ma *et al*., 1994) or to Ca^{2+} channels (Hao *et al*., 2000) and harbours four equivalent inhibitory domains (I-IV) (similar but not identical) (Leloup & Wells, 2011) (**Figure 1.3**). Wendt *et al*. (2004) reviewed the different ability of the four domains of calpastatin and the order from high to low effectiveness of their inhibitory abilities on calpains is as follow: domain I≥ domain IV> domain III≥ domain II. Thus, one calpastatin molecule, with its intrinsically unstructured nature, is capable of reversibly inhibiting four calpain heterodimers in the presence of Ca^{2+} (Wendt *et al*., 2004). Calcium-binding induces structural changes in calpain to allow the calpastatin to interact and inhibit calpains (Storr *et al*., 2011a). Each inhibitory domain possessing three conserved regions signed as regions A, B, and C (Leloup & Wells, 2011). Regions A and C are not inhibitory elements but for binding, while region B inhibit calpain by blocking its active site (Friedrich & Bozóky, 2005; Storr *et al*., 2011a; Campbell & Davies, 2012). Genomic sequencing indicated that each domain is encoded by four exons; three of them each encoding one region (A, B, or C) and the fourth encoding the sequence from subdomain C to the end of the domain (Maki *et al*., 1989).

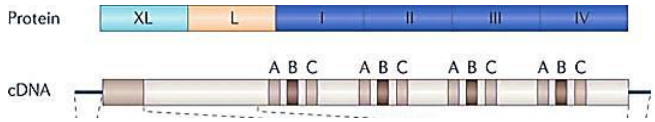


Figure 1.3 Schematic structure of calpastatin. Reproduced from (Storr *et al*., 2011a), Nature Reviews Cancer, 11, 364-374; Copyright© 2011 by Nature Publishing Group with permission conveyed through Copyright Clearance Centre Inc.

1.4.3 Calpain activation and its interaction with calpastatin

The review by Lebart & Benyamin (2006), focusing on muscle cells and myofibrils, summarised the regulation of calpains as being accomplished via calcium concentration, phosphorylation of substrates or the proteases themselves, intervention of phospholipids, presence and/or the regulation of a specific inhibitor (calpastatin), or formation of multiprotein complexes containing calpains. The main regulator, Ca^{2+}, tightly regulates calpain activation both spatially and temporally (Suzuki K *et al*., 2004).

Calpains require calcium and can be activated at neutral pH, in addition, calpains as a "modulator protease" (Sorimachi *et al*., 2011) mediate the activity of their target substrates by partial cleavage (non-digestive or degradation) similar to post-translational modification; whereas typical cellular proteases are usually calcium-independent, active at acidic pH and completely degrade their substrates (Friedrich & Bozóky, 2005; Cortesio *et al*., 2011; Baudry, Chou & Bi, 2013). Moreover calpain only needs one or two molecules to act on its substrate whilst proteasomal and lysosomal proteases need to follow a coordinated step-wise way with proteins mediating ubiquitination and with intracellular compartments (e.g. autophagosomes and endosomes) (Sorimachi *et al*., 2011). Calpains preferentially recognise PEST sequences (proline (P), glutamate (E), serine (S) and threonine (T) - rich polypeptide chain) and/or the patterns of primary/secondary protein structures around the scissile bond (Tompa *et al*., 2004; Moretti *et al*., 2014).

Calcium concentrations that can be achieved intracellularly are generally far less than the concentrations required for calpain activation. Calcium

concentrations in physiological conditions cannot activate m-calpain, but they can reach to the level for μ-calpain activation. This range of concentrations still cannot lead to obvious calpain activation either in the regulation of cell cycle (Schollmeyer, 1988) or in apoptosis (Sarin, Adams & Henkart, 1993). To explain how calpain is activated by physiological calcium levels, different activation mechanisms have been suggested and are reviewed in **section 4.2.1.**

The Ca^{2+} concentration required for calpastatin - calpain interaction has been shown to be less than the concentration required for half-maximal proteolytic activation of m-calpain or autolysed calpains (i.e. calpain-1 and -2), suggesting that increasing Ca^{2+} concentrations would first lead to the formation of calpastatin-calpain complex before proteolytic activation of calpains, if calpastatin co-localises in cells (Wendt *et al*., 2004). The increase of cytoplasmic Ca^{2+} concentration first reaches the point that allows calpastatins to bind to calpains and blocks calpain activity until the calpain content in the cell is stoichiometrically excess (Friedrich & Bozóky, 2005). Some studies suggest that calpastatin plays a role as the storage/anchoring site for calpains, and after the binding with calpastatin, calpain may be released in an active form at a comparatively lower Ca^{2+} concentration (Friedrich & Bozóky, 2005). In addition, the Ca^{2+} concentration required for the binding of calpastatin and conventional calpains appears to mainly depend on the calpains, since no evidence suggest that calpastatin binds Ca^{2+} (Wendt *et al*., 2004). From above, the calpain-calpastatin complex seems to work as a functional unit. Studies conducted on μ-calpain supported that region A and C of calpastatin (**Figure 1.3**) bind to domain IV and VI of calpain (**Figure 1.2**) respectively (Ma *et al*., 1994; Yang *et al*., 1994; Takano *et al*., 1995). Calpastatin was found to be generally more efficient in inhibiting m-calpain than μ-calpain, as the calpastatin IC50 for μ-calpain can be 2- to 10- fold higher than the IC50 for m-calpain (Wendt *et al*., 2004). In the cytosol, peptides containing only calpastatin region B by itself shows inhibitory effect on both μ–calpain and

m-calpain, though less effective, probably by binding at or near the active site of calpain (IIa and/or IIb) (Goll *et al*., 2003; Friedrich & Bozóky, 2005). By adjusting the length of peptides containing the interested regions of calpastatin, researches indicated that subdomain B seems to block or shield the active site of calpains rather than directly binding to the active site (Betts *et al*., 2003). Whereas, peptides only containing either region A or C alone cannot inhibit calpains; in other words, calpastatin only inhibits dimeric calpains (i.e. μ-calpain, m-calpain, and calpain 9) in the presence of both IV and VI domains (Goll *et al*., 2003, Friedrich & Bozóky, 2005).

1.4.4 Calpain family and cancers

1.4.4.1 Cellular localisation of calpain

Cellular locations of the calpain system have been revealed by IHC studies. Calpain-1 has been shown to be expressed on the cell membrane and in the cytoplasm in both gallbladder carcinomas and gallbladder tissue from cholecystitis patients (Luo *et al*., 2016). In gastro-oesophageal adenocarcinomas, calpain-1, calpain-2 and calpastatin showed cytoplasmic expression (Storr *et al*., 2013). Calpain-1, calpain-2 and calpastatin were also found predominantly expressed in cytoplasm in breast cancers (Storr *et al*., 2011c, 2012b, 2016). Calpain-4 expression has been primarily found in the cytoplasm in intrahepatic cholangiocarcinoma and ovarian carcinomas (Zhang *et al*., 2013; Yang X *et al*., 2017), whilst in melanoma tissues, granular distribution was observed in the nucleus and cytoplasm (Wang E., *et al*., 2017). In pancreatic, bile duct and ampullary carcinomas, calpastatin expression was found in both the cytoplasm and nucleus; additionally, cytoplasmic calpastatin expression showed strong correlation with nuclear calpastatin expression (Storr *et al*., 2012c).

1.4.4.2 Calpain expression/activity levels in tumour versus non-tumour tissue

Aberrant expression/activity of the conventional calpains and/or calpastatin occurs in various cancers; often high conventional calpains or low calpastatin levels are observed in cancers but there are exceptions. Several calpain family expression studies have focused on gynaecological cancers. Comparing with the benign tissue, endometrial cancer expressed higher level of calpastatin (P=0.037) (Salehin *et al*., 2010). Calpain-1 expression was of the same level between endometrial cancer (29/47) and benign endometrial tissue (18/47); also no statistically significant differences in calpain-2 expression were detected between benign and malignant endometrial tissue, although malignant tissue had higher calpain-2 expression in comparison to benign tissue (calpain-2 negative) (Salehin *et al*., 2010). The expression of calpain-1, calpain-2 and calpastatin were studied in both normal ovarian tissue and ovarian tumour (Salehin *et al*., 2011). Salehin *et al*., (2011) indicated that although no specific function could be linked with calpain-1, -2 and calpastatin, their expression levels in malignant tissue differed from those in benign tissue: comparing with normal tissue, ovarian tumours were found with lower calpastatin expression levels.

In cancer of the digestive system, calpain-1 expression was found elevated in gallbladder carcinoma (n=100) in comparison with cholecystistis patient tissue (n=30) ($\chi 2$=7.668; P=0.006) (Luo *et al*., 2016). Calpain activity (detected with casein as substrate) was significantly higher in colorectal adenocarcinoma than in normal mucosa (n=50, P<0.05) which were in parallel with the higher calpain-2 and lower calpastatin expression levels (P<0.05) detected by Western blotting in tumours than in matched normal colonic mucosa (Lakshmikuttyamma *et al*., 2004). In nasopharyngeal carcinoma, calpastatin mRNA was found down-regulated (P=0.00009, n=8) compared with normal nasopharyngeal epithelium in the study conducted by Sriuranpong *et*

al. (2004).

It seems that calpain-4 is usually expressed at a higher level in tumours than in non-tumour tissues. Calpain-4 was significantly higher compared with paired adjacent normal tissues at both mRNA (by qRT-PCR) (P=0.013) and protein (P=0.005) (by Western blotting) level in human clear cell renal cell carcinoma (n=40) (Zhuang *et al*., 2014), intrahepatic cholangiocarcinoma (mRNA: P=0.003, protein: P<0.001, n=33) (Zhang *et al*., 2013), gastric cancer (mRNA: P=0.026, n=33) (Peng *et al*., 2016) and nasopharyngeal carcinoma (normal tissues: n=2, tumour samples: n=7) (Zheng *et al*., 2014). Higher calpain-4 expression was found in ovarian carcinomas than in normal ovarian surface epithelium tissues (mRNA expression data from the GEO database) (Yang X *et al*., 2017). In addition, higher calpain-4 protein expression was observed in ovarian carcinomas (ovarian carcinomas: n=91, borderline ovarian tumour tissues n=5 and normal ovarian epithelial tissues: n=5, P<0.01, using IHC assessment) (Yang X *et al*., 2017), glioma (glioma: n=279 and control tissues: n=21, P<0.01, using IHC assessment) (Cai *et al*., 2014) non-small cell lung cancers (n=8, using Western blotting assessment) (Gu *et al*., 2015) and melanoma (melanoma: n=120 and normal tissues: n=34, P<0.01, using IHC assessment) (Wang E., *et al*., 2017) than in the normal or non-tumour tissues or borderline tumours.

1.4.4.3 Calpain expression/activity levels and patient outcomes

The relationship between the expression of the calpain system (i.e. restricted to calpastatin, calpain-1, -2 and -4) and patient outcome has been examined in a number of studies. Whether the expression and outcomes were significantly associated or whether the associations were positive or negative seem to depend on the context (e.g. tumour type, subtype and heterogeneity etc.).

Calpastatin

Low expression of calpastatin was significantly associated with adverse patient survival in inflammatory breast cancers of pre-chemotherapy (*P*=0.020, n=43) (Storr *et al*. 2016), cancers of the bile duct and ampulla (*P*=0.012, n=120) (Storr *et al*., 2012c) and in both the primary surgery and neo-adjuvant chemotherapy treated gastric/gastro-oesophageal cancers (*P*=0.013, n=140; *P*=0.012, n=88) (Storr *et al*., 2013). Whereas, high calpastatin expression was significantly associated with poor disease-specific survival in non-inflammatory breast cancers of pre-chemotherapy (*P*=0.035, n=94) (Storr *et al*. 2016). In breast cancers, neither protein nor mRNA level calpastatin expression was correlated with disease specific survival (*P*=0.204, n=53; *P*=0.094, n=56, respectively) (Storr *et al*., 2011b). Calpastatin expression was not significantly associated with patient survival in breast cancers treated with trastuzumab following adjuvant chemotherapy (*P*=0.298, n=93) (Storr *et al*., 2011c), in a total early-stage invasive breast cancer cohort (n=1371), in basal-like or triple-negative subtype of breast cancers (Storr *et al*., 2012b) nor in primary ovarian carcinomas (*P*=0.898, n=153) (Storr *et al*., 2012a).

Calpain-1

Calpain-1 expression has been shown to be positively associated with patient survival in both primary surgery and neo-adjuvant chemotherapy treated gastric/gastro-oesophageal cancer cases (*P*=0.001, n=140; *P*=0.004, n=88) (Storr *et al*., 2013) and in pre-chemotherapy inflammatory breast cancer cases (*P*=0.003, n=43) (Storr *et al*. 2016). In HER2-positive primary breast cancers treated with trastuzumab after adjuvant chemotherapy, the expression of calpain-1 was adversely associated with relapse-free survival (*P*=0.001, n=93, Storr *et al*., 2011c; *P*=0.01, n=194; 0.019, n=87, Pu *et al*., 2016a). Calpain-1 was not significantly associated with patient survival in the total early-stage invasive breast cancer cohort (n=1371), in basal-like (Storr et al., 2012b) or triple-negative subtype of breast cancers (Storr *et al*., 2012b; OS: *P*=0.88, recurrence-free survival: *P*=0.71, n= 55, Al-Bahlani *et al*., 2017) nor in

primary ovarian carcinomas (*P*=0.412, n=153) (Storr *et al*., 2012a).

Calpain-2

High calpain-2 expression has been shown to be significantly associated with high OS in pancreatic adenocarcinoma cases (*P*= 0.036, n=68) (Storr *et al*., 2012c), neo-adjuvant chemotherapy treated gastric/gastro-oesophageal cancer cases (*P*=0.001, n=88) (Storr *et al*., 2013) and in non-inflammatory breast cancer cases with pre-chemotherapy (*P*=0.031, n=94) (Storr *et al*. 2016). In primary ovarian carcinomas (*P*=0.006, n=153), basal-like (*P*=0.025, n=270 or *P*=0.020, n=149) and triple-negative (*P*=0.043, n=236 or *P*=0.033, n=253) subtype of breast cancers, high calpain-2 expression was associated with adverse survival (Storr *et al*., 2012a; Storr *et al*., 2012b). In the total early-stage invasive breast cancer cohort, the negative association between calpain-2 and patient survival was maintained (*P*=0.043, n=1371) (Storr *et al*., 2012b). Calpain-2 expression was not associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy (*P*=0.193, n=93) (Storr *et al*., 2011c).

Calpain-4

High calpain-4 expression has been shown to associate with shorter OS, PFS and/or advanced malignancy in ovarian carcinomas (*P*=0.033, n=91), clear cell renal cell carcinoma (*P*=0.006, n=40), nasopharyngeal carcinoma (PFS: *P*=0.003, OS: *P*=0.002; n=153), intrahepatic cholangiocarcinoma (*P*=0.006, n=140), primary hepatocellular carcinoma (*P*<0.001, n=323), glioma (*P*= 0.032, n=279), primary non-small cell lung cancer (*P*=0.001, n=208) (Yang *et al.*, 2017; Zhuang *et al*., 2014; Zheng *et al*., 2014; Zhang *et al*., 2013; Dai *et al*., 2014; Cai *et al*., 2014; Gu *et al*., 2015).

It is difficult, bearing in mind the above, to draw any generalisable conclusions regarding the association between calpain family members and

patient survival, as conflicting findings have emerged in different types, even subtypes, of cancers. Calpains are involved in various aspects of tumour progression including oncogenic transformation, cell survival and apoptosis, tumour growth and cancer dissemination which will be discussed in the following chapters. It is therefore necessary to understand the specificity of different isoforms of calpains and their cell-specific influence on tumour progression on a tumour type specific basis.

1.5. Hypotheses and aims

Several *in vitro* studies have been published regarding how the calpain system is involved in chemotherapeutic response: conventional calpain expression/activation was found either positively or negatively associated with resistance to platinum-based chemotherapy in these studies; only one study proposed that the calpain system is involved in hypoxia- rather than cisplatin-induced cell death. As mentioned in **section 1.4.4**, a previous IHC study conducted by our group (Storr S.J. *et al*., 2012) showed that high expression of calpain-2 significantly associated with ovarian cancer resistance to platinum-based adjuvant chemotherapy, poor PFS and OS. Results from multivariate analysis showed that calpain-2 expression was an independent prognostic factor and suggested that the association between high calpain-2 expression and OS was independent of platinum sensitivity – such data suggests that any effect calpain-2 expression had, in terms of patient survival, may not be solely due to the modulation of cellular response to platinum-based chemotherapy but that other processes might also be in operation. Based on this previous IHC study, only one hypothesis is addressed in the current study.

Although in the previous IHC study in ovarian cancers no significant association was observed between the expression of the calpain system and tumour stage, many studies support calpain system involvement in tumour

metastasis. In certain IHC studies the conventional calpain subunits were found positively associated with lymphatic metastasis and/or TNM stage in intrahepatic cholangiocarcinomas (calpain-4, Zhang *et al*., 2013), breast cancers (calpain-2, Storr *et al.*, 2012b) and HER2-positive primary breast cancers with trastuzumab treatment (calpain-1, Pu *et al.*, 2016a); however, negative associations were found in adjuvant chemotherapy treated gastric/gastro-oesophageal cancer patients (calpain-1, Storr *et al.*, 2013). Calpastatin expression showed positive association with tumour stage and vascular invasion in the bile duct and ampullary carcinomas (nuclear calpastatin, Storr *et al.*, 2012C) whilst a negative association was found in neo-adjuvant chemotherapy treated gastric/gastro-oesophageal cancer patients (Storr *et al.*, 2013) and breast cancer (Storr *et al*., 2012b). In certain *in vitro* studies the regulation of calpain activity and/or the expression of the calpain system were found to significantly influence cancer cell migration and many substrates of conventional calpains are related to focal adhesion complexes and the cytoskeleton (reviewed in chapter 4). As a result of such studies, the involvement of the calpain system in ovarian cancer cell migration was studied in the current study.

Thus, the hypotheses of this project were as follow:

1. Down-regulation of calpain-2 can sensitise chemo-resistant ovarian cancer cells to platinum-based chemotherapy (cisplatin and/or carboplatin). Regulation of calpain activity will be achieved by using inhibitors such as calpeptin; or by decreasing calpain expression using calpain shRNA.

2. Conventional calpains and/or calpastatin are involved in ovarian cancer cell migration and/or invasion.

The study aimed to:

1. Investigate whether differential regulation of calpain activity can influence the chemoresponse of ovarian cancer cells such as proliferation via *in vitro* assays (i.e. cell growth curve and clonogenic survival assay) in a variety of established ovarian cancer cell lines of varying sensitivities to platinum-based chemotherapy.

2. Verify the initial immunohistochemical data using a larger patient cohort and including calpain-4 expression.

3. Investigate whether differential regulation of calpain activity can influence the phenotypic behaviour (i.e. haptotaxis migration) of ovarian cancer cells via *in vitro* assays (i.e. scratch-wound migration assay) in established ovarian cancer cell lines.

4. Study the expression of proteins that have recently been found to be associated with calpain family members by IHC and determine their association with conventional calpain subunits, calpastatin, clinicopathological variables and survival in ovarian cancer patient samples.

Chapter 2. *In vitro* calpain expression and drug sensitivity of platinum-based chemotherapies

2.1. Abstract

INTRODUCTION: The calpain system is associated with cancer chemotherapeutic response in both *in vivo* and *in vitro* studies. Previous immunohistochemistry (IHC) data conducted in our group indicated that high calpain-2 expression associated with both resistance to platinum-based adjuvant chemotherapy and worse patient outcome.

OBJECTIVES: This chapter sought to determine if altering calpain activity could sensitise ovarian cancer cells to platinum-based chemotherapy.

METHODS: Five ovarian cancer cell lines, with varying platinum-based chemotherapy sensitivities, were chosen as *in vitro* models: the platinum-sensitive A2780 cells and its resistant counterpart A2780-cis cells; the platinum-resistant SKOV3 cells; and the platinum-sensitive PEO1 cells and its platinum-resistant counterpart PEO4 cells. Western blotting was used to assess the expression of calpain-1, -2, -4 and calpastatin in this panel of cell lines. Calpain-2 short hairpin RNA (shRNA) was used for calpain-2 knockdown and a calpain inhibitor, calpeptin, was used to inhibit calpain activity. For the optimisation of drug combination study, sensitivity of the cells (subIC50/IC50 values) towards calpeptin, cisplatin and carboplatin was investigated by growth curves respectively over the panel of cell lines. Calpain activity in live cells was assessed with a fluorescent plate reader assay using a fluorescent substrate CMAC, t-BOC-Leu-Met that can readily diffuse into cells. In addition, PEO1 and PEO4 survival was assessed by clonogenic assay. The combined action of calpeptin and platinum-based chemotherapy was assessed via cell proliferation (five cell lines) and clonogenic survival (PEO1 and PEO4).

RESULTS: Significant variations of calpain system protein expression levels were observed between the different cell lines. In comparison to the other 3 lines, the A2780 cell lines expressed very low levels of calpain system proteins. No significant difference of calpain system expression was detected between the chemo-sensitive and the chemo-resistant counterparts. Because SKOV3 and PEO4 expressed the highest levels of calpain-2, calpain-2 shRNA was used for calpain-2 knockdown in these two cell lines - unfortunately the numerous attempts proved unsuccessful. Calpeptin caused a similar inhibition of cell proliferation in chemo-sensitive ovarian cancer cells and their resistant counterparts. Using optimised concentrations and treatment durations, calpeptin could inhibit approximately 30% of calpain activity. Calpeptin combined with cisplatin or carboplatin did not futher alter cell proliferative ability (in SKOV3, PEO1 and PEO4 cells) or clonogenic survival (based upon a single experiment in PEO4 cells) in comparison with cells treated by cisplatin or carboplatin alone.

CONCLUSION: The current results did not support the initial hypothesis that calpain can sensitise the ovarian cancer cells to cisplatin/carboplatin. The roles that calpain system played in human cancer cells vary with cell context and it is difficult to draw a direct link between the results from *in vitro* and *in vivo* studies. To revisit the question as to whether calpain associates with chemoresponse and patient survival, a larger cohort was used to validate the previous study and is described in the subsequent chapter.

2.2. Introduction and aims

As discussed in **section 1.2.1.1**, the backbone of ovarian cancer treatment remains platinum-based chemotherapy (Markman, 2013), and the late stage of diagnosis, along with intrinsic and acquired chemo-resistance are major causes of the poor prognosis associated with ovarian cancer (40% 5-year OS) (Suh *et al*., 2016). Therefore, understanding the mechanisms that

underlie chemo-resistance is central to improving patient outcome.

Calpains belong to the family of calcium-dependent cysteine proteases. Calpain-1 (large subunit of protein heterodimer μ-calpain) and calpain-2 (large subunit of protein heterodimer m-calpain) are ubiquitous isoforms that are activated by Ca^{2+} at micromolar and millimolar concentrations *in vitro* respectively (Goll *et al*., 2003). Found in all eukaryotes, calpains have been implicated in various cellular processes, such as signal transduction, cell proliferation, differentiation, apoptosis and necrosis (Goll *et al*., 2003; Suzuki *et al*., 2004). The role of the calpain system has been studied in various tumour types and it has been suggested that calpains maybe associated with chemotherapeutic response in certain cancer cells.

2.2.1 Conventional calpains and chemotherapeutic response

The role of calpain in chemotherapeutic response has been studied in different cancer types. Studies that are reviewed in the following section are summarised in **Table S2.1**, including the cancer types of the cell line models, the method(s) of calpain regulation and assessment of calpain activity.

2.2.1.1 Calpains and differences between chemo-sensitive and –resistant cell lines.

By comparing matched chemo-sensitive and chemo-resistant cell lines, calpains have been suggested as an important protein family involved in the cellular response to chemotherapy. As a potential NF-κB inducer, calpain-2 has been found to be overexpressed both at the mRNA and protein levels in chemo-resistant colon cancer HT-29R cells in comparison to the parental line (Fenouille *et al*., 2012). Calpain activity was significantly higher in cisplatin-resistant MeWo-cis1 cells than in the parental MeWo cells (Młynarczuk-Biały *et al*., 2006), and in chemo-resistant HT-29R and RSN cells, down-regulation of calpain-2 caused reduced NF-κB activation, increased IκBα levels and the partial restoration of the sensitivity to CPT-11 chemotherapy

(Fenouille *et al*., 2012). Whilst the ubiquitin proteasomal pathway has been reported as an important pathway (Krappmann & Scheidereit, 2005), calpains have also been shown to be involved in a parallel pathway that regulates IκBα degradation (Han *et al*., 1999).

Calpain-4 expression was shown to be significantly lower in cisplatin-resistant gastric cancer SGC-7901/DDP and BGC-823/DDP cells than in parental cells (Zhang Y *et al*., 2016). In addition, up-regulated calpain-4 expression sensitised chemo-resistant cells while down-regulated calpain-4 expression increased the resistance of the parental cells (Zhang Y *et al*., 2016). From Western blotting studies, calpain-4 expression was found to positively correlate with calpain-1 and calpain-2 expression, the cleavage of caspase-3 and PARP1 which may be the pathway that links with chemo-induced apoptosis (Zhang Y *et al*., 2016).

2.2.1.2 Calpains and cancer cell chemotherapeutic response

1) Calpain mediated chemo-induced cell death

Various studies have suggested that cisplatin induces apoptosis in a calpain-dependent pathway across different cancer types (Anguissola *et al*. 2009; Liu *et al*., 2008; Liu *et al*., 2009; Al-Bahlani *et al*., 2016). Cisplatin has been shown to initially act on the ER (endoplasmic reticulum) and the cisplatin-induced ER stress increases cytosolic Ca^{2+} concentration (Xu *et al*., 2015; Mandic *et al*., 2003), which then induced calpain activation and calpain-mediated apoptosis in triple-negative breast, melanoma, cervical carcinoma cells and colon cancer cells (Tan *et al*., 2006; Mandic *et al*., 2003; Shen *et al*., 2016 and Al-Bahlani *et al*., 2016). Mandic *et al*. (2002) reported that calpain activity was induced after 5-hour cisplatin treatment in melanoma cells (also in U1285 lung carcinoma cells and MDA-MB-231 breast carcinoma cells). Similarly, cisplatin induced a sharp peak of calpain activity at 4–6 hour after cisplatin treatment in ASTC-a-1 lung adenocarcinoma cells (Liu *et al*.,

2008).

The cisplatin-induced increase of intracellular Ca^{2+} can be achieved by releasing intracellular Ca^{2+} stores (e.g. endoplasmic reticulum (ER)) via inositol 1,4,5- trisphosphate (IP3) receptor/Ca^{2+} release channel and/or the extracellular Ca^{2+} via voltage-insensitive Ca^{2+} channels/IP3 receptor in ovarian cancer cell lines (Sergeev, 2004; 2005; 2012; Al-Bahlani *et al*., 2011; Splettstoesser, Florea & Büsselberg, *et al*., 2007). Calpain activation was increased following the cisplatin-induced increase of Ca^{2+} levels in ovarian cancer OV2008 cells (Al-Bahlani *et al*., 2011; Woo *et al*., 2012), whilst in C13* cells (the resistant counterpart of OV2008 cells), Al-Bahlani *et al*. (2011) observed a cisplatin-induced Ca^{2+} level increase and calpain activation, but Woo *et al*. (2012) detected no increase in calpain activity. In breast cancer MDA-MB-231 cells, cisplatin treatment led to an up-regulation of calpain-1 activity levels and down-regulation of calpain-1 mRNA, but had no effect on calpain-1 protein expression (Al-Bahlani *et al*., 2016).

As stated above, ER appears as a non-nuclear target for cisplatin, which seems to induce apoptosis by a nucleus-independent pathway (Mandic *et al*., 2003; Martins *et al*., 2011; Al-Bahlani *et al*., 2016). Cisplatin induced apoptosis in enucleated melanoma 224, enucleated colon cancer HCT 116, HCT 116 wild-type and HCT 116 p53-/- cells indicating that cisplatin induces apoptosis in the absence of DNA damage (Mandic *et al*., 2003). ER stress was up-regulated by cisplatin in melanoma 224 cells (Mandic *et al*., 2003) and cervical carcinoma HeLa cells (Shen *et al*., 2016) and calpain was required for ER-mediated apoptosis (Shen *et al*., 2016; Selimovic *et al*., 2011). Cisplatin-induced ER stress and apoptosis was also observed in SKOV3 parental cells but not the cisplatin-resistant SKOV3/DDP cells (Xu *et al*., 2015).

The chemotherapy-induced increase of calpain activity may not always be accompanied by an increase in intracellular Ca^{2+} levels. In human metastatic

melanoma Me665/2/21 cells, cisplatin induced increases in calpain activity in adherent cells (with minimal signs of cell death) and increased further in detached apoptotic cells without any accompanied increase in intracellular Ca^{2+} levels, suggesting that calpain activity is increased early in pre-apoptotic cells but calcium fluxes are unimportant in cisplatin-induced calpain activation and apoptosis (Del Bello *et al*., 2007, 2013). Another study also pointed out the importance of time-point as calpains seem to be only involved in the early process of cisplatin-induced apoptosis. Cisplatin-induced Bid cleavage and apoptosis appeared to be inhibited by calpeptin when calpeptin was co-incubated with cisplatin early during treatment (20 hours treatment, in melanoma, lung and breast cancer cell lines) (Mandic *et al*., 2002), but calpeptin could not inhibit cisplatin-induced apoptosis when it was added 8 hours after cisplatin treatment (Mandic *et al*. 2002).

In ovarian cancer OV2008 cells, calpain activation rather than expression was induced by cisplatin with the induction being time- and concentration-dependent (Al-Bahlani *et al*., 2011; Woo *et al*., 2012). Woo *et al*., (2012) suggested that Ca^{2+}/calpain contributed to the down-regulation of cisplatin-induced PARC (p53-associated parkin-like cytoplasmic protein, a calpain substrate) and apoptosis in certain ovarian cancer cell lines. The pretreatment of calpeptin attenuated cisplatin-induced PARC down-regulation, α-fodrin cleavage and apoptosis in OV2008 cells (Woo *et al*., 2012). As PARC inactivation can induce nuclear localisation of p53 and p53-induced apoptosis (Nikolaev *et al*., 2003), cisplatin-induced calpain activation facilitated cell apoptosis in OV2008 cells.

In cervical carcinoma cells, the calpain system also plays an important role in chemotherapy induced cell death (Hill, Hu & Evans, 2008; Al-Bahlani *et al*., 2011). The intracellular Ca^{2+} increase was found to trigger calpain activation and apoptosis in cervical cancer HeLa-S3 cells but not in osteosarcoma U2-OS cells (Splettstoesser, Florea & Büsselberg, 2007). Human

8-oxoguanine-DNA glycosylase 1 (OGG1), a DNA repair enzyme, was a specific target of calpain-1 and can be truncated and further degraded by the protease (Hill, Hu & Evans, 2008). OGG1 degradation was found induced by cisplatin in HeLa cells and this process could be inhibited by calpeptin (Hill, Hu & Evans, 2008). In a separate cervical carcinoma cell based study, an early and significant increase of calpain activation was detected in response to oxaliplatin; while calpain inhibition decreased Bid cleavage, mitochondrial depolarisation, and oxaliplatin-induced activation of caspase-9, -3, -2, and -8 (Anguissola *et al*., 2009). Shen *et al*., (2016) also found that caspase-3 was involved in the cellular response to cisplatin: cisplatin induced the expression of calpain-1 and the cleavage/activation of caspase-3 in cervical carcinoma HeLa cells. Similarly, caspases appeared down-stream of calpains in melanoma cells during cisplatin-induced, calpain-mediated, apoptosis; a calpain inhibitor was able to down-regulate caspase-3/-7 activity and protect cells from p53-induced apoptotic cell death (Del Bello *et al*., 2007).

In lung adenocarcinoma cells, cisplatin-induced cell death has been shown to be delayed by several hours when pre-treated with calpain inhibitors, such as calpeptin or PD150606 (Liu *et al*., 2008, Liu, Xing & Chen; 2009). Cisplatin-induced calpain activation led to Bid cleavage, followed by Bid translocation, cytochrome c release, and caspase-3 activation resulting in cell death (Liu *et al*., 2008). In addition, in cisplatin-induced apoptosis, the original mitochondrial μ-calpain cleaves apoptotic inducing factor (AIF), which then translocates from the mitochondria to the cytosol, followed by caspase-9 and -3 activation and apoptosis (Liu, Xing & Chen; 2009). In prostate cancer cells, cisplatin also induced calpain activation (Wang *et al*., 2010). Galectin-3 (a protein with anti-apoptotic functions) expression was associated with cisplatin resistance and galectin-3 knockdown sensitised cells to cisplatin in a calpain-2-dependent way (Wang *et al*., 2010).

Few *in vitro* studies have focused on the level of calpain-4 in response to

platinum-based chemotherapy. The up-regulation of calpain-4 expression was found to sensitise gastric cancer cells to cisplatin-induced apoptosis; moreover calpain-4 expression positively correlated with calpain-1 and calpain-2 expression as well as the cleavage of caspase-3 and PARP1, which might be the pathway that led to apoptosis (Zhang Y *et al*., 2016). Together, in most of the cancer types the treatment of platinum-based chemotherapy induces an increase in calpain activity, although the mechanism/protein downstream of calpain varied across different cancer types.

2) Calpain inhibitor sensitisation of chemo-induced cell death

The above studies suggest that calpains are activated by chemotherapy treatment and play an important role in chemo-induced cell death; however, several studies have shown that calpain inhibitors can chemo-sensitise certain cancer cells. Viral oncoprotein E7, which is highly expressed in human papilloma virus transformed cervical carcinoma, and calpain mediated the degradation of retinoblastoma protein (Rb, a tumour suppressor protein); thus calpain inhibitors have been shown to restore p53 and p53-mediated cell death thereby synergising with cisplatin (Darnell *et al*., 2007). Down-regulation of calpain-2 attenuated calpain-mediated IκBα degradation/ depolymerisation and decreased NF-κB activation which sensitised both colon cancer HT-29R and RSN cells to CPT-11 (irinotecan) (Fenouille *et al*., 2012). A similar process was also observed in melanoma cells. Calpain inhibitors prevented TNF-induced IκBα degradation and NF-κB activation in melanoma MeWo and MeWo-cis1 cells (as indicated in **Table S2.1**); whilst completely suppressed NF-κB activation via stabilising IκBα, especially in MeWo-cis1 cells (Młynarczuk-Biały *et al*., 2006). NF-κB inhibited by calpain inhibitor resulted in apoptosis in MeWo-cis1 whilst inducing necrosis in MeWo, however, the combination of calpain inhibitor and cisplatin did not further reduce the cell viability compared with calpain inhibitor alone.

3) Lack of involvement of calpains in chemo-induced cell death

In contrast to the above studies, others have indicated that calpains were not among the main regulators of the process of chemotherapy induced cell death. It is known that cisplatin induced an apoptotic cell death whereas hypoxia induced a necrosis cell death (Kim *et al*., 2007). As indicated by Kim *et al*., (2007), in hepatoblastoma cells, the cleavage of calpain-2 (activate form) was observed after hypoxia treatment but no calpain cleavage was observed after cisplatin treatment which suggested that calpain signalling was more important in hypoxia-induced cell death than in cisplatin-induced DNA damage. Calpain inhibitors switched hypoxia-induced cell death to apoptotic cell death without affecting cell viability (Kim *et al*., 2007).

2.2.2 Other calpains and chemotherapeutic response

In addition to the conventional calpains, other calpain family members have been found to be associated with cell chemoresponse. The study by Moretti *et al*. (2009) suggests that calpain-3 and its variants play a pro-apoptotic role in the cisplatin-treated melanoma cells. Calpain-6 inhibited cisplatin-induced apoptosis in HeLa cells and facilitated VEGF-induced angiogenic properties of HUVECs such as cell invasion and the formation of more organised endothelial cell networks in Matrigel; hence, Rho, Byun & Park *et al*. (2008) suggested that calpain-6 may promote tumourigenesis via inhibiting apoptosis and facilitating angiogenesis. In addition, calpain-6 was found highly expressed in leiomyosarcomas, uterine sarcomas and uterine cervical cancers (6/8) compared to normal tissues (Lee *et al*., 2008). Moreover, calpain-6 expression was found increased along with the progression of cervical neoplasia, indicating an important role that calpain-6 might played in the progression of uterine cervical neoplasia (Lee *et al*., 2008).

2.2.3 Aims

In a previous IHC study from our group (Storr *et al*., 2012a), high

expression of calpain-2 significantly associated with resistance to platinum-based adjuvant chemotherapy (*P*=0.031, Pearson chi-square test of association), poor PFS (*P*=0.049, Kaplan–Meier analysis) and worse OS (*P*=0.006, Kaplan–Meier analysis). Results from multivariate analysis showed that calpain-2 expression was an independent prognostic factor (accounting for grade, stage, optimal debulking and platinum sensitivity). Based upon such data experiments described in the current chapter sought to address the following hypotheses:

1. Calpain expression and the cell response to calpeptin in platinum-sensitive cells differ from their resistant counterparts;
2. Inhibition of calpain-2 can sensitise ovarian cancer cells to platinum based chemotherapy.

The study aimed to:

1. Study calpain-1, -2, -4 and calpastatin protein expression levels across 5 ovarian cancer cell lines (SKOV3, A2780, A2780-cis, PEO1 and PEO4) with different sensitivities to platinum-based chemotherapies;
2. Downregulate calpain-2 expression in the cell lines that have high endogenous calpain-2 expression;
3. Study cell proliferation and calpain activity across the 5 ovarian cancer cell lines in response to calpain inhibitor (i.e. calpeptin);
4. Investigate if altering calpain activity can sensitise ovarian cancer cells to chemotherapy.

2.3. Materials and methods

2.3.1 Cell lines, subculture, cryopreservation and cell recovery

In the current study, 5 ovarian cancer cell lines with differing sensitivities towards platinum-based chemotherapies were used. The human ovarian carcinoma cell line SKOV-3 (platinum-resistant cell line), A2780 (platinum-sensitive cell line, the parental cell line of A2780-cis) and A2780-cis (platinum-resistant cell line), along with PEO1 (platinum-sensitive cell line) and PEO4 (platinum-resistant cell line) cells were all obtained from the American Type Culture Collection (ATCC). The SKOV3 human ovarian cancer cell line was derived from ascitic fluid from a moderately well-differentiated ovarian adenocarcinoma patient. The A2780 human ovarian cancer cell line was established from tumour tissue from an untreated patient. A2780-cis was developed by chronically exposing the parental A2780 cell line to increasing concentrations of cisplatin. PEO1 and PEO4 are a matched set originating from the same patient. PEO1 was derived from ascitic fluid of a patient with ovarian adenocarcinoma which was clinically responsive to cisplatin while PEO4 derived from the ascites at the time of relapse with acquired cisplatin resistance. Characteristics of the panel of ovarian cancer lines are listed in **Table 2.1**. The five cell lines used in this study all have wild-type BRCA but only A2780 and A2780-cis cells have wild-type p53 (**Table 2.2**). PEO1 and PEO4 cells have mutant P53 (missense, nonsynonymous substitution).

Table 2.1 Characteristics of ovarian cancer cell lines.

Cell line	Original Tumour Histology	Stage	Grade	Morphology	Histology	Putative Histology	Isolated from	Pre-isolation		Post-isolation	
								Treatment	Response	Treatment	Response
PEO1	Serous	3	3	Epithelial	HGSC	HGSC	Ascites	Cisplatin, Chlorambucil, 5-Fluorouracil	Complete Response	Cisplatin, Chlorambucil, 5-Fluorouracil	Complete Response
PEO4	Serous	3	3	Epithelial	HGSC	HGSC	Ascites	Cisplatin, Chlorambucil, 5-Fluorouracil	Complete Response	Higher dose of cisplatin	Progressive Disease
SKOV3	Adenocarcinoma	\	1/2	Epithelial	serous	Endometrioid, C/ Clear cell	Ascites	Thiotepa	Unknown	Unknown	Unknown
A2780 A2780-cis	Unknown	\	\	Round	\	Endometrioid, C/ Clear cell	Primary tumour	None	\	Unknown	Unknown

Data adapted from (Stordal *et al*., 2013; Beaufort CM *et al*., 2014)

Table 2.2 BRCA and p53 status of ovarian cancer cell lines.

Cell line	BRCA1			BRCA2		P53
	Loss of heterozygosity	Mutation status	Methylated	Loss of heterozygosity	Mutation status	
PEO1	Yes	Wild-type	No	Yes	Wild-type/mutated	Missense mutation
PEO4	Yes	Wild-type	No	Yes	Wild-type/mutated	Missense mutation
SKOV3	No	Wild-type	No	No	Wild-type	Null
A2780 / A2780-cis	No	Wild-type	No	No	Wild-type/mutated	Wild-type

Data adapted from (Stordal *et al*., 2013; Beaufort CM *et al*., 2014; Im-aram *et al*., 2013; Cooke SL, *et al*., 2010)

SKOV-3 was grown in McCoy's 5A Modified Medium (M9309, SIGMA, UK; $CaCl_2$ 110.98 g/mol, (900.6127 µM Ca^{2+})) supplemented with 10% iron supplemented donor bovine serum (DBS) (Gibco, Life Technologies, UK). DBS was heat-inactivated by incubating for 30 minutes in a 56°C water bath before use. Penicillin/streptomycin (SIGMA, UK) (with 10,000 units penicillin and 10 mg streptomycin/mL) was used. Cell lines A2780 and A2780-cis were grown in Roswell Park Memorial Institute (RPMI) medium (R8758, SIGMA, UK; 0.1 g/L Calcium Nitrate • $4H_2O$ (423.4597 µM Ca^{2+})) supplemented with 10% DBS. In order to retain resistance, cisplatin (1 µM) was added to A2780-cis culture media every 2-3 passages. The passage window used for SKOV3, A2780 and A2780-cis cell lines was 15. The PEO1 and PEO4 were grown in RPMI medium (R8758, SIGMA, UK) containing Sodium Pyruvate (SIGMA, UK) 2mM and 10% DBS. The passage window used for these cell lines was 10. Cells were cultivated at 37°C in 5% CO_2 atmosphere.

Short tandem repeating (STR) DNA profiling can identify human cell lines derived from the tissue of a single individual (Reid *et al*., 2013). Cell lines were verified, approximately every 6-8 months, using Promega Powerplex® 16 short tandem repeat (STR) system, conducted by postdoctoral researchers within the group, to ascertain there was no misidentification or cross-contamination of cell lines. PEO1 and PEO4 were derived from the same patient, as were A2780 and A2780-cis, so using STR cannot discriminate within these platinum-sensitive and -resistant counterpart. 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).

Tissue culture was performed in a class II biological safety hood, which was cleaned by swabbing with 70% ethanol before and after use. To subculture, the original medium was removed and cell layers were washed with an equivalent volume of Dulbecco's phosphate buffered saline (PBS, Sigma, UK). Following aspiration 0.5mg/mL trypsin-EDTA (Sigma, UK) was

added to the flask to ensure the monolayer was completely covered, and incubated for 30 seconds. Extra trypsin-EDTA was removed and the flask was put back to incubator at 37°C. SKOV-3, A2780 and A2780-cis cells need to be incubated for 2 minutes at 37°C, PEO1 for approximately 15 minutes and PEO4 for approximately 8 minutes. The cells were then checked under a microscope to ensure they rounded up. Complete medium was used to deactivate trypsin-EDTA prior to harvesting the cells. Cells were dispersed with repeated pipetting to ensure a single cell suspension. The harvested cells were split into a new tissue culture flask and fresh medium added. The density of cells at seeding varied depending on the level of confluence. Usually cultures were split when reaching 70-80% confluence in a subcultivation ratio of 1:4 to 1:6.

To control for genetic drift, limit risk of microbial, viral or cellular cross-contamination, and ensure low passage number for subsequent experiments, cells were initially obtained from a master cell stock then expanded and frozen down in liquid nitrogen as working cell stocks. 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 $1X10^6$ cells /mL. The cells were transferred in labelled cryovials and put into CoolCell ® alcohol-free cell freezing containers which ensure a -1°C/minute freezing rate before being placed at -70°C overnight, after which cells in cryovials were stored in liquid nitrogen (-180°C) for further use.

To recover the frozen cells, cells were thawed immediately in a 37°C water bath and then transferred into a centrifuge tube with 9 ml of pre-warmed complete cell culture medium. The mixture of cell culture

medium and cell frozen medium were removed by centrifuged at 170 x g for 5 minutes and the cell pellet was resuspended in fresh growth medium.

2.3.2 Cell lysis and protein extraction

Subconfluent cells were trypsinised as above (**section 2.3.1**). Trypsinisation was stopped by adding complete culture medium. Cells were collected by centrifuging the suspension at 170 x g for 5 minutes. Cell pellets were then resuspended in PBS and counted. The cell suspensions were then centrifuged again to wash away any residual serum proteins. After removing the supernatant, 5 x 10^6 cells was resuspended in 1 ml of RIPA buffer (R0278, SIGMA, UK) supplemented with 1X Halt Protease Inhibitor Cocktail containing protease inhibitors, phosphatase inhibitors and EDTA (ThermoFisher Scientific, USA) to maintain and preserve protein functionality following cell lysis. Tubes were incubated at 4°C for 10 minutes and then lysates were frozen at -20˚C or -70˚C for long-term storage. Lysates were clarified by centrifuging at 15,000 rpm in a microcentrifuge for 10 minutes at room temperature to pellet the cell debris after thawing the lysates for the first time. Thawed lysates were kept on ice prior to use and repeat freeze-thaw cycles were avoided.

2.3.3 Western blotting

Buffers used in Western blotting were prepared as follow:

1X SDS running buffer: Add 50 ml MOPS SDS Running Buffer (20×) to 950ml distilled water;

1X NuPAGE® Transfer buffer: 25ml 20X NuPAGE Transfer Buffer (5%), 100ml methanol (20% v/v) and 375 ml distilled water.

Cell lysate (13 volumes) was mixed with NuPAGE® LDS sample buffer (4X) (5 volumes) and the NuPAGE® Reducing Agent (2 volumes) according to manufacturer's instructions and incubated at 100 ˚C for 10 minutes to denature the proteins before being cooled on ice. Protein samples were then

loaded with equal amounts (20 µl) into wells of the SDS-PAGE gel (Novex® ready-made NuPAGE®4-12% Bis-Tis Protein Gels, USA). In addition, Rainbow Marker (GE Healthcare, UK) and Magic Marker (Invitrogen, NY, USA) were loaded into the gel to allow a comparison against known molecular weight proteins. SDS PAGE was run at 125 V for 120 minutes (during which time the current and buffer were checked to avoid excessive heating). Proteins that were separated by gel electrophoresis were transferred from the gel onto a 0.2 µm nitrocellulose membrane (Millipore, MA, USA) by gel electrophoresis at 25 V for 90 minutes in 1x NuPAGE®transfer buffer. The membrane was then transferred into a 50ml centrifuge tube and blocked with 10 ml of blocking buffer (i.e. 1.5 g non-fat milk (Marvel Dried Skimmed Milk, UK) in 50 ml 0.1% PBS-Tween 20) on a Tube Roller Mixer for 1 hour at room temperature. The blocking buffer was removed and the membrane incubated with primary antibody (listed in **Table 2.3**) (Thermo Scientific, UK) which was diluted in 5ml blocking buffer in a 50ml centrifuge tube overnight at 4°C on a Tube Roller Mixer. The membrane was washed for 5 minutes with 0.1% PBS/Tween 20 three times before being incubated with anti-β actin polyclonal antibody (1 in 1,000 dilution) (listed in **Table 2.3**), using different host species with the primary antibody, for 1 hour at room temperature. Anti-β-actin antibody was used to ensure equal loading of sample in the gel. As indicated by Greer *et al*. (2010), the heat-shock β-actin protein stayed constant without being influenced by cell densities which makes it a reliable internal loading control.

Following washing the membrane three times for 5 minutes each, secondary antibodies were added and incubated with the membrane for 1 hour (680 Donkey anti-Mouse IgG (H + L) (926-32222, IRDye®, LI-COR, USA) and 800CW Donkey anti-Rabbit IgG (H + L) (926-32213, IRDye®, LI-COR, USA)). The membrane was washed three times for 5 minutes each with 0.1% PBS/Tween before being visualised using an Odyssey FC Imager (LI-COR Biosciences). Experiments were repeated 3 times independently with lysates

from cells with different passage numbers. Images were obtained and the fluorescence intensity was quantified via software Image Studio Ver 4.0. The signals of calpains or calpastatin were normalised by β-actin signals. Data are presented as the average ± standard deviation (SD) of three independent experiments.

Table 2.3 Source and dilutions of primary antibodies used in Western blotting.

Name	Company	Catalogue number	Dilution
Mouse calpain 1 large subunit monoclonal (P-6) antibody	SANTA CRU BIOTECHNOLOGY, INC., USA	sc-81171	1 in 1,000
Rabbit anti-calpain-II (m-Calpain) polyclonal antibody	Chemicon® (Millipore, MA, USA)	AB1625	1 in 2,500
Mouse anti-calpain small subunit (μ-or m-Calpain) monoclonal antibody	EMD Millipore, Germany	MAB3083	1 in 1,000
Mouse anti-calpastatin monoclonal (PI-11) antibody	EMD Millipore, Germany	MAB3084	1 in 1,000
Mouse Anti-β-actin	Abcam	ab8226	1 in 1,000
Rabbit Anti-β-actin	Abcam	ab8227	1 in 1000

2.3.4 Drug preparation and treatment

Cisplatin (p4394, SIGMA) was initially diluted to 1mg/ml in sodium chloride solution (0.9%) (S8776, SIGMA). Carboplatin (c2538 SIGMA) was initially diluted in sterile distilled water (dH_2O) to reach 20 mM. Both stock solutions were protected from light, stored at room temperature and used within 2 months. Calpeptin (03-34-0051, Merck Millipore) was initially diluted to 100 mM in dimethyl sulfoxide (DMSO, D2650, SIGMA), then the aliquoted stock solution was stored at -20°C. The stock solution was sub-diluted using complete culture medium to achieve the final working concentrations for cell treatment. For vehicle controls, sodium chloride solution (0.9%), dH_2O or DMSO was used and diluted using cell culture medium in the same ratio as cisplatin, carboplatin or calpeptin dilution, respectively.

2.3.5 Cell growth curves and evaluation of cell proliferation and chemo-sensitivity

Growth curves were initially conducted to determine control cell characteristics. Subconfluent cultures were trypsinised and resuspended as single cells in complete cell culture medium. Two ml of cells suspension were seeded into each well of a 6-well tissue culture plate at different seeding densities: $4X10^4$/ml A2780, A2780-cis or SKOV3 cells, $2X10^5$/ml PEO1 cells and $5X10^5$/ml PEO4 cells) in the wells of 6-well plates. Triplicate wells for each time point. At 24, 48, 72, 96 and 168 hours after seeding, cells were trypsinised and the total cell number counted by haemocytometer.

Growth curves were subsequently used to determine the toxicity of cisplatin, carboplatin and calpeptin. The proliferation of the cells was assessed after 24-, 48-, and 72-hour treatment with cisplatin, carboplatin or calpeptin. The initial seeding density was chosen based on the treatment duration and the control growth curve data of each cell line aiming to obtain a subconfluent culture of cells for drug treatment and to ensure the cells were at log phase during the experiment without refreshing culture medium. Same timeframe was used for all cell lines in the current study. For A2780, A2780-cis, and PEO1, the initial seeding density was 2×10^5 cells per well of 6-well plates for 24-hour time course, 1×10^5 cells/well for 48- and 72-hour time course. For SKOV3, the initial seeding density was 1.6×10^5 cells per well for 24-hour, 8×10^4 cells/well for 48- and 72-hour time course. Cells were incubated for 1 day to allow them to attach. For PEO4, the initial seeding density was 1×10^5 cells per well and incubated 3 days to allow them to attach. Cells were then treated with cisplatin, carboplatin or calpeptin over a range of doses. Sodium chloride solution, sterile distilled water or DMSO was added to the vehicle control groups at the same volume equivalent to that in the drug treatment groups. Each treatment condition was performed in triplicate. Cells were collected every 24 hours for the 72-hour experiment and were counted with a

haemocytometer. Then the total cell numbers in drug treated wells were plotted as the percentage of the total cell numbers in vehicle control wells. Data represent the average ± SD of at least two independent experiments, with each experiment performed in triplicate.

2.3.6 Calpain inhibition by calpeptin assessed by calpain activity assay

Calpain activation requires Ca^{2+} and calpain translocation. Molinari and Carafoli (1997) indicated that calpain proteins become active after translocation from the cytosol to membranes and into the lumen of organelles (especially ER and Golgi apparatus). In addition, while preparing cell lysates, the subcellular structure can be all disrupted; in this condition, calpastatin can rapidly associate with active calpain (Han *et al*., 1999). In order to avoid introducing artificial errors, it is therefore best to measure calpain activity levels in intact cells (Del Bello *et al*., 2007). Calpain activity, in the current project, was measured in living cells by reading fluorescence intensity obtained from a membrane-permeant non-fluorescent calpain substrate t-BOC (CMAC, t-BOC-Leu-Met, 7-Amino-4-Chloromethylcoumarin, t-BOC-L-Leucyl-L-Methionine amide) that fluoresces upon cleavage by calpain. This calpain substrate consists of a lipid-soluble calpain substrate peptide Boc-Leu-Met and a glutathione binding fluorophore 7-amino-4-chloromethylcoumarin (CMAC). t-BOC is conjugated to glutathione by glutathione transferase on entering the cell and then becomes membrane-impermeant. Glutathione links to the coumarin moiety which releases fluorescence and allows the measurement of intracellular calpain activity (Sobhan *et al*., 2013; Farr & Berger, 2010). Treatment of cells with calpeptin has been shown to lead to a decrease in fluorescence intensity in this assay. Calpain-cleaved t-BOC can produce fluorescence with excitation 351 nm/emission 430 nm (Excitation filter: 350; Emission filter: 430). Initially, in the current work, $5X10^5$ SKOV3, A2780 and A2780-cis cells or $1X10^6$ PEO1 and PEO4 cells were seeded into T25 tissue culture flasks. After 24 hours, the cells

were treated with a sub-IC50 concentration or IC50 concentration (the concentration which can reduce half of the cell proliferative ability) of calpeptin for various times as indicated in the results (**section 2.4.6**). DMSO was added to vehicle control groups using the volume equivalent to that in the drug treatment groups. Cells were then harvested before diluting the collected cells to $5X10^5$/mL (for SKOV3, A2780 and A2780-cis) or $1X10^6$/mL (for PEO1 and PEO4) in 500 µL serum free RPMI-1640 medium. The 500 µL of resuspended cell suspension was aliquoted into two groups (250 µL each vial): in one group, 2 µL pre-warmed t-BOC (2 mM stock) per vial was added to assess the whole fluorescence intensity after the addition of calpain substrate, t-BOC; for the other group, 2 µL DMSO (final conc. 0.8%) per vial was added to assess the background fluorescence intensity. The samples were added into a 96-well fluorescence plate with optical bottom (100 µl/well in duplicate for each sample) and read using a BMG FLUOstar OPTIMA Microplate Reader (pre-heated and kept at 37°C). Fluorescence was continually recorded on a 10 cycle kinetic window (excitation/emission 350/430, gain 2000, ~4 minutes interval). The results were normalised using the reading of the first cycle and the reading of medium alone. The values from the background fluorescence intensity were also subtracted from the total fluorescence intensity to obtain the fluorescence intensity of cleaved t-BOC which represented calpain activity.

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 ± SD of at least two independent experiments.

2.3.7 Cell growth curves and evaluation of chemo-sensitisation of calpeptin

Cells were seeded at the same seeding density as mentioned in **section 2.3.5**. Cells were pre-treated for 1 hour or 90 minutes with calpeptin followed

by a 48-hour treatment with cisplatin or carboplatin (IC50 concentration) in the presence of calpeptin. Based on results from the calpain activity assay, 50 µM of calpeptin was chosen for calpain inhibition in SKOV3, PEO1 and PEO4. Cells in each well were trypsinised and counted by haemocytometer. Total cell numbers in drug treatment cultures was showed as the percentage of total cell numbers in vehicle cultures. Data represent the average ± SD of at least two independent experiments, each performed in triplicate.

2.3.8 Clonogenic assay of cisplatin/carboplatin with/without calpeptin

For the study of drug treatment and chemo-sensitivity measurements, cells were initially seeded in T25 tissue culture flasks at the same densities as used in the study of cell growth curves. Cells were then treated with cisplatin/carboplatin alone (concentration indicated in results) or with cisplatin/carboplatin plus calpeptin as described in **section 2.3.7**.

After collecting cells, they were counted and plated into T25 tissue culture flasks (seeding densities varied according to individual cell lines and respective plating efficiencies etc.). Flasks were incubated for 2-3 weeks (incubation periods varied according to individual cell lines and depended on cell doubling times etc.) at 37°C, 5% CO_2 to allow colony formation. Incubation periods were chosen to leave control colonies sufficient time to produce colonies consisting of at least 50 cells. Following incubation flasks were washed gently once with PBS. Colonies were fixed with 50% methanol in 0.9% saline solution for 5 minutes and then with pure methanol for 1 minute. The fixed colonies were stained with 0.01% crystal violet (in PBS) for 4 hours. Stained colonies that contained more than 50 cells were counted.

Prior to conducting chemo-sensitivity/chemo-resistance studies, the plating efficiency (PE), an inherent characteristic of the cells, was initially determined using the clonogenic survival assay. In addition, the PE for each cell line was used to determine the optimal plating densities for drug

treatment experiments. Briefly, sub-confluent cells were plated, at a series of cell densities (e.g. 200, 400, 800, 1000 or 2000 cells per flask depend on cell lines) and incubated for different time periods (depending on the cell type) without disturbance. The cell density and the incubation time that generated the highest PE and appropriate colony size was applied in the following studies (i.e. 1000/2000 cells per flask in 0.5μM carboplatin (PEO1), 0.5μM cisplatin (PEO1) and 6μM carboplatin (PEO4) treated groups; 2000/4000 per flask in 1μM carboplatin (PEO1) and 1μM cisplatin (PEO1) treated groups; 4000/8000 per flask in 15μM cisplatin (PEO4) and 10μM carboplatin treated (PEO4) groups; and 200/500 or 500/1000 per flask in the other groups).

The PE was generated from untreated controls and calculated as:

$$\mathrm{PE} = \frac{\text{Number of colonies formed}}{\text{Number of cells plated}} \times 100\%$$

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

$$\mathrm{SF} = \frac{\text{Number of colonies formed after treatment}}{\text{Number of cells plated} \times \mathrm{PE}}$$

(Woolston and Martin, 2011).

The SF of cells following drug combination treatment was calculated as:

$$\mathrm{SF}_{\mathrm{calpeptin}} = \frac{\text{Number of colonies formed after calpeptin treatment}}{\text{Number of cells plated} \times \mathrm{PE}}$$

$$\mathrm{SF}_{\mathrm{combination}} = \frac{\text{Number of colonies formed after cisplatin or carboplatin treatment in combine with calpeptin treatment}}{\text{Number of cells plated} \times \mathrm{PE} \times \mathrm{SF}_{calpeptin}}$$

All individual experiments were performed in triplicate. Data represent the average SF ± SD of at least two independent experiments unless otherwise specified, each performed in triplicate.

2.3.9 shRNA transfection

In order to specifically inhibit calpain-2, short hairpin RNA (shRNA) was used to knock-down endogenous calpain-2 expression. shRNA was used rather than siRNA due to the 5-day metabolic half-life of m-calpain (Zhang, Lane & Mellgren, 1996) which could not serve the time course of current study and, if used, would require multiple transfections over a period of time to ensure knockdown. Before transfection, the puromycin (Thermo Fisher Scientific, USA) concentration, used for selection, was optimised in both SKOV3 and PEO4 cells. For each cell line, the experiments were conducted twice with two different passage numbers. Puromycin concentrations at 0.2, 0.5, 1, 2, 3, 4 and 5μg/ml were included in the optimisation experiments. Cells were checked by microscopy every day to find out the lowest concentration that could kill all wild-type cells within 10 days.

Generally, cell lines with a higher expression level of the targeted gene(s) are better for the investigation of the knockdown effects (Xu *et al*., 2009). Knockdown of calpain-2 was performed by transfection of shRNA into PEO4 and SKOV3 cells which were pre-selected based on the high calpain-2 expression by Western blotting. Both Lipofetamine 3000 (life technologies, UK) and FuGENE® HD transfection reagent (Promega, USA) were used for delivering the shRNA plasmid into the cells. Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, USA) was used for the dilution of the transfection reagents. One set of HuSH 29mer shRNA constructs against calpain-2 in pRS plasmid (CAPN2-human, 4 unique 29mer shRNA constructs in retroviral untagged vector, TR314204, OriGene Technologies, USA); along with scrambled negative control non-effective shRNA cassette in pRS plasmid (TR30012, OriGene Technologies, USA) and negative control shRNA (pRS vector; TR20003, OriGene Technologies, USA) in pRS plasmid were purchased. The 4 unique shRNA sequences are:

TR314204A (TI356809) ACGCCAAGATCAACGGATGCTATGAAGCT;

TR314204B (TI356810) GCCATCAAGTACCTCAACCAGGACTACGA;

TR314204C (T1356811) CAACTCCACCAAGTCATCGTTGCTCGGTT;

TR314204D (TI356812) AGATCTCTGCCTTTGAGCTGCAGACCATC.

The pRS vector contains a puromycin-N-acetyl transferase sequence as a selection marker and puromycin can be used for selection once the suppressing function of a shRNA vector is established

To reach approximately 60-70% confluence before conducting transfection, $5X10^5$/ml PEO4 cells were seeded into 6-well (2ml/well) or 24-well (500µl/well) tissue culture plates. After 24 or 48 hours the cell medium was replaced with 1-2 ml (6-well) or 500 µl (24-well) fresh media, or Opti-MEM I Reduced Serum Medium (without antibiotics) for each well. The final DNA concentration in each well was 0.75µg/ml or 1.5µg/ml (0.1 µg/uL stock).

When using FuGENE® HD transfection reagent (Promega, USA), the reagent and DNA construct/ scrambled control/ pRS vector was mixed in Opti-MEM I Reduced Serum Medium following the manufacturer's instructions. Different reagent:DNA ratios (i.e. 4:1, 3:1 and 2:1) were used in attempts to successfully transfect constructs into PEO4 cells. A Reagent:DNA ratio of 3:1 was used for SKOV3 transfection. Mixtures were added to designated wells drop-wise whilst gently swirling the plate.

When using Lipofetamine 3000, the reagent was diluted in Opti-MEM® Medium. DNA was diluted in Opti-MEM® Medium and then P3000™ reagent added following the manufacturer's instructions. As above, different reagent:DNA ratios (i.e. 3:4 and 3:2) were used in attempt to successfully transfect constructs into PEO4 cells. Then diluted DNA was added to diluted reagent and incubated for 5 minutes at room temperature. After the incubation, the mixtures were added into the designated wells drop-wise

whilst gently swirling the plate. The final DNA concentration in each well was 2µg/ml or 2.5µg/ml (0.1 µg/uL stock) plasmid.

Expression periods of 48 or 72 hours were used post-transfection, before 0.5µg/mL or 1 µg/mL selective agent puromycin (Thermo Fisher Scientific, USA) was applied for the selection of transfected cells and cells incubated for 2-4 weeks during which time selective media was refreshed every 2-3 days. To evaluate the effect of transfection, the lysates of transfected cells, scrambled control, negative control and wild-type cells were collected and their protein levels were assessed by Western blotting. Meanwhile, the polyclonal mixed population was plated into 96-well plates with a dilution of 1 cell/well to establish monoclonal lines. Normally, the isolation of monoclones from polyclonal cell culture should be conducted after examining the calpain-2 protein expression. Due to time limitation, the expansion of polyclonal cells for lysate collection and the monoclone isolation was conducted during the same period of time.

2.3.10 Statistical analysis

Results from *in vitro* experiments were represented as average ± inter-experiment 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 more than groups) were chosen to evaluate whether data differ significantly between the control and the drug treatment groups when at least three independent experiments were conducted. SDs were calculated using the following formula:

$$\mathrm{SD}_{\mathrm{combined}}=\sqrt{\left\{\frac{\left[(n_1-1)\times {SD_1}^2+(n_2-1)\times {SD_2}^2\right]}{n_1+n_2-y}\right\}}$$

Where $\mathrm{SD}_{\mathrm{combined}}$ is the combined SDs from y (number of experiments) experiments, SD1 and SD2 are intra-experiment SDs in individual experiments and n_1 and n_2 are number of replicate in each experiment (Lewis Sarah, personal communication; Abhik Mukherjee, 2004).

IC50 values were estimated from the growth curves between inhibition ratios and concentration gradients and also obtained through the Probit regression model between inhibition ratios and concentration gradients. An average inhibition ratio corresponding to each concentration gradient was calculated from two or three independent experiments. A fitting line Y=A+BX was gained, plotted by log (concentration) as the horizontal axis and probit of inhibition ratio as the vertical axis. When Y was half of probit of inhibition ratio, the value of X was log (IC50). Then the value of IC50 was calculated from log (IC50). $P<0.05$ denoted statistical significance. Statistical analysis was performed using SPSS 22.0 software.

2.4. Results

2.4.1 Cell growth curves and doubling times

From the cell growth curves of the 5 cell lines (**Figure S2.1** & **Figure S2.2**), doubling times (summarised in **Table 2.4**) were calculated using the following the equation: Doubling time=T×log(2, [2])/(log(N2,[2])-log(N1, [2])), Where N1: initial cell number; N2: final cell number; T: time interval

Table 2.4 Doubling time calculated from current data and reviewed from publications

Cell lines	Doubling time (hours)	Doubling time (hours) from publications and reference
A2780	20	~20 (Kuang *et al*., 2001); 27.3 ± 1.6 (Haslehurst *et al*., 2012); 13.5±3.2 (Roberts *et al*., 2005); 18 (Round), 22(ECACC, Round) (Beaufort *et al*., 2014)
A2780-cis	20	45.7 ± 2.4 (Haslehurst *et al*., 2012); 25 (ECACC, Spindle) (Beaufort *et al*., 2014)
SKOV3	32	35 (ATCC, 2012); 32.4±3.6 (Roberts *et al*., 2005); 27 (Epithelial), 34 (ECACC, Spindle) (Beaufort *et al*., 2014)
PEO1	57	37 (passage 77 to 82) (Langdon *et al*., 1988); 25.4±6.4 (Roberts *et al*., 2005); 84 (Epithelial) (Beaufort *et al*., 2014);
PEO4	97	46 (passage 2 to 7), 36 (passage 48 to 54)(Langdon *et al*., 1988); 24.1±5.2 (Roberts *et al*., 2005); 106(Epithelial) (Beaufort *et al*., 2014);

2.4.2 Cell proliferation in response to platinum-based chemotherapy (cisplatin or carboplatin)

The cell lines were investigated for their sensitivity towards cisplatin and carboplatin by determining a dose response curve obtained from cell counts. Dose- and time-dependent inhibitory effects of cisplatin and carboplatin can be observed in A2780, A2780-cis (**Figure 2.1**), SKOV3 (**Figure 2.2**) and PEO1 and PEO4 cells (**Figure 2.3**).

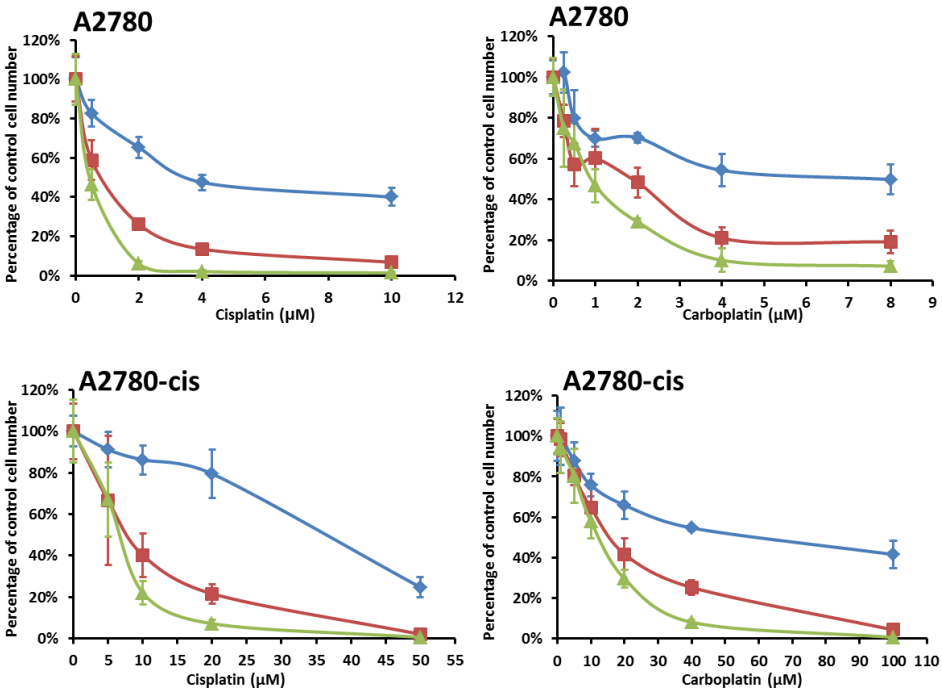


Figure 2.1 Effect of platinum-based chemotherapy on proliferation of ovarian cancer cells. Cell growth is presented as the percentage of vehicle control. A2780 and A2780-cis cells were treated with various concentrations of cisplatin or carboplatin for 24 (blue line), 48 (red line) and 72 (green line) hours. Doses ranged from 0–50 μM for cisplatin, and from 0–100 μM for carboplatin. Data represent the average ± SD of two independent experiments, with each experiment performed in triplicate.

A2780 cells were more sensitive than its resistant counterpart in response to cisplatin and carboplatin treatment. After 24-hour treatment, 10 μM of cisplatin induced more than 60% inhibitory effect on A2780 proliferation whereas 20 μM of cisplatin caused a 20% inhibition of A2780-cis proliferation. The dose-response curves at the 48-hour time point and the 72-hour time point were similar in both cell lines in response to cisplatin: a

dramatic decrease of proliferation occurred between 0 and 2 µM in A2780 cells, whereas A2780-cis cell proliferation decreased gradually between 0 and 20 µM. In response to carboplatin, the proliferation of A2780 cells dropped steadily between 0 to 4 µM at all-time points after which the inhibition stayed constant at round 40%, 80% and 95% at the 24-, 48- and 72-hour time point respectively. In response to carboplatin, A2780-cis cell proliferation dropped steadily between 0 to 40 µM carboplatin at all-time points after which the inhibition stayed constant at round 50% at the 24-hour time point whilst the inhibition dropped further at the 48- and 72- hour time point.

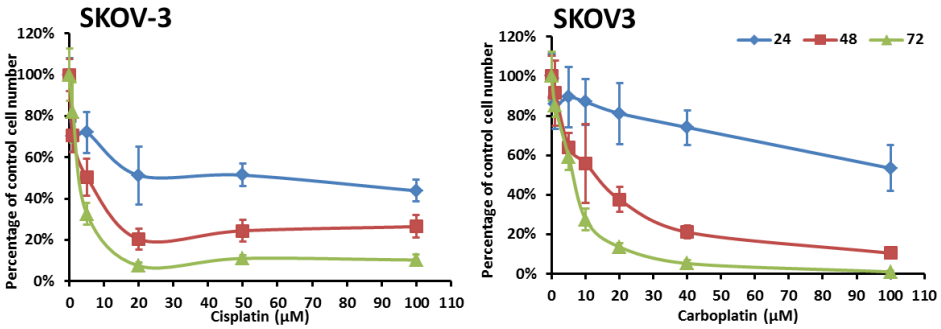


Figure 2.2 Effect of platinum-based chemotherapy on proliferation of ovarian cancer cells. Cell growth is presented as the percentage of vehicle control. SKOV3 cells were treated with various concentrations of cisplatin or carboplatin for 24, 48 and 72 hours. Doses ranged from 0–100 µM for cisplatin and carboplatin. Data represent the average ± SD of three independent experiments, with each experiment performed in triplicate.

Twenty four hours cisplatin treatment caused a 50% inhibition of SKOV-3 cell proliferation at approximately 20 µM whilst the inhibition of proliferation reaching 40% after increasing carboplatin concentration to 100 µM (**Figure 2.2**). Cisplatin caused a gradual decline of SKOV-3 cell proliferation between 0 to 20 µM at all-time points after which the inhibitory effect remained steady at 50%, 80% and 95% at the 24-, 48- and 72-hour time point respectively. SKOV-3 responded to the increase in carboplatin concentration with a moderate decrease in cell proliferation at the 24-hour time point. Cell proliferation steadily decreased at the 48- hour time point from 0 to 40 µM and plateaued at approximately 90% inhibition with carboplatin concentrations reaching 100 µM. At the 72-hour time point, a steep decrease

in cell proliferation occurred between 0 and 20 µM with the inhibitory effect reaching approximately 90%.

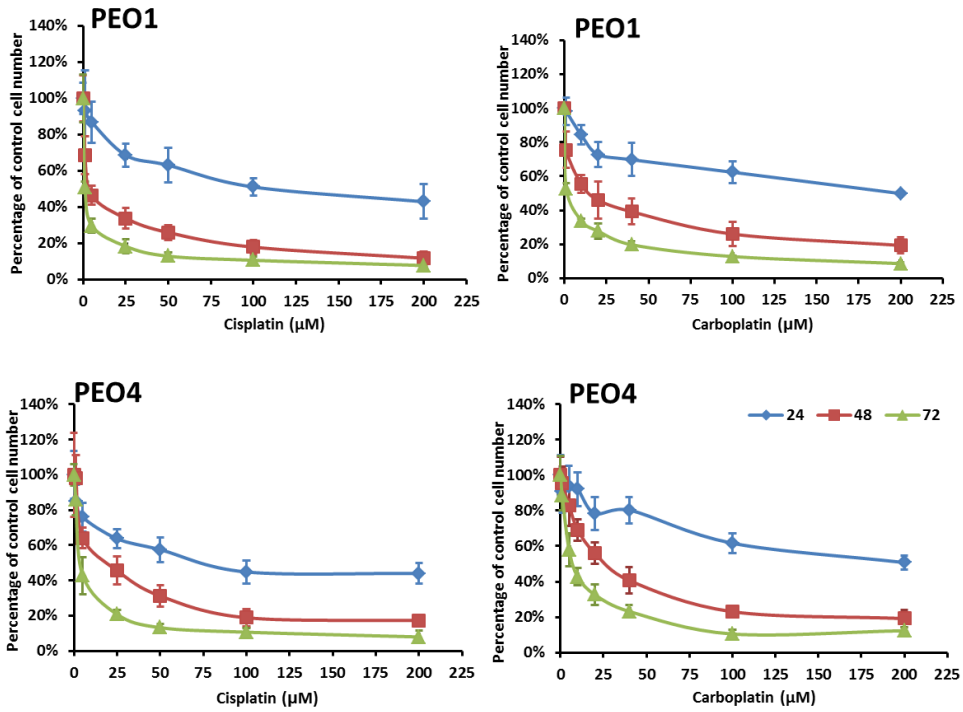


Figure 2.3 Effect of platinum-based chemotherapy on proliferation of ovarian cancer cells. Cell growth is presented as the percentage of vehicle control. PEO1 and PEO4 cells were treated with various concentrations of cisplatin or carboplatin for 24, 48 and 72 hours. Doses ranged from 0–200 µM for cisplatin and carboplatin. Data represent the average ± SD of three independent experiments, with each experiment performed in triplicate.

PEO1 and PEO4 cells responded to cisplatin concentration increasing with a moderate decrease in proliferation at the 24-hour time point between 0 and 100 µM; whereas at 72-hour the decrease was sharp between 0 to 5 µM and followed by a more moderate decrease up to 100 µM after which the inhibition was maintained at the same level (just above 80%) (**Figure 2.3**). At the 48-hour time point, a sharp decrease in cell proliferation was observed in cisplatin-treated PEO1 whilst in cisplatin-treated PEO4 the decrease was moderate. PEO1 and PEO4 responded to carboplatin in a similar pattern: a moderate decrease at the 24-hour time point between 0 and 200 µM; a marked decrease at 48- and 72-hour between 0 and 100 µM after which the inhibition was maintained at a steady level (of approximately 80%).

IC50 values were estimated from **Figure 2.1**, **Figure 2.2** and **Figure 2.3** and are shown in **Table 2.5** and **Table 2.6** together with the IC50 values from the published literature (results analysed by Probit Regression are listed in **Table S2.2**). As indicated in **Table 2.5** and **Table 2.6** other studies mainly applied high-throughput screening assays such as tetrazolium-based MTT and MTS assay and SRB assay to determine the IC50 values. The MTT assay determines cell viability rather than cell numbers and mainly relies on enzymatic reduction of the dye, tetrazolium compound, to water insoluble formazan crystals by dehydrogenases occurring in many organelles including mitochondria and endoplasmic reticulum (van Tonder *et al*., 2015). The sulforhodamine B assay determines cell numbers based on measuring the total cellular protein content rather than cell functionality (van Tonder *et al*., 2015). The comparability of the IC50 values may be limited by the different assays applied in these studies.

Cisplatin IC50 values have been intensively studied in the panel of ovarian cancer cell lines and results from current study fall within the range of existing values (**Table 2.5**). There are only limited published studies with carboplatin and IC50 values are invariably single time-point measurements that are more than 10-fold higher than the values obtained from current study (**Table 2.6**). As indicated above, MTT measures the cell viability rather than cell numbers which may contribute to such differences.

Table 2.5 IC50 of cisplatin in the panel of ovarian cancer cell lines in publications and the approximate IC50 of cisplatin in the current study

Cell line	Treatment period	IC50 (μM) assessed by MTT (unless otherwise indicated) and reference	Estimated IC50 from current study (μM)
SKOV 3	24h	\	20
	48h	9.09–12.08 (Wei *et al.*,2009); 10 (Wst-1 assay) (Vang *et al*. ,2013)	5
	72h	21.7 (Smith, 2005); 18.2±0.1 (Ganta *et al*., 2014); 6.7±2.1 (Roberts *et al*., 2005); 12.442 (Banerji *et al*. 2008); 341 nM (CellTiter 96 AQueous One Solution Cell Proliferation Assay, MTS) (Teoh D. *et al*., 2011); 42.9 (sulforhodamine B (SRB) assay) (Huynh *et al*., 2012); 28.8 (SRB) (Stronach *et al*., 2011)	3
A2780	24h	16.34 (Jendželovská *et al*., 2014); 11.5±1.6 (Dvořák *et al*., 2012)	3.5
	48h	5.04 (Jendželovská *et al*., 2014); 1.5±0.09 (Zamora *et al*., 2013)	0.8
	72h	2.81 (Jendželovská *et al*., 2014); 0.19 (Godwin AK. *et al*., 1992); 3.6±0.1 (Ganta *et al*., 2014); 4.53±1.9 (Koch *et al*., 2013); 0.4+0.1(Roberts *et al*., 2005); 6.177 (Banerji *et al*., 2008); 84.6 nM (CellTiter 96 AQueous One Solution Cell Proliferation Assay, MTS) (Teoh *et al*., 2011)	0.4
A2780-cis	24h	37.49 (Jendželovská *et al*., 2014); 30.5±6.1 (Dvořák *et al*., 2012)	36
	48h	12.05 (Jendželovská *et al*., 2014); 13±1(Zamora *et al*., 2013); 45.32–67.09 (Wei *et al*., 2009).	7.5
	72h	7.36 (Jendželovská *et al*., 2014); 28.50–47.41(Wei *et al*., 2009); 10.95±1.11 (Koch *et al*., 2013).	6.5
PEO1	24h	\	107
	48h	\	3
	72h	1.0±1.2 (Roberts *et al*., 2005); 1.33(SRB) (Stronach *et al*., 2011)	1
PEO4	24h	\	72
	48h	\	19
	72h	9.1±0.2 (Roberts *et al*., 2005); 11.6 (SRB) (Stronach *et al*., 2011)	3

Table 2.6 IC50 of carboplatin in the panel of ovarian cancer cell lines in publications and the approximate IC50 of carboplatin in the current study

Cell line	Treatment period	IC50 (μM) assessed by MTT (unless otherwise indicated) and reference	Estimated IC50 from current study (μM)
SKOV 3	24h	\	>100
	48h	\	11
	72h	23.8 (Smith, 2005); 96.8±4.6 (Roberts *et al*., 2005)	6
A2780	24h	\	6.5
	48h	\	1.8
	72h	6.8+3.0 (Roberts *et al*., 2005)	0.9
A2780-cis	24h	\	58
	48h	\	16
	72h	\	12
PEO1	24h	\	200
	48h	\	14
	72h	13.6±3.9 (Roberts *et al*., 2005)	1.5
PEO4	24h	\	200
	48h	\	25.5
	72h	120.5±24.7 (Roberts *et al*., 2005)	7

The anti-proliferative effects of cisplatin and carboplatin were linked to both concentration and treatment time in all 5 cell lines. All lines were more sensitive to cisplatin than carboplatin. Comparing IC50 values at each treatment time point, the IC50s of carboplatin were approximately doubled those of cisplatin. These measurements confirmed that the IC50 values of cisplatin and carboplatin in platinum-sensitive cells are lower than the IC50 values in their platinum-resistant counterparts.

2.4.3 Expression levels of calpains and calpastatin in ovarian cancer cell lines

Calpain system protein expression level was assessed across the 5 cell lines by Western blotting (**Figure 2.4**) and significant variations were observed between the different cell lines.

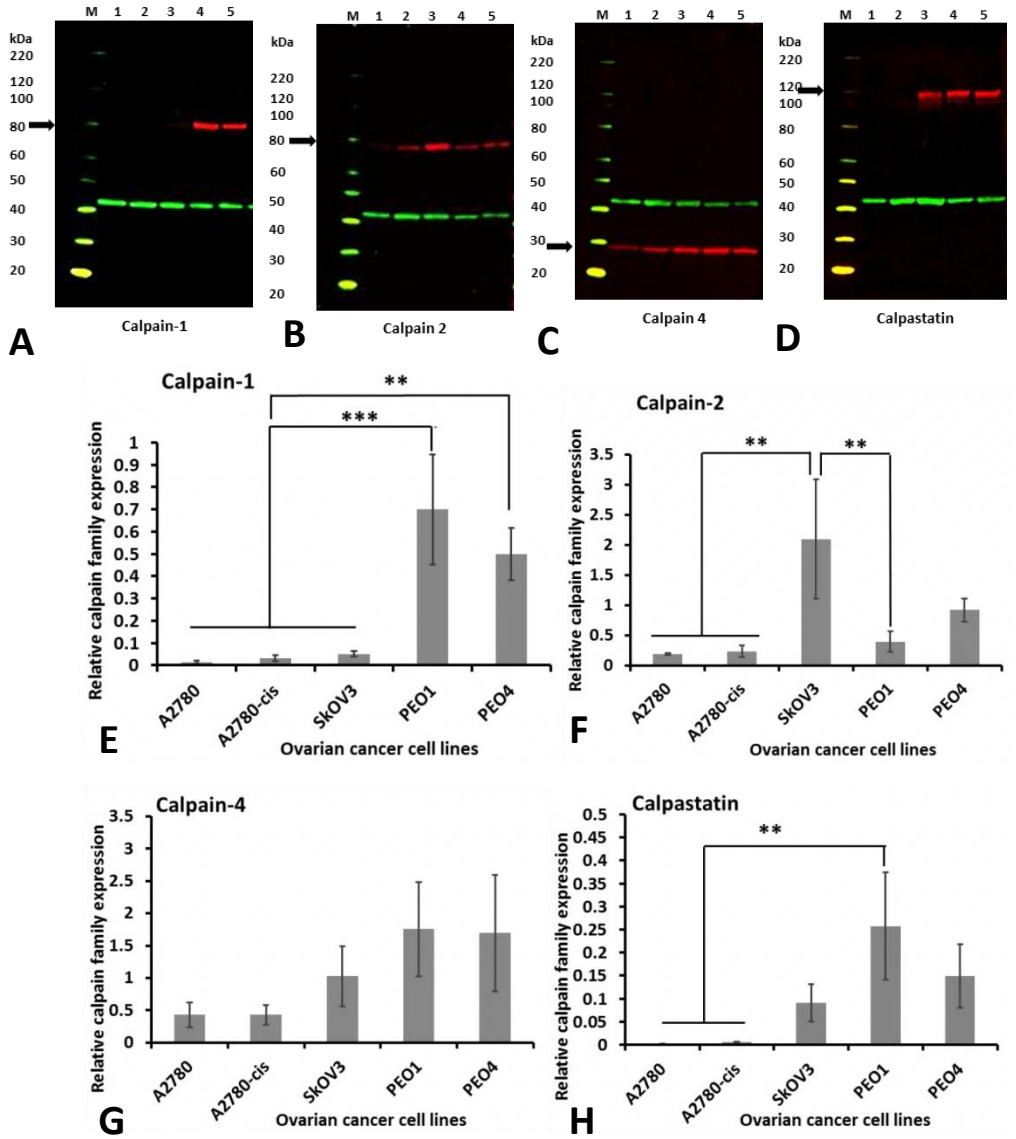


Figure 2.4 Quantification of calpain family protein expression across 5 different ovarian cancer cell lines by Western blotting analysis. Representative blots of three independent experiments are presented here. β-actin was used as the loading control (42 kDa). Arrows indicate **(A)** calpain-1 (82 kDa), **(B)** calpain-2 (80kDa), **(C)** calpain-4 (28-kDa) and **(D)** calpastatin (between 100-120 kDa) respectively. Lane M: Protein marker, Lane 1: A2780, Lane 2: A2780-cis, Lane 3: SKOV3, Lane 4: PEO1, and Lane 5: PEO4. The signals of **(E)** calpain-1, **(F)** calpain-2, **(G)** calpain-4 and **(H)** calpastatin were normalised by β-actin signals. Data are presented as the average ± SD of three independent experiments. Statistical significance determined by one-way ANOVA test is indicated by asterisk. **P*<0.05, ***P*<0.01 and *** *P*<0.001.

The expression levels of calpain-1 in PEO1 and PEO4 cells were significantly higher than the levels in A2780 (*P*<0.001, *P*=0.006), A2780-cis (*P*=0.001, *P*=0.008) and SKOV3 cells (*P*=0.001, *P*=0.01). SKOV3 cells expressed the highest level of calpain-2, followed by PEO4 cells. Calpain-2 level of SKOV3

cells was significantly higher than A2780 (*P*=0.004), A2780-cis (*P*=0.005) and PEO1 cells (*P*=0.009). As SKOV3 and PEO4 cells expressed the highest level of calpain-2, they were selected for calpain-2 knockdown (**section 2.4.4**). PEO1 and PEO4 cells expressed the highest level of calpain-4 followed by SKOV3 cells, then A2780 and A2780-cis cells. Calpastatin had duplicate bands between 100-120 kDa which could be the isoforms of calpastatin (Parr *et al*., 2001) and the duplicate bands were also observed in the study of Xuan (2016) in breast cancer cell lines. Calpastatin expression was hardly detectable in A2780 and A2780-cis cells. Calpastatin level in PEO1 cells was significantly higher than the calpastatin expression levels in A2780 (*P*=0.005) and A2780-cis cells (*P*=0.006).

No significant difference of calpain system protein expression was observed between cell lines and their resistant counterpart. A2780 cells and its resistant counterpart A2780-cis cells expressed similar levels of calpain-1, -2, -4 and calpastatin proteins. The expression levels were also similar between PEO1 and PEO4 cells. The calpain-2 expression in PEO4 cells was higher than the expression in PEO1 cells but the difference was not significant.

2.4.4 shRNA transfection

Based on the Western blotting results, PEO4 and SKOV3 cells were chosen for calpain-2 knockdown. Puromycin at 0.5 µg/mL was able to kill all the non-transfected PEO4 cells in 10 days and kill all the non-transfected SKOV3 cells within 7 days. Increasing the concentration to 1 µg/mL killed all the non-transfected PEO4 cells in 5 days. PEO4 cells were initially chosen for transfection due to their higher PE, which might offer an increased chance of successfully establishing monoclonal stable cell lines following cell selection. Four unique 29mer human calpain-2 shRNA constructs in retroviral untagged vector (OriGene Technologies, USA) were used in attempts to knock-down calpain-2 expression in PEO4. The following factors were adjusted to serve the

purpose: cell passage numbers, incubation media, reagent:DNA ratios, incubation times before and after transfection. Unfortunately no PEO4 cells survived puromycin selection which might be due to their long doubling times as mentioned above, although different recovery times were tried before and after adding the selection medium. SKOV3 cells were therefore subsequently tried. Although transfected SKOV3 cells survived puromycin selection, unfortunately none of the 29mer shRNA constructs were able to significantly reduce calpain-2 expression (**Figure 2.5**). This might be due to the initial high calpain-2 expression in SKOV3 cell lines.

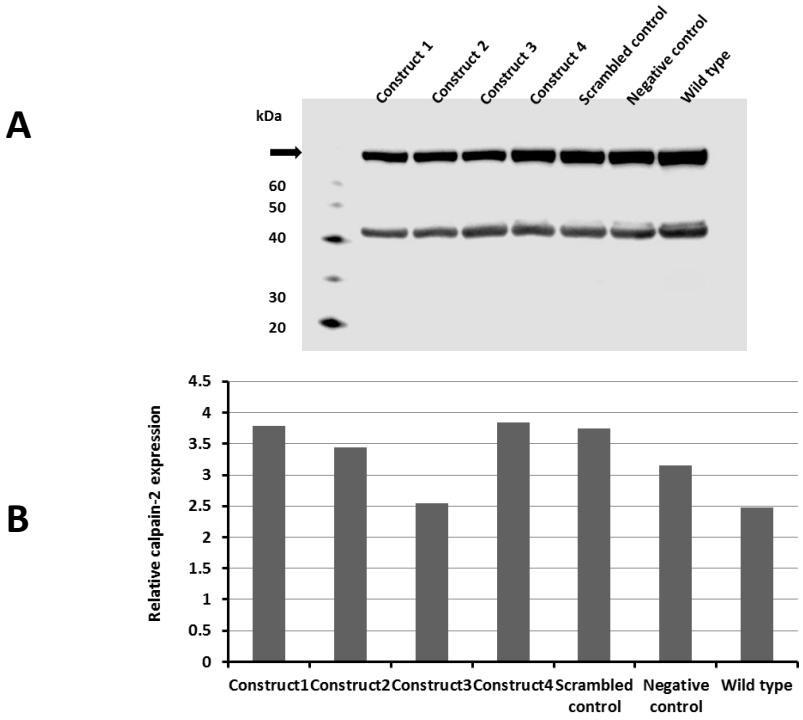


Figure 2.5 Calpain-2 expression levels after shRNA-transfection in SKOV3 cells. After 2- to 4- week expansion, the lysates of the transfected cells were collected. (A) Calpain-2 expression levels in SKOV3 cells transfected with vectors containing shRNA construct against calpain-2, scrambled control vector and empty vector (negative control) and wild-type SKOV3 cells were detected by Western blot analysis using specific antibody. Arrow indicates calpain-2 (80 kDa). β-actin was used as the loading control (42 kDa). (B) The bar charts showed the relative calpain-2 expression level normalised by β-actin expression level. Data represent the result from one representative experiment.

Since the mRNA levels of the transfected cells were not measured by reverse transcription polymerase chain reaction, whether the calpain-2 mRNA levels had been changed or not after transfection is unknown. It is difficult to

explain why the transfection was unable to down-regulate calpain-2 expression in the SKOV3 line even though cells were able to grow in the presence of selection agent. The establishment of monoclonal cell lines were attempted and terminated.

2.4.5 Cell proliferation in response to calpain inhibitor calpeptin

The calpain inhibitor, calpeptin, was used in the current study to inhibit calpain activity in *in vitro* drug sensitivity experiments. In order to find the highest concentration that each cell line can tolerate without significantly inhibiting cell proliferation, calpeptin concentrations were optimised for the following drug combination study. The proliferative responses of A2780, A2780-cis, SKOV3, PEO1 and PEO4 cells following calpeptin treatment were initially examined over a 72-hour time period. Growth curves were conducted following treatment of the cells with increasing concentrations of calpeptin (**Figure 2.6**). The general trend is that increased calpeptin concentrations were accompanied by decreased proliferation. The proliferation of A2780 and A2780-cis cells decreased gradually at all three time points. At the 48-hour time-point, A2780-cis cells gave quite variable results but appeared slightly more resistant to calpeptin than A2780 cells. At other time-points, both lines responded to calpeptin in a similar pattern.

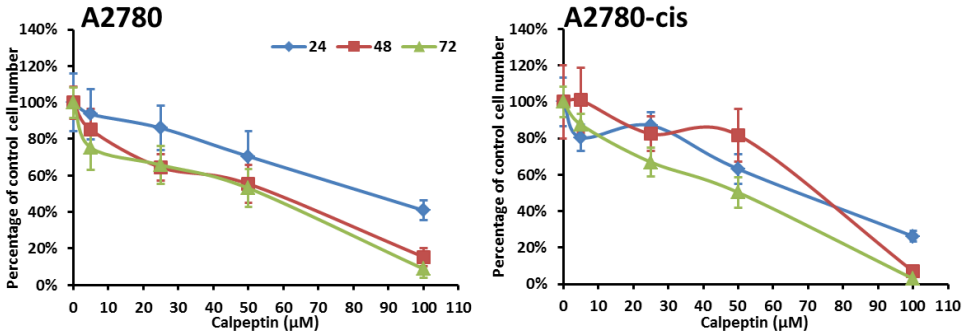


Figure 2.6 Effect of calpeptin on proliferation of ovarian cancer cells. A2780 and A2780-cis cells were treated with various concentrations of calpeptin for 24, 48 and 72 hours. Doses ranged from 0 to 100 μM. Data represent the average ± SD of at least two independent experiments, with each experiment performed in triplicate.

A2780 and A2780-cis cells seemed more sensitive to calpain inhibition than the other three cell lines (PEO1, PEO4 and SKOV3, **Figure 2.7**). As shown above, with increasing calpeptin concentration, A2780 and A2780-cis cells responded with a gradual decrease in proliferative ability; however, calpeptin did not show obvious inhibitory effects on PEO1, PEO4 or SKOV3 cells below 50 µM, above this dose a gradual decrease in proliferation was observed in PEO4 cells between 50 µM and 250 µM whereas SKOV3 and PEO1 cell proliferation ability decreased rapidly between 50 µM and 100 µM following with slow decreases at the remaining time-points.

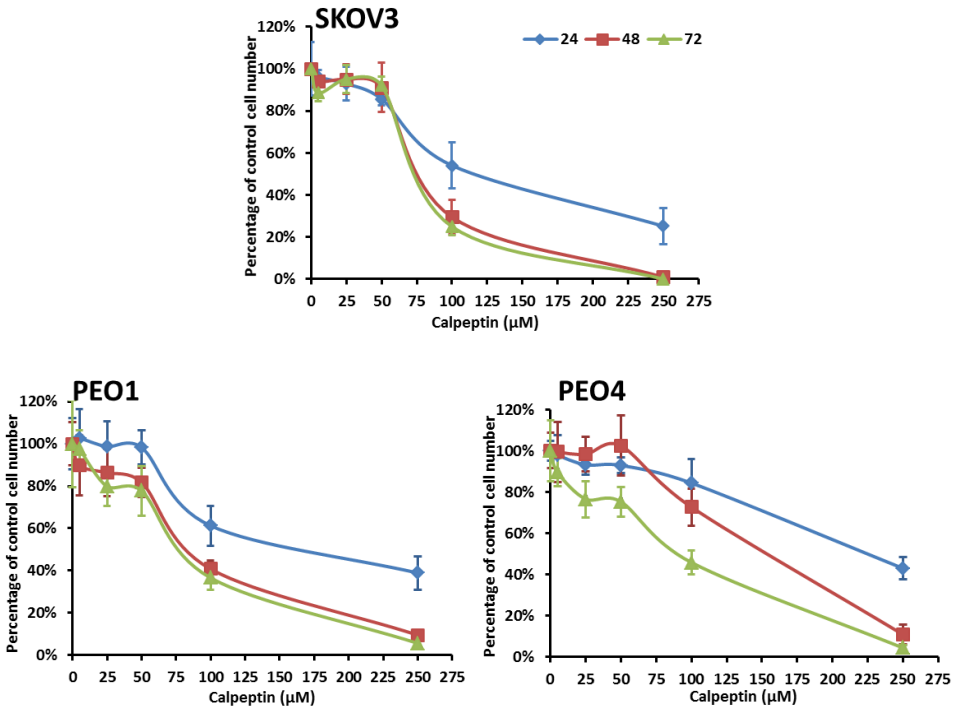


Figure 2.7 Effect of calpeptin on proliferation of ovarian cancer cells. SKOV3, PEO1 and PEO4 cells were treated with various concentrations of calpeptin for 24, 48 and 72 hours. Data represent the average ± SD of two independent experiments, with each experiment performed in triplicate.

The IC50 concentrations of calpeptin towards each cell line at three time points are summarised in **Table 2.7** (results analysed by Probit Regression are list in **Table S2.3**). The calpeptin-induced decrease of cell proliferation was concentration-dependent. But with an increase of calpeptin treatment duration, the inhibitory effect on cell proliferation was not increased.

Table 2.7 IC50 of calpeptin in the panel of ovarian cancer cell lines

Treatment period	IC50 (μM)		
	24h	48h	72h
A2780	84	58	54
A2780-cis	74	68	50
SKOV3	116	86	82
PEO1	160	82	76
PEO4	225	154	90

2.4.6 Calpeptin inhibition of calpain activity

Calpain activity assays were conducted for all 5 cell lines using both sub-IC50 and IC50 concentrations of calpeptin obtained from proliferation data (**section 2.4.5**). In SKOV3, PEO1, and PEO4 cells, 50 μM of calpeptin (sub-IC50 concentration) gave approximately 30% to 50% inhibitory effects on calpain activity (**Figure 2.8**, **Figure 2.9,** and **Figure 2.10**). Further increasing calpeptin concentration (IC50) did not increase the inhibition of calpain activity in these cell lines (**Figure S2.3, Figure S2.4,** and **Figure S2.5**). However, the instability and low reproducibility of the t-BOC assay makes it difficult to compare across experiments or to compare between different cell lines. In SKOV3 cells, the normalised average fluorescent reading of the controls at the 10th cycle various from 9000 to 15000. Similarly, in PEO1 and PEO4 cells, the variation of the normalised fluorescent reading of the controls were from 10000-12000 and 8000-11000 respectively at the 10th cycle.

The inhibitory effects of 30 minutes treatment with sub-IC50 (50 μM) calpeptin on SKOV3 cells were approximately 20% which increased to approximately 30% (1 hour treatment) with prolonged exposure. The inhibitory effects then remained steady at approximately 30% for 48 hours (**Figure 2.8**). The IC50 concentration of calpeptin was also tested in SKOV3 cells; with the increased calpeptin concentration the inhibitory effect increased to nearly 50% in cells that received 1 hour treatment (**Figure S2.3**), but the inhibition decreased to approximately 5% after 24 hours IC50 calpeptin treatment.

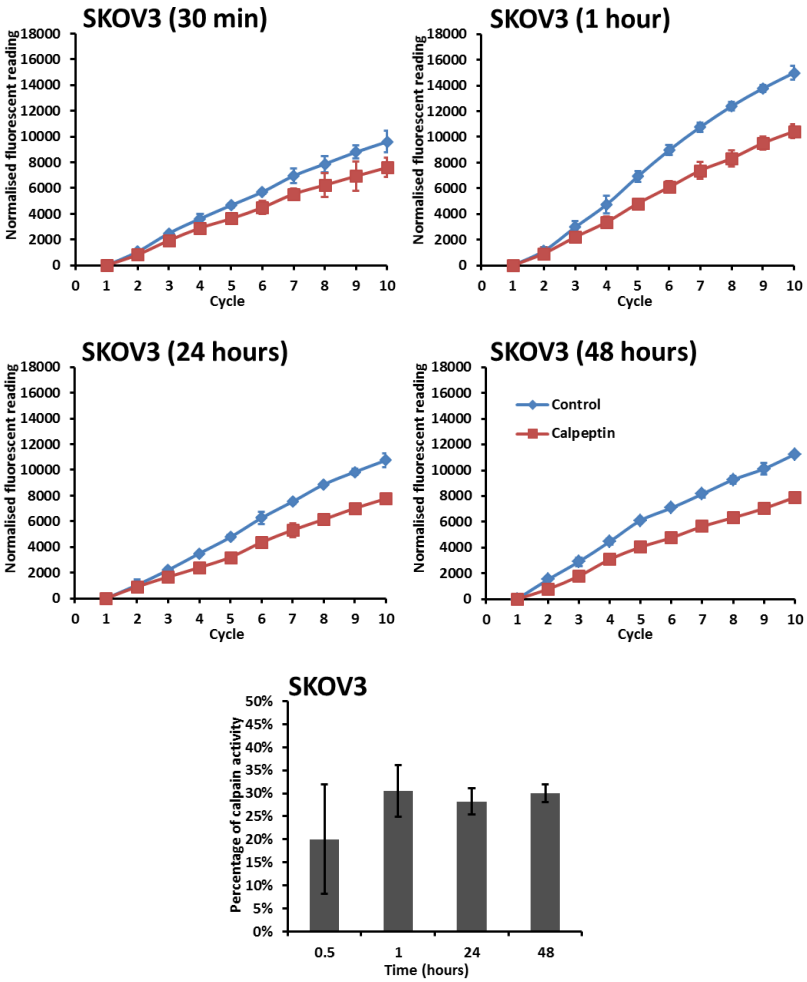


Figure 2.8 The inhibition of calpain activity by calpeptin (50 µM) in SKOV3 cells. SKOV3 cells were treated with 50 µM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of two independent experiments with each experiment performed in duplicate.

In PEO1, sub-IC50 (50 µM) calpeptin treatment resulted in a significant approximately 30% inhibition of calpain activity across 72-hour treatment of calpeptin apart from the 90-minute time point (**Figure 2.9**). The inhibition of calpain activity showed a slight increase with increasing treatment time between 90 minutes to 48 hours, after which the inhibition slightly dropped. Using the IC50 concentration of calpeptin did not increase the inhibitory effect in PEO1 cells (**Figure S2.4**) and no significant reduction in calpain activity was observed at all 5 time points.

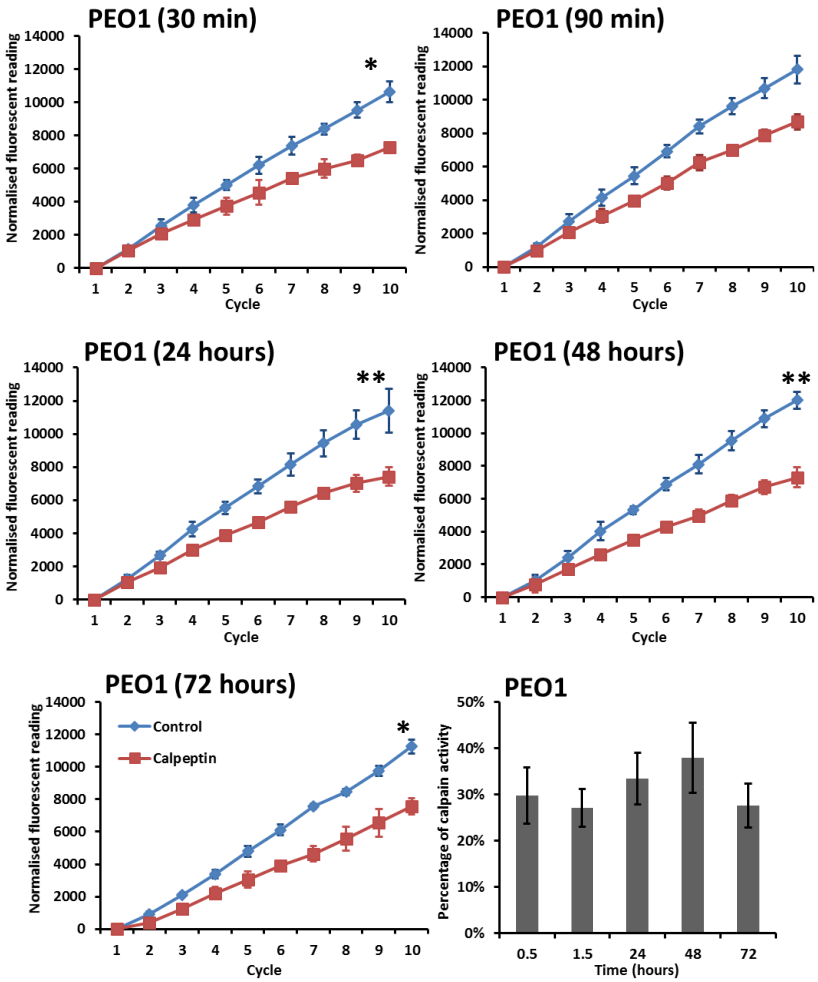


Figure 2.9 The inhibition of calpeptin (50 µM) on calpain activity of PEO1. PEO1 cells were treated with 50 µM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. Student T-test were used to compared the calpeptin treated group with control group at cycle ten (**P*<0.05, ***P*<0.01 vs control).

In PEO4 cells, both sub-IC50 (**Figure 2.10**) and IC50 concentration (**Figure S2.5**) of calpeptin can significantly inhibit calpain activity at 90-minute and the 24-hour time-point. Calpeptin with sub-IC50 concentration inhibited approximately 30% of calpain activity in PEO4 cells after 30-minute treatment. The inhibitory effect increased with the prolonged treatment time and reached a peak (49%) at the 24-hour time point. The inhibition of calpain activity remained stable at approximately 40% after 90-minute treatment and last for more than 48 hours. Increasing the calpeptin concentration did not increase the inhibitory effect (**Figure S2.5**). The IC50 concentration of

calpeptin inhibited approximately 30% of calpain activity in PEO4 cells across the 72-hour treatment period and significant inhibition was observed at 30-minutes, 90-minutes and 24-hours.

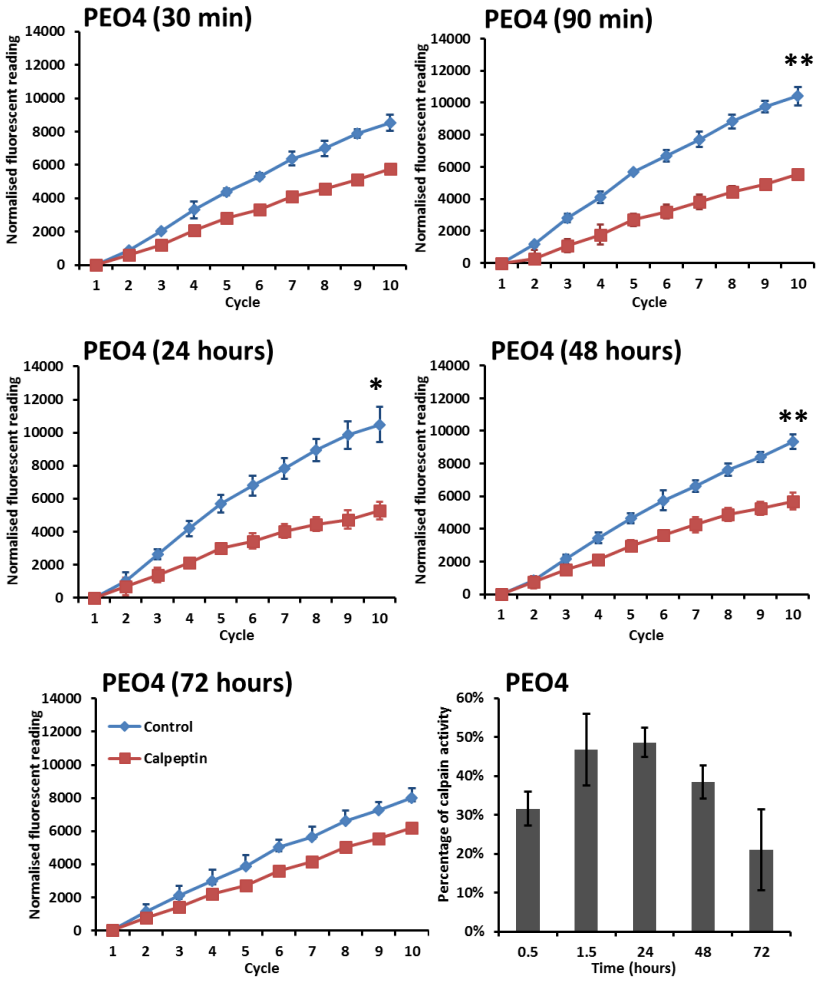


Figure 2.10 The inhibition of calpeptin (50 µM) on calpain activity of PEO4. PEO4 cells were treated with 50 µM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. Student T-test were used to compared the calpeptin treated group with control group at cycle ten (**P*<0.05, ***P*<0.01 vs control).

As mentioned in **section 2.4.5,** A2780 and A2780-cis cells were more sensitive to calpeptin in terms of its anti-proliferative effects, so 5 µM calpeptin (the sub-IC50 concentration) was used for calpain activity inhibition. MDA-MB-231 cells were included in these experiments as a control as they had been used previously in our research group. Calpeptin (50 µM) yielded approximately 20% inhibition of calpain activity in MDA-MB-231 cells which

fell within the range (20-30% inhibition) as indicated in a previous study (Pu, 2016). Four independent experiments were conducted, with calpain inhibition varying from -11.09% to 58.23% and -39.64% to 31.23% respectively. Due to the variation of calpain inhibition, A2780 and A2780-cis cells were not used as experimental models to study the effect of calpain inhibitor on cellular chemotherapeutic response.

Calpeptin was able to inhibit calpain activity in ovarian cancer PEO1, PEO4 and SKOV3 cell lines, although its inhibitory effects was not increased by increasing its concentration and treatment time. As no other suitable inhibitor is currently readily available and this agent is commonly used, calpeptin was used to study of the role of calpain in cell chemotherapeutic response. The reason and limitation of the calpain inhibitor and calpain activity assay is discussed in **section 2.5**.

2.4.7 Cell proliferation in response to platinum-based chemotherapy with or without calpeptin

The effects of calpeptin on the chemoresponse of SKOV3, PEO1 and PEO4 cell proliferation were assessed. Cells were pre-treated with 50 µM calpeptin for 90 minutes and IC50 doses of cisplatin/carboplatin added for another 48 hours. As showed in **Figure 2.11**, the quantification of cell numbers indicated that the IC50 concentration of cisplatin/carboplatin inhibited approximately 50% of cell proliferation when compared to control cells, whilst calpeptin treatment did not further inhibit the cell proliferation. SKOV3, PEO1 and PEO4 cells were not significantly more sensitive to the inhibition of proliferation induced by the cisplatin/carboplatin plus calpeptin when compare to cisplatin/carboplatin alone in the current experimental conditions.

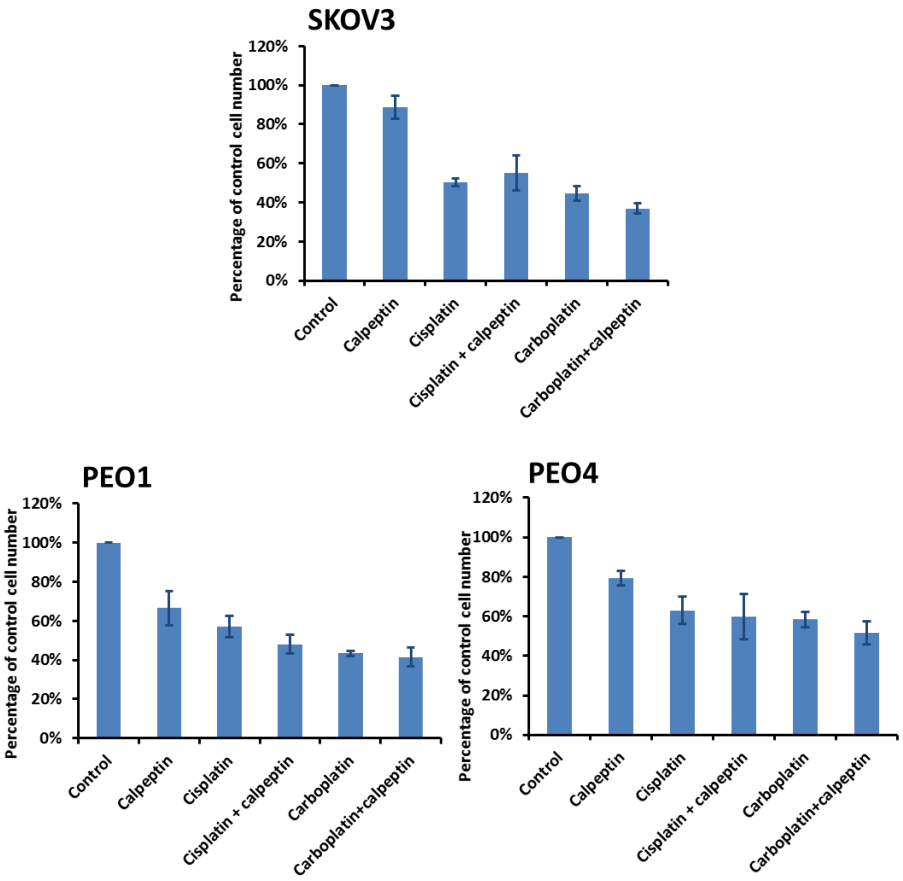


Figure 2.11 Effect of drug combinations (calpeptin and cisplatin/carboplatin) on cell proliferation. Total cell number of cells in treatment group present as the percentage of the total cell number of the cells in vehicle control group. Data represent the average ± SD of three independent experiments, with each experiment performed in triplicate.

2.4.8 Clonogenic survival following platinum-based chemotherapy combined with calpain inhibition

The incubation times for colony formation for A2780 and SKOV3 cells were 2 weeks whilst incubation times for PEO1 and PEO4 cells were 3 weeks. The PE of SKOV3 cells was approximately 5%; A2780, A2780-cis and PEO4 approximately 30%; with PEO1 varying between 20-30%. Initially, in order to improve the PE of SKOV3 cells, flasks were pre-coated with 0.2% gelatin, 1% collagen or diluted BD MatrigelTM, also flasks from different suppliers (i.e. FalconTM) were tested, but no improvements were observed (data not shown).

The concentrations of cisplatin and carboplatin were optimised for

clonogenic assays on the PEO1 and PEO4 cells (**Figure 2.12 A, B**). The PEs were 32%, 16.6% and 31.6% in 3 out of 9 experiments with PEO1 cells; the PEs were lower than 6% in the others 6 experiments and so data from such experiments were excluded.

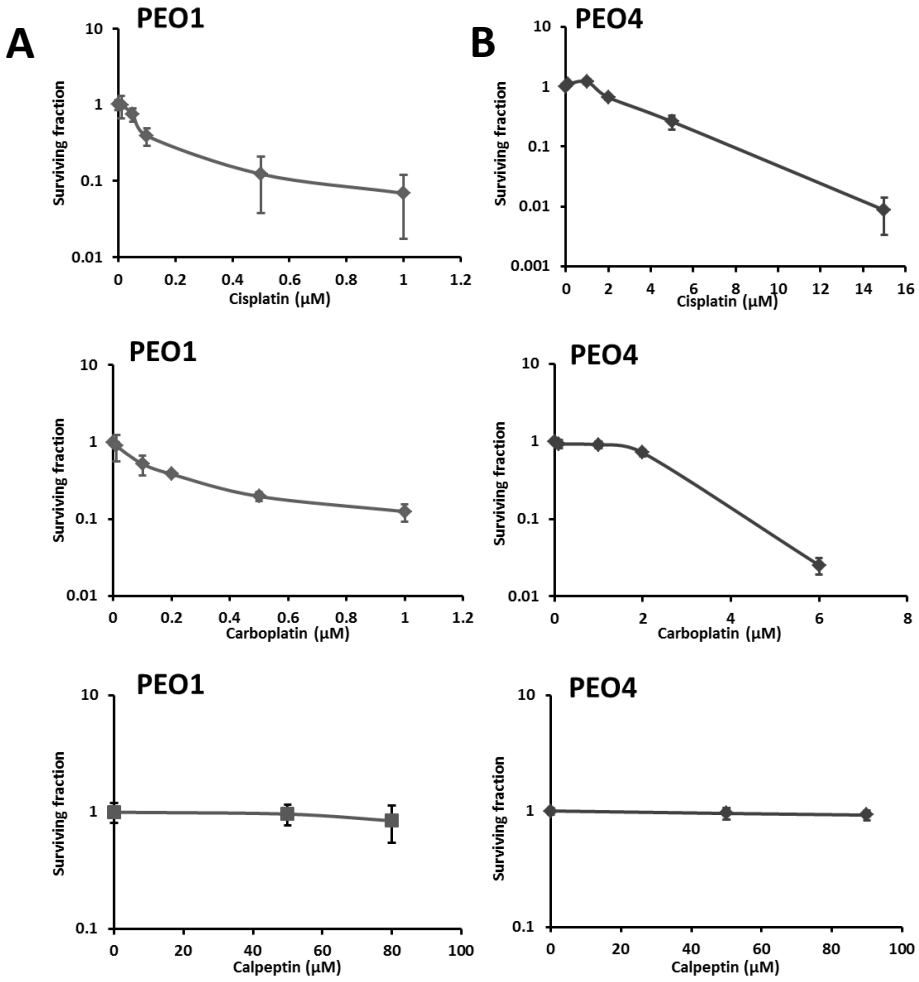


Figure 2.12 Effect of cisplatin, carboplatin or calpeptin on PEO1 and PEO4 clonogenic survival. (A) Survival fraction of PEO1 cells treated by cisplatin, carboplatin or calpeptin for 48 hours (experiments were included when the PE of the experiment was approximately 20% to 30%), data represented the average ± SD of two independent experiments, with each experiment performed in triplicate. (B) Survival fraction of cells treated by cisplatin, carboplatin or calpeptin for 48 hours (experiments were included when the PE of the experiment was approximately 30%). Data represented the average ± SD of three independent experiments, with each experiment performed in triplicate.

Results from the experiments with 20-30% PE were analysed and are presented in **Figure 2.12A**. In PEO4 cells, out of 19 experiments, 12 experiments had PE ranging from 24.4% to 36.7% and 7 experiments had PE's below 15%. The PE of PEO4 was 32.9±4.5% (n=12). Results from the

experiments with approximately 30% PE were analysed and are plotted in **Figure 2.12B**. As shown in **Figure 2.12**, 50 nM and 2.6 μM cisplatin for 48 hours decreased clonogenic survival in PEO1 and PEO4 cells and resulted in a survival fraction of 0.5; 100 nM and 3.2 μM carboplatin resulted in a survival fraction of 0.5 in PEO1 and PEO4 cells respectively. PEO1 cells were more sensitive to cisplatin and carboplatin than PEO4 cells. The combined effect of calpeptin and cisplatin/carboplatin treatment on colony formation was examined via clonogenic survival assay however due to problems in the laboratories (variable carbon dioxide levels), data was highly variable and so no definitive conclusions can be drawn from the drug combination experiments with only 1 experiment yielding data with an acceptable PE (**Figure 2.13**).

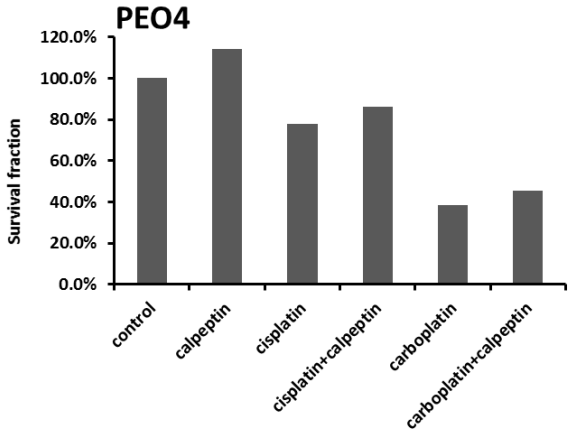


Figure 2.13 Effect of cisplatin/carboplatin with or without calpeptin on PEO4 clonogenic survival. Survival fractions of cells treated by calpeptin with or without cisplatin/ carboplatin are presented. Result from only one experiment was showed here, due to the low PE (below 10%) of other experiments which made those results unreliable. The PE of this experiment was 31.7%.

With the increase of calpeptin concentration from sub-IC50 to IC50, the variation of survival fraction increased but the clonogenic survival was maintained at the same level in both PEO1 and PEO4 cell line (**Figure 2.12 A, B**). PEO4 cells were treated with cisplatin/carboplatin (the concentration resulted in a survival fraction of 0.5) for 48 hours with or without sub-IC50 calpeptin for 90 minutes. No clear effect of calpeptin was observed on

chemo-sensitisation in clonogenic survival (**Figure 2.13**) and it is difficult to draw a firm conclusion based on the result from one experiment. Drug combination was also conducted a number of times with PEO1 cells, but due to low PE's no reliable and reproducible data were obtained.

2.5. Discussion

A previous IHC study conducted by our group (Storr S.J. *et al*., 2012) showed that high expression of calpain-2 significantly associated with resistance to platinum-based adjuvant chemotherapy, poor PFS and OS. To address the question as to whether the calpain family plays an important role in ovarian cancer chemotherapeutic response, 5 ovarian cancer cell lines (A2780, A2780-cis, SKOV3, PEO1 and PEO4), with different sensitivity towards platinum-based chemotherapies, were included in this study.

The protein expression levels of calpains and calpastatin in the 5 cell lines were studied by Western blotting. To our knowledge, this is the first time that the expression of calpain-1, -2, -4 and calpastatin was studied in a panel of ovarian cancer cell lines and the expression levels varied among these cell lines. The chemo-resistance of cells from certain cancer types is calpain-related. As reviewed in **section 2.2.1.1**, high calpain expression or calpain activity levels were observed in chemo-resistant cells when compared to their parental counterparts in colon cancer and melanoma cells; whilst gastric chemo-resistant cancer cells had a lower calpain expression than the parental counterpart. But in the current ovarian cancer study, both A2780 and A2780-cis cells expressed low levels of calpains indicating that the resistance of A2780-cis cells to platinum-based chemotherapy may not be related to calpain expression. A2780 cells and its resistant counterpart A2780-cis cells showed similar calpeptin sensitivities, which corresponded to the similar calpain expression patterns between both lines. The calpain expression patterns in PEO1 and PEO4 cells were also similar, only calpain-2 was

expressed at a higher level in PEO4 than in PEO1 (but the difference was not statistically significant). PEO1 appeared more sensitive to calpeptin than PEO4, especially at the 48-hour time point. The sensitivity of ovarian cancer cells towards calpeptin could be associated with calpain expression/activity levels suggesting that calpain may be important in cell proliferation and survival. As calpain-4 was found positively associated with the cell proliferation of transfected ovarian cancer cell lines (Yang X. *et al*., 2017). But the cisplatin resistance of A2780-cis and PEO4 cells comparing with A2780 and PEO1 cells may not be calpain-related.

It was reported that calpains and calpastatin have long metabolic half-lives. In the study of Zhang, Lane & Mellgren (1996), WI 38 human Caucasian fibroblast-like fetal lung cells were used as a cell model. Pulse-labeled with [35S] methionine, the remaining initial calpain in WI 38 cells were detected by the residual radioactivity. The major calpain isozymes, m-calpain and μ-calpain, had metabolic half-lives of about 5 days. In addition, calpains were long-lived proteins in cervical carcinoma-derived HeLa cells, A-431 epidermoid carcinoma cells, simian vacuolating virus 40 (SV-40) transformed lung epithelial cells (TE2 cells), and SV-40 transformed WI-38 cells (VA-13 cells) (Zhang, Lane & Mellgren, 1996). Calpastatin appeared to have long metabolic half-life in C-33A cervical carcinoma cells and TE2 cells (Zhang, Lane & Mellgren, 1996). Calpain cannot be completely knocked down by siRNA, because gene silencing resulting from siRNA often last from 5-7 days during which the calpains that have already been produced may not be rapidly degraded. Therefore, we chose shRNA to down-regulate calpain-2 level.

Comparatively, SKOV3 and PEO4 cells expressed the highest level of calpain-2 and so were chosen for calpain-2 knockdown. PEO4 cells were first chosen for transfection due to its higher PE which might has higher chance to survive during single cell selection. Before calpain-2 shRNA transfection, puromycin concentrations were optimised for the selection of stable

transfectants. PEO4 cells could not survive after selection which might be due to long doubling time although different recovery times were tried before adding the selection medium. Few studies use PEO4 cells as experimental model for transfection (Sakai W *et al*. 2009). Although four unique calpain-2 shRNA constructs were used and the transfected cells were able to survive puromycin selection, none of the four structures could down-regulate calpain-2 expression after transfection on SKOV3 cells which might be due to the initial high expression or the low targeting efficiency of shRNA structures. The attempt to specifically inhibit calpain-2 activity via down-regulation of calpain-2 level was unsuccessful, even after numerous attempts and significant time expended.

The calpain inhibitor, calpeptin, was used in this study for the inhibition of calpain activity in *in vitro* experiments. Other calpain inhibitors such as ALLN, ALLM, E-64, PD150606 and MDL28170 are also commercially available for the study of calpain, but none of them are sufficiently specific to inhibit calpain without targeting other proteases (e.g. cathepsin, papain and matrix metalloproteinase-2) (Ono *et al*., 2016). Preliminary studies in our group (conducted by Dr. Sarah Storr) indicated that calpeptin was more effective than PD150606 (Pu, 2016), so calpeptin was used in the current study.

As listed in **Table S2.1**, calpeptin is widely used in calpain and chemotherapeutic response studies. Calpeptin (12.5μM for 4 hours) pre-treatment effectively inhibited cisplatin-induced but not the intrinsic calpain activity in OV2008 cells and significantly inhibited cisplatin induced apoptosis (Al-Bahlani *et al*., 2011). Similar results were observed in breast cancer MDA-MB231 cells with 50μM calpeptin treatment for 4 hours (Al-Bahlani *et al*., 2016). Another study using OV2008 cells pre-treated cells with calpeptin for 1 hour followed by cisplatin, the maximal effect was observed at 25 μM calpeptin whilst 50 μM calpeptin alone caused significant apoptosis (Woo *et al*., 2012). Lapinska K *et al*., (2016) applied calpeptin on

CAOV-3 and SKOV-3 ovarian cancer cell lines but no direct information about the inhibitory effect of calpeptin on calpain activity was provided. The author indicated that 48 hours 20 μg/ml of calpeptin was not sufficient to completely inhibit Akt phosphorylation in ovarian cancer cells and the complete inhibition of calpain activity is usually achieved with about 40 μg/ml (approx. 110 μM) calpeptin, depending on the cell line used (Lapinska *et al*., 2016). In lung cancer studies, calpeptin (10μM) was able to significantly inhibit 20 to 50% of cisplatin-induced calpain activation (Liu *et al*., 2008; Liu, Xing & Chen; 2009). Although calpeptin is widely used in the studies of calpain and chemotherapeutic response, these studies did not address to what extent did calpeptin inhibite intrinsic calpain activity.

Studies regarding other functional roles of calpain also used calpeptin for calpain inhibition and give a range of effective concentration of calpeptin. In colorectal cancer cell lines, calpeptin (30 min 100 μM treatment) significantly caused a more than 50% inhibition of calpain activity (measured using t-BOC) (Thorpe *et al*., 2015). Autolysis of calpain-1 (i.e. activation) could be significantly suppressed by calpeptin (50 μM for 30 min) in MCF-7 (Hou *et al*., 2012). Calpeptin (50 μM for 12 h) was also found able to effectively inhibit calpain-induced IκBα depolymerisation in MCF7 cells (Kim *et al*., 2010). Immunoblotting results indicated that calpeptin (40 μM for 24 hours) effectively inhibited calpain activity and protected filamin A and androgen receptor from cleavage by calpain in human prostate cancer cell lines PC-3 (Liu *et al*., 2014).

The above suggests that 40 to 100 μM calpeptin treatment for at least 30 min could effectively inhibit calpain activity and calpain-induced protein cleavage. However there is no information indicating how the 5 ovarian cancer cell lines respond to calpeptin which has been covered in the current study. Firstly, the calpeptin concentrations were optimised for 5 cell lines using proliferation assay. A2780 and A2780-cis cells were more sensitive to calpeptin

than other 3 cell lines (PEO1, 4 and SKOV3), because 50 μM of calpeptin which caused no obvious toxicity on PEO1, -4 and SKOV3 cells was toxic to A2780 and A2780-cis cells. A2780 cells and its resistant counterpart A2780-cis cells showed same sensitive to calpeptin which suggested that the acquired chemo-resistance of A2780-cis cells may not be related to conventional calpains. Calpain activity assays were then conducted in all 5 cell lines at different time points. Wide variations were observed in the calpain activity assay. In PEO1, PEO4 and SKOV3 cells, 50 μM of calpeptin (sub-IC50 concentration) caused similar inhibitory effects on calpain activity. Higher concentration (IC50) did not increase these effects. Inconsistent results were also observed in the studies conducted by Dr. Xuan Pu, and the inhibitory effect of calpeptin was not in a dose-dependent way (Pu, 2016). Similarly, as mentioned above, in ovarian cancer OV2008 cells 25 μM calpeptin achieved the maximal inhibitory effects on cisplatin-induced, calpain mediated protein cleavage and apoptosis; and the inhibitory effects dropped after increase calpeptin concentration (Woo *et al*., 2012). In A2780 and A2780-cis cells, the effect of 5 μM (sub-IC50 concentration) calpeptin on calpain activity was very variable, with the observation of calpain activity increase after calepetin treatment, which was not observed in other cell line models after calpeptin (sub-IC50) treatment; although this might be due to the inherent variation of the calpain activity assay. Further experiments were conducted using PEO1, PEO4 and SKOV3 cells.

Fluorogenic calpain activity assay and Western blotting are generally used for the assessment of calpain activity (listed in **Table S2.1**). Four generally used calpain activity assays were tested by Dr Xuan Pu in our group: two assays measuring fluorescence intensity of cleaved products of fluorogenic calpain substrate Suc Leu-Leu-Val-Tyr-AMC and t-BOC, one assay detecting the cleaved α-fodrin by Western blotting and one using Casein zymography. Unlike substrate degradation (α-fodrin and casein), the results from fluorometric

assays can be more accurately quantified (Pu, 2016). As t-BOC assay showed favourable sensitivity over the other fluorogenic assays, it was applied in the previous study and current study (Pu, 2016) (further discussed in **section 4.5**).

In the current study, cells were pre-treated with calpeptin and then exposed to cisplatin or carboplatin. Comparing with cisplatin or carboplatin treatment alone, no chemo-sensitisation was detected in cell proliferation. As reviewed in **section 2.2.1.2**, the association between calpain and cell chemoresponse seems to depend on cell context. Most of the studies only focused on the association between calpain activity and cisplatin-induced apoptosis, but calpain is involved in the process of cell apoptosis, autophagy and survival. Current information cannot give a full picture of how different signalling pathways coordinate to influence cancer cell chemoresponse. Ideally, results generated from clonogenic assay would be more suitable for revealing the role of calpain in chemotherapeutic response in PEO-1 and -4 cells owing to the long doubling time of these cell lines, but limited by the condition, the current study lacks of reliable data generated from clonogenic assay. As the published literature used 72 hour-time point current timeframes enable meaningful comparisons with such data (references listed in **Table 2.5**) and 24- to 72-hour cisplatin/carboplatin treatment did result in cytoxicity in these cell lines.

In conclusion, the roles that calpain system played in human cancer cells vary with cell context and the current results did not support the initial hypothesis that calpain can sensitise the ovarian cancer cells to cisplatin/carboplatin. The calpain-inhibitor calpeptin caused 20 to 50% of calpain inhibition which did not sensitise the ovarian cancer lines, SKOV3, PEO1 and PEO4, to altered proliferation induced by cisplatin/carboplatin. In addition, chemo-sensitive ovarian cancer cells and their resistant counterparts expressed similar levels of calpain-1, -2, -4 and calpastatin and showed similar sensitivity to calpeptin treatment. It is difficult to draw a direct link between

the results from *in vitro* and *in vivo* studies. To revisit the question as to whether calpain associates with chemoresponse and patient survival, a larger cohort was used to validate the previous study and is described in the next chapter. In addition, clinical and pathological parameters were studied to address how calpain expression might influence patient outcome.

2.6. Summary

1. Although significant variations of calpain system protein expression levels were observed between the different cell lines, chemo-sensitive ovarian cancer cells and their resistant counterparts express similar levels of calpain-1, -2, -4 and calpastatin.
2. Calpain-inhibitor, calpeptin, caused a similar inhibitory effect on cell proliferation in chemo-sensitive ovarian cancer cells (i.e. A2780 and PEO1) and their resistant counterparts (i.e. A2780-cis and PEO4).
3. Calpeptin did not sensitise the SKOV3, PEO1 and PEO4 cells to cisplatin/carboplatin-induced proliferation inhibition.

Chapter 3. Calpain family expression in ovarian cancer

3.1. Abstract

INTRODUCTION: Although limited in number, studies have shown that calpain expression is either positively or adversely associated with patient survival in different tumour types (e.g. breast cancer, melanoma and colorectal cancer). A previous study by our group examined calpastatin, calpain-1 and calpain-2 expression in ovarian cancer and demonstrated that high calpain-2 expression was associated with resistance to platinum-based adjuvant chemotherapy and adverse patient survival.

OBJECTIVES: This chapter aimed to verifying the previous study using a larger patient cohort, adding more detailed analysis in different ovarian cancer subtypes in terms of the association between the protein expression and patient clinical outcomes. In addition to verifing the study on the expression of calpastatin, calpain-1 and calpain-2, calpain small subunit 1 (calpain-4) expression was added into the current study. Correlations between the expression of conventional calpain subunits and calpastatin were also investigated.

METHODS: Samples (n=575) from primary tumour sites of patients were evaluated for protein expression of calpain-1, calpain-2, calpain-4 and calpastatin by standard IHC.

RESULTS: The H-scores of calpain-1 and calpastatin expression in the current study were significantly correlated with the H-scores from matched cases in the previous study (n=80), however, the matched H-scores of calpain-2 expression from these two studies was not correlated with each other. The current study confirmed that calpain-2 is associated with patient OS (*P*=0.026), whilst calpain-1 was not associated with either OS or PFS in the current cohort.

The association between calpain-2 expression and OS was not significant in the 80 cases in the current study but was significant in the previous study. All cases (n=154) in the previous study received carboplatin-based adjuvant chemotherapy. Thus calpain-2 expression was exclusively analysed in patients who received carboplatin-containing treatment in the present cohort which also indicated that calpain-2 was associated with OS but not PFS. The expression of calpain-1, calpain-2, calpain-4 or calpastatin in this study, however, was not associated with resistance to platinum-based adjuvant chemotherapy. Different from the previous study, significant association was found between calpastatin expression (low) and OS (poor) in the current study (*P*=0.010). Moreover, low calpain-4 was significantly associated with poor OS (*P*=0.003). Although calpain-2 was positively associated with OS whilst calpain-4 and calpastatin negatively associated, the significant correlations between the expression of calpain-1/calpain-2 and calpain-4/calpastatin were positive according to Spearman's rank correlation coefficient test. The expression of calpastatin, calpain-1 and -4 was associated with histological subtype. Calpastatin expression was associated with age. Although calpain-1 expression was not associated with patient outcome, it was associated with tumour stage and the presence of residual disease. Moreover, calpain-1 showed significant association with the presence of tumour spread (stage 2, 3 and 4) ($\chi 2$=11.310, d.f.=1, *P*=0.001) but not with the appearance of distant metastasis (stage IV). In multivariate analysis, using the cut-point calculated from the whole cohort, neither calpain-2, calpain-4 nor calpastatin was found to be an independent marker of OS, when considering tumour stage, histological subtype, grade, platinum-sensitivity, patient age and residual disease. But calpain-4/calpastatin status appeared as an independent prognostic factor (Hazard Ratio (HR) 0.869, 95% confidence interval (Cl) 0.757-0.998; *P*=0.047) together with cancer stage, platinum-sensitivity, and residual disease. The expression of the calpain system was also assessed within different subgroups, but the association was not of statistic significance

in any of the subgroups.

CONCLUSION: In both the previous and the current study, calpain-2 expression was adversely associated with OS of ovarian cancer patients. In spite of the association found between calpain-2 and OS, calpain-2 status was not related to platinum-sensitivity, age, histological subtype, tumour grade, FIGO-sub-stage, or recurrent disease. Future studies are required to provide insight into understanding how calpain-2 expression influences patient outcome by considering calpain activity in addition to protein expression. Although there was no association between calpain-1 and patient outcome, calpain-1 was associated with tumour stage. Low calpain-1 expression was associated with tumours that were confined to the ovaries (stage 1) and the absence of residual disease. As stage reflects the extent of tumour metastatic spread, such data may suggest that conventional calpains and calpastatin may be involved with ovarian tumour migration and invasion.

3.2. Introduction and aims

3.2.1 Calpain family and ovarian cancer

The expression levels of calpain-1, -2 and calpastatin was previously shown to differ in ovarian tumours from those in normal tissues (Salehin *et al*. 2011). Compared with normal tissues, ovarian tumours had lower calpastatin expression levels (Salehin *et al*. 2011). High calpain-1 expression was associated with lower tumour grade (a similar pattern was found in breast cancer studies (Pu *et al*., 2016 a; Storr *et al*., 2012b)), whilst low calpain-2 expression was associated with increased lymph node metastasis (Salehin *et al*., 2011). Storr *et al*., (2012a) studied the expression of calpastatin, calpain-1 and calpain-2 in 154 primary ovarian carcinoma patients, all of whom received carboplatin-based adjuvant chemotherapy treatment. With overall survival-data-based cut-points calculated by X-tile (i.e. calpastatin: 165;

calpain-1: 25; calpain-2: 162) (Storr *et al*., 2012a), high levels of calpain-2 expression was significantly associated with platinum resistance ($\chi 2=4.658$, d.f.=1, $P=0.031$), poor PFS ($P=0.049$) and poor OS ($P=0.006$). Furthermore, calpain-2 expression was significantly associated with OS (hazard ratio=2.174; 95% confidence interval=1.144–4.130; $P=0.018$) from multivariate analysis including grade, stage, optimal debulking and platinum sensitivity (Storr *et al*., 2012a). Protein expression differed from one histological subtype to another with serous ovarian carcinomas being associated with high calpain-1, calpain-2 and calpastatin expression (Storr *et al*., 2012a).

Very little information is available regarding the association between calpain family members and clinicopathological factors in ovarian cancer. As conflicting findings have emerged in different types, even subtypes, of cancers (**section 1.4.4**), it is, therefore, necessary to understand the specificity of different isoforms of calpains on tumour progression in ovarian cancer with the consideration of their histological subtypes (i.e. HGSC, LGSC, CCC, mucinous and endometrioid carcinomas).

3.2.2 Aims

In a previously published study by our group, high calpain-2 expression was significantly associated with resistance to platinum-based adjuvant chemotherapy ($P=0.031$, Pearson chi-square test of association), PFS ($P=0.049$, Kaplan–Meier analysis) and OS ($P=0.006$, Kaplan–Meier analysis) (Storr S.J. *et al*., 2012a). Results from multivariate analysis showed that calpain-2 expression was an independent prognostic factor (including grade, stage, optimal debulking and platinum sensitivity). Based upon such findings the current chapter sought, using a large patient cohort, to address the following hypotheses:

1. Conventional calpain subunits (i.e. calpain -1, -2 and -4) and calpastatin expression associate with clinicopathological factors.

2. Calpain-2 expression is significantly associated with resistance to platinum-based adjuvant chemotherapy, poor PFS and OS.
3. The expression of conventional calpain subunits and calpastatin are correlated with each other.

The study aimed to:

1. Study the association between the expression of the calpain system and tumour stage, grade, histological subtypes, platinum-sensitivity and other clinical parameters in ovarian cancer.
2. Study the association between the expression of the calpain system and patient outcome.
3. Study the correlations between calpain-1, -2, -4 and calpastatin expression.

3.3. Materials and methods

3.3.1 Clinical samples

The ovarian tissue microarray (TMA) was composed of tumour cores from 575 ovarian cancer patients. Within these cases, 448 cases were chemo-naïve samples and 22 cases were sampled post chemotherapy (information on the rest cases were not available for various reasons). The clinicopathological variables of the cohort are listed in **Table 3.1**. Patients were diagnosed with ovarian cancer and received treatment at Nottingham University Hospital between 1991 and 2011. The median follow-up period was 8 years ranging between 3 years for the most recent cases and 20 years for the oldest cases. The mean age of the patients was 61±13 years, the median age was 62 years, and the median OS time was 44 months (ranging from 0 to 223 months). Ethical approval was obtained from Derbyshire Ethics Committee (07/H0401/156). This study is reported in accordance with REMARK (Reporting recommendations for tumour MARKer prognostic studies) criteria (McShane *et al*., 2005).

The adjuvant therapies that patients received are shown in **Table 3.2**. The platinum-based chemotherapy that the majority of the patients received was carboplatin with or without paclitaxel or docetaxel. Several cases were treated with triplet therapy: either one chemotherapeutic drug (e.g. irinotecan or topotecan) or one targeted therapy drug (e.g. bevacizumab) was added to the first-line doublet; 21 cases were treated with carboplatin, cyclophosphamide and doxorubicin; 5 cases were treated with cyclophosphamide, doxorubicin and cisplatin (CAP). Seven patients received radiotherapy and 2 of them received radiotherapy for their lung cancer. Two patients were treated by BIBF 1120 (Nintedanib): a novel triple angiokinase inhibitor that simultaneously inhibits three growth factor receptors which are crucial to angiogenesis: vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) (Hilberg

F *et al*., 2008).

Table 3.1 Clinicopathologic variables of patient cohort

Variable	Patient Number	Valid Percent (%)
Age		
≤62	295	52.4
>62	268	47.6
Histological subtypes		
High grade serous carcinoma (HGSC)	337	59.7
Mucinous carcinoma	60	10.6
Endometrioid carcinoma	68	12.1
Clear-cell carcinoma (CCC)	53	9.4
Low grade serous carcinoma (LGSC)	30	5.3
Serous borderline tumour	15	2.7
Grade (Shimizu-Silverberg grading system)		
1	48	8.5
2	90	16.0
3	425	75.5
Stage		
I	203	36.7
II	64	11.6
III	245	44.3
IV	41	7.4
Residual disease		
No residual tumour	311	62.2
Residual tumour (<2cm)	58	11.6
Residual tumour (>2cm)	131	26.2
Adjuvant therapy		
Platinum based chemotherapy	357	63.3
Non-platinum based chemotherapy	6	1.1
No chemotherapy	80	14.2
Information not available	121	21.5
Response to platinum-based chemotherapy		
Platinum refractory	40	10.7
Platinum resistance	26	7.0
Platinum sensitivity	307	82.3
Progression Status		
No recurrence	137	32.9
Recurred	280	67.1
Survival Status		
Living	234	42.0
Deceased	323	58.0

Table 3.2 Treatment received by the ovarian cancer patients in present study

Treatment	Patient Number	Frequency Percent (%)*
Carboplatin	148	25.7
Carboplatin and paclitaxel (taxol ®)	160	27.8
Carboplatin and docetaxel (Taxotere ®)	2	0.3
Carboplatin, docetaxel and irinotecan (Campto ®)	1	0.2
Carboplatin, paclitaxel and topotecan (Hycamtin ®)	3	0.5
Carboplatin, paclitaxel and bevacizumab	1	0.2
Carboplatin and cyclophosphamide	5	0.9
Carboplatin, cyclophosphamide and doxorubicin (adriamycin)	21	3.7
Carboplatin and topotecan	9	1.6
Carboplatin and radiotherapy (HGSC)	1	0.2
Carboplatin, paclitaxel and radiotherapy (HGSC)	1	0.2
CAP: cyclophosphamide, doxorubicin and cisplatin	5	0.9
Chlorambucil (Leukeran ®)	2	0.3
Topotecan (Hycamtin ®)	2	0.3
Gemcitabine (Gemzar ®)	1	0.2
No chemotherapy	74	12.9
Radiotherapy (HGSC and endometrioid carcinoma)	2	0.3
Radiotherapy and progesterone (endometrioid carcinoma)	1	0.2
Dexamethasone and radiotherapy for lung metastasis (endometrioid carcinoma)	1	0.2
BIBF 1120 (Nintedanib)	2	0.3

* Percentage of the total cohort (n=575).

3.3.2 TMA, IHC and interpretation

Protein expression was investigated via IHC using a formalin-fixed paraffin-embedded (FFPE) TMA provided by Dr S. Deen. For the majority of the cases, 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 the specialist gynaepathologist, Dr S. Deen. The specificity of calpain system antibodies was checked and working concentrations were optimised by Dr. X Pu using breast cancer cell lines and breast cancer TMA sections (anti-calpain-1 antibody: 1:2500, anti-calpain-2 antibody: 1:2500, anti-calpain-4 antibody: 1:100,000 dilution, anti-calpastatin antibody: 1:20,000 dilution). Antibodies generating

expected single bands without non-specific bands (**section 2.4.3**) were used for TMA staining. The working concentrations for IHC were verified; anti-calpain-1 and anti-calpastatin were further optimised (anti-calpain-1 antibody: 1: 1000 dilution; anti-calpastatin antibody: 1:30,000 and 1:50,000 dilution). Therefore, the antibodies and dilutions applied in the current study were: mouse calpain 1 large subunit monoclonal (P-6) antibody (dilution 1:1000, Santa Cruz Biotechnology, INC., USA), rabbit anti-calpain-II (m-Calpain) polyclonal antibody (dilution 1:2500, Chemicon® International Millipore, USA), mouse anti-calpain small subunit (μ- or m-Calpain) monoclonal antibody (dilution 1:100,000, Chemicon® International Millipore, USA), mouse anti-calpastatin monoclonal (PI-11) antibody (dilution 1:50,000, Chemicon® International Millipore, USA) (as mentioned in **section 2.3.3**). Sections (4 μm) were cut from each block and placed onto coated slides then dried for 2 days at room temperatures. Briefly, slides were heated at 60°C for 10 minutes then dewaxed in two xylene baths (5 minutes each) and sequentially rehydrated in three industrial methylated spirits baths (2 minutes each), followed by a 5-minute wash in running tap water. The whole procedure was performed automatically in an Autostainer XL staining System ST5010 (Leica, USA). The sections were pre-treated by heat-induced epitope retrieval in 0.01 mol/L sodium citrate buffer, pH 6, for 10 min (750W) + 10 min (450W) in a microwave oven. 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 after washing with Tris buffered saline (TBS). Primary antibody was diluted in Bond Primary Antibody Diluent (Leica, Denmark) and applied to the tissue overnight at 4°C. Post Primary reagent, a polymer penetration enhancer, was applied on

the slides for 30 minutes; followed by NovoLink Polymer (anti-mouse/rabbit IgG-Poly- horseradish peroxidase (HRP)) for another 30 minutes, with each step followed by a TBS wash. Immunohistochemical reactions were visualised with 3, 3'-diaminobenzidine (DAB) for 5 minutes and counterstained with haematoxylin for 6 minutes. Then the slides were loaded into the Autostainer for dehydration. Briefly, slides were washed for 5 minutes with running tap water which followed by three industrial methylated spirits baths (2 minutes each), and two xylene baths (5 minutes each). The coverslips were mounted with DPX (Leica, Germany) and left to dry overnight. Negative controls had primary antibody omitted and represented the background staining levels.

Positive staining for calpain-1, -2, -4 and calpastatin were confined to the membrane and cytoplasm of the tumour cells. Slides were scanned by high-resolution scanning (Nanozoomer Digital Pathology Scanner, Hamamatsu Photonics) with the objective lens at 20X magnification. The expression of calpain-1, -2, -4 and calpastatin in tumour cells was manually and semi-quantitatively assessed using an immunohistochemical H-score (following initial training by a specialist consultant gynaecological cancer pathologist, Dr S. Deen). Staining intensity was ranked as: none (0), weak (1), medium (2) and strong (3) and the percentage area of each staining intensity was multiplied by the intensity rank (H-score range: 0–300). Above 25% of the slides were examined by a second independent assessor (G. Chondrou) blinded to scores and clinicopathologic criteria. Single measure intraclass correlation coefficient (ICC) analysis was conducted to determine the level of agreement between independent scorers. The single measure ICCs between scores were 0.8, 0.814, 0.733 and 0.796 for anti-calpain 1, anti-calpain2, anti-calpain 4 and anti-calpastatin antibody stained samples respectively. X-tile software was used to determine a non-biased cut-point of the immunohistochemical scores dividing the data into high or low subsets by considering the strength of associations between tumour biomarker expression and patient outcomes (here cut-point was newly generated using

data from the current whole cohort with the consideration of patient OS) (Camp *et al*., 2004).

3.3.3 Statistical analysis

The relationships between categorised protein expression and clinicopathologic factors were examined using Pearson's chi-square test of association ($\chi 2$) or Fisher's exact test if a cell count was less than 5 in a 2 x 2 table. To assess the relationship between protein expression and survival outcomes, survival curves were calculated by the Kaplan–Meier method and statistical significance determined using the Log-rank test. When conducting Kaplan–Meier survival analysis in different subgroups of ovarian cancers, individual confidence intervals were adjusted by the Bonferroni correction. To assess the prognostic power of protein expression and other clinicopathological variables, multivariate survival analysis was applied using a proportional hazards model by Cox's regression analysis to estimate hazard ratios and 95% confidence intervals for OS. Spearman's rank test was performed to assess the correlations between the expression levels of different proteins. The correlation strength was interpreted as follows: Spearman rho (rs) less than 0.16 is too weak to be meaningful, ranging from 0.16 to 0.19 as very weak correlation; 0.20 to 0.39 as a weak correlation; 0.40 to 0.59 as a moderate correlation, 0.60 to 0.79 as strong correlation and 0.80 or greater as very strong correlation (Divaris K *et al*., 2012; Divaris K *et al*., 2012). One should be cautious when using them since these classifications applicable for linear associations and their interpretation depend on the specific subject area; however, higher absolute values and smaller associated *P* values are generally accepted as a stronger departure from a null hypothesis of no correlation (Divaris K *et al*., 2012). Statistical analyses were carried out using SPSS 22.0 software. Two-sided *P*-values < 0.05 were considered statistically significant.

3.4. Results

3.4.1 The previous ovarian cancer IHC study and the current study

Figure 3.1 shows the immunohistochemical staining patterns. Calpastatin, calpain-1, -2 and -4 expression were confined to the membrane and cytoplasm. As pointed in the previous study, calpains and calpastatin showed predominant granular/diffuse staining in the cytoplasm of the ovarian cancer cells with the appearance of membrane staining and there was 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. Some stromal cell staining was observed, however, this was not scored as part of this study. In total, there were 469 cases with valid H-scores for calpains and calpastatin expression after excluding cores that contained no tumour or missing cores which may due to the drop off of the tissue section from the slide or the depletion of successive sections in the original donor block. It is estimated that technical causes alone bring about 10–30% of core loss (Voduc *et al*., 2008).

Although, as indicated in **section 3.3.2**, the TMA was primarily constructed of single cores taken from patient tumours, there were 48 cases that had two cores per case and 3 that had 3 cores per case. After excluding invalid cores, in total, there were 29 cases that had two H-scores per case and one had three H-scores per case. The variability between H-scores of different tissue cores from the same case was assessed using the coefficient of variation (CV, which is the ratio of the SD to the mean). Out of the 30 cases, calpastatin expression of 16 cases had the variation higher than 25%; calpain-1 expression of 19 cases, calpain-2 expression of 8 cases and calpain-4 expression of 13 cases had CVs higher than 25%. The high variation revealed the heterogeneity of tumours and support sampling more cores to represent the expression of calpains and calpastatin in the whole tumours, or ensuring that sufficient

patient numbers are used to yield meaningful and repeatable results.

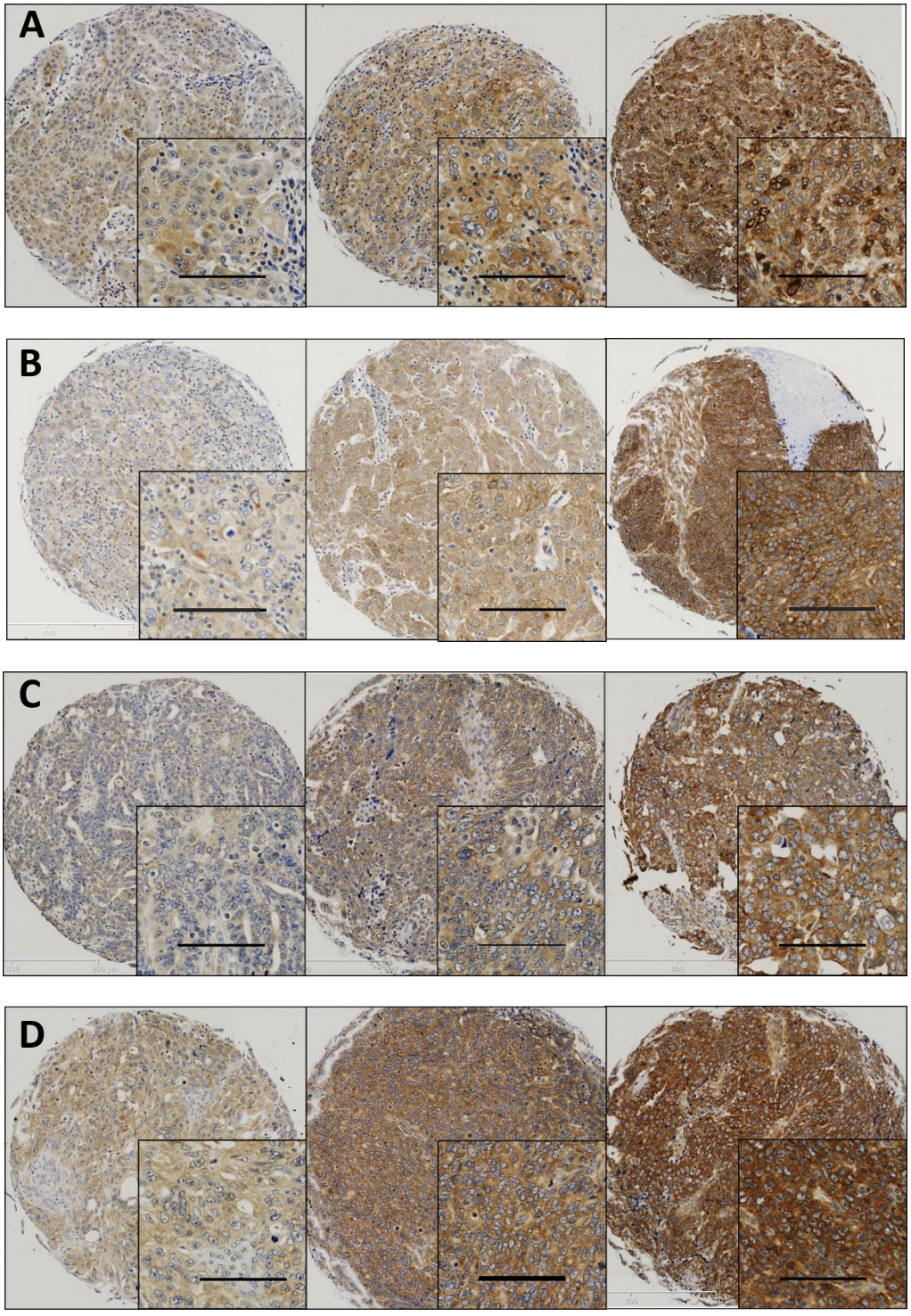


Figure 3.1 Examples of immunohistochemical expression and location of calpains and calpastatin in ovarian carcinoma cells. Staining pattern, including weak (left), medium (middle) and strong (right) staining, of (A) calpastatin (10×, 20× magnification), (B) calpain-1, (C) calpain-2 and (D) calpain-4 in the cytoplasm. Scale bar represents 100 µm.

Ninety-eight cases (80 valid cases) were included in both the previous cohort (Storr *et al*., 2012a) and the current cohort. Although tissues were from the same cases, different positions of a primary tumour were sampled for each of the two cohorts. Average protein expression from the previous study was compared with the protein expression in matched cases from the current cohort using Spearman's correlation test. We found that calpastatin and calpain-1 expression levels were significantly correlated with each other in these two studies (rs=0.602, $P<0.001$, n=67 and rs=0.512, $P<0.001$, n=69 respectively). No correlation was detected between these two studies on calpain-2 expression (rs=0.179, $P=0.141$, n=69) which might be due to the variability of polyclonal antibodies between different batches (further discussed in **section 3.5**).

3.4.2 Calpain system expression and clinicopathological factors

High calpastatin expression was observed in 256 (55%) out of 469 cases for this marker (cut-point: 80; mean±SE: 71±3; median±SD: 80±57). The median age of the patients with high calpastatin expression and low calpastatin expression tumours was 62 years (ranging from 3 to 83) and 65 years (ranging from 24 to 93); median OS time was 38 months (ranging from 0 to 165 months) and 24 months (ranging from 0 to 122 months); median PFS time was 14 months (ranging from 0 to 52 months) and 12 months (ranging from 1 to 53 months), respectively. Pearson's Chi-squared test was performed to evaluate the relationships between the expression of calpain-system and clinicopathologic characteristics (**Table 3.3**). High calpastatin expression was associated with younger patients ($\chi2=4.955$, d.f.=1, $P=0.026$) and HGSC ($\chi2=17.403$, d.f.=5, $P=0.004$) whilst low calpastatin expression was associated with clear-cell carcinoma (CCC). This association between calpastatin and ovarian subtype was also significant in chemo-naïve samples ($\chi2=15.958$, d.f.=5, P=0.007).

High calpain-1 expression was observed in 368 (79%) of the 469 available cases for this marker (cut-point: 55; mean±SE: 108±3; median±SD: 120±66). The median age of the patients with high calpain-1 expression and low calpain-1 expression tumours was 63 years (ranging from 3 to 93) and 64 years (ranging from 24 to 88); median OS time was 27 months (ranging from 0 to 165 months) and 30 months (ranging from 1 to 120 months); median PFS time was 12 months (ranging from 0 to 53 months) and 16 months (ranging from 1 to 48 months) respectively. Low calpain-1 expression was associated with low stage ($\chi2$=15.259, d.f.=3, *P*=0.002), no residual disease ($\chi2$=15.388, d.f.=2, *P*<0.001), CCC, endometrioid and mucinous carcinoma ($\chi2$=56.577, d.f.=5, *P*<0.001) whilst high calpain-1 expression was associated with the presence of residual disease and HGSC (**Table 3.3**). Here, calpain-1 expression was associated with residual disease, but different from tumour stage or grade, which unalterably reflects the tumour nature, residual disease reflects the experience and skills of surgeons (Münstedt & Franke, 2004). Significant associations between calpain-1 expression and ovarian subtype ($\chi2$=46.754, d.f.=5, *P*<0.001), stage ($\chi2$=17.449, d.f.=3, *P*=0.001) and residual disease ($\chi2$=14.723, d.f.=2, *P*=0.001) were also detected in chemo-naïve samples.

High calpain-2 expression was observed in 379 (81%) of the 469 available cases for this marker (cut-point: 10; mean±SE: 79±3; median±SD: 100±52). The median age of the patients with high calpain-2 expression and low calpain-2 expression tumours was 64 years (ranging from 3 to 93) and 62 years (ranging from 24 to 83); median OS time was 27 months (ranging from 0 to 165 months) and 33 months (ranging from 2 to 65 months); median PFS time was 12 months (ranging from 0 to 53 months) and 14 months (ranging from 1 to 43 months). Pearson's Chi-squared test was performed to examine the association between calpain expression and clinicopathologic criteria (**Table 3.3**). Calpain-2 status was not related to age, histological subtype, tumour grade, FIGO-sub-stages, recurrent disease or chemoresponse.

Table 3.3 The association between protein expression and clinicopathological variables.

Variable	Calpastatin			Calpain-1			Calpain-2			Calpain-4		
	Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Age												
≤62	97 (20.7%)	143 (30.5%)	**0.026**	50 (10.7%)	190 (40.5%)	0.705	45 (9.6%)	195 (41.7%)	0.880	67 (14.3%)	173 (36.9%)	0.215
>62	116 (24.7%)	113 (24.1%)		51 (10.9%)	178 (38.0%)		44 (9.4%)	184 (39.3%)		76 (16.2%)	153 (32.6%)	
Histological subtypes												
HGSC	122 (26.0%)	165 (35.2%)	**0.004**	38 (8.1%)	249 (53.1%)	**<0.001***	54 (11.5%)	233 (49.8%)	0.334*	83 (17.7%)	204 (43.5%)	**0.003***
Mucinous	24 (5.1%)	21 (4.5%)		14 (3.0%)	31 (6.6%)		11 (2.4%)	34 (7.3%)		14 (3.0%)	31 (6.6%)	
Endometrioid	25 (5.3%)	30 (6.4%)		20 (4.3%)	35 (7.5%)		6 (1.3%)	48 (10.3%)		14 (3.0%)	41 (8.7%)	
CCC	31 (6.6%)	13 (2.8%)		25 (5.3%)	19 (4.1%)		12 (2.6%)	32 (6.8%)		25 (5.3%)	19 (4.1%)	
LGSC	7 (1.5%)	17 (3.6%)		3 (0.6%)	21 (4.5%)		3 (0.6%)	21 (4.5%)		5 (1.1%)	19 (4.1%)	
SBOT	4 (0.9%)	10 (2.1%)		1 (0.2%)	13 (2.8%)		3 (0.6%)	11 (2.4%)		2 (0.4%)	12 (2.6%)	
Grade												
1	16 (3.4%)	21 (4.5%)	0.796	8 (1.7%)	29 (6.2%)	0.415	8 (1.7%)	29 (6.2%)	0.915	9 (1.9%)	28 (6.0%)	0.196
2	31 (6.6%)	42 (9.0%)		20 (4.3%)	53 (11.3%)		14 (3.0%)	59 (12.6%)		17 (3.6%)	56 (12.0%)	
3	166 (35.5%)	192 (41.0%)		73 (15.6%)	285 (60.9%)		67(14.3%)	290 (62.1%)		117 (25.0%)	241 (51.5%)	
Stage												
I	83 (18.0%)	86 (18.7%)	0.217	51 (11.1%)	118 (25.6%)	**0.002**	35 (7.6%)	133 (28.9%)	0.572	52 (11.3%)	117 (25.4%)	0.969
II	24 (5.2%)	30 (6.5%)		13 (2.8%)	41 (8.9%)		7 (1.5%)	47 (10.2%)		17 (3.7%)	37 (8.0%)	
III	82 (17.8%)	123 (26.7%)		28 (6.1%)	177 (38.4%)		40 (8.7%)	165 (35.9%)		59 (12.8%)	146 (31.7%)	
IV	18 (3.9%)	15 (3.3%)		8 (1.7%)	25 (5.4%)		5 (1.1%)	28 (6.1%)		10 (2.2%)	23 (5.0%)	

HGSC: high grade serous carcinoma; LGSC: low grade serous carcinoma; CCC: clear-cell carcinoma; SBOT: serous borderline ovarian tumour

*have expected count less than 5. Significant P-values are indicated by bold type.

Table 3.3 Continued

Variable	Calpastatin			Calpain-1			Calpain-2			Calpain-4		
	Low	**High**	**P-value**	**Low**	**High**	**P-value**	**Low**	**High**	**P-value**	**Low**	**High**	**P-value**
Residual disease												
No residual tumour	114 (27.5%)	143 (34.5%)	0.958	73 (17.6%)	184 (44.3%)	**<0.001**	55 (13.3%)	201 (48.6%)	0.677	76 (18.3%)	181 (43.6%)	0.982
Residual tumour (<2cm)	23 (5.5%)	27 (6.5%)		5 (1.2%)	45 (10.8%)		11 (2.7%)	39 (9.4%)		15 (3.6%)	35 (8.4%)	
Residual tumour (>2cm)	47 (11.3%)	61 (14.7%)		14 (3.4%)	94 (22.7%)		19 (4.6%)	89 (21.5%)		33 (8.0%)	75 (18.1%)	
Response to platinum-based chemotherapy												
Platinum refractory	8 (2.7%)	22 (7.3%)	0.087	4 (1.3%)	26 (8.7%)	0.360*	3 (1.0%)	27 (9.0%)	0.347*	5 (1.7%)	25 (8.3%)	0.240
Platinum resistance	11 (3.7%)	9 (3.0%)		6 (2.0%)	14 (4.7%)		5 (1.7%)	15 (5.0%)		7 (2.3%)	13(4.3%)	
Platinum sensitivity	114 (38.0%)	136 (45.3%)		53 (17.7%)	197 (65.7%)		49 (16.4%)	200 (66.9%)		77 (25.7%)	173(57.7%)	

High calpain-4 expression was observed in 326 (70%) out of 469 available cases for this marker (cut-point: 95; mean±SE: 114±3; median±SD: 120±70). The median age of the patients with high calpain-4 expression and low calpain-4 expression tumours was 63 years (ranging from 3 to 93) and 66 years (ranging from 24 to 84); median OS time was 32.5 months (ranging from 0 to 165 months) and 24 months (ranging from 0 to 107 months); median PFS time was 12 months (ranging from 0 to 52 months) and 12 months (ranging from 1 to 53 months). Pearson's Chi-squared test was performed to examine the association between calpain expression and clinicopathologic criteria **Table 3.3**. HGSC was associated with high calpain-4 expression whilst CCC was associated with low calpain-4 expression ($\chi 2=18.181$, d.f.=5, $P=0.003$). This association between calpain-4 and ovarian subtype was also significant in chemo-naïve samples ($\chi 2=15.420$, d.f.=5, $P=0.009$). Original tables of the significant associations in **Table 3.3** (Crosstab and Chi-Square Tests) from SPSS software with the expected and observed count are listed in the Appendix (**Table S3.1**, **Table S3.2**, **Table S3.3**, **Table S3.4** and **Table S3.5**).

Calpain-1, -4 and calpastatin expression were all associated with ovarian cancer histological subtypes, whereas previous results (Storr *et al*., 2012a) showed no association between the expression of calpain-1, -2 or calpastatin and tumour stages which could be due to the smaller sample size. In the present study, calpain-1 was also associated with stage and residual disease. Based on the information from tumour stage, patients were then grouped according to whether they had an organ-confined tumour (i.e. group 1: stage 1 and group 2: stage 2-4) or according to whether they had distant metastasis (i.e. group 1: stage 1-3 and group 2: stage 4). Calpain-1 showed significant association with organ-confined ovarian cancers rather than cancers with distant metastases, with low calpain-1 being associated with organ-confined ovarian cancer ($\chi 2=11.310$, d.f.=1, $P=0.001$) (**Table S3.6**).

3.4.3 The expression of calpain system and clinical outcomes

3.4.3.1 Kaplan-Meier analysis in the current whole cohort

Calpain-2, calpain-4 and calpastatin expression were significantly associated with OS (**Figure 3.2**). Kaplan-Meier analysis of OS demonstrated that high calpain-2 expression significantly associated with adverse OS (*P*=0.026) while high calpain-4 and calpastatin were significantly associated with better OS (with *P*=0.003 and *P*=0.010 respectively). No significant association was found between calpain-1 expression and OS (*P*=0.153). In addition, calpain-1, -2, -4 and calpastatin were not associated with PFS (**Figure 3.3**). As ovarian cancer progresses rapidly, recording the PFS for each case during follow-up would be challenging. As fewer cases are recorded with PFS comparing with cases recorded with OS in the current cohort; thus, no association was detected between the protein expression and PFS.

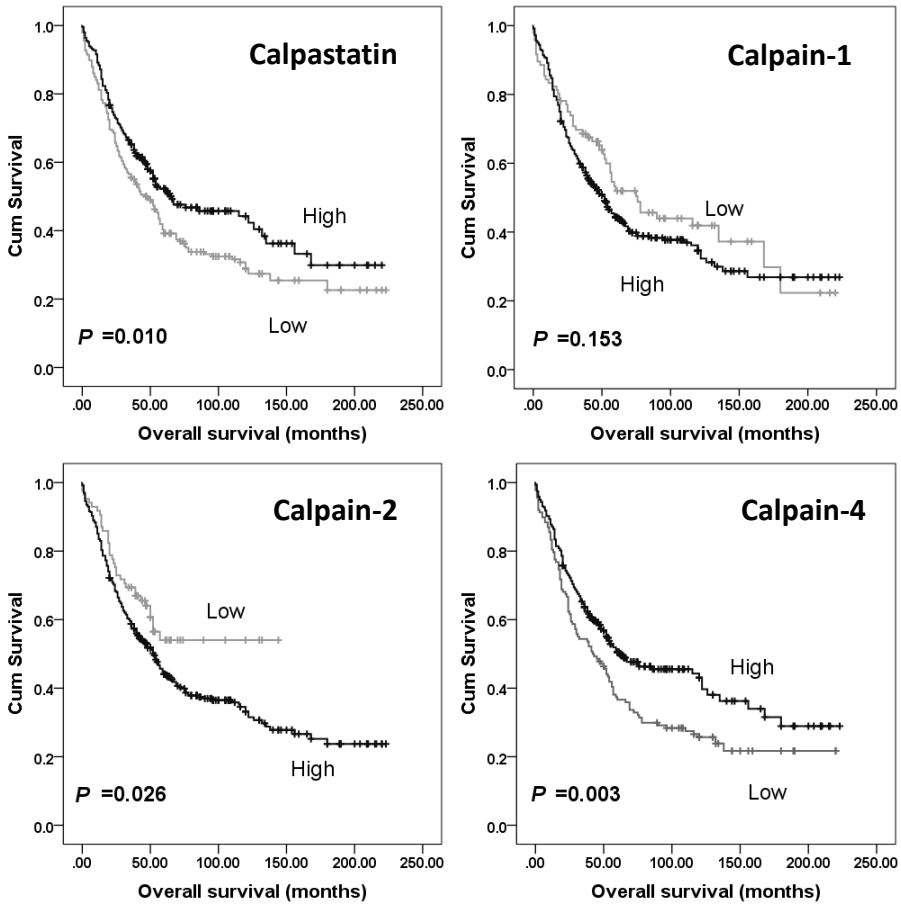


Figure 3.2 Kaplan-Meier survival curves show the impact of calpain-1, -2, -4 and calpastatin expression on OS.

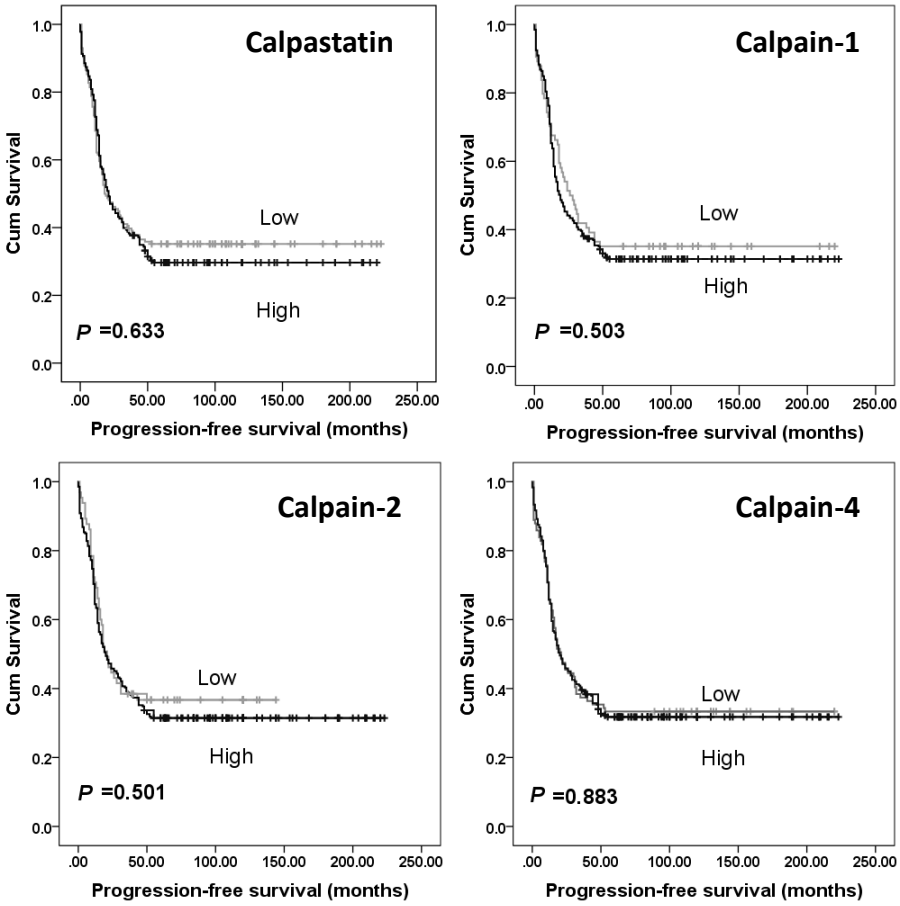


Figure 3.3 Kaplan-Meier survival curves show the impact of calpain-1, -2, -4 and calpastatin expression on PFS.

All cases (n=154) that comprised the clinical cohort used in the previous study received carboplatin-based adjuvant chemotherapy. In consideration of this, Kaplan-Meier survival analysis was conducted on 352 cases in the current cohort who received carboplatin-based adjuvant chemotherapy using the same cut-point calculated from the current whole cohort (figure not shown). High calpain-2 was associated with poor OS (*P*=0.030) but not PFS (*P*=0.381). Calpastatin, calpain-1 and calpain-4 expression was not associated with either OS (*P*=0.129, 0.080 and 0.060, respectively) or PFS (*P*=0.181, 0.161 and 0.470, respectively). Using X-tile, the recalculated cut-points of calpastatin (80), calpain-1 (60), calpain-2 (5) and calpain-4 H-scores (110) were generated from these 352 cases respectively. Kaplan-Meier analysis was used to assess the associations between OS and the expression of calpastatin and the conventional calpain subunits (figure not shown), with P-values as follow: *P*=0.202 (calpastatin), *P*=0.045 (calpain-1), *P*=0.026 (calpain-2) and *P*=0.025

(calpain-4). There was no significant association between PFS and the expression of calpastatin (*P*=0.271), calpain-1 (*P*=0.095), calpain-2 (*P*=0.372) or calpain-4 (*P*=0.956) (figure not shown). Thus, consistent with the previous finding, calpain-2 expression significantly associated with the OS of ovarian cancer patients.

3.4.3.2 Kaplan-Meier analysis in cases common to both current and previous studies

Kaplan-Meier survival analysis was conducted in the 80 valid cases that were involved in both the current and previous (Storr *et al*. 2012a) studies. Using the cut-points that was applied for the previous cohort (calpastatin: 165; calpain-1: 25; calpain-2: 162), calpain-2 expression remained associated with OS (*P*=0.016) in the 80 cases using the data from the previous study whilst the expression of calpastatin and calpain-1 was not associated with OS (*P*=0.575, *P*=0.382 respectively) (Storr *et al*., 2012a). In the current study, when using the same cut-point as the current whole cohort, high calpain-4 was associated with better OS (*P*=0.030), whereas the expression of calpain-1, calpain-2 and calpastatin were not significantly associated with patient outcome. When using the cut-points calculated from the 80 cases in the current study (i.e. calpastatin: 110; calpain-1: 85; calpain-2: 90; calpain-4: 70), neither calpastatin nor calpain (-1, -2 or -4) was associated with OS (*P*=0.387, *P*=0.061, *P*=0.086 and *P*=0.084 respectively).

3.4.3.3 Kaplan-Meier analysis in the cases unique to the current study

Kaplan-Meier survival analysis was also conducted using the cut-point of the current whole cohort in the remaining cases after excluding the 80 cases. In the remaining cases, high calpain-2 was associated with poor OS (*P*=0.024). Calpastatin, calpain-1, calpain-2 or calpain-4 expression was not associated with PFS (*P*=0.449, 0.335, 0.424 and 0.361, respectively). Low calpastatin and low calpain-4 was associated with poor OS (*P*=0.007, *P*=0.039 respectively) as

above. Again calpain-1 was not associated with either OS (*P*=0.523) or PFS (*P*=0.335). When using the recalculated cut-points from the remaining cases in the current study after excluding the 80 cases that appeared previously (i.e. calpastatin: 80; calpain-1: 40; calpain-2: 105 and calpain-4: 5), as above, high calpastatin (*P*=0.008), low calpain-2 (*P*=0.021) and high calpain-4 (*P*=0.008) was significantly associated with better OS respectively. Still, there was no significant association between calpain-1 and OS (*P*=0.361).

3.4.3.4 Multivariate analysis in the current whole cohort

Before multivariate analysis, age, grade, FIGO stage, histological subtypes，tumour residue and platinum sensitivity were assessed by Kaplan-Meier method and their significances were determined by log-rank test in the whole cohort (*P* values are listed in **Table 3.4**). In the univariate analysis, advanced stage, high grade, old age, platinum-resistance, the presence of residual disease, HGSC, high calpain-2, low calpain-4 and low calpastatin expression were significantly associated with a shorter OS than early stage, low grade, young age, platinum-sensitive, the absence of residual disease, low grade serous and endometrioid carcinoma, low calpain-2, high calpain-4 and high calpastatin expression respectively. Therefore, these parameters were used in multivariate analysis.

Table 3.4 Univariate (log-rank test) analysis

Variables associate with OS	Number of patients	P (value)
Age (median 62)	548	**<0.001**
Grade	548	**<0.001**
FIGO stage	539	**<0.001**
Histological subtypes	549	**<0.001**
Platinum sensitivity	313	**<0.001**
Tumour residue	486	**<0.001**
Calpain-2 expression	456	**0.026**
Calpain-4 expression	456	**0.003**
Calpastatin expression	456	**0.010**

Significant P-values are indicated by bold type.

In multivariate analysis, using the cut-point calculated from the whole

cohort, neither calpain-2/-4 nor calpastatin was found to be an independent marker of OS, with the above parameters being considered (**Table 3.5** and **Table S3.7**). Whereas, cancer stage, platinum-sensitivity and residual disease were independent prognostic factors in the current cohort.

Table 3.5 Multivariate (Cox proportional hazard regression) analysis

Variables	Multivariate (OS) (239 patients)			
	P-value	Exp (B)	95% Confidence Interval for Exp (B)	
			Lower	Upper
Age (median 62)	0.225	1.253	0.870	1.805
Grade	0.177	1.445	0.847	2.465
FIGO stage	**0.001**	1.513	1.192	1.919
Histological subtypes	0.833	0.983	0.836	1.155
Platinum sensitivity	**<0.001**	3.142	2.024	4.879
Tumour residue	**0.029**	1.298	1.028	1.639
Calpastatin expression	0.061	0.711	0.498	1.015
Variables	**Multivariate (OS) (238 patients)**			
	P-value	**Exp (B)**	**95% Confidence Interval for Exp (B)**	
			Lower	**Upper**
Age (median 62)	0.059	1.404	0.987	1.998
Grade	0.138	1.527	0.873	2.673
FIGO stage	**<0.001**	1.540	1.209	1.962
Histological subtypes	0.959	1.004	0.855	1.180
Platinum sensitivity	**<0.001**	3.272	2.107	5.082
Tumour residue	**0.031**	1.283	1.023	1.610
Calpain-2 expression	0.070	1.524	0.967	2.404
Variables	**Multivariate (OS) (239 patients)**			
	P-value	**Exp (B)**	**95% Confidence Interval for Exp (B)**	
			Lower	**Upper**
Age (median 62)	0.164	1.296	0.900	1.867
Grade	0.201	1.427	0.828	2.462
FIGO stage	**0.001**	1.497	1.178	1.904
Histological subtypes	0.801	0.979	0.832	1.152
Platinum sensitivity	**<0.001**	3.088	1.987	4.798
Tumour residue	**0.023**	1.303	1.037	1.639
Calpain-4 expression	0.232	0.793	0.542	1.160

Significant P-values are indicated by bold type.

Using the cut-point calculated from the remaining cases of the current cohort after excluding the 80 cases, multivariate analysis indicated that neither calpain-2/-4 nor calpastatin was an independent marker of OS when

considering age, stage, histological subtype, platinum-sensitivity, and residual disease.

3.4.4 Correlations between calpain-1, -2, -4 and calpastatin expression

Heterodimer μ-calpain and m-calpain consist of the large subunit of calpain, calpain-1 and calpain-2, and the regulatory small subunit, calpain-4 (Storr *et al*., 2011a). The endogenous calpain inhibitor calpastatin can inhibit four calpain heterodimers at the same time (Storr *et al*., 2011a). Presumably, calpain-4 expression should be linearly associated with calpain-1 and calpain-2 expression. To validate the existence of correlations between conventional calpain subunits and calpastatin expression levels, Spearman's rank correlation coefficient (Spearman's rho) test was used in assessing the correlation between matched H-scores. The correlation strength was interpreted based on **section 3.3.3**. As listed in **Table 3.6**, conventional calpain subunits and calpastatin either weakly or moderately correlated with each other (calpain-1 and calpastatin, calpain-4 and calpastatin, calpain-1 and calpain-4 moderately correlated).

Table 3.6 Spearman's rank correlation coefficient between calpain-1, -2, -4 and calpastatin expression

		Calpain-1	Calpain-2	Calpain-4
Calpastatin	**Correlation Coefficient**	**0.480****	**0.205****	**0.514****
	Sig. (2-tailed)	2.4147E-28	0.000008	6.783E-33
Calpain-1	**Correlation Coefficient**	\	**.300****	**0.481****
	Sig. (2-tailed)	\	3.3557E-11	1.5002E-28
Calpain-2	**Correlation Coefficient**	\	\	**0.296****
	Sig. (2-tailed)	\	\	6.4717E-11

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Significant P-values are indicated by bold type.

Tumours were re-categorised according to expression status of calpain-1 and -2, calpain-1 and -4, calpain-2 and -4 or calpastatin and one of the conventional calpain subunits respectively to assess whether their expression had additional predictive values. With each pair of proteins, tumours were

grouped into 4 groups. Kaplan-Meier survival analysis showed that better OS was detected in cases with low calpain-1/high calpastatin (*P*=0.005), high calpain-4/low calpain-1 (*P*=0.002), or high calpain-4/ high calpastatin expression (*P*=0.009). High calpain-2/low calpastatin (*P*=0.004) expression and high calpain-2/low calpain-4 expression (*P*=0.002) was associated with adverse patient OS (**Figure S3.1**). The Cox-regression model was used to evaluate the clinicopathological parameters and the combined expression status of calpains and calpastatin to predict patient OS. Despite the association between calpain-4/calpastatin status and OS was not stronger than the association between calpain-4 alone and OS, using the same variables as listed in **Table 3.4**, multivariate analysis showed that calpain-4/calpastatin status (Hazard Ratio (HR) 0.869, 95% confidence interval (CI) 0.757-0.998; *P*=0.047) appeared as an independent prognostic factor together with cancer stage, platinum-sensitivity, and residual disease.

3.4.5 Calpain expression and OS in patient subgroups

Further analysis was conducted to investigate the prognostic significance of conventional calpains and calpastatin in the individual subgroups divided by histological subtypes, stage, the presence or absence of residual disease, platinum-sensitive and platinum-resistant groups etc. The *P* values of Kaplan-Meier survival analysis are summarised in **Table S3.8**. Although in certain subgroups the expression of conventional calpain subunit or calpastatin had stronger association with OS, none of the association was statistically significant after the correction.

Chemotherapy treatment is likely to induce changes in protein expression in tumours, thus associations between protein expression and survival outcomes were analysed in 448 cases, which clearly recorded as chemo-naïve samples. Calpain-4 (*P*=0.030) and calpastatin (*P*=0.022) expression were significantly associated with OS, however, the association between calpain-2

expression and OS was no longer significant (*P*=0.532, use same cut-point as the whole cohort; *P*=0.341, use recalculated cut-point 120).

3.5. Discussion

As mentioned above, previously published results from our research group suggested that high calpain-2 expression associated with resistance to platinum-based adjuvant chemotherapy (*P*=0.031), adverse PFS (*P*=0.049) and OS (*P*=0.006) in ovarian cancer (n=154; Storr S.J. *et al*., 2012a). Results from multivariate analysis showed that calpain-2 expression was an independent prognostic factor (among grade, stage, optimal debulking and platinum sensitivity). The present study used a larger cohort (n=575) to verify the previous study and further study the relationships between calpain-1, -2, -4 and calpastatin expression across different ovarian cancer histological subtypes.

Although the previous study was somewhat limited in number (n=154), it assessed two tumour cores per patient. The current study has a larger patient number (n=575) but one limitation is that most patients only had one core per patient. Using Spearman's correlation test on IHC data from the previous study and matched data from the current study, we found that calpain-1 and calpastatin expression levels were significantly correlated between these two studies, although the correlation of calpain-1 expression levels was marginal (calpain-1: rs=0.512, *P*<0.001, n=69; calpastatin: rs=0.602, *P*<0.001, n=67). No correlation was detected between these two studies on calpain-2 expression (rs=0.179, P=0.141, n=69) which might be due to the variability of polyclonal antibodies between different batches. The CV was used to assess the variability between IHC data from the same patient but different tissue cores. The percentages of CV above 25% were 53.3 % (16/30) for calpastatin expression, 63.3% (19/30) for calpain-1, 26.7% (8/30) for calpain-2, and 43.3% (13/30) for calpain-4. The high variations between some samples indicated

that one core is not ideal to represent the calpain expression level of a tumour. The difference of IHC data between cohorts and within cohort indicated that it would be more precise to have multiple cores taken from each case for the representative of a tumour. But using multiple cores per case rapidly increases workload in TMA construction and biomarker scoring. As indicated by Torhorst *et al*. (2001), in sufficiently large patient cohorts (n=553), single core TMAs (one core per case) can reproduce important clinical associations. When the case number is limited (n=171), Khouja *et al*. (2010) indicated that the data derived from both TMA (3 cores per case, cores 1, 2 and 3, which were analysed separately rather than take the means) and the whole tissue sections were significantly correlated to disease-related survival; however, in multivariate analysis only the whole tissue sections retained an independent prognostic significance. Voduc *et al*., (2008) concluded that generally biomarkers of true clinical significance will display relatively consistent staining in tumours and retain important clinicopathological associations. Thus the current TMAs were considered adequate to serve this purpose. But variations of the calpain expression were observed between cores from same cases in the current study; moreover, calpain-2 expression between the current and the previous studies lacked of correlation. Hence, the current TMA might not be ideal for the study of calpain expression. Future studies should initially use, if available, whole sections to check marker heterogeneity of expression of calpain and other relevant proteins within each tumour to decide if a TMA approach is acceptable, especially if the TMA is comprised of only one core per tumour. If this is the case then the number of patient samples must be sufficiently high to account for any inherent variability. Ideally more than a single core should be sampled per case to adequately capture variability in protein expression. Whole section studies also supply information on expression patterns that may be associated with focal events such as tumour-stroma interactions and angiogenesis, which could not be obtained by TMA studies.

The current results confirmed that low calpain-2 expression significantly associates with better OS (*P*=0.026) while there was no significant association between calpastatin and PFS (*P*=0.633). In both studies, calpain-1 was not significantly associated with either PFS (*P*=0.503) or OS (*P*=0.153). In the current study, conventional calpain subunits and calpastatin expression status were combined for the better understanding of how they influence patient survival. Kaplan-Meier survival analysis showed a similar or an improved relevance between OS and the combined expression status compared with that seen between OS and individual protein expression status alone. The combination of calpain-1 and calpain-2 expression status was not significantly associated with patient OS (*P*=0.092). These findings suggest that the role that calpain-1 plays may not be complementary to those of calpain-2.

In contrast to the previous findings, calpain-2 was not associated with PFS, whilst calpain-4 and calpastatin was associated with OS (*P*=0.003 and *P*=0.010 respectively) (**Figure 3.3**). All cases (n=154) included in the previous study received carboplatin-based adjuvant chemotherapy, thus Kaplan-Meier survival analysis was conducted in 352 cases from the current cohort who received carboplatin-based adjuvant chemotherapy. Again, high calpain-2 was associated with poor OS but no significant association was found between calpain-2 expression and PFS. In the current study, calpain-2 was not associated with patient response to platinum-based chemotherapy, which may be due to the variability of polyclonal antibodies between different batches and/or due to the different composition of patients. The percentage of platinum-sensitive patients was different between the previous cohort (67.5%) and the current cohort (82.3%). Patients were then divided into platinum-resistant and platinum-sensitive groups, and Kaplan–Meier analysis was conducted in these two groups separately. Conventional calpain subunits and calpastatin did not show significant association with OS in either of these two groups. By Pearson's Chi-squared test, neither conventional calpain

subunits nor calpastatin was associated with the resistance to platinum-based adjuvant chemotherapy in the current study. Such data may also help explain the results in **section 2.4** where inhibition of calpain did not significantly alter *in vitro* platinum sensitivity.

Calpain-1, calpain-4 and calpastatin all showed significant association with histological subtypes. HGSCs were associated with high expression of calpain-1, -4 and calpastatin whilst CCCs were associated with low expression of calpain-1, -4 and calpastatin. The above findings suggest that in different histological subtypes the conventional calpains and calpastatin may have different expression pattern and associate with patient outcome in different ways. There are two important notes about the histological subtypes of ovarian cancers when interpreting the IHC data, concerning mucinous carcinomas and CCCs respectively. As indicated in **section 1.1.3.1**, some mucinous carcinomas originate from the gastrointestinal tract (i.e. colorectum, appendix or stomach), pancreas, biliary tract or cervix and spread to the ovary as metastatic mucinous carcinoma. To discriminate between the primary and metastatic mucinous carcinomas, markers such as cytokeratin (i.e. CK7 and CK20), CA125 and oestrogen receptor could be used (McCluggage, 2012). But previously used primary ovarian mucinous carcinoma markers overlapped with metastatic mucinous carcinoma markers. Since these facts were not known in those days, therefore, the mucinous carcinomas in these TMA may be metastatic mucinous carcinoma. Most primary ovarian mucinous carcinomas at diagnosis are confined to the ovary which means a mucinous carcinoma with higher stage is likely to be a metastatic mucinous carcinoma and originate from elsewhere (McCluggage, 2012). In the current study, 45 mucinous carcinoma cases were at stage 1 and 13 cases at stage 2-4; presumably, the majority of the mucinous ovarian carcinoma cases in the current study are primary mucinous carcinomas. The morphologic characteristics of CCCs include clear cytoplasm (DeLair *et al*., 2011). In the

study of the cytoplasmic expression of protein markers, this character could be the main reason that CCCs showed low staining intensity/protein expression, even though the CCCs included in the current studies were all grade 3 tumours.

In conclusion, calpain-2 expression was adversely associated with OS in ovarian cancer patients in both the previous and current study. However calpain-2 was not associated with platinum resistance in the current study. The expression of calpastatin, calpain-1 and -4 was associated with histological subtype. Calpain-1 expression was associated with the presence of residual disease and tumour stage. Calpastatin expression was associated with age. Conventional calpain subunits and calpastatin expression were positively associated with each other (Spearman's rho), with calpain-2 positively associated with OS whilst calpain-4 and calpastatin negatively associated with OS. None of the conventional calpain subunits or calpastatin expression was suggested as an independent prognostic factor in multivariate analysis of the current study. Although there was no association between calpain-1 and patient outcome, calpain-1 was associated with tumour stage. Low calpain-1 expression was associated with tumours that confined to the ovaries (stage 1) and the absence of residual disease. As stage reflects the extent of tumour metastatic spread, such data may suggest that conventional calpains and calpastatin may be involved with tumour migration and invasion. Hence, the following chapter intended to study whether altering calpain activity can influence cell haptotactic migration.

3.6. Summary

1. H-scores of calpain-1 and calpastatin expression in the current study were significantly correlated with matched H-scores from the previous study. The matched H-scores of calpain-2 expression from these two studies were not correlated with each other.

2. The expression of calpain-1, calpain-2, calpain-4 or calpastatin was not associated with resistance to platinum-based adjuvant chemotherapy in this study.

3. The expression of calpastatin, calpain-1 and -4 was associated with histological subtype. Calpain-1 expression was associated with the presence of residual disease and tumour stage. Calpastatin expression was also associated with age.

4. The current study confirmed that calpain-2 is associated with patient OS, whilst calpain-1 was not associated with either OS or PFS.

5. Calpain-4 and calpastatin were positively associated with OS but were not associated with PFS in this cohort.

6. In multivariate analysis of the current study, neither calpain (-2 or -4) nor calpastatin was found to be an independent marker of OS, with the following parameters being considered: tumour stage, histological subtype, grade, platinum-sensitivity, patient age and residual disease.

7. The expression of calpain-1, calpain-2, calpain-4 and calpastatin was significantly associated with each other.

Chapter 4. Phenotypic effects of modulating calpain activity.

4.1. Abstract

INTRODUCTION: Cellular calpain activity is highly regulated by multiple mechanisms. The calpain proteolytic processing of its substrates is a mechanism of modulation rather than degradation (Perrin & Huttenlocher, 2002) in which way calpain regulates cell migration; and calpain substrates are involved in destabilising cell adhesion to the ECM, promoting rear detachment, cell spreading, membrane protrusion, chemotaxis, and adhesion complex formation and turnover. Numerous proteins have been identified as calpain substrates related to focal adhesions and migration *in vitro* (e.g. focal adhesion kinase (FAK) and talin etc.). A large majority of studies have indicated that calpain activity is positively associated with cell migration and that inhibition of calpain inhibits cell motility.

OBJECTIVES: This chapter sought to determine if altering calpain activity could influence ovarian cancer cell migration.

METHODS: In SKOV3, PEO1 and PEO4 cells, the effect of A23187 (a calcium ionophore) and calpeptin (a widely used calpain inhibitor) on calpain activity and cell haptotactic migration was assessed using t-BOC calpain activity assay and scratch wound migration assay respectively. A novel calpain inhibitor 'compound-1', obtained via collaboration with Prof R Allemann (Cardiff University), was tested on SKOV3 cells, with breast cancer MDA-MB-231 cells used as a comparator.

RESULTS: A23187 increased calpain activity in SKOV3 cells which was, unexpectedly, accompanied by a significant inhibition of cell migration.

Inhibitory effects of calpeptin on both calpain activity and cell migration were observed in SKOV3 cells. A23187 offered no significant change on calpain activity and cell haptotactic migration in ovarian cancer PEO1 and PEO4 cell lines. Although calpeptin significantly inhibited calpain activity in PEO1 and PEO4 cells, no significant inhibition in cell migration was detected. Compound-1 (5μM) did not significantly alter either calpain activity or cell migration in SKOV3 and MDA-MB-231 cells (used as a positive control); however, calpeptin caused significant inhibition of cell migration in MDA-MB-231 cells.

CONCLUSION: Inhibiting calpain activity leads to inhibition of cell migration in SKOV3 and MDA-MB-231 cells which agree with the suggested positive association between calpain activity and cell migratory ability in publications. Similar calpain activity levels in PEO1 and PEO4 cells with or without A23187 treatment may also in parallel with the similar migration rate between the treated and untreated cells. The A23187-increased calpain activity in SKOV3 cells was accompanied by a significant inhibition of cell migration. Moreover, significant calpain inhibition was not accompanied with decreased migration rate in calpeptin-treated PEO1 and PEO4 cells. Such conflicting results indicate that the associations between calpain and cell haptotactic migration depend on the context and could be tumour subtype specific. Due to the limitation of the current calpain activity assay, it is difficult to draw a firm conclusion on the effect of A23187, calpeptin and compound-1 on calpain activity regulation. Compound-1 could not significantly inhibit cell migration in SKOV3 and MDA-MB-231 cell line which might be due to drug concentrations and treatment periods applied in the initial, and preliminary, experiments.

4.2. Introduction and aims

4.2.1 Regulation of calpain activity

Calpain activity has been reported to be important for a wide array of cellular responses including apoptosis, proliferation, necrosis, autophagy, cell morphology, migration and adhesion (Storr *et al*., 2011a; Franco & Huttenlocher, 2005; Kim JY *et al*., 2017). Most of these responses can be accomplished by the two ubiquitous isoforms, μ- and m-calpain (Franco & Huttenlocher, 2005). Calpain activity is, therefore, tightly controlled by multiple mechanisms including the most widely studied mechanism, activation by calcium ions, which causes conformational changes in calpains (Guroff, 1964). In the cytosol, calpain exists in an inactive form until the increases of the cellular Ca^{2+} level lead to its translocation to membranes where it is activated in the presence of Ca^{2+} and phospholipids (Suzuki K *et al*., 2004).

The Ca^{2+} concentration required for calpain activation is generally far greater for cells than that achieved intracellularly (Hood *et al*., 2004; Morita *et al*., 2006; Xu & Deng, 2006). The levels of calcium required for calpain activation exceed the maximal levels in living cells *in vitro*: to reach half-maximal activity, μ-calpain needs 2-75μM Ca^{2+} whilst m-calpain needs 200–1000μM Ca^{2+}, however, normal intracellular Ca^{2+} concentrations of the cytosol is estimated to range from 50 to 300nM (Maravall *et al*., 2000; Friedrich, 2004) which is much lower than the extracellular calcium concentration (around 2 mM) (Baudry, Chou & Bi, 2013). Intracellular calcium is stored in organelles such as the endoplasmic reticulum (ER) and mitochondria (Baudry, Chou & Bi, 2013). After stimulation, intracellular calcium concentrations can be elevated by releasing Ca^{2+} from such organelles or importing it into cells via plasma membrane ionotropic receptors and voltage-gated Ca^{2+} channels (Baudry, Chou & Bi, 2013). The intracellular

calcium concentration can be rapidly increased by 10 to 100 fold which is estimated to reach tens of μM but cannot achieve mM, unless it is under pathological conditions (Baudry, Chou & Bi, 2013): Ca^{2+} influx was found regulated by proteins such as KCa3.1 channel in pancreatic stellate cells (Storck *et al*., 2017); L-type calcium channels in human cancer cells (Jacquemet *et al*. 2016); Ca^{2+}-sensing receptor in androgen receptor-deficient prostate cancer cells (Huang *et al*., 2017) and stromal-interaction molecule 1, an endoplasmic reticulum Ca^{2+} sensor, which triggered store-operated Ca^{2+} entry activation in cervical cancer cell (Chen *et al*., 2011).

To reach the Ca^{2+} threshold for calpain activation, there is a hypothesis that calpains and their regulatory molecules may closely interact with organelles storing Ca^{2+} i.e. endoplasmic reticulum and Golgi apparatus (Hood, Brooks& Roszman, 2006). Although not knowing the physiological relevance, adding synthetic A and C regions of calpastatin facilitated the binding of Ca^{2+} to calpain which markedly activated calpains (Tompa, 2002a). In addition, several other regulatory modes have been identified that can lower calpains requirement for Ca^{2+}, such as: the autolysed-form of conventional calpain subunits, binding of phospholipids, intracellular distribution of conventional canpains and calpastatin, escaping from endogenous inhibition, regulation of protein-protein interactions (protein coactivators were found in animal models) and phosphorylation at several sites of calpains (e.g. extracellular signal–regulated kinase (ERK) phosphorylates m-calpain at serine 50 both *in vitro* and *in vivo*) (Elce *et al*., 1997; Li *et al*., 2004; Huang *et al*., 2004; Glading *et al*., 2000; Franco & Huttenlocher, 2005; Qin *et al*., 2010; Baudry, Chou & Bi, 2013; Chai *et al*., 2014). On one hand, calpain can be phosphorylated and activated by MAPK (mitogen-activated protein kinase) -ERK cascade (Glading *et al*., 2000; Glading *et al*., 2004; Huang *et al*., 2004; Satish *et al*., 2005; Zadran *et al*., 2010; Xu, Bismar & Su, *et al*. 2010; Qin *et al*., 2010; Kovacs *et al*., 2016) and protein kinase Ciota (PKCiota) (Xu & Deng, 2006); however, calpain

activity can also be inhibited by phosphorylation: cyclic-AMP-mediated activation of protein kinase A (PKA) can block EGF-induced activation of calpain-2 through phosphorylation of calpain-2 into an inactive conformation (Shiraha *et al*., 2002; Smith *et al*., 2003; Budnar *et al*., 2006). Du and collogues (2017) reported that PKA phosphorylation of calpain-1 and alkaline phosphatase dephosphorylation of calpain-1 promoted calpain autolysis and activation.

4.2.2 Calpain and cancer cell migration

Chemical inhibition of calpain activity using calpain inhibitors such as ALLN, PD150606 and/or calpeptin significantly reduced cell migration and invasion (Carragher *et al*., 2001) in melanoma (Byzova *et al*., 2000), prostate carcinoma (Mamoune A. *et al*., 2003), fibrosarcoma (Carragher *et al*., 2006), pancreatic cancer (Yoshida *et al*., 2016) and bladder carcinoma cells (Castiglioni & Maier, 2012; Kassis *et al*., 2002). A positive linear correlation between calpain activity and migration was found in rhabdomyosarcoma cells and non-tumour human myoblasts cells (Roumes *et al*., 2010). μ- and m-calpain may perform distinct functions in different cell types and/or under different physiologic conditions (Chen B *et al*., 2013). For example, in keratinocytes, both μ- and m-calpain regulate cell migration, but through different signalling pathways: m-calpain and μ-calpain was activated by EGF receptor and interferon-inducible protein 9 respectively (Glading *et al*., 2004; Satish *et al*., 2005).

To specifically inhibit each calpain isoform, siRNA or shRNA has been applied in many studies. Knockdown of calpain-1 significantly suppressed cell migration (and invasion) in breast cancer (Rodríguez-Fernández *et al*., 2016), hepatoma (Chen B *et al*., 2013), oral squamous cell carcinoma (Ma *et al*., 2017) cells and undifferentiated human primary keratinocytes (Vandenberghe *et al*., 2013). Wound-healing (haptotaxis) assays indicate that calpain-1 knockdown

decreased migration of breast cancer MCF-7 and MDA-MB-231 cells by 50% compared with control cells, whereas, cell migration was not affected by calpain-2 knockdown (Rodríguez-Fernández *et al*., 2016). Inhibition of calpain activity or knockdown of calpain-2 expression using shRNA did not cause inhibition of cell migration but reduced invasion in glioblastoma cells (Jang *et al*., 2010). Knockdown of calpain-2 significantly suppressed cell migration in pancreatic carcinoma (Yoshida *et al*., 2016), hepatoma (Chen B *et al*., 2013), mesenchymal-like colon carcinoma cells (Carragher *et al*., 2006) and caspase-8 expressing lung carcinoma (Barbero S *et al*., 2009) cells. Following the same trend, calpain-4 knockdown was able to decrease cell migration and invasion in melanoma (Wang *et al*., 2017), intrahepatic cholangiocarcinoma (Zhang C *et al*., 2013), nasopharyngeal (Zheng PC *et al*., 2014), hepatocellular carcinoma (Bai *et al*., 2009), ovarian cancer cells (Yang *et al*., 2017) and mouse embryonic fibroblast (Dourdin *et al*., 2001) cells. Moreover, calpain-4 knockdown abolished oncoprotein hepatitis B X-interacting protein HBXIP-mediated cell migration in MCF-7 cells (Li *et al*., 2014). Contradictory, increasing calpain inhibition by introducing rabbit calpastatin into melanoma B16-F10 cells increased cell migration (Raimbourg *et al*., 2013).

In addition to the regulation of calpain expression, upregulated calpain activity has also been found associated with cellular migration. Nicotine induced activation of μ-calpain and m-calpains associated with increased migration and invasion in lung cancer cells (Xu & Deng, 2004; 2006). In colon cancer cells the activation of PI3K/Akt/Rac1/calpain pathway induced Store Operated Calcium Entry amplification which promotes migration of colon cancer cell following the formation of a lipid raft ion channel complex (Guéguinou M *et al*., 2016). On the contrary, neferine induced ER stress and apoptosis while significantly upregulated the expression of certain proteins including calpain-2 and caspase-12 which reduced migration of hepatocellular carcinoma Hep3B cells (Yoon JS *et al*., 2013). Similarly, TMC (potential

chemotherapeutic agents) caused DNA double-strand breaks while significantly upregulated the expression of proteins including caspase-3 and -9, cytochrome c, calpain-1 and -2, but reduced the migration potential (Lai CK *et al*., 2014). The upregulation of calpain might be involved in apoptosis which links with decreased cell migratory ability and contributes to the negative association between calpains and cellular migratory ability.

A variety of explanations and potential regulatory mechanisms have been put forward to explain the positive association between the conventional calpains and migration. In thyroid cancer cells, calpains were found to mediate the secretion of MMP2 (matrix metalloproteinase) and MMP9 which are secreted by cells for the degradation of ECM during invasion and migration (Kalhori & Törnquist, 2015; Jabłońska-Trypuć *et al*., 2016). Hepatitis B virus X protein promotes breast cancer and hepatoma cell migration by upregulating calpain-4 at mRNA and protein levels (Li *et al*., 2014; Zhang X *et al*., 2012). In addition, calpain-4 downregulation was associated with increased E-cadherin expression in melanoma (Wang E *et al*., 2017) and nasopharyngeal carcinoma cells (Zheng PC *et al*., 2014); whilst changes in Snail, vimentin, MMP9, N-cadherin, or β-catenin depended on the cell line studied. In addition, calpains regulate cell migration via their various substrates, that are found involved in cellular processes such as destabilising cell adhesion to the ECM and promoting cell spreading (reviewed in **section 4.2.3**).

The association between calpain and tumour invasion and metastasis has been examined in patient samples via RT-PCR, Western blotting and/or IHC. High calpain-4 at both mRNA and protein levels significantly correlated with the presence of lymphatic metastasis of intrahepatic cholangiocarcinoma and the tumour-node-metastasis (TNM) stage in 33 matched cases (tumours and matched peritumor samples, Zhang C *et al*., 2013). mRNA levels of calpain-4 were higher in breast tumours than those in their adjacent normal tissues (n = 28) (Li *et al*., 2014). IHC studies indicated that, in epithelial ovarian

tumours, high calpain-4 expression was significantly associated with the presence of lymph node metastasis (*P*=0.009) and high FIGO stage (*P*=0.001, n=91) (Yang *et al*., 2017). In gastric tumours (n=174), high expression of calpain-4 was positively associated with vessel invasion, lymph node metastasis, advanced TNM (Tumour Node Metastasis) stage and adverse OS (Peng *et al*., 2016); in hepatocellular carcinomas (n=192), high calpain-4 also significantly correlated with invasive phenotype (Bai *et al*., 2009). Calpastatin, but not calpain-1 or -2, at both mRNA (only exon 3-containing calpastatin) and protein levels were significantly associated with the presence of lymphovascular invasion in breast cancer specimens (mRNA: n=56, *P*=0.014 and protein: n=53, *P*=0.025 respectively) (Storr *et al*., 2011b). In a breast cancer IHC study conducted in our group, low calpastatin expression was found significantly associated with both peri-tumoural ($\chi2$=5.572, d.f.=1, *P*=0.018) and intra-tumoural lymphovascular invasion ($\chi2$=4.003, d.f.=1, *P*=0.045) (Storr *et al*., 2011b). A separate study (n=1371) also pointed out the association between high calpastatin expression and the absence of intra-tumoural lymphovascular invasion ($\chi2$=6.1; d.f.=1; *P*=0.014); moreover, high calpain-2 expression has been found associated with peri-tumoural lymphovascular invasion ($\chi2$=5.2; d.f.=1; *P*=0.022) (Storr *et al*., 2012b).

4.2.3 Calpain substrates and cancer cell migration

The end result of proteolytic processing by calpain on its substrates is to modulate rather than degrade (Perrin & Huttenlocher, 2002). It is via such processing that calpains regulate cell migration by various means such as destabilising cell adhesion to the ECM, promoting rear detachment, cell spreading, membrane protrusion, chemotaxis, and adhesion complex formation and turnover (Franco & Huttenlocher, 2005). Friedl P et al., (2012) comprehensively reviewed different migration modes including single-cell migration, multicellular streaming and collective cell migration, with single-cell migration being subdivided into amoeboid and mesenchymal

migration (Sun & Zaman, 2017). Mesenchymal mode was more likely to be dependent on β1-integrin and MMP whilst amoeboid mode was more dependent on actin and myosin (Storr et al., 2011a). Calpains were found acting upstream of MMPs to contribute to cell migration and invasion (Kalhori & Törnquist, 2015; Jabłońska-Trypuć et al., 2016; Storr et al., 2011a); moreover, integrins are calpain substrates whilst neither actin nor myosin is. As further discussed in section 4.5, calpain isoforms, especially calpain-2, appear to be more involved in the mesenchymal mode than the amoeboid mode. Calpains are, as reviewed by Franco & Huttenlocher (2005), associated with cell motility with numerous proteins being identified as calpain substrates related to focal adhesions and migration *in vitro*: α-actinin, β-integrins, β-catenin, ezrin, cadherins, cortactin, EGFR, focal adhesion kinase (FAK), filamin, paxillin, PTP-1B, RhoA, spectrin, Src, talin, vinculin. However, research in this area lacks information for *in vivo* substrates of calpain related to focal adhesions and detachment (Perrin & Huttenlocher, 2002).

The focal adhesion complex contains an interacting multi-protein matrix linking ECM with the actin cytoskeleton inside the cell and controlling the network of signalling cascades underlying the dynamic process of cell migration (Barbero *et al*., 2009; Li *et al*., 2016), which requires temporal and spatial regulation of integrin activation and focal adhesion turnover (Huang *et al*., 2009; Perrin & Huttenlocher, 2002). The multi-protein matrix consists of (1) catalytic proteins and (2) adaptor and actin-binding proteins (e.g. talin, paxillin, α-actinin, filamin, vinculin and tensin) (Wozniak *et al*., 2004; Wu, 2007). Catalytic proteins include kinases (e.g., nonreceptor protein tyrosine kinases (e.g. Src, FAK)), cytosolic phosphatases (e.g., protein tyrosine phosphatase‐PEST), proteases (e.g., calpain), and other enzymes (e.g., phospholipase C‐g) (Barbero *et al*., 2009; Wu, 2007).

Calpain promotes focal adhesion assembly/ disassembly by the cleavage of focal adhesion proteins (Barbero *et al*., 2009) which may change the

cytoskeletal anchoring or remodel the adhesive structures (e.g. changing the composition of adhesion) thus stimulating cancer cell migration, invasion, and metastasis formation by promoting adhesion turnover, rear detachment and destabilising the structure of focal contact (Perrin & Huttenlocher, 2002; Xu, Bismar & Su *et al*. 2010; Park *et al*., 2012). To explain how the calpain system regulates both the assembly and disassembly of focal adhesion in the same cell, Glading et al. (2002) proposed that the regulation might be mediated via spatiotemporal heterogeneous distribution of calpain substrates and/or the spatiotemporal heterogeneous distribution and activation of calpain isoforms. Thus, it is more likely that calpain isoforms generally regulate adhesion turnover, rather than specifically regulating assemble and disassemble adhesions. The inhibition of calpain activity was found to attenuate the production of calpain cleaved ezrin, FAK, filamin A and vinculin in metastasising mouse adenocarcinoma TS/A cells (Lee & Shyur, 2012). Studies using calpain inhibitors, or calpain-deficient cells, support that, as a regulator of focal-adhesion turnover, calpain is necessary for cell migration (Carragher *et al*., 2003).

FAK is the most studied calpain substrate among the catalytic proteins. Calpain has been found able to cleave FAK (Carragher *et al*., 2003; Huang *et al*., 2004) which caused inhibition of FAK activity, FAK signalling termination, cytoskeletal protein degradation, focal adhesion disassembly and an increase of focal adhesion turnover during transformation and migration (Carragher *et al*., 2003; Carragher *et al*., 2001; Xu, Bismar & Su *et al*. 2010; Chan *et al*., 2010; Franco *et al*., 2004; Vandenberghe *et al*., 2013). In addition, FAK was required for recruiting both m-calpain and p42 ERK/MAPK (mitogen-activated protein kinase) to peripheral adhesion sites, maximising calpain activity (Carragher *et al*., 2003).

Talin is a main molecule of focal adhesion complexes that links intracellular networks (actin cytoskeleton) with the ECM via transmembrane

heterodimer integrins (Haining AW *et al*., 2016; Dourdin N *et al*., 2001). Talin is essential in integrin activation (Huang *et al*., 2009) and focal adhesion turnover (Barbero *et al*., 2009). Calpain-mediated talin cleavage (Wu J *et al*., 2015; Rodríguez-Fernández *et al*., 2016; Dourdin N *et al*., 2001; Chang SJ *et al*., 2017) plays a central role in focal adhesion assembly (Huang *et al*., 2009) and disassembly (Li *et al*., 2016) in motile cells (Chan *et al*., 2010; Franco *et al*., 2004). The calpain-cleaved talin was able to accelerate focal adhesion disassembly, inhibited cell adhesion and increased cell migration (Li *et al*., 2016; Barbero *et al*., 2009).

Other adaptor and actin-binding proteins, in addition to talin, have been found to be cleaved by calpain and be involved in cell migration. Extracellular Ca^{2+} induced the up-regulated expression and cleavage of filamin A and promoted the migration via a calpain dependent pathway in highly metastatic prostate cancer cells (androgen receptor –deficient) (Huang *et al*., 2017). Paxillin is an adaptor protein localising to focal adhesions. Phosphorylated paxillin has been shown to promote migration (López-Colomé *et al*., 2017) whereas the calpain-generated proteolytic fragments (e.g. paxillin delta) of paxillin were found to negatively regulate focal adhesion turnover and migration (Cortesio *et al*., 2011; Tumbarello *et al*., 2005).

4.2.4 Aims

Calpain-1 was one of several key regulators of cell adhesion and migration that had a changed expression in a quantitative proteomics study associated with loss of BRCA1 function in epithelial ovarian tumours (Gau *et al*., 2015). Calpain-1 has been shown to proteolyse RhoA which can inhibit cell spreading (Kulkarni *et al*., 2002) and regulate actin cytoskeleton and cell migration (Wells *et al*., 2005). Although calpain-1 expression was not associated with patient survival, the IHC study presented in **section 3.4.2** indicated that high calpain-1 expression significantly associated with high

tumour stage. Since stage reflects how ovarian cancer has spread, calpain-1 may be involved with tumour migration and invasion *in vitro*. As indicated in **section 4.2.2**, the calpain system appears closely associated with cancer cell migration in *in vitro* studies. Calpain inhibition via pharmacological inhibition or genetic knockdown associated with increased stability of cell-substrate adhesion and reduced cell migration (Dourdin *et al*., 2001; Huttenlocher *et al*., 1997). To our knowledge, the effect of calpain on ovarian cancer cell migration remains poorly understood which will be addressed in this chapter. The current chapter sought to address the following hypotheses:

1. Regulation of calpain activity (using A23187 or calpeptin) influences ovarian cell migration.
2. A novel calpain inhibitor compound-1 inhibits cell migration via inhibiting calpain activity.

The study aimed to:

1. Assess the effect of A23187 in the regulation of calpain activity in ovarian cancer cell lines.
2. Study the effect of calpeptin and A23187 in the regulation of ovarian cell haptotactic migration.
3. Study the regulatory effect of a novel calpain inhibitor, compound-1, using a recommended working concentration of 5μM, on calpain activity and cell migration.

4.3. Materials and methods

4.3.1 Chemicals and drug preparation

t-BOC-Leu-Met-CMAC (Invitrogen, USA) stock solution was prepared in DMSO (Sigma, UK) at a concentration of 2 mM and stored as aliquots at -20°C. Calpeptin (Merck Millipore Corporation, Germany) was dissolved in DMSO at 100 mM and stored as aliquots at -20°C. The calcium ionophore A23187 (Sigma, UK) was stored in 10 mM stock solution in DMSO at -20°C. Compound-1 ($(C_6H_4BrCHCOOHCSe-)_2$, a kind gift from Prof. Rudolf K. Allemann (Cardiff University)) was stored at 50mM stock in DMSO. The recommended working concentration for the compound was 5µM (unpublished results provided by Prof Dr Rudolf K. Allemann). Stock solutions were sub-diluted in complete culture medium to achieve working concentrations for individual treatments. DMSO was added to the negative control groups at the same volume equivalent to that in the drug-treated group (the final percentage of DMSO was < 0.1‰ v/v).

4.3.2 Cell proliferation in response to mitomycin-C, cytochalasin D and A23187

Growth curves were used to determine the toxicity of mitomycin-C and cytochalasin D in SKOV3, PEO1 and PEO4 cells. The toxicity of A23187 was determined in SKOV3 cells. Cell proliferation was assessed after 24- and/ or 48-hour mitomycin-C treatment using the same protocol as mentioned in **section 2.3.5**. As for cytochalasin D and A23187, cells were treated with the drug for 24 hours and then incubated with fresh culture medium followed by cell counting every 24 hours. Data represent the average of total cell numbers ± SD of at least two independent experiments, each performed in triplicate.

4.3.3 Calpain activity assay

For the study of calpain inhibition, cells were treated with or without calpain inhibitor (5µM compound-1 or 50µM calpeptin) for 1 hour or 24 hours,

after which cells were collected and calpain activity was assessed as described in **section 2.3.6**. In order to study the calpain activation, cells were treated with or without 0.5µM A23187 for 1 hour or 24 hours. Calpain activity was either assessed immediately after treatment, termed "immediate measurement", or incubated with fresh culture medium for another 24 hours or 48 hours before measurement, termed the "prolonged measurement". All measurements were assessed in duplicate and data is represented as the average ± SD of at least two independent experiments.

In addition, calpain-cleaved t-BOC substrate fluorescence, in control and drug treated cells, was monitored visually via confocal microscopy. Coverslips (Scientific Laboratory Supplies Coverslips No 1 22x26mm, MIC3220) were sterilised by autoclaving. SKOV3 cells in growth medium were seeded onto coverslips located in 6-well dishes (1 coverslip/well) and then returned to the incubator for 24 hours to allow adequate adhesion. In each well, $1X10^5$ cells/ml, 2ml/well were seeded. Cells were then treated with or without calpain inhibitor (5µM compound-1 or 50µM calpeptin) for one hour. Each condition was performed in duplicate. After treatment, medium with or with calpain inhibitor was removed and cells were washed twice with phosphate buffered saline (PBS). As each condition was performed in duplicate, fresh medium with t-BOC (20µM) was added in one well whilst fresh medium with the same volume of DMSO was added to the other well for 40 minutes. From this step forward samples were protected from light. Cells were washed with PBS 3 times, each time for 5 minutes. Paraformaldehyde/PBS (4%, 1ml/well) was used to fix the cells for 10 minutes at room temperature after which cells were washed again with PBS 3 times. The coverslips were carefully removed from the wells with a pair of tweezers and tilted to remove excess PBS. The coverslips were mounted with 1:1 glycerol/PBS (50% glycerol in PBS) with the cells facing towards the microscope slide and slides were sealed with clear nail polish. Prepared slides were visualised using a Leica SP8 Confocal Microscope using LEICA LAS-AF software. Slides were subsequently stored in a slide box

at 4 °C for later examination.

4.3.4 Haptotaxis (scratch wound) migration assay

The seeding density was initially optimised for the migration assay. To form a confluent monolayer, cells with different seeding densities were plated in 12-well plates and incubated for 24 hours. The seeding densities of SKOV3 cells used for optimisation were 1×10^5/ml, 1.5×10^5/ml, 2×10^5/ml and 2.5×10^5/ml. For PEO1 cells, 5×10^5/ml, 6×10^5/ml and 7×10^5/ml cells were plated for the optimisation. PEO4 cells were plated at 7×10^5/ml, 8×10^5/ml, 9×10^5/ml, 10×10^5/ml and 11×10^5/ml and the incubation time extend to 96 hours for cells to reach to > 90% confluence.

Different cell lines were seeded into a 12-well tissue culture plate in normal growth medium. When cells reached a suitable level of confluence (time intervals are listed in **Table 4.1**), the normal growth media was refreshed. Cells were pre-incubated with calcium ionophore A23187 (Sigma-Aldrich, UK), calpeptin (Calbiochem®, Merck Millipore, UK), cytochalasin D (Sigma-Aldrich, UK) or compound-1 and incubated at 37°C (concentrations and incubation times are listed in **Table 4.1**). After treatment, a 200 µl pipette tip was used at a 45° or 90° angle (**Table 4.1**) to scratch a wound on the confluent cell monolayers through the centre of the well with one flowing movement to give a clean straight edge. The monolayer was then rinsed twice with 500 µL of PBS to remove cellular debris and the released stimuli (e.g. extracellular signal-regulated kinase, growth factors etc. Dieckgraefe *et al*., 1997; Xu *et al*., 2004; Yin *et al*. 2007) caused by cell rupture. Fresh medium with mitomycin-C (Sigma-Aldrich, UK) (concentration listed in **Table 4.1**) was added to inhibit cellular proliferation for the duration of the assay. In the experiments that studied the effect of calpeptin on cell migration, each experiment had three groups: one vehicle control group, two calpeptin-treated groups. Both calpeptin-treated groups were incubated with

50μM calpeptin for 24 hours before wounding the monolayer and monitoring the wound closure; during the wound closure, one group was incubated with mitomycin-C alone whilst 50μM calpeptin was added together with mitomycin-C in the other group (pre-treatment time and duration of experiment were picked based on the calpain activity tested in **section 2.4.6** to ensure its inhibition during each experiment). Images were captured under a 20× objective at 0, 2, 4, 6, 24 and 48 hours after the scratch to monitor the wound closure. Two areas were pictured and measured in each well. The wound area was measured using Image J 1.43u software (National Institute Health, USA) and the healed area after a defined period of time was represented as the percentage of the initial wound area. Each condition was performed in triplicate and repeated at least three times. Data represent the average ± SD of three independent experiments.

Table 4.1 Experimental conditions for migration assay

Cell lines	SKOV3	PEO1	PEO4
Seeding density	2×10^5/ml	7×10^5/ml	1×10^6/ml
Incubation time (the interval between plating and drug treatment)	24 hours	24 hours	96 hours (change media in between)
Create wound	90°	45°	45°
Mitomycin C (cellular proliferation inhibitor)	30μM (10μg/ml)	1μM	10μM
Cytochalasin D (actin-disrupting agent as positive control)	0.1μM (24 hours)	0.01μM (24 hours)	0.1μM (24 hours)
Calpeptin (calpain inhibtor)	50 μM (24 hours)	50 μM (24 hours)	50 μM (24 hours)
A23187 (calcimycin, mobile ion-carrier that increase intracellular Ca^{2+} levels)	0.5 μM (1 hour or 24 hours)	0.5 μM (1 hour or 24 hours)	0.5 μM (1 hour or 24 hours)

4.3.5 Statistics

Results from *in vitro* experiments were represented as average ± SD (inter-experiment SD) of at least two independent experiments with each performed in duplicate or triplicate. The SDs were calculated using the formula mentioned in **section 2.3**. The Student t-test (comparing two groups)

and ANOVA one-way test (comparing more than groups) were chosen to evaluate whether data differ significantly between the control and the drug treatment groups when at least three independent experiments were conducted.

4.4. Results

4.4.1 Calpain activity in response to calpeptin and A23187

Calpeptin and A23187 were used to alter calpain activity (**Table S2.1**). A23187 concentration was optimised in SKOV3 cells (**Figure 4.10**). The optimisation of calpeptin concentration was discussed in **section 2.4.5**. The highest concentrations of calpeptin and A23187 that did not harm the integrity of cell monolayers were used in the current study as listed in **Table 4.1**. It is known that the free intracellular calcium concentrations range from 100 nM to 1 µM and extracellular free calcium was approximately 1.2mM (Clapham, 2007; Shen H *et al*., 2012). The calcium concentrations in the serum-free medium were listed in **section 2.3.1**. As mentioned in **section 4.3.3**, cells were treated with A23187 (for either 1 hour or 24 hours), after which cells were collected for "immediate measurement" or A23187 was removed and cells were incubated for another 24 hours or 48 hours with fresh culture medium before the "prolonged measurement".

The inherent variation of the t-BOC assay was observed again in the current section which fell into the same range as explained in **section 2.4.6.** In SKOV3 cells, the normalised average fluorescent reading of the controls at the 10th cycle various from 10000 to 14000. In PEO1 and PEO4 cells, the variation of the normalised fluorescent readings of the controls were from 10000-12000 and 8000-10000 respectively at the 10th cycle. Limited by this variation, it is difficult to make comparisons across experiments and draw any firm conclusion from this experiment alone.

In SKOV3 cells, the "immediate measurement" of calpain activity showed that the 1-hour and the 24-hour A23187 treatment increased <10% and <30% of the basal activity respectively. The "prolonged measurement" of cells that received the same treatment showed an approximately 50% increase in calpain activity (**Figure 4.1**). The increase of calpain activity in cells that were continuously exposed under A23187 for 24 hours was less than the increase in cells that were exposed under A23187 for 1 hour followed by another 24 hours incubation with fresh medium (**Figure 4.1**). Measuring the cytosolic free calcium concentration may provide relative information for the interpretation of the upregulated calpain activity after the removal of A23187 treatment which currently cannot be explained.

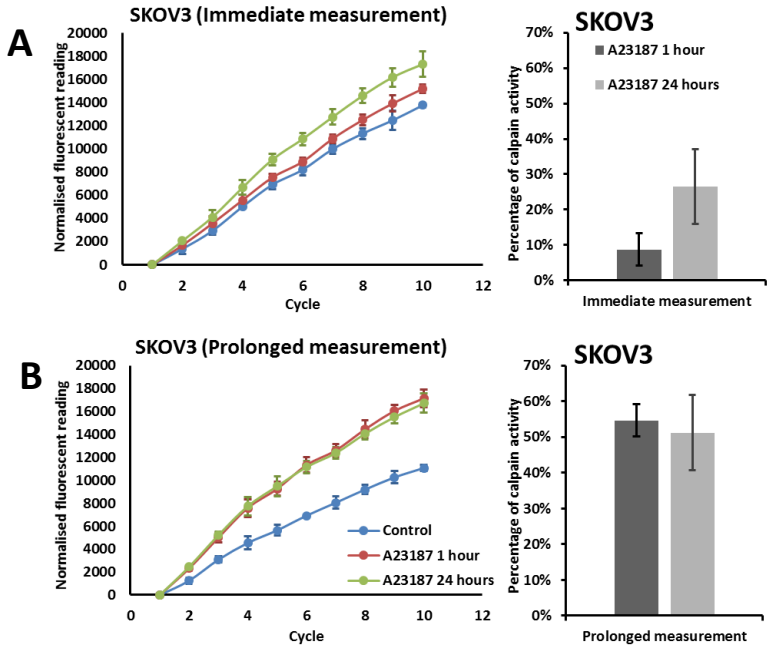


Figure 4.1 The activation of A23187 (0.5µM) on calpain activity in SKOV3 cells. SKOV3 cells were treated with 0.5µM A23187 for 1 hour or 24 hours, and then measured (A) immediate after treatment (i.e. "immediate measurement") or (B) 24 hours after treatment (i.e. "prolonged measurement"). The fluorescence readings were plotted against the cycle number in line graphs. The percentage increase of fluorescence readings at cycle 10 in each treated cells from that of the control cells was presented in the bar charts. Data represent the average ± SD of two independent experiments with each experiment performed in duplicate.

In PEO1 cells, no increase in calpain activity levels was detected immediately after cells being exposed to A23187 for 1 hour or 24 hours. A 30%

increase of calpain activity was detected 24 hours after the 24-hour A23187 treatment, but the upregulation of calpain activity did not last till 48 hours after treatment (**Figure 4.2**). Although the prolonged treatment induced a higher level of calpain activation at the 24-hour time point, the difference was not of statistical significance.

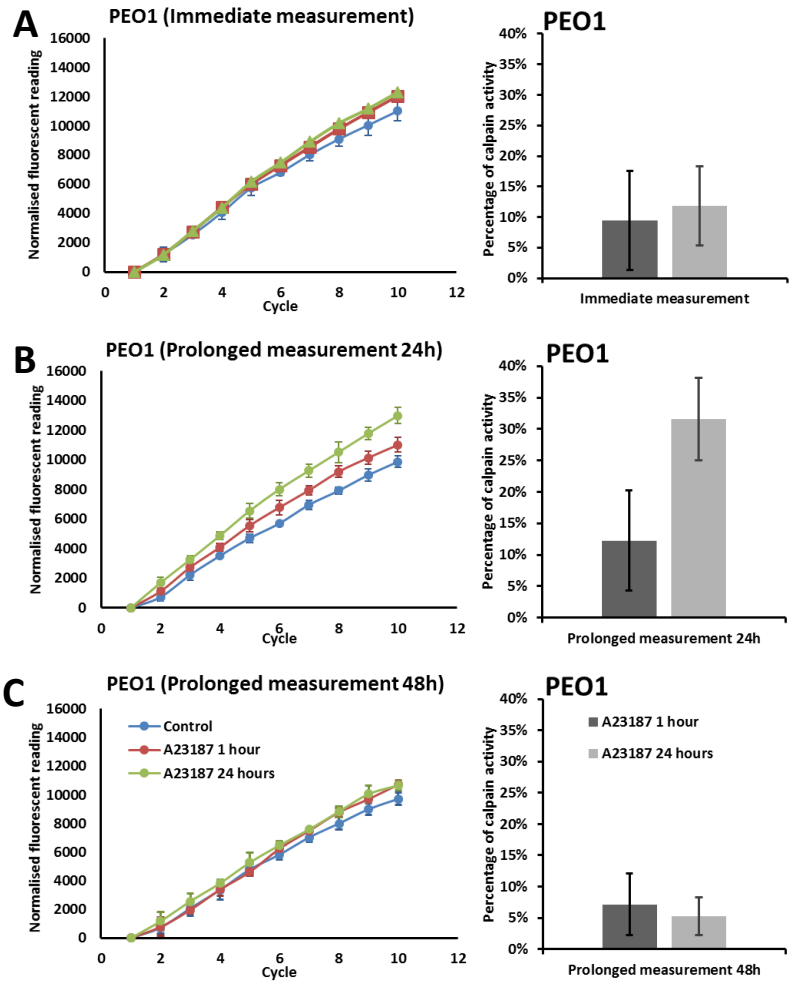


Figure 4.2 The activation of A23187 (0.5µM) on calpain activity in PEO1 cells. PEO1 cells were treated with 0.5µM A23187 for 1 hour or 24 hours and then either (A) measured immediately after treatment (i.e. "immediate measurement") or incubate the cells with fresh medium for another (B) 24 hours or (C) 48 hours before the measurement (i.e. "prolonged measurement"). The fluorescence readings were plotted against the cycle number in line graphs. The percentage increase of fluorescence readings at cycle 10 in each treated cells from that of the control cells was presented in the bar charts. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. Student T-test was used to compare the A23187 treated group with the control group at cycle ten.

The highest upregulation of calpain activity was observed 24 hours after the removal of A23187 in PEO4 cells as in PEO1 cells. In PEO4 cells 1-hour

A23187 treatment induced 15 to 20% average increases when compare to the basal activity, whist 24-hour treatment showed less increase, but no significant increase in calpain activity levels was observed at any of the time-points (**Figure 4.3**).

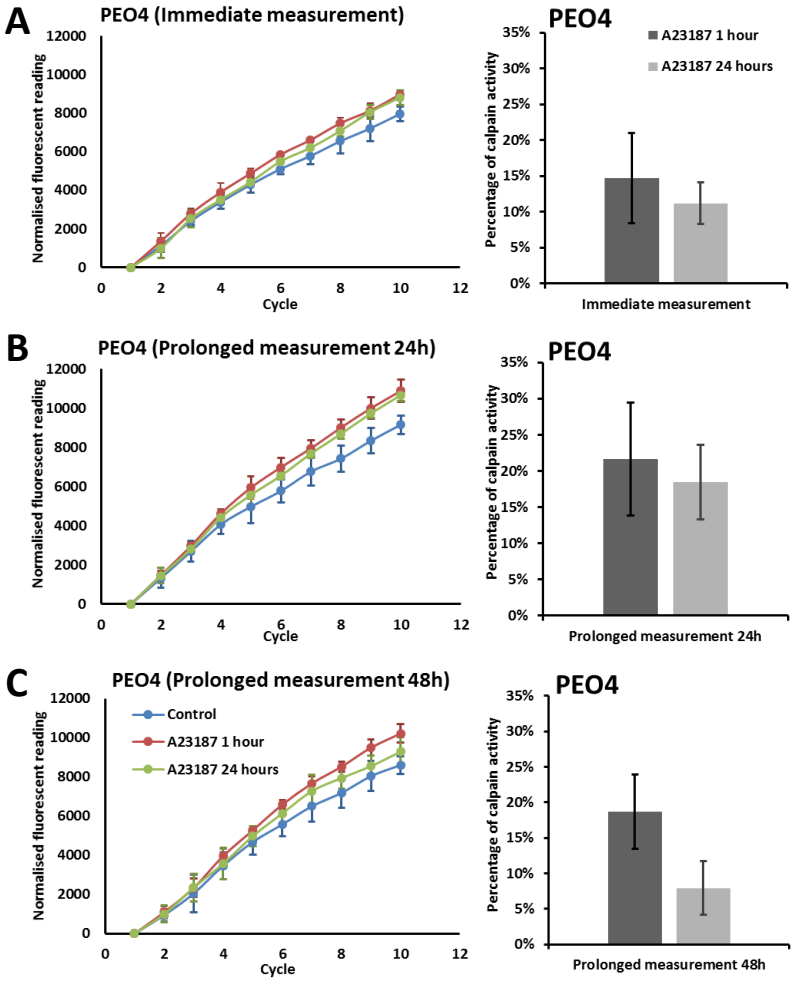


Figure 4.3 The activation of A23187 (0.5µM) on calpain activity in PEO4 cells. PEO4 cells were treated with 0.5µM A23187 for 1 hour or 24 hours and then either (A) measured immediately after treatment (i.e. "immediate measurement") or incubate the cells with fresh medium for another (B) 24 hours or (C) 48 hours before the measurement (i.e. "prolonged measurement"). The fluorescence readings were plotted against the cycle number in line graphs. The percentage increase of fluorescence readings at cycle 10 in each treated cells from that of the control cells was presented in the bar charts.. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. Student T-test was used to compare the A23187 treated group with control group at cycle ten.

As the widely used calpain inhibitor calpeptin caused no statistically significant inhibitory effect on ovarian cancer cells, a novel inhibitor 'compound-1' was introduced into the current project. The effects of

compound-1 and calpeptin on calpain activity were assessed in SKOV3 cells (**Figure 4.4**). The inherent variability in the t-BOC assay makes interpretation very difficult: cells showed decreased calpain activity after calpeptin treatment as expected, however, increased calpain activity was observed following compound-1 treatment.

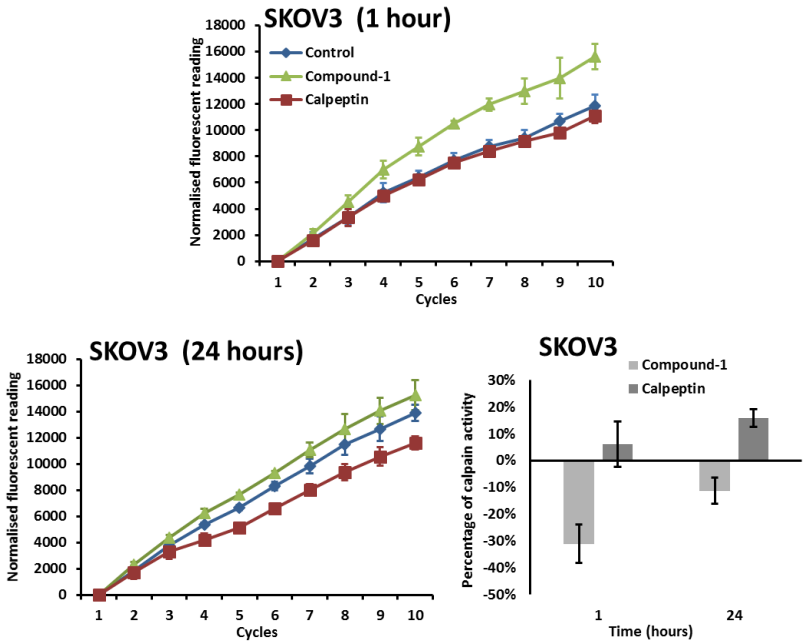


Figure 4.4 The inhibitory effect of calpain inhibitor on calpain activity in SKOV3 cells. SKOV3 cells were treated with or without calpain inhibitor (50µM calpeptin or 5µM compound-1) for 1 hour or 24 hours. The fluorescence readings were plotted against the cycle number in line graphs. The decrease of fluorescence readings at cycle 10 in treated cells were presented as the percentage of the control in bar chart. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. One-way ANOVA was used to compare the experimental groups with control group at cycle ten.

In an attempt to get an interpretation of the effect of compound-1 on calpain activity, confocal fluorescence microscopy was used to visualise the compound-1 treated SKOV3 cells in comparison with the calpeptin-treated cells and vehicle control cells (**Figure 4.13**). Results from this experiment cannot be quantified but they showed that the compound-1-treated cells had fluorescence signal without the adding of t-BOC. It is therefore difficult to draw any firm conclusion on the effect of compound-1 on calpain activity regulation, given that the variation of the current calpain activity assay and the background fluorescence generated by compound-1 fall into the

wavelength for the detection of cleaved t-BOC in the current assay. More details will be discussed in **section 4.5.**

4.4.2 Migration in response to calpain inhibitors and A23187

As calpain activity has been shown to be associated with cancer cell migration, albeit, in other cancer types (**section 4.2.2** and **4.2.3**), experiments were designed to assess if altering calpain activity could affect ovarian cancer cell migration, using the calcium ionophore A23187 as an activator and calpain inhibitors, calpeptin and compound-1. Calcium ionophores such as calcimycin (A23187) and ionomycin are known to raise intracellular Ca^{2+} levels which then activate ubiquitous calpains (Gil-Parrado *et al*., 2002; Molinari *et al*., 1995; Pelley RP *et al*., 2006; Sivanandam *et al*., 2011). The A23187 induces Ca^{2+} increase via promoting the release of intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+} ions (Mtango *et al*., 2008).

The t-BOC calpain activity assay showed that 1) calpeptin was able to significantly inhibit the calpain activity in PEO1 and PEO4 cells (**section 2.4.6**) whilst the inhibitory effect on SKOV3 cells was not of statistical significance; 2) A23187-treated cells had increased calpain activity which was, however, not significantly higher than the basal activity; 3) compound-1 seems to increase calpain activity. Limited by the variation of the t-BOC assay, it is difficult to make comparisons across experiments and draw any firm conclusion based on the results from this assay alone. Thus, the assessment of the effects of A23187, calpeptin and compound-1 on cell migration was continued. Working concentrations of A23187 were optimised in SKOV3 cells (**Figure 4.10**) and applied to PEO1 and PEO4 cells.

The antitumour antibiotic mitomycin-C is known to cause DNA cross-linking and growth arrest (Barlogie & Drewinko, 1980; An *et al*., 2015), thus has been used in scratch-wound migration assay to inhibit cell proliferation: cells were commonly exposed to 5 or 10 µg/ml mitomycin-C for

1 to 2 hours before creating the wound after which cells were incubated in serum free medium during wound closure (Wu, Yang & Zheng *et al*., 2016; Shikatani *et al*., 2012; Chow *et al*. 2011). In the current study, cells were incubated with mitomycin-C during the whole process of wound closure. The working concentration of mitomycin-C in each cell line was optimised over a 24-hour period in SKOV3 whilst over a 48-hour period for PEO1 and PEO4 cell lines (**Figure 4.12**). The Inhibition of SKOV3, PEO1 and PEO4 cell proliferation occurred at 30μM, 1μM and 10μM of mitomycin-C treatment respectively, at which concentration the total cell numbers during the whole incubation times were still close to the initial cell numbers. Hence these concentrations were applied in migration assay in each cell lines (**Table 4.1**). Cytochalasin D is known to inhibit endocytic pathways through its inhibition of actin polymerisation (Escrevente *et al*. 2011). As an actin-disrupting agent, cytochalasin D induces the polymerisation and depolymerisation of actin filaments (Jarrett CR *et al*., 2001; Shoji *et al*., 2012) and its IC90 value (96-hour treatment) of SKOV3 cells was 0.08μM (Trendowski M *et al*., 2015). Here, cytochalasin D was used as a positive control; the highest concentration that did not cause a significant inhibition of cell proliferation at both 24-hour and 48-hour time points was 0.1μM in SKOV3 and PEO4 cells and 0.01μM in PEO1 cells (**Figure 4.11**) which was utilised in cell migration assays (**Table 4.1**).

Representative images are shown in **Figure 4.5A**, presenting the inhibitory effect of cytochalasin D on SKOV3 cell migration as determined by scratch wound assay. Quantitative analysis of the wound closure indicated that at all four time points, the inhibition effect of cytochalasin D was significant (P<0.001) **Figure 4.5B.** Cytochalasin D-treated SKOV3 cells exhibited approximately 20% reduction in average migration when compared to those of the untreated cells 22 hours after wounding **Figure 4.5B**. As a positive control, a similar extent of inhibition was observed in the study of the effect of calpeptin and A23187 on cell migration: data analysis showed a significant

decrease in the percentage of wound closure in the cytochalasin D-treated group as compared to the controls 22 hours after scratch **Figure 4.5C, D**. Calpeptin caused a decrease in wound healing in SKOV-3 cells when compared to the untreated cells, moreover, the inhibitory effect on wound healing increased with the increase of calpeptin treatment period **Figure 4.5C**, but the inhibitory effect was not significant, even in the group with prolonged calpeptin treatment after wounding. Significant decreases were, however, observed in the percentage of wound closure at 6 and 22 hours (P<0.01) in groups with 1 hour or 24 hours 0.5µM A23187 treatment. To be precise, data indicate that the increase in calpain activation leads to a significantly 'decreased' percentage closure in both 1 hour (P=0.0057) and 24-hour (P=0.0076) A23187-treated groups at 22 hours (**Figure 4.5 D**).

Scratch-wound migration assay was performed over a 48-hour interval in PEO1 and PEO4 cells. The current study showed that it took longer time for PEO1 and PEO4 cells to attach to the plastic surfaces and form monolayers than the other cell lines used in the current study. SKOV3 cells have a greater migratory ability comparing to PEO1/PEO4 cells; SKOV3 cells took 22 hours to reach 70-80% wound closure, whilst the wound closure of PEO1 and PEO4 only reached to 30% and 40% respectively 72 hours after creating the wounds. In scratch wound migration assay, the epithelia cells of the wounded monolayer tended to migrate onto the wound bed and help to restore the epithelial barrier (Grada *et al*., 2017). Thus the low migration rate in the scratch wound migration assay might reflect the loss of epithelial cell features and the loss of ability to maintain the integrity of the epithelial barrier. Although calpeptin was able to significantly inhibit calpain activity in both PEO1 and PEO4 cells (**section 2.4.6**), their migratory ability was not inhibited by calpeptin. PEO1 and PEO4 cells treated with calpeptin, A23187 or cytochalasin D closed the wound at a rate similar to the cells in the vehicle control group (**Figure 4.6** & **Figure 4.7**).

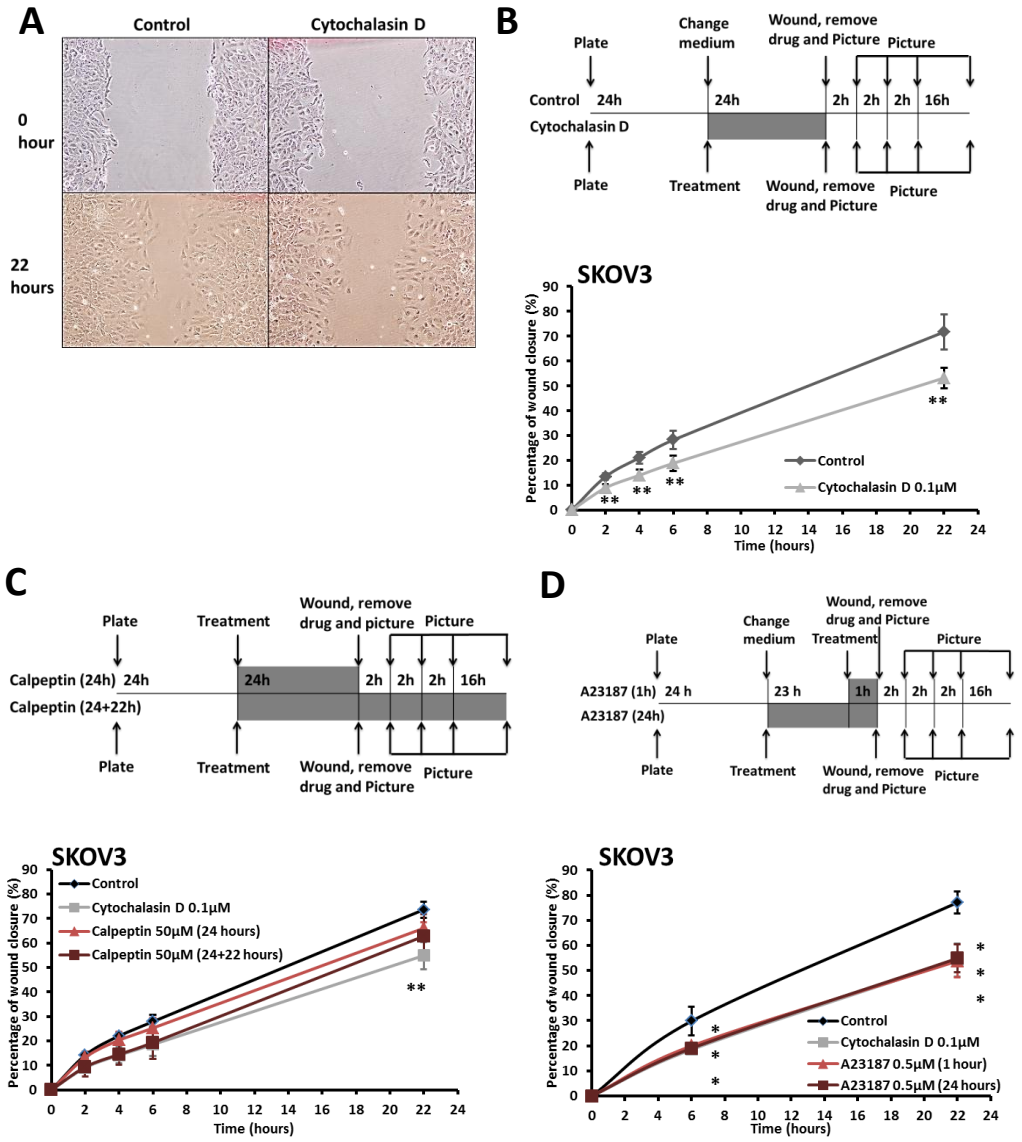


Figure 4.5 Effects of A23187 and calpeptin on cell migration of SKOV3 cells detected by scratch-wound assay. SKOV3 cells were treated with or without 0.01μM cytochalasin D for 24 hours: (A) representative images at indicated time points are shown (magnification, ×200); (B) cells were treated and wound closure was quantified at the indicated time points. (C) SKOV-3 cells were treated with or without calpeptin for 24 hours. Following a scratch wound, the wound closure was recorded over a 22-hour time period with or without calpeptin. (D) SKOV-3 cells were treated with or without A23187 for 1 hour or 24 hours. Following a scratch wound, the wound closure was recorded over a 22-hour time period. Time scale for treatment and measurement is listed above each figure (grey bars over the hours indicate the treatment period). After the creation of the wounds, mitomycin-C was used for the inhibition of cell proliferation in all migration assays. Data represent the average ± SD of three independent experiments with each experiment performed in triplicate. Student T-test and one-way ANOVA was used to compare the percentage of wound closure among control group and experimental group (*P<0.01, ** P<0.001 vs control).

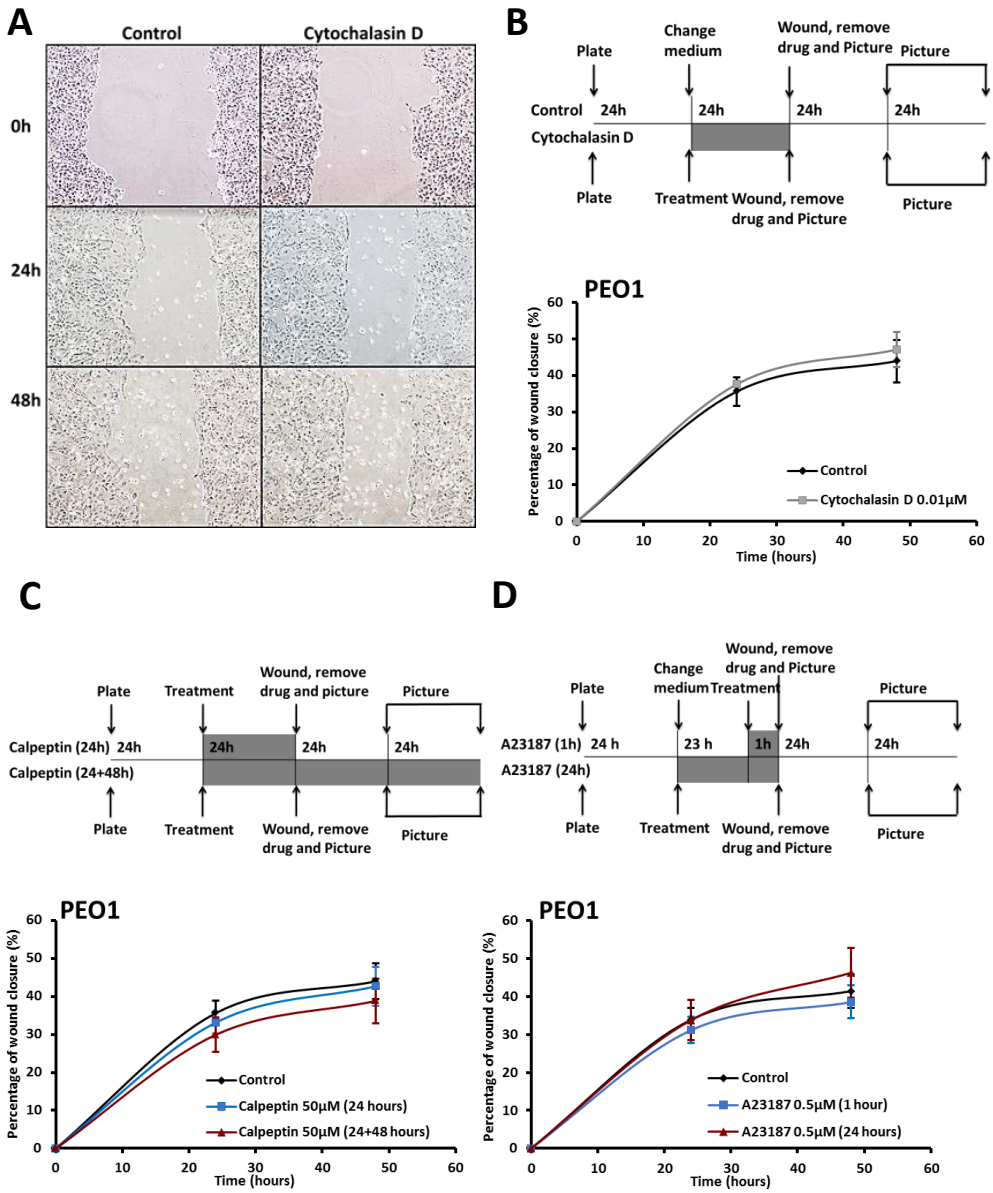


Figure 4.6 Effects of calpeptin and A23187 on cell migration of PEO1 cells detected by scratch-wound assay. Confluent monolayers of PEO1 cells were treated with or without 0.01μM cytochalasin D for 24 hours: (A) representative images at indicated time points are shown (magnification, ×200); (B) cells were treated and wound closure was quantified at the indicated time points. (C) PEO1 cells were treated with or without calpeptin for 24 hours. Following a scratch wound, the wound closure was recorded over a 48-hour time period with or without calpeptin. (D) PEO1 cells were treated with or without A23187 for 1 hour or 24 hours. Time scale for treatment and measurement is listed above each figure (grey bars over the hours indicate the treatment period). After the creation of the wounds, mitomycin-C was used for the inhibition of cell proliferation in all migration assays. Data represent the average ± SD of three independent experiments with each experiment performed in triplicate. Student T-test and one-way ANOVA was used to compare the percentage of wound closure among control group and experimental group (*P<0.01, ** P<0.001 vs control).

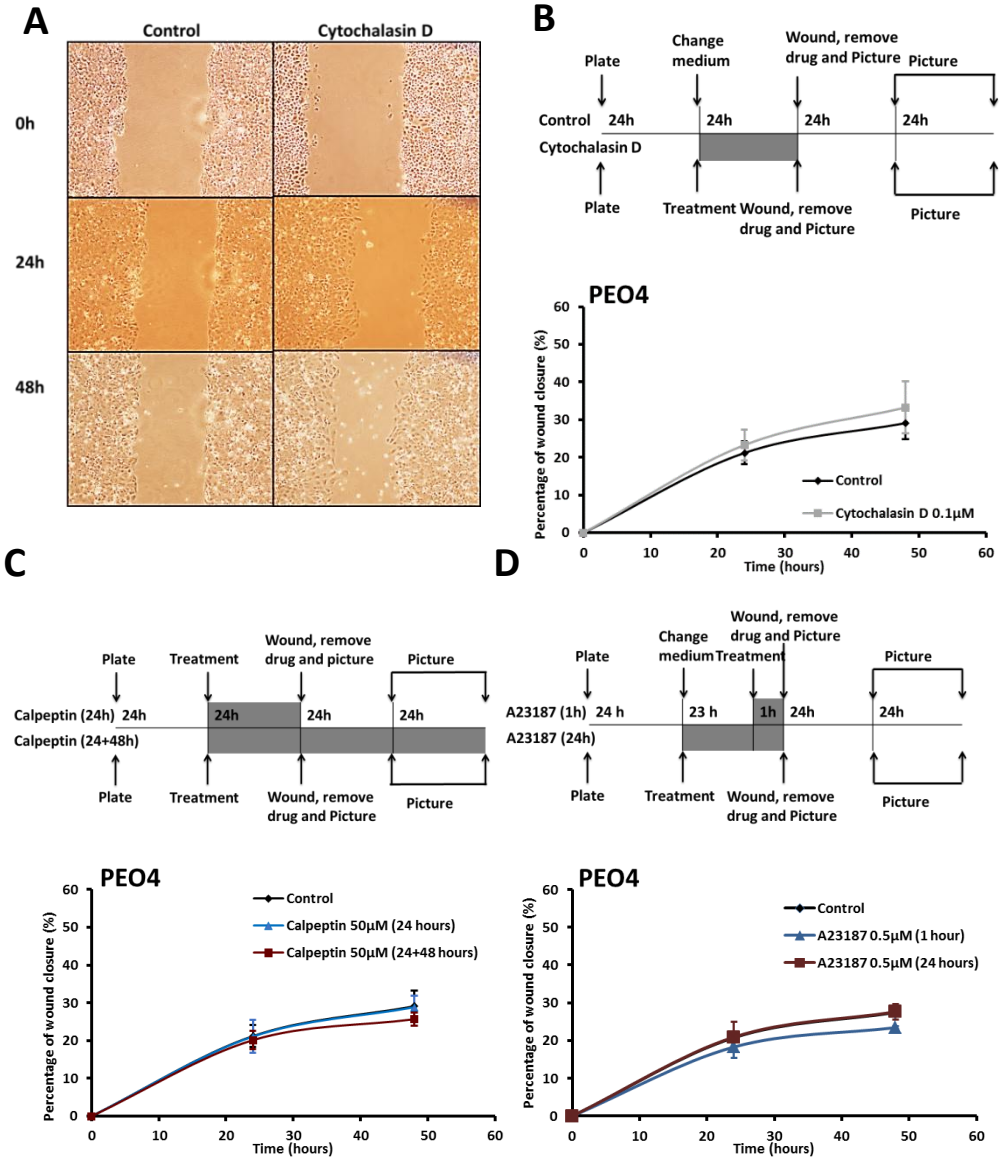


Figure 4.7 Effects of calpeptin and A23187 on cell migration of PEO4 cells detected by scratch-wound assay. Confluent monolayers of PEO4 cells were treated with or without 0.1μM cytochalasin D for 24 hours: (A) representative images at indicated time points are shown (magnification, ×200); (B) cells were treated and wound closure was quantified at the indicated time points. (C) PEO4 cells were treated with or without calpeptin for 24 hours. Following a scratch wound, the wound closure was recorded over a 48-hour time period with or without calpeptin. (D) PEO4 cells were treated with or without A23187 for 1 hour or 24 hours. Time scale for treatment and measurement is listed above each figure (grey bars over the hours indicate the treatment period). After the creation of the wounds, mitomycin-C was used for the inhibition of cell proliferation in all migration assays. Data represent the average ± SD of three independent experiments with each experiment performed in triplicate. Student T-test and one-way ANOVA was used to compare the percentage of wound closure among control group and experimental group (*P<0.01, ** P<0.001 vs control).

In PEO1 cells, calpeptin treatment (24 hours before wounding and 48 hours after wounding) seems to slightly inhibit (<5%) cell migratory ability (**Figure** 4.7**C**), whilst 24-hour A23187 treatment induced a small increase in cell migration (**Figure** 4.7**D**) but none of the effect was of statistical significance. In both PEO1 and PEO4, 1-hour A23187 treatment induced a small decrease in cell migration at 24-hour and 48-hour endpoints; again there was no statistical significance (**Figure** 4.7**D** & **Figure 4.7D**). Cytochalasin D which was used in SKOV3 as a positive control did not affect PEO1 or PEO4 cell migration.

Based on the studies conducted by group members Dr Storr and Ms Bhudsaban Sukkarn, significant inhibition of cell migration was observed in calpeptin-treated breast cancer MDA-MB-231 cells; and therefore the MDA-MB-231 cell line was introduced in the current ovarian cancer study as a reference. Although calpeptin did not significantly inhibit cell migration in ovarian cancer cells, significant calpeptin inhibition of cell migration was observed in MDA-MB-231 cells (*P*=0.002) (**Figure 4.8**).

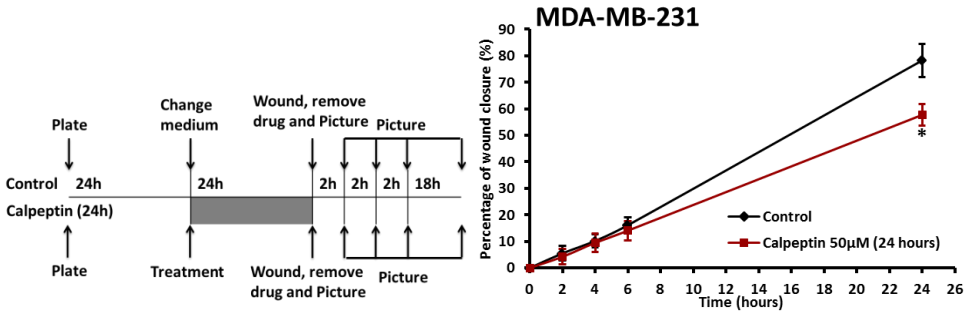


Figure 4.8 Effects of calpeptin on cell migration of MDA-MB-231 cells detected by scratch-wound assay. MDA-MB-231 cells were treated with or without calpeptin for 24 hours. Time scale for treatment and measurement is listed above each figure (grey bars over the hours indicate the treatment period). After the creation of the wounds, mitomycin-C was used for the inhibition of cell proliferation in all migration assays. Data represent the average ± SD of three independent experiments with each experiment performed in triplicate. Student T-test was used to compare the percentage of wound closure among control group and experimental group (**P*<0.01 vs control).

We evaluated SKOV3 cell migration upon exposure to the novel calpain

inhibitor 'compound-1'. Compound-1 at the recommended concentrations (5μM) did not inhibit SKOV3 migration (**Figure 4.9C**). In MDA-MB-231 cells, the migration was significantly inhibited by 50μM calpeptin for 24-hour treatment (*P*=0.03) (**Figure 4.9A, B**). One-hour calpeptin treatment also inhibited cell migration but the inhibition was not significant (**Figure 4.9B**). With the recommended dose, 1-hour compound-1 treatment could not significantly inhibit cell migration in the MDA-MB-231 cell line (**Figure 4.9B**), which might be due to the low concentration applied in the current study. Increasing the treatment time to 24 hours attenuated the limited inhibition effect of compound-1 on MDA-MB-231 cell migration (**Figure 4.9B**). In other words, the cells were relatively less responsive to 24-hour compound-1 treatment than 1-hour treatment.

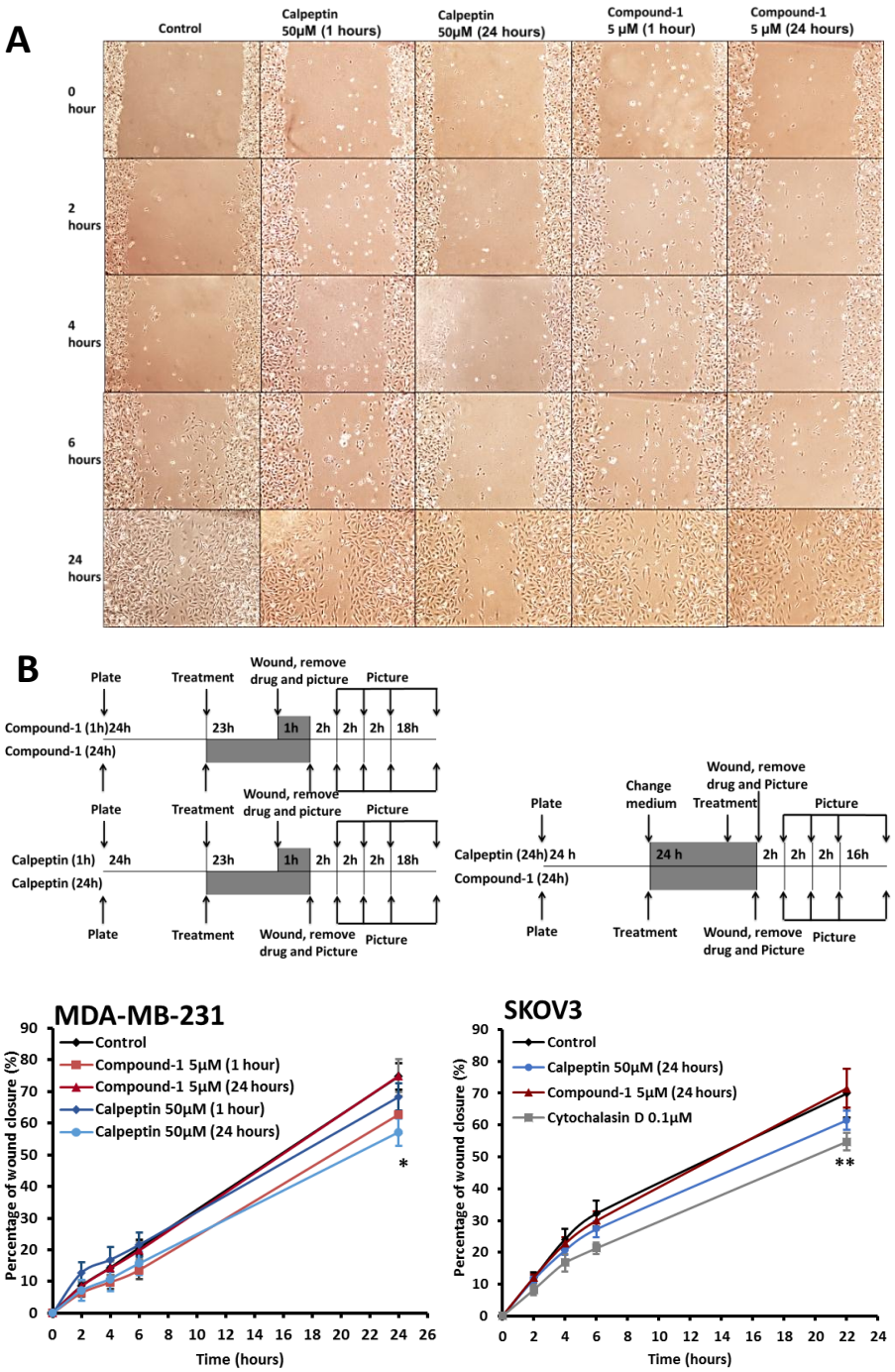


Figure 4.9 Effects of calpeptin and compound-1 on cell migration of MDA-MB-231 and SKOV3 cells detected by scratch-wound assay. MDA-MB-231 cells were treated with or without calpain inhibitor for 1 hour or 24 hours: (A) representative images at indicated time points are shown (magnification, ×200); (B) wound closure was quantified at the indicated time points (n=5). (C) SKOV-3 cells were treated with or without calpain inhibitor for 24 hours and wound closure was quantified at the indicated time points (n=3). Data represent the average ± SD of the independent experiments with each experiment performed in triplicate. One-way ANOVA was used to compare the percentage of wound closure among control group and experimental group (*P<0.01, ** P<0.001 vs control).

4.5. Discussion

Invasion and metastasis are major malignant characteristics of ovarian cancer. With the progression of tumours into higher stages (i.e. more invasive and metastatic states) mortality is increased. As discussed previously, elevated calpain expression and activity levels have been reported in a variety of tumour cells compared to non-malignant cells (**section 1.4.4.2**, Kimura *et al*., 2000; Witkowski *et al*., 2002; Carragher *et al*., 2004; Lakshmikuttyamma *et al*., 2004; Wu *et al*., 2006). Both inhibiting calpain activity by a pharmacological agent or genetically disrupting calpain expression by gene silencing can induce inhibition of cell migration and invasion, indicating that calpain is a positive regulator of the invasive and metastatic processes (Mamoune *et al*.,2003; Wu *et al*., 2006; Xu & Deng, 2006). This, however, seems to be context and tumour type specific.

Although results from chapter 2 and 3 did not support the idea that calpain can sensitise ovarian tumours/cancer cells to platinum-based chemotherapy, calpain-2 expression was significantly associated with patient outcome in both the current and previous studies (Storr *et al*., 2012a). To investigate the correlation between calpain and other determinants of a malignant phenotype, we tested both calpain inhibitors (i.e. calpeptin and compound-1) and an activator (i.e. A23187) for their ability to influence haptotactic migration in a number of ovarian cancer cell lines. In PEO1 and PEO4 cells no significant increase of calpain activity was observed after A23187 treatment which tracks with the non-significant changes in cell migration after treatment. Although calpeptin significantly inhibited calpain activity in PEO1 and PEO4 cells (**section 2.4.6**), no inhibition of cell migration was detected, which might be due to their low migratory ability (i.e. the wound closure of PEO1 and PEO4 only reached to 30% and 40% respectively 72 hours after creating the wounds). A23187 stimulated calpain activation

Figure 4.1 yet inhibited migration in SKOV3 cells (**Figure 4.5**), in a non-time dependent manner. With the limited regulation level of calpain activity, no significant change of cell migratory ability had been observed in calpeptin-treated SKOV3 although positive control drug cytochalasin-D significantly inhibited SKOV3 cell migration.

Compound-1 was tested in both SKOV3 and MDA-MB-231 cells, rather than showing inhibitor effect on calpain activity, the t-BOC assay suggested that compound-1 increased calpain activity. Compound-1 treated SKOV3 cells were visualised in comparison with the calpeptin-treated cells and vehicle control cells with t-BOC/DMSO (vehicle) for assessing calpain activity. Results from this experiment cannot be quantified but they showed that the compound-1-treated cells had fluorescence without the addition of t-BOC. It is therefore difficult to draw any firm conclusion on the effect of compound-1 on calpain activity regulation, given that the variation of the current t-BOC calpain activity assay and the background fluorescence generated by compound-1 falling into the wavelength for the detection of cleaved t-BOC (**Figure 4.13**). Compound-1 might be a fluorophore compound and fluorescence resonance energy transfer (FRET) might have occured between compound-1 and t-BOC: the energy of one fluorophore, as a donor, transfers to the other fluorophore, as a receptor, and largely enhanced the fluorescence intensity of the receptor. Thus, the influence could not be entirely removed by normalisation (using the reading of the first cycle and the reading of medium alone) which could have created the false impressions. In the future study, alternative ways of assessing calpain activity should be used before testing the inhibitory effect of these novel inhibitors. Other ways could be considered for assessing calpain activity in future studies to validate these findings. Although the calpain system has numerous endogenous targets, only α-Fodrin and spectrin αII (Table S2.1) were generally used for the study of calpain activity via Western blotting. Further studies (e.g. the expression and fragment size of

calpain specific substrates) are needed to develop other calpain substrates (section 4.2.3) for this purpose. As pointed out by Prof. P Davies and colleagues (Queen's university, Canada) in the FASEB conference, conventional colourimetric or fluorimetric calpain activity assays lack sensitivity and/or specificity. They designed an optimal FRET-based protein reporter substrate (different from t-boc, it can be excitation at 434 nm (± 10 nm) and emissions at 485 nm (± 10 nm) and 528 nm (± 10 nm), McCartney et al., 2017) for measuring calpain activity which could be used in future studies. Although inhibition of haptotactic migration was observed in compound-1 treated cell, the difference between control and treated groups was not significant. The concentration and treatment period of compound-1 might also need optimisation to significantly alter calpain activity.

The migratory ability of the cell lines tested in the current study from high to low was SKOV3, PEO1 and PEO4, and their calpain activity levels appeared to follow the same order. It seems that there is a positive linear correlation between calpain activity and migratory ability as has been reported out in rhabdomyosarcoma cells and non-tumour human myoblasts cells (Roumes *et al*., 2010). Limited by the variations of the t-BOC assay and the number of cell lines that have been tested, this correlation lacks statistical support in the current study.

The cell numbers of 0.5μM A23187-treated or 50μM calpeptin-treated cells were similar to those of control cells (**section 2.4.6**), suggesting that altering calpain-activity with these agents does not significantly affect the proliferation of SKOV3, PEO1 or PEO4 cells, thus excluding the possibility that the observed changes in cell migration was caused by different cell growth rates between the experimental groups and control group.

As indicated in **section 4.2.2** and **4.2.3**, calpain activation is generally positively associated with cell migration – this is in line with other published

studies indicating that with decreases in calpain activation cell migratory ability also decreases. Perrin & Huttenlocher (2002) reviewed and emphasised that the effects of calpain on cell motility are greatly influenced by cellular context. Calpain is involved in cell migration by modulating the architecture and dynamics of cell adhesions and cytoskeletal components, in addition via involving in intracellular signalling pathways (Perrin & Huttenlocher, 2002). Depending on the cell type, the observed effects of calpain inhibition were very different, but of all the cell types calpain inhibition seems to consistently attenuate the migration rate and invasiveness of cells (Perrin & Huttenlocher, 2002).

A large number of studies have indicated that the inhibition of calpain leads to decreased migration, but information is lacking on how cell migration may be changed after an increase in calpain activity (**section 4.2.2**). Results from a recent study using both transwell migration and wound-healing assays in ovarian cancer cells revealed that downregulating calpain-4 decreased the migratory ability, whilst, overexpressing calpain-4 increased the migration rate (Yang *et al*., 2017). In previous and current studies, calpeptin is able to inhibit cell migration of MDA-MB-231 cells in agreement with the published literature. The current study, however, also showed that increased calpain activation led to significant inhibition of cell migration in SKOV3 cells. This could be due to the following possibilities: (1) this was the initiation of cell death; (2) proteins that promote cell spreading/migration, such as integrins, were cleaved by calpain which led to the inhibition of cell spreading/migration (reviewed in **section 5.5**); or (3) A23187 can regulate other migration-related proteins besides calpains (e.g. S100A4 and calcineurin which are further discussed in **section 6.2.1**). One study indicated that calpain activity was upregulated by disulfiram which is an approved drug for the treatment of alcohol dependence and was able to kill MDA-MB-231 cells (Kim JY *et al*., 2017). Calpain co-localised with vimentin and talin in the cytoplasm and its upregulation of

activity increased the cleavage of vimentin and talin (1-hour treatment) at an early stage (Kim JY *et al*., 2017). Disulfiram-induced calpain activation but inhibited cell migration and invasion in MDA-MB-231 cells (3-hour treatment), caused by the initial collapse of cytoskeletal structure vimentin filaments, the degradation of focal adhesion molecules and cell detachment, eventually resulting in apoptosis with the increase of treatment time (Kim JY *et al*., 2017). Different from the current study, the concentration they used that can significantly inhibit cell migration reduced cell viability significantly.

Unlike the association between cell migration and calpain-1/-4, the inhibition of which uniformly associated with attenuated cell migration, the way that calpain-2 expression associated with cell migration seems more dependent on cellular context (**section 4.2.2**). Intracellular calpain-2 proteolytic activity is usually necessary for turnover of integrin-linked adhesions during two-dimensional planar migration / mesenchymal invasion (Carragher & Frame, 2004; Franco *et al*., 2004). Notably, an amoeboid invasion is independent of intracellular calpain-2 proteolytic activity (Carragher & Frame, 2004; Franco *et al*., 2004). The invasion of cells with mesenchymal-like features was substantially impaired by calpain-2 depletion (by siRNA and calpain inhibitors) whilst amoeboid-like cells were relatively insensitive to calpain-2 inhibition when invading through Matrigel (Carragher *et al*., 2006b). In glioblastoma cells, calpain-2-knockdown did not influence cell morphology or the organisation of actin cytoskeleton or migration but inhibited the proteolysis of an actin-binding protein (i.e. cortactin) and cytoskeletal proteins (i.e. talin and filamin) and decreased MMP-2 secretion which inhibited the cell invasion by 90% (Jang, Lal and Greenwood, 2010). Hence calpain-2 appears to be more involved in the mesenchymal type of migration/invasion than the amoeboid type of invasion which emphasises the necessity of using different cell models and different assays (such as using 3D ECM gel or Boyden chamber) for the understanding of how calpain is involved in cell migration and invasion

from different perspectives.

In conclusion, alteration of calpain activity in ovarian cancer SKOV3 cells by the ion-carrier A23187 induced significant suppression of the two-dimensional planar cell migration. The calpain inhibitor calpeptin was able to inhibit calpain activity which was paralleled by a decreased migration rate in SKOV3 cells. The similar levels of calpain activity in PEO1 and PEO4 cells with or without A23187 treatment may also in parallel with the similar migration rate between the treated and untreated cells. But significant inhibition of calpain activity in PEO1 and PEO4 cells induced by calpeptin did not significantly inhibite cell migration. Since SKOV3 and PEO1/4 cell lines belong to different ovarian cancer subtypes (further discussed in **section 6.1**), μ- and m-calpain may play important roles in the progression of certain ovarian cancer subtypes via migration. Future studies on the spatial and temporal nature of calpain activation (Franco & Huttenlocher, 2005) will be necessary to dissect and understand the role of calpain-mediated proteolysis in different types of migration. Understanding the role of calpain may benefit the development of novel therapeutic strategies for treating tumour metastasis. Cancer metastasis requires more determinants than cell migration alone, so proteins involved in other aspects of metastasis were studied in the following chapter.

4.6. Summary

1. A23187 increased calpain activity in SKOV3 cells which was paralleled by a significant inhibition of cell migration. An inhibitory effect of calpeptin on calpain activity and cell migration was observed in SKOV3, but the results were non-significant. With limited upregulation of calpain activity, A23187 induced no significant change on cell migration in ovarian cancer cell lines PEO1 and PEO4. Although calpeptin significantly inhibited calpain activity in PEO1 and PEO4 cells, no significant inhibition of cell migration was detected.

2. Compound-1 could not significantly inhibit cell migration in SKOV3 or MDA-MB-231 cells which might be due to drug concentrations or treatment periods used. Due to variations in the t-BOC calpain activity assay, it is difficult to draw a firm conclusion on the effect of compound-1 on calpain activity regulation.

Chapter 5. Potentially calpain-related proteins- MAP4 and Syk

5.1. Abstract

INTRODUCTION: The calpain system is involved in cancer progression via various cellular processes including apoptosis, migration and invasion. In the 2016 FASEB summer conference, calpains were found correlated with microtubule-associated protein 4 (MAP4) and spleen tyrosine kinase (Syk) with potential involvement in the processes of angiogenesis and EMT respectively. MAP4 is a ubiquitously-expressed microtubule-associated protein which functions as a microtubule stabiliser. Syk is a non-receptor tyrosine kinase that phosphorylates a variety of cytoskeletal proteins. Widely expressed in a variety of hematopoietic cells, Syk has also been detected in normal and tumourigenic mammary epithelial cells. A few recent studies have suggested potential interactions between Syk and the conventional calpains. In addition, MAP4 has been reported to be regulated by Syk.

OBJECTIVES: This chapter focuses on whether MAP4 or Syk expression, in ovarian cancer specimens, is associated with patient clinical outcomes and/or other clinicopathological factors; and if there are any correlations between the expression of MAP4, Syk, the calpain system and other proteins that potentially contribute to tumour invasion and metastasis (i.e. proteins that are associated with angiogenesis and EMT).

METHODS: Samples (n=575) from primary ovarian tumour sites of patients (using the same cohort as **Chapter 3**) were evaluated for protein expression of MAP4 and Syk by standard IHC.

RESULTS: MAP4 expression was significantly associated with ovarian cancer histological subtype, stage, grade and residual tumour. Cytoplasmic and

nuclear Syk expression was significantly associated with cancer histological subtype. Low cytoplasmic Syk expression was significantly associated with lower stage whilst low nuclear Syk expression was associated with chemo-resistance in patients treated by therapy containing taxane. Patients were also grouped according to whether they had an organ-confined tumour (i.e. group 1: stage 1 and group 2: stage 2-4) or according to whether they had distant metastasis (i.e. group 1: stage 1-3 and group 2: stage 4). Low cytoplasmic Syk, low nuclear Syk and low MAP4 showed significant association with organ-confined tumour status, but no significant association was detected between their expression and cancer distant metastasis.

Neither MAP4 nor Syk expression was significantly associated with OS in the whole cohort. Patients were then grouped by other clinicopathological variables. No significant association was detected between MAP4 expression and OS in any of the subgroups tested, whereas, high nuclear Syk expression was associated with better OS in certain subgroups. The specimens in the whole cohort were subsequently recategorised according to the expression of any two of CXCL4, CXCR3 (provided by Dr S. Deen), MAP4, Syk, calpain-1, -2, -4 and calpastatin into four subgroups each time. On the Kaplan–Meier Univariate survival analysis for OS, the combined marker expressions were not able to predict patient outcome with improved significance comparing with single calpain system members.

In the whole cohort, between the calpain system and MAP4, Syk, CXCR3 and CXCL4, the strongest correlation was found between calpain-1 and MAP4. The expression of a panel of proteins was studied on 87 HGSC patients from the whole cohort including vascular endothelial growth factor (VEGF), CXC chemokine ligands: CXCL1, CXCL4 and CXCL8, CXC chemokine receptor: CXCR3, silent/decoy CXC chemokine receptor: Duffy antigen receptor for chemokines (DARC), integrin α2β3, N-, E- and P-cadherin (provided by Dr S. Deen). Most of the protein pairs were weakly correlated or lack of correlations. Integrin α2β3

was moderately correlated with calpain-1, MAP4 and cytoplasmic DARC expression.

CONCLUSION: Although MAP4 and Syk expression were not significantly associated with either OS or PFS, both MAP4 and Syk were associated with tumour stages. The current study also observed the paradoxical roles of Syk, both as tumour suppressor and pro-survival factor and indicated that high Syk expression might facilitates the tumour spreading whilst high nuclear Syk sensitises tumour cells to taxane-containing chemotherapy. The expression of integrin α2β3 was found to be significantly correlated with MAP4, Syk, and calpain-1 expression. Together, results from the current chapter suggest that in addition to paclitaxel resistance, integrin, MAP4, Syk and the calpain system might also be involved in cancer cell spreading. How they interconnect in tumour cell spreading/migration and cell response to taxane-containing chemotherapy remains to be elucidated.

5.2. Introduction and aims

Prof. Stewart Martin and Dr Sarah Storr attended the Biology of Calpains in Health and Disease conference - the 2016 Federation of American Societies for Experimental Biology (FASEB) summer conference devoted to the calpain system at which novel, unpublished data was presented. Two presenters described data that was of potential relevance to the current project. One presentation (Dorothy E Croall and colleagues) mentioned that calpain-2 was associated with MAP4 as a major interacting protein in endothelial cells (both bovine aortic endothelial cells and human umbilical vein endothelial cells) using affinity capture coupled with LC-Ms/MS and proteomic analysis. Another presentation (Robert L. Geahlen and colleagues) suggested a correlation between calpain, Syk and EMT. EMT has been suggested as one of the pathways though which the calpain system affects cancer progression (further

discussed in **section 5.5**). Syk is a negative regulator of the EMT process which is expressed at a higher level in well differentiated than in poorly differentiated breast and pancreatic carcinoma cells; inhibiting the expression of Syk is likely to decrease the epithelial gene products and the increase expression of characteristic genes of mesenchymal cells (Krisenko & Geahlen, 2015). The above suggested that calpains were correlated with MAP4 and Syk and potentially involved in the processes of angiogenesis and EMT respectively which inspired us to include the studies of MAP4 and Syk into the current project; hence their correlations with the proteins related to these processes (provided by Dr S. Deen) were also studied.

5.2.1 Microtubule-associated protein 4 (MAP4)

Microtubules, which are composed of α- and β-tubulin heterodimers subunits, are major components of the eukaryotic cytoskeleton (Zhou *et al*., 2015). In many eukaryotic cells, microtubules are highly dynamic and have been involved in a variety of physiological and pathological cellular processes, including cell motility, polarisation, change of cell shape, protein synthesis, transmembrane signals, hyperpermeability, organelle transportation and vesicles (Zhou *et al*., 2015; Wei & Birrer, 2015).

5.2.1.1 MAPs

Microtubule-associated proteins (MAPs) regulate microtubule dynamics by interacting with tubulin polymers and microtubules (Orr *et al*., 2003). MAPs consist of MAP1A, MAP1B, MAP2, MAP4 and tau subtypes (Honore *et al*., 2005) among which MAP-tau, MAP2 and MAP4 proteins bind and stabilise the microtubules by regulating dynamic instability and actin filament treadmilling in all living cells (Honore *et al*., 2005). MAP4 is the predominant human non-tubulin component of microtubule-associated protein in non-neuronal tissues (Bash-Babula J. *et al*., 2002; Orr *et al*., 2003; De Conto *et al*., 2012). MAP4 is conserved throughout evolution and ubiquitously expressed in

mammalian tissues (Poruchynsky *et al*., 2001). MAP4 functions as a microtubule stabiliser by binding to tubulin microtubules (Olmsted *et al*., 1989), regulating the assembly level of microtubules (Nguyen *et al*., 1999; Hait & Yang, 2006) and promoting microtubule polymerisation (Bash-Babula J. *et al*., 2002; Kavallaris *et al*., 2001; Orr *et al*., 2003). Inhibition of MAP4 expression by an antisense approach decreases microtubule polymer levels in HeLa cells, whereas overexpression of MAP4 increases microtubule stability (Orr *et al*., 2003).

MAPs are regulated by post-translational modification i.e. phosphorylation, which can destabilise microtubules by dissociating MAP from the microtubule and/or tubulin in general (Honore *et al*., 2005; McGrogan *et al*., 2008). It is microtubule affinity regulating kinase (MARK) that phosphorylates MAP4, MAP2c, and tau on their microtubule-binding domain *in vitro* (Ebneth *et al*., 1999). In addition, calpain has been found as a regulator of MAPs. MAP2 and MAP tau are known as a very sensitive physiological substrate of calpain in neuroblastoma cells (Tompa *et al*., 2001b; Guttmann & Johnson, 1998). In neuroblastoma cells, ionophore A23187-mediated calpain activation induces the phosphorylation of the MAP tau (Ekinci & Shea, 1999). The precise relation between MAP4 and the calpain system remained to be fully defined.

5.2.1.2 MAP4 and tumour sensitivity to taxanes

Platinum- and taxane-based chemotherapy regimens remain the standard care for adjuvant treatment in early-stage epithelial ovarian cancers and first-line treatment for advanced stage diseases (Webber & Friedlander, 2017). As an anti-tumour drug, taxane induces microtubule stabilisation and reduces microtubule dynamics which promotes mitotic arrest and cell death (Aoki *et al*., 2009). Paclitaxel is a representative agent of taxanes (Aoki *et al*., 2009). Based on the mechanism of taxane on tumour cells, proteins that are involved in the regulation of cellular microtubule dynamics via interacting with

tubulin dimers or polymerised microtubules clearly possess the potential for modulating the cell sensitivity towards paclitaxel (Orr *et al*., 2003).

The association between MAP4 and paclitaxel resistance has been observed in different cancer types including ovarian, breast, and lung cancer (Poruchynsky *et al*., 2001; Orr *et al*., 2003; Xiao H *et al*., 2012; Aoki *et al*., 2009; McGrogan *et al*., 2008; Dorman *et al*., 2016; Martello *et al*., 2003). The drug sensitivity profile of ovarian cancer paclitaxel-resistant sublines parallels the regulation of MAP4 modification: MAP4 phosphorylation and dissociation from microtubules decrease the sensitivity of paclitaxel-resistant ovarian cell lines towards paclitaxel (Poruchynsky *et al*., 2001; Orr *et al*., 2003). However, down-regulation of MAP4 in SKOV3 cells did not influence the sensitivity to either paclitaxel or cisplatin; and MAP4 status was not correlated with the survival of patients treated with either taxane-based regimen or taxane-free regimen (Aoki *et al*., 2009). The precise role for MAP4 in tumour sensitivity to taxanes remains to be defined.

5.2.2 Spleen tyrosine kinase (Syk)

Syk is a cytoplasmic non-receptor tyrosine kinase (Baldock *et al*., 2000) that phosphorylates a variety of cytoskeletal proteins (e.g., paxillin, tubulin and cortactin) (Fernandez & Suchard, 1998) and is involved in signalling cascades of integrins (e.g. αIIbβ3) in platelets (Gao *et al*., 1997). Syk is widely expressed in a variety of hematopoietic cells, including B- and T-cells (at various stages of development and activation), mast cells, basophils, platelets, neutrophils, erythrocytes, dendritic cells, macrophages and natural killer cells (Singh *et al*., 2012; Sada *et al*., 2001; Krisenko & Geahlen, 2015). In addition, Syk expression has been detected in osteoclasts, normal and tumourigenic mammary epithelial cells, melanocytes, human nasal fibroblasts, hepatocytes, neuronal cells and vascular endothelial cells suggesting that Syk also has a general physiological function in a wide variety of non-hematopoietic cells

(Tsuchida *et al*., 2000; Coopman & Mueller, 2006; Yanagi *et al*., 2001; Singh *et al*., 2012).

Syk has been found as a pro-survival factor in many hematopoietic malignancies (Rickert, 2013; Krisenko & Geahlen, 2015) and some epithelial original tumours (Geahlen, 2014). The presence of activated Syk has been suggested to enhance the ability of cancer cell to resist apoptosis, although the signalling events downstream Syk are still unclear (Geahlen, 2014; Krisenko & Geahlen, 2015). In addition, Syk was required for malignant virus-induced transformation and enhanced motility (e.g. in breast and nasopharyngeal epithelial cells; Katz *et al*., 2005; Krisenko & Geahlen, 2015), growth and survival of cancer cells such as breast cancer, ovarian cancer (Prinos *et al*., 2011), Syk-positive small cell lung cancer (Udyavar *et al*., 2013), certain subsets of retinoblastoma and pancreatic carcinoma cells (Fei *et al*., 2013). In ovarian cancer, Syk expression level was elevated with the increase of tumour grade and silencing Syk expression significantly inhibited anchorage-independent growth and induced apoptosis in Syk-expressing ovarian cancer cells (Prinos *et al*., 2011). Besides, Syk appeared as a preferentially expressed protein in recurrent HGSCs (Jinawath *et al*., 2010). In prostate cancers, elevated Syk expression was associated with both invasive growth and dissemination (Ghotra *et al*., 2015). In the study of patients with squamous cell carcinomas of the head and neck, high Syk expression (mRNA level, n=45; protein level, n=38) significantly correlated with worse survival, enhances cell migration and metastases to the lymph nodes (Luangdilok *et al*., 2007).

In cancers of non-immune cells, Syk can be a pro-survival factor but has also been found to play a contradictory role, as a tumour suppressor (Moroni *et al*., 2004; Coopman *et al*., 2000; Coopman & Mueller, 2006; Krisenko & Geahlen, 2015). The decrease of Syk expression in high malignant and invasive tumour cells has been observed in breast, lung, melanoma, pancreatic,

bladder, colorectal, gastric, nasopharyngeal, hepatocellular cancer, oral squamous cell and esophageal squamous cell carcinoma (Bailet *et al*., 2009; Krisenko & Geahlen, 2015; Chuanliang *et al*., 2016; Dong *et al*., 2011; Yuan *et al*., 2006; Hong *et al*., 2012; Layton *et al*., 2009; Geahlen, 2014; Ma *et al*., 2010; Ogane *et al*., 2009; Dong *et al*., 2013). In some breast cancer studies, although the tendency of the Syk loss along with the increase of cancer invasiveness was retained, no statistically significant differences were detected between normal tissues and invasive tumours (Coopman & Mueller, 2006). Syk was found negatively associated with cell proliferation, division and invasive/anchorage-independent growth; moreover, there were negative associations between Syk and cell migration, lymphovascular invasion, microvessel density and/or metastasis formation in cancers (Ogane *et al*., 2009; Fei *et al*., 2013; Nakashima *et al*., 2006; Coopman & Mueller, 2006; Peng *et al*., 2013; Chuanliang *et al*., 2016). Syk may increase cell-cell interactions and limit EMT and migration (Krisenko & Geahlen, 2015). Thus, Syk functions as a tumour suppressor and its absence from many highly aggressive epithelial cell-derived tumours can be explained (Krisenko & Geahlen, 2015; Coopman & Mueller, 2006).

5.2.2.1 Regulation of Syk

Syk can be activated by either auto-phosphorylation or phosphorylation through binding with upstream kinases (e.g. Lyn) (Bohnenberger *et al*., 2011; Yi *et al*., 2014; de Castro., 2011; Krishnan *et al*., 2008; Zyss *et al*., 2005). In the immune system, Syk plays an important role in distinguishing self from non-self via antigen and immune receptors (Baldock *et al*., 2000; Krisenko & Geahlen, 2015). Both in immune and non-immune cells, Syk can be activated by crosslinking activated cell surface receptors such as integrins (Fei *et al*., 2013). For example, Syk is closely associated with the morphological changes of neutrophils mediated by non-immune receptors, β_2-integrins (Fernandez & Suchard, 1998; Baldock *et al*., 2000; Gonscherowski *et al*., 2006). Similarly, the

crosslinking of integrins leads to the activation of Syk (Ulanova *et al*., 2005; Fei *et al*., 2013), such as β1-integrins in airway epithelial cells, the HB2 mammary epithelial cells and the MCF7 breast carcinoma cell line (Coopman & Mueller, 2006). After binding to the receptors (Baldock *et al*., 2000; Rolli V *et al*., 2002), the activation of Syk allows it to phosphorylate downstream substrates (Lowell *et al*., 2011) such as cytoskeletal proteins including α-tubulin, cortactin, E-cadherin, α-catenin and MAP1B and phospholipase, phosphatidylinositol 3-kinase (PI3-K) (Lowell *et al*., 2011; Stewart & Pietenpol, 2001; Krisenko & Geahlen, 2015).

5.2.2.2 Syk and calpain

Certain studies in the literature have suggested potential interactions between Syk and the conventional calpains, which negatively regulate Syk protein/activity levels. The calpain inhibitor ALLN is able to completely suppress H_2O_2-induced NF-κB activation via Syk-mediated tyrosine phosphorylation of IκBα in myeloid cells (Takada *et al*., 2003). In breast cancer cells, Syk enhances TNF-α-induced NF-κB activation, possibly via inhibiting calpain mediated proteolysis on the NF-κB subunit RelA (Fei *et al*., 2013). In basophils, calpain inhibitors (i.e. calpain inhibitor V and ALLM) had no detectable effect on Syk levels by Western blotting analysis (Youssef *et al*., 2002) which might be due to the site of the antibody failed to discriminate the fragments or the phosphorylated form of Syk. Western analysis has detected a C-terminal fragment (40-45 kDa) of Syk generated by calpain cleavage of human recombinant Syk produced by a recombinant baculovirus expression system (Baldock *et al*., 2000) which suggests that Syk is also a calpain substrate. The regulation of Syk by calpain has been pointed out in another study, using human umbilical vein endothelial cells (HUVECs), which indicates that calpain inhibitors significantly increased full-length Syk (72 kDa) in the membrane fraction of HUVECs; the possible mechanisms of which are as follow: 1) the impaired-degradation of Syk induces an accumulation of Syk on

the membrane; 2) calpain inhibition prevents the Syk detachment from the membrane where the Syk can stay active (Gonscherowski *et al*., 2006). Following the inhibition of calpain, the accumulation of Syk increased at the edge of the spreading in breast cancer MCF7 cells (Fei *et al*., 2013). As pointed out by Gonscherowski *et al*. (2006), the latter is supported by the findings that calpain mediated proteolysis of β3-integrin on its cytoplasmic tail where Syk binds to β3-integrin when β3-integrin interacting with the extracellular matrix. Calpain inhibitors also cause translocation and activation of Rho family of G proteins (e.g. RhoA and Rac1) in HUVEC (Kulkarni *et al*., 2002; Somlyo, 2002) together with the increased Syk on cell membrane which may be the cause of the observed morphological changes of HUVEC and the coronary leak in isolated guinea pig hearts (Gonscherowski *et al*., 2006).

In two other studies, Syk appears upstream of calpains instead of being a calpain substrate and Syk regulated calpain activity via altering the level of calcium and calpastatin expression. But the effect of Syk on calpain activity seems contradictory and might depend on the cellular context. Integrin engagement induced activation of the tyrosine kinases c-Src and Syk which led to the activation of calpain and subsequent cleavage of integrin β3 cytoplasmic tail, which seemed to be an early event in detachment, followed by apoptosis, in endothelial cells (Meredith *et al*., 1998; Maile LA *et al*., 2008). In breast cancer cells, the expression of Syk resulted in a significant reduction of calpain activity when using cell lysates for assessment (Fei *et al*., 2013). Controversially, the same study also indicated that intracellular calcium concentrations, calpain activity levels (in intact cells) and calpastatin expression levels were significantly higher in Syk-positive MCF7 cells than Syk-deficient MCF7 cells, especially in the cytosolic fraction; however, the highest calpastatin expression and lower calpain activity level was observed in breast cancer MDA-MB-231 cells where Syk was not detectable when compared with MCF7 cells (Fei *et al*., 2013).

5.2.3 Associations between Syk and MAP4

A study conducted by Yu *et al*. (2015) revealed an association between Syk and MAP4 and a link with paclitaxel resistance. Proteome comparison showed that Syk was one of the preferentially expressed proteins in recurrent HGSCs after chemo-therapeutic treatment in comparison with the primary tumours from the same patients (Jinawath *et al*., 2010). Syk expression/activity levels were positively associated with the resistance to paclitaxel, moreover, Syk inhibition significantly enhanced paclitaxel sensitivity in the established paclitaxel-resistant (not carboplatin-resistant) ovarian cancer SKOV3 and MPSC1 cell clones and among cells collected from 25 primary ovarian tumours (Yu *et al*., 2015). Pharmacological and genomic Syk inhibitors had a synergistic effect with paclitaxel both *in vitro* and *in vivo* especially in cells that are resistant to paclitaxel (Yu *et al*., 2015). When comparing Syk inhibitor treated paclitaxel-resistant SKOV3 cells with vehicle control cells, phosphoproteomic screening identified several proteins essential for microtubule dynamics which are Syk substrates including MAP1B, MAP4, α-tubulin, and β-tubulin (Krisenko *et al*., 2014; Yu *et al*., 2015). The activated Syk mutant (Syk-130E) which has been shown to have a high basal kinase activity significantly increased cell resistance to paclitaxel and the levels of phosphorylated MAP1B and MAP4 whilst the inhibited Syk enhanced the microtubule stabilisation and sensitivity of paclitaxel-resistant tumour cells toward paclitaxel (Yu *et al*., 2015). Syk-mediated tyrosine phosphorylation of microtubule associated proteins (MAP1B and MAP4) and tubulin could be one of the paclitaxel resistant mechanisms (Wei & Birrer, 2015).

5.2.4 Aims

Results from chapter 3 indicated that calpain-2, -4 and calpastatin expression were significantly associated with patient OS, but the role that the calpain system played in ovarian cancer progression has not yet been fully elucidated. As calpain-1 expression showed significant association with

tumour stage in the current study, this implicated that calpain-1 might mediate tumour spreading. As stated above, Prof. Stewart Martin and Dr Sarah Storr attended and shared information from the 2016 FASEB summer conference devoted to the calpain system. Two presentations (delivered by Dorothy E Croall and colleagues and Robert L. Geahlen and colleagues respectively) mentioned two potentially calpain-related proteins, MAP4 and Syk, in addition to their potential involvement in the processes of angiogenesis and EMT respectively. Moreover, current data in the literature has suggested some potential interactions between Syk and the calpain system. Especially in the study conducted by Fei *et al*., (2013), where the level of Syk significantly influenced the expression of calpastatin, intracellular calcium concentrations and calpain activity levels (**section 5.2.2.2**). The study of the interactions of calpain with angiogenesis and EMT biomarkers, in addition to MAP4 and Syk, might shed some light on how the calpain system is involved in tumour spreading. MAP4, as a substrate of Syk, was involved in regulating microtubule stability and paclitaxel resistance in ovarian cancer cells (Yu *et al*., 2015). Based upon such findings and the information from the FASEB conference, the current chapter sought to address the following hypotheses:

1. The expression of MAP4 or Syk is associated with the clinicopathological factors (e.g. patient outcome, stage and grade) of ovarian cancer patients;
2. MAP4 and/or Syk expression is correlated with the expression of the calpain system (i.e. calpain-1, -2, -4 and calpastatin);
3. The calpain system, MAP4 or Syk expression correlate with biomarkers related to ovarian tumour angiogenesis/EMT.

The study aimed to:

1. Study how MAP4 and Syk expression is associated with patient clinical outcomes and other clinicopathological factors by IHC;
2. Study the correlation between MAP4, Syk, the calpain system (i.e.

calpain-1, -2, -4, and calpastatin) and other proteins that potentially contribute to tumour metastasis (e.g. proteins that are associated with angiogenesis and EMT).

5.3. Materials and methods

5.3.1 Western blotting

The methodology was described in **section 2.3.3**. Antibodies used were Rabbit polyclonal anti-human MAP4 antibody targeting AA1102-1152 (BETHYL LABORATORIES, INC, UK; A301-489A) and Mouse monoclonal anti-human Syk antibody (4D10.1) targeting AA313-339 (Thermo Fisher SCIENTIFIC, UK; MA5-13087). Initially, rabbit polyclonal anti-human MAP4 antibody (Proteintech Group Inc, US; 11229-1-AP) was tested; but due to the presence of extra bands, this antibody was replaced by the anti-MAP4 antibody mentioned above. In the Western blotting study, primary antibodies were diluted in 1:1000 dilution.

5.3.2 Clinical samples, TMA, IHC and interpretation

Anti-human MAP4 antibody and anti-human Syk antibody were mentioned in **section 5.3.1**. Other materials and all methodologies were described in **section 3.3.2.** The cytoplasmic staining intensity was visually scored and an H-score calculated by adding the percentage of area stained at each intensity level multiplied by the corresponding staining intensity (i.e. 0: no staining, 1: weak, 2: moderate, and 3: strong staining). The nuclear staining intensity was also visually scored and the percentage of the stained nucleus recorded. More than 25% of the slides were examined by a second independent assessor (Anqi Yao) blinded to scores and clinicopathologic criteria. Single measure intraclass correlation coefficients (ICC) analysis was conducted to determine the level of agreement between independent scorers. The single measure ICCs between scores were 0.925, 0.833 and 0.887 for anti-Syk antibody cytoplasmic and nuclear stained samples, and anti-MAP4 antibody stained samples respectively. X-tile software was used to determine a non-biased cut-point of the immunohistochemical scores.

5.3.3 Statistical analysis

The association between the categorised protein expression and clinicopathologic factors was examined using Pearson's chi-square test of association (χ2) or Fisher's exact test. Survival analysis was conducted using the Kaplan–Meier method and the statistical significance of difference between groups was determined by the Log-rank test. Multivariate survival analysis was conducted using Cox's multivariate proportional hazard model analysis. Correlation between proteins was determinate using Spearman's rank test (**section 3.3.3**).

5.4. Results

5.4.1 Antibody specificity and IHC optimisation

The expression of MAP4 and Syk was investigated in ovarian tumours by standard IHC using a TMA. The specificity of anti-MAP4 antibody and anti-Syk antibody was initially assessed by Western blotting before conducting IHC. A single band corresponding to the molecular mass of the intended target was observed in the Western blotting studies which indicated the specificity of both anti-MAP4 (BETHYL LABORATORIES, UK) and anti-Syk (Thermo Fisher SCIENTIFIC, UK) antibody. Results from the current study (**Figure 5.1**) agree with reports in the literature that MAP4 is ubiquitously found in all cell types (Honore *et al*., 2005) with the expected size, 210 kDa (West *et al*., 1991). Syk with the size of 72 kDa (Tsuchida *et al*., 2000; Baldock D *et al*., 2000; Grädler *et al*., 2013) was expressed in SKOV3, PEO1 and PEO4 cells, whereas, Syk was not detected in A2780 and A2780-cis cells (**Figure 5.1**).

The optimal concentration of each antibody to be used in IHC was determined as follow: dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1600 for anti-MAP4 antibody and dilutions of 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 for anti-Syk antibody were assessed. Based on the results, to achieve specific

staining with minimal background, a dilution of 1:400 for anti-MAP4 (4°C overnight) and 1:1600 for anti-Syk antibodies (4°C overnight) were used on TMA s. Representative staining patterns of different antibody concentrations during optimisation are presented in **Figure S5.1** and **Figure S5.2**.

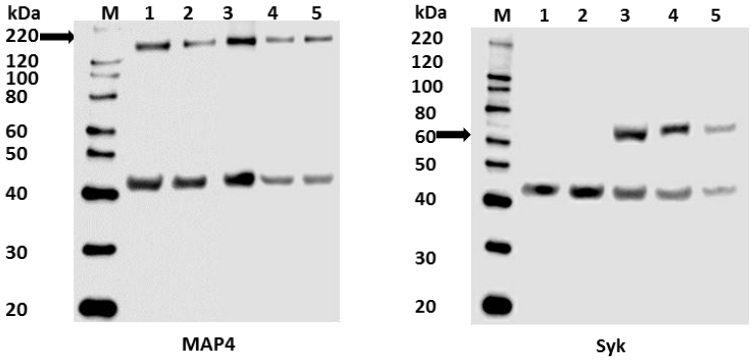


Figure 5.1 Optimisation of anti-MAP4 and anti-Syk antibody in ovarian cancer cells. MAP4 and Syk expression across the panel of cell lines were detected by Western blotting analysis using specific antibodies. Figures present the representative blots of 2 independent experiments with lysates from cells with different passage numbers. Arrows indicate MAP4 (210 kDa) and Syk (72 kDa), respectively. β-actin was used as the loading control (42 kDa). Lane M: Protein marker, Lane 1: A2780, Lane 2: A780-cis, Lane 3: SKOV3, Lane 4: PEO1, and Lane 5: PEO4.

MAP4 showed predominant staining in the cytoplasm of the ovarian cancer cells with the appearance of membrane staining. Syk was found to be expressed in both the cytosol and the nucleus as reported by Zhang X. *et al*. (2009) and Fei *et al*. (2013). Both MAP4 and Syk showed granular/diffuse staining with heterogeneity between adjacent cancer cells. In addition to the staining of tumour cells, MAP4 and Syk staining was observed in some parts of tumour-adjacent normal tissues; moreover, Syk staining was also observed in immune cells, but the current study focused only on the staining of tumour cells. Representative staining patterns of weak, moderate, and strong staining intensity are shown in **Figure 5.2**.

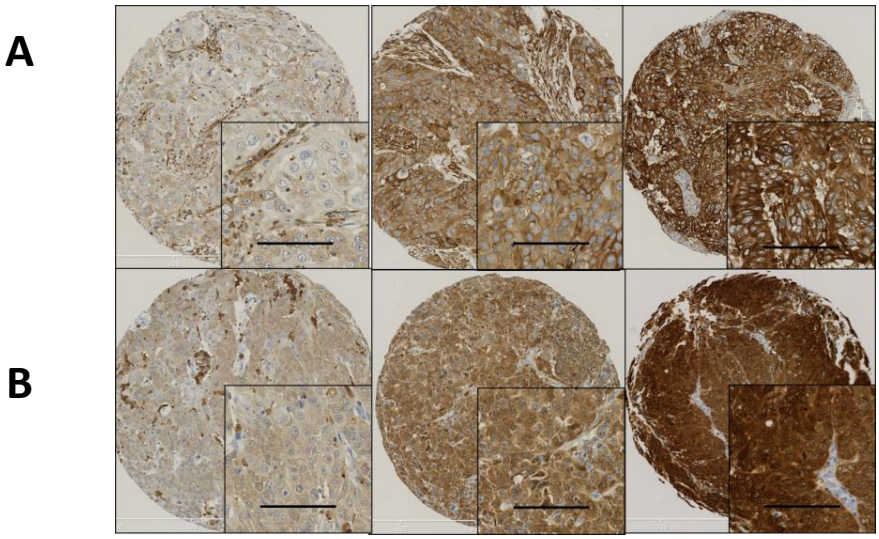


Figure 5.2 Examples of immunohistochemical expression and location of MAP4 and Syk in ovarian carcinoma cells. Staining pattern, including weak (left), medium (middle) and strong (right) staining of (A) MAP4 (10×, 20× magnification) and (B) Syk. Scale bar represents 100 μm.

5.4.2 Relationships between the expression of MAP4 and Syk and clinicopathological factors

High MAP4 expression was observed in 291 (66%) out of 442 available cases for this marker (cut-point: 152; mean±SE: 177±3; median±SD: 200±70). Pearson's Chi-squared test was performed to evaluate the associations of clinicopathological factors with MAP4 expression (**Table 5.1**). Among the ovarian cancer histological subtypes, HGSCs tended to express MAP4 of a higher level whilst mucinous, CCCs and LGSCs had lower MAP4 expression ($\chi2$=56.386, d.f.=5, *P*=6.7681E-11). Low expression was significantly associated with lower stage ($\chi2$=15.857, d.f.=3, *P*=0.001) and lower grade ($\chi2$=22.933, d.f.=2, *P*<0.001). In addition, a significant association was detected between high MAP4 expression and the presence of residual tumour ($\chi2$=10.426, d.f.=2, *P*=0.005). However, no statistically significant correlation was detected between MAP4 expression and chemo-resistance in patients treated by taxane-containing chemotherapy ($\chi2$=3.243, d.f.=1, *P*=0.072). The above analysis was also conducted in chemotherapy naïve cases (n=448). Significant associations were found between MAP4 expression and subtype ($\chi2$=45.528, d.f.=5, *P*<0.001), grade ($\chi2$=14.209, d.f.=2, *P*=0.001), stage ($\chi2$=15.050, d.f.=3,

P=0.002), residual disease (χ2=9.965, d.f.=1, *P*=0.007) and tumour response to taxane-containing chemotherapy regimens (χ2=4.596, d.f.=1, *P*=0.032; high MAP4 expression associated with chemo-sensitisation).

Table 5.1 Association between the MAP4 expression and clinicopathological criteria.

Variable	MAP4		
	Low	High	*P*-value
Age			
≤62	82 (18.6%)	136 (30.8%)	0.169
>62	70 (15.9%)	153 (34.7%)	
Histological subtypes			
HGSC	59 (13.3%)	211 (47.7%)	**<0.001***
Mucinous	25 (5.7%)	16 (3.6%)	
Endometrioid	22 (5.0%)	33 (7.5%)	
CCC	29 (6.6%)	16 (3.6%)	
LGSC	11 (2.5%)	8 (1.8%)	
SBOT	6 (1.4%)	6 (1.4%)	
Grade			
1	16 (3.6%)	19 (4.3%)	**<0.001**
2	38 (8.6%)	28 (6.3%)	
3	97 (22.0%)	243 (55.1%)	
Stage			
I	75 (17.2%)	89 (20.5%)	**0.013**
II	12 (2.8%)	36 (8.3%)	
III	52 (12.0%)	140 (32.2%)	
IV	10 (2.3%)	21 (4.8%)	
Residual disease			
No residual tumour	97 (24.8%)	144 (36.8%)	**0.005**
Residual tumour (<2cm)	10 (2.6%)	38 (9.7%)	
Residual tumour (>2cm)	27 (6.9%)	75 (19.2%)	
Response to platinum-based chemotherapy			
Chemo-refractory	8 (2.8%)	22 (7.8%)	0.509
Chemo-resistance	8 (2.8%)	11 (3.9%)	
Chemo-sensitivity	72 (25.5%)	161 (57.1%)	
Response to taxane-containing (i.e. paclitaxel, docetaxel) chemotherapy regimens			
Chemo-resistance	9 (7.5%)	10 (8.3%)	0.072
Chemo-sensitive	27 (22.5%)	74 (61.7%)	

HGSC: high grade serous carcinoma; LGSC: low grade serous carcinoma; CCC: clear-cell carcinoma; SBOT: serous borderline ovarian tumour

* Expected count less than 5. Significant *P*-values are indicated by bold type.

Out of 441 available cases, high cytoplasmic Syk expression was observed

in 374 (85%) cases and high nuclear Syk expression was observed in 329 (75%) cases for this marker (cytoplasmic Syk cut-point: 65; mean±SE: 154±4; median±SD: 150±85; nuclear Syk cut-point: 12%; mean±SE: 59±2%; median±SD: 20±40%). Pearson's Chi-squared test was performed to evaluate the associations of clinicopathological factors with Syk expression (**Table 5.2**). Among the ovarian cancer histological subtypes, HGSCs often displayed higher Syk expression in the cytoplasm and nucleus whilst CCCs tended to have lower cytoplasmic and nuclear Syk expression (cytoplasmic Syk: $\chi2=58.835$, d.f.=5, $P=2.115E-11$ and nuclear Syk: $\chi2=29.874$, d.f.=5, $P=0.000016$ respectively). Low cytoplasmic Syk expression was found associated with lower stage ($\chi2=10.725$, d.f.=3, $P=0.013$). The cytoplasmic Syk expression did not show significant association with patient response to taxane-containing chemotherapy ($\chi2=0.082$, d.f.=1, $P=0.774$), whilst low nuclear Syk expression significantly associated with chemo-resistance in patient treated by therapy containing taxane ($\chi2=7.582$, d.f.=1, $P=0.006$). Original tables of significant associations in **Table 5.1** and **Table 5.2** (Crosstab and Chi-Square Tests) from SPSS software with the expected and observed counts are listed in the Appendix (**Table S5.1**, **Table S5.2**, **Table S5.3**, **Table S5.4**, **Table S5.5**, **Table S5.6**, **Table S5.7**and **Table S5.8**).

The above analysis was also conducted in chemotherapy naïve cases (n=448). Significant associations were found between Syk expression and subtype (cytoplasmic Syk: $\chi2=46.443$, d.f.=5, $P<0.001$; nuclear Syk: $\chi2=26.744$, d.f.=5, $P<0.001$), stage (only cytoplasmic Syk: $\chi2=11.520$, d.f.=3, $P=0.009$), and tumour response to taxane-containing chemotherapy regimens (only nuclear Syk: $\chi2=7.582$, d.f.=1, $P=0.006$).

Table 5.2 Association between the Syk expression and clinicopathological criteria.

Variable	Cytoplasmic Syk expression			Nuclear Syk expression		
	Low	High	P-value	Low	High	P-value
Age						
≤62	30 (6.8%)	184 (41.8%)	0.492	51 (11.6%)	163 (37.0%)	0.447
>62	37 (8.4%)	189 (43.0%)		61 (13.9%)	65 (37.5%)	
Histological subtypes						
HGSC	25 (5.7%)	247 (56.0%)	**<0.001***	57 (12.9%)	215 (48.8%)	**<0.001***
Mucinous	9 (2.0%)	34 (7.7%)		10 (2.3%)	33 (7.5%)	
Endometrioid	9 (2.0%)	45 (10.2%)		16 (3.6%)	38 (8.6%)	
CCC	23 (5.2%)	21 (4.8%)		25 (5.7%)	19 (4.3%)	
LGSC	1 (0.2%)	17 (3.9%)		4 (0.9%)	14 (3.2%)	
SBOT	0 (0.0%)	10 (2.3%)		0 (0.0%)	10(2.3%)	
Grade						
1	4 (0.9%)	29 (6.6%)	0.813	7 (1.6%)	26 (5.9%)	0.174
2	9 (2.0%)	56 (12.7%)		11 (2.5%)	54 (12.2%)	
3	54 (12.2%)	289 (65.5%)		94 (21.3%)	249 (56.5%)	
Stage						
I	36 (8.3%)	123 (28.3%)	**0.013***	50 (11.5%)	109 (25.1%)	0.209
II	6 (1.4%)	43 (9.9%)		12 (2.8%)	37 (8.5%)	
III	20 (4.6%)	175 (40.3%)		42 (9.7%)	153 (35.3%)	
IV	5 (1.2%)	26 (6.0%)		8 (1.8%)	23 (5.3%)	
Residual disease						
No residual tumour	44 (11.3%)	195 (50.0%)	0.227	72 (18.5%)	167 (42.8%)	0.076
Residual tumour (<2cm)	5 (1.3%)	41 (10.5%)		10 (2.6%)	36 (9.2%)	
Residual tumour (>2cm)	13 (3.3%)	92 (23.6%)		20 (5.1%)	85 (21.8%)	
Response to platinum-based chemotherapy						
Chemo-refractory	6 (2.1%)	24 (8.5%)	0.589*	13 (4.6%)	17 (6.0%)	0.154
Chemo-resistance	2 (0.7%)	19 (6.7%)		6 (2.1%)	15 (5.3%)	
Chemo-sensitivity	35 (12.4%)	196 (69.5%)		61 (21.6%)	170 (60.3%)	
Response to taxane-containing (i.e. paclitaxel, docetaxel) chemotherapy regimens						
Chemo-resistance	3 (2.5%)	18 (14.8%)	0.774*	11 (9.0%)	10 (8.2%)	**0.006**
Chemo-sensitivity	17 (13.9%)	84 (68.9%)		23 (18.9%)	78 (63.9%)	

HGSC: high grade serous carcinoma; LGSC: low grade serous carcinoma; CCC: clear-cell carcinoma; SBOT: serous borderline ovarian tumour

* Expected count less than 5. Significant *P* values are indicated by bold font.

Patients were then grouped according to whether they had an organ-confined tumour (i.e. group 1: stage 1 and group 2: stage 2-4) or according to whether they had distant metastasis (i.e. group 1: stage 1-3 and group 2: stage 4). Higher significance was observed for MAP4 and Syk expression on tumour spreading when grouping patients into two broader categories by organ-confined status than grouping patients into the four categories by FIGO staging classifications (**Table S5.9**). Low cytoplasmic Syk, low nuclear Syk and low MAP4 showed significant association with organ-confined status of tumour (cytoplasmic Syk: $\chi2=9.975$, d.f.=1 , $P=0.002$; nuclear Syk: $\chi2=4.169$, d.f.=1, $P=0.041$; and MAP4: $\chi2=15.402$, d.f.=1, $P=0.000087$), whereas no significant association was detected between the protein expression and cancer distant metastasis. In chemotherapy naïve cases (n=448), low cytoplasmic Syk, low nuclear Syk and low MAP4 showed significant association with organ-confined status of tumour (cytoplasmic Syk: $\chi2=10.551$, d.f.=1, $P=0.001$; nuclear Syk: $\chi2=5.041$, d.f.=1, $P=0.025$ MAP4: $\chi2=14.322$, d.f.=1, $P=0.000154$).

5.4.3 The expression of MAP4 and Syk and clinical outcomes

5.4.3.1 Single marker expression

Kaplan-Meier analysis for OS and PFS were conducted to investigate whether expression of MAP4, nuclear or cytoplasmic Syk had any prognostic significance. As shown in **Figure 5.3**, patients with high MAP4 expression tended to have poor OS and PFS as compared with patients with weak or no MAP4 staining, but no significant association between MAP4 expression and patient outcome was detected ($P=0.064$, $P=0.442$, respectively).

Syk expression in the cytoplasm was associated with neither OS nor PFS (**Figure 5.4**). Patients with high nuclear Syk expression tended to have a better OS and PFS as compared with those with low nuclear expression but no

significant association was detected (*P*=0.113, *P*=0.053, respectively; **Figure 5.5**). After grouping patients into the following 4 groups (i.e. high nuclear Syk and high cytoplasmic Syk; high nuclear Syk and low cytoplasmic Syk; low nuclear Syk and high cytoplasmic Syk; low nuclear Syk and low cytoplasmic Syk) there was still no significant association between Syk expression and OS (*P*=0.424, **Table S5.10**).

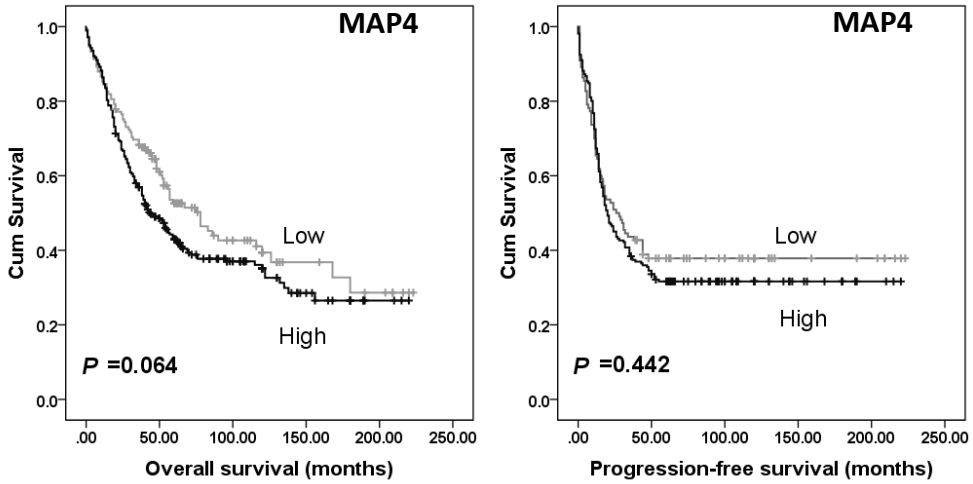


Figure 5.3 Kaplan-Meier survival curves show the impact of MAP4 on the OS and PFS.

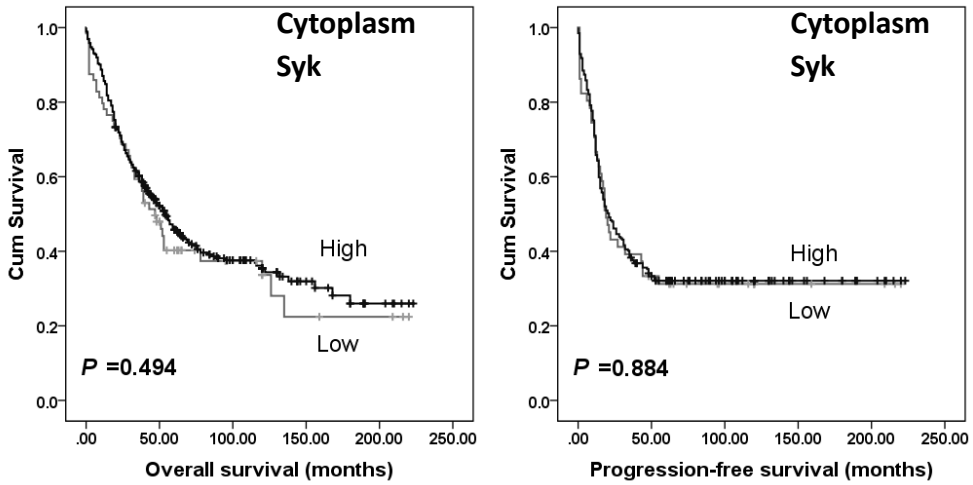


Figure 5.4 Kaplan-Meier survival curves show the impact of cytoplasmic Syk expression on the OS and PFS.

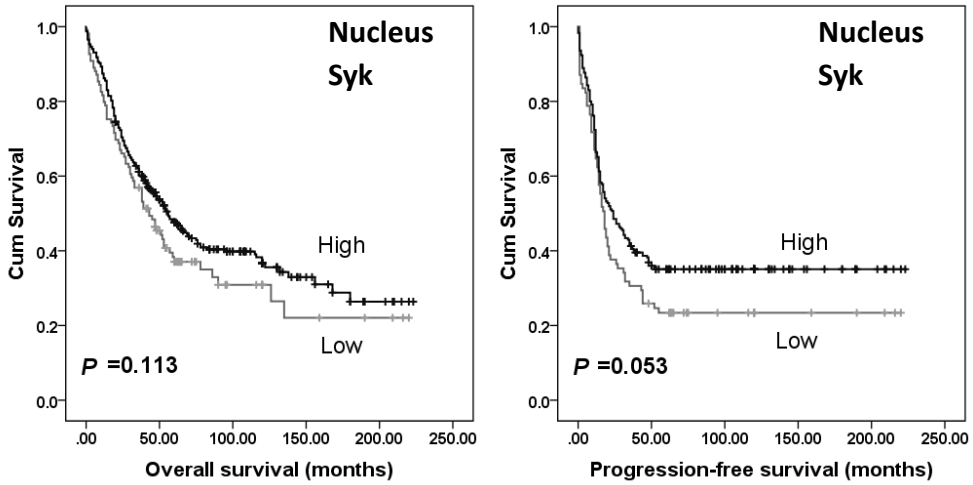


Figure 5.5 Kaplan-Meier survival curves show the impact of nuclear Syk expression on the OS and PFS.

5.4.3.2 The association between protein expression and OS in subgroups

Patients were divided into subgroups based on clinicopathological variables and Kaplan-Meier analysis was then conducted in the subgroups of patients. The *P* values of the Kaplan-Meier survival curves for OS in association with each protein expression are listed in **Table S5.11**. No significant association was detected between MAP4 expression and OS in any of the subgroups tested. Although high nuclear expression seemed to associate with better OS in several subgroups, no statistically significant association was detected between cytoplasmic Syk expression and OS in any subgroup. High nuclear Syk expression indicated a better OS among platinum resistant patients (*P*=0.001, **Figure 5.6A**), patients with tumours confined to the ovaries (stage 1 ovarian cancer) (*P*=0.001, **Figure 5.6B**) or patients with no residual disease (*P*=0.001) (**Figure 5.6C**).

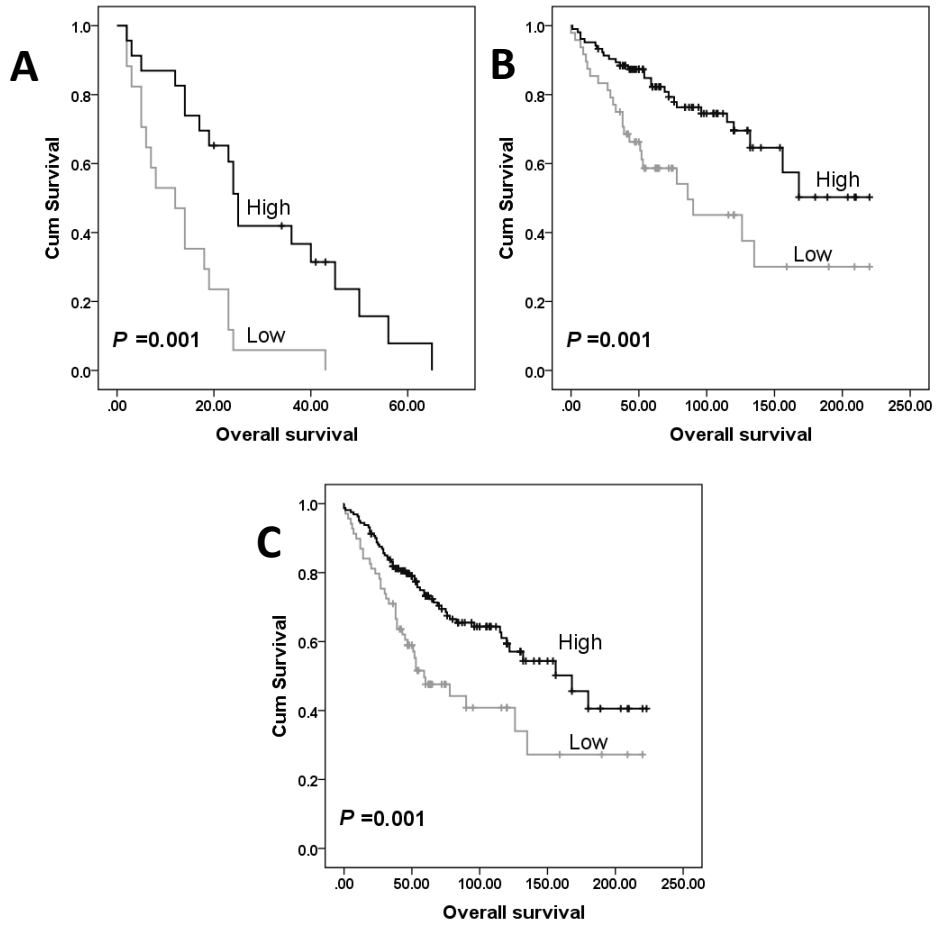


Figure 5.6 Kaplan-Meier survival curves show the impact of Syk expression on OS in different subgroups. (A) The impact of Syk expression in the nucleus on the OS of patients resistant to platinum-based chemotherapy. (B) The impact of Syk expression in nucleus on the OS of patients with tumour confined to the ovaries. (C) The impact of Syk expression in nucleus on the OS of patients with no residual carcinoma.

5.4.3.3 Combined marker expression

Due to the heterogeneous expression of tumour-related genes, it is generally believed that combined marker expression is more reliable, sensitive, and specific than a single-marker assay. Multiple molecular markers might predict the survival of ovarian cancer more significantly than any single molecular marker. Kaplan–Meier Univariate survival analyses of OS was conducted in relation to combinational protein expression in the whole cohort (**Table S5.10**). In this chapter, MAP4, Syk and the calpain system were studied as potential biomarkers, and their relations with other potential biomarker

CXCR3 and CXCL4 (data provided by Dr S. Deen) were also studied. The correlations between the potential biomarkers mentioned here were analysed and will be fully discussed in **section 5.4.4.1**. As mentioned above, using as single marker, neither MAP4 nor Syk expression was significantly associated with OS. Tumours were subsequently recategorised according to expression of any two of MAP4, Syk, calpain-1, -2, -4 and calpastatin into four groups each time; for example, using calpastatin and MAP4 expression the recategorised groups are: tumour with low expression of both MAP4 and calpastatin; tumour with high expression of both MAP4 and calpastatin; tumour with low expression of MAP4 and high expression of calpastatin; tumour with high MAP4 expression and low calpastatin expression. On the survival analysis, tumours with high MAP4 and low calpastatin expression had the shortest OS when compared with the other three groups (P=0.024); tumours with low MAP4 and low calpain-2 expression or low MAP4 and high calpain-4 expression had the highest OS when compared with the other three groups (P=0.037, P=0.034, respectively), but the results were less significant than those obtained with single calpain system members. Other ways of combining the protein expression status did not show prognostic significance in patient survival. When combining CXCL4 or CXCR3 expression with other potential markers, no significant association was detected between OS and the combinational protein expression (**Table S5.10**).

5.4.4 Correlations between calpain family expression and calpain-associated protein expression

5.4.4.1 Protein expression correlations in the whole cohort and in each histological subtype

The correlations between any two of the following proteins were studied in both the whole cohort and in each group divided by the 5 main histological subtypes respectively using Spearman's rank test; the proteins including the

calpain system (i.e. calpain-1, -2, -4 and calpastatin), MAP4, Syk and other proteins that are related to tumour progression (e.g. CXCR3 and CXCL4 study conducted by Dr S. Deen) (**Table 5.3**).

Table 5.3 Spearman's rank correlation coefficient between the expression of calpain system and the expression of potential correlated proteins

		Calpastatin	Calpain-1	Calpain-2	Calpain-4
MAP4	**Correlation Coefficient**	**.244****	**.537****	.143**	**.286****
	Sig.	4.8817E-7	2.3144E-32	.004	2.8108E-9
	N	414	415	414	415
Cytoplasmic Syk	**Correlation Coefficient**	.135**	**.396****	.127**	.145**
	Sig.	.006	4.3041E-17	.010	.003
	N	415	416	415	416
Nuclear Syk	**Correlation Coefficient**	.160**	**.346****	.082	.129**
	Sig.	.001	4.0912E-13	.097	.009
	N	415	416	415	416
CXCL4	**Correlation Coefficient**	.065	.130*	.007	.040
	Sig.	.245	.019	.898	.469
	N	326	327	327	327
CXCR3	**Correlation Coefficient**	-.004	**.317****	.149**	.059
	Sig.	.939	3.4196E-10	.004	.253
	N	374	375	375	375

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Significant P-values are indicated by bold type.

In agreement with the findings reported in 2016 FASEB summer conference, calpain expression was found moderately or weakly correlated with MAP4 and Syk expression respectively. In the whole cohort, most of the protein pairs were weakly correlated or lack of correlations, except that MAP4 expression was moderately correlated with calpain-1 (rs=0.537, *P*<0.001); in addition, cytoplasmic and nuclear Syk expression were strongly associated with each other (rs=0.809, *P*<0.001) (**Table S5.12** & **Table S5.13**). The

correlation was studied in each histological subtype separately. The moderate correlation between calpain-1 and MAP4 was also observed in HGSCs (rs=0.472, *P*<0.001, n=255) and CCCs (rs=0.516, *P*<0.001, n=42), whilst strong correlation was observed in endometrioid (rs=0.687, *P*<0.001, n=50) and mucinous tumours (rs=0.708, *P*<0.001, n=36), but no significant correlation was detected between calpain-1 and MAP4 in LGSC (data not shown). Between calpain-1 and Syk expression, weak correlation was found in the whole cohort and also in HGSCs (cytoplasmic Syk: rs=0.347, *P*<0.001, n=257; nuclear Syk: rs=0.313, *P*<0.001, n=257) and CCCs (cytoplasmic Syk: rs=0.378, *P*=0.014, n=42; nuclear Syk: rs=0.327, *P*=0.035, n=42), whilst a moderate correlation was found in mucinous tumours (cytoplasmic Syk: rs=0.425, *P*=0.007, n=39; nuclear Syk: rs=0.471, *P*=0.002, n=39), no correlation was detected between calpain-1 and Syk expression in LGSCs or endometrioid tumours (data not shown). Either no correlation or weak correlation was detected between MAP4 and nuclear Syk expression in the whole cohort and in the 5 groups of histological subtypes (data not shown). Between MAP4 and cytoplasmic Syk expression, weak correlation was found in the whole cohort and also in HGSCs (rs=0.312, *P*<0.001, n=265) and mucinous tumours (rs=0.376, *P*=0.018, n=39), whilst a moderate correlation was found in CCCs (rs=0.576, *P*<0.001, n=44); no correlation was detected between MAP4 and Syk expression in LGSCs or endometrioid tumours (data not shown). It is noticeable that CXCR3 and Syk expression lacked correlation or was weakly correlated in the whole cohort and 4 of the histological subtypes; only in LGSC type, strong correlation was detected (cytoplasmic Syk: rs=0.640, *P*=0.006, n=17; nuclear Syk: rs=0.606, *P*=0.010, n=17). These findings suggest that the role this panel of proteins played and how they cooperated with each other seems to be histological subtype-dependent.

5.4.4.2 Protein expression correlations in 87 HGSC cases of the whole cohort

Additional biomarkers were available in 87 HGSC cases of the cohort and a similar analysis (to what was mentioned in **section 5.4.4.1**) was conducted in this cohort which included angiogenic-related proteins: vascular endothelial growth factor (VEGF), CXC chemokine ligands: CXCL1, CXCL4 and CXCL8; CXC chemokine receptor: CXCR3; silent/decoy CXC chemokine receptor: Duffy antigen receptor for chemokines (DARC); integrin α2β3; N-, E- and P-cadherin. CXCR3 is the receptor of CXC chemokines CXCL9, CXCL10, CXCL11 and CXCL4 which are involved in inducing inflammation and inhibiting angiogenesis (Lasagni *et al*. 2003; Furuya *et al*., 2011; 2012). VEGF (Ai *et al*., 2016; Devery *et al*., 2015; Liang *et al*, 2006), CXCL1 and CXCL8 (Bièche *et al*., 2007; Devapatla *et al*., 2015; Kristjánsdóttir *et al*., 2014; Singha *et al*., 2014) are involved in neovascularisation, tumour cell proliferation and metastasis which lead to ovarian cancer progression. The expression switch from E-cadherin to P- and N- cadherin is involved in EMT and tumour malignant progression (Roggiani *et al*., 2016). Spearman's rank correlations between proteins in this panel and calpain-1, -2, -4, calpastatin, Syk and MAP4 were analysed and listed in **Table S5.13**.

In the 87 HGSC patients from the whole cohort, the correlations between the MAP4/Syk and the EMT- or angiogenesis-associated proteins were assessed based on the information from FASEB summer conference (**section 5.2**). No significant correlation was found between Syk and cadherin. Significant correlations were found between Syk and nuclear DARC (cytoplasmic Syk: rs=0.399, *P*=0.001; nuclear Syk: rs=0.240, *P*=0.037). Nuclear CXCL8 was weakly correlated with cytoplasmic Syk (rs=0.320, *P*=0.012) whilst cytoplasmic CXCL8 was weakly correlated with MAP4 (rs=0.274, *P*=0.031). Weak correlation was also observed between VEGF and MAP4 (rs=0.307,

P=0.014). No strong correlation was found between Syk expression and EMT or MAP4 expression and angiogenesis in the present study. Different isoforms of VEGF could be considered in the future studies to see whether there are correlations between MAP4 and specific VEGF isoforms.

The extent of correlations between calpain-1, -2, -4, calpastatin, Syk, MAP4, CXCR3, and CXCL4 in 87 HGSC cases were similar when compared to the measuring extent of correlations calculated from the whole cohort. Notably, calpain-1 expression showed stronger correlation with both MAP4 and cytoplasmic Syk (rs=0.650 and 0.607, *P*<0.001) in the 87 HGSCs (**Table S5.13**). In addition, calpain-4 showed a stronger correlation with MAP4 (rs=0.451, *P*<0.001) in the 87 HGSCs when compared with the correlation from the whole cohort. Both cytoplasmic and nuclear Syk moderately correlated with MAP4 (rs=0.560 and 0.503 respectively, both *P*<0.001) (**Table S5.14**). However, these stronger correlations observed in the 87 HGSCs were not observed when including all HGSCs cases from the whole cohort into the calculation (**section 5.4.4.1**). Additional protein expression data from the 87 HGSC patients (**Table S5.13** & **Table S5.14**) indicated that integrin α2β3 was significantly correlated with calpain-1 (rs=0.517, *P*<0.001), MAP4 (rs=0.501, *P*<0.001) and cytoplasmic Syk (rs=0.286, *P*=0.030). No other significant correlation was detected between the calpain system and cadherins (i.e. E-, P- and N-cadherin) except that calpastatin was weakly and negatively correlated with N-cadherin (rs=-0.378, *P*=0.001). Other pairwise correlations were either weak or not significant.

5.5. Discussion

In the current study, a significant association was detected between high MAP4 expression and the presence of residual tumour ($\chi 2$=10.426, d.f.=2, *P*=0.005). Low MAP4 expression was significantly associated with lower stage

($\chi 2$=15.857, d.f.=3, *P*=0.001) which agreed with the study of esophageal squamous cell carcinomas (n=364), in which MAP4 expression was associated with tumour stage and lymph node metastasis (Jiang *et al*., 2016). Low MAP4 expression was significantly associated with lower grade ($\chi 2$=22.933, d.f.=2, *P*<0.001) which was also observed in bladder cancer (n=34) where high cancer grade was correlated with significant high MAP4 expression (Ou *et al*., 2014). The invasion of bladder cancer cells was found markedly inhibited by the knockdown of MAP4 (Ou *et al*., 2014). Ou *et al*., (2014) suggested a mechanism involved in inhibiting cancer cell invasion, as follows: the phosphorylation of MAP4 induced by cyclic adenosine monophosphate/ protein kinase A pathway dissociates MAP4 from microtubules which disrupts the stability of microtubule cytoskeleton. The positive association between MAP4 expression and cancer cell invasion and migration was also observed in esophageal squamous cell carcinoma (Jiang *et al*., 2016) where MAP4 was found upregulating vascular endothelial growth factor A expression by ERK (extracellular signal-regulated kinases)-c-Jun signalling pathway (Jiang *et al*., 2016).

In the present study, Kaplan-Meier analysis indicated that the expression of MAP4, nuclear Syk and cytoplasmic Syk had no prognostic significance in predicting patient OS or PFS in the whole cohort. Patients were then grouped by histological subtypes and clinicopathological variables. Although a higher level of MAP4 mRNA was observed in non-small cell lung carcinomas when compared to normal lung tissues (Cucchiarelli *et al*., 2008) and MAP4 expression was associated with shorter survival of the esophageal squamous cell carcinoma patients (n=364) (Jiang *et al*., 2016), no significant association was detected in the present study between MAP4 expression and OS in either the whole cohort or any of the subgroups tested. As mentioned in **section 5.2.1.2**, the association between MAP4 and paclitaxel resistance has been

observed in different cancer types, but in the present study the correlation between low MAP4 and chemo-resistance was not significant in patients treated by taxane-containing chemotherapy ($\chi2$=3.243, d.f.=1, *P*=0.072). To understand the association between MAP4 and paclitaxel resistance, the levels of phosphorylated forms of MAP4 (inactivated form of MAP4) should be considered. In chemotherapy-naïve cases, the correlation between high MAP4 expression and chemo-sensitisation was statistically significant in patients treated by taxane-containing chemotherapy ($\chi2$=7.813, d.f.=1, P=0.020).

The paradoxical roles of Syk, reviewed in **section 5.2.2,** were also observed in the current study. Among the ovarian cancer histological subtypes, HGSCs displayed higher Syk expression in cytoplasm and nucleus whilst CCCs tended to have lower cytoplasmic and nuclear Syk expression (cytoplasmic Syk: $\chi2$=58.835, d.f.=5, *P*=2.115E-11 and nuclear Syk: $\chi2$=29.874, d.f.=5, *P*=0.000016 respectively). The high Syk expression in HGSCs is against the finding from other studies that Syk was absent from many highly aggressive epithelial cell-derived tumours (Krisenko & Geahlen, 2015; Coopman & Mueller, 2006), but might be explained by the pro-survival function of Syk involving anti-apoptosis and invasive growth, cancer cell migration and dissemination (Luangdilok *et al*., 2007; Ghotra *et al*., 2015) as reviewed in **section 5.2.2**. The low Syk expression in CCCs, as mentioned in **section 3.5**, might be mainly due to the clear cytoplasm. In addition, the present study indicated that low cytoplasmic Syk expression was associated with lower tumour stage ($\chi2$=10.725, d.f.=3, *P*=0.013; **section 5.4.2**). Different from the positive association between cancer spreading and Syk expression observed in the current study, Krisenko & Geahlen (2015) indicated that Syk may increase cell-cell interactions and limit EMT and migration. Syk knockdown in more well differentiated cancers was found to enhance invasive/anchorage-independent growth, and motility (Fei *et al*., 2013) whilst its re-introduction in more

malignant, invasive cancer cells decreased cancer malignancy through increasing adhesion and reducing tumour cell growth, motility, invasion and metastasis (Ogane *et al*., 2009; Fei *et al*., 2013; Peng *et al*., 2013; Krisenko & Geahlen, 2015). Paradoxically, in the current study, the tumour-suppressive effect of nuclear Syk was reflected in its negative association with chemo-resistance and positive association with OS when patients have tumours confined in ovaries (stage 1 ovarian cancer) (P=0.001). High Syk expression in the nucleus was associated with better OS in the subgroup of platinum resistant patients (P=0.001) and in the group of patients with no residual disease (P=0.001). The association between high Syk expression and better OS in these subgroups agrees with the tumour suppressive function of Syk (as reviewed in **section 5.2.2**). Together, the current results suggest that high Syk expression might facilitate tumour spreading whilst high nuclear Syk sensitises tumour cells to taxane-containing chemotherapy.

The discrepancy might be due to the localisation of Syk, despite cytoplasmic and nuclear Syk expression being strongly and positively correlated with each other (rs=0.809, P<0.001). Full-length Syk is known as Syk long isoform, Syk(L), whilst the shorter gene product SykB omits a stretch of 23 amino acids in linker B which is also known as Syk(S) (Krisenko & Geahlen, 2015). Syk(L) and Syk(S) were found in both nucleus and cytoplasm in B lymphocytes and breast cancer cells (Zhou et al., 2006; Luangdilok S. et al., 2007) with Syk(L) predominantly presented in nucleus whilst Syk(S) in cytoplasm in untreated tumour cells (Prinos et al., 2011). Yet “the basis of the differential localization of Syk(L) and Syk(S) is not entirely clear” (Krisenko & Geahlen, 2015). Wang et al. (2003) suggest that the subcellular distribution of Syk can be affected by Syk splicing due to a nonclassical nuclear localization signal located in the alternative exon on Syk(L). Consistently, it is Syk(L), rather than Syk(S), that can enter into nucleus where it interacts with histone

deacetylases and the transcription factor Sp1 to downregulate the transcription of Sp1-regulated oncogenes (e.g. CCD1 (cyclin D1) and FRA1 (FOSL1)) (Wang et al., 2005). Corresponding to the above mechanism, the presence of nuclear Syk was of positive prognostic significance in gastric cancer (Nakashima et al. 2006) and in certain subgroups in the current ovarian cancer study. In addition, via altering gene expression, Syk has been found to regulate EMT by influencing cellular differentiation programs (Krisenko & Geahlen, 2015) which agreed with the positive association between high cytoplasmic Syk expression and high tumour stage in the current study. The function of Syk(L) appeared to vary with its localisation: nuclear Syk(L) can repress JUN expression and promote cell survival whereas cytoplasmic Syk(L) can interact with the centrosome and microtubules to affect mitotic spindle assembly and cytokinesis (Prinos et al., 2011). Specifically inhibiting Syk(L)/ changing the alternative splicing of *SYK* increased cells with abnormal nuclear morphology (i.e. aberrant, larger or/and multiple nuclei) indicating that Syk(L) is involved in mitotic arrest (Prinos et al., 2011). And this Syk(L)–dependent effect on mitosis was compensated by the increase of Syk(S) expression (Prinos et al., 2011). Syk(S) was found frequently expressed in primary breast tumours but not in matched normal mammary tissues, suggesting a contribution of Syk(S) to tumour progression (Wang et al. 2003). Prinos et al., (2011) concluded that altering the splicing pattern/cellular localization of Syk may induce apoptosis whereas the alteration of both Syk splicing and expression affects mitosis and cell proliferation. As for anchorage-independent growth is mainly regulated by overall Syk expression rather than splicing. Luangdilok S. et al., (2007) detected Syk(S) expression in six Syk-positive cell lines via RT-PCR or Western blot, however, no selective antibodies are available to discriminate the specific isoforms and clarify their localization via immunohistochemistry. Syk splicing pattern was linked to ovarian but not

breast cancer (Klinck et al., 2008; Venables et al. 2008; Prinos et al., 2011) and SKOV3 cells were found preferentially expressing Syk(L) (Prinos et al., 2011). The above highlighted the importance of assessing Syk with the consideration of its isoform/localisation in addition to its expression. Although Syk(L) and Syk(S) cannot be discriminated via IHC, nuclear Syk and cytoplasmic Syk were studied separately in the current study; however, neither nuclear/cytoplasmic Syk nor the total Syk expression was of prognostic value. The correlation between Syk nuclear localisation and the invasion suppression function of full-length Syk indicated that nuclear Syk possesses biological activities associated with tumour suppression in mammary epithelial cells (Wang L. *et al*., 2003). In the current study, the tumour-suppressive effect of nuclear Syk also reflected in its negative association with chemo-resistance. HGSC and endometrioid carcinomas tend to have high intrinsic chemo-sensitivity whilst the intrinsic chemo-sensitivity of CCC, mucinous and LGSC is lower (Hollis & Gourley, 2016; Mabuchi *et al*., 2016). Thus, the significant association between low nuclear Syk expression and CCCs ($\chi2=29.874$, d.f.=5, $P=0.000016$) agreed with the significant association between low nuclear Syk expression and chemo-resistance in patient treated by therapy containing taxane ($\chi2=7.582$, d.f.=1, $P=0.006$), although cytoplasmic Syk expression was not significanty associated with patient chemoresponse to either platinum-containing or taxane-containing therapy. By contrast, the study conducted by Yu *et al*. (2015) found that high Syk was associated with chemo-resistance in ovarian cancer cells. Moreover, nuclear Syk can also act as pro-survival factor; restriction of Syk to the nucleus diminishes the stress-induced activation of caspase 3 in B-cells (Zhou *et al*., 2006; Mohammad *et al*., 2016) and tumour cells (Wang L. *et al*., 2005). As the cohort used in the current study was constructed using one core per patient, the analyses conducted in each subgroup were limited by the case number that

the heterogeneity of Syk might not be fully revealed. Thus the findings of this study should be validated in larger cohort with the whole section study. The association with clinical parameters and patient outcome might be further revealed by study the ratio of phosphorylated Syk (activate form of Syk) and total Syk (**section 5.2.2.1**).

Different histologic subtypes of epithelial ovarian cancer can be seen from protein expression profiles (Jia *et al*., 2012; Foss *et al*., 2014). As indicated in **section 3.4.2** and **5.4.2**, histologic subtypes of ovarian cancer significantly associated with the expression of calpain-1 ($\chi2=56.577$, d.f.=5, $P<0.001$), calpain-4 ($\chi2=18.181$, d.f.=5, $P=0.003$), calpastatin ($\chi2=17.403$, d.f.=5 , $P=0.004$), MAP4 ($\chi2=56.386$, d.f.=5, $P<0.001$), cytoplasmic and nuclear Syk expression (cytoplasmic Syk: $\chi2=58.835$, d.f.=5, $P<0.001$ and nuclear Syk: $\chi2=29.874$, d.f.=5, $P<0.001$ respectively). And as a panel of proteins, calpain-1, -2, -4, calpastatin, MAP4 and Syk, they presented different expression pattern in different histological subtypes. The cross-tabulation (Chi-Squared) suggested that HGSC is associated with high calpain-1, -4, calpastatin, MAP4 and Syk expression, whilst LGSCs is associated with low MAP4 expression without any clear association with other markers, which could be due to the small patient numbers. CCCs were distinct from HGSCs as all the five proteins tended to be expressed at a lower level in this histological subtype of ovarian cancer. Mucinous carcinomas were associated with low calpain-1 and MAP4 expression. Endometrioid carcinomas only showed association with low calpain-1 expression. As indicated in **section 3.5**, caution should be made when interpreting the association between protein expression and histological subtypes, especially CCCs and mucinous tumours.

To the best of our knowledge, this is the first report studying the expression of Syk, MAP4 and the calpain system in human ovarian carcinomas.

MAP4, Syk and calpain-1 appeared to be closely associated with each other in influencing tumour spreading. Patients were grouped according to whether they had an organ-confined tumour (i.e. group 1: stage 1 and group 2: stage 2-4) or according to whether they had distant metastasis (i.e. group 1: stage 1-3 and group 2: stage 4). Low cytoplasmic Syk, low nuclear Syk, low MAP4 (**Table S5.9**) and low calpain-1 (cytoplasmic Syk: $\chi2$=9.975, d.f.=1, *P*=0.002; nuclear Syk: $\chi2$=4.169, d.f.=1 , *P*=0.041; and MAP4: $\chi2$=15.402, d.f.=1, *P*=0.000087; $\chi2$=11.310, d.f.=1, *P*=0.001; calpain-1: $\chi2$=11.310, d.f.=1, *P*=0.001) expression showed significant association with organ-confined status of tumour, but no significant association was detected between their expression and cancer distant metastasis.

In addition, integrin α2β3 seems also associated with calpain-1 and MAP4. In the whole cohort with 469 valid cases, between the calpain system and MAP4, Syk, CXCR3 and CXCL4, the most significant correlation was found between calpain-1 and MAP4 (rs=0.537 and *P*<0.001). A panel of protein expression was studied on a limited number of HGSC patients from the whole cohort including vascular endothelial growth factor (VEGF), CXC chemokine ligands: CXCL1, CXCL4 and CXCL8, CXC chemokine receptor: CXCR3, silent/decoy CXC chemokine receptor: DARC, integrin α2β3, N-, E- and P-cadherin. Integrin α2β3 was moderately correlated with calpain-1, MAP4 and cytoplasmic DARC expression (rs=0.517, *P*<0.001; rs=0.501, *P*<0.001; rs=0.519, *P*<0.001). Both low calpain-1 and low MAP4 expression was found significantly associated with low cancer stage. The results implied that calpain-1, integrin, and MAP4 play important roles in tumour spreading.

As mentioned in **section 5.2.2.1** integrins are important regulator of Syk activity. The integrins are heterodimeric cell-surface receptors that are fundamental in cell-cell and cell-matrix adhesion. Calpain regulates integrin by

1) cleaving the associated regulatory proteins of integrin such as integrin adaptor protein talin which stimulates integrin activation (Liu *et al*., 2013; Yan *et al*., 2001; Huang *et al*., 2009; Bate *et al*., 2012); and 2) cleaving the cytoplasmic domain of integrin β3 subunit (Du *et al*., 1995; Pfaff *et al*., 1999; Xi X *et al*., 2006; Kumagai *et al*., 2008; Huang *et al*., 2016; Shi *et al*., 2016). Calpain-mediated cleavage of integrin β3 subunit at C-terminal Tyr(759) can switch the functional outcome of integrin signalling from cell spreading to retraction in Chinese hamster ovary cells (Flevaris *et al*., 2007). Inhibition of calpain cleavage of the β3 promoted integrin signalling and cell spreading (Xi X *et al*., 2006; Kulkarni *et al*., 2002). The activation of Src and Syk is upstream of calpain activation and is required for calpain-mediated cleavage of β3 (Maile *et al*., 2008). Calpain activation negatively regulates integrin-mediated adhesion, signalling, and cytoskeleton association during apoptosis of human umbilical vein endothelial cells (Meredith *et al*., 1998) and, at least partially, contributed to integrin αIIbβ3 inactivation (Liu F. *et al*., 2013; Mattheij *et al*., 2013; van Geffen *et al*., 2016). On the contrary, several studies indicated that the inhibition of calpain resulted in inhibited integrin-dependent cell spreading (Rosenberger *et al*., 2005). Calpain inhibitors were also found able to inhibit β1 and β3 integrin-mediated cell migration by decreasing the retraction rate of integrin receptors at the cell's rear and cell rear detachment during migration (Huttenlocher *et al*., 1997; Leitinger B. *et al*., 2000). For a clearer understanding of how calpain-1 and integrin interact with each other, future studies could consider evaluating calpain-cleaved forms of integrin in addition to the full-length integrins. Comparison studies between primary tumour site and matched metastases might identify how the protein expression changed to facilitate cancer progression.

To study whether the calpain system has cooperative participation with angiogenesis and EMT in the development of ovarian cancer, in the 87 HGSC

patients from the whole cohort, the correlations between the calpain system and biomarkers were assessed. Between the calpain system and cadherins, the only significant correlation was found between calpastatin and N-cadherin (rs=-0.378, *P*=0.001). Found in adhesion junctions, E-cadherin is mostly present in polarised epithelial cells whilst N-cadherins present in some mesenchymal tissues and mesothelium in addition to neural tissues, cardiac and skeletal muscle (Derycke & Bracke, 2004; McLachlan & Yap, 2007). Calpain has been shown to cleave E-cadherin (Konze *et al*., 2014; Ye *et al*., 2013; Rios-Doria *et al*., 2003) and is related to the decrease in E-cadherin expression or activation levels (Weber *et al*., 2009; Schaar A *et al*., 2016; Tan *et al*., 2017; Rios-Doria & Day, 2005; Trillsch *et al*., 2016). Calpain cleaved off the C terminus of E-cadherin under three-dimensional culture conditions (Konze et al., 2014) and calpain-cleaved E-cadherins (100kDa) were released from their traditional cadherin-based adherens junctions so that they can distribute along adjacent non-cadherin-based adherens junction areas which directly resulted in the spheroid-genesis and formation of lymphovascular embolus (Ye et al., 2013). Cleaved E-cadherins (100kDa) was found up-regulated in both localised and metastatic prostate tumours accompanied with calpain-2 accumulation (Rios-Doria et al., 2003). Rodríguez-Fernández et al. (2016) also indicated that calpain-2 was the only isoform co-localising with E-cadherin in cell junctions but it was calpain-1 that cleaved adhesion proteins and mediated cell migration in breast cancer cell line. Via this calpain-mediated E-cadherin truncation and functional inactivation, calpain disrupted the adhesion complex during the tumour progression (Rios-Doria et al., 2003; Rios-Doria & Day, 2005). In the study of epithelial ovarian cancer, by treating lysates from SKOV3 cells and epithelial ovarian tumours with calpain, another cleaved form of E-cadherin was detected and appeared mediating a potential mechanism for intraperitoneal epithelial ovarian cancer progression (Trillsch et

al., 2016). N-cadherin has been found as a calpain substrate in neural cells (Covault *et al*., 1991; Jang *et al*., 2009) and cardiomyocytes, where calpain led to disassembling of cell-cell adhesion (Kudo-Sakamoto *et al*., 2014). In calpain-4 shRNA-infected cells, E-cadherin expression was increased and N-cadherin expression was decreased compared with that of controls (Wang *et al*., 2017) which coincides with the negative association between calpastatin and N-cadherin in the current study. As we know, HGSC metastases include metastatic tumours growing in the solid stromal matrices and multicellular aggregates floating and growing in the malignant ascites (Roggiani et al., 2016). P-cadherin has been considered as the predominant cadherin subtype expressing in the aggregates of the peritoneal effusion. In addition, P-cadherin contributed to the adhesion of epithelial ovarian cancer cells to the peritoneum and the metastasis formation with its inhibition reducing tumour growth, ascites formation and metastasis in *in vivo* pre-clinical models (Roggiani et al., 2016). In the current study, no significant correlation was detected between cadherins and conventional calpains which suggest that the calpain system does not influence the expression level of cadherins in HGSCs, however, studying only the expression at the protein level cannot fully depict the interactions between calpain and cadherins; so it might be necessary to study the calpain-cleaved form of cadherins and the level of the cleaved form in addition to the full-length cadherins.

VEGF can mediate m-calpain activation (Yates-Binder et al., 2012). Calpains (e.g. calpain-4, Chillà et al., 2013; calpain-2, Chakrabarti et al., 2010; reviewed in Zhang Y et al., 2017) mediated VEGF-induced angiogenesis in human endothelial cells (Su et al., 2006; Youn et al. 2009; Hoang et al., 2010). During the regulation of VEGF-induced angiogenesis, besides the primary signalling pathway which involves VEGFR2, calpain and PI3K/AMPK/Akt, calpain negatively regulated VEGFR2 in a feedback loop (Zhang Y et al., 2017).

In the current study, no association can be detected between the calpain system and VEGF. But VEGF expression was significantly correlated with MAP4 (rs=0.307, P=0.014). As mentioned above, studying the activated or cleaved form of the proteins might provide a better understanding on protein functional characterisation and the interaction between these proteins.

The high expression of CXCL1, CXCL8, and/or VEGF by cancer cells was reported able to enhance inflammation and tumour growth, proliferation, migration, invasion and colony formation, especially angiogenesis (Dhawan & Richmond, 2002; Bolitho C et al., 2010; Singha B et al., 2014; Kavandi et al., 2012; Devapatla et al., 2015; Mikuła-Pietrasik et al., 2016a; 2016b; Duckworth et al., 2016). Overexpression of CXCR3 was found promoting ovarian tumour metastasis by enhancing cell migration and invasion (Lau TS et al., 2014; Windmüller et al., 2017). CXCL4 promoted prostate cancer cell motility and invasiveness partially via μ-calpain activation and CXCR3 inhibited cell motility seemed partially due to m-calpain inhibition through CXCR3B signal transduction (Wu, Dhir & Wells, 2012). Moreover, the migration of endothelial cell, keratinocyte and fibroblast was regulated by growth factors and chemokines through the modulation of calpain activity (Leloup L et al., 2010). CXCR3 has been suggested able to activate calpain (Bodnar et al., 2009; 2013); paradoxically, CXCR3 ligands inhibited cell migration by blocking m-calpain activity (Leloup L et al., 2010). In the current study, the lack of associations suggested that conventional calpain subunits and calpastatin expression did not synergise with the biomarkers involving in angiogenesis. Notably, studies indicated that there are at least three CXCR3 variants including CXCR3A, CXCR3B and CXCR3-alt; CXCR3A reflected inflammatory conditions and increased cell survival whilst CXCR3B has been reported with anticancer properties such as inducing apoptosis, inhibiting cellular proliferation and neovascularisation (Lasagni et al., 2003; Furuya et al., 2011). Study focused on

clear cell type ovarian cancers, including endometriosis-associated ovarian cancers (EAOC), endometriosis and normal ovaries, indicated that the expression of CXCR3A/CXCR3-alt and ligand CXCL11 expression was upregulated and CXCR3B and its ligand CXCL4 was downregulated in cancers. CXCL11–CXCR3-alt might play an opposite role to CXCL4–CXCR3B (Furuya et al., 2011; 2012). In like manner, in prostate cancer specimens, tumour metastasis was promoted by CXCR3A mRNA level upregulation and CXCR3B mRNA downregulation stimulated cell migration and invasion (Wu, Dhir & Wells, 2012). Thereby, it would be more precise and functional relevant to study the expression of different CXCR3 variants separately.

The migration of endothelial cells, keratinocytes and fibroblasts has been reported to be regulated by growth factors and chemokines through the modulation of calpain activity (Leloup L *et al*., 2010). To our knowledge, no study has investigated the correlations between the expression of the calpain system and the expression of chemokine CXCL1, CXCL4, CXCL8 together with the chemokine receptor CXCR3 and decoy receptor DARC in addition to VEGF which are all involved with neovascularisation. The present study assessed protein correlation using Spearman's correlation coefficient, which indicated that the expression of calpain-1 was weakly correlated with CXCR3 (rs=0.317, $P<0.001$), cytoplasmic and nuclear DARC (rs=0.303, $P=0.013$; rs=0.287, $P=0.019$) expression; calpastatin was weakly correlated with nuclear DARC expression (rs=0.247, $P=0.046$), besides which no stronger correlation was detected among these proteins. DARC is a glycosylated membrane protein and an atypical chemokine binder that selectively binds angiogenic chemokines (Latini *et al*., 2013). Various cancer studies (e.g. prostate, breast and ovarian cancers) have demonstrated that DARC inhibits tumour growth and progression by acting as silent/decoy CXC chemokine receptor (Smolarek *et al*., 2010; Maeda *et al*., 2017; Wang *et al*., 2006) and clearing the pro‑malignancy

chemokines (Lentsch, 2006). Increased DARC expression has been associated with better prognosis, lower metastatic potential, and inhibition of tumour neovascularity both *in vivo* and *in vitro* (Latini *et al*., 2013). In epithelial ovarian cancer, chemokines are known to interact with cancer cells and promote angiogenesis, which is essential for tumour growth (Zhu *et al*., 2017). DARC overexpression in epithelial ovarian cancer inhibited cell viability *in vitro* and inhibited tumour growth mainly via inhibiting angiogenesis in xenograft tumours (Zhu *et al*, 2017).

Data from **section 5.4.4** indicated that among calpain-1, -2, -4 and calpastatin, it was the expression of calpain-1 that was more closely associated with integrin α2β3, CXCR3, MAP4 and Syk expression; furthermore, it was the calpain-1 expression that significantly associated with residual disease and tumour stage, especially with the organ-confined status. It is likely that calpain-1 plays an important role in tumour spreading via different pathways such as the decrease of cell adhesion and angiogenesis. It is noteworthy that many studies used draining blood samples from peripheral and tumours to assess the serum levels of chemokines (Mikuła-Pietrasik *et al*., 2016a; 2016b; Spaks A *et al*., 2016) which might need to be included in future studies of protein correlations. Limited by the case number, the observations on account of the 87 HGSC patients from the whole cohort need further verification.

In conclusion, although MAP4 and Syk expression were not significantly associated with either OS or PFS using the same cohort as **Chapter 3**, both MAP4 and Syk were associated with tumour stages and the associations were more related to whether cancer cells had spread out of ovaries or still confined to the ovaries rather than whether ovarian cancer underwent distant metastasis or not. MAP4 and cytoplasmic Syk expression did not show

significant association with patient chemotherapeutic response. The current study also observed the paradoxical roles of Syk, both as tumour suppressor and pro-survival factor. High Syk expression indicated a better OS in certain subgroups of patients divided by the clinical variables. Low nuclear Syk expression significantly associated with chemo-resistance in patients treated by therapy containing taxane which support the role of Syk as a tumour suppressor. Contradictory, low cytoplasmic Syk expression was associated with low stage. Together, it appears from this study of ovarian cancers that high Syk expression might facilitates the tumour spreading (higher stage) whilst high nuclear Syk sensitises tumour cells to taxane-containing chemotherapy. In the whole cohort, MAP4 and Syk expression showed strongest correlations with calpain-1 when compared to calpain-2, calpain-4 and calpastatin. The correlation between MAP4 and Syk expression was also statistically significant. In the 87 HGSC patients from the whole cohort, there was strong correlation between MAP4 and calpain-1 expression both of which were moderately correlated with integrin α2β3. In addition, calpain-1 expression was strongly correlated with cytoplasmic Syk in these 87 HGSC patients. Numerous studies have indicated that integrins are calpain substrates, and most studies have suggested that calpains inhibit integrin-mediated cell spreading/migration (reviewed in **section 4.2.3** and **5.2.2**). Together, these findings suggest that in addition to paclitaxel resistance, integrin, calpain, Syk and MAP4 might be also involved in cancer cell spread. How MAP4, Syk, calpain-1 and integrin interconnect in tumour cell spreading/migration and in cell responding to taxane-containing chemotherapy remained to be further investigated.

5.6. Summary

1. MAP4 expression associated with grade and residual disease. Syk and MAP4 expression associated with histological subtype and tumour stage. Syk

and MAP4 were more closely related to whether the cancer cell had spread out of the ovaries or was confined to the ovaries rather than whether ovarian cancer underwent distant metastasis or not.

2. Low nuclear Syk expression significantly associated with chemo-resistance in patients treated by therapy containing taxane.

3. MAP4 and Syk expression showed no significant association with either OS or PFS using the same cohort as in Chapter 3. But high Syk expression in either the nucleus or cytoplasm indicated a better OS in certain subgroups of patients divided by clinical variables.

4. In the whole cohort, MAP4, calpain-1 and Syk significantly correlated with each other. A correlation study was conducted in the 87 HGSC cases from the whole cohort involving the following proteins: VEGF, CXCL1, CXCL4,CXCL8, CXCR3, DARC, integrin α2β3, N-, E- and P-cadherin, in addition to calpain-1, -2 and -4, calpastatin, MAP4 and Syk. Most of the protein pairs were weakly correlated or lacked correlations. There was strong correlation between MAP4 and calpain-1 expression both of which were moderately associated with integrin α2β3. In addition, calpain-1 expression was strongly correlated with cytoplasmic Syk in these 87 HGSC patients. DARC expression was significantly associated with calpain-1 and Syk expression.

Chapter 6. General discussion

6.1. General discussion

Ovarian cancer is one of the five most common cancers in females in the UK and with a 35% 10-year survival rate it is the leading cause of gynaecological cancer death (Cancer Research UK). Around 75% of ovarian cancer cases have progressed to advanced stages (with intra-abdominal or distant metastasis) at diagnosis contributing to its high rate of mortality (Dinh *et al*., 2008). Current standard regimen is platinum-based chemotherapy, but most patients experience disease recurrence after primary therapy and require further treatment. Chemotherapy resistance, tumour metastasis and recurrence are main obstacles hindering the improvement of patient outcome, it is therefore important to understand the mechanisms behind these processes and find novel prognostic factors and/or treatment approaches.

The conventional calpains, including μ-calpain (heterodimer of calpain-1 and -4) and m-calpain (heterodimer of calpain-2 and -4), belong to the family of cytoplasmic cysteine proteases which are involved in various cellular processes whose deregulation has been associated with various pathological conditions, including cancer (**section 1.4**). A previous IHC study from our group showed that high expression of calpain-2 was significantly associated with resistance to platinum-based adjuvant chemotherapy, poor PFS and OS (Storr S.J. *et al*., 2012). To address the question as to whether the calpain family plays an important role in ovarian cancer chemotherapeutic response, 5 ovarian cancer cell lines (**section 6.2.2**), with different sensitivities towards platinum-based chemotherapy, were included in this study. The protein expression levels of calpastatin and conventional calpain subunits in the 5 cell lines were examined by Western blotting. To our knowledge, this is the first

time that expression of these proteins was studied in a panel of ovarian cancer cell lines. As reviewed in **section 2.2.1.1**, the chemo-resistance of cells from certain cancer types is calpain-related; high calpain expression or calpain activity levels were observed in colon cancer and melanoma chemo-resistant cells when compared to their parental counterparts; whilst chemo-resistant gastric cancer cells had lower calpain expression than the parental counterpart. In the current ovarian cancer study, calpastatin and conventional calpain subunits were, when platinum-sensitive/platinum-resistant matched cell line pairs (i.e. A2780/A280-cis, PEO1/PEO4) were compared, expressed at similar levels suggesting that the resistance to platinum-based chemotherapy may not be calpain-related in this setting. This was in parallel with the similar response to calpeptin in cell proliferation between matched cell line pairs (**section 2.4.5**).

Western blotting results indicated that calpain-2 expression in SKOV3 cells was significantly higher than A2780 (P=0.004), A2780-cis (P=0.005) and PEO1 cells (P=0.009), with PEO4 cells expressing the highest level. Based upon such results, PEO4 and SKOV3 cells were chosen for calpain-2 knockdown. As indicated in **section 2.5**, calpain could not be completely knockdown by siRNA, as gene silencing resulting from siRNA often lasts from 5-7 days during which time the calpains that were already produced cannot be rapidly degraded (Zhang, Lane & Mellgren, 1996). Therefore, shRNA was chosen for the downregulation of calpain-2 expression. Unfortunately PEO4 cells could not survive selection which might be due to their long doubling times, although different recovery times were tried before adding the selection medium; and it should be noted that, from the literature, only very few studies have used PEO4 cells in transfection studies (Sakai W *et al*. 2009). Although in the current study four unique calpain-2 shRNA constructs were used and the transfected cells were able to survive puromycin selection, none of the four structures

could down-regulate calpain-2 expression after transfection on SKOV3 cells which might be due to the initial high expression level or the low targeting efficiency of shRNA structures. The attempt to specifically inhibit calpain-2 activity via down-regulation of calpain-2 level was unsuccessful, even after numerous attempts and significant time expended. If the calpain-2 level was successfully downregulated, for future colony selection, cloning cylinder selection might be a better way for cells with low PE (e.g. SKOV3).

Calpeptin, as a calpain inhibitor, is widely used in studying calpain involvement in cancer chemotherapeutic response (**section 2.5**). Preliminary studies in our group (conducted by Dr. Sarah Storr) indicated that calpeptin was more effective than PD150606 (Pu, 2016), so was used in the current study. Although calpeptin is widely used in studies of calpain-chemotherapeutic response, these studies invariably did not address to what extent calpain activity was inhibited. Calpeptin has also been used for calpain inhibition in the study of other calpain-related cellular processes (reviewed in **section 2.5**) with such studies suggesting that 40 to 100μM calpeptin treatment for ≥30 min can effectively inhibit calpain activity and calpain-induced protein cleavage. However there is no information indicating how the panel of 5 ovarian cancer cell lines used in the current study respond to calpeptin.

In terms of cell proliferation, A2780 and A2780-cis cells seemed more sensitive to calpeptin than PEO1, PEO4 and SKOV3 cells. A2780 cells and its resistant counterpart A2780-cis cells showed similar sensitivity towards calpeptin which corresponded to the similar calpain expression patterns between both lines suggesting that the acquired chemo-resistance of A2780-cis cells may not be related to calpastatin and conventional calpains. PEO1 cells appeared more sensitive to calpeptin than PEO4 cells which could

possibly due to the higher calpastatin and calpain-1 expression and/or lower calpain-2 expression in PEO1 cells when comparing to PEO4 cells.

Calpain activity assays were conducted in all 5 cell lines at different time points. Wide variations were observed when using the t-BOC based calpain activity assay. In PEO1, PEO4 and SKOV3 cells, 50μM of calpeptin (sub-IC50 concentration) gave similar inhibitory effects on calpain activity. Higher concentrations (IC50) did not increase these effects. The studies conducted by Dr. X. Pu, working with breast cancer *in vitro* models, reported similar inconsistent results and that the inhibitory effect of calpeptin did not exhibit a dose-dependent relationship (Pu, 2016). In A2780 and A2780-cis cells, the effect of 5μM (sub-IC50 concentration) calpeptin on calpain activity was very variable, with calpain activity increasing after calpeptin treatment, which was not observed in other cell line models after calpeptin (sub-IC50) treatments. Thus A2780 and A2780-cis were excluded from further experiments.

In the current study, although 30-50% calpain inhibition was observed in SKOV3, PEO1 and PEO4 cells after calpeptin treatment, calpeptin did not sensitise these cells to cisplatin or carboplatin in term of cell proliferation. As reviewed in **section 2.2.1**, the association between calpain and cell chemoresponse seems to depend on cell context. Most studies only focused on the association between calpain activity and cisplatin-induced apoptosis, but calpain is also involved in other process such as cell autophagy and survival. Many studies have revealed that calpain facilitates platinum-based chemotherapy-induced apoptosis (Mandic *et al*., 2003; Del Bello *et al*., 2007; Shen *et al*., 2016 and Al-Bahlani *et al*., 2016) whilst others suggested that calpain inhibitors (e.g. by stabilising P53 or IκBα proteins) can chemo-sensitise certain cancer cells (Darnell *et al*., 2007; Fenouille *et al*., 2012; Młynarczuk-Biały *et al*., 2006). It is unclear from the current study which

calpain related signalling pathways might be involved and coordinate to influence ovarian cancer cell chemoresponse.

Interestingly, Kim MJ *et al*., (2007) pointed out that in hepatoblastoma cells, calpain signalling was more important in hypoxia-induced cell death than in cisplatin-induced DNA damage. In routine laboratory based cell culture, the "normal" oxygen level used is 20–21% oxygen (i.e. normoxia), whereas a more accurate approximation of tissue oxygenation should be 5% oxygen (i.e. physoxia, physiological hypoxia 2–6% oxygen); almost all oxygen levels in tumours fall below 2% (i.e. hypoxia, pathological hypoxia 0.3-4.2% oxygen) (McKeown, 2014). If the oxygen level used during *in vitro* culture condition were restored to *in vivo* physiological or pathological conditions, under the stress of both low oxygen and the platinum-based chemotherapy, the calpain system might be shown to contribute to the chemotherapeutic response of cancers – it would be of interest to investigate this in future experiments, perhaps by using spheroid 3D models. 3D models, as multicellular aggregates, can generate hypoxic gradients which might help elucidate the mechanisms of calpain-related cancer cell chemo-resistance.

In ovarian carcinomas, reviewed in **section 3.2.1**, one study found that high calpain-1 expression was associated with lower tumour grade and low calpain-2 expression was associated with increased lymph node metastasis (Salehin *et al*., 2011); however, another study found that high calpain-2 expression significantly associated with platinum resistant tumours, poor PFS and OS, neither calpastatin expression nor the expression of conventional calpain large subunits was significantly associated with tumour grade or stage (Storr *et al*., 2012a). Furthermore, univariate analysis revealed that grade, FIGO stage, optimal debulking, platinum sensitivity and calpain-2 expression were predictors for OS; while in multivariate Cox proportional hazards model,

stage was not an independent prognostic factor for OS, whilst, platinum sensitivity and calpain-2 expression were independent prognostic factors for OS (Storr *et al*., 2012a). Agreed with the previous study (Storr *et al*., 2012a), the current study indicating that low calpain-2 expression was significantly associated with better OS (P=0.026) with no significant association being found between patient outcome and calpain-1 expression; however calpain-2 expression was not associated with either tumour platinum resistance or PFS. Likewise, neither calpastatin expression nor the expression of conventional calpain subunits showed significant association with tumour grade. Notably in the present study, low calpain-1 expression was significantly associated with organ-confined ovarian cancer. In the previous study from our group, serous ovarian carcinomas were associated with high calpastatin, calpain-1 and -2 expression (Storr *et al*., 2012a). In the present study, although LGSCs showed no clear association with either calpastatin or calpain (-1, -2 and -4) which could be due to the small patient number, HGSCs were associated with high calpastatin, calpain-1 and -4 expression. Univariate analysis revealed that age, grade, FIGO stage, histologic types, tumour residue and platinum sensitivity and the expression of calpastatin, calpain-2 and-4 were predictors for OS. In multivariate Cox proportional hazards model, FIGO stage, platinum sensitivity and tumour residue, but not the expression of calpastatin and calpains, were independent prognostic factors for OS. As indicated in **section 3.4.1** the discrepancy between the present (80/469) and previous (80/154) studies may be due to the fact that different numbers (i.e. previously 2 cores/case, currently mostly 1 core/case) and different positions of the primary tumour were sampled for each of the two cohorts; but, as discussed in **section 3.5**, use sample size large enough, one core per tumour should be sufficient to reproduce the important clinical associations (Torhorst *et al*., 2001; Khouja *et al*., 2010).

The expression of calpastatin, calpain-1 and calpain-2 has been studied in other cancer types and correlations assessed using the Spearman's rank correlation coefficient. All the correlation studies suggest that calpastatin and the conventional calpain subunits were positively correlated with each other. Examples include a HER2-positive primary breast cancer study (n=93) where a weak biological correlation existed between calpastatin and calpain-2 expression (rs=0.231, p=0.027) (Storr *et al*., 2011c). Studying the pre-chemotherapy core biopsy from breast cancer patients (n=137), calpastatin and calpain-1 expression showed a weak correlation (rs=0.283, *P*=0.001) (Storr *et al*. 2016). In endometrial cancer cases (n=29), calpain-1 and calpain-2 were moderately correlated (rs=0.488, *P*=0.040), moreover calpastatin and calpain-1 showed stronger correlation both in cancer (rs=0.579, p=0.001, n=29) and benign endometrial tissues (rs=0.508, *P*=0.032, n=18), however, there were no significant correlations between calpastatin and calpain-2 (Salehin *et al*., 2010). Significant correlations were detected between calpain-1 and calpain-2 expression (rs=0.281, *P*=0.026), calpastatin and calpain-1 expression (cytoplasmic: rs=0.327, *P*=0.010; nuclear: rs=0.346, *P*=0.006) in pancreatic cohort (n=68), while in the bile duct and ampullary carcinomas (n=120), calpain-1 expression significantly correlated with calpastatin (cytoplasmic: rs=0.425, *P*<0.001; nuclear: rs=0.295, *P*=0.002), but not with calpain-2 expression (Storr *et al*., 2012c). Calpain-2 expression was correlated with only the cytoplasmic calpastatin expression (rs=0.250, *P*=0.009) in the bile duct and ampullary carcinomas. Adding calpain-4 into the study of the correlations between conventional calpains and calpastatin, the present study indicated that the expression of calpastatin, calpain-1 and calpain-4 moderately correlated with each other (calpain-1/calpastatin: rs=0.480, *P*<0.001; calpain-4/calpastatin: rs=0.514, *P*<0.001; calpain-1/calpain-4: rs=0.481, *P*<0.001), whilst calpain-2 weakly correlated with calpain-1 (rs=0.300,

P<0.001), calpain-4 (rs=0.296, *P*<0.001) and calpastatin (rs=0.205, *P*<0.001). In the current correlation study, calpastatin and conventional calpain subunits were all positively correlated with each other (**section 3.4.4**).

μ-calpain and m-calpain are known as two isozymes, and their large subunits, calpain-1 and calpain-2, share the same regulatory subunit-calpain-4. (Murachi *et al*., 1990; Zhang W. *et al*., 1996). As reviewed in **section 1.4.4.3**, high calpain-4 expression was associated with adverse patient outcomes in various cancer types, whilst both positive and adverse associations have been observed between patient outcomes and calpain-1/-2 or calpastatin expression in different cancer types or subtypes. Contradictory results emerged when assessing patient survival in the current study, with data indicating that low rather than high expression of calpain-4 was associated with adverse patient OS, whilst high calpain-2 and low calpastatin expression were associated with adverse OS. It should be borne in mind that the current immunohistochemistry study only reflects protein expression rather than activity, so results cannot be directly compared to the results from the *in vitro* studies. It might be useful to study calpain activity in the FFPE sections by assessing expression levels of certain calpain cleaved substrates. However, as indicated in Pu *et al*. (2016b), antibodies for calpain-cleaved substrates have not been tested to serve these purposes.

Accumulating evidence suggests that ovarian cancer subtypes have distinct non-ovarian tissues/precursor lesions: endometrioid and clear-cell carcinoma mostly originating from endometrium/endometriosis; whilst HGSC from fimbriated end of the fallopian tube/ serous tubal intraepithelial carcinoma (Nezhat *et al*., 2015; Scheib, 2017). Different origins infer differential mechanisms of pathogenesis and genetic alterations in the major histological subtypes of ovarian cancer (Nolen and Lokshin, 2012). Moreover

how the expression of the calpain system is associated with breast cancer patient survival was largely dependent on cancer subtypes (**section 1.4.4**), therefore, it is necessary to address this question in ovarian cancer study which was previously limited by the size of the cohort. In the current study, calpastatin calpain-1 and -4 expression, in addition to MAP4 and Syk expression (**section 5.4.3**), were significantly associated with ovarian cancer histological subtypes: HGSC was associated with high expression of calpastatin ($\chi2=17.403$, d.f.=5, $P=0.004$), calpain-1 ($\chi2=56.577$, d.f.=5, $P<0.001$), calpain-4 ($\chi2=18.181$, d.f.=5, $P=0.003$), MAP4 ($\chi2=56.386$, d.f.=5, $P<0.001$) and Syk (cytoplasmic Syk: $\chi2=58.835$, df=5, $P<0.001$ and nuclear Syk: $\chi2=29.874$, df=5, $P<0.001$ respectively) whilst CCCs tended to express the 5 proteins at lower levels. Low calpain-1 expression was also associated with mucinous and endometrioid subtypes, but calpastatin, calpain-2 and -4 expression levels showed no clear association with LGSC, mucinous or endometrioid subtypes. As indicated in **section 3.5**, cautions should be made when interpreting the association between protein expression and histological subtypes, especially CCCs and mucinous tumours; as CCCs typically characterised by clear cytoplasm which influence the assessment of cytoplasmic staining intensity, whereas mucinous carcinomas might not be primary mucinous carcinoma.

In the current *in vitro* studies, PEO1 and PEO4 cells are HGSC cell lines whilst A2780 and SKOV3 cell lines are most likely endometrioid carcinoma rather than HGSC cells. The Western blotting study (**section 2.4.3**) showed that calpastatin, calpain-1 and -4 were expressed at a higher level in PEO1/4 than A2780/SKOV3 cells: 1) PEO1 cells expressed significantly higher level of calpastatin than A2780 cells whilst calpastatin expression was at similar level between SKOV3 and PEO4 cells; 2) PEO1 and PEO4 cells expressed significantly higher levels of calpain-1 than A2780 and SKOV3 cells; 3) PEO1 and PEO4 cells expressed higher level of calpain-4 than SKOV3 and A2780 cells, but the

differences were not significant. SKOV3 cells expressed higher level of calpain-2 than A2780, PEO1 and PEO4 cells, which hinted that calpain-2 expression levels may not relate with subtypes in this setting. Although limited by the number of cell lines for each cancer subtype, the protein expression pattern agreed with the results from the IHC study and hinted that HGSC cells express higher levels of conventional calpains and calpastatin than other subtypes. The above findings support the review from Nolen and Lokshin (2012) that high-grade serous carcinoma is associated with a larger array of aberrantly expressed biomarkers than other subtypes. These findings suggest that in different histological subtypes the conventional calpains and calpastatin have different expression patterns and are involved in differential mechanisms that influence clinical outcome.

Although calpain-1 expression was not associated with patient survival, the IHC study (**section 3.4.3**) indicated that high calpain-1 expression significantly associated with high tumour stage. Similar findings were reported by Al-Bahlani *et al*. (2017) in triple-negative breast cancers; calpain-1 expression was not significantly associated with patient outcome (n=55) but significant association was found between calpain-1 expression and the lymph node status (*P*= 0.02). In ovarian cancer, tumour stage appears as the most important clinical parameter of prognostic relevance among the established prognostic factors such as age, histology type (e.g. serous carcinoma and clear cell carcinomas are considered of poor prognosis; Uharcek, 2008), grade, performance status, volume of ascites, extent of residual disease following cytoreductive surgery (Colombo *et al*., 2007; Holschneider & Berek, 2000; Agarwal & Kaye, 2005). In the current study, stage was also an independent prognostic factor based on Kaplan–Meier and Cox proportional hazard regression analysis. Since stage reflects the extent of ovarian tumour spread *in vivo*, calpain-1 may be involved in tumour migration and invasion *in vitro*. As

indicated in **section 4.2.2**, inhibiting calpain via pharmacological inhibition or genetic knockdown, the calpain system appears closely associated with cancer cell migration in *in vitro* studies.

Haptotactic migration in response to the alteration of calpain activity was studied using the scratch wound migration assay. The current study showed that SKOV3 cells have a greater migratory ability comparing to PEO1/PEO4 cells; SKOV3 cells took 22 hours to reach 70-80% wound closure, whilst the wound closure of PEO1 and PEO4 only reached to 30% and 40% respectively at 72 hours post-wounding (**section 4.4.2**). A23187 increased calpain activity in SKOV3 cells and significantly inhibited SKOV3 migration which might be due to calpain-related apoptosis and inhibition of cell spreading/migration or A23187 can regulate other migration-related proteins besides calpains **section 4.5**. Inhibitory effects of calpeptin on calpain activity and cell migration were observed in SKOV3, but the results were not statistically significant. By contrast, with limited downregulation or upregulation of calpain activity, neither calpeptin nor A23187 could induce any alteration to PEO1 or PEO4 cell migration. How PEO1 and PEO4 cells differ from SKOV3 in maintaining calpain homeostasis is yet to be explained. Notably, as mentioned above, the difference between SKOV3 and PEO1/-4 cells is that SKOV3 cells expressed higher level of calpain-2 than PEO1 and PEO4 cells; moreover, SKOV3 cells expressed significantly lower level of calpain-1 than PEO1 and PEO4 cells (**section 2.4.3**). Knowing that PEO1, PEO4 and SKOV3 were all isolated from ascites and do not express wild-type P53 (**section 2.3.1**); in addition, PEO4 and SKOV3 cells expressed similar level of calpastatin and no significant difference of calpain-4 expression was detected between SKOV3 and PEO1/PEO4 cells; hence, calpain-2 might be critical in regulating the haptotactic migration leading to the greater migratory ability of SKOV3 cells. Specific knockdown of calpain-1 and/or calpain-2 in ovarian cancer cell lines might offer an insight

into isoform-specific functions.

Several studies from the literature have suggested potential interactions between the calpain system and Syk (Jinawath *et al*., 2010; Fei *et al*., 2013; **section 5.2.2**). Such links between Syk, and also MAP4, with the calpain system were further enhanced by data presented at the 2016 FASEB conference held in Montana (S Martin/ S Storr personal communications, **section 5.2**). Thus, the relationship between conventional calpain subunits, calpastatin, Syk and MAP4 expression in ovarian cancer was investigated in the current study by immunohistochemistry. As shown and discussed in **section 5.4.2**, neither MAP4 nor Syk expression was significantly associated with OS or PFS. Patients were grouped by clinicopathological variables. No significant association was detected between MAP4 expression and OS in all the subgroups tested. High Syk expression in nucleus was significantly associated with better outcomes in patients with platinum-resistant tumours, patients with tumours that were confined to ovaries (stage 1 ovarian cancer) or patients with no residual disease (**section 5.4.3.2**). The associations between high cytoplasmic Syk expression and better OS were not statistically significant. In many studies, Syk seems to function as a tumour suppressor and its re-introduction into more malignant, invasive cancer cells decreases cancer malignancy by increasing adhesion and reducing tumour cell growth, motility, invasion and metastasis (Ogane *et al*., 2009; Fei *et al*., 2013; Peng *et al*., 2013; Krisenko & Geahlen, 2015). Results from the current study tend to suggest that Syk may functioned as a tumour suppressor in certain subgroups of patients, although these subgroups cannot be generalised and summarised as more malignant or invasive subgroups.

By contrast, the current study also indicated that low Syk expression was associated with low tumour stage. Low calpain-1, low MAP4 expression and

low Syk expression in the cytoplasm was associated with low stage (calpain-1: χ2=15.259, d.f.=3, *P*=0.002; MAP4: χ2=15.857, d.f.=3, *P*=0.001; cytoplasmic Syk: χ2=10.725, d.f.=3, *P*=0.013) (**section 3.4.2; section 5.4.2**). To be precise, low calpain-1, low MAP4, low cytoplasmic Syk and low nuclear Syk showed significant association with organ-confined status of tumour (calpain-1: χ2=11.310, d.f.=1, *P*=0.001; MAP4: χ2=15.402, d.f.=1, *P*=0.000087; cytoplasmic Syk: χ2=9.975, d.f.=1, *P*=0.002 and nuclear Syk: χ2=4.169, d.f.=1, *P*=0.041), but no significant association was detected between their expression and cancer distant metastasis. It may be likely that, in light of such data, Syk promotes tumour spread via increasing cell invasion and migration (Krisenko & Geahlen, 2015; Ghotra *et al*., 2015; Luangdilok *et al*., 2007, **section 5.2.2**) at the early stage of ovarian carcinogenesis. This needs further investigation to understand the contradictory function of Syk in consideration of cellular context and tumour microenvironment.

As mentioned in **section 5.2.1.2**, the association between MAP4 and paclitaxel resistance has been observed in different cancer types. Furthermore, an interesting study revealed an association between Syk and MAP4 and their link with microtubule-targeting agent paclitaxel chemo-resistance (Yu *et al*., 2015). It is therefore of interest in the IHC study to look at patients who have been treated with regimen including taxanes. In the present study the association between low MAP4 expression and chemo-resistance was significant in chemo-naïve cases treated by taxane-containing chemotherapy. Low Syk expression in nucleus was significantly associated with chemo-resistance in the whole cohort and in chemo-naïve cases. A recent study has found that Syk-mediated tyrosine phosphorylation of MAP1B and MAP4 can induce paclitaxel resistant via reducing microtubule stabilisation (Yu *et al*., 2015; Wei & Birrer, 2015). In the whole cohort, among conventional calpain subunits, calpastatin, MAP4, Syk, CXCR3 and CXCL4, the most

significant correlation was found between calpain-1 and MAP4 expression. Syk expression significantly correlated with both MAP4 and calpain-1 expression. To understand the association between paclitaxel resistance and the expression of MAP4, Syk and potentially calpain-1, the levels of phosphorylated forms of MAP4 (inactivated form of MAP4) and Syk (activated form of Syk) should be considered.

Moreover, a panel of protein expression (i.e. angiogenic related proteins: VEGF, CXC chemokine ligands: CXCL1, CXCL4 and CXCL8; CXC chemokine receptor: CXCR3; silent/decoy CXC chemokine receptor: DARC; integrin α2β3; EMT associated proteins: N-, E- and P-cadherin) was examined in light of conventional calpain subunits, MAP4 and Syk expression in 87 HGSC patients from the current cohort, amongst which calpain-1, Syk and MAP4 expression were moderately or strongly correlated with each other; moreover, both MAP4 and calpain-1 expression was moderately correlated with integrin α2β3 expression whilst Syk weakly correlated with integrin α2β3. These findings mesh with the functional associations between calpain, integrin and Syk as reviewed in **section 5.2.2** and discussed in **section 5.5**. Numerous studies have indicated that integrins are calpain substrates, and most studies have suggested that conventional calpains inhibit integrin-mediated cell spreading/migration (reviewed in **section 5.5**) which agrees with the data in **section 4.4.2** that A23187 inhibit cell migration. Thus, in addition to paclitaxel resistance, integrin, calpain, Syk and MAP4 might also be involved in cell motility. For a clearer understanding of how calpain-1 and integrin interact with each other, future studies could consider evaluating calpain-cleaved form of integrin in addition to the full-length integrins and other integrin isoform. Comparison between primary tumours and matched metastases might also help reveal the protein function in context and identify how protein expression changes to facilitate cancer progression.

6.2. Limitations and future work

6.2.1 Regulation of calpain activity

Studies have shown that intracellular calcium ions, the most abundant second messenger in human body, have a substantial diversity of roles in fundamental cellular physiology, including gene transcription, cell cycle/cell proliferation control (via regulating proteins such as calmodulin, calcineurin and NF-κB), cell motility, autophagy and apoptosis (e.g. Bad and B-cell lymphoma-2) (Cui *et al*. 2017; Farfariello *et al*., 2015). Accumulating evidence has demonstrated that the alteration of intracellular Ca^{2+} homeostasis in cancer cells is involved in oncogenesis, increased cell proliferation, escaping cell death and immune-attack, neovascularisation, cell motility and invasion, progression and metastasis (Cui *et al*. 2017). Both A23187 and ionomycin have most often been used in biological studies to increase the cytosolic Ca^{2+} concentration by transport Ca^{2+} across biological membranes from extracellular Ca^{2+} and/or Ca^{2+} in internal stores, intracellular organelles (Fasolato & Pozzan, 1989; Abraham *et al*., 2002). As carboxylic ionophores, A23187 and ionomycin promote electroneutral exchange diffusion controlled by the cation chemical gradient and external pH (Fasolato & Pozzan, 1989). In addition to A23187 and ionomycin, reagents such as maitotoxin (a known calcium channel opener) (Guingab-Cagmat *et al*., 2012), Bay K 8644 (a Ca^{2+} channel activator/ agonist) (Klett *et al*., 1993) (Ohbayashi K. *et al*., 1998; Kroeber *et al*., 2000; Yamamura *et al*., 2016), thapsigargin and cyclic ADP-ribose (regulating the intracellular calcium stores; Mueller *et al*., 1999) that increase intracellular Ca^{2+} via different pathways could be considered.

To what extent the calpain system is involved in A23187-induced apoptosis has not been addressed in ovarian cancer studies. Although the working concentration of A23187 was optimised by prolonged treatment (up

to 24 hours) and assessment (assessed cell proliferation by trypan blue), it cannot exclude the possibility that using the current concentration for 60 to 90 min treatment might have triggered apoptosis. Further optimisation of A23187 working concentrations, using additional assays that can study the early stages of apoptosis (e.g. terminal deoxy transferase transferase-mediated dUTP nick end labelling (TUNEL) or measuring propidium iodide and Annexin V immunostaining by flow cytometry, Archana *et al*., 2013) would be of interest.

It is noteworthy that using A23187 for the induction of calpain activity might introduce uncertain factors, as it has been reported that A23187 was able to regulate other proteins involved in cellular migration. For example, S100A4, a calcium-binding protein, is a central mediator of the cellular processes associated with metastasis formation in colon cancer via inducing cell migration and invasion (Sack *et al*., 2011). In epithelial ovarian cancer, the expression of S100A4 was found positively associated with the clinical stage and *in vitro* cell mobility and metastatic capacity (Horiuchi *et al*. 2012; Yan *et al*., 2016). It is worth noting that A23187 was identified as a transcriptional inhibitor of S100A4 in a high-throughput screen study in colon cancer cells and A23187 treatment (1μM for 24 hours) inhibited 30% of S100A4 expression comparing to that of the vehical control without affecting cell viability (Sack *et al*., 2011). A23187 treatment inhibited both S100A4 and β-catenin expression at mRNA and protein level; moreover, A23187 inhibited S100A4-induced colon cancer cell migration and invasion *in vitro* and also inhibited metastasis formation in xenografted immunodeficient mice (Sack *et al*., 2011). Protein kinase C has been found activated by A23187 (0.3μM for 60 to 90 minutes) which significantly enhanced breast cancer cell adhesion to type IV collagen and vitronectin (Palmantier *et al*., 1996). Calcineurin was found expressed in ovarian cancer cells (Hata *et al*., 2009). Elevated Ca^{2+} by A23187 (1.0 μM)

significantly downregulated human melanoma cell chemotaxis migration to type IV collagen via a calcineurin-mediated pathway (Hodgson & Dong, 2001). VEGF has been shown to upregulate the regulator of calcineurin, RCAN-1, in a variety of endothelial cell lines; moreover, knockdown of RCAN1 in endothelial cells inhibited VEGF-induced migration *in vitro*, and VEGF-induced angiogenesis, *in vivo* (Holmes *et al*., 2010). Thereby, A23187-induced inhibition of SKOV3 cell migration in the current study might not due to a calpain-specific modulation.

Due to the complexity of Ca^{2+}-signalling, regulating intracellular Ca^{2+} concentration cannot specifically modulate calpain activity. There are calpain activators been reported such as calpastatin subdomains A and C (19 amino acids each) which can strongly activate calpain *in vitro* (Tompa *et al*., 2002b; Banóczi *et al*., 2007; Világi *et al*., 2008; Toke, *et al*., 2009); dibucaine, a local amide anesthetic and sodium channel blocker (Risher *et al*., 2011), was also used as calpain activator (Wang Z. *et al*., 2010; Zhang W. *et al*., 2011; Zhang P. *et al*., 2013). High-energy compounds (HECs) such as ATP, phosphoenolpyruvate, phosphocreatine, acetyl coenzyme A, guanosine triphosphate and pyrophosphate were found able to promote the intracellular calpain activity (Nguyen & Chen, 2014). Because the pharmacological inhibitors/activators used for calpain studies are not specific to calpain and the inhibition might not be sufficient to induce phenotypic changes, it is necessary to use several different inhibitors/activators to determine whether calpains are involved in specific phenomena. In the FASEB conference regarding calpains, other calpain inhibitors such as BDA-410 and selective calpain-2 inhibitor, Z-Leu-Abu-CONH-CH2-C6H3 (3, 5-(OMe)2 (C2I), were mentioned (Nabeshima *et al*., 2014; Liu Y. *et al*. 2016; Li *et al*., 1996). Novel mercaptoacrylate calpain inhibitors were synthesised and tested by Prof. R Allemann and colleagues (Cardiff University); results indicated that those

compound were able to inhibit calpain activity and Ca^{2+}-related neutrophil spreading. Three newly synthesised inhibitors were offered by the group of Prof. R Allemann and one of them (i.e. compound 1) was tested in the current study. Different from the mercaptoacrylates, these new inhibitors have diselenide bound instead of forming disulphide bound. The diselenide "compound-1" tested in the current study has similar structure with the mercaptoacrylate calpain inhibitors.

Together, the calpain activity cannot be specifically modulated by the pharmacological inhibitors/activators. And the IHC result from **section 3.4** and **5.4** suggested that calpain-1, calpain-2 and calpain-4 play different roles in tumour progression. Thus it would be useful to specifically knockdown conventional calpain subunit (calpain-1, -2 or -4) or calpastatin using shRNA or CRISPR-Cas9 to establish cell models for the future study of the role of calpain in ovarian cancer cell chemoresponse, adhesion, migration and invasion. Although in the current study, the shRNAs against calpain-2 were not able to inhibit calpain-2 expression, other shRNAs, transfection reagents, and/or cell lines could be tested for this purpose.

6.2.2 Cell lines and other models

A range of ovarian cancer cell lines were used in the present study. It is worth noting that ovarian cancer A2780, SKOV3 and IGROV1 cell lines are most likely to be endometrioid carcinoma histological subtype rather than HGSC subtype based on the genomic data (Domcke *et al.*, 2013), immunohistochemistry markers (Anglesio *et al.*, 2013), mutation analyses, histology and grade of the tumour of origin (Beaufort *et al.*, 2014). Hence, if the future study is subtype-specific, cautions should be taken when choosing the representative cell lines. Results from the present *in vitro* study need to be interpreted in consideration of the subtype difference within the panel of cells. Moreover, the established cells lines were often derived from ascites instead

of primary tumour and have high passage numbers indicating that cells have been cultured in non-physiological conditions for many years (Alkema *et al*., 2016). More mutations have been found in the established cell lines than in primary ovarian cancer samples (Domcke *et al*., 2013). Therefore, future *in vitro* ovarian cancer research should use cell lines that have been well-defined and characterised and should perhaps consider using patient derived primary lines or xenografts.

It is known that the high mortality associated with ovarian carcinoma is linked to chemo-resistance and metastasis. The metastasis of ovarian serous carcinomas is generally considered to disseminate through transcoelomic route, hematogenous and/or lymphatic route, with transcoelomic route as the most frequently observed dissemination mechanism (Cannistra, 2004). Although ovarian cancer cells can also spread via ovary-draining lymphatics to pelvic and para-aortic lymph nodes (Greer *et al*., 2004), hematological and lymphatic metastasis in ovarian cancer is rare (Weidle *et al*., 2016). Thus, further study of cell motility should include both human mesothelial cells (e.g. non-malignant pleural mesothelial cell line Met-5A and primary peritoneal mesothelial cell line LP-9, Morone *et al*., 2012; Musrap *et al*., 2014) and human endothelial cells (e.g. lymphatic endothelial cells hTERT-LEC, macrovascular endothelial cell line EAhy926, micro vessel endothelial cells HMEC-1 and umbilical vein endothelial cells HUVEC) in the cell-adhesion or trans-membrane migration assays (Morone *et al*., 2012).

3-dimensional (3D) models resemble the clinical tumour micro-environment and should be considered in future drug resistance studies, but they are complex and laborious and require extensive optimisation (Alkema *et al*., 2016). To mimic the *in vivo* microenvironment, 3D ovarian cancer models consist of tumour cells and ECM, stromal, endothelial and/or immune cells (Fuller & Howell, 2014; Lengyel *et al*., 2014, Alkema *et al*., 2016).

Initial strategies of establishing 3D models are embedding ovarian cancer multicellular aggregates in purified ECM proteins (e.g. collagen) or in extract containing a mixture of ECM proteins (e.g. Matrigel); improved strategies incorporate mesothelial cells, fibroblast, adipocytes, and/or macrophages into the models (Kenny *et al*., 2009; White *et al*., 2014; Lengyel *et al*., 2014). 3D models have been shown to alter the expression of proteins involved in proliferation, matrix penetration and adhesion when compared to 2D models and can reflect the structure and oxygen conditions, growth factor/ immune factor/ cytokine gradient of the tumour microenvironment (Fuller & Howell, 2014; Lengyel *et al*., 2014).

6.2.3 The calpain system and taxanes

Several studies have reported a role for calpain in response to taxanes; the majority of which focused on the association between calpain and the side effect of taxanes, however the relationship between calpain and taxane resistance in ovarian cancer has not been well documented. Calpain has been found involved in the pathway of paclitaxel-induced peripheral neuropathy, which is a side effect of paclitaxel treatment for breast and ovarian cancer (Benbow *et al*., 2011; 2012). Paclitaxel activated μ-calpain and triggered calpain-dependent proteolysis of neuronal calcium sensor-1 (NCS-1), leading to decreased affinity of NCS-1 for Ca^{2+} and reduced Ca^{2+}-signalling within the neuroblastoma cell (Blachford *et al*., 2009) which appeared to be critical in inducing the paclitaxel-induced peripheral neuropathy (Blachford *et al*., 2009). In lymphoma cells, paclitaxel was found able to activate calpain and paclitaxel-induced apoptosis can be inhibited by the calpain inhibitor calpeptin (Liao *et al*., 2008). Rather than only focusing on chemo-induced cell apoptosis, recently, Liu L. *et al*., (2017) reported that docetaxel treatment increased calpain activation in prostate cancer cells, whilst calpain inhibitor sensitised docetaxel-resistant prostate cancer cell sublines to docetaxel

treatment by inhibiting both cell proliferation and cell migration which suggests that calpain may be a factor in promoting docetaxel resistance. Unpublished data presented at the 2016 FASEB summer conference indicated that conventional calpains were associated with Syk and MAP4. In addition, Yu et al., (2015) reported that Syk and MAP4 were involved in regulating microtubule stability and paclitaxel resistance in ovarian cancer cells. Thus, the expression of MAP4 and Syk were examined in the current study.

Based on the IHC results in **section 3.4** and **5.4**, the expression of calpain-1, MAP4 and Syk was significantly correlated with each other; moreover, their expression significantly associated with ovarian cancer stage. All the patients who received taxane-containing chemotherapy were also treated by the platinum-based chemotherapy which might be the reason that no significant association could be detected between the protein expression (i.e. calpain, MAP4 and Syk) and the chemoresponse of patients who received taxane-containing chemotherapy. As indicated in **section 5.5**, Yu *et al*. (2015) revealed an association between the expression of Syk and MAP4 and paclitaxel resistance. In addition to the present study which focused on the association between calpain and platinum-based chemotherapy, it would be interesting to further study the relation between the calpain system (especially calpain-1) and the microtubule microtubule-interacting drugs (e.g. taxanes) and/or anti-microtubule drugs (i.e. microtubule-depolymerising agents such as Vinca alkaloids).

6.2.4 The calpain system and EMT

Recent studies suggest that the calpain system plays an important role in the process of EMT and providing a starting point to elucidate the mechanism of how calpains involved in chemo-resistance and tumour metastasis. Western blotting studies have revealed that in ovarian cancer cells 100 kDa

N-terminal fragments (NTF) of E-cadherin (full-length 120 kDa) was produced by calpain directly, resulting in spheroidgenesis and lymphovascular emboli formation (Ye *et al*., 2013). EMT could be induced by autocrine growth factors, such as epidermal growth factor-and tumour growth factor (TGF) β, secreted by tumour cells (Brozovic, 2017). In lung epithelial cell line, TGF-β1 treatment significantly enhanced calpain-1 expression and downregulated E-cadherin whilst upregulated α-smooth muscle actin expression which was reversed by calpain inhibitor (Tan *et al*., 2017). Recent studies have also shown that, in breast cancer MCF-7 cells, fibronectin induced calpain-2 expression and calpain was involved in fibronectin-induced EMT response (Li *et al*., 2017). Calpain-4 plays an important role in the EMT pathway in human melanoma cells (Wang E. *et al*., 2017). The downregulation of epithelial markers and the upregulation of mesenchymal markers can be inhibited by calpain inhibitors during EMT (Li. *et al*., 2017; Wang E. *et al*., 2017).

From the IHC study in chapter 5, no significant correlation was detected between the expression of the calpain system and cadherins except a negative weak correlation between the expression of calpastatin and P-cadherin (rs=-0.378, P=0.001). Further study is necessary before a firm conclusion can be drawn, because, as mentioned in **section 5.5**, one limitation of IHC is that only protein expression is assessed rather than activity, or cleaved forms of proteins; thus functional correlations cannot be drawn but only inferred. Previous study indicated an association between the expression of the calpain system and ovarian cancer chemo-resistance (Storr *et al*., 2012a), whilst in the current study an association was found between the calpain expression and cancer stages (indicating the extent of tumour spread, **section 3.4.2**). Although there are discrepancies between these two studies, calpain might be involved in the process of EMT, which was found able to contribute to both cancer cell chemo-resistance and tumour metastasis

(reviewed in Brozovic, 2017). Hence, the Human EMT RT² Profiler PCR Array profiles could be used in the future study to analyse the difference in EMT-associated gene expression between calpain-1/-2 knockdown cells and empty vector-transfected cells in order to study the role of calpain isoform in tumour metastasis and development. As the array includes genes mediating cell adhesion, migration, growth, proliferation, as well as genes controlling cell signalling and transcription, it could potentially enlighten us on which processes the conventional calpain subunits involved in EMT.

In conclusion, the roles that calpain system played in human cancer cells vary with cell context and the current results did not support the initial hypothesis that calpain can sensitise ovarian cancer cells to cisplatin/carboplatin. The calpain-inhibitor calpeptin did not sensitise the ovarian cancer cell lines to cisplatin/carboplatin-induced proliferation inhibition. In addition, chemo-sensitive ovarian cancer cells (i.e. A2780 and PEO1) and their resistant counterparts (i.e. A2780-cis and PEO4) expressed similar levels of conventional calpain subunits and calpastatin. Moreover calpeptin caused a similar inhibitory effect on cell proliferation in chemo-sensitive ovarian cancer cells and their resistant counterparts. Calpain-2 expression was adversely associated with OS in ovarian cancer patients in both the previous and current IHC study. Calpain-4 and calpastatin expression were positively associated with patient OS in the current IHC study. Although there was no association between calpain-1 expression and patient outcome, calpain-1 was associated with tumour stage. Low calpain-1 expression was associated with tumours that were confined to the ovaries (stage 1) and the absence of residual disease. As stage reflects the extent of tumour metastatic spread, conventional calpains and calpastatin could be involved with tumour migration. Ion-carrier A23187 activated calpain in SKOV3 cells but induced significant suppression of the two-dimensional planar cell

migration, whilst the inhibition of calpain activity caused by calpeptin also suppressed cell migration. To further pursue the study on the role of calpain in the tumour metastatic spread and chemoresponse, the correlation between calpains and potentially related proteins were studied. In the whole cohort, among conventional calpain subunits, calpastatin, MAP4, Syk, CXCR3 and CXCL4 the most significant correlation was found between calpain-1 and MAP4. Syk significantly correlated with both MAP4 and calpain-1 expression. A correlation study was conducted in the 87 HGSC cases from the whole cohort involving the following proteins: VEGF, CXCL1, CXCL4 and CXCL8, CXCR3, DARC, integrin α2β3, N-, E- and P-cadherin, in addition to calpain-1, -2 and -4, calpastatin, MAP4 and Syk. In the 87 HGSC patients, there was strong correlation between MAP4 and calpain-1 expression both of which were moderately correlated with integrin α2β3. In addition, calpain-1 expression was strongly correlated with cytoplasmic Syk in these 87 HGSC patients. These findings suggest that in addition to paclitaxel resistance, integrin, calpain, Syk and MAP4 might also involve in cancer cell spreading. How MAP4, Syk, calpain-1 and integrin interconnect in tumour cell spreading/migration and cell responding to taxane-containing chemotherapy remained to be further investigated in the future.

The conventional calpains and calpastatin have been confirmed to play an important role in ovarian cancer but the precise mechanisms whereby they exert effects remain to be elucidated.

References

ABE, K. & TAKEICHI, M. (2007) NMDA-receptor activation induces calpain-mediated beta-catenin cleavages for triggering gene expression. Neuron 53, 387–397.

ABRAHAM, S.A. *et al*., (2002). Formation of transition metal-doxorubicin complexes inside liposomes. Biochim Biophys Acta. 1565(1):41-54.

AI B. *et al*., (2016). Paclitaxel targets VEGF-mediated angiogenesis in ovarian cancer treatment. Am J Cancer Res. 6(8):1624-35. eCollection 2016.

AL-BAHLANI, S.M. *et al*., (2011). P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. Oncogene. Oct 13;30(41):4219-30.

AL-BAHLANI, S.M. *et al*., (2016). Cisplatin Induces Apoptosis Through the Endoplasmic Reticulum-mediated, Calpain 1 Pathway in Triple-negative Breast Cancer Cells. Clin Breast Cancer. 17(3):e103-e112.

AL-BAHLANI, S.M. *et al*., (2017). Calpain-1 Expression in Triple-Negative Breast Cancer: A Potential Prognostic Factor Independent of the Proliferative/Apoptotic Index. Biomed Res Int. 2017:9290425.

ALBERTS, D.S., *et al*. (2002). Intraperitoneal therapy for stage III ovarian cancer: a therapy whose time has come! J Clin Oncol. 20(19):3944-6.

ALKEMA, N.G. *et al*., (2016). Studying platinum sensitivity and resistance in high-grade serous ovarian cancer: Different models for different questions. Drug Resist Updat. 24:55-69.

ANGLESIO, M.S. *et al*., (2013). Type-specific cell line models for type-specific ovarian cancer research. PLoS One. 8(9):e72162.

ANGUISSOLA, S. *et al*., (2009). Bid and calpains cooperate to trigger oxaliplatin-induced apoptosis of cervical carcinoma HeLa cells. Mol Pharmacol.76(5):998-1010.

AN, Q. *et al*., (2015). *In vitro* effects of mitomycin C on the proliferation of the non-small-cell lung cancer line A549. Int J Clin Exp Med. 8(11):20516-23.

AOKI, D. *et al*., (2009). Overexpression of class III beta-tubulin predicts good response to taxane-based chemotherapy in ovarian clear cell adenocarcinoma. Clin Cancer Res. 15(4):1473-80.

APPS, M.G., CHOI, E.H. & WHEATE, N.J. (2015). The state-of-play and future of platinum drugs. Endocr Relat Cancer. 22(4):R219-33.

ARCHANA, M. *et al*., (2013). Various methods available for detection of apoptotic cells--a review. Indian J Cancer. 50(3):274-83.

ARAVANTINOS, G. and PECTASIDES, D. (2014). Bevacizumab in combination with chemotherapy for the treatment of advanced ovarian cancer: a systematic review. J Ovarian Res. 7:57.

ARTHUR, J. S. *et al*. (2000). Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol. Cell. Biol. 20:4474–4481.

ATCC, (2012), Protocol for Thawing, Propagating and Cryopreserving of NCI-PBCF-HTB77 (SK-OV-3; ATCC®HTB-77 ™) cells ovarian adenocarcinoma Version 1.6. available from: https://physics.cancer.gov/docs/bioresource/ovary/NCI-PBCF-HTB77_SK-OV-3_SOP-508.pdf April 12, 2017.

AURE, J.C., HOEG, K. & KOLSTAD, P. (1971). Clinical and histologic studies of ovarian carcinoma. Long-term follow-up of 990 cases. Obstet Gynecol. 37(1):1–9.

AVERNA, M. *et al*. (2003). Changes in calpastatin localization and expression during calpain activation: a new mechanism for the regulation of intracellular Ca 2+ -dependent proteolysis. Cell. Mol. Life Sci. 60, 2669–2678.

BADGWELL, D and BAST, R.C. Jr. (2007). Early detection of ovarian cancer. Dis Markers. 23(5-6):397-410.

BAI, D.S. *et al*., (2009). Capn4 overexpression underlies tumor invasion and metastasis after liver transplantation for hepatocellular carcinoma. Hepatology. 49(2):460-70.

BAILET, O. *et al*., (2009). Spleen tyrosine kinase functions as a tumor suppressor in melanoma cells by inducing senescence-like growth arrest. Cancer Res. 69(7):2748-56.

BALCH, C., *et al*., (2004). The epigenetics of ovariancancer drug resistance and resensitization. Am. J. Obst. Gynecol. 191, 1552–1572.

BALDOCK, D. *et al*., (2000). Purification and characterization of human Syk produced using a baculovirus expression system. Protein Expr Purif. 18(1):86-94.

BANERJEE, S., BOOKMAN, M. & GORE, M. (2011). Systemic therapy for ovarian cancer, current treatment, recent advances, and unmet needs. In: Kaye S,

BANERJI, U. *et al*. (2008). An *in vitro* and *in vivo* study of the combination of the heat shock protein inhibitor 17-allylamino-17-demethoxygeldanamycin and carboplatin in human ovarian cancer models. Cancer Chemother Pharmacol. 62(5):769-78.

Banóczi Z. *et al*. (2007). Synthesis of cell-penetrating conjugates of calpain activator peptides. Bioconjug Chem. 18(1):130-7.

BARBERO, S. *et al*., (2009). Caspase-8 association with the focal adhesion complex promotes tumor cell migration and metastasis. Cancer Res. 69(9):3755-63.

BARLOGIE, B. & DREWINKO, B. (1980). Lethal and cytokinetic effects of mitomycin C on cultured human colon cancer cells. Cancer Res. 40(6):1973-80.

BASH-BABULA, J. *et al*. (2002). A Phase I/pilot study of sequential doxorubicin/vinorelbine: effects on p53 and microtubule-associated protein 4. Clin Cancer Res. 8(5):1057-64.

BAST, Jr RC, HENNESSY, B. & MILLS, G.B. (2009). The biology of ovarian cancer:

new opportunities for translation. Nat Rev Cancer 9:415–428

BATE, N. *et al*., (2012). Talin contains a C-terminal calpain2 cleavage site important in focal adhesion dynamics. PLoS One. 7(4):e34461.

BAUDRY M., CHOU M.M. & BI X. (2013). Targeting calpain in synaptic plasticity. Expert Opin Ther Targets. 17(5):579-92.

BEAUFORT, C.M. *et al*. (2014). Ovarian cancer cell line panel (OCCP): clinical importance of *in vitro* morphological subtypes. PLoS One. 9(9):e103988.

BELL, D.A. (2005). Origins and molecular pathology of ovarian cancer. Modern Pathology.(Suppl 2):S19–S32.

BELLACOSA, A., DE FEO, D., GODWIN, A.K., *et al*. (1995).Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer;64:280-5.

BENBOW, J.H. *et al*., (2011). Protection of neuronal calcium sensor 1 protein in cells treated withpaclitaxel. J Biol Chem. 286(40):34575-82.

BENBOW, J.H. *et al*., (2012). Inhibition of paclitaxel-induced decreases in calcium signaling. J Biol Chem. 287(45):37907-16.

BENYAMIN Y. (2006). The structural basis of calpain behavior. FEBS J. 273(15):3413-4.

BERAL, V. *et al*., (2008). Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23,257 women with ovarian cancer and 87,303 controls, Lancet 371 (9609). 303–314.

BERCHUCK, A. & CARNEY, M. (1997). Human ovarian cancer of the surface epithelium. Biochem Pharmacol;54:541-4.

BEREK, J.S. *et al*. (1999). Advanced epithelial ovarian cancer: 1998 consensus statements. Ann Oncol 10: 87−92.

BETTS, R. *et al*. (2003). Structural determinants of the calpain inhibitory activity of calpastatin peptide B27-WT. J. Biol. Chem. 278, 7800–7809.

Bièche I *et al*., (2007). CXC chemokines located in the 4q21 region are up-regulated in breast cancer. Endocr Relat Cancer. 14(4):1039-52.

BLACHFORD, C. *et al*., (2009). Discrete proteolysis of neuronal calcium sensor-1 (NCS-1) by mu-calpain disrupts calcium binding. Cell Calcium. 46(4):257-62.

BLOEMBERG, D. & QUADRILATERO, J. (2016). Caspase activity and apoptotic signaling in proliferating C2C12 cells following cisplatin or A23187 exposure. Data Brief.;7:1024-30.

BOHNENBERGER, H. *et al*., (2011). Complex phosphorylation dynamics control the composition of the Syk interactome in B cells. Eur J Immunol. 41(6):1550-62.

BOOKMAN, M.A. *et al*. (1996). Carboplatin and paclitaxel in ovarian carcinoma: a phase I study of the Gynecologic Oncology Group. J Clin Oncol.14:1895-902.

BROWN JS, KAYE SB, YAP TA. (2016). PARP inhibitors: the race is on. Br J Cancer. 114(7):713-5.

BROZOVIC, A. (2017). The relationship between platinum drug resistance and epithelial-mesenchymal transition. Arch Toxicol. 91(2):605-619.

BRUSTMANN H & Naudé S. (2002). Vascular endothelial growth factor expression in serous ovarian carcinoma: relationship with high mitotic activity and high FIGO stage. Gynecol Oncol. 84(1):47-52.

BYZOVA, T.V. *et al*., (2000). Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp Cell Res. 254(2):299-308.

CAI J.J. et al., (2014). Increased expression of Capn4 is associated with the malignancy of human glioma. CNS Neurosci Ther. 20(6):521-7.

CAMPBELL R.L. & DAVIES P.L. (2012). Structure-function relationships in calpains. Biochem J. 447(3):335-51.

CANCER RESEARCH UK (2016a) Ovarian cancer Key Facts. [Online] Cancer Research UK. Available from: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/ovary/incidence/ [Accessed: 8th September 2014]

CANCER RESEARCH UK (2016b) Ovarian cancer incidence statistics. Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/ovarian-cancer/incidence#heading-Ten (Accessed: 27 January 2017).Lifetime risk estimates calculated by the Statistical Information Team at Cancer Research UK. Based on data provided by the Office of National Statistics, ISD Scotland, the Welsh Cancer Intelligence and Surveillance Unit and the Northern Ireland Cancer Registry, on request, December 2013 to July 2014.

CANNISTRA, S.A. (2004). Cancer of the ovary. N Engl J Med. 351(24):2519-29.

CARRAGHER, N.O. *et al*. (2001). Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J Biol Chem. 276(6):4270-5.

CARRAGHER, N.O. *et al*., (2003). A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr Biol. 13(16):1442-50.

CARRAGHER, N.O. *et al*. (2002). v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. Mol Cell Biol. 22:257–269.

CARRAGHER, N.O., Fonseca BD, Frame MC. (2004). Calpain activity is generally elevated during transformation but has oncogene-specific biological functions. Neoplasia. 6(1):53-73.

CARRAGHER N.O. & FRAME M.C. (2002). Calpain: a role in cell transformation and migration. Int J Biochem Cell Biol. 34(12):1539-43.

CARRAGHER, N.O. & FRAME, M.C. (2004). Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol. 14(5):241-9.

CARRAGHER, N.O. (2006a). Calpain inhibition: a therapeutic strategy targeting multiple disease states. Curr Pharm Des. 12(5):615-38.

CARRAGHER, N.O. (2006b). Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. Oncogene. 25(42):5726-40.

CASTIGLIONI, S. & MAIER, J.A. (2012). The tyrosine phosphatase HD-PTP (PTPN23) is degraded by calpains in a calcium-dependent manner. Biochem Biophys Res Commun. 421(2):380-3.

CHAI, H.H. *et al*., (2014). Homology modeling study of bovine μ-calpain inhibitor-binding domains. Int J Mol Sci. 15(5):7897-938.

CHANG, S.J. *et al*., (2017). Revealing the three dimensional architecture of focal adhesion components to explain Ca2+-mediated turnover of focal adhesions. Biochim Biophys Acta. 1861(3):624-635.

CHAN, J.K. *et al*. (2014). Ovarian cancer rates after hysterectomy with and without salpingooophorectomy, Obstet. Gynecol. 123 (1).p. 65–72.

CHAN, J.K. *et al*. (2016). Weekly vs. Every-3-Week Paclitaxel and Carboplatin for Ovarian Cancer. N Engl J Med.374(8):738-48.

CHAN, K.T. *et al*. (2010).Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). J Biol Chem. 285(15):11418-26.

CHAN WY, CHEUNG KK, SCHORGE JO *et al*. (2000). Bcl-2 and p53 protein expression, apoptosis, and p53 mutation in human epithelial ovarian cancers. Am J Pathol;156:409-17.

CHEN, B. *et al*., (2013). Calpains are required for invasive and metastatic potentials of human HCC cells. Cell Biol Int. 2013 Jul;37(7):643-52.

CHENG JQ, *et al*. (1992). AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci U S A;89:9267-71.

CHESTER C, *et al*. (2015). Immunotherapeutic approaches to ovarian cancer treatment. J Immunother Cancer.pp.3:7.

CHI, D.S. *et al*. (2001). Identification of prognostic factors in advanced epithelial ovarian carcinoma. Gynecol Oncol. 82:532–7.

CHIEN, J. *et al*. (2013). Platinum-sensitive recurrence in ovarian cancer: the role of tumour microenvironment. Front Oncol. 3:251.

CHO, K. R. & SHIH, I. (2009). OVARIAN CANCER. Annu Rev Pathol. 4: 287–313.

CHOW, S.E. *et al*. (2011). Downregulation of p57kip² promotes cell invasion via LIMK/cofilin pathway in human nasopharyngeal carcinoma cells. J Cell Biochem. 112(11):3459-68.

CHUANLIANG, P. *et al*., (2016). Syk expression in non-small-cell lung cancer and its relation with angiogenesis. J Cancer Res Ther. 12(2):663-6.

CLAPHAM, D.E. (2007). Calcium signaling. Cell. 131(6):1047-58.

CLARKE, B. A. & GILKS, B. (2011). Ovarian Carcinoma: Recent Developments in Classification of Tumour Histological Subtype. Canadian Journal of Pathology

COLOMBO, N. *et al*. & ESMO Guidelines Working Group. (2012). Non-epithelial ovarian cancer: ESMO Clinical Practice Guidelines for diagnosis,

treatment and follow-up. Ann Oncol.23 Suppl 7:vii20-6.

CONTEDUCA, V, *et al*. (2014). The emerging role of anti-angiogenic therapy in ovarian cancer (review). Int J Oncol. 44(5):1417-24.

CONNOLLY JL, *et al*. (2003). Role of the Surgical Pathologist in the Diagnosis and Management of the Cancer Patient. In: Kufe DW, Pollock RE, Weichselbaum RR, *et al*., editors. Holland-Frei Cancer Medicine. 6th edition. Hamilton (ON): BC Decker; Available from: https://www.ncbi.nlm.nih.gov/books/NBK13237/

COOKE, S.L., *et al*. (2010). Genomic analysis of genetic heterogeneity and evolution in high-grade serous ovarian carcinoma. Oncogene. 29(35):4905-13.

COOKE, S.L. and BRENTON, J.D. (2011). Evolution of platinum resistance in high-grade serous ovarian cancer. Lancet Oncol. 12(12):1169-74.

COOPMAN, P.J., *et al*., (2000). The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature. 406(6797):742-7.

COOPMAN, P.J. & MUELLER, S.C. (2006). The Syk tyrosine kinase: a new negative regulator in tumor growth and progression. Cancer Lett. 241(2):159-73. Epub 2006 Jan 27.

CORTESIO, C.L. *et al*. (2011). Calpain-mediated proteolysis of paxillin negatively regulates focal adhesion dynamics and cell migration. J Biol Chem. 286(12):9998-10006.

COVAULT, J. *et al*. (1991). Calcium-activated proteolysis of intracellular domains in the cell adhesion molecules NCAM and N-cadherin. Brain Res Mol Brain Res. 11(1):11-6.

CRUM, C.P, *et al*. (2007). Lessons from BRCA: the tubal fimbria emerges as an origin for pelvic serous cancer. Clinical Medicine & Research. 5, pp.35–44.

CUCCHIARELLI, V. *et al*., (2008). Beta-tubulin isotype classes II and V expression patterns in nonsmall cell lung carcinomas. Cell Motil Cytoskeleton. 65(8):675-85.

CUI, C. *et al*. (2017). Targeting calcium signaling in cancer therapy. Acta Pharm Sin B. 7(1):3-17.

DAI, Z. et al. (2014). Capn4 contributes to tumour growth and metastasis of hepatocellular carcinoma by activation of the FAK-Src signalling pathways. J Pathol. 234(3):316-28.

DARNELL, G.A. *et al*., (2007). Human papillomavirus E7 requires the protease calpain to degrade the retinoblastoma protein. J Biol Chem. 2007 Dec 28;282(52):37492-500.

DE ANGELIS, R *et al*. (2014). Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population-based study. Lancet Oncol. 15(1):23-34.

DE CASTRO, R.O. (2011). Regulation and function of syk tyrosine kinase in mast cell signaling and beyond. J Signal Transduct. 2011:507291.

DE CONTO, F. *et al*., (2012). Highly dynamic microtubules improve the

effectiveness of early stages of human influenza A/NWS/33 virus infection in LLC-MK2 cells. PLoS One. 7(7):e41207.

DELAIR, D. *et al*. (2011). Morphologic spectrum of immunohistochemically characterized clear cell carcinoma of the ovary: a study of 155 cases. Am J Surg Pathol. 35(1):36-44.

DEL BELLO, B. *et al*. (2007). Cross-talk between calpain and caspase-3/-7 in cisplatin-induced apoptosis of melanoma cells: a major role of calpain inhibition in cell death protection and p53 status. Oncogene.26(19):2717-26.

DEL BELLO, B. *et al*. (2013). Cisplatin-induced apoptosis inhibits autophagy, which acts as a pro-survival mechanism in human melanoma cells. PLoS One. 8(2):e57236.

DELLA PEPA, C. *et al*. (2015). Ovarian cancer standard of care: are there real alternatives? Chin J Cancer. 34(1):17-27.

DERYCKE, L.D. & Bracke M.E. (2004). N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling. Int J Dev Biol. 48(5-6):463-76.

DEVAPATLA, B. *et al*., (2015). CXCR2 Inhibition Combined with Sorafenib Improved Antitumor and Antiangiogenic Response in Preclinical Models of Ovarian Cancer. PLoS One. 10(9):e0139237.

DEVERY, A.M. *et al*., (2015). Vascular endothelial growth factor directly stimulates tumour cell proliferation in non-small cell lung cancer. Int J Oncol. 47(3):849-56.

DHILLON, K.K., Swisher EM, Taniguchi T. (2011). Secondary mutations of BRCA1/2 and drug resistance. Cancer Sci. 102(4):663-9.

DIECKGRAEFE, B.K. *et al*. (1997). ERK and p38 MAP kinase pathways are mediators of intestinal epithelial wound-induced signal transduction. Biochem Biophys Res Commun. 233(2):389-94.

DINH, P.*et al*. (2008). New therapies for ovarian cancer: cytotoxics and molecularly targeted agents. Crit. Rev. Oncol. Hematol. 67, pp. 103–112.

DIVARIS, K. *et al*. (2012). Examining the accuracy of caregivers' assessments of young children's oral health status. J Am Dent Assoc. 143(11):1237-47.

DOMCHEK, S. M. and REBBECK, T. R. (2007). Prophylactic oophorectomy in women at increased cancer risk. Curr Opin Obstet Gynecol 19 , 27 – 30.

DOMCKE, S. *et al*., (2013). Evaluating cell lines as tumour models by comparison of genomic profiles. Nat Commun 4: 2126.

DONG, S.W. *et al*., (2011). Research on the reactivation of Syk expression caused by the inhibition of DNA promoter methylation in the lung cancer. Neoplasma. 58(1):89-95.

DONG, S.W. *et al*., (2013). Expression of putative tumor suppressor gene spleen tyrosine kinase in esophageal squamous cell carcinoma. Clin Lab. 59(5-6):647-53.

DORMAN, S.N. *et al*., (2016). Genomic signatures for paclitaxel and gemcitabine resistance in breast cancer derived by machine learning.

Mol Oncol. 10(1):85-100.
DOURDIN, N. *et al*., (2001).Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. J Biol Chem. 276(51):48382-8.
DU BOIS, A *et al*. (1997). Carboplatin/paclitaxel versus cisplatin/paclitaxel as first-line chemotherapy in advanced ovarian cancer: an interim analysis of a randomised phase III trial of the Arbeitsgemeinschaft Gynäkologische Onkologie Ovarian Cancer Study Group. Semin Oncol. 24(5 Suppl 15):S15-44-S15-52.
DU M. *et al*. (2017). Effects of phosphorylation on μ-calpain activity at different incubation temperature. Food Res Int. 100(Pt 2):318-324.
DU, X. *et al*., (1995). Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. J Biol Chem. 270(44):26146-51.
Dvořák, Z. *et al*. (2012). Evaluation of *in vitro* cytotoxicity of one-dimensional chain [Fe(salen)(L)](n) complexes against human cancer cell lines. Toxicol *In vitro*. 26(3):480-4.
EBNETH, A. *et al*., (1999). Phosphorylation of MAP2c and MAP4 by MARK kinases leads to the destabilization of microtubules in cells. Cell Motil Cytoskeleton. 44(3):209-24.
ECKSTEIN, N. (2011). Platinum resistance in breast and ovarian cancer cell lines. J Exp Clin Cancer Res. 4;30:91.
EKINCI, F.J. & SHEA T.B. (1999).Free PKC catalytic subunits (PKM) phosphorylate tau via a pathway distinct from that utilized by intact PKC. Brain Res. 850(1-2):207-16.
EGUCHI, R. *et al*., (2010). Calpain is involved in cisplatin-induced endothelial injury in an *in vitro* three-dimensional blood vessel model. Int J Oncol. 37(5):1289-96.
El BAIRI, K *et al*., (2016). Emerging diagnostic, prognostic and therapeutic biomarkers for ovarian cancer. Cell Oncol (Dordr). [Epub ahead of print]
ELCE, J.S., HEGADORN, C. & ARTHUR, J.S. (1997). Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. J Biol Chem. 272(17):11268-75.
ERICKSON, B.K., CONNER, M.G. & LANDEN, C.N. Jr. (2013). The role of the fallopian tube in the origin of ovarian cancer. Am J Obstet Gynecol. 209(5):409-14.
ESCREVENTE, C. *et al*. (2011). Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer. 11:108.
FALCONER H, *et al*. (2015).Ovarian cancer risk after salpingectomy: a nationwide population-based study. J Natl Cancer Inst. 107(2). pii: dju410.
FARFARIELLO, V. *et al*., (2015). Calcium homeostasis in cancer: A focus on senescence. Biochim Biophys Acta. 1853(9):1974-9.
FARKAS A, TOMPA, P, FRIEDRICH, P. (2003). Revisiting ubiquity and tissue specificity of human calpains. Biol Chem. 384(6):945-9.
FARR, C. & BERGER, S. (2010). Measuring calpain activity in fixed and living

cells by flow cytometry. J Vis Exp. (41). pii: 2050.

FASOLATO, C. & POZZAN, T. (1989). Effect of membrane potential on divalent cation transport catalyzed by the "electroneutral" ionophores A23187 and ionomycin. J Biol Chem. 264(33):19630-6.

FEI, B. *et al*. (2013). Modulation by Syk of Bcl-2, calcium and the calpain-calpastatin proteolytic system in human breast cancer cells. Biochim Biophys Acta. 1833(10):2153-64.

FENOUILLE, N. *et al*., (2012). Calpain 2-dependent IκBα degradation mediates CPT-11 secondary resistance in colorectal cancer xenografts. J Pathol. 227(1):118-29.

FERLAY, J. *et al*. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; Available from: http://globocan.iarc.fr, accessed on 05/09/2017.

FERNANDEZ, R., SUCHARD, S.J. (1998). Syk activation is required for spreading and H2O2 release in adherent human neutrophils. J Immunol. 160(10):5154-62.

FLEVARIS, P. *et al*., (2007). A molecular switch that controls cell spreading and retraction. J Cell Biol. 179(3):553-65.

FOSS, C.D. *et al*., (2014). Protein profiling of ovarian cancers by immunohistochemistry to identify potential target pathways. Gynecol Oncol Res Pract. 1:4.

FRANCIS-THICKPENNY, K.M. *et al*. (2001). Analysis of the TGF beta functional pathway in epithelial ovarian carcinoma. Br J Cancer.;85:687-91.

FRANCO, S.J. *et al*., (2004).Calpain-mediated proteolysis of talin regulates adhesion dynamics. Nat Cell Biol. 6(10):977-83.

FRANCO, S. J. & HUTTENLOCHER, A. (2005). Regulating cell migration: Calpains make the cut. J. Cell Sci. 118 (Pt 17): 3829–3838.

FRANK, T. S. *et al*. (1998) Sequence analysis of BRCA1 and BRCA2: correlation of mutations with family history and ovarian cancer risk. J Clin Oncol 16 , 2417 – 2425.

FRIEDL, P et al., (2012). Classifying collective cancer cell invasion. Nat Cell Biol. 14(8):777-83.

FRIEDRICH, P. & Bozóky Z. (2005). Digestive versus regulatory proteases: on calpain action *in vivo*. Biol Chem. 386(7):609-12.

FRIEDRICH, P. (2004). The intriguing Ca^{2+} requirement of calpain activation. Biochem Biophys Res Commun. 323(4):1131-3.

FUJIWARA K. (2012). Three ongoing intraperitoneal chemotherapy trials in ovarian cancer. J Gynecol Oncol. 23(2):75-7.

FULLER, E.S. & HOWELL, V.M. (2014). Culture models to define key mediators of cancer matrix remodeling. Front Oncol. 4:57.

FURUYA, M. *et al*., (2011). Differential expression patterns of CXCR3 variants and corresponding CXC chemokines in clear cell ovarian cancers and endometriosis. Gynecol Oncol. 122(3):648-55.

FURUYA, M. *et al*., (2012). Impaired CXCL4 expression in tumor-associated macrophages (TAMs) of ovarian cancers arising in endometriosis. Cancer Biol Ther. 13(8):671-80.

GAO, J. *et al*., (1997). Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3. EMBO J. 16(21):6414-25.

GAU, D.M. *et al*., (2015). BRCA1 deficiency in ovarian cancer is associated with alteration in expression of several key regulators of cell motility - A proteomics study. Cell Cycle. 14(12):1884-92.

GALLUZZI, L., *et al*. (2012). Molecular mechanisms of cisplatin resistance. Oncogene 31,1869–1883.

GANTA, S. *et al*. (2014).Development of EGFR-targeted nanoemulsion for imaging and novel platinum therapy of ovarian cancer. Pharm Res. 31(9):2490-502.

GEAHLEN, R.L. *et al*., (2014). Getting Syk: spleen tyrosine kinase as a therapeutic target. Trends Pharmacol Sci. 35(8):414-22.

GENTRY-MAHARAJ A & MENON U. (2012). Screening for ovarian cancer in the general population. Best Pract Res Clin Obstet Gynaecol. 26(2):243-56.

GERTIG D.M.*et al*. (2000). Prospective study of talc use and ovarian cancer. J Natl Cancer Inst. 92(3):249-52.

GERSHENSON D.M. *et al*., (2006). Clinical behavior of stage II-IV low-grade serous carcinoma of the ovary, Obstet. Gynecol. 108 (2): 361–368.

GHOTRA, V.P. *et al*., (2015). Syk is a candidate kinase target for the treatment of advanced prostate cancer. Cancer Res. 75(1):230-40.

GIL-PARRADO, S. *et al*., (2002). Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. J Biol Chem. 277(30):27217-26.

GLADING, A. *et al*. (2000). Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J. Biol. Chem. 275, 2390-2398.

GLADING, A., LAUFFENBURGER, D. A. & WELLS, A. (2002) Cutting to the chase: Calpain proteases in cell motility. Trends Cell Biol. 12, 46–54.

GLADING, A. *et al*. (2004). Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol. Cell Biol. 24, 2499-2512.

GODWIN, A.K. *et al*. (1992). High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci U S A. 89(7):3070-4.

GOFF, B.A., *et al*. (1996). Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. Gynecol Oncol. 60:412–417.

GOLL, D.E. *et al*. (2003). The calpain system. Physiol Rev. 83(3):731-801.

GONSCHEROWSKI, V. *et al*., (2006). Calpains: a physiological regulator of the endothelial barrier? Am J Physiol Heart Circ Physiol. 290(5):H2035-42.

GRADA, A. *et al*., (2017). Research Techniques Made Simple: Analysis of

Collective Cell Migration Using the Wound Healing Assay. J Invest Dermatol. 137(2):e11-e16.

Grädler U *et al*., (2013). Structural and biophysical characterization of the Syk activation switch. J Mol Biol. 425(2):309-33.

GRAFFEP, R. *et al*., (2016). Time to incorporate germline multigene panel testing into breast and ovarian cancer patient care. Breast Cancer Res Treat. 160(3):393-410.

GREER, B.E. *et al*., (2004). Surgery for ovarian cancer: rationale and guidelines. J Natl Compr Canc Netw. 2(6):561-8.

GREER, S. *et al*., (2010). Housekeeping genes; expression levels may change with density of cultured cells. J Immunol Methods. 2010 Apr 15;355(1-2):76-9.

GRIFFITHS, C.T. (1975). Surgical resection of tumour bulk in the primary treatment of ovarian carcinoma. Natl Cancer Inst Monogr.42:101–4.

GRIFFITHS, C.T. & FULLER, A.F. (1978). Intensive surgical and chemotherapeutic management of advanced ovarian cancer. Surg Clin North Am 58(1):131−42.

GUINGAB-CAGMAT, J.D. *et al*. (2012). In vitro MS-based proteomic analysis and absolute quantification of neuronal-glial injury biomarkers in cell culture system. Electrophoresis. 33(24):3786-97.

GU, J. et al., (2015). Capn4 promotes non-small cell lung cancer progression via upregulation of matrix metalloproteinase 2. Med Oncol. 32(3):51.

GUTTMANN, R.P. & JOHNSON, G.V. (1998). Oxidative stress inhibits calpain activity in situ. J Biol Chem. 273(21):13331-8.

Guéguinou M *et al*., (2016). SK3/TRPC1/Orai1 complex regulates SOCE-dependent colon cancer cell migration: a novel opportunity to modulate anti-EGFR mAb action by the alkyl-lipid Ohmline. Oncotarget. 7(24):36168-36184.

GUROFF, G. (1964). A neutral, calcium-activated proteinase from the soluble fraction of rat brain. J. Biol. Chem. 239, 149-155.

HAINING, A.W. *et al*., (2016). Talin: a mechanosensitive molecule in health and disease. FASEB J. 30(6):2073-85.

HAIT, W.N., Yang J.M. (2006).The individualization of cancer therapy: the unexpected role of p53. Trans Am Clin Climatol Assoc. 117:85-101; discussion 101.

HAN, Y. *et al*. (1999). Tumor necrosis factor-a-inducible InBa proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factornB activation. J Biol Chem. 274:787–794.

HAN, E.S., WAKABAYASHI, M. & LEONG, L. (2013). Angiogenesis inhibitors in the treatment of epithelial ovarian cancer. Curr Treat Options Oncol. 14(1):22-33.

HASLEHURST, A.M. *et al*. (2012). EMT transcription factors snail and slug directly contribute to cisplatin resistance in ovarian cancer. BMC Cancer.

12:91.

HATA, K. *et al*., (2009). Association of metastin/a G-protein-coupled receptor signaling and Down syndrome critical region 1 in epithelial ovarian cancer. Anticancer Res. 29(2):617-23.

HATA, S. *et al*. (2001). Domain II of m-calpain is a Ca 2+ -dependent cysteine protease. FEBS Lett 501:111–114.

HAVELOCK, J.C., RAINEY, W.E. & CARR, B.R. (2004). Ovarian granulosa cell lines. Mol Cell Endocrinol. 30;228(1-2):67-78.

HESS, V, *et al*. (2004). Mucinous epithelial ovarian cancer: a separate entity requiring specific treatment. J Clin Oncol. 22: 1040–1044.

HILBERG, F. *et al*., (2008). BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy. Cancer Res. 68(12):4774-82.

HILDEBRAND, J.S. *et al*., (2015). Moderate-to-vigorous physical activity and leisure-time sitting in relation to ovarian cancer risk in a large prospective US cohort. Cancer Causes Control. 26(11):1691-7.

HILL, J.W., HU, J.J. & EVANS, M.K. (2008). OGG1 is degraded by calpain following oxidative stress and cisplatin exposure. DNA Repair (Amst). 7(4):648-54.

HIWASA, T. *et al*., (2002). Regulation of transformed state by calpastatin via PKCepsilon in NIH3T3 mouse fibroblasts. Biochem Biophys Res Commun. 290(1):510-7.

HODGSON, L. & DONG, C. (2001). [Ca2+]i as a potential downregulator of alpha2beta1-integrin-mediated A2058 tumor cell migration to type IV collagen. Am J Physiol Cell Physiol. 281(1):C106-13.

HOLLIS, R.L.& GOURLEY, C. (2016). Genetic and molecular changes in ovarian cancer. Cancer Biol Med. 13(2):236-47.

HOLMES, K. *et al*., (2010). VEGF stimulates RCAN1.4 expression in endothelial cells via a pathway requiring Ca2+/calcineurin and protein kinase C-delta. PLoS One. 5(7):e11435.

HONG, J. *et al*., (2012). CHK1 targets spleen tyrosine kinase (L) for proteolysis in hepatocellular carcinoma. J Clin Invest. 122(6):2165-75.

HONORE, S. *et al*. (2005). Understanding microtubule dynamics for improved cancer therapy. Cell Mol Life Sci. 62(24):3039-56.

HOOD, J.L., *et al*. (2004).Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus. J Biol Chem. 279(41):43126-35. Epub 2004 Aug 9.

HOOD J.L., Brooks W.H. & Roszman T.L. (2006). Subcellular mobility of the calpain/calpastatin network: an organelle transient. Bioessays. 28(8):850-9.

HORIUCHI, A. *et al*. (2012). Hypoxia upregulates ovarian cancer invasiveness via the binding of HIF-1α to a hypoxia-induced, methylation-free hypoxia response element of S100A4 gene. Int J Cancer. 131(8):1755-67.

HOSFIELD C.M. *et al*. (1999). Crystal structure of calpain reveals the structural

basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. Embo J. 18:6880–9.

HOU, J. (2012). 17beta-estradiol induces both up-regulation and processing of cyclin E in a calpain-dependent manner in MCF-7 breast cancer cells. FEBS Lett. 586(6):892-6.

HOWLADER, N. *et al*. (eds). SEER Cancer Statistics Review, 1975-2013, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2013/, based on November 2015 SEER data submission, posted to the SEER web site, April 2016.

HUANG, C. *et al*. (2004). MAP kinases and cell migration. J Cell Sci. 117(Pt 20):4619-28.

HUANG, C. *et al*., (2009). Talin phosphorylation by Cdk5 regulates Smurf1-mediated talin head ubiquitylation and cell migration. Nat Cell Biol. 11(5):624-30.

HUANG, C, *et al*. (2017). Signaling regulation and role of filamin A cleavage in Ca^{2+}-stimulated migration of androgen receptor-deficient prostate cancer cells. Oncotarget. 8(3):3840-3853.

HUANG, J. *et al*. (2016). Roles of integrin β3 cytoplasmic tail in bidirectional signal transduction in a trans-dominant inhibition model. Front Med. 10(3):311-9.

HUTTENLOCHER, A., *et al*. (1997). Regulation of cell migration by the calcium-dependent protease calpain. J Biol Chem. 272:32719-22.

HUYNH, V.T. *et al*. (2012). Block copolymer micelles with pendant bifunctional chelator for platinum drugs: effect of spacer length on the viability of tumor cells. Biomacromolecules. 13(4):1010-23.

IM-ARAM, A., *et al*. (2013). The mTORC2 component rictor contributes to cisplatin resistance in human ovarian cancer cells. PLoS One. 8(9):e75455.

INOMATA M, *et al*. (1993). A variety of calpain/calpastatin systems in mammalian erythrocytes. Biochim Biophys Acta. 1178(2):207-14.

Jabłońska-Trypuć A, Matejczyk M & Rosochacki S. (2016). Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J Enzyme Inhib Med Chem. 31(sup1):177-183.

JACOBY, V.L., *et al*. (2009). Factors associated with undergoing bilateral salpingo-oophorectomy at the time of hysterectomy for benign conditions. Obstet Gynecol. 113(6):1259-67.

JACQUEMET, G. *et al*., (2016). L-type calcium channels regulate filopodia stability and cancer cell invasion downstream of integrin signalling. Nat Commun.7:13297.

JANG, H.S, Lal S, Greenwood JA. (2010). Calpain 2 is required for glioblastoma cell invasion: regulation of matrix metalloproteinase 2. Neurochem Res. 35(11):1796-804.

JANG, Y.N. *et al*., (2009). Calpain-mediated N-cadherin proteolytic processing

in brain injury. J Neurosci. 29(18):5974-84.

JARRETT, C.R. *et al*., (2001). Human APC2 localization and allelic imbalance. Cancer Res. 61(21):7978-84.

JEON SY, Hwang KA, Choi KC. (2016). Effect of steroid hormones, estrogen and progesterone, on epithelial mesenchymal transition in ovarian cancer development. J Steroid Biochem Mol Biol. 158:1-8.

Jendželovská, Z. *et al*. (2014). Single pre-treatment with hypericin, a St. John's wort secondary metabolite, attenuates cisplatin- and mitoxantrone-induced cell death in A2780, A2780cis and HL-60 cells. Toxicol *In vitro*. 28(7):1259-73.

JIA, L. *et al*., (2012). Proteomic analysis reflects different histologic subtypes of epithelial ovarian cancer. Med Hypotheses. 78(3):407-9.

JIANG, Y.Y. *et al*., (2016). Microtubule-associated protein 4 is an important regulator of cell invasion/migration and a potential therapeutic target in esophageal squamous cell carcinoma. Oncogene. 35(37):4846-56.

KALHORI, V. & Törnquist K. (2015). MMP2 and MMP9 participate in S1P-induced invasion of follicular ML-1 thyroid cancer cells. Mol Cell Endocrinol. 404:113-22.

KARNEZIS, A.N., *et al*. (2017). The disparate origins of ovarian cancers: pathogenesis and prevention strategies. Nat Rev Cancer. 17(1):65-74.

KASSIS, J. *et al*. (2002). Motility is rate-limiting for invasion of bladder carcinoma cell lines. Int J Biochem Cell Biol. 34(7):762-75.

KATSUMATA N. (2011). Dose-dense therapy is of benefit in primary treatment of ovarian cancer? In favor. Ann Oncol. 22 Suppl 8:viii29-viii32.

KATSUMATA N. (2015). Dose-dense approaches to ovarian cancer treatment. Curr Treat Options Oncol. 16(5):21.

KATZ, E. *et al*., (2005). MMTV Env encodes an ITAM responsible for transformation of mammary epithelial cells in three-dimensional culture. J Exp Med. 201(3):431-9.

KAVALLARIS, M. *et al*., (2001). Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells. Cancer Res. 61(15):5803-9.

KEHOE S, *et al*. Chemotherapy or upfront surgery for newly diagnosed advanced ovarian cancer: results from the MRC CHORUS trial. J Clin Oncol, 2013,31 suppl:abstr 5500. Available at: http://meetinglibrary.asco.org/content/112631-132.

KELEMEN, L.E. & KOBEL, M. (2011). Mucinous carcinomas of the ovary and colorectum: different organ, same dilemma. Lancet Oncology. 12:1071–1080.

KENNY, H.A. *et al*., (2009). Organotypic models of metastasis: A three-dimensional culture mimicking the human peritoneum and omentum for the study of the early steps of ovarian cancer metastasis. Cancer Treat Res. 149:335-51.

KHOUJA, M.H. *et al*., (2010).Limitations of tissue microarrays compared with

whole tissue sections in survival analysis. Oncol Lett. 1(5):827-831. Epub 2010 Sep 1.

KIM, D.S. *et al*., (2010). I-kappaBalpha depletion by transglutaminase 2 and mu-calpain occurs in parallel with the ubiquitin-proteasome pathway. Biochem Biophys Res Commun. 399(2):300-6.

KIM, M.J. *et al*., (2007). Hypoxia-induced cell death of HepG2 cells involves a necrotic cell death mediated by calpain. Apoptosis.12(4):707-18.

KIM, J.Y., *et al*. (2017). Disulfiram induces anoikis and suppresses lung colonization in triple-negative breast cancer viacalpain activation. Cancer Lett. 386:151-160.

KIMURA, Y. *et al*. (2000). Calpain-dependent proteolysis of NF2 protein: involvement in schwannomas and meningiomas. Neuropathology. 20(3):153-60.

KLINCK, R. et al., (2008). Multiple alternative splicing markers for ovarian cancer. Cancer Res. 68(3):657-663.

KOBAYASHI E *et al*., (2012). Biomarkers for screening, diagnosis, and monitoring of ovarian cancer. Cancer Epidemiol Biomarkers Prev. 21(11):1902-12.

KOCH, M. *et al*. (2013). Overcoming chemotherapy resistance of ovarian cancer cells by liposomal cisplatin: molecular mechanisms unveiled by gene expression profiling. Biochem Pharmacol. 85(8):1077-90.

KOHLER MF, *et al*. (1993). Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. J Natl Cancer Inst;85:1513-9.

KOHN, E.C. & HURTEAU, J. (2013). Ovarian cancer : making its own rules-again. Cancer.;119(3):474-6.

KOVACS, L. *et al*., (2016). Activation of Calpain-2 by Mediators in Pulmonary Vascular Remodeling of Pulmonary Arterial Hypertension. Am J Respir Cell Mol Biol. 54(3):384-93.

KRAPPMANN, D. & SCHEIDEREIT, C. (2005). A pervasive role of ubiquitin conjugation in activation and termination of InB kinase pathways. EMBO Rep 2005;6:321–6.

KRISENKO, M.O., *et al*. (2014). Nanomechanical property maps of breast cancer cells as determined by multiharmonic atomic force microscopy reveal Syk-dependent changes in microtubule stability mediated by MAP1B. Biochemistry 54, 60–68.

KRISENKO, M.O. & GEAHLEN, R.L. (2015).Calling in Syk: Syk's dual role as a tumor promoter and tumor suppressor in cancer. Biochim Biophys Acta. 1853(1):254-63.

KRISHNAN, S. *et al*., (2008). Differential expression and molecular associations of Syk in systemic lupus erythematosus T cells. J Immunol. 181(11):8145-52.

Kristjánsdóttir B *et al*., (2014). Early inflammatory response in epithelial ovarian tumor cyst fluids. Cancer Med. 3(5):1302-12.

KROEBER, S. *et al*. (2000). Analyses of signal transduction cascades reveal an

essential role of calcium ions for regulation of melatonin biosynthesis in the light-sensitive pineal organ of the rainbow trout (Oncorhynchus mykiss). J Neurochem. 74(6):2478-89.

KUANG, J. *et al*. (2001). Bimodal effects of 1R,2R-diaminocyclohexane(trans-diacetato)(dichloro)platinum(IV) on cell cycle checkpoints. Clin Cancer Res. 7(11):3629-39.

KUDO-SAKAMOTO, Y. *et al*., (2014). Calpain-dependent cleavage of N-cadherin is involved in the progression of post-myocardial infarction remodeling. J Biol Chem. 289(28):19408-19.

KULKARNI, S, *et al*. (2002). Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. J Biol Chem. 277(27):24435-41.

KUMAGAI, K *et al*., (2008). Role of mu-calpain in human decidua for recurrent miscarriage. Am J Reprod Immunol. 59(4):339-46.

KUMAR, V. & ALI, A., (2017). Targeting calpains: A novel immunomodulatory approach for microbial infections. Eur J Pharmacol. pii: S0014-2999(17)30506-X.

KUO KT, MAO TL, JONES S, *et al*. (2009). Frequent activating mutations of PIK3CA in ovarian clear cell carcinoma. Am J Pathol 174:1597–601.

KUPRYJANCZYK, J. *et al*. (1993).p53 gene mutations and protein accumulation in human ovarian cancer. Proc Natl Acad Sci U S A. 90:4961-5.

KURMAN RJ. (2013). Origin and molecular pathogenesis of ovarian high-grade serous carcinoma. Ann Oncol. 24 Suppl 10:x16-21.

KURMAN RJ, SHIH IeM. (2010). The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol. 34(3):433-43.

KWA, M. & MUGGIA, F. (2014). Ovarian cancer: a brief historical overview of intraperitoneal trials. Ann Surg Oncol. 21(5):1429-34.

LAI, C.K. *et al*., (2014). 3,3',4', 5'-Tetramethoxychalcone inhibits human oral cancer cell proliferation and migration via p53-mediated mitochondrial-dependent apoptosis. Anticancer Res. 34(4):1811-9.

LAKSHMIKUTTYAMMA, A. *et al*., (2004). Overexpression of m-calpain in human colorectal adenocarcinomas. Cancer Epidemiol Biomarkers Prev. 13(10):1604-1609.

LAPINSKA, K., *et al*. (2016). The Effects of Histone Deacetylase Inhibitor and Calpain Inhibitor Combination Therapies on Ovarian Cancer Cells. Anticancer Res. Nov;36(11):5731-5742.

LANDEN, Jr C.N., BIRRER, M.J. & SOOD, A.K. (2008). Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol 26:995–1005

LANGDON, S.P. *et al*. (1988). Characterization and properties of nine human ovarian adenocarcinoma cell lines. Cancer Res. 48(21):6166-72.

LASAGNI, L. *et al*. (2003). An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. J Exp Med.

197(11):1537-49.
LATINI, F.R. *et al*., (2013). DARC (Duffy) and BCAM (Lutheran) reduced expression in thyroid cancer. Blood Cells Mol Dis. 50(3):161-5.
LAYTON T., *et al*., (2009). Syk tyrosine kinase acts as a pancreatic adenocarcinoma tumor suppressor by regulating cellular growth and invasion. Am J Pathol. 175(6):2625-36.
LEBART M.C. & BENYAMIN Y. (2006). Calpain involvement in the remodeling of cytoskeletal anchorage complexes. FEBS J. 273(15):3415-3426.
LEE, J.Y. *et al*., (2013).Ovarian cancer biomarker discovery based on genomic approaches. J Cancer Prev. 18(4):298-312.
LEE, K.R. & YOUNG, R.H. (2003). The distinction between primary and metastatic mucinous carcinomas of the ovary: gross and histologic findings in 50 cases. American Journal of Surgical Pathology. 27:281–292.
LEE, S.J. *et al*. (2008). Increased expression of calpain 6 during the progression of uterine cervical neoplasia: immunohistochemical analysis. Oncol Rep. 19(4):859-63.
LEITINGER, B. *et al*., (2000). The regulation of integrin function by Ca(2+). Biochim Biophys Acta. 1498(2-3):91-8.
LELOUP, L. *et al*., (2010). m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate. J Biol Chem. 285(43):33549-66.
LELOUP L. & WELLS A. (2011). Calpains as potential anti-cancer targets. Expert Opin Ther Targets. 15(3):309-23.
LENTSCH, A.B. (2006). CXC chemokines and prostate cancer: growth regulators and potential biomarkers. Future Oncol. 2(5):651-8.
LIANG, Y. *et al*. (2006). Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. Endocr Relat Cancer. 13(3):905-19.
LIAO, P.C. *et al*., (2008). Involvement of endoplasmic reticulum in paclitaxel-induced apoptosis. J Cell Biochem. 104(4):1509-23.
LI, C.L. *et al*., (2017). Fibronectin induces epithelial-mesenchymal transition in human breast cancer MCF-7 cells via activation of calpain. Oncol Lett. 13(5):3889-3895.
LI, H., *et al*. (2004). Effects of autolysis on properties of mu- and m-calpain. Biochim Biophys Acta. 1691(2-3):91-103.
LIU, F. *et al*., (2013). Mitochondrially mediated integrin αIIbβ3 protein inactivation limits thrombus growth. J Biol Chem. 288(42):30672-81.
LIU, L. *et al*., (2008). Calpain-mediated pathway dominates cisplatin-induced apoptosis in human lung adenocarcinoma cells as determined by real-time single cell analysis. Int J Cancer. 122(10):2210-22.
LIU, L., XING, D. & CHEN, W.R. (2009). Micro-calpain regulates caspase-dependent and apoptosis inducing factor-mediated caspase-independent apoptotic pathways in cisplatin-induced apoptosis. Int J Cancer. 125(12):2757-66.

LIU, L. *et al*., (2017). Calpain and AR-V7: Two potential therapeutic targets to overcome acquired docetaxel resistance in castration-resistant prostate cancer cells. Oncol Rep. 37(6):3651-3659.

LIU, T. *et al*., (2014). Prolonged androgen deprivation leads to overexpression of calpain 2: implications for prostate cancer progression. Int J Oncol. 44(2):467-72.

LIU, Y. *et al*. (2016). A calpain-2 selective inhibitor enhances learning & memory by prolonging ERK activation. Neuropharmacology. 105:471-477.

LI, Y. *et al*., (2014). The oncoprotein HBXIP enhances migration of breast cancer cells through increasing filopodia formation involving MEKK2/ERK1/2/Capn4 signaling. Cancer Lett. 2014 Dec 28;355(2):288-96.

LI, Y. *et al*., (2016). High Stoichiometry Phosphorylation of Talin at T144/T150 or S446 Produces Contrasting Effects on Calpain-mediated Talin Cleavage and Cell Migration. J Cancer. 7(12):1645-1652.

LI, Z. *et al*., (1996). Novel peptidyl alpha-keto amide inhibitors of calpains and other cysteine proteases. J Med Chem. 39(20):4089-98.

López-Colomé A.M. *et al*., (2017). Paxillin: a crossroad in pathological cell migration. J Hematol Oncol. Feb 18;10(1):50.

Longuespée R *et al*., (2012). Ovarian cancer molecular pathology. Cancer Metastasis Rev. 31(3-4):713-32.

LOWELL, C.A. *et al*., (2011) .Src-family and Syk kinases in activating and inhibitory pathways in innate immune cells: signaling cross talk. Cold Spring Harb Perspect Biol. 3(3). pii: a002352.

LUANGDILOK, S. *et al*., (2007). Syk tyrosine kinase is linked to cell motility and progression in squamous cell carcinomas of the head and neck. Cancer Res. 67(16):7907-16.

LUO W. *et al*., (2016). Clinical correlation of calpain-1 and glypican-3 expression with gallbladder carcinoma. Oncol Lett. 11(2):1345-1352. Epub 2016 Jan 7.

LUVERO, D., MILANI, A. & LEDERMANN, J.A. (2014). Treatment options in recurrent ovarian cancer: latest evidence and clinical potential. Ther Adv Med Oncol. 6(5):229-39.

MABUCHI, S. *et al*., (2016). Clear cell carcinoma of the ovary: molecular insights and future therapeutic perspectives. J Gynecol Oncol. 27(3):e31.

MCCARTNEY, C.E. *et al.,* (2017). An easy-to-use FRET protein substrate to detect calpain cleavage in vitro and in vivo. Biochim Biophys Acta. 1865(2):221-230.

MA, D. *et al*., (2017). High level of calpain1 promotes cancer cell invasion and migration in oral squamous cell carcinoma. Oncol Lett. 13(6):4017-4026.

MAILE, L.A. *et al*., (2008). Modulation of integrin antagonist signaling by ligand binding of the heparin-binding domain of vitronectin to the alphaVbeta3 integrin. J Cell Biochem. 105(2):437-46.

MA, H., *et al*. (1994). Amino-terminal conserved region in proteinase inhibitor domain of calpastatin potentiates its calpain inhibitory activity by interacting with the calmodulin-like domain of the proteinase. J. Biol. Chem. 269, 24430–24436.

MA, L. *et al*., (2010). The relationship between methylation of the Syk gene in the promoter region and the genesis of lung cancer. Clin Lab. 56(9-10):407-16.

MAEDA, S. (2017). Duffy antigen receptor for chemokines (DARC) expressing in cancercells inhibits tumor progression by suppressing CXCR2 signaling in human pancreatic ductal adenocarcinoma. Cytokine. 95:12-21.

MAKI, M., *et al*. (1989). Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. J. Biol. Chem. 264, 18866–18869.

MALPICA, A. (2008). Grading of ovarian cancer: a histotype-specific approach. Int J Gynecol Pathol 27;175–81.

MAMOUNE, A. *et al*., (2003). Calpain-2 as a target for limiting prostate cancer invasion. Cancer Res. 63(15):4632-40.

MANDAI, M. *et al*. (2016). Anti-PD-L1/PD-1 immune therapies in ovarian cancer: basic mechanism and future clinical application. Int J Clin Oncol. 21(3):456-61.

MANDIC, A. *et al*., (2002). Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. Mol Cell Biol. 22(9):3003-13.

MANDIC, A. *et al*., (2003). Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem. 278(11):9100-6.

MARAVALL, M. *et al*. (2000). Estimating intracellular calcium concentrations and buffering without wavelength ratioing. Biophys J. 78:2655–2667.

MARENGO, B. *et al*., (2016). Redox Homeostasis and Cellular Antioxidant Systems: Crucial Players in Cancer Growth and Therapy. Oxid Med Cell Longev. 2016:6235641.

MARKMAN, M. (2013). Current standards of care for chemotherapy of optimally cytoreduced advanced epithelial ovarian cancer. Gynecol Oncol. 2013 Oct;131(1):241-5.

MARTINS, I. *et al*. (2011).Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. Oncogene. 30(10):1147-58.

MARTELLO, L.A., *et al*. (2003). Elevated levels of microtubule destabilizing factors in a Taxol-resistant/dependent A549 cell line with an {alpha}-tubulin mutation, Cancer Res. 63(6): 1207–1213.

MASUDA, H., TANAKA, T. & TAKAHAMA, U. (1994). Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. Biochem Biophys Res Commun. 203(2):1175-80.

MATTHEIJ, N.J. *et al*., (2013). Dual mechanism of integrin αIIbβ3 closure in procoagulant platelets. J Biol Chem. 288(19):13325-36.

MATULONIS, U.A. *et al*. (2011). High throughput interrogation of somatic mutations in high grade serous cancer of the ovary. PLoS One. 6:e24433.

MCCLUGGAGE, W.G. (2011). Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis. Pathology. 43(5), pp.420-432.

MCCLUGGAGE, W.G. (2012). Immunohistochemistry in the distinction between primary and metastatic ovarian mucinous neoplasms. J Clin Pathol. 65(7):596-600.

MCGROGAN, B.T. *et al*. (2008). Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta. 1785(2):96-132. Epub 2007 Nov 12.

MCGUIRE WP, *et al*. (1996). Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med. 334:1-6. CrossRefMedline.

MCKEOWN, S.R. (2014). Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br J Radiol. 87(1035):20130676.

MCLACHLAN, R.W. & YAP, A.S. (2007). Not so simple: the complexity of phosphotyrosine signaling at cadherin adhesive contacts. J Mol Med (Berl). 85(6):545-54.

MCSHANE, L.M. *et al*. (2005). REporting recommendations for tumour MARKer prognostic studies (REMARK). Br. J. Cancer. 93; 387–391.

MEINHOLD-HEERLEIN, I. *et al*., Kommission Ovar of the AGO. (2015). Statement by the Kommission Ovar of the AGO: The New FIGO and WHO Classifications of Ovarian, Fallopian Tube and Primary Peritoneal Cancer. Geburtshilfe Frauenheilkd. 75(10):1021-1027.

MENON, U, GRIFFIN M & GENTRU-MAHARAJ A. (2014). Ovarian cancer screening--current status, future directions. Gynecol Oncol. 132(2):490-5.

MEREDITH, J. Jr, *et al*. (1998). Cleavage of the cytoplasmic domain of the integrin beta3 subunit during endothelial cell apoptosis. J Biol Chem. 273(31):19525-31.

MERRITT, M.A. & CRAMER, D.W. (2010). Molecular pathogenesis of endometrial and ovarian cancer. Cancer Biomark. 9:287–305.

Mikuła-Pietrasik J *et al*., (2016a). Biochemical composition of malignant ascites determines high aggressiveness of undifferentiated ovarian tumors. Med Oncol. 33(8):94.

Mikuła-Pietrasik J *et al*., (2016b). Senescent peritoneal mesothelium induces a pro-angiogenic phenotype in ovarian cancer cells *in vitro* and in a mouse xenograft model *in vivo*. Clin Exp Metastasis. 33(1):15-27.

MOHAMMAD, D.K. *et al*., (2016). Protein kinase B (AKT) regulates SYK activity and shuttling through 14-3-3 and importin 7. Int J Biochem Cell Biol. 78:63-74.

MODUGNO, F. *et al*. (2012). Hormone response in ovarian cancer: time to reconsider as a clinical target? Endocr Relat Cancer. 19(6):R255-79.

MOLDVEANU T. *et al*. (2002). A Ca 2+ switch align the active site of calpain. Cell 108:649 – 660.

MOLDOVEANU T., JIA Z. & DAVIES P.L. (2004). Calpain activation by cooperative Ca2+ binding at two non-EF-hand sites. J Biol Chem. 279:6106–14.

MOLINARI, M., ANAGLI, J. & CARAFOLI, E. (1995). PEST sequences do not influence substrate susceptibility to calpain proteolysis. J Biol Chem. 270(5):2032-5.

MOLINARI, M., CARAFOLI, E. (1997). Calpain: a cytosolic proteinase active at the membranes. J Membr Biol 156: 1–8.

MORENO-Bueno G, *et al*. (2001). beta-Catenin expression pattern, beta-catenin gene mutations, and microsatellite instability in endometrioid ovarian carcinomas and synchronous endometrial carcinomas. Diagn Mol Pathol. 10:116-22.

MORETTI, D. *et al*., (2009). Novel variants of muscle calpain 3 identified in human melanoma cells:cisplatin-induced changes *in vitro* and differential expression in melanocytic lesions. Carcinogenesis. 2009 Jun;30(6):960-7.

MORETTI, D. *et al*., (2014). Calpains and cancer: friends or enemies? Arch Biochem Biophys. 564:26-36.

MORITA, M. *et al*., (2006). Mu-calpain is involved in the regulation of TNF-alpha-induced matrix metalloproteinase-3 release in a rheumatoid synovial cell line. Biochem Biophys Res Commun. 343(3):937-42.

MORONE, S. *et al*., (2012). Overexpression of CD157 contributes to epithelial ovarian cancer progression by promoting mesenchymal differentiation. PLoS One. 7(8):e43649.

MORONI, M., *et al*. (2004). Progressive loss of Syk and abnormal proliferation in breast cancer cells. Cancer Res. 64(20):7346–7354.

MTANGO, N.R., POTIREDDY, S. & LATHAM, K.E. (2008). Oocyte quality and maternal control of development. Int Rev Cell Mol Biol. 268:223-90.

MUELLER, A. *et al*., (1999). Subcellular distribution of S100 proteins in tumor cells and their relocation in response to calcium activation. Histochem Cell Biol. 111(6):453-9.

MUGGIA, F.M. *et al*. (2000). Phase III randomised study of cisplatin versus paclitaxel versus cisplatin and paclitaxel in patients with suboptimal stage II or IV ovarian cancer: a gynecologic oncology group study, J. Clin. Oncol. pp.18, 15–106.

MURACHI, T. *et al*. (1980). Intracellular Ca 2 + -dependent protease (calpain) and its high-molecular-weight endogenous inhibitor (calpastatin). Adv. Enzyme Regul. 19, 407–424.

MURACHI, T. *et al*. (1989). The calpain-calpastatin system in hematopoietic cells. Adv Exp Med Biol.255:445-54.

MURACHI, T. *et al*. (1990). Gene expression for calpain isozymes in human hematopoietic system cells. Prog Clin Biol Res. 344:477-94.

MUSRAP, N. *et al*., (2014). Proteomic analysis of cancer and mesothelial cells reveals an increase in Mucin 5AC during ovarian cancer and peritoneal interaction. J Proteomics. 103:204-15.

MUTCH, D.G. & PRAT, J. (2014). 2014 FIGO staging for ovarian, fallopian tube and peritoneal cancer. Gynecol Oncol. 133(3):401-4.

Młynarczuk-Biały I *et al*., (2006). Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells. Cancer Res. 66(15):7598-605.

Münstedt K. & Franke F.E. (2004). Role of primary surgery in advanced ovarian cancer. World J Surg Oncol. 2:32.

NABESHIMA, Y. *et al*. (2014). Calpain 1 inhibitor BDA-410 ameliorates α-klotho-deficiency phenotypes resembling human aging-related syndromes. Sci Rep. 4:5847.

NAKAGAWA K. *et al*. (2001). Dissociation of m-calpain subunits occurs after autolysis of the N-terminus of the catalytic subunit, and is not required for activation. J Biochem. 130:605–11.

NAKASHIMA, H. *et al*., (2006). Clinical significance of nuclear expression of spleen tyrosine kinase (Syk) in gastric cancer. Cancer Lett. 236(1):89-94.

NAROD, S. *et al*. (1995) Genetic heterogeneity of breast-ovarian cancer revisited. Breast Cancer Linkage Consortium. Am J Hum Genet 57 , 957 – 958

NEIJT, J.P. *et al*. (1991). Long-term survival in ovarian cancer. Mature data from The Netherlands Joint Study Group for Ovarian Cancer. Eur J Cancer. 27(11):1367–72.

NEZHAT, F.R. *et al*., (2015). New insights in the pathophysiology of ovarian cancer and implications for screening and prevention. Am J Obstet Gynecol. 213(3):262-7.

NEZHAT, F. *et al*. (2008). The relationship of endometriosis and ovarian malignancy: a review. Fertility and Sterility. 90:1559–1570.

NGUYEN, H.L. *et al*. (1999). Microtubule-associated protein 4 (MAP4) regulates assembly, protomer-polymer partitioning and synthesis of tubulin in cultured cells. J Cell Sci. 112 (Pt 12):1813-24.

NGUYEN, H. N. *et al*. (1994) Ovarian carcinoma. A review of the significance of familial risk factors and the role of prophylactic oophorectomy in cancer prevention. Cancer 74 , 545 – 555.

NGUYEN, H.T. & Chen M. (2014). High-energy compounds mobilize intracellular Ca2+ and activate calpain in cultured cells: is calpain an energy-dependent protease? Brain Res Bull. 102:9-14.

NIKOLAEV, A.Y. *et al*., (2003).Parc: a cytoplasmic anchor for p53. Cell. 112(1):29-40.

NOLEN BM and LOKSHIN AE. (2012). Protein biomarkers of ovarian cancer: the forest and the trees. Future Oncol. 8(1):55-71.

OGANE, S. *et al*., (2009). Spleen tyrosine kinase as a novel candidate tumor suppressor gene for human oral squamous cell carcinoma. Int J Cancer.

124(11):2651-7.
OHBAYASHI, K. *et al*., (1998). Stimulation of L-type Ca2+ channel in growth cones activates two independent signaling pathways. J Neurosci Res. 51(6):682-96.
OLMSTED, J.B. *et al*., (1989). Cell cycle-dependent changes in the dynamics of MAP 2 and MAP 4 in cultured cells. J Cell Biol. 109(1):211-23.
ONO, Y., SAIDO, T.C. & SORIMACHI, H. (2016). Calpain research for drug discovery: challenges and potential. Nat Rev Drug Discov. 15(12):854-876.
ORR, G.A. *et al*., (2003).Mechanisms of Taxol resistance related to microtubules. Oncogene. 22(47):7280-95.
OZOLS R.F., *et al*. (2003). Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. J Clin Oncol.21:3194-200.
OZOLS, R.F. (2006). Challenges for chemotherapy in ovarian cancer. Ann Oncol. Suppl 5:v181-7.
PALMANTIER, R. *et al*., (1996). Regulation of the adhesion of a human breast carcinoma cell line to type IV collagen and vitronectin: roles for lipoxygenase and protein kinase C. Cancer Res. 56(9):2206-12.
PARK, J.J. *et al*., (2012). Effects of lysophosphatidic acid on calpain-mediated proteolysis of focal adhesion kinase in human prostate cancer cells. Prostate. 72(15):1595-610.
PARR, T., *et al*. (2001). Calpastatin expression in porcine cardiac and skeletal muscle and partial gene structure. Archives of Biochemistry and Biophysics, 395, 1-13.
PATCH AM *et al*., (2015). Whole-genome characterization of chemoresistant ovarian cancer. Nature. 521(7553):489-94.
PECORELLI, S., ODICINO, F.& FAVALLI, G. (2002). Interval debulking surgery in advanced epithelial ovarian cancer. Best Pract Res, Clin Obstet Gynaecol.16:573–83.
PECTASIDES D, *et al*. (2005). Advanced stage mucinous epithelial ovarian cancer; the Hellenic Cooperative Oncology Group experience. Gynecol Oncol. 99: 7988–7990.
PECTASIDES D, *et al*. (2006). Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. Gynecol Oncol 102:285–291.
PELLEY, R.P. *et al*. (2006). Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. Cancer Res. 2006 Dec 15;66(24):11754-62.
PENG, C.L. *et al*., (2013). Inhibitory effects of syk transfection on lung cancer cell invasion. Asian Pac J Cancer Prev. 14(5):3001-3.
PENG, P. *et al*., (2016). Elevated Expression of Calpain-4 Predicts Poor Prognosis in Patients with Gastric Cancer after Gastrectomy. Int J Mol Sci.

17(10). pii: E1612.
PERMUTH-Wey J & SELLERS TA. (2009). Epidemiology of ovarian cancer. Methods Mol Biol. 472:413-37.
PERRIN, B.J. & HUTTENLOCHER, A. (2002). Calpain. Int J Biochem Cell Biol. 34(7):722-5.
PFAFF, M. *et al*. (1999). Calpain cleavage of integrin beta cytoplasmic domains. FEBS Lett. 460(1):17-22.
PFISTERER, J. & LEDERMANN, J.A. (2006). Management of platinum-sensitive recurrent ovarian cancer. Semin Oncol. 33:S12–6.
PICCART, M.J. *et al*., (2000). Randomised intergroup trial of cisplatin–paclitaxel versus cisplatin–cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results, J. Natl. Cancer Inst. pp.92, 699–708.
PIGNATA, S. *et al*. (2011). Chemotherapy in epithelial ovarian cancer. Cancer Lett. 303(2):73-83.
PISANO, C. *et al*. (2005). Activity of chemotherapy in mucinous epithelial ovarian cancer: a retrospective study. Anticancer Res. 25: 3501–3505.
PORUCHYNSKY, M.S. *et al*., (2001).Accompanying protein alterations in malignant cells with a microtubule-polymerizing drug-resistance phenotype and a primary resistance mechanism. Biochem Pharmacol. 62(11):1469-80.
PRAT, J. & FIGO Committee on Gynecologic Oncology. (2014). Staging classification for cancer of the ovary, fallopian tube, and peritoneum. Int J Gynaecol Obstet. 124(1), pp.1-5.
PRAT, J, RIBE A, GALLARDO A. (2005). Hereditary ovarian cancer. Hum Pathol ;36:861-70.
PRINOS, P. *et al*., (2011). Alternative splicing of Syk regulates mitosis and cell survival. Nat Struct Mol Biol. 18(6):673-9.
PU, X. (2016). 'Role of calpain in breast cancer and in regulation of therapeutic response to targeted treatment', PhD thesis, University of Nottingham, viewed 15 Feb 2017 http://eprints.nottingham.ac.uk/id/eprint/37664
PU X. *et al*., (2016 a). Calpain-1 is associated with adverse relapse free survival in breast cancer: a confirmatory study. Histopathology. 68(7):1021-9.
PU X. *et al*., (2016 b). Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival. Apoptosis. [Epub ahead of print]
QIN, Q. *et al*., (2010). Role of calpain-mediated p53 truncation in semaphorin 3A-induced axonal growth regulation. Proc Natl Acad Sci U S A. 107(31):13883-7.
RAIMBOURG, Q. *et al*., (2013). The calpain/calpastatin system has opposing roles in growth and metastatic dissemination of melanoma. PLoS One. 8(4):e60469.
RAVULAPALLI R, *et al*. (2009). Distinguishing between calpain heterodimerization and homodimerization. FEBS J. 276(4):973-82.
RAWLINGS N.D. (2015). Bacterial calpains and the evolution of the calpain (C2) family of peptidases. Biol Direct. 10:66.

REED, N. *et al*. & ESMO Guidelines Working Group. (2010). Non-epithelial ovarian cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 21 Suppl 5:v31-6.

REID, Y., *et al*. (2013). Authentication of Human Cell Lines by STR DNA Profiling Analysis. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

RHO, S.B., BYUN, H.J. & PARK, S.Y. *et al*., (2008). Calpain 6 supports tumorigenesis by inhibiting apoptosis and facilitating angiogenesis. Cancer Lett. Nov 28;271(2):306-13.

RICKERT, R.C. (2013). New insights into pre-BCR and BCR signalling with relevance to B cell malignancies. Nat Rev Immunol. 13(8):578-91.

RIOS-DORIA, J *et al*., (2003). The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. J Biol Chem. 278(2):1372-9.

RIOS-DORIA, J. & DAY, M.L. (2005). Truncated E-cadherin potentiates cell death in prostate epithelial cells. Prostate. 15;63(3):259-68.

RISHER, W.C. *et al*., (2011). Dibucaine mitigates spreading depolarization in human neocortical slices and prevents acute dendritic injury in the ischemic rodent neocortex. PLoS One. 6(7):e22351.

ROBERTS, D. *et al*. (2005). Identification of genes associated with platinum drug sensitivity and resistance in human ovarian cancer cells. Br J Cancer. 92(6):1149-58.

Rodríguez-Fernández L *et al*., (2016). Isoform-specific function of calpains in cell adhesion disruption: studies in postlactational mammary gland and breast cancer. Biochem J. 473(18):2893-909.

ROGGIANI, F. *et al*., (2016).Guidance of Signaling Activations by Cadherins and Integrins in Epithelial Ovarian Cancer Cells. Int J Mol Sci. 17(9). pii: E1387.

ROLLI, V. *et al*., (2002). Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. Mol Cell. 10(5):1057-69.

ROMERO, I. & BAST, R.C. Jr. (2012). Minireview: human ovarian cancer: biology, current management, and paths to personalizing therapy. Endocrinology. 153(4):1593-602.

ROSENBERGER, G. *et al*., (2005). AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. J Biol Chem. 280(8):6879-89. Epub 2004 Dec 20.

ROSENBERG SA & RESTIFO NP. (2015). Adoptive cell transfer as personalised immunotherapy for human cancer. Science. 348:62–8.

ROSEN DG *et al*., (2010). Morphological and molecular basis of ovarian serous carcinoma. J Biomed Res. 24(4):257-63.

ROSS JS, Yang F, Kallakury BV, *et al*. (1999). HER-2/neu oncogene amplification by fluorescence in situ hybridization in epithelial tumors of the ovary. Am J Clin Pathol;111:311-6.

ROUMES, H. *et al*., (2010). Calpains: markers of tumor aggressiveness? Exp

Cell Res. 316(9):1587-99.
SADA, K. *et al*., (2001). Structure and function of Syk protein-tyrosine kinase. J Biochem. 130(2):177-86.
SAEZ M.E. *et al*. (2006). The therapeutic potential of the calpain family: new aspects. Drug Discov Today. 11(19-20):917-23.
SAKAI, W. *et al*., (2009). Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. Cancer Res. Aug 15;69(16):6381-6.
SALEHIN, D. *et al*., (2010). Immunhistochemical analysis for expression of calpain 1, calpain 2 and calpastatin in endometrial cancer. Anticancer Res. 30(7):2837-43.
SALEHIN, D. *et al*., (2011). Immunhistochemical analysis for expression of calpain 1, calpain 2 and calpastatin in ovarian cancer. Eur J Gynaecol Oncol. 32(6):628-35.
SANKARANARAYANAN, R., and FERLAY, J. (2006). Worldwide burden of gynaecological cancer: the size of the problem. Best Pract Res Clin Obstet Gynaecol. 20 , 207 – 225
SARIN A., Adams D.H. & Henkart P.A. (1993). Protease inhibitors selectively block T cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. J Exp Med. 178:1693–700.
SATISH, L. *et al*. (2005). Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of mu-calpain. Mol. Cell Biol. 25, 1922-1941.
SACK, U. *et al*., (2011). S100A4-induced cell motility and metastasis is restricted by the Wnt/β-catenin pathway inhibitor calcimycin in colon cancer cells. Mol Biol Cell. Sep;22(18):3344-54.
SCHAAR, A. *et al*., (2016). TRPC1-STIM1 activation modulates transforming growth factor β-induced epithelial-to-mesenchymal transition. Oncotarget. 7(49):80554-80567.
SCHEIB, S.A. (2017). A Laparo-endoscopic Single Site Surgical Approach to Laparoscopic Salpingectomy. J Minim Invasive Gynecol.. pii: S1553-4650(17)30223-6.
SCHOLLMEYER J E. (1988). Calpain II involvement in mitosis. Science. 240:911–13.
SCHWAB CL, *et al*. (2014). Past, present and future targets for immunotherapy in ovarian cancer. Immunotherapy. 6(12):1279-93.
SCULLY R.E., *et al*. (1998). Tumours of the Ovary, Maldeveloped Gonads, Fallopian Tube and Broad Ligament. Atlas of Tumour Pathology. Third Series, Fascicle 23 Armed Forces Institute of Pathology, Washington, DC.
SELIMOVIC, D. *et al*. (2011) Apoptosis-related protein-2 triggers melanoma cell death by a mechanism including both endoplasmic reticulum stress and mitochondrial dysregulation. Carcinogenesis. 32(8):1268-78.
SERGEEV, IN. (2004). Calcium as a mediator of 1,25-dihydroxyvitamin D3-induced apoptosis. J Steroid Biochem Mol Biol. 89-90(1-5):419-25.

SERGEEV, IN. (2005). Calcium signaling in cancer and vitamin D. J Steroid Biochem Mol Biol. 97(1-2):145-51.

SERGEEV, IN. (2012). Vitamin D and cellular Ca^{2+} signaling in breast cancer. Anticancer Res. 32(1):299-302.

SHEN, H. *et al*., (2012). Lower blood calcium associates with unfavorable prognosis and predicts for bone metastasis in NSCLC. PLoS One. 7(3):e34264.

SHEN, D.W. *et al*. (2012). Cisplatin resistance: acellular self-defense mechanism resulting from multiple epigenetic and geneticchanges. Pharmacol. Rev. 64, 706–721.

SHEN, D. *et al*., (2014). Sedentary behavior and incident cancer: a meta-analysis of prospective studies. PLoS One. 9(8):e105709.

SHEN, L. *et al*., (2016). Calcium efflux from the endoplasmic reticulum regulates cisplatin-induced apoptosis in human cervical cancer HeLa cells. Oncol Lett. 11(4):2411-2419.

SHIH, IeM, & KURMAN, R.J. (2004). Ovarian tumourigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 164:1511–1518.

SHIKATANI, E.A. *et al*., (2012). Inhibition of proliferation, migration and proteolysis contribute to corticosterone-mediated inhibition of angiogenesis. PLoS One. 7(10):e46625.

SHINKAI-OUCHI, F. *et al*., (2016). Predictions of Cleavability of Calpain Proteolysis by Quantitative Structure-Activity Relationship Analysis Using Newly Determined Cleavage Sites and Catalytic Efficiencies of an Oligopeptide Array. Mol Cell Proteomics. 15(4):1262-80.

SHIRAHA, H. *et al*. (2002). Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. Mol. Cell Biol. 22, 2716-2727.

SHI, X. *et al*., (2016). Functional Effect of the Mutations Similar to the Cleavage during Platelet Activation at Integrin β3 Cytoplasmic Tail when Expressed in Mouse Platelets. PLoS One. 11(11):e0166136.

SHOJI, K. *et al*., (2012). Cytochalasin D acts as an inhibitor of the actin-cofilin interaction. Biochem Biophys Res Commun. 424(1):52-7.

SIEGEL RL, Miller KD & Jemal A. (2016). Cancer statistics, 2016. CA Cancer J Clin. 66(1):7-30.

SIEH W *et al*., (2013). Tubal ligation and risk of ovarian cancer subtypes: a pooled analysis of case-control studies. Int J Epidemiol. 42(2):579-89.

SILVERBERG, S.G. (2000). Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol. 19: 7–15.

SINGHA, B. *et al*., (2014). Proteasome inhibition increases recruitment of IκB kinase β (IKKβ), S536P-p65, and transcription factor EGR1 to interleukin-8 (IL-8) promoter, resulting in increased IL-8 production in ovarian cancer cells. J Biol Chem. 289(5):2687-700.

SINGH, R., MASUDA, E.S., PAYAN, D.G. (2012).Discovery and development of

spleen tyrosine kinase (Syk) inhibitors. J Med Chem. 55(8):3614-43.

SINGH R, *et al*. (2014). Calpain 5 is highly expressed in the central nervous system (CNS), carries dual nuclear localization signals, and is associated with nuclear promyelocytic leukemia protein bodies. J Biol Chem. 289(28):19383-94.

Sivanandam A *et al*., (2011). Calmodulin protects androgen receptor from calpain-mediated breakdown in prostate cancer cells. J Cell Physiol. 2011 Jul;226(7):1889-96.

SMITH, S. D., *et al*. (2003). Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation. FEBS Lett. 542, 115-118.

SMITH, J.A. (2005). An evaluation of cytotoxicity of the taxane and platinum agents combination treatment in a panel of human ovarian carcinoma cell lines. Gynecol Oncol. 98(1):141-5.

SMITH, H.O. *et al*. (2006). Incidence and survival rates for female malignant germ cell tumours. Obstet Gynecol. 107(5): 1075–1085. SYRIOS, J., BANERJEE, S. & KAYE, S.B. (2014) Advanced epithelial ovarian cancer: from standard chemotherapy to promising molecular pathway targets--where are we now? Anticancer Res. 34(5):2069-77.

SOBHAN, P.K., *et al*. (2013). Calpain and reactive oxygen species targets Bax for mitochondrial permeabilisation and caspase activation in zerumbone induced apoptosis. PLoS One. 8(4):e59350.

SOMLYO, A.V. (2002). New roads leading to Ca2+ sensitization. Circ Res. 91(2):83-4.

SOPIK V, *et al*. (2015). Why have ovarian cancer mortality rates declined? Part I. Incidence. Gynecol Oncol. 138(3):741-9.

SORIMACHI H, HATA S & ONO Y. (2011). Impact of genetic insights into calpain biology. J Biochem. 150(1):23-37.

SPAKS, A. *et al*., (2016). CXC chemokine ligand 4 (CXCL4) is predictor of tumour angiogenic activity and prognostic biomarker in non-small cell lung cancer (NSCLC) patients undergoing surgical treatment. Biomarkers. 21(5):474-8.

SPLETTSTOESSER, F. FLOREA, A.M. & Büsselberg, D. (2007). IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca^{2+}-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis. Br J Pharmacol. 151(8):1176-86.

SRIURANPONG V *et al*., (2004). Global gene expression profile of nasopharyngeal carcinoma by laser capture microdissection and complementary DNA microarrays. Clin Cancer Res. 10(15):4944-58.

STEWART, Z.A. & PIETENPOL, J.A. (2001).Syk: a new player in the field of breast cancer. Breast Cancer Res. 3(1):5-7. Epub 2000 Nov 2.

STORCK, H. *et al*. (2017). Ion channels in control of pancreatic stellate cell migration. Oncotarget. 8(1):769-784.

STROBL S. *et al*. (2000). The crystal structure of calcium-free human m-calpain

suggests an electrostatic switch mechanism for activation by calcium. Proc Natl Acad Sci U S A 97:588 –592.

STRONACH, E.A. *et al*. (2011). DNA-PK mediates AKT activation and apoptosis inhibition in clinically acquired platinum resistance. Neoplasia. 13(11):1069-80.

STORR SJ. *et al*. (2011a). The calpain system and cancer. Nat Rev Cancer. 11(5):364-74.

STORR SJ *et al*. (2011b). Calpastatin is associated with lymphovascular invasion in breast cancer. Breast. 20(5):413-8.

STORR, S.J. *et al*. (2011c). Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. Int J Cancer. 129(7):1773-80.

STORR S.J. *et al*. (2012a). Calpain-2 expression is associated with response to platinum based chemotherapy, progression-free and overall survival in ovarian cancer. J Cell Mol Med. 16(10):2422-8.

STORR S.J. *et al*. (2012b).Calpain system protein expression in basal-like and triple-negative invasive breast cancer. Ann Oncol.23(9):2289-96.

STORR S.J. *et al*. (2012c). Calpain system protein expression in carcinomas of the pancreas, bile duct and ampulla. BMC Cancer.12:511.

STORR SJ. *et al*. (2013). Expression of the calpain system is associated with poor clinical outcome in gastro-oesophageal adenocarcinomas. J Gastroenterol. 48(11):1213-21.

STORR SJ *et al*. (2016). The calpain system is associated with survival of breast cancer patients with large but operable inflammatory and non-inflammatory tumours treated with neoadjuvant chemotherapy. Oncotarget. Jul 26;7(30):47927-47937.

SUGIYAMA T, Kamura T, Kigawa J *et al*. (2000). Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer. 88:2584–2589.

SUH, D.H., *et al*. (2014). Major clinical research advances in gynecologic cancer in 2013. J Gynecol Oncol. 25(3):236-48.

SUH, D.H. *et al*. (2015). Major clinical research advances in gynecologic cancer in 2014. J Gynecol Oncol. 26(2):156-67.

SUH, D.H. *et al*. (2016). Surgical management of recurrent ovarian cancer. Gynecol Oncol. 142(2):357-67.

SUN, M. & ZAMAN, M.H. (2017). Modeling, signaling and cytoskeleton dynamics: integrated modeling-experimental frameworks in cell migration. Wiley Interdiscip Rev Syst Biol Med. 9(1).

SUZUKI K *et al*. (2004). Structure, activation, and biology of calpain. Diabetes; 53 (Suppl 1):S12–8.

SWISHER EM, *et al*. (2008). Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. Cancer Res. 68(8):2581-6.

Sölétormos G *et al*.,(2016). Clinical Use of Cancer Biomarkers in Epithelial

Ovarian Cancer: Updated Guidelines From the European Group on Tumour Markers. Int J Gynecol Cancer. 26(1):43-51.

TAKADA, Y. *et al*., (2003). Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. J Biol Chem. 278(26):24233-41.

TAKANO, E. *et al*. (1984). Molecular diversity of calpastatin in mammalian organs. Biochem Biophys Res Commun. 122(3):912-7.

TAKANO, E. *et al*. (1993). Molecular diversity of calpastatin in human erythroid cells. Arch Biochem Biophys. 303(2):349-54.

TAKANO, E. *et al*. (1995). Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains. FEBS Lett. 362, 93–97.

TAKANO J. *et al*. (2011). Vital role of the calpain-calpastatin system for placental-integrity-dependent embryonic survival. Mol Cell Biol. 31(19):4097-106.

TANDAY S. (2016). Weekly dose-dense chemotherapy for ovarian cancer. Lancet Oncol. 2016 Mar 3. pii: S1470-2045(16)00154-6.

TAN, W.J. *et al*., (2017). Calpain 1 regulates TGF-β1-induced epithelial-mesenchymal transition in human lung epithelial cells via PI3K/Akt signaling pathway. Am J Transl Res. 9(3):1402-1409. eCollection 2017.

TAN, Y. *et al*. (2006). Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. J. Biol. Chem. 281, 16016–16024.

TAVASSOLI, F. A. & DEVILEE, P. (Eds.) (2003) World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. The first Edition. Lyon: IARC Press.

TEOH, D. *et al*. (2011). Dasatinib (BMS-35482) has synergistic activity with paclitaxel and carboplatin in ovarian cancer cells.Gynecol Oncol. 121(1):187-92.

TERRY, K.L. *et al*. (2013). Genital powder use and risk of ovarian cancer: a pooled analysis of 8,525 cases and 9,859 controls. Cancer Prev Res (Phila). 6(8):811-21.

TEWARI, D. *et al*., (2015). Long-term survival advantage and prognostic factors associated with intraperitoneal chemotherapy treatment in advanced ovarian cancer: a gynecologic oncology group study. J Clin Oncol. 2015 May 1;33(13):1460-6.

THIGPEN T., *et al*. Gynecologic Cancer InterGroup. (2011). First-line therapy in ovarian cancer trials. Int J Gynecol Cancer. 21(4):756-62.

THORPE, H. et al. (2015). Multiple pathways regulate Cten in colorectal cancer without a Tensin switch. Int J Exp Pathol. 96(6):362-9.

TOKE, O. *et al*., (2009). Folding transitions in calpain activator peptides studied by solution NMR spectroscopy. J Pept Sci. 15(6):404-10.

TOMPA P. *et al*. (2001a). Domain III of calpain is a ca2+-regulated phospholipid-binding domain. Biochem Biophys Res Commun. 280:1333–9.

TOMPA, P., *et al*. (2001b). Frequency decoding of fast calcium oscillations by calpain. Cell Calcium. 29(3):161-70.

TOMPA, P. (2002a). Intrinsically unstructured proteins. Trends Biochem. Sci. 27, 527–533.

TOMPA, P. *et al*., (2002b). Calpastatin subdomains A and C are activators of calpain. J Biol Chem. 277(11):9022-6.

TOMPA P. *et al*. (2004). On the sequential determinants of calpain cleavage. J Biol Chem. 279(20):20775-85.

TOSS A *et al*., (2013). Ovarian cancer: can proteomics give new insights for therapy and diagnosis? Int J Mol Sci. Apr 15;14(4):8271-90.

TORHORST, J. *et al*. (2001). Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol. 159(6):2249-56.

TRENDOWSKI, M. *et al*., (2015). Effects of cytochalasin congeners, microtubule-directed agents, and doxorubicin alone or in combination against human ovarian carcinoma cell lines *in vitro*. BMC Cancer. 2015 Sep 10;15:632.

TRILLSCH, F. *et al.,* (2016). E-Cadherin fragments as potential mediators for peritoneal metastasis in advanced epithelial ovarian cancer. Br J Cancer. 114(2):213-20.

TSUCHIDA, S. *et al*., (2000). Purification of a 72-kDa protein-tyrosine kinase from rat liver and its identification as Syk: involvement of Syk in signaling events of hepatocytes. J Biochem. 127(2):321-7.

TUMBARELLO, D.A. *et al*., (2005). Regulation of paxillin family members during epithelial-mesenchymal transformation: a putative role for paxillin delta. J Cell Sci. 118:4849–4863.

UDYAVAR, A.R. *et al*., (2013). Co-expression network analysis identifies Spleen Tyrosine Kinase (Syk) as a candidate oncogenic driver in a subset of small-cell lung cancer. BMC Syst Biol. 7 Suppl 5:S1.

ULANOVA, M. *et al*., (2005). Syk tyrosine kinase participates in beta1-integrin signaling and inflammatory responses in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol. 288(3):L497-507.

VANDENBERGHE, M. *et al*., (2013). ORAI1 calcium channel orchestrates skin homeostasis. Proc Natl Acad Sci U S A. 110(50):E4839-48.

VAN GEFFEN, J.P., SWIERINGA, F., HEEMSKERK, J.W. (2016). Platelets and coagulation in thrombus formation: aberrations in the Scott syndrome. Thromb Res. 141 Suppl 2:S12-6.

VANG, R, SHIH, IE. M. & KURMAN, R.J. (2009). Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems. Adv Anat Pathol. 16:267–282.

VANG, S. *et al*. (2013). Identification of ovarian cancer metastatic miRNAs.

PLoS One. 8(3):e58226.
VAN TONDER, A., JOUBERT, A.M. & CROMARTY, A.D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Res Notes. 8:47.
VAUGHAN, S., Coward JI, Bast RC, *et al*. (2011). Rethinking ovarian cancer: recommendations for improving outcomes. Nat Rev Cancer.11:719–25.
VENABLES, J.P. et al. (2008). Identification of alternative splicing markers for breast cancer. Cancer Res. 68(22):9525–9531.
VERGOTE, I. *et al*. (2011). Primary surgery or neoadjuvant chemotherapy followed by interval debulking surgery in advanced ovarian cancer. Eur J Cancer. 47 Suppl 3:S88-92.
VERGOTE I, *et al*.; European Organization for Research and Treatment of Cancer-Gynaecological Cancer Group; NCIC Clinical Trials Group. (2010). Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. N Engl J Med. 363(10):943-53.
VODUC, D., KENNEY, C. & NIELSEN, T.O. (2008). Tissue microarrays in clinical oncology. Semin Radiat Oncol. 18(2):89-97.
Világi I. *et al*., (2008). Synthetic calpain activator boosts neuronal excitability without extra Ca2+. Mol Cell Neurosci. 38(4):629-36.
WANG, D. & LIPPARD, S.J. (2005). Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov. 4(4):307–20.
WANG, E. *et al*., (2017). Capn4 promotes epithelial-mesenchymal transition in human melanoma cells through activation of the Wnt/β-catenin pathway. Oncol Rep. 37(1):379-387.
WANG, J. *et al*., (2006). Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. Oncogene. 25(54):7201-11.
WANG, L. *et al*., (2003). Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. Cancer Res. 63(15):4724-30.
WANG, L. *et al*., (2005). Transcription repressor activity of spleen tyrosine kinase mediates breast tumor suppression. Cancer Res. 65(22):10289-97.
WANG, Y. *et al*., (2010). Calpain activation through galectin-3 inhibition sensitizes prostate cancer cells to cisplatin treatment. Cell Death Dis. 1:e101
WANG, Z. *et al*., (2010). The role of calpain in the regulation of ADAM17-dependent GPIbalpha ectodomain shedding. Arch Biochem Biophys. 495(2):136-43.
WEBER, H. *et al*., (2009). Calpain-mediated breakdown of cytoskeletal proteins contributes to cholecystokinin-induced damage of rat pancreatic acini. Int J Exp Pathol. 90(4):387-99.
WEBBER, K. & FRIEDLANDER, M. (2017). Chemotherapy for epithelial ovarian,

fallopian tube and primary peritoneal cancer. Best Pract Res Clin Obstet Gynaecol. 41:126-138.

WEBB, P.M. and JORDAN, S.J. (2016). Epidemiology of epithelial ovarian cancer. Best Pract Res Clin Obstet Gynaecol. pii: S1521-6934(16)30091-8.

WEIDLE, U.H. *et al*., (2016). Mechanisms and Targets Involved in Dissemination of Ovarian Cancer. Cancer Genomics Proteomics. 13(6):407-423.

WEI, L. *et al*. (2009). Roles of clusterin in progression, chemoresistance and metastasis of human ovarian cancer. Int J Cancer. 125(4):791-806.

WEISSMAN, S.M., *et al*. (2011). Genetic counseling considerations in the evaluation of families for Lynch syndrome- a review. J Genet Couns;20: 5-19.

WEI,i W. & BIRRER, M.J., (2015). Spleen Tyrosine Kinase Confers Paclitaxel Resistance in Ovarian Cancer. Cancer Cell. 28(1):7-9.

WELLS, A., HUTTENLOCHER, A. & LAUFFENBURGER, D.A. (2005). Calpain proteases in cell adhesion and motility. Int Rev Cytol.245:1-16.

WENDT, A. *et al*. (2004). Interaction of calpastatin with calpain: a review. Biol Chem. 385(6):465-72.

WENTZENSEN N. *et al*. (2016). Ovarian cancer risk factors by histologic subtype: an analysis from the Ovarian Cancer Cohort Consortium. J Clin Oncol;34(24):2888-98.

WEN WH, Reles A, Runnebaum IB *et al*. (1999). p53 mutations and expression in ovarian cancers: correlation with overall survival. Int J Gynecol Pathol 18:29-41.

WEST, R.R., TENBARGE, K.M. & OLMSTED, J.B. (1991). A model for microtubule-associated protein 4 structure. Domains defined by comparisons of human, mouse, and bovine sequences. J Biol Chem. 266(32):21886-96.

WHITE, E.A. *et al*., (2014). Three-dimensional modeling of ovarian cancer. Adv Drug Deliv Rev. 79-80:184-92.

WILKINSON, N. & MCCLUGGAGE, W.G. (2008). Datasets for the Histopathological Reporting of Neoplasms of the Ovaries and Fallopian Tubes and Primary Carcinomas of the Peritoneum. London: Royal College of Pathologists.

WINTER-ROACH, B.A, KITCHENER, H.C, LAWRIE, T.A. (2012). Adjuvant (post-surgery) chemotherapy for early stage epithelial ovarian cancer. Cochrane Database Syst Rev. 14;3:CD004706.

WITKOWSKI, J.M. *et al*., (2002). Modulation of the activity of calcium-activated neutral proteases (calpains) in chronic lymphocytic leukemia (B-CLL) cells. Blood. 100(5):1802-9.

WONG, E. & GIANDOMENICO, C.M. (1999). Current status of platinum-based antitumour drugs. Chem Rev. 99(9):2451–66.

WOODRUFF, T. K. (2010). Oncofertility: Ethical, legal, social, and medical perspectives. New York: Springer.

WOOLSTON, C.M. *et al*., (2010). Redox protein expression predicts

progression-free and overall survival in ovarian cancer patients treated with platinum-based chemotherapy. Free Radic Biol Med. 49(8):1263-72.

WOOLSTON, C. & MARTIN, S. (2011). Analysis of tumor and endothelial cell viability and survival using sulforhodamine B and clonogenic assays. Mammalian Cell Viability. Springer.

WOO, M.G. *et al*., (2012). Calpain-mediated processing of p53-associated parkin-like cytoplasmic protein (PARC) affects chemosensitivity of human ovarian cancer cells by promoting p53 subcellular trafficking. J Biol Chem. 287(6):3963-75.

WOZNIAK, M.A. *et al*. (2004).Focal adhesion regulation of cell behavior. Biochim Biophys Acta. 1692(2-3):103-19.

WU, M. *et al*., (2006). Functional dissection of human protease mu-calpain in cell migration using RNAi. FEBS Lett. 580(13):3246-56.

WU, X., Yang L., Zheng Z. *et al*., (2016). Src promotes cutaneous wound healing by regulating MMP-2 through the ERK pathway. Int J Mol Med. 37(3):639-48.

WU, Z.Z. & CHAO, C.C. (2010). Knockdown of NAPA using short-hairpin RNA sensitizes cancer cells to cisplatin: implications to overcome chemoresistance. Biochem Pharmacol. 80(6):827-37.

XIAO, H. *et al*., (2012). Structural evidence for cooperative microtubule stabilization by Taxol and the endogenous dynamics regulator MAP4. ACS Chem Biol. 7(4):744-52.

XI, X. *et al*., (2006). Tyrosine phosphorylation of the integrin beta 3 subunit regulates beta 3 cleavage by calpain. J Biol Chem. 281(40):29426-30.

XU, K.P. *et al*. (2004). Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. Invest Ophthalmol Vis Sci. 45(3):813-20.

XU, L., Deng X. *et al*., (2004). Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induces phosphorylation of mu- and m-calpain in association with increased secretion, cell migration, and invasion. J Biol Chem. 279(51):53683-90.

XU L. & DENG, X. (2006). Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. J Biol Chem. 281(7):4457-66.

XU, X.M. *et al*., (2009). Simultaneous knockdown of the expression of two genes using multiple shRNAs and subsequent knock-in of their expression. Nat Protoc.;4(9):1338-48.

XU, Y. *et al*., (2015). Tolerance to endoplasmic reticulum stress mediates cisplatin resistance in human ovarian cancer cells by maintaining endoplasmic reticulum and mitochondrial homeostasis. Oncol Rep. 34(6):3051-60.

XU, Y., Bismar TA, Su J. *et al*. (2010). Filamin A regulates focal adhesion disassembly and suppresses breast cancer cell migration and invasion. J Exp Med.207(11):2421-37.

YAMADA, M. *et al*., (1999). Cellular sensitization to cisplatin and carboplatin with decreased removal of platinum-DNA adduct by glucose-regulated stress. Cancer Chemother Pharmacol. 44(1):59-64.

YAMAMURA, A. *et al*., (2016). Molecular Mechanism of Dihydropyridine Ca2+ Channel Blockers in Pulmonary Hypertension. Yakugaku Zasshi. 136(10):1373-1377.

YANAGI, S. *et al*., (2001). Syk expression and novel function in a wide variety of tissues. Biochem Biophys Res Commun. 288(3):495-8.

YAN, B. *et al*., (2001). Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J Biol Chem. 276(30):28164-70.

YANG, H.Q., *et al*.(1994). Analysis of calcium-dependent interaction between amino-terminal conserved region of calpastatin functional domain and calmodulin-like domain of m-calpain large subunit. J. Biol. Chem. 269, 18977–18984.

YANG, X., *et al*., (2017). Capn4 Enhances Osteopontin Expression through Activation of the Wnt/β-Catenin Pathway to Promote Epithelial Ovarian Carcinoma Metastasis. Cell Physiol Biochem. 42(1):185-197.

YAN, W. *et al*., (2016). Deregulated miR-296/S100A4 axis promotes tumor invasion by inducing epithelial-mesenchymal transition in human ovarian cancer. Am J Cancer Res. 6(2):260-9. eCollection 2016.

YAP, T.A., CARDEN, C.P. & KAYE, S.B. (2009). Beyond chemotherapy: targeted therapies in ovarian cancer. Nat Rev Cancer. 9(3):167-81.

YE, Y. *et al*., (2013). The genesis and unique properties of the lymphovascular tumor embolus are because of calpain-regulated proteolysis of E-cadherin. Oncogene. 32(13):1702-13.

YIN, J *et al*. (2007). Wound-induced ATP release and EGF receptor activation in epithelial cells. J Cell Sci. 120(Pt 5):815-25.

YI, Y.S. *et al*., (2014). Functional roles of Syk in macrophage-mediated inflammatory responses. Mediators Inflamm. 2014:270302.

YOON, JS *et al*., (2013). Neferine isolated from Nelumbo nucifera enhances anti-cancer activities in Hep3B cells: molecular mechanisms of cell cycle arrest, ER stress induced apoptosis and anti-angiogenic response. Phytomedicine. 20(11):1013-22.

YOSHIDA, M. *et al*., (2016). Calpain inhibitor calpeptin suppresses pancreatic cancer by disrupting cancer-stromal interactions in a mouse xenograft model. Cancer Sci. 2016 Oct;107(10):1443-1452.

YOUSSEF, L.A, WILSON, B.S, OLIVER, J.M. (2002).Proteasome-dependent regulation of Syk tyrosine kinase levels in human basophils. J Allergy Clin Immunol. 110(3):366-73.

YU, Y, *et al*., (2015). Inhibition of Spleen Tyrosine Kinase Potentiates Paclitaxel-Induced Cytotoxicity in Ovarian Cancer Cells by Stabilizing Microtubules. Cancer Cell. 28(1):82-96.

YUAN, Y., *et al*., (2006). Frequent epigenetic inactivation of spleen tyrosine kinase gene in human hepatocellular carcinoma. Clin Cancer Res. 12(22):6687-95.

YUN, J. *et al*., (1995). Glucose-regulated stresses confer resistance to VP-16 in human cancercells through a decreased expression of DNA topoisomerase II. Oncol Res. 7(12):583-90.

ZADRAN, S. *et al*., (2010). Brain-derived neurotrophic factor and epidermal growth factor activate neuronal m-calpain via mitogen-activated protein kinase-dependent phosphorylation. J Neurosci. 30(3):1086-95.

ZAINO, R.J. *et al*. (2011). Advanced stage mucinous adenocarcinoma of the ovary is both rare and highly lethal: a Gynecologic Oncology Group study. Cancer. 117:554–562.

ZAMORA, A. *et al*. (2013).New steroidal 7-azaindole platinum(II) antitumor complexes. J Inorg Biochem. 128:48-56.

ZEIMET, A.G. *et al*. (2009). Pros and cons of intraperitoneal chemotherapy in the treatment of epithelial ovarian cancer. Anticancer Res. 29(7):2803-8.

ZHANG C *et al*., (2013). Prognostic significance of Capn4 overexpression in intrahepatic cholangiocarcinoma. PLoS One.;8(1):e54619.

ZHANG, LANE & MELLGREN. (1996). The major calpain isozymes are long-lived proteins. Design of an antisense strategy for calpain depletion in cultured cells. J Biol Chem. 1996 Aug 2;271(31):18825-30

ZHANG, P. *et al*., (2013). The role of intraplatelet reactive oxygen species in the regulation of platelet glycoprotein Ibα ectodomain shedding. Thromb Res. 132(6):696-701.

ZHANG, W. *et al*., (1996). The major calpain isozymes are long-lived proteins. Design of an antisense strategy for calpain depletion in cultured cells. J Biol Chem. 271(31):18825-30.

ZHANG, W. *et al*., (2011). Calpain activator dibucaine induces platelet apoptosis. Int J Mol Sci. 12(4):2125-37.

ZHANG, X *et al*, (2009). Role of the protein tyrosine kinase Syk in regulating cell-cell adhesion and motility in breast cancer cells. Mol Cancer Res. 7(5):634-44.

ZHANG, X. *et al*., (2012). Involvement of hepatitis B virus X gene (HBx) integration in hepatocarcinogenesis via a recombination of HBx/Alu core sequence/subtelomeric DNA. FEBS Lett. 586(19):3215-21.

ZHANG, Y., XU, W. & NI, P. *et al*., (2016). MiR-99a and MiR-491 Regulate Cisplatin Resistance in Human Gastric Cancer Cells by Targeting CAPNS1. Int J Biol Sci. 12(12):1437-1447. eCollection 2016.

ZHAO S., *et al*. (2012). Massive expansion of the calpain gene family in unicellular eukaryotes. BMC Evol Biol. 2012; 12:193.

ZHENG, P.C. *et al*., (2014). Capn4 is a marker of poor clinical outcomes and promotes nasopharyngeal carcinoma metastasis via nuclear factor-κB-induced matrix metalloproteinase 2 expression. Cancer Sci. 105(6):630-8.

ZHOU, F. *et al*., (2006). Nucleocytoplasmic trafficking of the Syk protein tyrosine kinase. Mol Cell Biol. 26(9):3478-91.

ZHOU, Z. *et al*., (2015). The p38/mitogen-activated protein kinase pathway is implicated in lipopolysaccharide-induced microtubule depolymerization via up-regulation of microtubule-associated protein 4 phosphorylation in human vascular endothelium. Surgery. 157(3):590-8.

ZHUANG, Q. et al., (2014). Capn4 mRNA level is correlated with tumour progression and clinical outcome in clear cell renal cell carcinoma. J Int Med Res. 42(2):282-91.

ZHU, Q. *et al*. (2017). The expression of Duffy antigen receptor for chemokines by epithelialovarian cancer decreases growth potential. Oncol Lett. 13(6):4302-4306.

ZIMMERMAN U.J. *et al*. (2000). The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life. 50(1):63-8.

ZYSS, D. *et al*., (2005). The Syk tyrosine kinase localizes to the centrosomes and negatively affects mitotic progression. Cancer Res. 65(23):10872-80.

Appendix Supplementary information

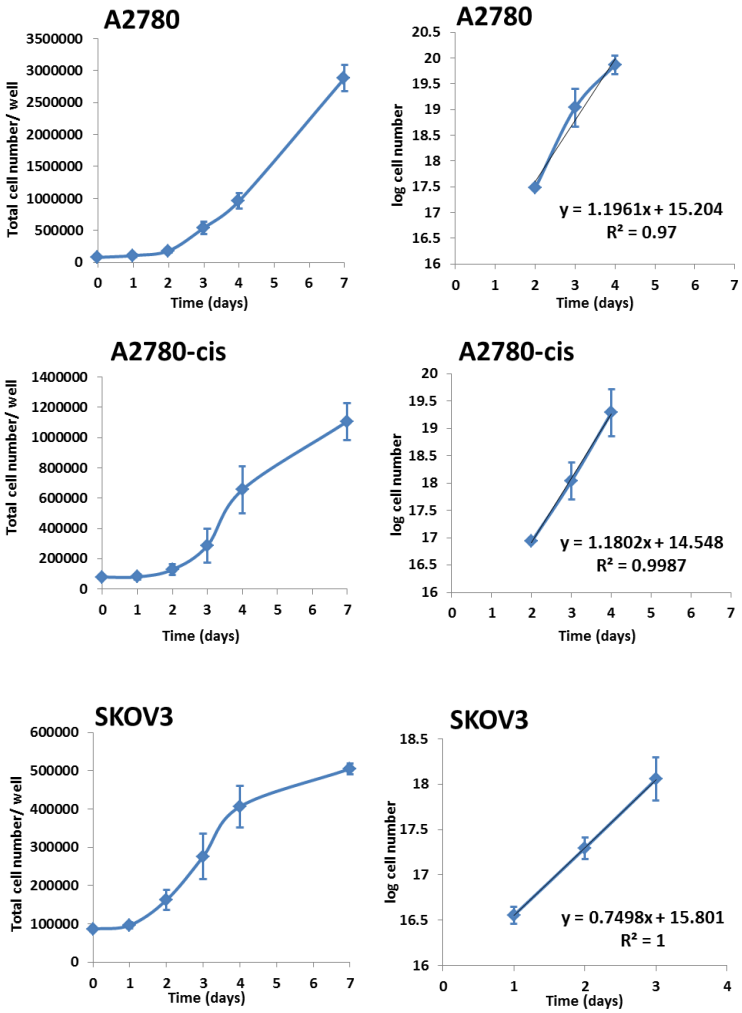


Figure S2.1 Growth curves of five ovarian cancer cell lines A2780, A2780-cis and SKOV3. Figures on the left showed the raw data of cell count while figures on the right showed the log cell numbers and indicated the time period that was chosen for the calculation of doubling time. Data represented the average ± SD of three independent experiments

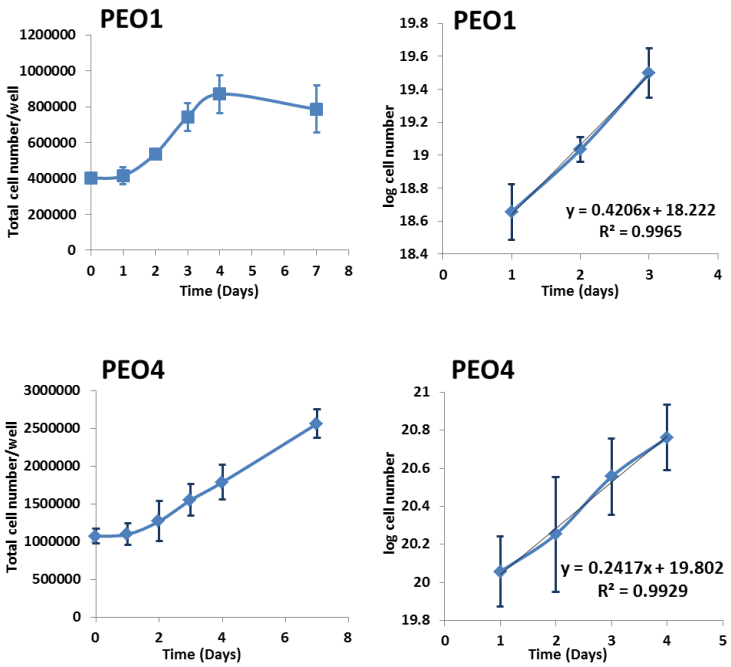


Figure S2.2 Growth curves of ovarian cancer cell lines PEO1 and PEO4. Figures on the left showed the raw data of cell count while figures on the right showed the log cell numbers and indicated the time period that was chosen for the calculation of doubling time. Data represented the average ± SD of three independent experiments

Table S2.1 Summary of studies with regard to calpain and chemotherapeutic response.

Pt-based chemotherapy	Model	Calpain regulation	Assessment of calpain regulation	Reference
Cisplatin	Human lung adenocarcinoma cell lines ASTC-a-1 and A549	Calpeptin (synthetic and specific calpain inhibitor, 10 μM) or PD150606 (selective inhibitor of calpain)	Ac-LLY-AFC (incubate with cell lysate)	Liu *et al*., 2008
Cisplatin	Human lung adenocarcinoma cell lines ASTC-a-1 and A549	Calpeptin (10 μM) or PD150606; calpain-1 and calpain-2 siRNA	Ac-LLY-AFC (incubate with cell lysate)	Liu, Xing & Chen, 2009.
Cisplatin	Human triple-negative breast cancer cell line MDA-MB231	Calpeptin (25μM, 50μM); calpain-1 siRNA; CPA	Cleavage of α-Fodrin and Caspase-12 assessed by Western blotting	Al-Bahlani *et al*., 2016
Cisplatin	Human ovarian cancer cell lines OV2008 and its chemo-resistant isogenic counterparts C13*	Calpeptin (0-50 μM); calpain siRNA; CPA	Cleaved α-Fodrin assessed by Western blotting	Al-Bahlani *et al*., 2011
Cisplatin	Human ovarian cancer cell lines: OV2008 and A2780s; cisplatin-resistant C13*, OVCA420, OVCA433, and HEY. IOSE397	Calpeptin (6.25-50 μM, 1 hour)	Cleaved α-Fodrin assessed by Western blotting	Woo *et al*., 2012
Cisplatin	Human cervix adenocarcinoma cell line HeLa-S3; human osteosarcoma cell line U2-OS	\	Calpain-1 active form (58 Kda) assessed by Western blotting	Splettstoesser, Florea & Büsselberg, 2007
Cisplatin	Human cervical cancer cell lines HeLa, SAOS-2, Caski and A549	PD 150606 (experiments included its inactive homologue PD 145305); calpain inhibitor VI	the fluorogenic calpain activity assay kit and a fluorogenic-calpain substrate H-K(FAM)-EVY~GMMK(DABCYL)-OH	Darnell *et al*., 2007

Table S2.1 Continued.

Pt-based chemotherapy	Model	Calpain regulation	Assessment of calpain regulation	Reference
Oxaliplatin	Human cervical carcinoma HeLa and HCT116 cells	Calpeptin (30 μM)	Calpain-4 autocatalytically cleaved form after calpain activation which was assessed by Western blotting	Anguissola *et al*., 2009
Cisplatin	Human cervical carcinoma HeLa cells	Calpeptin (5 or 10 μM) for 2 hours	\	Hill, Hu & Evans, 2008
Cisplatin	Human cervical carcinoma HeLa cells	BAPTA/AM (2.5 μM; 12 h) and 2‑APB (100 μM; 12 h)	\	Shen *et al*., 2016
Cisplatin	Human gastric cancer cell lines SGC-7901 and BGC-823; and their cisplatin resistant counterparts SGC-7901/DDP and BGC-823/DDP	siRNA	\	Zhang Y *et al*., 2016
Cisplatin	Human metastatic melanoma cell line Me665/2/21; unselected cell lines, derived from metastases of human melanomas: Me665/2/60, HT-144 and SK-Mel-28	Calpain inhibitors MDL-28170 (Z-Val-Phe-aldehyde) and calpeptin (Z-Leu-norLeu-aldehyde) both 50 μM	Measured in intact cells by using fluorescent substrates: N-suc-Leu-Tyr-AMC and the more cell-permeable t-BOC-Leu-Met-CMAC	Del Bello *et al*., 2007
Cisplatin	Human metastatic melanoma cell line, 224; melanoma cell line DFW	Calpeptin (10 μM); PD150606 (calpeptin acts on the active site of calpain, PD150606 blocks its calcium-binding site); BAPTA-AM	Calpain substrate N-Suc-Leu-Tyr-AMC with cell lysates; Boc-Leu-Met-CMAC (10 μM) with intact cells	Mandic *et al*., 2002

BAPTA/AM: bis‑(o‑aminophenoxy)ethane‑N,N,N',N'‑tetra‑acetic acid acetoxymethyl ester, a cell-permeant chelator regulating the intracellular Ca^{2+} level. It is a highly selective for Ca^{2+} over Mg^{2+} (more selective for Ca^{2+} than EDTA and EGTA).

Table S2.1 Continued.

Pt-based chemotherapy	Model	Calpain regulation	Assessment of calpain regulation	Reference
Cisplatin	Human melanoma cell line 224 and colon cancer cell line HCT 116	Calpeptin (10 μM); calcium chelator BAPTA-AM	\	Mandic *et al*., 2003
Cisplatin	Human melanoma cells MeWo and cisplatin-resistant cell line MeWo-cis1	Calpain inhibitors PD150606 and E64	Measured in cell lysates using the calpain substrate suc-LY-AMC	Młynarczuk-Biały *et al*., 2006
Cisplatin	Human prostate cancer cells PC3 and LNCaP (galectin-3 null cells)	Calpain inhibitor PD150606 and calpain-1/-2 siRNA	Western blotting analysis of calpain substrate spectrin αII 150 kD fragments; zymography analysis of calpain substrate casein	Wang *et al*., 2010
Cisplatin	Human embryonic kidney cells HEK293 cells	\	\	Wu & Chao, 2010
Cisplatin	Human hepatoblastoma cell line HepG2 (containing wild-type p53)	Calpain inhibitors, N-Acetyl-Lue-Lue-Norleu-al (ALLM) and N-Acetyl-Leu-Leu-Met-al (ALLN)	Cleaved protein products of μ-calpain (from 84 kD to 65 kD) and m-calpain assessed by Western blotting	Kim MJ *et al*., 2007
Cisplatin	Human umbilical vein endothelial cells (HUVECs)	μ- and m-calpain inhibitor MDL28170 (MDL)	Bax (21 kDa) and its cleaved form (18 kDa) assessed by Western blotting	Eguchi *et al*., 2010
Cisplatin	Mouse skeletal muscle myoblasts C2C12	A23187 (5, 10, or 15 μM; 3 h or 6 h)	Fluorogenic substrates Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) using cell lysates	Bloemberg & Quadrilatero, 2016

Table S2.2 Approximate IC50 of cisplatin and carboplatin in the panel of ovarian cancer cell lines

Cell line	Treatment period	IC50							
		Cisplatin				Carboplatin			
		Read from figure	**Probit Regression** using SPSS 95% Confidence Limits for concentration			Read from figure	**Probit Regression** using SPSS 95% Confidence Limits for concentration		
			Estimate	Lower Bound	Upper Bound		Estimate	Lower Bound	Upper Bound
SKOV 3	24h	20	47.402	23.504	155.781	>100	421.055	66.363	1.08676E+26
	48h	5	4.546	.029	17.952	11	10.935	8.890	13.332
	72h	3	3.232	.131	10.685	6	4.957	4.070	5.917
A2780	24h	3.5	3.798	1.432	9.619	6.5	7.438	4.593	18.951
	48h	0.8	.721	.514	.936	1.8	1.194	.634	2.098
	72h	0.4	.450	.336	.558	0.9	.824	.685	.977
A2780-cis	24h	36	29.384	16.487	57.249	58	52.112	40.090	72.783
	48h	7.5	7.916	6.711	9.126	16	15.354	13.157	17.891
	72h	6.5	6.471	5.654	7.258	12	10.618	6.922	15.576
PEO1	24h	107	115.828	80.918	185.349	200	183.290	122.017	331.811
	48h	3	4.702	2.772	7.110	14	13.239	8.673	19.025
	72h	1	.981	.401	1.797	1.5	1.651	.687	2.968
PEO4	24h	72	95.644	59.291	184.861	200	269.568	111.797	2548.342
	48h	19	19.118	8.093	38.323	25.5	27.880	22.668	34.487
	72h	3	5.380	1.671	11.391	7	8.386	6.434	10.624

Table S2.3 IC50 of calpeptin in the panel of ovarian cancer cell lines

Cell line	Treatment period	Calpeptin IC50 (μM)			
		Read from figure	Estimate	Lower Bound	Upper Bound
SKOV 3	24h	116	124.897	71.893	347.711
	48h	86	78.745	\	\
	72h	82	73.642	\	\
A2780	24h	84	87.939	\	\
	48h	58	37.703	\	\
	72h	54	30.629	\	\
A2780-cis	24h	74	50.562	\	\
	48h	68	57.607	\	\
	72h	50	32.485	\	\
PEO1	24h	160	136.751	\	\
	48h	82	73.709	4.481	381.070
	72h	76	69.453	43.621	103.533
PEO4	24h	225	152.557	\	\
	48h	154	121.446	\	\
	72h	90	64.257	13.696	179.358

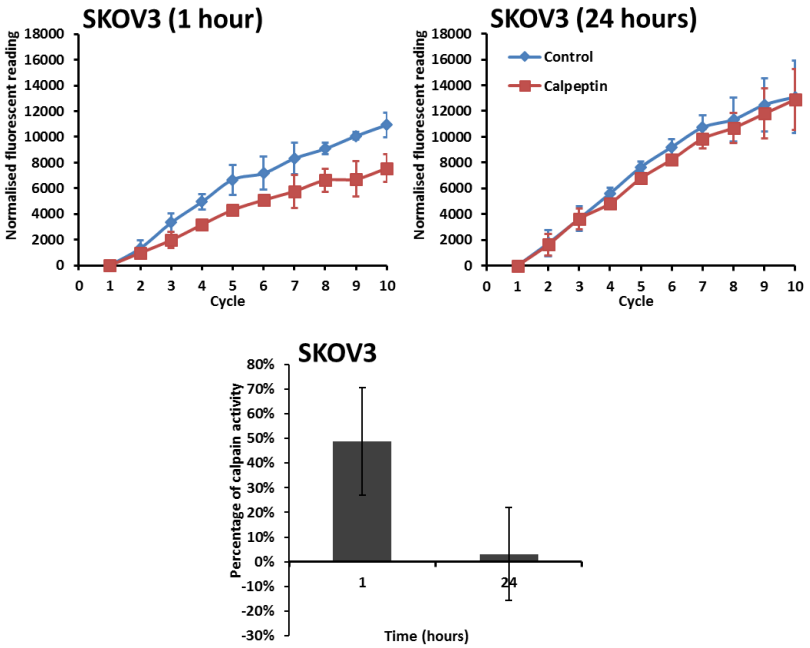


Figure S2.3 The inhibition of IC50 (80 μM) calpeptin on calpain activity of SKOV3. SKOV3 cells were treated with 80 μM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of two independent experiments with each experiment performed in duplicate. Student T-test were used to compared the calpeptin treated group with control group at cycle ten.

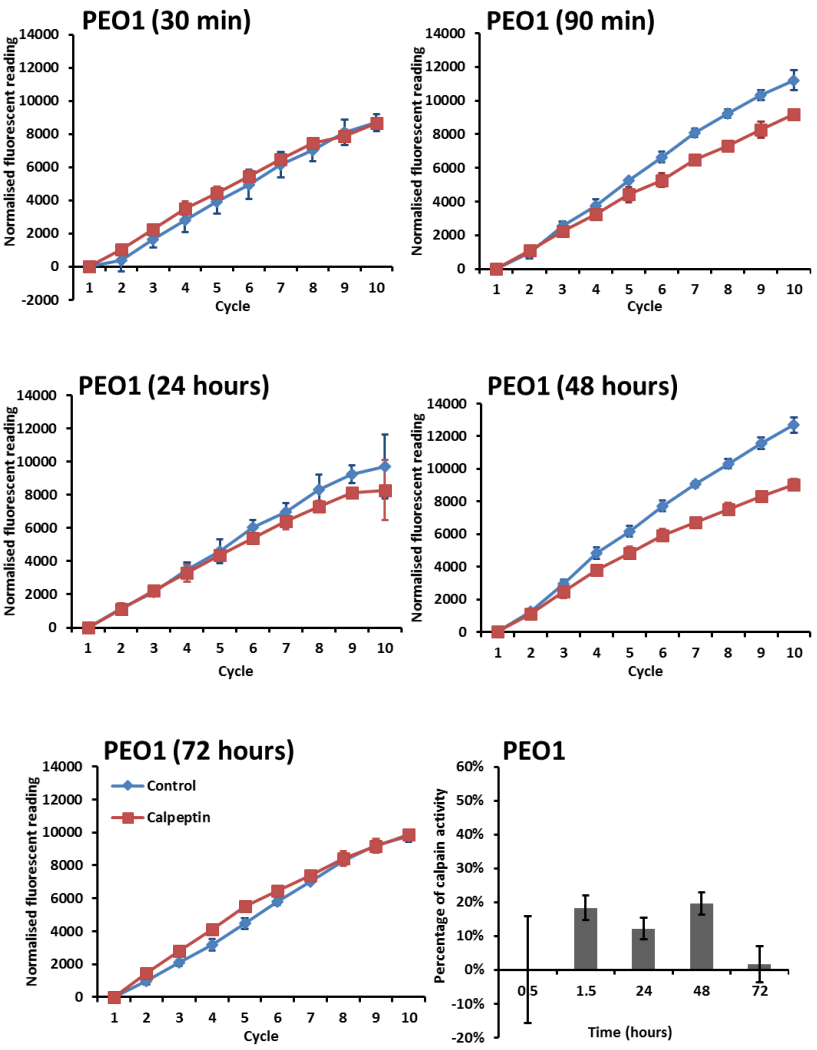


Figure S2.4 The inhibition of IC50 (80 µM) calpeptin on calpain activity of PEO1. PEO1 cells were treated with 80 µM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate.

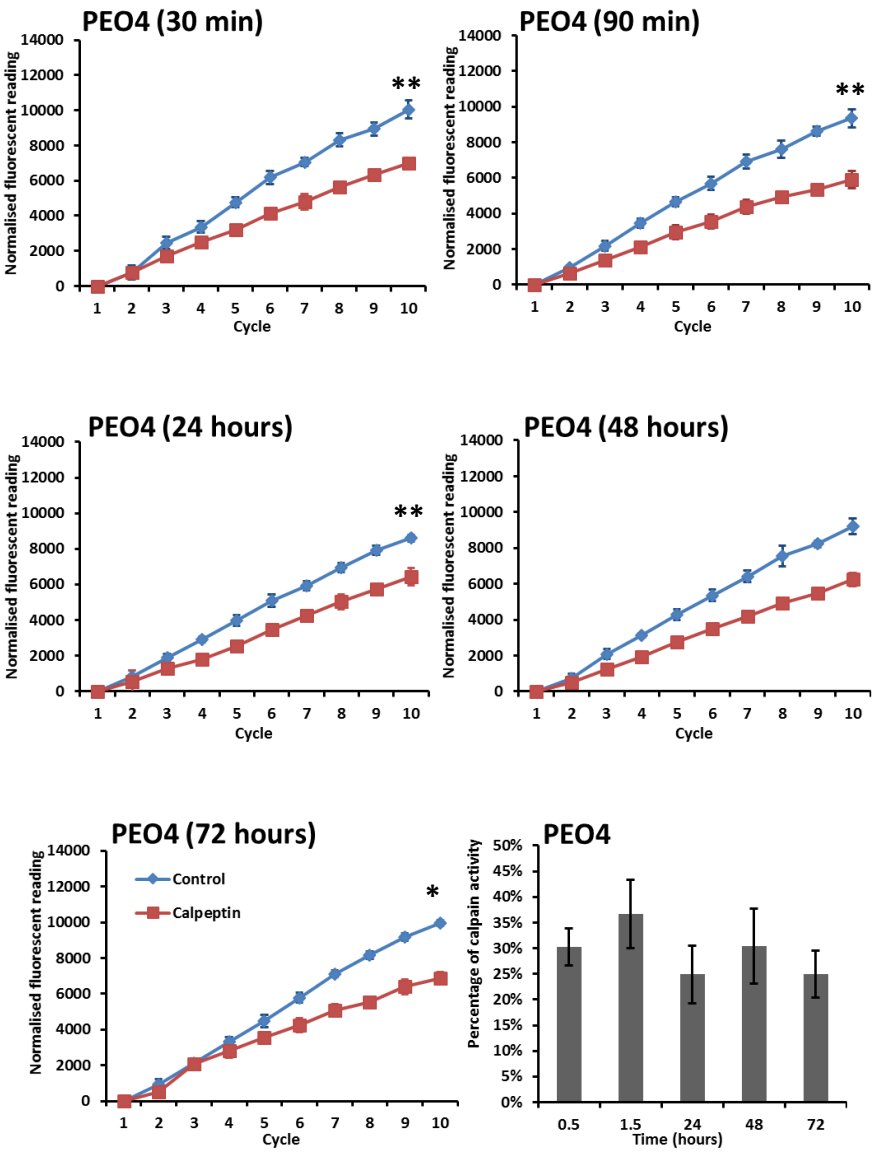


Figure S2.5 The inhibition of IC50 (90 µM) calpeptin on calpain activity of PEO4. PEO4 cells were treated with 90 µM calpeptin for 30 minutes, 90 minutes, 24 hours, 48 hours or 72 hours. The fluorescence readings at the cycle 10 were presented in bar chart. Data represent the average ± SD of three independent experiments with each experiment performed in duplicate. Student T-test were used to compared the calpeptin treated group with control group at cycle ten (*P<0.05, **P<0.01 vs control).

Table S3.1 The association between calpastatin expression and histological subtypes of ovarian cancers

Crosstab

			Histological subtypes					
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT
Calpastatin	Low	Count	122	24	25	31	7	4
		Expected Count	130.3	20.4	25.0	20.0	10.9	6.4
		% of Total	26.0%	5.1%	5.3%	6.6%	1.5%	0.9%
	High	Count	165	21	30	13	17	10
		Expected Count	156.7	24.6	30.0	24.0	13.1	7.6
		% of Total	35.2%	4.5%	6.4%	2.8%	3.6%	2.1%
Total		Count	287	45	55	44	24	14
		Expected Count	287.0	45.0	55.0	44.0	24.0	14.0
		% of Total	61.2%	9.6%	11.7%	9.4%	5.1%	3.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	17.403[a]	5	0.004
Likelihood Ratio	17.711	5	0.003
Linear-by-Linear Association	.026	1	0.871
N of Valid Cases	469		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.36.

Table S3.2 The association between calpain-1 expression and histological subtypes of ovarian cancers

Crosstab

			Histological subtypes						
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT	Total
Calpain-1	Low	Count	38	14	20	25	3	1	101
		Expected Count	61.8	9.7	11.8	9.5	5.2	3.0	101.0
		% of Total	8.1%	3.0%	4.3%	5.3%	0.6%	0.2%	21.5%
	High	Count	249	31	35	19	21	13	368
		Expected Count	225.2	35.3	43.2	34.5	18.8	11.0	368.0
		% of Total	53.1%	6.6%	7.5%	4.1%	4.5%	2.8%	78.5%
Total		Count	287	45	55	44	24	14	469
		Expected Count	287.0	45.0	55.0	44.0	24.0	14.0	469.0
		% of Total	61.2%	9.6%	11.7%	9.4%	5.1%	3.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	56.577[a]	5	<0.0001
Likelihood Ratio	50.899	5	<0.0001
Linear-by-Linear Association	5.876	1	0.015
N of Valid Cases	469		

a. 1 cells (8.3%) have expected count less than 5. The minimum expected count is 3.01.

Table S3.3 The association between calpain-4 expression and histological subtypes of ovarian cancers

Crosstab

			Histological subtypes						
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT	Total
Calpain-4	Low	Count	83	14	14	25	5	2	143
		Expected Count	87.5	13.7	16.8	13.4	7.3	4.3	143.0
		% of Total	17.7%	3.0%	3.0%	5.3%	1.1%	0.4%	30.5%
	High	Count	204	31	41	19	19	12	326
		Expected Count	199.5	31.3	38.2	30.6	16.7	9.7	326.0
		% of Total	43.5%	6.6%	8.7%	4.1%	4.1%	2.6%	69.5%
Total		Count	287	45	55	44	24	14	469
		Expected Count	287.0	45.0	55.0	44.0	24.0	14.0	469.0
		% of Total	61.2%	9.6%	11.7%	9.4%	5.1%	3.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.181[a]	5	0.003
Likelihood Ratio	17.191	5	0.004
Linear-by-Linear Association	<0.0001	1	0.987
N of Valid Cases	469		

a. 1 cells (8.3%) have expected count less than 5. The minimum expected count is 4.27.

Table S3.4 The association between calpain-1 expression and ovarian cancer stage

Crosstab

			Figo stage				
			1	2	3	4	Total
Calpain-1	Low	Count	51	13	28	8	100
		Expected Count	36.7	11.7	44.5	7.2	100.0
		% of Total	11.1%	2.8%	6.1%	1.7%	21.7%
	High	Count	118	41	177	25	361
		Expected Count	132.3	42.3	160.5	25.8	361.0
		% of Total	25.6%	8.9%	38.4%	5.4%	78.3%
Total		Count	169	54	205	33	461
		Expected Count	169.0	54.0	205.0	33.0	461.0
		% of Total	36.7%	11.7%	44.5%	7.2%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.259[a]	3	0.002
Likelihood Ratio	15.574	3	0.001
N of Valid Cases	461		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 7.16.

Table S3.5 The association between calpain-1 expression and residual disease

Crosstab

			residual disease			
			No residual tumour	Residual dis <2cm	Residual dis >2cm	Total
Calpain-1	Low	Count	73	5	14	92
		Expected Count	57.0	11.1	23.9	92.0
		% of Total	17.6%	1.2%	3.4%	22.2%
	High	Count	184	45	94	323
		Expected Count	200.0	38.9	84.1	323.0
		% of Total	44.3%	10.8%	22.7%	77.8%
Total		Count	257	50	108	415
		Expected Count	257.0	50.0	108.0	415.0
		% of Total	61.9%	12.0%	26.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.388[a]	2	<0.0001
Likelihood Ratio	16.561	2	<0.0001
Linear-by-Linear Association	12.517	1	<0.0001
N of Valid Cases	415		

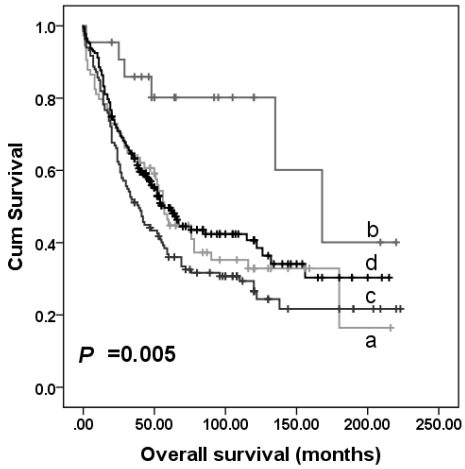
a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.08.

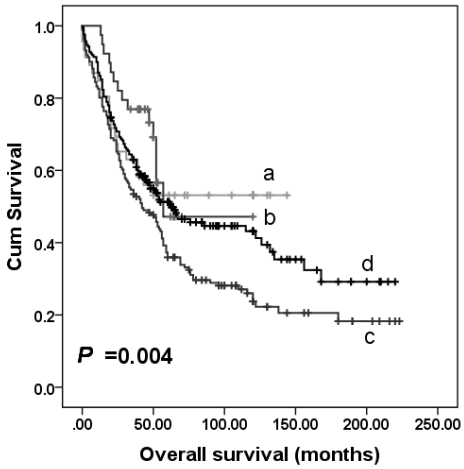
Table S3.6 The association between protein expression and tumour stage.

Variable	Calpastatin			Calpain-1			Calpain-2			Calpain-4		
	Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Stage												
I	83 (18.0%)	86 (18.7%)	0.217	51 (11.1%)	118 (25.6%)	**0.002**	35 (7.6%)	133 (28.9%)	0.572	52 (11.3%)	117 (25.4%)	0.969
II	24 (5.2%)	30 (6.5%)		13 (2.8%)	41 (8.9%)		7 (1.5%)	47 (10.2%)		17 (3.7%)	37 (8.0%)	
III	82 (17.8%)	123 (26.7%)		28 (6.1%)	177 (38.4%)		40 (8.7%)	165 (35.9%)		59 (12.8%)	146 (31.7%)	
IV	18 (3.9%)	15 (3.3%)		8 (1.7%)	25 (5.4%)		5 (1.1%)	28 (6.1%)		10 (2.2%)	23 (5.0%)	
Stage												
Tumour confined to the ovaries (stage 1)	83 (18.0%)	86 (18.7%)	0.167	51 (11.1%)	118 (25.6%)	**0.001**	35 (7.6%)	133 (28.9%)	0.425	52 (11.3%)	117 (25.4%)	0.766
Tumour spread out of ovaries (stage 2, 3 and 4)	124 (26.8%)	168 (36.4%)		49 (10.6%)	243 (52.7%)		52 (11.3%)	240 (52.2%)		86 (18.7%)	206 (44.7%)	
Stage												
Without distant metastasis(Stage 1,2 and 3)	189 (41.0%)	239 (51.8%)	0.248	92 (20.0%)	336 (72.9%)	0.712	82 (17.8%)	345 (75.0%)	0.567	128 (27.8%)	300 (65.1%)	0.962
Distant metastasis occurred (stage 4)	18 (3.9%)	15 (3.3%)		8 (1.7%)	25 (5.4%)		5 (1.1%)	28 (6.1%)		10 (2.2%)	23 (5.0%)	

Table S3.7 Multivariate (Cox proportional hazard regression) analysis

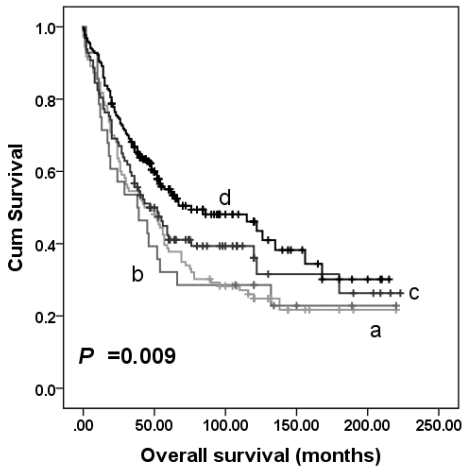
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Age (median 62)	.226	.186	1.472	1	.225	1.253	.870	1.805
Grade	.368	.273	1.820	1	.177	1.445	.847	2.465
FIGO stage	.414	.122	11.590	1	.001	1.513	1.192	1.919
Histological subtypes	-.017	.082	.044	1	.833	.983	.836	1.155
Platinum sensitivity	1.145	.225	26.005	1	<.0001	3.142	2.024	4.879
Tumour residue	.261	.119	4.794	1	.029	1.298	1.028	1.639
Calpastatin expression	-.341	.182	3.518	1	.061	.711	.498	1.015
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Age (median 62)	.339	.180	3.553	1	.059	1.404	.987	1.998
Grade	.424	.286	2.201	1	.138	1.527	.873	2.673
FIGO stage	.432	.123	12.244	1	<.0001	1.540	1.209	1.962
Histological subtypes	.004	.082	.003	1	.959	1.004	.855	1.180
Platinum sensitivity	1.186	.225	27.876	1	<.0001	3.272	2.107	5.082
Tumour residue	.249	.116	4.637	1	.031	1.283	1.023	1.610
Calpain-2 expression	.421	.232	3.289	1	.070	1.524	.967	2.404
	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Age (median 62)	.259	.186	1.936	1	.164	1.296	.900	1.867
Grade	.356	.278	1.637	1	.201	1.427	.828	2.462
FIGO stage	.404	.123	10.865	1	.001	1.497	1.178	1.904
Histological subtypes	-.021	.083	.064	1	.801	.979	.832	1.152
Platinum sensitivity	1.127	.225	25.112	1	<.0001	3.088	1.987	4.798
Tumour residue	.265	.117	5.138	1	.023	1.303	1.037	1.639
Calpain-4 expression	-.232	.194	1.432	1	.232	.793	.542	1.160

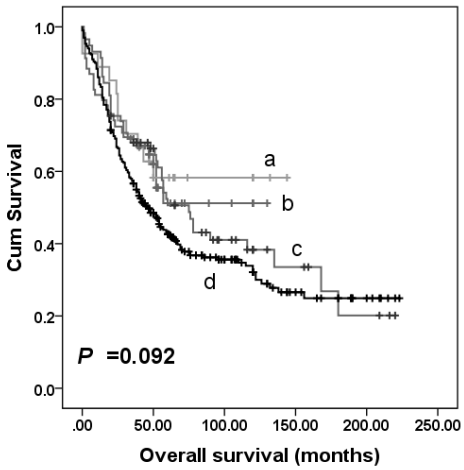




a Low calpain-1, low calpastatin
b Low calpain-1, high calpastatin
c High calpain-1, low calpastatin
d High calpain-1, high calpastatin

a Low calpain-2, low calpastatin
b Low calpain-2, high calpastatin
c High calpain-2, low calpastatin
d High calpain-2, high calpastatin

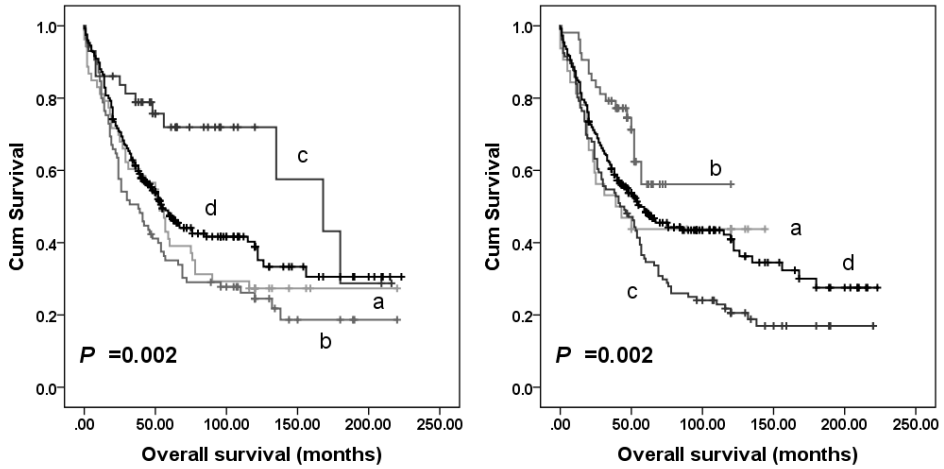




a Low calpain-4, low calpastatin
b Low calpain-4, high calpastatin
c High calpain-4, low calpastatin
d High calpain-4, high calpastatin

a Low calpain-2, low calpain-1
b Low calpain-2, high calpain-1
c High calpain-2, low calpain-1
d High calpain-2, high calpain-1

Figure S3.1 Kaplan-Meier survival curves show the impact of conventional calpains and calpastatin expression on the OS by combining any two of the calpain-1, -2, -4 and calpastatin expression.



a Low calpain-4, low calpain-1
b Low calpain-4, high calpain-1
c High calpain-4, low calpain-1
d High calpain-4, high calpain-1

a Low calpain-2, low calpain-4
b Low calpain-2, high calpain-4
c High calpain-2, low calpain-4
d High calpain-2, high calpain-4

Figure S3.1 Continued.

Table S3.8 Kaplan–Meier survival analysis in different subgroups of ovarian cancer

	***P* value (confidence interval: 99%)**			
	Calpastatin	Calpain-1	Calpain-2	Calpain-4
Histological subtypes				
High grade serous carcinoma (n=265)	0.002	0.658	0.011	0.064
Mucinous carcinoma (n=45)	0.497	0.448	0.556	0.014
Endometrioid carcinoma (n=53)	0.415	0.617	0.713	0.899
Clear-cell carcinoma (n=41)	0.564	0.308	0.826	0.461
Low grade serous carcinoma (n=24)	0.930	0.866	0.257	0.987
Stage				
Stage 1 (confined tumour) (n=163)	0.019	0.997	0.634	0.011
Stage 2, 3 & 4 (tumour spread) (n=286)	0.008	0.766	0.025	0.044
Stage 1, 2 & 3 (without distant metastasis) (n=416)	0.019	0.195	0.078	0.004
Stage 4 (with distant metastasis) (n=33)	0.976	0.254	0.024	0.963
Sensitivity to platinum-based chemotherapy				
Sensitive (n=213)	0.050	0.049	0.435	0.036
Resistant (n=39)	0.798	0.815	0.076	0.433
Residual disease				
No residual tumour (n=250)	0.085	0.988	0.244	0.026
residual tumour (n=153)	0.002	0.361	0.594	0.012

Individual confidence interval was adjusted by the Bonferroni correction.

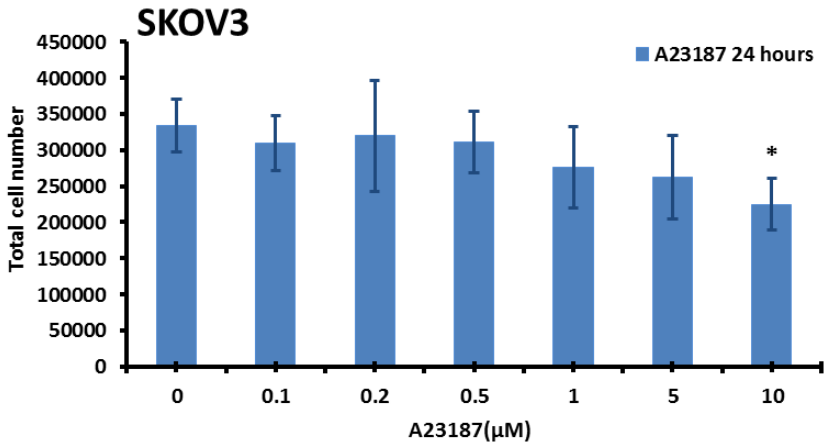


Figure 4.10 The effect of A23187 on SKOV3 cells proliferation. SKOV3 cells were treated by A23187 for 24 hours and incubated for another 24 hours in fresh growth media, after which cell numbers were counted. Data are expressed as the total cell number and refer to three separate experiments each conducted in triplicate ± SD. Student T-test were used to compared the treated group with control group (*P<0.05 vs control).

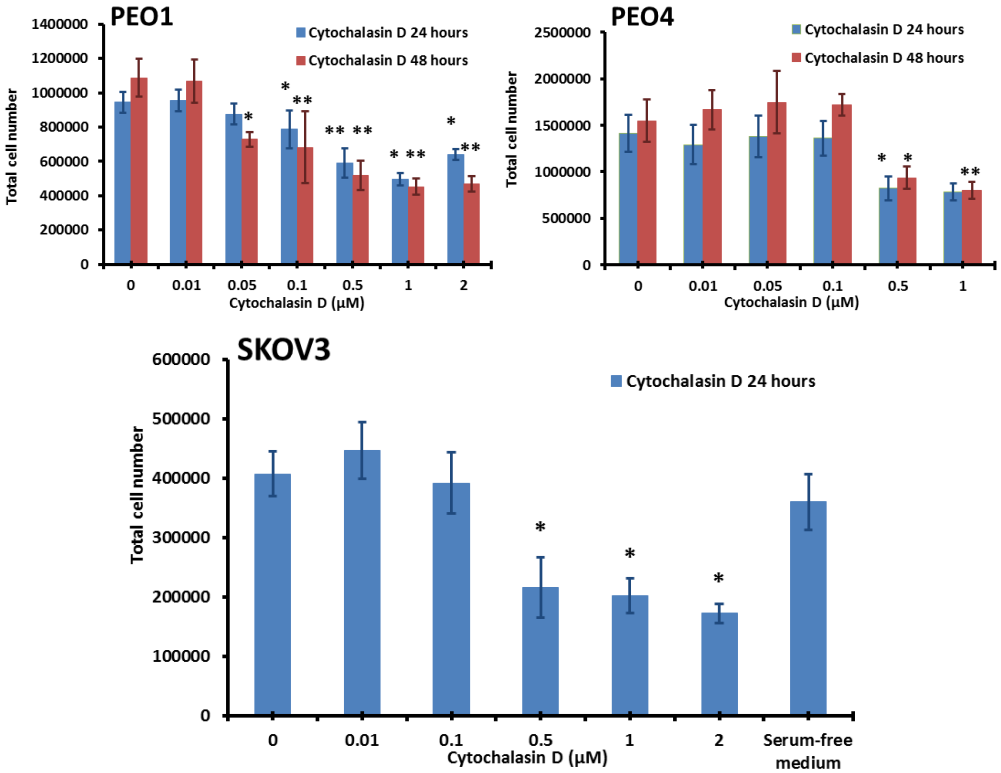


Figure 4.11 The effect of cytochalasin D on cell proliferation. Cells were treated by 0.01μM, 0.05μM, 0.1μM, 0.5μM and 1μM (and 2μM for SKOV3 and PEO1) cytochalasin D for 24 hours after which cells were incubated in fresh growth media and counted every 24-hours. SKOV3 were also tested with serum free medium rather than complete growth medium. Data are expressed as the total cell number and refer to three separate experiments each conducted in triplicate ± SD. Student T-test were used to compared the treated group with control group (*P<0.05, **P<0.01 vs control).

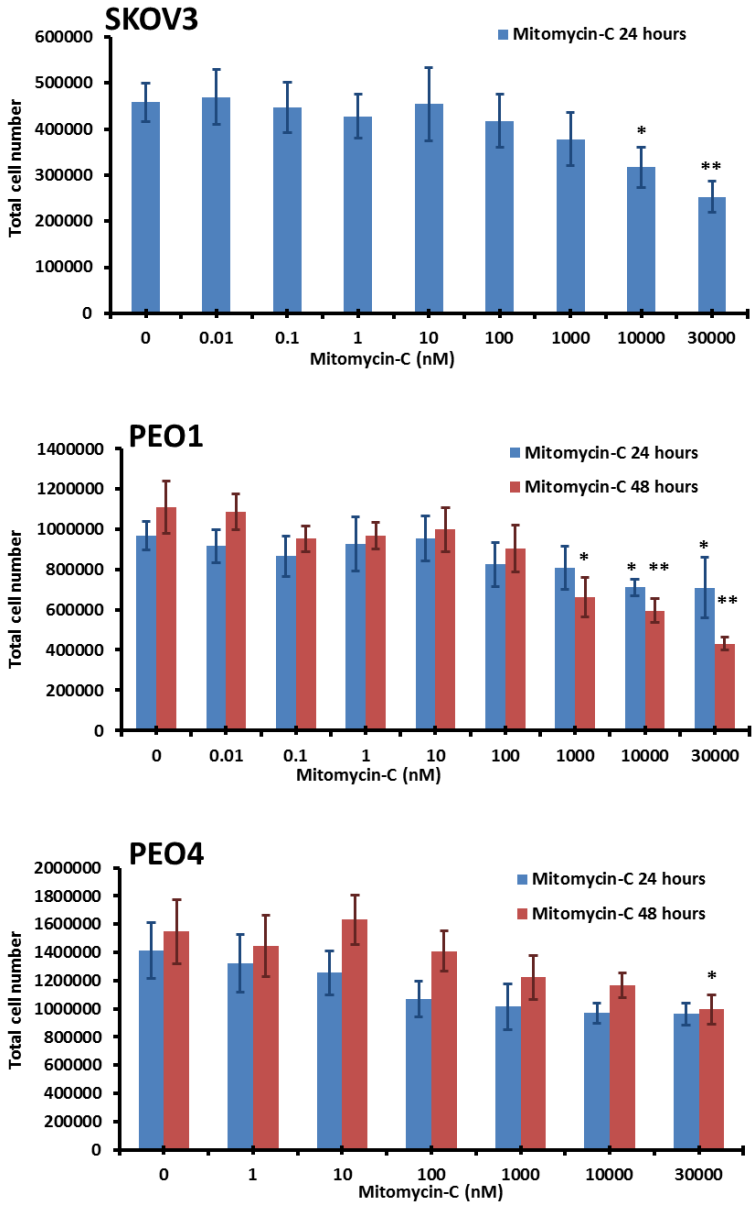


Figure 4.12 Growth inhibitory effect of mitomycin-C on ovarian cancer cells. Cells were treated with various concentrations of mitomycin-C (0.01nM, 0.1nM, 1nM, 10nM, 100nM, 1µM, 10µM and 30µM) for 24 to 48 hours. The initial cell number was 200000, 700000 and 1000000 respectively. Inhibition of SKOV3, PEO1 and PEO4 cell proliferation occurs at 30µM, 1µM and 30µM concentration of mitomycin-C respectively, at which the total cell numbers after 24 to 48 hours were still close to the initial cell numbers. Data are expressed as the total cell number and refer to three separate experiments each conducted in triplicate ± SD. Student T-test were used to compared the treated group with control group (*P<0.05, **P<0.01 vs control).

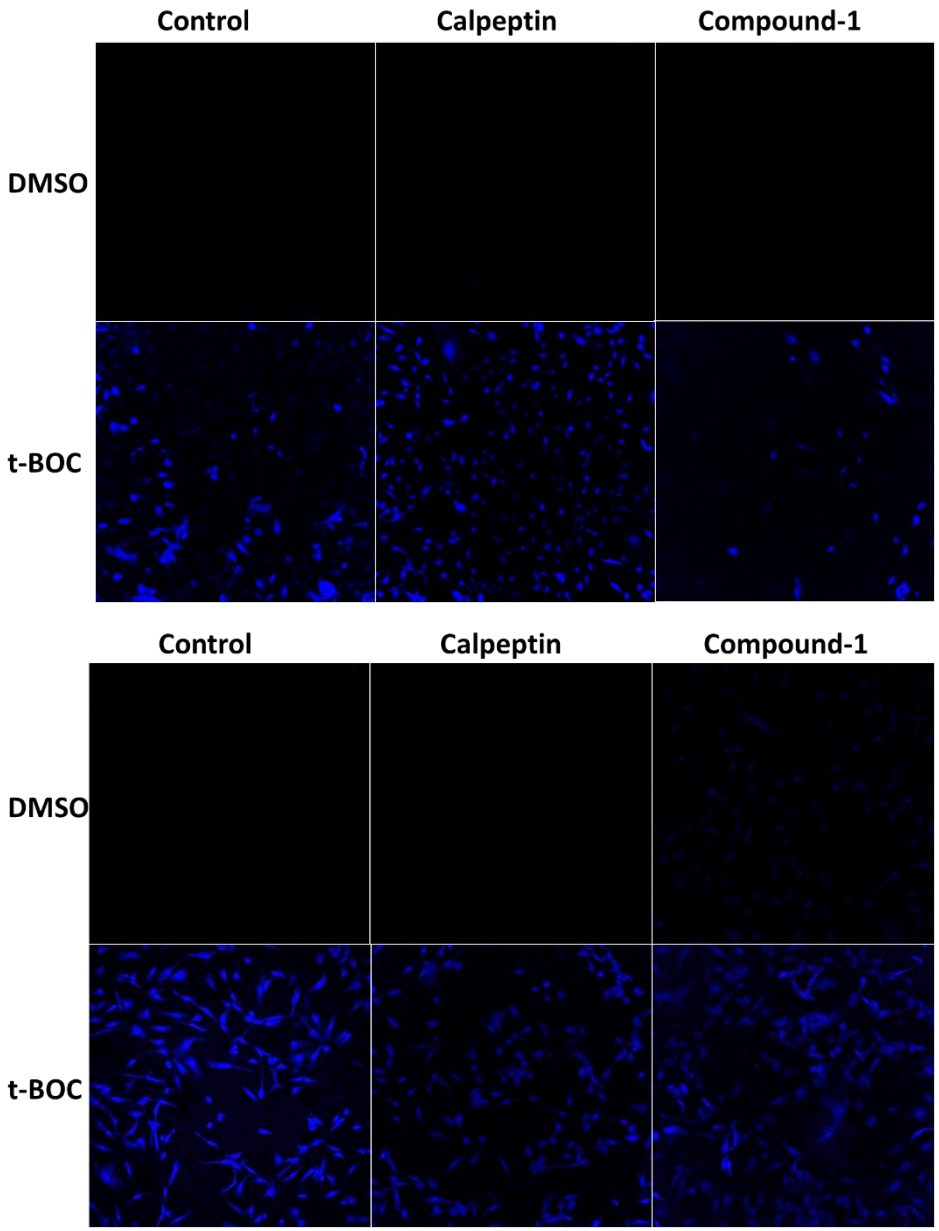


Figure 4.13 The inhibitory effect of calpain inhibitor on calpain activity in SKOV3 cells. Fluorescence intensity corresponding to calpain activity was captured (40x) by fluorescence microscopy. Blue fluorescence generated by calpain-cleaved t-BOC is indicative of calpain activity. Representative fluorescent image from two independent experiments are presented showing calpain activity in SKOV3 cells after exposure to calpain inhibitor (5µM compound-1 or 50µM calpeptin) for one hour.

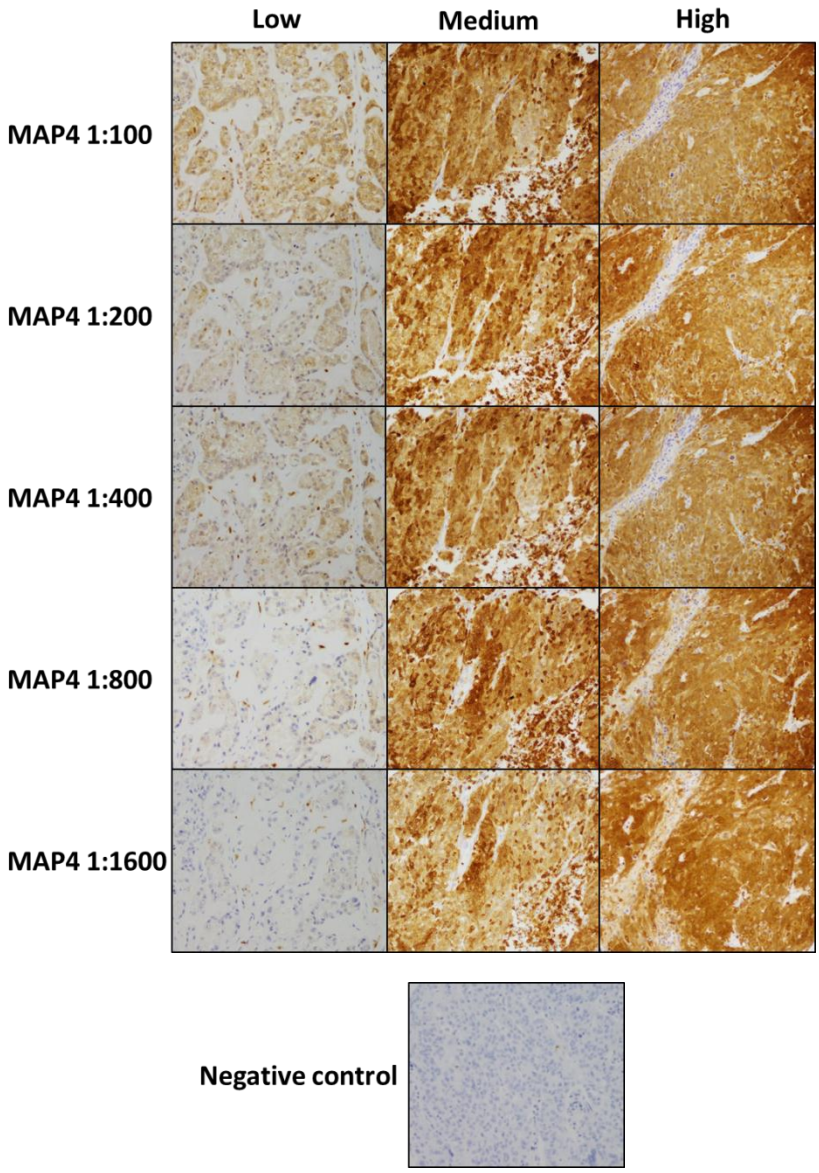


Figure S5.1 Optimisation of anti-MAP4 antibody on ovarian cancers. Ovarian cancer TMAs were subjected to immunohistochemistry. The figures are the representative photomicrographs of low, medium and high MAP4 protein expression with 1:100, 1:200, 1:400, 1:800 and 1:1600 dilutions of primary antibody in addition to the no staining negative control. One core is used for each column to present the serial dilution. Photomicrographs are at X10 magnification. The optimal dilution chosen was 1:1600.

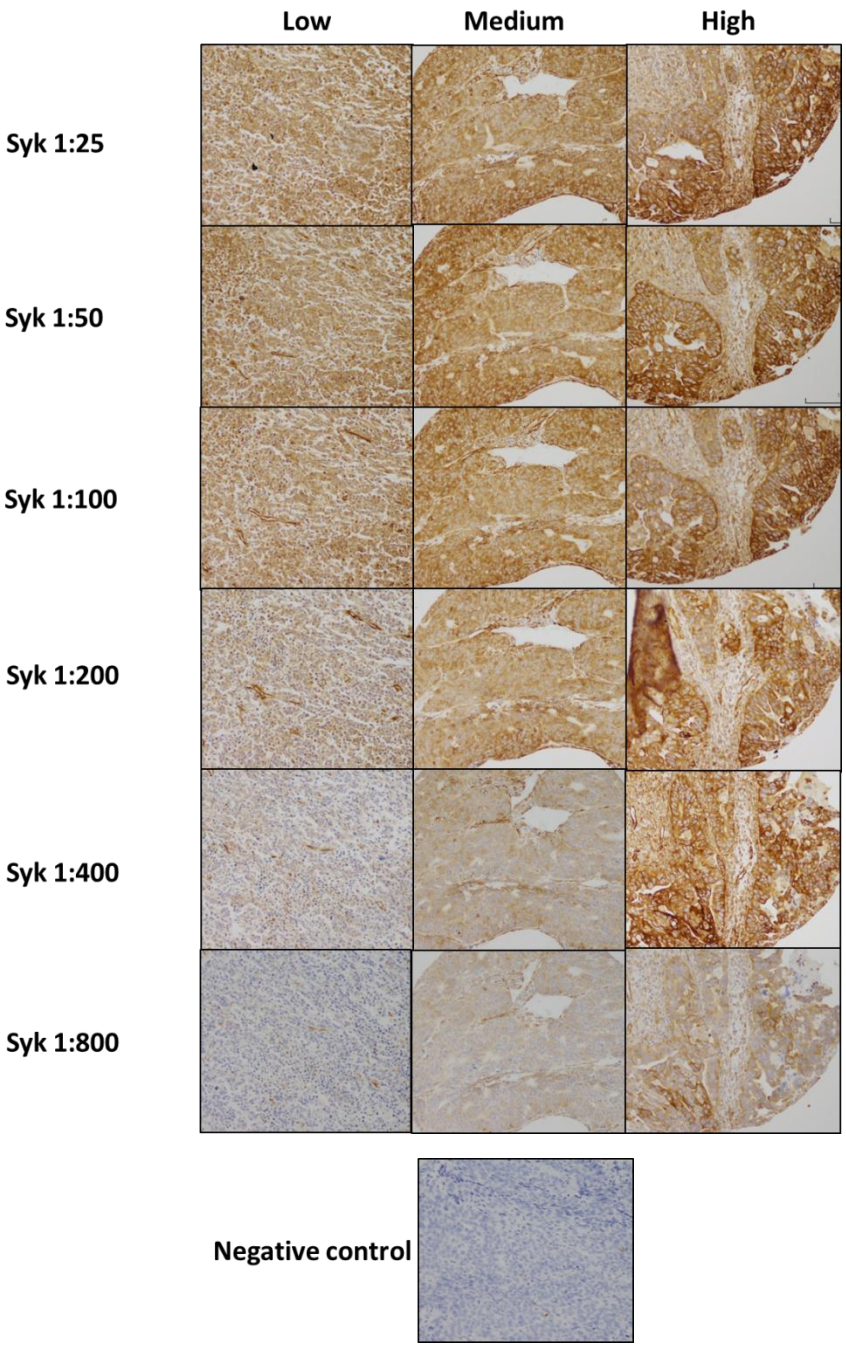


Figure S5.2 Optimisation of anti-Syk antibody on ovarian cancers. Ovarian cancer TMAs were subjected to immunohistochemistry. The figures present the representative photomicrographs of low, medium and high Syk protein expression with 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions of primary antibody in addition to the no staining negative control. Photomicrographs are at X10 magnification. The optimal dilution chosen was 1:400.

Table S5.1 The association between MAP4 expression and histological subtypes

Crosstab

			Histological subtypes						
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT	Total
MAP4	Low	Count	59	25	22	29	11	6	152
		Expected Count	92.9	14.1	18.9	15.5	6.5	4.1	152.0
		% of Total	13.3%	5.7%	5.0%	6.6%	2.5%	1.4%	34.4%
	High	Count	211	16	33	16	8	6	290
		Expected Count	177.1	26.9	36.1	29.5	12.5	7.9	290.0
		% of Total	47.7%	3.6%	7.5%	3.6%	1.8%	1.4%	65.6%
Total		Count	270	41	55	45	19	12	442
		Expected Count	270.0	41.0	55.0	45.0	19.0	12.0	442.0
		% of Total	61.1%	9.3%	12.4%	10.2%	4.3%	2.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	56.386[a]	5	6.7681E-11
Likelihood Ratio	55.462	5	<0.0001
Linear-by-Linear Association	29.890	1	<0.0001
N of Valid Cases	442		

a. 1 cells (8.3%) have expected count less than 5. The minimum expected count is 4.13.

Table S5.2 The association between MAP4 expression and ovarian cancer grade

Crosstab

			Tumour grade			
			1	2	3	Total
MAP4	Low	Count	16	38	97	151
		Expected Count	12.0	22.6	116.4	151.0
		% of Total	3.6%	8.6%	22.0%	34.2%
	High	Count	19	28	243	290
		Expected Count	23.0	43.4	223.6	290.0
		% of Total	4.3%	6.3%	55.1%	65.8%
Total		Count	35	66	340	441
		Expected Count	35.0	66.0	340.0	441.0
		% of Total	7.9%	15.0%	77.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	22.933[a]	2	<0.0001
Likelihood Ratio	21.990	2	<0.0001
Linear-by-Linear Association	14.831	1	<0.0001
N of Valid Cases	441		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.98.

Table S5.3 The association between MAP4 expression and ovarian cancer stage

Crosstab

			Figo stage				
			1	2	3	4	Total
MAP4	Low	Count	75	12	52	10	149
		Expected Count	56.2	16.4	65.8	10.6	149.0
		% of Total	17.2%	2.8%	12.0%	2.3%	34.3%
	High	Count	89	36	140	21	286
		Expected Count	107.8	31.6	126.2	20.4	286.0
		% of Total	20.5%	8.3%	32.2%	4.8%	65.7%
Total		Count	164	48	192	31	435
		Expected Count	164.0	48.0	192.0	31.0	435.0
		% of Total	37.7%	11.0%	44.1%	7.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.857[a]	3	0.001
Likelihood Ratio	15.733	3	0.001
Linear-by-Linear Association	10.988	1	0.001
N of Valid Cases	435		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 10.62.

Table S5.4 The association between MAP4 expression and residual disease

Crosstab

			Residual disease			
			no residual tumour	residual dis <2cm	residual dis >2cm	Total
MAP4	Low	Count	97	10	27	134
		Expected Count	82.6	16.5	35.0	134.0
		% of Total	24.8%	2.6%	6.9%	34.3%
	High	Count	144	38	75	257
		Expected Count	158.4	31.5	67.0	257.0
		% of Total	36.8%	9.7%	19.2%	65.7%
Total		Count	241	48	102	391
		Expected Count	241.0	48.0	102.0	391.0
		% of Total	61.6%	12.3%	26.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.426[a]	2	0.005
Likelihood Ratio	10.788	2	0.005
Linear-by-Linear Association	7.543	1	0.006
N of Valid Cases	391		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 16.45.

Table S5.5 The association between cytoplasmic Syk expression and histological subtypes

Crosstab

			Histological subtypes						
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT	Total
Cytoplasmic Syk	Low	Count	25	9	9	23	1	0	67
		Expected Count	41.3	6.5	8.2	6.7	2.7	1.5	67.0
		% of Total	5.7%	2.0%	2.0%	5.2%	0.2%	0.0%	15.2%
	High	Count	247	34	45	21	17	10	374
		Expected Count	230.7	36.5	45.8	37.3	15.3	8.5	374.0
		% of Total	56.0%	7.7%	10.2%	4.8%	3.9%	2.3%	84.8%
Total		Count	272	43	54	44	18	10	441
		Expected Count	272.0	43.0	54.0	44.0	18.0	10.0	441.0
		% of Total	61.7%	9.8%	12.2%	10.0%	4.1%	2.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	58.835[a]	5	2.115E-11
Likelihood Ratio	47.379	5	<0.0001
Linear-by-Linear Association	6.033	1	0.014
N of Valid Cases	441		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is 1.52.

Table S5.6 The association between cytoplasmic Syk expression and ovarian cancer stage

Crosstab

			Figo stage				
			1	2	3	4	Total
Cytoplasmic Syk	Low	Count	36	6	20	5	67
		Expected Count	24.5	7.6	30.1	4.8	67.0
		% of Total	8.3%	1.4%	4.6%	1.2%	15.4%
	High	Count	123	43	175	26	367
		Expected Count	134.5	41.4	164.9	26.2	367.0
		% of Total	28.3%	9.9%	40.3%	6.0%	84.6%
Total		Count	159	49	195	31	434
		Expected Count	159.0	49.0	195.0	31.0	434.0
		% of Total	36.6%	11.3%	44.9%	7.1%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.725[a]	3	0.013
Likelihood Ratio	10.546	3	0.014
Linear-by-Linear Association	7.484	1	0.006
N of Valid Cases	434		

a. 1 cells (12.5%) have expected count less than 5. The minimum expected count is 4.79.

Table S5.7 The association between nuclear Syk expression and histological subtypes

Crosstab

			Histological subtypes						
			HGSC	Mucinous	Endometrioid	CCC	LGSC	SBOT	Total
Nuclear Syk	Low	Count	57	10	16	25	4	0	112
		Expected Count	69.1	10.9	13.7	11.2	4.6	2.5	112.0
		% of Total	12.9%	2.3%	3.6%	5.7%	0.9%	0.0%	25.4%
	High	Count	215	33	38	19	14	10	329
		Expected Count	202.9	32.1	40.3	32.8	13.4	7.5	329.0
		% of Total	48.8%	7.5%	8.6%	4.3%	3.2%	2.3%	74.6%
Total		Count	272	43	54	44	18	10	441
		Expected Count	272.0	43.0	54.0	44.0	18.0	10.0	441.0
		% of Total	61.7%	9.8%	12.2%	10.0%	4.1%	2.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	29.874[a]	5	0.000016
Likelihood Ratio	28.995	5	<0.0001
Linear-by-Linear Association	2.298	1	0.130
N of Valid Cases	441		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is 2.54.

Table S5.8 The association between nuclear Syk expression and the response to taxane-containing chemotherapy regimens

Crosstab

			Chemo-response (taxane-containing)		
			resistant	sensitive	Total
Nuclear Syk	Low	Count	11	23	34
		Expected Count	5.9	28.1	34.0
		% of Total	9.0%	18.9%	27.9%
	High	Count	10	78	88
		Expected Count	15.1	72.9	88.0
		% of Total	8.2%	63.9%	72.1%
Total		Count	21	101	122
High syk chemo-sensitive		Expected Count	21.0	101.0	122.0
		% of Total	17.2%	82.8%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.582[a]	1	.006		
Continuity Correction[b]	6.181	1	.013		
Likelihood Ratio	6.938	1	.008		
Fisher's Exact Test				.014	.008
Linear-by-Linear Association	7.520	1	.006		
N of Valid Cases	122				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.85.

b. Computed only for a 2x2 table

Table S5.9 The association between protein expression and tumour stage

Variable	Cytoplasmic Syk			Nuclear Syk			MAP4		
	Low	High	P-value	Low	High	P-value	Low	High	P-value
Stage									
Tumour confined to ovaries (stage 1)	36(8.3%)	123 (28.3%)	**0.002**	50 (11.5%)	109 (25.1%)	**0.041**	75 (17.2%)	89 (20.5%)	**0.000087**
Tumour spread out of ovaries (stage 2, 3 and 4)	31 (7.1%)	244 (56.2%)		62 (14.3%)	213 (49.1%)		74 (17.0%)	197 (45.3%)	
Stage									
Without distant metastasis (stage 1,2 and 3)	62 (14.3%)	341 (78.6%)	0.912	104 (24.0%)	299 (68.9%)	1.000	139 (32.0%)	265 (60.9%)	0.808
Distant metastasis occurred (stage 4)	5 (1.2%)	26 (6.0%)		8 (1.8%)	23 (5.3%)		10 (2.3%)	21 (4.8%)	

Significant *P* values are indicated by bold font.

Table S5.10 Kaplan–Meier univariate survival analyses of OS related to combinational protein expression.

	MAP4 (*P*=0.064)	C-Syk (*P*=0.494)	N-Syk (*P*=0.113)	CXCL4 (*P*=0.031)	CXCR3 (*P*=0.182)
Calpastatin (*P*=0.010)	0.024	0.174	0.178	0.225	0.188
Calpain-1 (*P*=0.153)	0.283	0.273	0.292	0.309	0.445
Calpain-2 (*P*=0.026)	0.037	0.087	0.056	0.285	0.152
Calpain-4 (*P*=0.003)	0.034	0.139	0.061	0.074	0.068
C-Syk (*P*=0.494)	0.220	\	0.424	0.358	0.468
N-Syk (*P*=0.113)	0.112	\	\	0.166	0.208
MAP4 (*P*=0.064)	\	\	\	0.084	0.097

Table S5.11 Kaplan–Meier survival analyses of OS in different subgroups

Variables	*P* value (confidence interval: 99%)		
	MAP4	Cytoplasmic Syk	Nuclear Syk
Histological subtypes			
High grade serous carcinoma	0.699.	0.581	0.506
Mucinous carcinoma	0.791	0.026	0.115
Endometrioid carcinoma	0.110	0.188	0.006
Clear-cell carcinoma	0.988	0.822	0.453
Low grade serous carcinoma	0.212	0.549	0.196
Stage			
Stage 1 (confined tumour) (n=152)	0.679	0.002	**0.001**
Stage 2, 3 & 4 (tumour spread) (n=269)	0.404	0.688	0.224
Stage			
Stage 1, 2 & 3 (without distant metastasis) (n=390)	0.104	0.568	0.107
Stage 4 (with distant metastasis) (n=31)	0.061	0.402	0.509
Sensitivity to platinum-based chemotherapy			
Sensitive (n=195)	0.885	0.486	0.464
Resistant (n=40)	0.996	0.016	**0.001**
Residual disease			
No residual tumour (n=232)	0.867	0.054	**0.001**
residual tumour (n=147)	0.540	0.349	0.571

Individual confidence interval was adjusted by the Bonferroni correction.

Significant *P* values are indicated by bold font.

Table S5.12 Spearman's rank correlation coefficient between the expression of proteins that potentially correlated with the calpain system (469 valid cases)

		C-Syk	N-Syk	CXCL4	CXCR3
MAP4	**rs**	**0.385****	**0.290****	0.116*	**0.208****
	Sig.	1.350E-16 (n=429)	9.765E-10 (n=429)	0.038 (n=323)	8.000E-5 (n=355)
C- Syk	**rs**		**0.809****	0.145**	0.199**
	Sig.		3.189E-103 (n=441)	0.010 (n=319)	1.693E-4 (n=353)
N-Syk	**rs**			0.121*	0.137*
	Sig.			0.030 (n=319)	0.010 (n=353)
CXCL4	**rs**				0.037
	Sig.				0.520 (n=297)

rs: Spearman's correlation coefficient; N-: Nuclear; C-: Cytoplasmic.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Significant *P* values are indicated by bold font.

Table S5.13 Spearman's rank correlation coefficient between the expression of calpain system and the expression of potential correlated proteins (87 HGSC patients)

		N	Calpastatin	Calpain-1	Calpain-2	Calpain-4
MAP4	**rs**		**0.267***	**0.650****	0.067	**0.451****
	Sig.	65	0.032	4.5667E-9	0.598	1.6403E-4
C- Syk	**rs**		**0.282***	**0.607****	0.036	**0.349****
	Sig.	64	0.024	1.0773E-7	0.776	0.005
N-Syk (%)	**rs**		**0.244***	**0.401****	0.030	**0.266***
	Sig.	70	0.042	0.001	0.804	0.026
Integrin	**rs**		0.067	**0.517****	0.125	0.156
α2β3	**Sig.**	62	0.607	1.7009E-5	0.333	0.225
E-cadherin	**rs**		-0.260	-0.023	0.238	-0.275
	Sig.	33	0.144	0.898	0.182	0.121
P-cadherin	**rs**		-0.265	-0.061	0.140	-0.160
	Sig.	33	0.137	0.736	0.439	0.373
N-cadherin	**rs**		**-0.378****	0.109	-0.148	-0.215
	Sig.	70	0.001	0.369	0.222	0.074
VEGF	**rs**		-0.082	0.108	-0.147	-0.133
	Sig.	64	0.517	0.395	0.243	0.291
CXCL1	**rs**		0.218	0.026	0.073	0.088
	Sig.	70	0.070	0.832	0.549	0.469
CXCL4	**rs**		-0.190	0.135	0.167	-0.049
	Sig.	59	0.149	0.308	0.206	0.713
C-CXCL8	**rs**		-0.067	0.115	0.083	0.115
	Sig.	60	0.609	0.381	0.529	0.383
N-CXCL8 (%)	**rs**		0.153	0.150	-0.017	0.097
	Sig.	60	0.245	0.252	0.896	0.461
CXCR3	**rs**		0.132	0.219	0.071	0.042
	Sig.	58	0.324	0.099	0.596	0.754
C-DARC	**rs**		-0.167	**0.303***	0.006	-0.203
	Sig.	66	0.181	0.013	0.960	0.103
N-DARC	**rs**		**0.247***	**0.287***	0.196	0.130
	Sig.	66	0.046	0.019	0.116	0.298

rs: Spearman's correlation coefficient; N-: Nuclear; C-: Cytoplasmic.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Significant *P* values are indicated by bold font.

Table S5.14 Spearman's rank correlation coefficient between the expression of MAP4 and Syk and the expression of potential correlated proteins (87 HGSC patients)

		MAP4	C-Syk	N-Syk
MAP4	**rs**	\	**0.560****	**0.503****
	Sig.	\	8.2705E-7 (n=67)	1.0531E-5 (n=69)
C-Syk	**rs**	\	\	**0.876****
	Sig.	\	\	1.2595E-22 (n=68)
integrin α2β3	**rs**	**0.501****	**0.286***	0.201
	Sig.	4.609E-5 (n=60)	0.030 (n=58)	0.085 (n=74)
E-cadherin	**rs**	-0.088	-0.094	0.142
	Sig.	0.631 (n=32)	0.617 (n=31)	0.375 (n=41)
	N	32	31	41
P-cadherin	**rs**	-0.286	-0.131	0.030
	Sig.	0.112 (n=32)	0.483 (n=31)	0.852 (n=41)
N-cadherin	**rs**	0.157	0.103	0.033
	Sig.	0.196 (n=69)	0.403 (n=68)	0.760 (n=87)
C-DARC	**rs**	0.236	0.234	0.189
	Sig.	0.058 (n=65)	0.065 (n=63)	0.101 (n=76)
N-DARC	**rs**	0.136	**0.399****	**0.240***
	Sig.	0.278 (n=65)	0.001 (n=63)	0.037 (n=76)
VEGF	**rs**	**0.307***	0.113	0.023
	Sig.	0.014 (n=64)	0.378 (n=63)	0.843 (n=78)
CXCL1	**rs**	0.021	0.050	0.166
	Sig.	0.862 (n=69)	0.685 (n=68)	0.125 (n=87)
CXCL4	**rs**	0.022	0.215	0.130
	Sig.	0.868 (n=59)	0.106 (n=58)	0.283 (n=70)
C-CXCL8	**rs**	**0.274***	0.156	0.081
	Sig.	0.031 (n=62)	0.230 (n=61)	0.496 (n=73)
N-CXCL8 (%)	**rs**	0.166	**0.320***	0.096
	Sig.	0.198 (n=62)	0.012 (n=61)	0.418 (n=73)
CXCR3	**rs**	0.008	0.206	0.175
	Sig.	0.953 (n=60)	0.122 (n=58)	0.148 (n=70)

rs: Spearman's correlation coefficient; N-: Nuclear; C-: Cytoplasmic.

**. Correlation is significant at the 0.01 level (2-tailed).

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