

**Functional Tumour Heterogeneity in Advanced
Ovarian Cancer**

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of Doctor of Philosophy**

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Declaration of Originality

I declare that the content of this thesis is the product of my own work and that all the sources used for the preparation of this thesis and all the assistance received to complete this work has been acknowledged.

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This thesis is dedicated to my wonderful family
To my beloved husband, daughters and my parents
For their patience, endless encouragement and support

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Abstract

High-grade serous ovarian cancer (HGSOC) is the most lethal gynaecological malignancy. It is characterised by high degrees of genomic instability and heterogeneity, with many patients initially responding to chemotherapy but ultimately relapsing as they develop resistance to platinum chemotherapy. The diversity of platinum resistance mechanisms and complete lack of predictive biomarkers means that delivering the best treatment for ovarian cancer patients remains challenging. Novel therapies targeting the molecular pathways dysregulated in HGSOC are being explored with the goal of personalised treatment and improved outcome for patients. This study aims to understand the extent of intra-tumour heterogeneity (ITH) in HGSOC and define the relationship between ITH at the phenotypic and molecular levels with clinical outcomes.

Multiple tumour samples were collected from defined anatomical regions during maximal effort primary debulking surgery for HGSOC, and relapse samples were collected for comparative studies. Tumour cells were extracted, placed in short-term cultures, treated with cisplatin and/or DNA-PKcs inhibitor, and levels of apoptosis and viability were then measured. Cisplatin chemotherapy-induced signalling changes were investigated in primary tumour cells using reverse phase protein array (RPPA) proteomics. Phylogenetic trees were constructed, and clonal expansion indices (CEI) were quantified using the Minimum Event Distance for Intra-tumour Copy-number Comparisons (MEDICC) algorithm.

Data from phenotypic apoptosis assays demonstrated spatial and temporal heterogeneity in response to cisplatin across different disseminated tumours. There was a trend of higher variability in response to cisplatin showing a better progression-free survival in our cohort. We observed that the combination of DNA-PKcs inhibitor and cisplatin enhanced apoptosis in

primary ovarian tumour cells. Our results also demonstrated vast proteomic heterogeneity in our cohort by examining platinum-induced protein signaling changes in our samples. Linear regression analyses of proteomic data showed significant alterations of HSP27, p38, and Chk1 proteins amongst others following cisplatin treatment. Pathway enrichment analysis showed the PI3K-AKT signaling pathway as a significantly enriched pathway induced by cisplatin. eEF2K and PR were identified as potential biomarkers of prognosis. Inhibition of Chk1 using prexasertib was demonstrated to enhance and restore platinum sensitivity in combination with cisplatin treatment in platinum sensitive and resistant HGSOC cells. Furthermore, CNV data analysis revealed vast heterogeneity across our sample cohort, with a higher clonal expansion index showing association with worse survival outcomes.

Taken together, the results in this thesis illustrates clearly the inter- and intratumour heterogeneity that exists in HGSOC. While it has been demonstrated before at the CNV level, this is the first time showing phenotypic heterogeneity and to some extent proteomic heterogeneity in HGSOC. Our research supports further investigation into the prognostic biomarker selection and clinical development of DNA-PK and Chk1 inhibitors in combination with cisplatin to overcome platinum resistance in ovarian cancer. The association of the clonal expansion index with survival outcomes warrants further investigation for CEI as a predictive factor of platinum-response in HGSOC.

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List of Abbreviations

ABC - ATP-binding cassette

ABCA1 - ATP binding cassette subfamily A member 1

ABCA6 - ATP binding cassette subfamily A member 6

ABCA8 - ATP binding cassette subfamily A member 8

ABCA9 - ATP binding cassette subfamily A member 9

ABCB1 - ATP binding cassette subfamily B member 1

ABCC1 - ATP binding cassette subfamily C member 1

ABCC2 - ATP binding cassette subfamily C member 2

ABCC4 - ATP binding cassette subfamily C member 4

ACT - adoptive cell therapy

ACTA2 - Actin Alpha 2

AGO OVAR - Arbeitsgemeinschaft Gynäkologische Onkologie Studiengruppe Ovarialkarzinom

AML - acute myeloid leukaemia

ANGPTL1 - angiopoietin like 1

ANGPTL2 - angiopoietin like 2

APE1- apurinic/apyrimidinic endonuclease 1

AR - androgen receptor

ASCAT - Allele-Specific Copy Number Analysis of Tumours

ATM - ataxia telangiectasia mutated

ATP7A - ATPase copper transporting alpha

ATP7B - ATPase copper transporting beta

ATR - ataxia-telangiectasia and Rad3-related

AUC - area under the curve

AURELIA - Avastin Use in Platinum-Resistant Epithelial Ovarian Cancer

BAF - B-allelic frequency

BCL2 - B-Cell Lymphoma 2

BER - base excision repair

BSA - Bovine serum albumin

BRAF - Serine/threonine-protein kinase B-Raf

BRCA1 - breast cancer associated 1 gene

BRCA2 - breast cancer associated 2 gene

CA125 - cancer antigen 125

CAF - cancer-associated fibroblast
CAR - chimeric antigen receptor
CCNE1 - cyclin E1
CCND1 - cyclin D1
CD8A - cluster differentiation 8A
CD31 - cluster differentiation 31
CD90 - cluster differentiation 90
CDK2 - cyclin-dependent kinase 2
CEI - clonal expansion index
CGP - Cancer Genome Project
Chk1 - Checkpoint kinase 1
CIC - cortical inclusion cyst
CK7 - cytokeratin 7
CPTAC - Clinical Proteomic Tumour Analysis Consortium
CNA - copy number alterations
CNV - Copy number variation
CSA - Cockayne syndrome proteins A
CSB - Cockayne syndrome proteins B
CSC - cancer stem cell
CT - computerised tomography
CTLA-4 - cytotoxic T-lymphocyte-associated antigen 4
CTNNB1 - Catenin Beta 1
CTR1 - copper transporter 1
CTR2 - copper transporter 2
CTX - chemotherapy
CV - coefficient of variation
CXCL9 - C-X-C Motif Chemokine Ligand 9
DAB - 3,3'-diaminobenzidine
DDR - DNA Damage Repair
DMSO - dimethyl sulfoxide
DSB - double-strand break
EGFR - epidermal growth factor receptor
EOC - epithelial ovarian cancer
EMT - epithelial-to-mesenchymal transition

EpCAM - Epithelial cell adhesion molecule
ER - estrogen receptor
ERCC1 - excision repair cross-complementation group 1
ERK - extracellular signal-related kinase
EORTC - European Organisation for Research and Treatment of Cancer
EVII - ecotropic virus integration site 1
EXO1 - Exonuclease 1
FAK - Focal adhesion kinase
FAP - Fibroblast activation protein
FasL - Fas ligand
FBI - foldback inversion
FDA - Food and Drug Administration
FDR - false discovery rate
FEN1- flap endonuclease 1
FIGO - International Federation of Gynaecology and Obstetrics
FDG - fluorodeoxyglucose
FSH - follicle stimulating hormone
Gadd45a - Growth Arrest and DNA Damage Inducible Alpha
GCIG - Gynaecologic Cancer InterGroup
GF-AFC - glycyl-phenylalanyl-aminofluorocoumarin
GGR - global genomic repair
GOG - Gynaecologic Oncology Group
GST - glutathione S-transferase
HE4 - human epididymis secretory protein 4
HGS - high grade serous
HGSOC - High-grade serous ovarian cancer
Hif - Hypoxia-induced factor
HMGA2 - high mobility group AT-hook 2
HNPCC - hereditary nonpolyposis colorectal cancer
HR - homologous recombination
HRD - homologous recombination repair deficiency
HRP - horseradish peroxidase
HRT - hormone replacement therapy
ICGC - the International Cancer Genomics Consortium

ICHTB - Imperial College Healthcare Tissue Bank
 ICON7 - International Collaborative Group for Ovarian Neoplasm-7
 IDS - interval debulking surgery
 IGF1R - insulin like growth factor 1 receptor
 IL-6 - Interleukin 6
 IL-10 - Interleukin 10
 IMO - Intraoperative Mapping of Ovarian Cancer
 IQR - interquartile range
 ITH - Intra-tumour heterogeneity
 JNK - c-Jun N-terminal kinase
 KRAS - Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
 LGS - low grade serous
 LH - luteinizing hormone
 LOH - loss-of-heterozygosity
 LRR - Log R ratio
 MAPK - mitogen-activated protein kinase
 MCM2 - minichromosome maintenance protein 2
 MDR1 - multidrug-resistant protein 1
 MDS - multidimensional scaling
 MDS1 - myelodysplasia syndrome-associated protein 1
 MECOM - MDS1 And EVI1 Complex Locus
 MEDICC - Minimum Event Distance for Intra-tumour Copy-number Comparisons
 MIG - Monokine induced by gamma interferon
 MLH1 - MutL Homolog 1
 MSH2 - MutS Homolog 2
 MSH3 - MutS Homolog 3
 MSH6 - MutS Homolog 6
 MMR - mismatch repair
 MOFA - Multi-Omics Factor Analysis
 MSC - Mesenchymal Stem Cell
 MSI - create microsatellite instability
 mTOR - Mammalian target of rapamycin
 MUC1 - Mucin 1
 MUC16 - Mucin 16

NAC - neoadjuvant chemotherapy

NBS1 - Nijmegen Breakage Syndrome 1

NER - nucleotide excision repair

NF1 - Neurofibromin 1

NHEJ - non-homologous end joining

NRES - National Research Ethics Service

OCEANS - Ovarian Cancer Study Comparing Efficacy and Safety of Chemotherapy and Anti-Angiogenic Therapy in Platinum-Sensitive Recurrent Disease

OCSI - ovarian cancer symptom index

ORR - an overall response rate

OS - overall survival

OSE - ovarian surface epithelium

PAR - poly(ADP-ribose)

PARP - Poly(ADP-Ribose) polymerase

PCA - principal component analysis

PLCO - Prostate, Lung, Colorectal and Ovarian

PCNA - proliferating cell nuclear antigen

PD-1 - programmed death 1

PD-L1 - programmed death ligand 1

PECAM1 - Platelet and endothelial cell adhesion molecule 1

PET - positron emission topography

PFS - progression-free survival

PI3K - Phosphoinositide 3-kinase

PIK3CA - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PIKK - PI3K-related protein kinase

PMS1 - PMS1 Homolog 1

PMS2 - PMS1 Homolog 2

PTEN - Phosphatase and tensin homolog

QC - quality control

RAD23B - RAD23 homolog B

RB1 - Retinoblastoma transcriptional corepressor 1

RECIST - response evaluation criteria in solid tumours

RMI - Risk of Malignancy Index

ROCKeTS - Refining Ovarian Cancer Test Accuracy Scores

ROCA - Risk of Ovarian Cancer Algorithm
ROMA - Risk of Ovarian Malignancy Algorithm
ROS - reactive oxygen species
RPA - replication protein A
RPPA - reverse phase protein array
RTK - receptor tyrosine kinase
SEE-FIM - Sectioning and Extensively Examining the FIMbria
SDS - sodium dodecyl sulphate
SLC25A40 - solute carrier family 25 member 40
SNP - single nucleotide polymorphism
SOX2 - sex determining region Y-box 2
SOX11 - sex determining region Y-Box 11
SSB - single-strand break
STAT3 - signal transducer and activator of transcription 3
STIC - serous tubal intraepithelial carcinoma
TAM - tumour-associated macrophage
TBS - Tris Buffered Saline
TCGA - the Cancer Genome Atlas Project
TCR - transcription-coupled repair
TFIIH - transcription Factor II H
TIL - tumour-infiltrating lymphocyte
TVS - transvaginal ultrasound
UKFOCCS - UK Familial Ovarian Cancer Screening Study
UKCTOCS - UK Collaborative Trial of Ovarian Cancer Screening
VEGF - vascular endothelial growth factor
WHO - World Health Organisation
WT1 - Wilms tumour 1
YB1 - Y-box binding protein 1
XLF - XRCC4-like factor
XPA - Xeroderma pigmentosum complementation group A
XPB - Xeroderma pigmentosum complementation group B
XPC - Xeroderma pigmentosum group C-complementing protein
XPD - Xeroderma pigmentosum complementation group D
XPF - xeroderma pigmentosum complementation group F

XPG - xeroderma pigmentosum complementation group G

XRCC1 - X-ray repair cross-complementing protein 1

XRCC4 - X-ray repair cross-complementing protein 4

Chapter 1

Introduction

1.1 Ovarian Cancer

1.1.1 Epidemiology

According to the Globocan 2012 database, ovarian cancer is the seventh most common cancer in women worldwide. The estimated global incidence of this cancer in 2012 was approximately 240,000 cases, and it contributed to 152,000 deaths (Ferlay *et al.*, 2015). In the UK, ovarian cancer is both the sixth most common malignancy and cause of female malignancy-related death. Around 20 new cases are diagnosed every day, with approximately 7,270 new cases were diagnosed in 2015 (Cancer Research UK, 2018). Ovarian cancer remains one of the most highly lethal diseases among all gynaecological malignancies despite advancements in therapy. More than 70% of patients are diagnosed at a late stage due to the absence of specific signs and symptoms and the lack of an effective early detection strategy. At stages III and IV, the five year survival rates are 35% and 20%, respectively (Chornokur *et al.*, 2013). Conversely, if the diagnosis is made while the disease is still localised to the ovaries, 84% of patients can be treated successfully using the currently available treatments (Chan *et al.*, 2008).

Risk factors identified for the development of ovarian cancer are early age of menarche, late age of menopause, infertility, nulliparity, having never breastfeed, obesity, the use of hormone replacement therapy (HRT) and lack of oral contraceptive use (Sueblinvong and Carney, 2009). A family history of ovarian and breast cancer, particularly in first-degree relatives, also increases the risk of developing ovarian cancer. Around 10-15% of ovarian cancer patients have inherited mutations in carcinoma predisposing genes, including breast cancer associated 1 (*BRCA1*) and breast cancer associated 2 (*BRCA2*) genes (Osman, 2014). The lifetime risk of suffering ovarian cancer in *BRCA1* mutation carriers lies between 30-44%, where the lifetime risk for *BRCA2* carriers are 27% (Hunn and Rodriguez, 2012). Also, mutations in genes related to hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome predispose to

cancer, namely MutL Homolog 1 (*MLH1*), MutS Homolog 2 (*MSH2*), MutS Homolog 3 (*MSH3*), MutS Homolog 6 (*MSH6*), PMS1 Homolog 1 (*PMS1*) and PMS1 Homolog 2 (*PMS2*) genes (Nakamura *et al.*, 2014). Ovarian cancers in Lynch syndrome are mostly endometrioid or clear cell and diagnosed in stage I or II. In terms of prognosis, the survival rates of ovarian cancer in Lynch syndrome were better from those of sporadic ovarian cancers (Toss *et al.*, 2015).

1.1.2 Classification, Pathogenesis and Molecular Pathology of Ovarian Cancer

The normal ovary derives from the gonadal ridge and contains three main cell types: germ cells which originates from the endoderm and migrate to the gonadal ridge where they differentiate into oocytes; endocrine and interstitial cells which produce oestrogen and progesterone; and epithelial cells, a flat-to-cuboidal layer of mesothelial cells covering the exterior surface of the ovary (Romero and Bast, 2012). Ovarian tumours can arise from each of the three ovarian cell types. Germ cell tumours arise most frequently in the age group of 20 and 30 years and account for 2-3% of ovarian malignancies (Brown *et al.*, 2014). Sex-cord-stromal tumours arise from the ovarian somatic cells, particularly granulosa cells and theca cells, comprising approximately 7% of all ovarian cancer (Horta and Cunha, 2015). Epithelial ovarian cancers (EOC) generally develop in perimenopausal age and accounts for 85-90% of ovarian cancers.

Histologically, the World Health Organisation (WHO) classifies EOC into: serous tumour, mucinous tumour, endometrioid tumour, clear cell carcinoma, Brenner tumour, seromucinous tumour and undifferentiated carcinoma (Figure 1.1) (Meinhold-Heerlein *et al.*, 2016). These subtypes vary greatly, with different site of origin, pathologies, responses to therapy, prognoses and likely different molecular drivers. A new classification system for EOC has recently been proposed based on pathogenetic mechanism, which classifies EOC into: Type I and Type II

(Kurman and Shih, 2010). Type I consists of low grade serous (LGS), endometrioid, clear cell, and mucinous tumours with Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), Serine/threonine-protein kinase B-Raf (*BRAF*), Catenin Beta 1 (*CTNNB1*), Phosphatase and tensin homolog (*PTEN*) or Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) mutations. This type usually develops from well-described, borderline tumours and genetically stable precursor lesions. In contrast, Type II consists of high grade serous (HGS) and endometrioid tumours and undifferentiated carcinoma which usually have 97% *TP53* (also called *P53*) (Ahmed *et al.*, 2010) mutations and BRCA1/2 dysfunction. The activation of wild type p53 promotes tumour suppression function, including senescence, cell cycle inhibition, DNA repair and autophagy and apoptosis (Cole *et al.*, 2016). Mutant p53 leads to loss of these tumour suppressive actions. Both BRCA1 and BRCA2, work in common pathway of genome protection with its specific role in different stage. BRCA1 plays role in both checkpoint activation and DNA repair, whereas BRCA2 is a mediator of homologous recombination (Roy, Chun and Powell, 2011). Type II tumours usually emerges *de novo* from adnexal epithelium and shows aggressive behaviour. Type I tumours generally lack *TP53* mutations and are not sensitive to platinum-based therapy, whereas Type II tumours usually present at an advanced stage and are initially sensitive to chemotherapy.

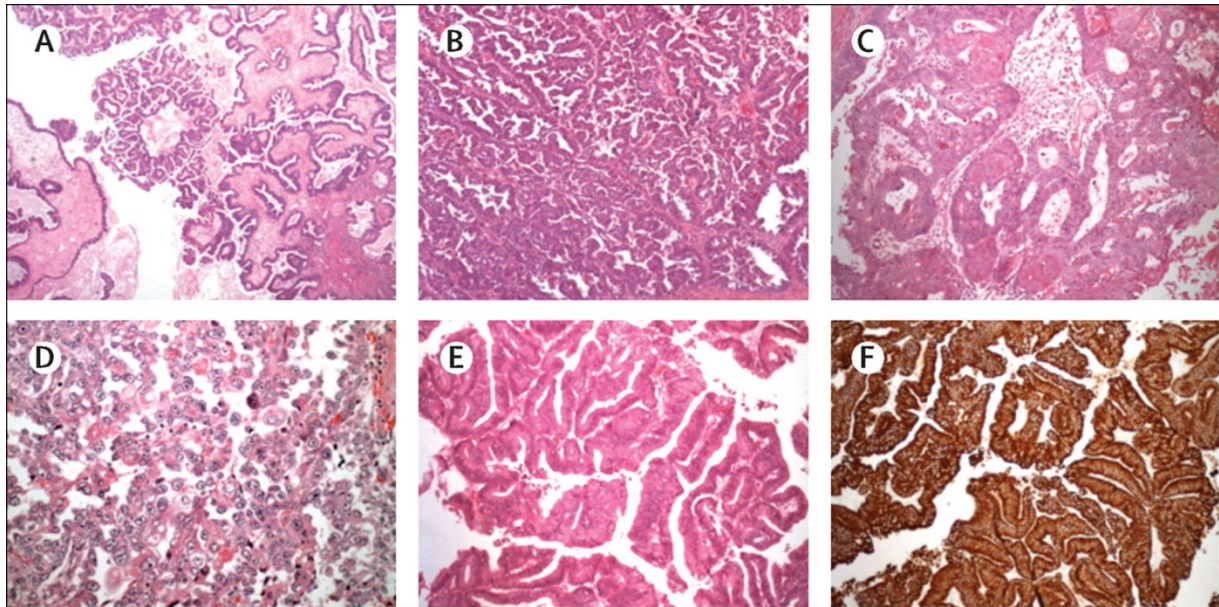


Figure 1. 1: Histology of the different subtypes of epithelial ovarian cancer. A) Borderline tumour: serous epithelium with progressive branching, without high-grade atypia or stromal invasion. B) Typical HGSOC with a papillary pattern and high nuclear grade. C) Endometrioid carcinoma: foci of squamous metaplasia, the glands are crowded and fused. D) Clear cell carcinoma of the ovary: papillary pattern. E) Mucinous carcinoma: well-differentiated with a labyrinthine pattern. F) Mucinous carcinoma: cytokeratin 7 (CK7) – diffuse staining of tumour cells. Taken from Jayson *et al.*, 2014., with permission from Elsevier.

The most common EOC is a serous subtype and the focus of the present study is high grade serous ovarian cancer subtype (HGSOC). HGSOC is the most lethal subtype, contributing to more than 70% of ovarian cancer-related deaths (Bowtell *et al.*, 2015). Due to its intra-abdominal location, the cell of origin as well as the early molecular and genetic events associated with ovarian carcinogenesis remains unclear. The conventional theory describes that HGSOC arise from ovarian surface epithelium (OSE) under incessant ovulation model (Fathalla, 1971). During ovulation, follicular rupture and oocyte release cause physical trauma upon the ovarian surface, creating a damage and subsequent repair of the OSE. OSE cells are also exposed to inflammatory mediators induced by ovulation which promotes extensive tissue remodelling and increases the risk of mutations (Fleming *et al.*, 2006). In addition, the incidence of TP53 mutations associates with the number of lifetime ovulations (Hakim *et al.*, 2009); thus, it may lead to cumulative errors in DNA repair and increase ovarian cancer

susceptibility. Furthermore, the ovarian surface develops age-related surface invaginations into the cortical stroma, forming circular OSE-lined structures namely ‘cortical inclusion cysts’ (CICs) (Fleszar *et al.*, 2018). Once inside the ovary, the epithelial cells lining CICs are exposed to the rich hormonal environment which able to trigger metaplastic changes within the coelomic epithelium, causing a change in its differentiation lineage from mesothelium to tissue type similar to those lining the fimbriae of Fallopian tube (Dubeau and Drapkin, 2013). This epithelial type has a common embryological origin from the Müllerian ducts; hence, the term Müllerian metaplasia often used to designate these changes. Moreover, OSE has a phenotypic plasticity features which demonstrated by its reactivity with antibodies to both epithelial (cytokeratin, laminin and collagen IV) and mesothelial (vimentin, collagen I and III) antigens. Also, OSE cells can be induced to shift between epithelial and mesenchymal morphology *in vitro* (Mehra *et al.*, 2011). Several hormones acting upon the ovary (follicle stimulating hormone (FSH), luteinizing hormone (LH), oestrogens, and androgens) have growth-promoting properties that may increase cell division as well as mutations of the epithelial cells within CICs (Rossing *et al.*, 1994; Lukanova and Kaaks, 2005). This model is also consistent with epidemiologic data suggesting that a decrease in ovulatory cycles is the greatest risk-reducing factor for ovarian cancer (Permuth-Wey and Sellers, 2009). However, the OSE-CIC hypothesis fails to account for the fact that a precursor lesion to ovarian cancer could not be clearly detected on the OSE despite extensive histopathological investigations.

Another hypothesis proposes that HGSOC arises from cells originating in the Fallopian tube. This insight came from studies of women with germline *BRCA1/2* mutations, which increase the risk of several malignancies, particularly breast, ovarian, peritoneal and Fallopian tube malignancies (Finch *et al.*, 2014). Thus, the standard risk-reducing prophylactic procedure has become removal of the ovaries and Fallopian tubes (bilateral salpingo-oophorectomies)

(Walker *et al.*, 2015). This procedure enables pathologists to examine ovarian and Fallopian tube tissues for occult cancer. An early observation suggesting the Fallopian tube as the origin for HGSOC came from a study of 12 high-risk women including *BRCA1* carriers which found that 50% of these women displayed dysplasia in the epithelium of their Fallopian tubes (Piek *et al.*, 2001). In 2006, Medeiros *et al.* employed a specific protocol for Sectioning and Extensively Examining the FIMbria (SEE-FIM) to systematically evaluate the Fallopian tubes of *BRCA* mutation carriers. This study identified early serous carcinomas in fimbria, the distal end of the Fallopian tube termed serous tubal intraepithelial carcinoma (STIC) (Medeiros *et al.*, 2006). The STIC lesions were proliferative, indicated by Ki67 (a proliferative marker) immunohistochemistry (IHC) staining and also stained strongly for p53. Other studies confirmed that STICs were also found in 50% - 60% of women with sporadic ovarian cancer (Kindelberger *et al.*, 2007; Przybycin *et al.*, 2010). Further support of this hypothesis is the finding that there are short stretches (7 – 10 cells) of benign-appearing secretory cells in Fallopian tube that have p53 signatures which refers to intense nuclear p53 staining, positive γ -H2AX staining (a marker of DNA damage), and lack of Ki-67 staining (Lee *et al.*, 2007). These patches harboured *TP53* mutations but were not proliferative. As p53 signatures lack histological features of STIC, p53 signatures are considered earlier lesions preceding STICs. Importantly, the p53 signature and the STIC lesion harbour the same *TP53* mutation identical to HGSOC, which indicates a clonal relationship between them (Figure 1.2).

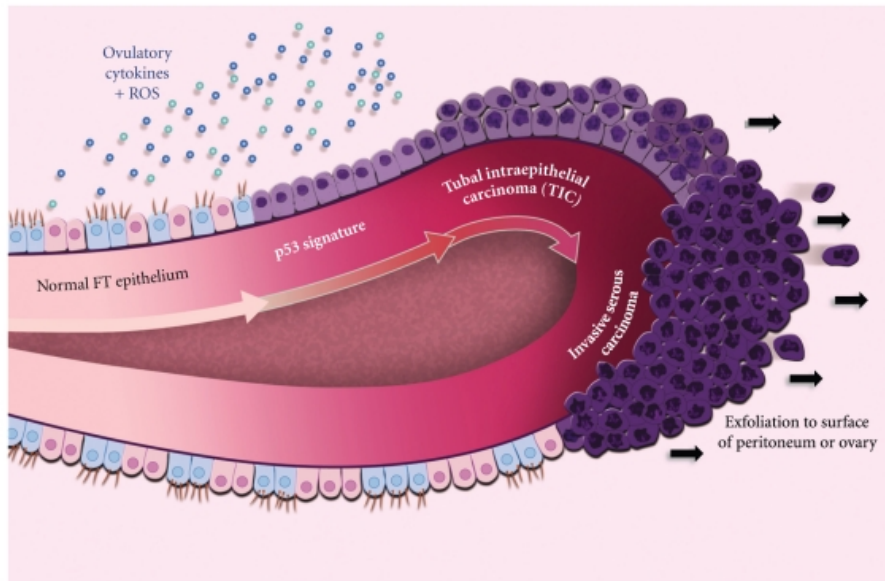


Figure 1. 2: Pathogenesis of HGSOC. This process involves the fimbria of the Fallopian tube (FT), in which a combination of genotoxic stress and TP53 mutation result in the clonal expansion of secretory epithelial cells, forming a pre-neoplastic precursor lesion termed p53 signature. The acquisition of nuclear pleomorphism, mitoses, and loss of polarity enable these cells to acquire a proliferative capacity, facilitating progression to STIC. STICs are able to spread rapidly, moving from the fimbria to adjacent pelvic structures or exfoliating into the peritoneal cavity. Taken from Karst and Drapkin, 2010., under Creative Commons License.

Molecularly, HGSOC is classified as a C-class (chromosomally unstable) malignancy (Ciriello *et al.*, 2013). This classification is characterised by a high level of genomic instability, despite the low frequency of focal point mutations. *TP53* mutations are present in almost 100% of HGSOC patients (Ahmed *et al.*, 2010). Approximately 50% of HGSOCs are defective in the homologous recombination (HR) DNA repair pathway, which arises mainly from germline, somatic mutations and epigenetic modifications in the *BRCA1* and *BRCA2* genes, and a small proportion of other mutations such as Retinoblastoma transcriptional corepressor 1 (*RBI*) (2%), Neurofibromin 1 (*NFI*) (4%) mutations and *PTEN* deletion (8%) (Cancer Genome Atlas Research Network, 2011; Konstantinopoulos *et al.*, 2015; Mittempergher, 2016). Approximately 30% of those that do not exhibit defects in the HR repair pathway, have amplification in *CCNE1*. *CCNE1* encodes Cyclin E1, a protein which drives the shift from G1

to S phase by binding and activating cyclin-dependent kinase 2 (CDK2) and subsequently promoting DNA synthesis (Hwang and Clurman, 2005).

HGSOC is also classified into four molecular subtypes which have been identified and validated by gene expression profiling: mesenchymal, immunoreactive, differentiated and proliferative (Tothill *et al.*, 2008; Cancer Genome Atlas Research Network, 2011). These classifications are related to the different clinical outcomes, distinct biological and microenvironment features. The mesenchymal subtype is characterised by increased stromal components, including microvascular myofibroblast (Actin Alpha 2 (*ACTA2*), Fibroblast activation protein (*FAP*)), vascular endothelial cells (Platelet and endothelial cell adhesion molecule 1 (*PECAMI*) or also known as cluster differentiation 31 (CD31) antigen), and microvascular pericytes (angiopoietin like 2 (*ANGPTL2*), angiopoietin like 1 (*ANGPTL1*)). Enrichment of pathways and gene ontology groups defining extracellular matrix production and remodelling, cell adhesion, cell signalling, and angiogenesis are also present in this subtype. It is associated with primary treatment failure and poor survival. Observation from previous studies reveal that the mesenchymal features in this subtype is derived from the tumour cells itself and the surrounding stromal cells, suggesting its important role in HGSOC behaviour (Zhang, Wang and Cliby, 2019).

The immunoreactive subtype tumours generally have a more favourable prognosis and are characterised by overexpression of genes involved in T-cell activation (such as cluster differentiation 8A (CD8A) and Granzyme B) and T-cell trafficking (C-X-C Motif Chemokine Ligand 9 (CXCL9)/Monokine induced by gamma interferon (MIG)). The differentiated subtype is characterised by a high expression of ovarian tumour markers such as Mucin 16 (MUC16) or known as cancer antigen 125 (CA125) and Mucin 1 (MUC1). Finally, the

proliferative subtype is defined by overexpression of transcription factors such as high mobility group AT-hook 2 (HMGA2) and SRY-Box 11 (SOX11) and proliferation markers such as minichromosome maintenance protein 2 (MCM2) and proliferating cell nuclear antigen (PCNA), and low expression of CA125. Similar to the mesenchymal subtype, the proliferative subtype is associated with poor outcomes.

1.1.3 Diagnosis, Screening and Staging

Ovarian cancer has been termed the ‘silent killer’ due to its lack of specific signs and symptoms. However, a study reported that more than 80% of patients are symptomatic, even when the disease is still in the early stages (Goff *et al.*, 2000). Most of these symptoms resemble gastrointestinal, genitourinary, and other gynaecological problems, leading to incorrect initial diagnoses. The Gynaecologic Cancer Foundation, the Society of Gynaecologic Oncologists and the American Cancer Society released a consensus statement to guide the diagnosis of ovarian cancer called the Ovarian Cancer Symptom Index (OCSI), which consists of four categories including pelvic or abdominal pain, difficulty eating or feeling full, urinary frequency or urgency, and increased abdominal size or bloating (Goff *et al.*, 2007; McLemore *et al.*, 2009). The presence of any symptom within 12 months and occurring for more than 12 days per month indicates a positive result. The sensitivity and specificity of this index in women younger than 50 years old are 86.7% and 86.7%, respectively. In women older than 50 years, the sensitivity and specificity are 66.7% and 90%, respectively (Goff *et al.*, 2007). Currently, a prospective phase III trial namely Refining Ovarian Cancer Test Accuracy Scores (ROCKeTS) study, aiming to validate risk prediction models which estimate the probability of having ovarian cancer in women with symptoms is ongoing (Sundar *et al.*, 2016). This study also aims to identify threshold of best models that inform decision to guide patient management.

Diagnosis of ovarian cancer was made based on symptoms and clinical assessment, biomarker test (particularly CA125, a large transmembrane glycoprotein also known as mucin 16), family history, imaging assessment and laparoscopy. The National Institute for Health and Care Excellence in the UK and the US based National Comprehensive Cancer Network guidelines recommend sequential testing using serum CA125 followed by pelvic ultrasound scan in women presenting to the GP with ovarian cancer symptoms if the serum CA125 level is 35 IU/L or more (Sundar, Neal and Kehoe, 2015). However, some benign conditions such as endometriosis, uterine myoma, pelvic inflammatory disease and pregnancy also cause an elevated level of CA125 biomarker (Medeiros *et al.*, 2009). As a result, CA125 cannot be used as a single marker for diagnosing ovarian cancer. Currently, human epididymis secretory protein 4 (HE4) is the only biomarker apart from CA125 which has been approved by Food and Drug Administration (FDA) as a diagnostic marker for ovarian cancer (Leung *et al.*, 2016). HE4, a member of the Wey acidic protein gene family is mainly expressed in the reproductive and respiratory tracts and is overexpressed in ovarian cancer cells, especially in serous or endometrioid cancers (Hamed *et al.*, 2013). An algorithm called Risk of Ovarian Malignancy Algorithm (ROMA) which incorporates CA125 and HE4 concentration in serum has showed a significant improvement of sensitivity and specificity in identifying ovarian cancer patients (Chudecka-Głaz, 2015). In this algorithm, the CA125 cut-off value for ovarian cancer diagnosis is 35 U/ml, while the HE4 cut-off values are > 70 pmol/l (premenopausal women) and > 140 pmol/l (postmenopausal women).

Despite the presence of this diagnosis tool, the diagnosis of ovarian cancer has not shifted into an earlier stage. Thus, an effective screening strategy is necessary to improve the outcome of ovarian cancer. The US Prostate, Lung, Colorectal and Ovarian (PLCO) study showed that

screening with a single threshold value of CA125 and transvaginal ultrasound (TVS, a type of pelvic ultrasound via the vagina used to examine female reproductive organs) did not result in reduction of mortality. Moreover, diagnostic evaluation following false-positive results was associated with complications in 15% patients (Buys *et al.*, 2011). The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), which evaluated the benefit of annual CA125 measurement and TVS in 202,638 postmenopausal women demonstrated no significant decrease in mortality in screened group compared to the unscreened group (Jacobs *et al.*, 2016). However, a subset analysis excluding prevalent cases found 20% reduction in deaths after seven years of follow-up. This study also found that screening using longitudinal CA125 measurements interpreted via Risk of Ovarian Cancer Algorithm (ROCA) doubles the number of screen-detected EOC compared with testing using single thresholds of CA125 (Menon *et al.*, 2015). A sister trial of UKCTOCS, The UK Familial Ovarian Cancer Screening Study (UKFOCCS) evaluated CA125 measurement using ROCA based screening and TVS in women considered at least 10% risk of developing ovarian/Fallopian tube cancer. A change in stage at diagnosis has been evidenced as a result of this study, but a significant mortality benefit is still being investigated (Rosenthal *et al.*, 2017). The ROCA based screening could be an option for women at high risk of ovarian cancer who do not wish to undergo risk reducing salpingo-oophorectomy, given its high sensitivity. Recent study has offered a potential alternative of screening strategy in women with *BRCA1/2* mutation via detection of sex determining region Y-box 2 (SOX2) overexpression in Fallopian tube (Hellner *et al.*, 2016). This study revealed that SOX2 expression is increased in Fallopian tube of *BRCA1/BRCA2* carriers and in HGSOC, irrespective of tumour stage. However, safe screening methods to detect SOX2 overexpression in clinical setting has yet to be developed.

Staging of ovarian cancer is based on operative findings during surgery, typically laparotomy. Ovarian cancer usually spreads trans-peritoneally through the abdominal fluid; however, the lymphatic route is also often seen. Thus, careful assessment of micrometastasis in the abdominal cavity including the paracolic gutters, omentum and diaphragm is mandatory. Ascites and peritoneal washings are also cytologically assessed to determine the presence of malignant cells. Clinical staging is very important for treatment planning and to predict the prognosis. According to the International Federation of Gynaecology and Obstetrics (FIGO), ovarian cancer is divided into four different stages as follows (Meinhold-Heerlein *et al.*, 2015):

Table 1. 1 The staging of ovarian cancer according to FIGO

<i>FIGO stage</i>	<i>Description</i>
<i>Stage I</i>	Tumour limited to the ovaries or tube(s) ^a
<i>IA</i>	One ovary or tube is involved, capsule intact and no tumour on the external surface, no ascites present with malignant cells
<i>IB</i>	Growth limited to both ovaries or tubes, capsule intact and no tumour on the ovarian or tubal surface, no ascites present with malignant cells
<i>IC</i>	Tumour in one or both ovaries or tube(s) with one of the following: IC1: ruptured capsule intraoperatively IC2: ruptured capsule preoperatively or tumour on ovarian or tubal surface IC3: ascites or peritoneal washing present containing malignant cells
<i>Stage II</i>	Tumour involving one of both ovaries or tube(s) with extension to the pelvic cavity or primary peritoneal carcinoma ^b
<i>IIA</i>	Metastases to uterus and/or tubes and/or ovary/ovaries
<i>IIB</i>	Extension to other pelvic intraperitoneal tissue
<i>Stage III</i>	Tumour in the ovary/ovaries or tube(s) or primary peritoneal carcinoma with cytologically peritoneal metastases outside the pelvis and/or retroperitoneal lymph node metastases which are histologically confirmed
<i>IIIA1</i>	Positive retroperitoneal lymph nodes only which are cytologically or histologically confirmed IIIA1(i): metastases, up to diameter of 10 mm IIIA(ii): metastases, with diameter more than 10 mm
<i>IIIA2</i>	Microscopic extra pelvic peritoneal involvement with or without positive retroperitoneal lymph nodes.
<i>IIIB</i>	Macroscopic peritoneal metastasis up to 2 cm in diameter with or without retroperitoneal lymph node involvement (including the capsule of the liver/spleen but without parenchymatous involvement)
<i>IIIC</i>	Macroscopic extrapelvic peritoneal metastasis more than 2 cm in diameter with or without extension to retroperitoneal lymph node (including capsule of the liver/spleen but without parenchymatous involvement)
<i>Stage IV</i>	Distant metastases beyond the peritoneal area
<i>IVA</i>	Cytologically proven pleural effusion
<i>IVB</i>	Parenchymatous metastases and metastases to extraabdominal organs (including inguinal lymph node metastases and extraabdominal lymph node metastases) ^c

^a There is no stage I peritoneal cancer

^b Dense adhesions with histologically confirmed tumour metastases justify upstaging from stage I to II

^c Extraabdominal metastases include transmural bowel infiltration and umbilical metastases.

1.1.4 Ovarian Cancer Treatments

Surgery plays an essential role in the management of ovarian cancer, both in accurate staging and in treatment to resect the tumour. It provides information about the prognosis and affects the clinical decision making on whether chemotherapy is necessary. During surgery, a frozen section is collected to identify malignant tumour so that the necessary surgical staging can be completed on the first attempt. Optimal surgical cytoreduction of the tumour deposit or ‘optimal debulking surgery’ is preferable to minimise the risk of relapse. In early ovarian cancer (stage I-IIA), adequate surgery consists of peritoneal washing, hysterectomy, bilateral salpingo-oophorectomy, multiple biopsy sampling of all abdominal fields, appendectomy (for mucinous tumour), at least infracolic omentectomy, pelvic and para-aortic lymphadenectomy up to renal vessel (Ledermann *et al.*, 2013). Fertility-sparing surgery could be an option for young patients with ovarian cancer; however, patients must be informed about the risk of relapse and completion of surgery should be performed after childbearing.

In advanced ovarian cancer (stage IIB-IV), the goal of primary surgery is complete cytoreduction (complete debulking), defined as having no visible residual disease at the end of surgery. The Gynaecologic Oncology Group (GOG) defines optimal cytoreduction as a residual disease with a maximum residual tumour diameter of 1 cm or less, and suboptimal cytoreduction as when the residual tumour is larger than 1 cm (Seward and Winer, 2015). Complete cytoreduction includes peritoneal and diaphragmatic stripping, para-aortic lymphadenectomy, intestinal resection, and splenectomy when necessary. Studies have shown that this procedure significantly improves overall survival (OS) and progression-free survival (PFS) (du Bois *et al.*, 2009; Polterauer *et al.*, 2012).

When patients present with a bulky disease and a radical procedure to achieve optimal tumour removal is needed, neoadjuvant chemotherapy (NAC), followed by interval debulking surgery (IDS) to minimise the morbidity are considered. IDS is usually performed after 3 cycles of chemotherapy; however, this approach is still debatable. The European Organisation for Research and Treatment of Cancer (EORTC) Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Cancer trial and Primary Chemotherapy Versus Primary Surgery for Newly Diagnosed Advanced Ovarian Cancer (CHORUS) trial showed lower surgical morbidity and comparable mortality scores in patients with NAC-IDS, compared with primary debulking surgery (Vergote *et al.*, 2010; Kehoe *et al.*, 2015). These findings led to the broad implementation of NAC in HGSOc. There are indications of selection bias in these studies, in which many patients believed to be inoperable were recruited into both trials (Fotopoulou *et al.*, 2017). Thus, whether these results can be implemented in patients with good performance status in specialised, high-volume centre remains unclear. The ability to classify the patients who are at high risk of not being optimally debulked is still controversial. In addition, the capability of the surgeon and availability of adequate facilities must be taken into consideration. Moreover, defining patient criteria to predict recovery from the extensive surgery remains difficult to measure. The Trial on Radical Upfront Surgery in Advanced Ovarian Cancer (TRUST) and Arbeitsgemeinschaft Gynäkologische Onkologie Studiengruppe Ovarialkarzinom (AGO OVAR) OP 7 are investigating these issues and address the optimal time point of surgery, which hopefully provide a guideline for more individualised surgical approach for HGSOc patients (Mahner *et al.*, 2017).

Currently, treatments for ovarian cancer consist of debulking surgery followed by systemic therapy, including chemotherapy and targeted therapy, especially for patients with FIGO stage II-IV. The combination of platinum (carboplatin) and a taxane (paclitaxel) is considered as the

first line of chemotherapy for ovarian cancer. Platinum binds to N7 position of purines, leading to the formation of DNA adducts that causes DNA damage, causing the activation of several signal transduction pathways including DNA repair systems and thus, when effective, inducing the apoptotic cascade (Kelland, 2007). Whereas taxane binds to the beta-tubulin of microtubules, preventing microtubules depolymerisation, leading to cell cycle arrest during M phase and finally, to apoptosis (Perez, 2009). The concurrent use of taxane with platinum in ovarian cancer is supported by several theoretical arguments: the dual drugs approach may help to lower the doses, and thus lower the toxicity of each of the single drug; a greater apoptosis might be achieved at the start of the treatment, which may mean more patients becoming tumour-free at induction chemotherapy; the addition of taxane may reduce the possibility of development of resistant clones (Kumar *et al.*, 2010). The Gynaecologic Cancer InterGroup (GCIG) recommended the use of paclitaxel 175 mg/m² and carboplatin at a dose adjusted to produce an area under plasma concentration-time curve (AUC) of 5-7.5 mg/ml/minute, both given intravenously every three weeks for six cycles (Stuart *et al.*, 2011).

1.2 DNA Damage Repair (DDR) Pathways

Damage to DNA can be caused by several factors, such as from the cells themselves (reactive oxygen species (ROS) and replication errors), from chemical agents or high energy electromagnetic radiation (UV and X-rays). The accumulation of DNA damage can lead to genomic instability and induce carcinogenesis. However, the cells must also be able to tolerate DNA damage by losing DNA damage signalling and checkpoint pathways such as those controlled by TP53, and by disabling one of the major DNA repair pathways (Bartkova *et al.*, 2005; Macaluso *et al.*, 2005; Kennedy and D'Andrea, 2006). Defective DNA repair mechanisms not only predispose cells to becoming cancerous but also influence sensitivity to treatment. Most cytotoxic agents induce DNA damage in order to destroy cancer cells. The

ability to sustain proliferation through loss of checkpoint function can cause resistance to these agents. On the other hand, it can also result in increased sensitivity to treatment, as the cells go through the cell cycle in a damaged condition, which leads to apoptosis (Curtin, 2012). There are five major pathways responsible for the repair of DNA damage: single-strand break (SSB) repair pathways consisting of base excision repair (BER) and nucleotide excision repair (NER); double-strand break (DSB) repair pathways consisting of homologous recombination (HR) and non-homologous end joining (NHEJ); and the mismatch repair (MMR) pathway (Bernstein *et al.*, 2002).

1.2.1 Base Excision Repair (BER)

BER is the main pathway for repairing faulty bases within the DNA due to cellular metabolism such as ROS, methylation, deamination and hydroxylation (Hoeijmakers, 2001). Failure to repair these lesions leads to point mutations after DNA replication. BER is subdivided into the removal of either a single damaged nucleotide (short patch repair) or a few (between 2-15) nucleotides (long patch repair) (Figure 1.3). The essential proteins in BER are Poly [ADP-ribose] polymerase 1 (PARP-1), X-ray repair cross-complementing protein 1 (XRCC1), DNA ligase III and apurinic/apyrimidinic endonuclease 1 (APE1). In both pathways, the damaged base is recognised and removed by BER glycolases to produce an abasic site (or apurinic or apyrimidinic (AP) site), which is then cleaved by BER endonucleases (commonly APE1), generating an SSB.

In short patch BER, PARP-1 binds to the SSB, resulting in the production of poly(ADP-ribose) (PAR), which attaches to histones or to PARP-1 itself. PARP-1 mediated recruitment of XRCC1 followed by DNA polymerase B (Pol β) replaces the damaged nucleotide, and DNA ligase III ligates the gap (Krokan and Bjørås, 2013). In long patch BER, the patch is processed

by proliferating cell nuclear antigen (PCNA), 9–1–1 complex and flap endonuclease 1 (FEN1), the nucleotides are replaced by Pol δ/ϵ and ligated by ligase I (Gee *et al.*, 2018). This repair process can contribute to chemo-resistance and can also be a potential therapeutic target. The most extensively studied protein in this pathway is PARP-1, which regulates histones and transcription factors directly through PARylation. PARP inhibitors were developed to target the DNA repair pathway and overcome chemo-resistance in ovarian cancer using the synthetic lethality concept in BRCA deficient conditions (Banerjee and Kaye, 2013).

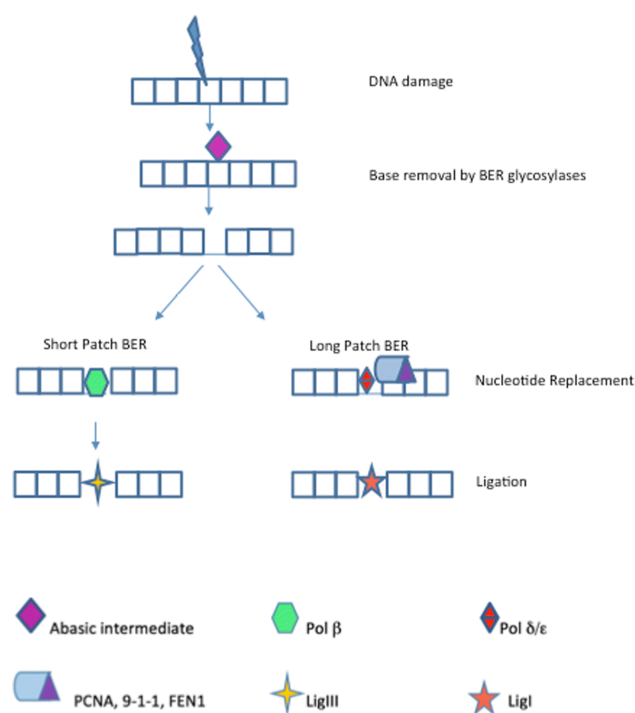


Figure 1. 3: Base excision repair (BER) pathway. BER is divided into two pathways based on the size of the damaged nucleotide: short-patch repair or long-patch repair. The repair consists of base removal, nucleotide replacement and ligation. Taken from Gee *et al.*, 2018., under Creative Commons License.

1.2.2 Nucleotide Excision Repair (NER)

NER repairs helix-distorting lesions that interfere with base pairing, DNA replication and transcription including DNA damage caused by platinum agents (Hoeijmakers, 2001). Two NER subtype pathways have been described to repair different types of damages: the

transcription-coupled repair (TCR) pathway which targets lesions in transcriptionally active domains and the global genomic repair (GGR) pathway which surveys the entire genome for distorting lesions (Figure 1.4). These pathways differ in the damage recognition step. In TCR, Cockayne syndrome proteins A and B (CSA and CSB) are engaged in recruiting transcription factor II H (TFIIH), Xeroderma pigmentosum complementation group A (XPA), and replication protein A (RPA) to the damaged site; whereas in GGR, damage recognition is dependent on Xeroderma pigmentosum group C-complementing protein (XPC) and RAD23 homolog B (RAD23B). After the damage recognition step, the processes are similar: the xeroderma pigmentosum complementation group F (XPF) and XPA helicases and the multi-unit transcription factor TFIIH open the damaged region, followed by the binding of RPA to the corresponding undamaged strand and stabilising it. Next, endonucleases xeroderma pigmentosum complementation group G (XPG) and XPF/excision repair cross-complementation group 1 (ERCC1) are recruited, cleaving 3' and 5' respectively at the borders of the opened stretch only in the damaged strand. Then, the removal of the damaged oligonucleotide occurs with the help of xeroderma pigmentosum complementation group B (XPB)/ xeroderma pigmentosum complementation group D (XPD) helicases, after which the gap is filled by DNA polymerase δ/ϵ together with PCNA. The final step is ligation using DNA ligase 3 (Hoeijmakers, 2001; Curtin, 2012).

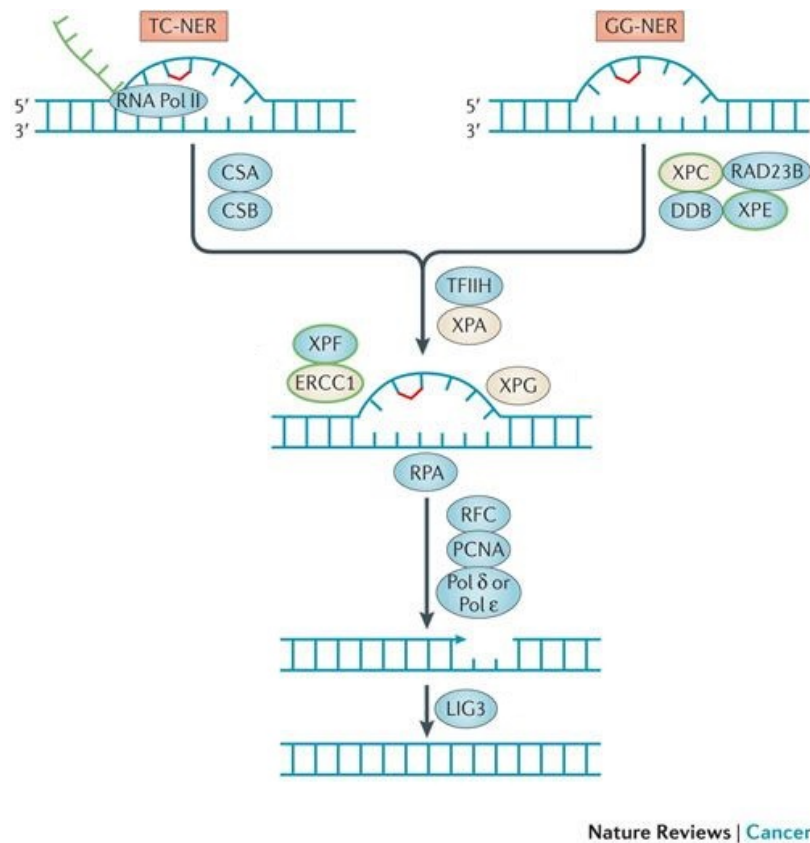


Figure 1. 4: Nucleotide excision repair (NER) pathway. Briefly, the NER process consists of several steps: DNA damage detection, the accumulation of DNA repair factors at the site of damage, and the physical excision and repair of the lesion. Taken from Curtin, 2012., with permission from Springer Nature.

1.2.3 Homologous Recombination (HR)

HR is the repair pathway for DSBs which occur during the S and G2 phases of the cell cycle and thus is an important pathway for genomic stability maintenance (Curtin, 2012). This pathway uses the sister chromatid as a template as the genetic information is identical (Weterings and Chen, 2008); thus, the repair system can effectively restore the genetic arrangement in an error-free manner (Figure 1.5). Mediated by BRCA1, the damaged DNA ends of the DSB are resected by MRN complex which consist of the MRE11 homolog (MRE11, double strand break repair nuclease), DNA repair protein Rad50 and Nibrin/Nijmegen Breakage Syndrome 1 (NBS1); resulting in a 3'-OH single-stranded tail. The

single-stranded DNA (ssDNA) overhang is then coated with RPA, to prevent degradation (Curtin, 2012). Mediated by BRCA2, Rad51 then displaces RPA and binds to the ssDNA to form a nucleoprotein filament, which is used to find the homologous sequence on the sister chromatid. The homologous sequence is then used by the cell as a template to guide repair followed by DNA synthesis. Holliday junction formation is a key step in this process as it allows both broken DNA strands to be repaired concurrently according to the equivalent homologous sequences. Finally, Holliday junction resolution is used to produce the final repaired chromosome (San Filippo, Sung and Klein, 2008).

In ovarian cancer, the role of the HR pathway became a pathway of increased significance due to the function of tumour suppressor genes *BRCA1* and *BRCA2*. These genes are best known as breast, and ovarian cancer susceptibility genes and inactivation of these genes leads to the accumulation of genetic alterations (San Filippo, Sung and Klein, 2008). *BRCA1/2* mutations were reported in 23 % of HGSOC cases and associated with improved survival after surgery, as well as platinum/taxane-based chemotherapy (Mahner *et al.*, 2017).

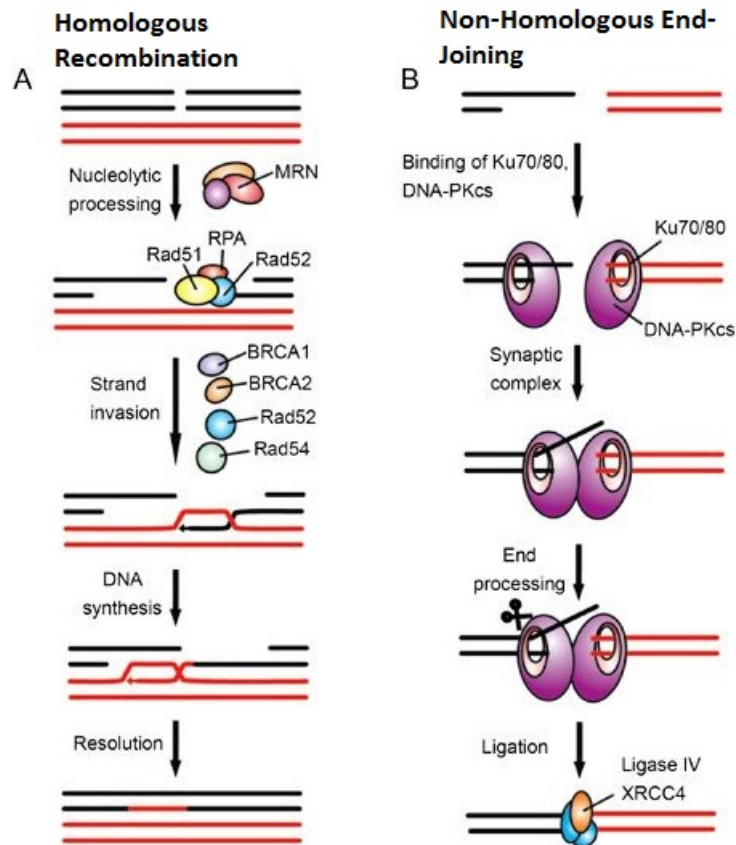


Figure 1. 5: Homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways. (A) HR uses a homologous sequence on a sister chromatid to precisely repair the DSB. (B) The mechanism of the NHEJ consists of a set of proteins that mediates the capture of both ends of the broken DNA, followed by molecular bridge formation that brings the two DNA ends back together, and finally re-ligation of the broken DNA. Taken from Weterings & Chen, 2008., with permission from Springer Nature.

1.2.4 Non-Homologous End Joining (NHEJ)

NHEJ ligates DSBs with minimal end processing, it lacks a homologous sequence to guide the repair and may contain insertions, deletions and translocations at the site of DNA damage. Thus, it is usually regarded as error-prone, but is active in all phases of the cell cycle, mainly in G0 and G1 phase, with some activity in late S and G2 (Shrivastav, De Haro and Nickoloff, 2008). Initially, DNA binding proteins (Ku70/Ku80) bind the DSBs and maintain their stability (Figure 1.5). This step is then followed by recruitment of the catalytic subunit of DNA-PKcs and subsequently initiates NHEJ repair (Hers, Vincent and Tavaré, 2011). DNA-PKcs acts as a central player in the NHEJ DNA repair pathway. DNA-PKcs is a member of the

Phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family which also contains ataxia telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR) and Mammalian target of rapamycin (mTOR). The other main component of NHEJ is Artemis, which has endo- and exonuclease activity and is involved in resecting 3' and 5' overhangs at the break sites where they exist when complexed with DNA-PK (Ma *et al.*, 2002). Following appropriate end processing, the DNA is ligated by DNA ligase IV, supported by two components called X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF) (Ahnesorg, Smith and Jackson, 2006). Defects in NHEJ are related to the development of chemotherapeutic resistance. A recent study has reported that NHEJ dysfunction was found in up to 40% of primary ovarian cancer cultures, is independent of HR function and appears to confer resistance to rucaparib, a PARP inhibitor *in vitro* (McCormick *et al.*, 2017).

1.2.5 Mismatch Repair (MMR)

The MMR pathway is a mechanism for correcting the base substitution mismatches and insertion-deletion errors generated during replication. Defects in this system correlate with genomic instability, owing to the high rates of spontaneous mutation, and thus create microsatellite instability (MSI) (Umar *et al.*, 2004). MMR is essential in DNA repair, and is also necessary for inducing cell cycle arrest and cell death in severely damaged cells (Li, 2008).

In the MMR pathway, either MSH2-MSH6 (hMutS α) or MSH2-MSH3 (hMutS β) complexes are involved in the DNA damage recognition process (Figure 1.6). hMutS α specially recognises base-base mismatches, and insertion/deletion mispairs of one or two nucleotides, whereas hMutS β identifies larger insertion/deletion mispairs (Li, 2008). These complexes then recruit a second heterodimer, comprised of MLH1 and PMS2. The two protein complexes initiate the

repair of the lesion, and each protein has overlapping functions and specificities which ensure that replication errors are recognised and efficiently processed. Exonuclease 1 (EXO1) then removes the regions up and down-stream of the mismatch site, followed by re-synthesis by DNA polymerase δ and re-ligation of the DNA strand by DNA ligase (McCormick *et al.*, 2017).

A defect in the MMR pathway is associated with increased cancer incidence, most notably due to defects in *MLH1* and *MSH2* genes, manifested as Hereditary Non-Polyposis Colorectal Cancer (HNPCC) or Lynch syndrome (Pal, Permuth-Wey and Sellers, 2008). Endometrial and ovarian cancers are also common in patients with this syndrome and often occur at a relatively young age (Vasen, 2005). Loss of *MLH1* has been observed in 50% of stage III/IV ovarian cancers (Scartozzi *et al.*, 2003).

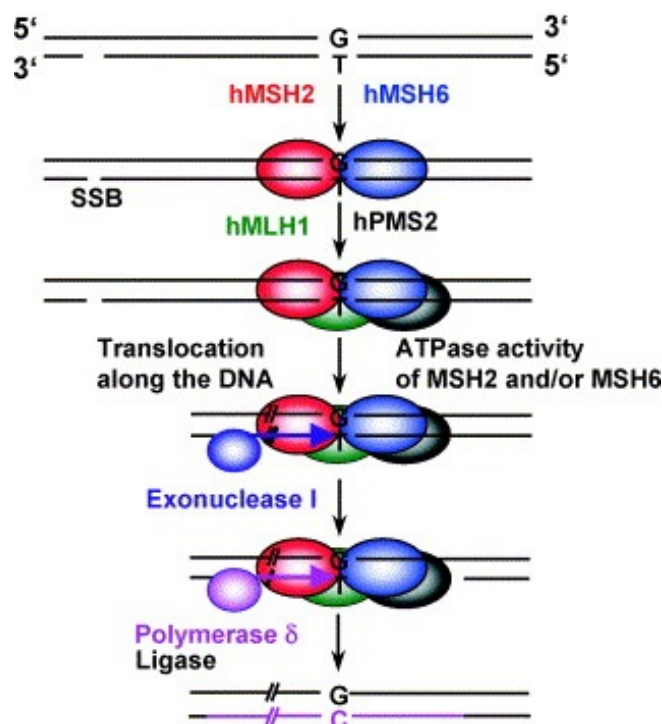


Figure 1. 6: Mismatch Repair (MMR) pathway. This pathway is initiated by the mismatch recognition factor MSH2-MSH6 and wrapped by DNA polymerase δ , which refills the DNA gap and DNA ligase I which ligates the DNA. Taken from Christmann, Tomicic, Roos, & Kaina, 2003., with permission from Elsevier.

1.3 Mechanism of Action of Platinum Compounds

Platinum compounds comprise of several drugs including cisplatin (cis-diammine-dichloro-platinum (II)), carboplatin ((cis-diammine (1,1-cyclobutanedicarboxylato) platinum (II)), oxaliplatin, satraplatin and picoplatin. The discovery and administration of these drugs has had significant clinical success in several solid tumours, such as ovarian, lung, colorectal, head and neck and testicular cancer (Kelland, 2007). The platinum-based agents used in ovarian cancer therapy are mainly carboplatin and cisplatin.

The cytotoxic effect of cisplatin on the cancer cells is primarily mediated by the induction of DNA damage. Cisplatin is activated inside the cell through the mono-aquation process, in which the chloride ions in cisplatin are replaced with water molecules (Figure 1.7). Once activated, cisplatin covalently binds purine N7 sites to form 1,2 G-G intrastrand crosslink (60–65% of all crosslink) and A-G 1,2 intrastrand crosslink (20–25%) in the DNA (Lemaire *et al.*, 1991). Other less common intrastrand crosslink are the 1,3 G-T-G intrastrand crosslink (in which two platinated guanines is separated by another base; around 2%), intrastrand C-G, and also less than 1% of monofunctional adducts on guanines (Fichtinger-Schepman *et al.*, 1987). Additionally, approximately 2% of adducts involve guanines on the other DNA strands, namely G–G interstrand crosslinks (Kelland, 2007). The cisplatin-DNA adducts lead to various cellular responses such as replication arrest, suppression of RNA transcription, cell-cycle arrest, DNA repair and induction of apoptosis (Wang and Lippard, 2005). These adducts are recognised by DNA damage induced proteins and subsequently activate DNA damage signalling pathways. The main DNA damage recognition proteins are MMR proteins hMSH2 or hMutS α and high mobility group proteins HMG1 and HMG2 (Siddik, 2003).

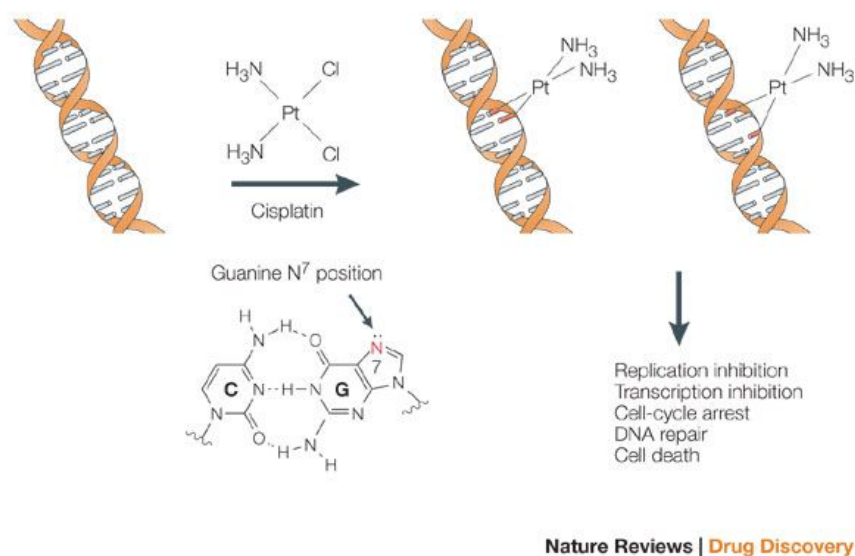


Figure 1. 7: Activation and effects of cisplatin. Cisplatin undergoes aquation once inside the cell. The platinum atom of cisplatin binds to the N7 position of guanine to form an intra- and inter-strand crosslinks adduct. Cisplatin-DNA adduct formation results in several cellular responses, including replication inhibition, transcription inhibition, cell-cycle arrest, inducing DNA repair mechanism and apoptosis. Taken from Wang & Lippard, 2005., with permission from Springer Nature.

The major apoptotic pathways involved in cisplatin-mediated cytotoxicity are ATM and ATR/Checkpoint kinase 1 (Chk1) induced p53 activation, which induces the extrinsic pathway of apoptosis Fas cell surface death receptor (Fas)/ Fas ligand (FasL), and the intrinsic Bax dependent caspase pathway (Figure 1.8). Induction of p53 also appears to be facilitated by HMG1/2, and its downstream effects also include the initiation of cell cycle arrest via p21 and DNA repair via the growth arrest and DNA damage inducible alpha (Gadd45a) protein (Jayaraman *et al.*, 1998). In addition, another protein product of a p53-related gene, p73, can also mediate cisplatin-induced apoptosis. Initiation of this pathway depends on both activated c-Abl tyrosine kinase and MMR status (Siddik, 2003). The involvement of the mitogen-activated protein kinase (MAPK) signalling pathway in cisplatin-induced apoptosis is of significant interest, in particular when it involves the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNKs) and p38 kinases. Cisplatin-induced DNA damage is

mainly repaired via the NER mechanism, by inducing the ATM pathway which is recruited to the damaged DNA site through XPC (Basu and Krishnamurthy, 2010).

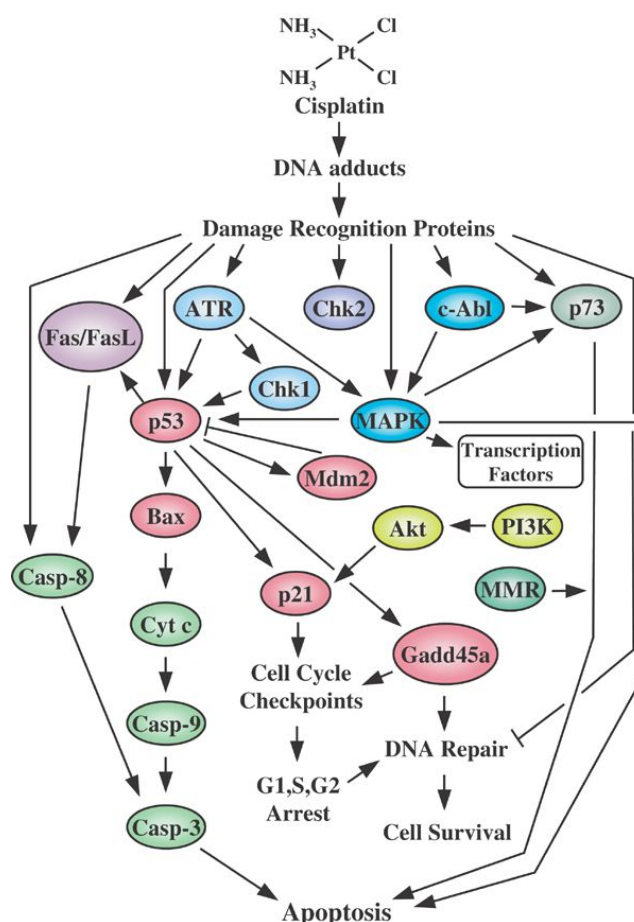


Figure 1. 8: Cellular effect of cisplatin-induced DNA damage. Apoptosis and cell survival pathways are activated simultaneously following cisplatin exposure. The final fate of the cell is influenced by the signal's intensity/duration and the crosstalk between the downstream pathways. Taken from Siddik, 2003., with permission from Springer Nature.

1.4 Platinum Resistance in Ovarian Cancer

Although 70–80% of patients initially respond to platinum-based chemotherapy, more than 60% of advanced ovarian cancer patients will eventually develop platinum resistance disease and die (Carden *et al.*, 2012). Tumour progression or recurrence is defined as the presence of progressive disease on a CT scan, elevated CA125 levels, or clinical evidence. Clinically, patients who have six months or more of disease-free survival following treatment with platinum are defined as having a platinum-sensitive disease (Progression free survival (PFS) $\geq$

6 months). Platinum-refractory is defined by disease recurrence prior to cessation of therapy, whereas platinum resistant relapse occurs within six months of finishing first-line treatment.

The development of platinum-resistance in ovarian cancer is still poorly understood. There are three possible theories for the development of chemoresistance in HGSOc (Figure 1.9). First, the tumour is genetically heterogeneous, where both resistant and sensitive clones exist prior to chemotherapy, and the therapy itself preferentially eradicates the sensitive clone, and eventually the resistant clone is able to survive and become predominant. Second, the chemoresistance is related to tumour renewing cancer stem cells which have higher tumourigenic potential and are insensitive to the chemotherapy due to relative quiescence. Third, chemoresistance is due to the growth of both the resistant clones and cancer stem cells (Agarwal and Kaye, 2003; Cooke and Brenton, 2011; Shah and Landen, 2014).

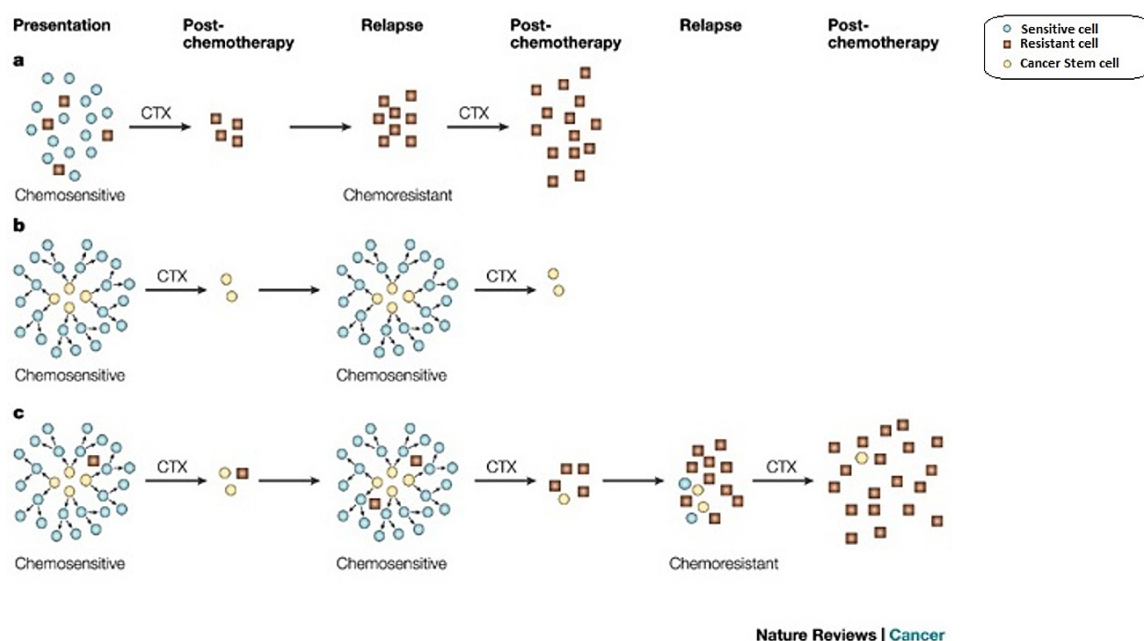


Figure 1. 9: Models of chemoresistance in ovarian cancer. **a)** The somatic mutation model of relapse proposes growth of resistant tumour-cell clones that remain at the end of chemotherapy and provides the rationale for combination and/or sequential chemotherapy. **b)** Subsequent relapse is due to the re-growth of persistent stem cells that were predominantly in G0 during chemotherapy. **c)** In practice, it seems likely that both models presented in (a) and (b) underlie clinical drug resistance in ovarian cancer. CTX, chemotherapy. Taken from Agarwal and Kaye, 2003., with permission from Springer Nature.

Several mechanisms underlie cellular resistance to platinum compounds. These mechanisms consist of those that limit the formation of platinum-DNA adducts and those that prevent apoptosis following platinum-adduct formation, including downregulation of pro-apoptotic pathways and up-regulation of survival/anti-apoptotic pathways, and increased efficiency of DNA repair (Davis, Tinker and Friedlander, 2014). The formation of cisplatin-DNA adducts can be restricted either by reduced uptake of cisplatin into cells, increased efflux, or inactivation of intracellular cisplatin by alteration into cisplatin-thiol conjugates. Initially, cisplatin was thought to enter cells via passive diffusion. In the past few years, copper transporter 1 (CTR1) has been shown to be involved in the uptake of cisplatin, and loss of CTR1 led to a threefold resistance to cisplatin (Holzer, Manorek and Howell, 2006). In a further study, the same author suggests that cisplatin can cause CTR1 downregulation through internalisation and degradation by a proteasome-mediated mechanism (Holzer and Howell, 2006). Higher expression of copper transporter 2 (CTR2), ATPase copper transporting alpha (ATP7A) and ATPase copper transporting beta (ATP7B) has also been linked to cisplatin resistance in ovarian cancer via cisplatin efflux (Kilari, Guancial and Kim, 2016).

A recent study found that increased ATP binding cassette subfamily B member 1 (ABCB1) gene expression is associated with acquired resistance in HGSOV (Patch *et al.*, 2015). The high level of ABCB1 expression is due to a structural variation involving the fusion of the promoter and non-coding exon 1 of solute carrier family 25 member 40 (SLC25A40) to the coding exons 2 of ABCB1 (Patch *et al.*, 2015; Christie and Bowtell, 2017). The ABCB1 gene encodes the multidrug-resistant protein 1 (MDR1), an ATP-binding cassette member involved in the cellular efflux of chemotherapeutic agents used in ovarian cancer treatment including cisplatin and paclitaxel (Patch *et al.*, 2015; Chohanadisai *et al.*, 2016). An increased efflux rate of cisplatin from cancer cells by members of the ATP-binding cassette (ABC) transporters family

is also considered to be important mechanisms behind the development of platinum resistance. These transporters function as either importers which bring substrates across membranes, ranging from ions to macromolecules, including drugs. The role of the ABC transporter family such as ATP binding cassette subfamily A member 1 (ABCA1), ATP binding cassette subfamily C member 1 (ABCC1), ATP binding cassette subfamily C member 2 (ABCC2) and ATP binding cassette subfamily C member 4 (ABCC4) in ovarian cancer chemoresistance has been well documented in several studies (Faggad *et al.*, 2009; Auner *et al.*, 2010; Chou *et al.*, 2011; Bagnoli *et al.*, 2013; Ween *et al.*, 2015). Additionally, a study revealed that a high expression of ABCA1, ATP binding cassette subfamily A member 6 (ABCA6), ATP binding cassette subfamily A member 8 (ABCA8) and ATP binding cassette subfamily A member 9 (ABCA9) in primary tumours was associated with shorter OS in serous ovarian cancer patients (Hedditch *et al.*, 2014). This study also confirmed the previous finding by Tothill *et al.*, which found that ABCA1, ABCA6, ABCA8, and ABCA9 were each more highly expressed in C1 tumours (an ovarian cancer subtype with poor prognosis).

The conversion of cisplatin into cisplatin-thiol conjugates is catalysed by glutathione S-transferases (GSTs), leading to more anionic compounds and easier to be exported from cells (Kelland, 2007). A previous study which utilises ovarian cancer cell lines derived from the same patient before and after the onset of drug resistance showed a 2.9-fold increase of GST in drug-resistant cells (Lewis, Hayes and Wolf, 1988).

As mentioned, the NER system is the major pathway involved in the repair of DNA damage following cisplatin treatment. Therefore, dysregulation of this system can modulate sensitivity or resistance to cisplatin treatment. One of the proteins involved in the NER pathway is ERCC1, which forms a heterodimer with XPF plays an important role in the removal of DNA

intra-strand crosslinks. This complex is a two subunit structure-specific nuclease, cuts double-stranded DNA 5' specifically near junctions between single-stranded and double-stranded DNA. A previous study has found that ERCC1 mRNA expression is higher in the ovarian cancer tissues from platinum-resistant patients compared to platinum-sensitive patients (Dabholkar *et al.*, 1994). Further, transfection experiments using ERCC1 antisense vectors in ovarian cancer cell lines and mice have shown enhanced cisplatin sensitivity (Selvakumaran *et al.*, 2003).

In addition, cisplatin-induced DNA lesions are recognised by the MMR pathway. If this mechanism fails in its attempt to repair DNA damage, the pro-apoptotic signal is transmitted (Kelland, 2007). Methylation of MLH1 causes the loss of MMR function and leads to microsatellite instability (MSI). MLH1 is a protein involved in recognising several types of drug-induced DNA adducts and thus initiate the MMR DNA repair pathway. In line with this, MLH1 methylation has been shown to be associated with increased MSI in plasma DNA in relapse cases of ovarian cancer. Moreover, the presence of MLH1 methylation at relapse predicts poor overall survival in ovarian cancer patients (Gifford *et al.*, 2004). Cisplatin-DNA inter-strand adducts lead to DSBs, which are repaired by the HR system (Tapia and Diaz-Padilla, 2013). BRCA1 and BRCA2 are essential components of the HR pathway. HR-deficient cancers due to mutations of BRCA1/2, as seen in HGSOC are known to be more sensitive to platinum (Yang *et al.*, 2011; Dann *et al.*, 2012). Reversions of germline BRCA1/2 mutations and loss of BRCA1 promoter methylation have been found to be correlated with acquired resistance in HGSOC (Patch *et al.*, 2015).

Cisplatin resistance can occur through dysregulation of proteins involved in apoptotic signalling pathways including p53, anti-apoptotic and pro-apoptotic members of the B-Cell

Lymphoma 2 (BCL2) family, and JNK (Gadducci *et al.*, 2002). The upregulation of pro-survival pathways such as HER-2 and its downstream effectors in the PI3K/AKT pathway also affect cisplatin sensitivity (Siddik, 2003). A previous study from our group revealed that the AKT pathway is involved in the development of platinum resistance through DNA-PK mediated activation (Stronach *et al.*, 2011). AKT is a serine/threonine kinase which regulates several cellular functions such as growth, proliferation, angiogenesis, motility, metabolism and survival (Hers, Vincent and Tavaré, 2011). In response to cisplatin-induced DNA damage, AKT was translocated into the nucleus of resistant cells where it was phosphorylated at serine 473 (S473) by DNA-PK, leading to inhibition of caspase-independent cisplatin-induced apoptosis. Other cell cycle control genes likely contribute to the development of platinum resistance, such as cyclin E (CCNE1) which its amplification in BRCA1 competent tumours promotes cell cycle progression in the presence of platinum (Etemadmoghadam *et al.*, 2013; Patch *et al.*, 2015). Gene breakages which inactivates *RBI*, *NF1*, *RAD51B* and *PTEN* also contributes to acquired resistance to chemotherapy in HGSOC (Patch *et al.*, 2015).

The tumour microenvironment also contributes to the development of platinum resistance in ovarian cancer. Tumours consist not only of cancer cells but have many different types of cells, such as cancer-associated fibroblasts (CAF), mesenchymal cells, macrophages and other immune cells, which all play an important role in cancer biology and drug sensitivity (Rebucci and Michiels, 2013). A recent study demonstrated that glutathione and cysteine released by CAFs contribute to cisplatin resistance in ovarian cancer by chelating cisplatin and subsequently effluxing it from the cells (Wang *et al.*, 2016). Mesenchymal Stem Cells (MSCs) recruited by ovarian cancer cells can secrete unsaturated fatty acids during platinum treatment that protect tumour cells against chemotherapy (Roodhart *et al.*, 2011). Also, tumour-associated macrophages (TAM) secretes cytokines such as Interleukin 6 (IL-6) and Interleukin

10 (IL-10) which activate the signal transducer and activator of transcription 3 (STAT3) pathway, resulting in the inhibition of apoptosis (Pogge von Strandmann *et al.*, 2017).

1.5 Targeted Therapies for the Management of Ovarian Cancer

An increased understanding of tumour biology in recent years has aided the development of a number of novel therapeutic targets to improve ovarian cancer treatments. These include anti-angiogenic therapy, growth factor inhibitors, immunotherapy and agents that target DNA repair pathways. However, currently there are only two targeted therapies used in the clinical settings for the treatment of ovarian cancer in the UK: bevacizumab, an anti-angiogenic agent targeting vascular endothelial growth factor (VEGF); and agents targeting a key regulator of DNA damage repair, poly(ADP-ribose) polymerase (PARP) e.g. olaparib and rucaparib.

Angiogenesis is important in the persistence of ovarian cancer and has been a key target for clinical research. Bevacizumab is a monoclonal antibody that specifically blocks the binding of VEGF to its high-affinity receptors and lead to regression of immature tumour vasculature, and inhibition of further tumour angiogenesis (Amit *et al.*, 2013). Four clinical trials have tested the efficacy of bevacizumab in ovarian cancer: the International Collaborative Group for Ovarian Neoplasm-7 (ICON7) phase III trial (Perren *et al.*, 2011) and GOG protocol 218 (Burger *et al.*, 2011) which evaluated the benefit of bevacizumab as first-line therapies in combination with platinum and paclitaxel; Ovarian Cancer Study Comparing Efficacy and Safety of Chemotherapy and Anti-Angiogenic Therapy in Platinum-Sensitive Recurrent Disease (OCEANS) trial (Aghajanian *et al.*, 2012) which assessed bevacizumab in a chemosensitive recurrent setting and the Avastin Use in Platinum-Resistant Epithelial Ovarian Cancer (AURELIA) trial (Pujade-Lauraine *et al.*, 2014) which examined bevacizumab in a chemoresistant recurrent setting. Although each of these trials showed improvements in PFS,

none showed extensions in OS, except the ICON7 trial which suggested improvement in OS when patients were stratified into a ‘high risk’ group, defined as those patients with stage III-IV with poor prognosis and sub-optimally debulked.

PARP inhibitors have been suggested for the treatment of ovarian cancer with germline or sporadic BRCA1/2 mutations. This drug uses the concept of synthetic lethality in BRCA-mutated patients, where unrepaired SSBs resulting from PARP inhibition are converted into the more genotoxic DSBs, which can be repaired effectively in cells with functional homologous recombination repair, whereas cancer cells with homologous recombination repair deficiencies (HRDs), such as BRCA mutations cannot repair this damage, leading to cell death. A study has shown that the PARP inhibitor olaparib prolonged the PFS in BRCA-mutated patients compared to BRCA wild-type (Ledermann *et al.*, 2014). Olaparib is currently approved as monotherapy for advanced ovarian cancer patients with a germline BRCA1/2 mutation, and as maintenance therapy for patients with platinum-sensitive, relapsed ovarian cancer and a germline or somatic BRCA1/2 mutation (Pujade-Lauraine *et al.*, 2017).

Apart from PARP inhibitors, another disruption to DNA repair pathways has been identified as an attractive approach to increasing therapeutic responses to DNA-damaging agents, including inhibition of AKT and DNA-PK. GSK2141795, an ATP-competitive pan-AKT inhibitor, was evaluated in a Phase I trial in patients with platinum-resistant ovarian cancer and showed reduced fluorodeoxyglucose (FDG) metabolism in the majority of tumours, downregulation of the proliferative marker Ki67 was also observed (Gungor *et al.*, 2011). Previous work by our centre regarding the effectivity of GSK2141795 (GSK795) revealed that the combination of AKT inhibition with cisplatin was beneficial both *in vitro* and *in vivo*. Proteomic analysis of this potential drug identified a signature of pathway inhibition which

included changes in phosphorylation of AKT and p38 and total androgen receptor (AR), Bcl-2-like protein 11 (Bim), insulin like growth factor 1 receptor (IGF1R), and Y-box binding protein 1 (YB1) levels. This protein signature was significantly predictive of CA125 response to GSK795 thus could aid patient stratification in the clinic (Cheraghchi-Bashi *et al.*, 2015). In a phase IB study of another AKT inhibitor GSK2110183 (afusertib) in combination with carboplatin and paclitaxel in patients with recurrent and platinum-refractory ovarian cancer showed an overall response evaluation criteria in solid tumours (RECIST) (v1.1) response rate of 32% with a PFS of 7.1 months was observed, showing the promising anti-tumour activity of this drug in the clinic (Blagden *et al.*, 2018).

NU7026 and NU7441 are two of the most widely studied DNA-PKcs inhibitors. NU7026 is a specific DNA-PK inhibitor which has been shown to potentiate the growth inhibition effect of a variety of chemotherapeutic agents including doxorubicin, etoposide and mitoxantrone in acute myeloid leukaemia (AML) cells lines (Willmore *et al.*, 2004). It was demonstrated that the inhibition of DNA-PK using NU7026 enables platinum-resistant ovarian cancer cell lines to become re-sensitised to cisplatin treatment (Stronach *et al.*, 2011). However, poor solubility of NU7026 remains problematic for clinical application of this compound. NU7441 is a small molecule DNA-PK inhibitor with improved potency compared to NU7026. NU7441 increased the cytotoxicity of ionising radiation and etoposide in colon cancer cell lines (Zhao *et al.*, 2006). NU7441 also improved the efficacy of ionising radiation and doxorubicin in prostate cancer (Shaheen *et al.*, 2011). More recently, this compound was shown to delay the repair of DSBs and increase the sensitivity of breast cancer cell lines to ionising radiation and doxorubicin, and as well as increase accumulation of cells in G2/M phase after DNA damage (Ciszewski *et al.*, 2014). The ability of this compound to enhance the effect of chemotherapy *in vitro* and *in vivo* has made it a promising candidate for improving ovarian cancer treatment outcomes.

Further, DNA-PKcs inhibitors are currently in clinical trials for advanced solid tumours and hematologic malignancies (NCT03724890, NCT02516813, and NCT01353625).

Another area in the development of new therapeutic strategies in ovarian cancer is anti-tumour immunotherapy. A meta-analysis of ten studies reported that a lack of tumour-infiltrating lymphocytes (TILs) is significantly correlated with a worse survival among ovarian cancer patients (Hwang *et al.*, 2012). Malignant cells evade the host immunity by disrupting immune checkpoint pathways and other immune-regulatory mechanisms. As a result, antibodies that target checkpoint proteins such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) and its ligand (PD-L1) are being developed to activate T-cell mediated immune response. Several immune-oncologic agents including anti-PD-1 antibody Pembrolizumab and anti-CTLA-4 antibody Ipilimumab are currently under investigation in ovarian cancer (Gaillard, Secord and Monk, 2016). These agents have shown a good response and are approved in melanoma and other solid tumours (Odunsi, 2017). Apart from checkpoints inhibitor, adoptive cell therapy (ACT) in which tumour-specific T cells are re-infused into patients to target and destroy tumour cells are being developed. These T cells can be genetically modified to express chimeric antigen receptor (CAR). Phase I studies of ACT-CAR targeting MUC16 (NCT02498912) and mesothelin (NCT01583686) are also on-going in ovarian cancer.

1.6 Tumour Heterogeneity

In numerous malignancies, the extent of tumour heterogeneity is not completely understood. The way in which molecular heterogeneity contributes to the tumour evolution and clinical progression is still being questioned. In addition, the interaction with the unique tumour environment that can influence the levels of heterogeneity is also yet to be addressed. Tumour heterogeneity is observed at several levels including genetic, proteomic, epigenetic, and leads

to a number of phenotypic features, such as cellular morphology, metabolism, motility, proliferative potential, immunogenic, angiogenic, metastatic potential, and response to treatment (Marusyk and Polyak, 2010; Ramon y Cajal *et al.*, 2018).

Tumour heterogeneity can be categorised into inter-tumour heterogeneity, in which variation exists between individuals with the same tumour type (for example the histological variation seen in ovarian cancer patients or different molecular profiles between serous ovarian cancers) and intra-tumour heterogeneity, where diversity is observed within tumours from the same patient (for example sub-clonal variation seen within a tumour deposit) (Figure 1.10). Interestingly, both inter- and intra-tumour heterogeneity appear to share a fundamental similarity, namely genomic instability (Burrell *et al.*, 2013). Accumulation of different mutations may result in the tumorigenesis of different normal cells from either a different type or location, thus promoting the development of different tumour types and contributing to inter-tumour heterogeneity. Likewise, dysregulated proliferation and genetic instability may lead to phenotypic variation amongst cancer cell sub-populations within a single tumour. Moreover, the genetic aberrations in cancer clones have been shown to promote tumour heterogeneity by modifying numerous key cellular pathways that control tumour progression such as the PIK3CA (Van Keymeulen *et al.*, 2015), epidermal growth factor receptor (EGFR) (Majem and Remon, 2013), mTOR and PTEN pathways (Gerlinger *et al.*, 2012). In addition, some clones may confer survival advantages that are responsible for the presence of cancerous sub-populations during and after treatment, leading to different responses to treatment (Blagden, 2015). This evidence illustrates the importance of understanding inter- and intra-tumour heterogeneity, and also how it can aid in designing more effective treatment strategies.

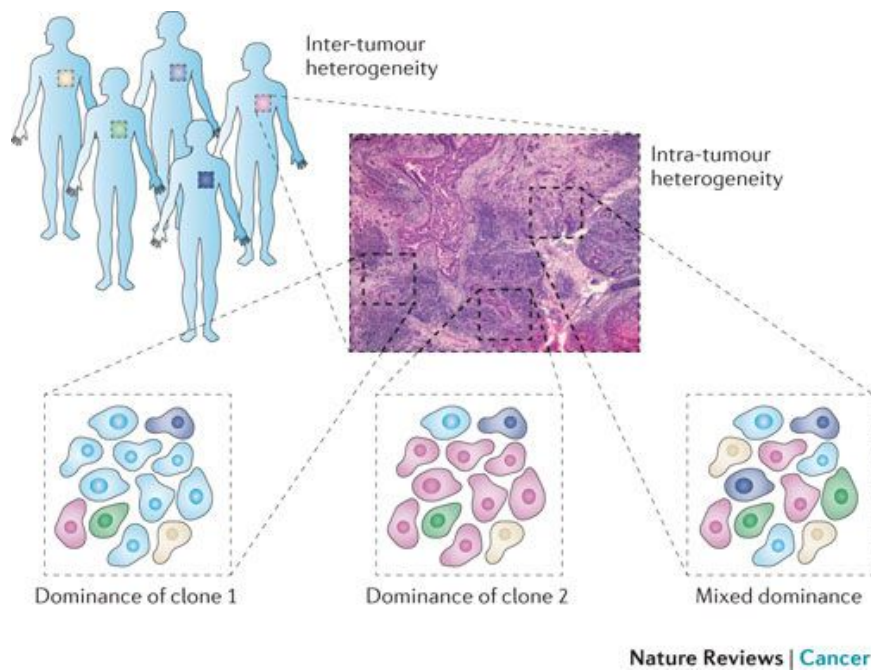


Figure 1. 10: Multiple levels of cancer heterogeneity. Inter-tumour heterogeneity may account for different types of cancer or different sub-types of a specific cancer, whereas intra-tumour heterogeneity is manifested by the existence of different sub-population/clones of cancer cells within a single tumour. Taken from Marusyk, Almendro, & Polyak, 2012., with permission from Springer Nature.

1.6.1 Major Concepts and Discoveries

The term tumour heterogeneity has long been recognised, as early as 1958 when evolutionary biologist Julian Huxley stated that the extent and rate of variance in tumour genetic inhomogeneity will be interesting to explore (Huxley, 1958). In 1965, it was postulated that effective cancer treatments depends on a better understanding of the diversity found within the disease (Foulds, 1965). However, in the 1970s that the field of intra-tumoral heterogeneity gained momentum. In 1976, Peter Nowell proposed a ‘clonal evolution model’, whereby the selection of a fitter sub-clone which has survival advantages, is required for tumour evolution (Nowell, 1976). The core of Nowell's hypothesis is the view that tumours are intrinsically ‘genetically unstable’, in line with Darwinian concept of cancer evolution, where the diversification of heritable types is tested by natural selection. This ecological view of tumour heterogeneity has become widely accepted and an essential notion in cancer research (Merlo

et al., 2006). This tumour evolution model is demonstrated in cancer by subclonal driver mutations and convergent evolution. However, not all cancers have these features, leaving the possibility of neutral evolution model. Neutral evolution first proposed by Motoo Kimura, this model hypothesises that there is no selection during most of the lifetime of the tumour (Kimura, 1991). Neutral evolution model proposes that after malignant transformation, random mutations accumulate over time causing genetic drift and vast tumour heterogeneity (Davis, Gao and Navin, 2017).

Another major concept in the field of intra-tumoral heterogeneity involves epigenetic mechanisms where changes in gene transcription do not involve alterations to the genome, as the source of tumour variation (Frost and Kerbel, 1983). The importance of epigenetic regulation in generating diversity apart from genetic variance has been shown in numerous systems. For example, epigenetic aberrations have been shown to regulate oestrogen receptor (ER) expression, and thus clinically differentiate between the two common ER positive and negative breast cancer types (Huynh *et al.*, 2012). In addition, heterogeneity based on dynamic variation in epigenome configurations is thought to contribute to the selection of drug-resistant cells (Wilting and Dannenberg, 2012).

However, another hypothesis proposes that tumour diversification is not a property of tumour cells. Instead, it is similar to normal embryonic development, in which a small population of initiating cells can give rise to a multitude of different cell phenotypes (Nicolson, 1987). Adding to the recognised importance of the field of tumour heterogeneity was the concept that intra-tumoural heterogeneity influences the chemotherapy response (Heppner *et al.*, 1978) and promotes metastatic processes (Fidler, 1978). Later, the concept of intra-tumoural

heterogeneity was considered as a necessary component in cancer progression contributing to the ‘the Hallmarks of Cancer’ (Hanahan and Weinberg, 2011).

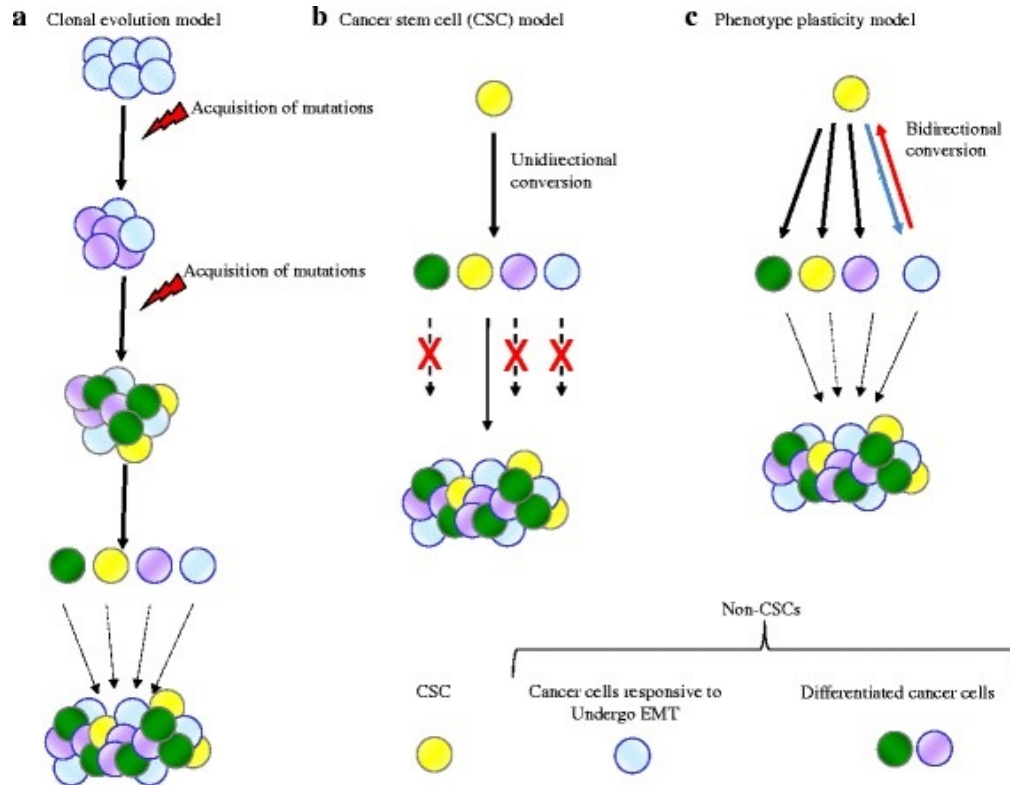


Figure 1. 11: Different models of tumour heterogeneity. **a)** The clonal evolution or stochastic model suggests that sequential acquired mutations generate heterogeneity and all cells are capable of self-renewal and inducing tumorigenesis. **b)** According to the CSC model, tumour cells are systematised into a hierarchy of heterogeneous cell populations, and only a small subset of cells called CSC within a tumour have the ability to sustain tumour formation and proliferation by unidirectional conversion. **c)** The phenotype plasticity model suggests that irreversibly differentiated cells can be converted back to an undifferentiated state or stem cell-like state under the influence of an appropriate stimulus. This dynamic bidirectional conversion between CSC and non-CSC leads to tumour heterogeneity. Taken from Jayachandran, Dhungel, & Steel, 2016., under Creative Commons License.

Nowell’s concept of tumour heterogeneity was challenged by the ‘cancer stem cell (CSC) model’ where cancer develops from the transformation of a stem cell that has the ability to self-renew and differentiates into a diverse population of daughter cells with limited self-renewal capability (Polyak and Hahn, 2006). This theory also provides convincing explanations for the

mechanisms behind malignancy and chemosensitivity, in which small subpopulations of CSC remain throughout chemotherapy and are responsible for the development of intra-tumour heterogeneity post-therapy, leading to drug resistance (Soltysova, Altanero and Altaner, 2005; Maugeri-Saccà, Vigneri and De Maria, 2011). Another concept termed ‘phenotypic plasticity’ further challenged the traditional hierarchy of ‘one-way’ differentiation proposed by the CSC model (Figure 1.11). According to this perception, most tumour cells can contain stem cell properties with various degrees of ‘stemness’ influenced by the tumour microenvironment and cell-autonomous mechanisms (Hill, 2006).

After decades of research, and with the advent of high throughput technologies allowing genomic, epigenetic, and proteomic profiling, a much more complex picture of tumour heterogeneity has been revealed. The first inter-tumoural comparison for understanding the nature and heterogeneity of human cancers was whole exome sequencing in colorectal and breast cancer (Wood *et al.*, 2007). Since then, the genetic characterisation of various tumour types using whole exome and genome sequencing has improved dramatically (Gerlinger *et al.*, 2012). Collaborative initiatives, including The Cancer Genome Atlas Project (TCGA), the Cancer Genome Project (CGP), the International Cancer Genomics Consortium (ICGC), and Clinical Proteomic Tumor Analysis Consortium (CPTAC) have pooled sequencing data, large-scale proteome or proteogenomics data, enabling a more comprehensive understanding of both inter and intra-tumour heterogeneity (Blagden, 2015).

1.6.2. Inter- and Intra-tumour Heterogeneity in Ovarian Cancer

Ovarian cancer is not considered to be a single disease, it is very heterogeneous both molecularly and histologically (Braicu *et al.*, 2012). The heterogeneity in EOC was first formally reported in 1979. Chromosomal analysis was performed on cancer cells taken from

patients' ascites prior to and after cytostatic treatment such as Leukeran, Mannogranol and Cytembena. Chemosensitivity was assessed using thymidine incorporation in *ex vivo* cells. The authors concluded that a wide range of ploidy chromosome (diploid to hyperdiploid) was present after chemotherapy and that the primary tumour and its metastases or ascitic tumour-cell population showed different chemosensitivities levels (Siracky, 1979).

With the development of DNA analysis technology, genetic studies have become more advanced. One study analysed restriction fragment length polymorphisms in ovarian carcinoma tissue and found significant chromosomal loss of heterozygosity in chromosomal segments 3p, 6q, and 11p, whereas another study showed allelic loss of chromosomes 6q, 11p, and 17q (Ehlen and Dubeau, 1990; Lee *et al.*, 1990). Chromosome 17q was found to harbour tumour suppressor genes, including *BRCA1* and *TP53* (Presneau *et al.*, 2005). In HGSOC, the high frequency of *TP53* mutations is likely to contribute to the high level of genomic instability which results in frequent chromosomal aberrations (Zhang *et al.*, 2017). In another study, multiple regions of tissue from 16 patients were used to assess the extent of genetic intra-tumour heterogeneity in HGSOC (Khalique *et al.*, 2007). The authors found that the most common genetic changes were chromosome 13 and 17 deletions, where *BRCA1*, *BRCA2*, and *TP53* genes are located. There was also considerable genetic inter- and intra-tumour heterogeneity, demonstrated as microsatellite instability and single nucleotide polymorphism (SNP) variation (Khalique *et al.*, 2007). In addition, evolutionary analysis of microsatellite data suggested that tumours were derived from a monoclonal origin, with subsequent evolutionary divergence creating mixed populations of genetically distinct cells within a tumour.

Using microarray gene expression profiling on tumour tissue collected from 285 patients at the time of debulking surgery, the Bowtell group in Melbourne attempted to characterise the

molecular profile of ovarian cancer and found that serous and endometrioid cancers display a high degree of molecular heterogeneity (Tothill *et al.*, 2008). This study identified six molecular subtypes of ovarian cancer and correlated their findings with clinical outcomes. Four of them representing HGSOC: C1 (high stromal response), C2 (immunoreactive), C4 (low stromal response), and C5 (mesenchymal). Based on this result, TCGA performed the same analysis and found four subtypes related to HGSOC, termed: ‘immunoreactive’, ‘differentiated’, ‘proliferative’, and ‘mesenchymal’ (Cancer Genome Atlas Research Network, 2011). Of particular interest, rather than identifying genes involved in the DNA repair, this method of sub-classification identified immunoregulatory and stromal genes, as detailed in section 1.1.2. Thus, it highlights the importance of the tumour microenvironment to the outcome in HGSOC and indicates an opportunity for a personalised medicine approach to treatment.

The data gathered by TCGA Research Network has improved the understanding of the molecular composition of HGSOC dramatically. This project sequenced exons from coding genes in 316 HGSOC samples. This project also analysed mRNA and miRNA expression, promoter methylation and DNA copy number in 489 HGSOC cases. The results of this project revealed that mutations in *TP53* are present in almost all tumours; statistically recurrent genetic mutations with low prevalence in further 8 genes (*BRCA1*, CUB And Sushi Multiple Domains 3 (*CSMD3*), *NF1*, cyclin dependent kinase 12 (*CDK12*), FAT Atypical Cadherin 3 (*FAT3*), gamma-aminobutyric acid type A receptor alpha 6 subunit (*GABRA6*), *BRCA2*, *RBI*); 122 missense mutations with oncogenic potential but not necessarily recurrent; 113 significant somatic copy number alterations; and promoter methylation in 168 genes, demonstrating the extensive heterogeneity of genetic mutations in HGSOC (Cancer Genome Atlas Research Network, 2011). This was supported by other studies using high-density SNP arrays and paired-

end next generation sequencing, which showed high copy number and frequent structural variations (Malek *et al.*, 2011; McBride *et al.*, 2012).

From demonstrating the presence of tumour heterogeneity, investigations of EOC have evolved to define its patterns and clinical impact of heterogeneity. A study using samples from primary and metastatic sites, based on 27 samples of 3 treatment-naïve advanced EOC cases aimed to probe intra-tumour heterogeneity using whole-genome mate-pair sequencing, topographic mapping of somatic breakpoints, and transcriptional profiling (Hoogstraat *et al.*, 2014). They observed a high degree of genetic heterogeneity between primary and secondary sites and showed different pathways activated between tumours in the same patient, suggesting that tumour lesions at different sites seem to evolve independently and adapt to the environment. Their findings highlight the plasticity of ovarian cancer genomes, which may contribute to the high relapse rate following standard therapy.

While a linear clonal tumour evolution model has been suggested, a genomics study in HGSOC has indicated another model showing a branching process represented by an evolutionary tree (Bashashati *et al.*, 2013). This study measured the degree of genomic diversity within primary cases to examine the natural state of tumour evolution using 31 spatially and temporally separated samples taken from six patients prior to therapy. Using exome sequencing, copy number analysis, target amplicon deep sequencing, and gene expression profiling, they found vast intra-tumoural variation in mutation, copy number and gene expression profiles. Except for *TP53*, mutations in the key driver genes such as *PIK3CA*, *CTNNB1*, and platelet-derived growth factor receptor (*PDGFR*), were present in a subset of samples, underlining the fact that a single tumour sample is not representative of an entire tumour. The branch-like evolutionary pattern revealed by this study suggests that tumour cells in secondary sites undergo additional

mutations which enable them to acquire an ongoing metastatic potential. The mutations present in early clonal progenitors such as *TP53* mutation are called ‘trunk’ mutations (Bashashati *et al.*, 2013), whereas branch mutations in HGSOC include *PIK3CA*, *CTNNB1*, and *PDGFR* mutations which appear in some metastatic sites.

Relatively little is known about the HGSOC genomic evolution under the selective pressure of chemotherapy. A study using whole genome sequence analysis of 114 samples from 92 HGSOC patients which compare resistant, refractory and sensitive primary disease has been published (Patch *et al.*, 2015). First, this study confirmed previous findings that *TP53* mutations were prevalent in HGSOC (Ahmed *et al.*, 2010; Cancer Genome Atlas Research Network, 2011) and that inactivating mutations in genes associated with homologous recombination or *BRCA1* methylation, occurred in about 50% of primary tumours. Furthermore, *CCNE1* gain/amplification occurred 19% of patients and was in large part exclusive of *BRCA1/2* pathway disruption. This amplification was also associated with primary treatment failure, and inactivating gene breakages at the level of the tumour suppressors *RBI*, *NFI*, *RAD51B* and *PTEN* were found to be correlated with acquired resistance to chemotherapy. Temporal heterogeneity was also observed in this study, in which mutational burden (SNVs and indels) at the coding and non-coding level in the relapse samples were higher compared to the matched primary samples. Several molecular events associated with acquired resistance were observed including reversion in *BRCA1/2* mutations, loss of *BRCA1* hypermethylation, and *MDR1* upregulation. Together, their findings underline the heterogeneity and adaptability of the HGSOC genome under the selective pressure of chemotherapy and indicate that tackling resistance to conventional chemotherapy will require a diversity of approaches.

A study has quantified the extent of intra-tumour heterogeneity and correlate this with clinical outcome (Schwarz *et al.*, 2015). Copy number profiles from 135 spatially and temporally separated samples were obtained from 17 HGSOC patients who had received neo-adjuvant platinum-based chemotherapy. They used a novel algorithm called ‘minimum event distance for intra-tumour copy number comparisons’ (MEDICC) to construct an evolutionary tree and quantify heterogeneity as the degree of clonal expansion between tumours (Schwarz *et al.*, 2014). The results demonstrated significant intra-tumour genetic heterogeneity and branched patterns of evolution were also found in the majority of samples, consistent with the Bashashati *et al.*'s findings. In this study, the patients were split into two groups based on their degree of clonal expansion (CE), CE-high and CE-low. Those classified as CE-high had a shorter PFS (10.1 months compared to 12.7 months) and a shorter OS (23.5 months compared to 42.6 months) than those classified as CE-low. Lastly, phylogenetic and NF1 deletion analysis of samples taken from two patients before and after disease progression indicated that a resistant sub-clonal population was already present before chemotherapy. This suggests that Nowell's clonal diversity model is correct - a resistant sub-clonal cellular population is present at diagnosis - and that clonal expansion occurs throughout the disease progression (Cooke *et al.*, 2010).

A recent integrated analysis of whole-genome points mutation and structural variation patterns in 59 HGSOC cases has shown that HGSOC can be subdivided into different prognostically relevant mutational subgroups (Wang *et al.*, 2017). There were two subgroups comprised primarily HGSOC cases: the foldback inversion (FBI) structural variation signature (41%) and the homologous-recombination-deficient (HRD) mutation signature (53%). The FBI subgroup bears a distinct pattern of high-level amplifications colocalised with foldback rearrangements typical of breakage-fusion-bridge processes. Also, *CCNE1* amplification and *PTEN* focal copy

number deletion were frequent in FBI tumours. The HRD subgroup characterises by *BRCA1/2* somatic or germline mutations, *BRCA1* promoter methylation, myelodysplasia syndrome-associated protein 1 (MDS1) And ecotropic virus integration site 1 (EVI1) Complex Locus (*MECOM*), proto-oncogene *MYC* and Cyclin D1 (*CCND1*) amplification and *RBI* focal copy number deletion. In this study, FBI subgroup was significantly associated with a poor response to platinum-based chemotherapy, superseding BRCA mutation status and gene expression biomarkers.

Also in 2017, a study in one HGSOC patient who was treated with multiple types of chemotherapy showed that tumour-immune microenvironments are heterogeneous, between the primary tumour and metastases and between metastases (Jiménez-Sánchez *et al.*, 2017). This patient displayed a differential progression rate of metastasis during the off-treatment period; some of the metastatic deposits regressed, while some progressed. The immunogenomic analysis in this study revealed that immune infiltration and activation were diverse in each tumour; immune cell exclusion was found in progressing deposits, while CD8+ and CD4+ T-cell infiltration was present in regressing and stable metastases. These findings indicate that inter-site immune heterogeneity may prove an essential challenge in treatment strategies to overcome inter and intra-tumour heterogeneity in HGSOC.

Another study has addressed the role of the immune system in metastatic heterogeneity of HGSOC (Zhang *et al.*, 2018). Three immunologic subtypes across samples and extensive within-patient diversity were identified through the multi-region study of 212 samples from 38 patients with whole-genome sequencing, immunohistochemistry, histologic image analysis, gene expression profiling, and T and B cell receptor sequencing. These subtypes were: N-TIL (tumours with no or limited TILs infiltration), S-TIL (tumours dominated by stromal TILs),

and ES-TIL (tumours with combined epithelial and stromal TILs). This classification was correlated with malignant clone diversity properties. Regions with the highest levels of epithelial immune infiltration exhibit the lowest malignant clone diversity and had a better outcome. Lastly, the authors showed prognostically relevant interaction between mutational processes and immune infiltration with foldback inversions associated with poor outcomes even in the presence of high cytotoxicity.

1.7 Advantages of Primary cell culture for HGSOC studies

Cell lines derived from tumours provide invaluable models for the study of cancer biology at the cellular and molecular level. Established immortalised ovarian cancer cell lines have the benefits of high proliferative potential, prolonged lifespan, clonogenicity and ease of maintenance (Cunnea and Stronach, 2014). Nevertheless, they appear to lose their original tumour characteristics due to the accumulation of genetic alterations during cell culture. In addition, there is evidence that some of ovarian cancer cell lines demonstrate a significant loss of integrity, with flat copy-number profiles, wild-type *TP53* and hypermutated genotype (Korch *et al.*, 2012; Domcke *et al.*, 2013). Thus, established cell lines may not reflect the clinical condition and the biology of advanced ovarian cancer. Primary ovarian cancer cells derived from ovarian tumours represent a unique tool for studying the disease and provide an invaluable source closer representing the patient's condition.

Broadly, there are two main sources of ovarian cancer patient material that can be used to derive primary cultures: solid tumour tissue and ascitic fluid. Ascitic fluid is relatively easy to obtain, especially since advanced HGSOC patients usually present with large volumes of ascites. The extraction of tumour cells from this resource is also technically more straightforward compared to extraction from solid tumour tissue, which requires dissection and

digestion of the tissues. Primary cell cultures generated from a solid tumour can facilitate the investigation of both inter and intra-tumoural heterogeneity at phenotypic and functional level.

1.8 Aims

The association between diverse molecular profiles of ovarian cancer and the prognosis of the disease remains necessary to be elucidated. In the present study, we are using tumour tissue specimens taken from patients undergoing primary debulking surgery. We believe that by isolating primary cancer cells from these patients, it may more precisely correspond to the biology of patients with advanced ovarian cancer. The primary aim of this study is characterising intra-tumour heterogeneity at phenotypic, proteomic and genomic levels using diverse sampling of HGSOC tumours from different anatomical sites. The strategies for achieving this were:

1. To understand the extent of phenotypic inter- and intra-tumoural heterogeneity in HGSOC using primary tumour cells extracted from disseminated tumour deposits and determine the relationship to chemotherapy response, as well as explore its relationship to clinical outcome. To determine the heterogeneity in PAX8 expression as a marker of HGSOC and its correlation with phenotypic response.
2. To investigate changes in protein signalling cascades following platinum treatment in different tumour deposits, to assess the level of heterogeneity of platinum-induced cell signalling protein expression in our primary tumour cell cultures, and to investigate the differences between platinum-induced protein changes in samples related to platinum resistant and sensitive relapse.
3. To investigate whether quantitative measures of genetic heterogeneity using CNV data can predict clinical outcome in HGSOC chemo-naïve patients using MEDICC approach.

Chapter 2

Material and Methods

2.1 Patient Selection

Ovarian cancer patients were recruited for this study between 2013 and 2016 at Hammersmith Hospital, London, UK. Inclusion criteria for patients in this study were histologically confirmed advanced EOC at FIGO stage III or IV, grade 3 tumours, and a resectable disease as identified by a multidisciplinary team, which consisted of gynaecological oncologists, clinical and medical oncologists, pathologists, radiologists, and clinical nurse specialists. The exclusion criteria were patients who had neoadjuvant chemotherapy and had a previous malignancy. Patients were also followed up for any subsequent relapses.

Full informed consent was collected from all patients in the study. The human samples used in this project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB). ICHTB is approved by National Research Ethics Service (NRES) to release human material for research (12/WA/0196), and the samples for this project (R14142-3A) were issued from sub-collection reference number Gyn_HG_13_020. The detailed objective surgical and histopathological data from each patient was documented in a validated system, 'Intraoperative Mapping of Ovarian Cancer' (IMO), to provide a comprehensive picture of the tumour pattern in the abdomen and accurate staging (Fotopoulou *et al.*, 2010). The clinical data collected as part of this study included FIGO stage; grade; histological type; debulking surgery status: tumour free, <0.5cm, >0.5cm, 1-2cm and >2cm; progression free survival (PFS) data: progression status and time to progression; overall survival (OS) data: survival status and time to death; the type of chemotherapy given; as well as response to treatment: chemosensitive relapse, chemoresistant relapse or no relapse. Progression free survival was described as the time from surgery to the first relapse or death. Overall survival was described as the time from surgery to death from any cause. Figure 2.1 outlines the design of this ITH study, which refers

to intra-tumour heterogeneity study, investigating the differences between tumours of a patient and between cancer cells within a tumour.

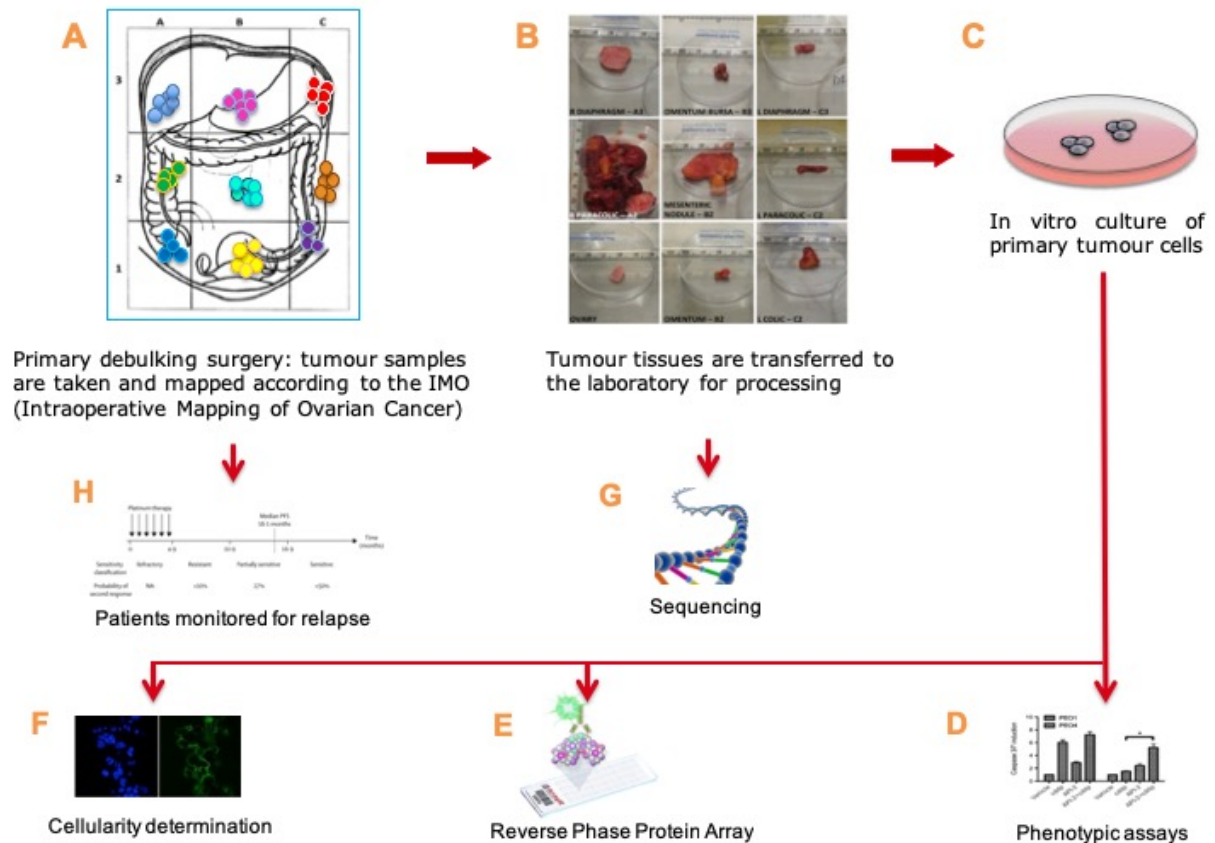


Figure 2. 1: Intra-tumour heterogeneity (ITH) study design. During primary debulking surgery, tumour deposits are mapped according to ‘Intraoperative Mapping of Ovarian Cancer’ (IMO), (A) fresh tumour samples were taken and transferred to the lab immediately for processing (B). Tumour cells were extracted, and short-term primary cultures were established (C) for phenotypic (D) and proteomic (E) assessment. Tumour cell content was determined in each culture (F). Representative frozen tumour pieces were genomically characterised by SNP arrays to investigate clonal expansion (G). Patients were then tracked throughout the treatment journey and samples were collected if undergoing surgery for relapse (H).

2.2 Laboratory Methods

2.2.1 Isolation of Tumour Cells from Tissue Samples

Primary and disseminated tumour tissue deposits were collected from patients undergoing primary maximal effort debulking surgery for HGSOc in Hammersmith Hospital. Each sample

was anonymised and given a unique code from the tissue bank, starting with T for tumour, followed by the year and a sample code. Samples were collected fresh from surgery in cold RPMI 1640 media (Sigma-Aldrich, UK) containing 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies, UK) and rapidly transported to the laboratory to be processed. Each tumour tissue was dissected into 1 mm pieces and depending on tissue size, incubated in 5-10 ml of 2.4 U/ml Dispase II (Roche, UK) for 1 hour at 37°C, 5% CO₂ with gentle shaking every five minutes. Complete tissue culture media (RPMI media with 20% Foetal Calf serum (FCS) (First Link, Wolverhampton, UK), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco-BRL, Life Technologies), 2 mM L-glutamine (Life Technologies, UK), 2.5 µg/ml Insulin (Sigma-Aldrich) and 2 mM Sodium Pyruvate (Sigma-Aldrich)) was then added to the tissue dispase mixture in order to dilute the dispase sufficiently to stop the digestion of the tissue.

Cells were collected by filtering the digested tumour pieces through a 70 µm filter using the bottom of a 10 ml syringe plunger, followed by centrifugation at 1500 rpm for 15 minutes. Red blood cell (RBC) lysis was carried out to remove any remaining erythrocytes using an RBC lysis buffer (Miteny Biotec, UK). Cells were then washed to remove any traces of RBC lysis buffer and resuspended in complete media. Cell populations were incubated for 30 minutes on plastic to remove populations of stromal cells including fibroblast and mesothelial cells. The non-adherent tumour cells were then placed into tissue culture flasks in complete media for subsequent growth and analysis (Figure 2.2).

2.2.2 Isolation of Tumour Cells from Ascites

Freshly obtained ascitic fluid drained from the peritoneum of ovarian cancer patients was also collected during surgery. The fluid was then centrifuged for 20 minutes at 2500 rpm to produce

a cell pellet, which was resuspended in an appropriate volume of phosphate-buffered saline (PBS), depending on the size of the pellet. Red blood cells were removed using an RBC lysis buffer (Milteny-Biotec). Cells were then washed and resuspended in an appropriate volume of tissue culture medium. Cells were then left for 30 minutes in flasks, allowing fibroblasts/mesothelial cells to adhere. The media was aspirated and re-plated into fresh flasks for subsequent culture and analysis (Figure 2.2).

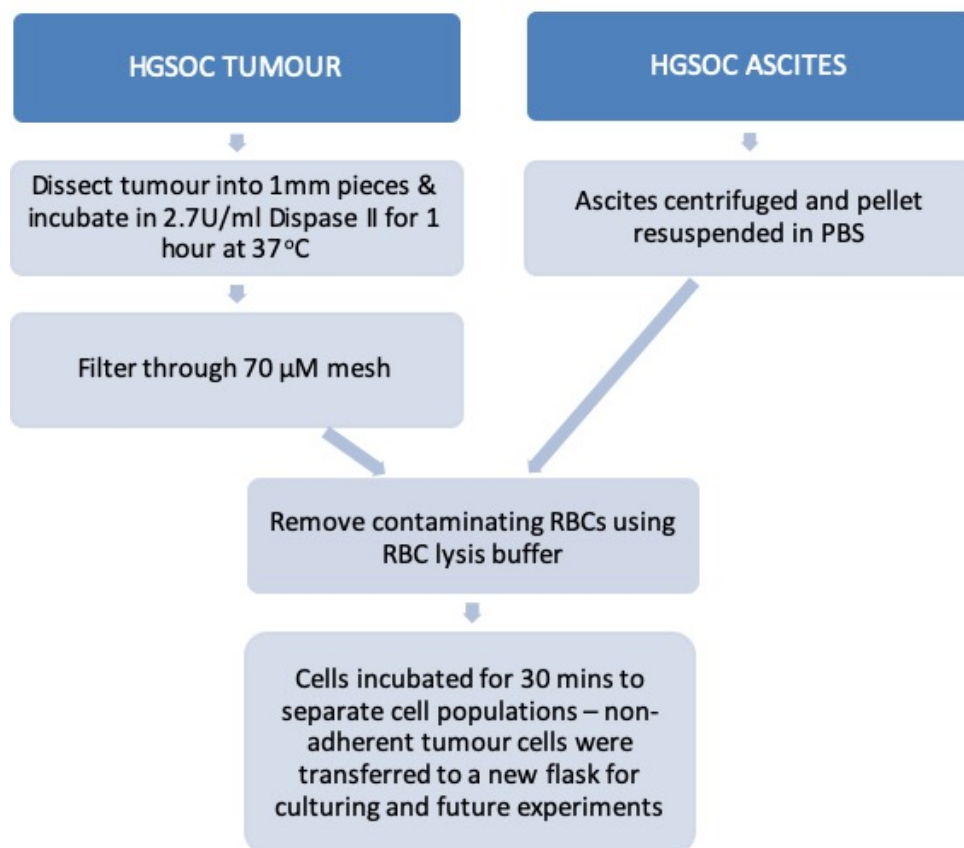


Figure 2. 2 Tumour cells isolation. Tumour cells were extracted from solid tumour and ascites, cultured and used for subsequent assays.

2.2.3 Immortalised Cell Culture

Ovarian cancer cell lines PEO1 and PEO4 were provided by Dr Simon Langdon (University of Edinburgh, UK). PEO1 and PEO4 were derived from HGSOC patients when they were

chemosensitive and after relapse with chemoresistant disease, respectively. Further characteristics of these cell lines are shown in Table 2.1.

Table 2. 1 Summary of cell lines' characteristics

Cell line	Histology	Source	Mutation status
PEO1	Poorly differentiated serous adenocarcinoma	Ascites	P53 Mutant BRCA2 mutant
PEO4	Poorly differentiated serous adenocarcinoma	Ascites	P53 Mutant

All cells were maintained in RPMI-1640 (Sigma) media supplemented with 10% FCS (First Link, Wolverhampton, UK), 2 mM L-glutamine (Life Technologies, UK), 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies, UK). Cells were grown at 37 °C in 95% humidified air containing 5% CO₂ and underwent regular mycoplasma testing. Cells were passaged at 75-90% confluency using an aseptic procedure in a level II laminar flow biosafety cabinet. Splitting of cells was performed by aspirating the medium, washing the cells with PBS and incubating them with 1% trypsin - EDTA at 37°C until detachment. The trypsin reaction was then neutralised with the addition of fresh media. The cell suspension was centrifuged at 1500 rpm for five minutes and passaged into a tissue culture flask (Corning, New York, USA) at the desired dilution. When required for subsequent experiments, cells were detached from the flasks using the previously described method. Cells were counted using a hemocytometer and seeded into plates (Corning, New York, USA) at the appropriate density, depending on each experiment's requirement.

2.2.4 Drug Treatments

The DNA-PKcs inhibitor NU7441, Chk1 inhibitor prexasertib (LY2606368) and p38 MAPK inhibitor SB203580 were purchased from Selleck Chemicals LLC (Houston TX, USA). All inhibitors were re-constituted in dimethyl sulfoxide (DMSO), aliquoted and stored at -20 °C. Each inhibitor (concentrations varied and are stated in the results sections) or DMSO as a vehicle control was added 1 hour prior to cisplatin treatment (1 mg/ml, Hammersmith Hospital Pharmacy). Drug treatments were performed by replacing the medium with the appropriate volume of drug which diluted to the desired concentration in the fresh medium. Cells were then incubated for 24 hours at 37 °C, 5% CO₂.

2.2.5 Apoptosis and Viability Assays

Cells were seeded into 96 well plates at a density of 1×10^4 cells/well (primary tumour cells) or 5×10^3 cells/well (immortalised cell lines). After either 24 or 48 hours, cells were treated with inhibitor or vehicle for 1 hour, followed by the addition of cisplatin as previously described, or media as a control. Levels of cell viability and caspase-dependent apoptosis within each well were determined using ApoTox-Glo™ Triplex Assay (Promega, US), following the manufacturer's instructions. This assay measures the protease activity of viable cells using a peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC). The fluorogenic, cell-permeant substrate penetrates intact cells and is cleaved by the live-cell protease activity. This reaction leads to a fluorescent signal generation, linear to the viable cell numbers. The live-cell protease is inactivated once the cell loses its membrane integrity and the protease leaks into the surrounding medium. To determine the cell viability, 10 µl of viability reagent containing GF-AFC substrate was added into each well, the plates were incubated for a minimum of 30 minutes at 37 °C, and fluorescence was measured by the FLUOstar OPTIMA (BMG LabTech, UK). In the apoptosis part of the assay, cells were lysed using Caspase-Glo® 3/7 reagent. This

reaction was then followed by caspase cleavage of DEVD substrate and production of luciferase substrate called aminoluciferin. Luciferase then oxidised the aminoluciferin and caused a generation of a luminescent signal proportional to the amount of caspase activity. For this purpose, 50 µl of Caspase-Glo® 3/7 reagent was added per well and incubated for a minimum of 30 minutes at room temperature. Luminescence was then measured using LUMIstar OPTIMA.

2.2.6 Slide Preparation for Tumour Cellularity Determination

Tumour cellularity is defined as the estimated proportion of tumour cells within each primary cell culture. Tumour cells for cellularity determination were grown parallel to the phenotypic assays performed for each case. Tumour cells from each deposit were seeded into 12 well plates at a density of 3×10^4 cells/well and allowed to adhere for 48 hours. Cells were then trypsinised with 1x Trypsin (Sigma-Aldrich) diluted with 0.02% EDTA (Sigma-Aldrich) and collected with the addition of 700 µl of complete media. The cells were centrifuged at 1500 rpm for 5 minutes, the supernatant discarded and resuspended in 600 µl of PBS. Three Superfrost Plus slides (VWR) were labelled per tumour deposit, attached to the funnel and placed into the cytospin centrifuge, then 200 µl of each cell suspension was added to a funnel attached to the corresponding labelled slide and centrifuged at 200 rpm for five minutes to attach the cells to the slide. Slides were air dried and stored at -20° C prior to staining.

2.2.7 Immunofluorescent Microscopy for PAX8

Cytospin slides were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, CA) at room temperature for 10 minutes. Slides were washed three times with 1x1xTris Buffered Saline (TBS), blocked and permeabilised using 10% Normal Goat Serum + 0.5% Triton-X in TBS for 1 hour at room temperature. Slides were then incubated with Pax8 primary antibody

(ab189249, Abcam, UK), diluted at 1:50 in 10% Normal Goat Serum + 0.1% Triton-X with 1xTBS at 4 °C overnight, then washed three times with TBS. After this, slides were incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibodies diluted at 1:1000 in 10% Normal Goat Serum + 0.1% Triton-X with 1xTBS for 2 hours in dark conditions at 37 °C. Slides were washed three times in 1xTBS and mounted using ProLong[®] Gold Antifade Mountant with DAPI (Life Technologies, UK), then left 24 hours at room temperature in dark conditions to dry. Coverslips were then sealed with nail polish and stored at 4°C in dark conditions. Imaging was performed using TCS SP5 confocal microscope (Leica, Germany).

2.2.8 Tumour Cellularity Determination

A minimum of 10 random microscopy fields of each tumour sample was captured at 40x magnification using confocal microscopy. The total number of cells was acquired by counting all nuclei stained by DAPI in the blue channel, while the number of tumour cells was obtained by manually counting positive cells according to Paired box 8 (PAX8) staining in the green channel. The percentage of tumour cells for each tumour deposit in culture was then determined.

2.2.9 Image Analysis Using CellProfiler

CellProfiler 2.2.0 (<http://www.cellprofiler.org/>) was used to analyse the PAX8 staining intensity. A pipeline was constructed based on individual modules that were placed in a sequential order. Cell cultures which have >60% cellularity according to the manual counting were included in this analysis. For confocal analysis, both component channels (blue and green) of each image were imported and cell nuclei (blue channel) were detected as primary objects. Clumped nuclei were resolved using the ‘intensity’ module. The detected nuclei were used as

seeds for PAX8 staining detection in green channel. In each image, the mean intensity of nuclei and PAX8 staining were measured. The measured PAX8 intensity was normalised to DAPI intensity. Samples were included for further analysis if the filtered positive cells measured above 60, or the filtered total cells measured above 100, after automatic counting using CellProfiler.

2.2.10 BCA Protein Assay

Protein concentration was quantified using the colorimetric based micro-bicinchoninic acid reagent (BCA) assay (Pierce, Thermo Scientific, Massachusetts, USA). This assay works by detecting Cu^{+1} , as a result of Cu^{+1} reduction by protein in alkaline condition. The bonding of two BCA molecules with one Cu^{+1} forms a purple-coloured reaction product, which shows a strong absorbance at 562 nm, linear with increasing protein concentrations. The micro-BCA assay preparation was carried out based on the manufacturer's instructions. A set of nine diluted bovine serum albumin (BSA) standards was prepared, with a concentration range between 0 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$. The working reagent was prepared by combining 25 parts of Reagent MA, 24 parts of Reagent MB and one part of Reagent MC. Briefly, 150 μL of each diluted standard and sample were pipetted into a microplate well. The equal volume of the working reagent was added to each well, and the plate was incubated for 2 hours at 37 °C. The plate was then allowed to cool at room temperature and the absorbance was measured at 562 nm using OPTImax plate reader (Molecular Devices, Wokingham, UK).

2.2.11 Reverse-Phase Protein Array (RPPA)

Primary tumour cells from each deposit were seeded into 6 well plates at a density of 2×10^5 cells/well and allowed to adhere for 48 hours, after which media was removed, and cells were washed once with PBS. Cells were then treated with either 25 μM cisplatin or media and

incubated for 4 hours at 37 °C. Cells were lysed using RPPA lysis buffer (1% Triton X-100, 50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM Na pyruvate, 1 mM Na₃VO₄, 10% glycerol with protease (Roche Diagnostics, UK) and phosphatase inhibitors (Calbiochem, Germany)) for 20 minutes on ice and shaken every five minutes. Cells were then scraped and centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatant was collected and stored at -80 °C for subsequent RPPA analysis. Protein concentration was determined using the BCA assay (Pierce, Thermo Scientific, Massachusetts, USA, see section 2.2.10). Prior to shipping to the Functional Proteomics RPPA Core Facility, MD Anderson Cancer Center, the protein concentration was adjusted and denatured through the addition of 4x sodium dodecyl sulphate (SDS) sample buffer (40% Glycerol, 8% SDS, 0.25M Tris-HCl and 10% beta-Mercaptoethanol).

In the RPPA Core Facility, lysates were diluted in five two-fold serial dilutions with lysis buffer containing 1% SDS and spotted on nitrocellulose-coated slides (Grace Biolab) using an Aushon Biosystems 2470 arrayer (Figure 2.2). Each slide also contained negative and positive controls spots, which were prepared from dilution buffer and mixed samples lysates, respectively (Neeley, Baggerly and Kornblau, 2012). Slides were probed with $\pm$ 300 validated primary antibodies and matched biotinylated secondary antibodies. In the antibody validation process, a single or dominant band on Western blotting is necessary. As well, the Pearson correlation coefficient between the RPPA and Western blotting should be greater than 0.7. The signal received was augmented using the Dako Cytomation-catalyzed system (Dako) and visualised by a secondary streptavidin-conjugated HRP and 3,3'-diaminobenzidine (DAB) colorimetric reaction. Array-Pro Analyzer software (Media Cybernetics) was used to scan, analyse and quantify the slides to generate spot intensity as level 1 data. The protein level in log₂ scale was estimated using SuperCurve GUI (Hu *et al.*, 2007). Raw spot intensity values were altered to

rectify spatial bias before model fitting with positive and negative controls. Level 2 data was generated from slides with the highest quality control (QC) levels (minimum 0.8 on 0 -1 scale, (Ju *et al.*, 2015)). Level 3 data was obtained by normalising protein concentration using the median-centering method, which was adjusted across samples according to the linear expression values, using the median expression levels of all antibodies used to compute a loading correction factor for the individual sample (Gonzalez-Angulo *et al.*, 2011).

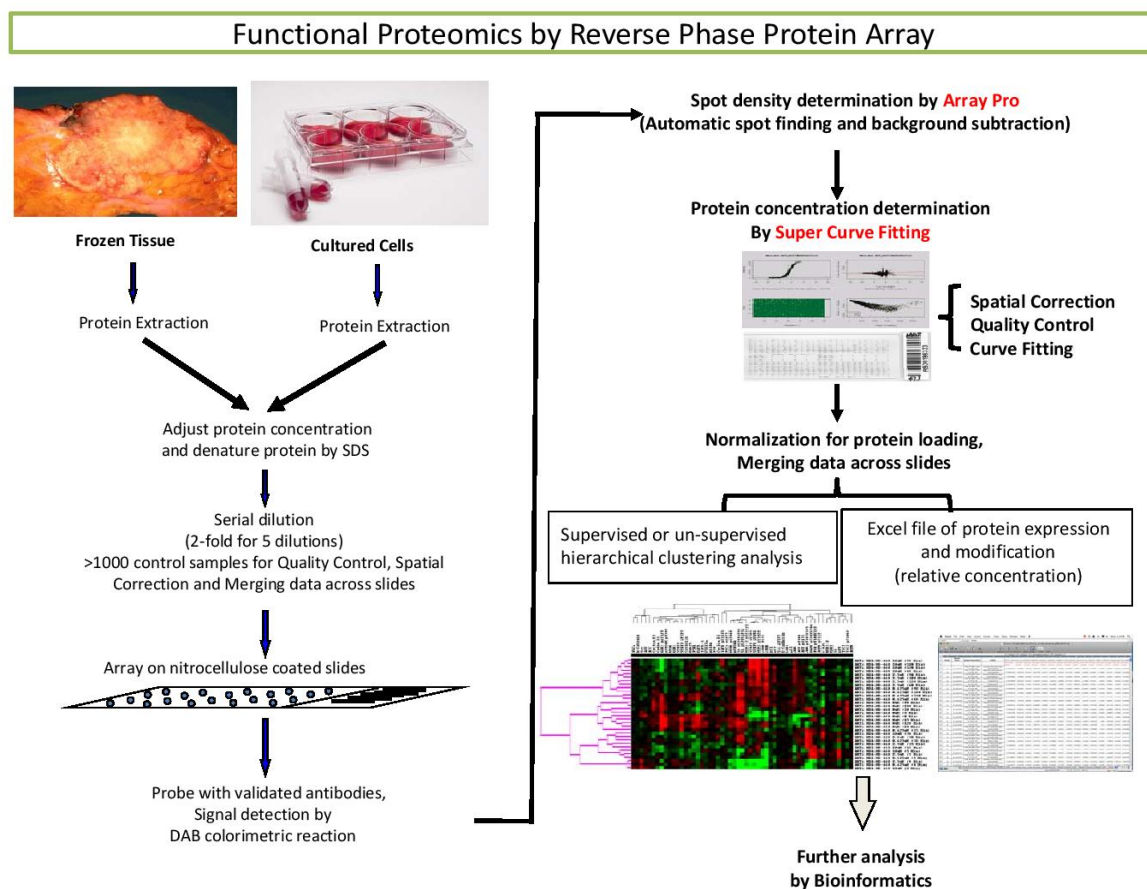


Figure 2. 3: Summary of RPPA Work-flow. Following protein extraction, protein lysates were denatured by SDS. Samples were printed on nitrocellulose-coated slides and probed with validated antibodies. Spot density was measured using Array-Pro following determination of protein concentration using SuperCurve fitting. Figure from www.mdanderson.org

2.2.12 Western Blotting

Equal amounts of protein lysates (5-10 µg) were diluted in distilled water and mixed with 4X Laemmli buffer (0.04% bromophenol blue; 20 mM Tris-HCl, pH 6.8; 40% glycerol and 8 %

SDS), 40X 2 M Dithiothreitol (DTT) and denaturated at 95°C for 5 minutes. Samples were loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Table 2.2) and separated at 100 V for 1-2 hours using a Mini-Protean Tetra Cell (Bio-Rad, Hempstead, UK) in 1x SDS running buffer (248 mM Trizma base, 1.92 M glycine, and 1% w/v SDS). Proteins were then transferred onto nitrocellulose membranes (median pore size 0.2 µm, Bio-Rad) by electroblotting using chilled (4°C) 1X Transfer Buffer (10X Transfer Buffer: 250 mM Tris Base and 1.92 M glycine) with 10% methanol at 90V for 1 hour. Membranes were blocked with 5% non-fat milk in 1x TBS-T (blocking buffer) at room temperature for 1 hour with agitation. Membranes were probed using antibodies diluted with either blocking buffer or 5% BSA in 1xTBS-T (Table 2.3) overnight, with shaking, at 4 °C. Following the incubation with primary antibody, membranes were washed three times in 1xTBS-T for 10 minute periods, at room temperature. Incubation with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Stockport, UK) diluted in blocking buffer (1:2000 dilution) was performed for 1 hour at room temperature. After incubation, membranes were then washed in TBS-T as previously described. Membranes were visualised with Immobilon ECL reagent (Millipore, Watford, UK) and exposed to photographic films (SLS, Nottingham, UK). The films were developed using a Konica Minolta SRX101 developer (Konica Minolta, Basildon, UK). Films were scanned, and the positive bands were analysed by ImageJ.

Table 2. 2 Gel components for western blotting

Resolving gel (10%)	
Stock Solutions	Volume
ddH ₂ O	5.9 ml
30% acrylamide/0.8% bis-acrylamide	5 ml
1.5M Tris pH 8.8	3.8 ml
10% SDS	150 µl
10% ammonium persulfate	150 µl
TEMED	6 µl
Stacking gel	
Stock Solutions	Volume
ddH ₂ O	4.2 ml
30% acrylamide/0.8% bis-acrylamide	1 ml
1.5M Tris pH 6.8	760 µl
10% SDS	60 µl
10% ammonium persulfate	60 µl
TEMED	6 µl

Table 2. 3 List of antibodies used in western blot analysis

Antibody	Dilution	Size	Isotype	Manufacturer
p38	1:1000 in 5% BSA in TBS-T	43 KDa	Rabbit, Polyclonal	Cell Signalling Technology, Cat. No: 9212
Phospho-p38 MAPK (Thr180/Tyr182)	1:1000 in 5% BSA in TBS-T	43 KDa	Rabbit, Polyclonal	Cell Signalling Technology, Cat. No : 9211
Phospho-HSP27 (Ser82)	1:500 in 5% BSA in TBS-T	27 KDa	Rabbit, monoclonal	Cell Signalling Technology, Cat. No : 9709
Chk1	1: 5000 in 5% milk in TBS-T	56 KDa	Rabbit, Monoclonal	Abcam, Cat. No: ab40866
Phospho-Chk1 (Ser296)	1:500 in 5% BSA in TBS-T	56 KDa	Rabbit, Polyclonal	Cell Signalling Technology, Cat. No: 2349
Vinculin	1: 500 in 5% BSA in TBS-T	124 KDa	Rabbit, Polyclonal	Cell Signalling Technology, Cat. No: 46505
Anti-Rabbit Immunoglobulins	1: 2000 in 5% milk in TBS-T	-	Goat, Polyclonal	Dako, Cat. No: P044801-2

2.2.13 Buffy Coat Isolation

Peripheral blood samples were collected from patients during optimal debulking surgery in standard phlebotomy tubes containing heparin. The samples were transported to the laboratory and processed as soon as possible. The blood was mixed by inverting eight times, then spun in a centrifuge at 1100 x g for 10 minutes, at deceleration rate 9, and acceleration rate 9. This step allowed separation of the plasma, buffy coat (leucocytes and platelets), and erythrocytes. The thin, whitish layer of buffy coat was collected and aliquoted in two tubes. Each tube was labelled with the subject number and type of sample, placed into the box in an upright position and kept in -80 °C.

2.2.14 DNA Extraction from Buffy Coats

Buffy coat samples were thawed at room temperature prior to extraction. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Briefly, each buffy coat sample (200 µl) was poured into a tube containing 20 µl of Proteinase K, and Buffer AL was then added. The tubes were mixed thoroughly by vortexing and incubated at 56 °C for 10 minutes. Ethanol (96–100%) was added to the tubes followed by vortexing again. The mixture was then pipetted into a DNeasy Mini spin column placed into a collection tube. Centrifugation at 6000 x g (8000 rpm) was performed for 1 minute, and the spin column was then placed in a new collection tube. After the addition of Buffer AW1, the tubes were centrifuged for 1 min at 6000 x g. The spin column was placed into a new collection tube, and Buffer AW2 was added, followed by centrifugation for 3 min at 20,000 x g (14,000 rpm). To obtain higher concentrations, DNA was eluted by adding Buffer AE to the centre of the spin column membrane. The tubes were incubated for 1 minute at room temperature (15–25°C), then spun for 1 minute at 6000 x g. DNA concentration was assessed using a Quant-

iTTM PicoGreenTM dsDNA Assay Kit (Thermo Scientific). These DNA samples were used as germline control for sequencing analysis.

2.2.15 DNA Extraction from Tissue

DNA extraction was performed as a service by Imperial College Healthcare Tissue Bank (ICHTB). First, cryotomy was used to collect frozen tumour sections for haematoxylin and eosin staining. For each frozen tumour tissue, tissues larger than 5mm³ were cut into pieces not larger than 3 mm³ and placed into an Eppendorf containing RLT buffer and Retsch cone. The tubes were then placed into 24 well adapters of the TissueLyser II, and the tissue was lysed at 15 Hz for 2 minutes. The ATL buffer was added to the tubes to open cell and nuclear membranes. Proteinase K was then added to denature the hydrolytic DNases enzymes and keep the DNA intact. The tubes were then vortexed and incubated at 56 °C overnight, allowing the tissue to lyse. Once the tissue was completely lysed, the tube was vortexed thoroughly, and Buffer AL was added followed by another mixing. Ethanol (100%) was also added, and the steps described in section 2.2.14 were then carried out on the samples. The DNA was quantified using NanoDrop and QuBit.

2.2.16 DNA Quantitation

A PicoGreen dsDNA quantitation kit which contained Quant-iTTM PicoGreen dsDNA reagents and Lambda DNA standard (100 µg/ml in TE) was obtained from Life Technologies, UK. Tris-EDTA buffer solution (TE buffer, 1.0 M Tris-HCl pH 8.0, containing 0.1M EDTA) and nuclease-free water were purchased from Sigma-Aldrich®, UK. The PicoGreen solution was diluted 200x using 1 x TE before being added to DNA standards and unknown samples. Lambda DNA standard (100 µg/ml) was diluted 50 x using 1 x TE to make a working solution

of 2 µg/ml. The working solution (2µg/ml) was used to make a series of dilutions ranging from 0.05-75 ng/µl of lambda DNA using 1 x TE. Two microliters of DNA standard or unknown samples and 98 µl 1 x TE, plus 100 µl PicoGreen working solutions were pipetted into 96 microplate wells in triplicate. The plate was then incubated for 5 minutes at room temperature, protected from light. Following the incubation, fluorescence was determined using FLUOstar OPTIMA (BMG LabTech, UK) plate reader at 480 nm. The reading from a blank well was subtracted from each well before analysing the data.

2.2.17 Statistical Analysis

Experimental data was analysed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). A paired Student's t-test was performed to reveal the statistical significance between two groups. For cell viability and apoptosis using ApoTox-Glo™ Triplex Assay, cell-free controls were deducted from all other values and resulting values were normalised to vehicle control values. In addition, caspase-3/7 values were normalised to corresponding viability values to account for variation in cell numbers. In addition, all graphical representations of experimental data were generated using this software.

2.3 Bioinformatics Methods

All library processing was performed in the Unix Shell environment. Data visualisation and statistical testing, unless otherwise mentioned, was performed in the R version 3.4.4 programming environment (R Core Team, 2013).

2.3.1 RPPA Analysis

Using the ‘SuperCurve’ method, relative levels for each sample were determined, followed by data normalisation for protein loading and transformation to linear values. Afterwards, data was altered into Log2 values as described previously in section 2.2.11, and finally, median-centred (level 3 data). This data, provided by the RPPA Core Facility, was then used to compare protein expression between control and cisplatin-treated cells. Outlier sample data was excluded from further analysis. Linear regression was used to determine the statistical differences and fold changes in protein expression using the lmFit function in the limma package from Bioconductor (Smyth, Ritchie and Thorne, 2015). Protein-protein interactions and pathway enrichment analysis were then performed using STRING v10 (Szklarczyk *et al.*, 2015).

2.3.2 CNV Analysis

Copy number variation (CNV) of the tumour sample cohort was assessed using Single nucleotide polymorphism (SNP) sequencing at whole genome coverage. The hybridisation of DNA to SNP arrays was performed by the Australian Genome Research Facility, using Illumina HumanOmniExpress-24 arrays and Illumina GenomeStudio 2.0.3 for analysis. There were two main outcome values for each sample from these arrays: the log-2 ratio or Log R ratio (LRR) B-allelic frequency (BAF). The LRR is related to the total probe intensity of a given SNP, relative to a reference database of SNP hybridisation values ($\log_2(\text{observed intensity}/\text{reference intensity})$). The BAF is determined using the ratio of the measured intensities from the two alleles (A and B), which depicts the relative quantity of one allele compared to the other.

2.3.2.1 Pre-processing of the SNP data

The detailed pre-processing pipeline is described in a GitHub repository at <https://github.com/luslab/multiregion-cnv-phylogenetics>. The first step in the analysis was mirroring the BAF along the 0.5 axis (mBAF), to facilitate the identification of allelic imbalance and subsequently remove the non-informative homozygous SNPs. For this purpose, estimations of aberrant cell fraction generated by R package called Allele-Specific Copy Number Analysis of Tumours (ASCAT) were used (Van Loo *et al.*, 2010). In this platform, LRR genomic waviness (patterns of signal intensities across all chromosomes, in which different samples may show different magnitudes of waviness) were detected and corrected from all arrays. The copy number profiles underwent further pre-processing including genomic segmentation and compression of copy number calls. The ASCAT also provide the percentage of aberrant tumour cells, tumour ploidy, and the whole-genome allele-specific copy number profile of each chromosome per patient (ASCAT profile, in FASTA format).

2.3.2.2 Quantification of Heterogeneity using MEDICC

The FASTA formatted files were interpreted using the MEDICC (Minimum Event Distance for Intra-tumour Copy-number Comparisons) algorithm (Schwarz *et al.*, 2014). This algorithm was used to generate the clonal evolution of tumour samples in each patient, where normal tissue or blood samples were used as the root of phylogenetic trees. The phylogenetic extrapolation in MEDICC consists of three steps: 1) phasing major and minor alleles ((the larger and the smaller of the two copy-numbers, respectively), 2) prediction of pairwise distances between samples followed by tree inference, and 3) ancestral genome reconstruction. The reconstruction of ancestral genomes also determines the lengths of the last branches of tree, which agree to the number of events between genomes. The phylogenetic trees were used

to compute an evolutionary value for each case, called the clonal expansion index (CEI). This index measures the degree of local clustering in a phylogenetic tree.

2.3.3 Statistical Analysis

The significance of Kaplan-Meier survival plots was measured using the log-rank test. The hazard ratio measurement in survival analysis was determined using the Cox proportional hazards regression model. Correlation was measured using the Spearman or Pearson coefficients (as described in the results section). The significance level was defined at $p < 0.05$ for all analyses. Multiple testing corrections for P-values were performed where appropriate using the false discovery rate (FDR) method.

Chapter 3

Investigation of Phenotypic

Heterogeneity in HGSOC

3.1 Introduction

Ovarian cancer typically presents at a disseminated stage and is treated with a combination of platinum and taxane-based therapy. Many HGSOC patients initially respond to chemotherapy but ultimately relapse with the development of resistance to platinum chemotherapy (Bowtell *et al.*, 2015). It has been proposed that tumour heterogeneity contributes to this drug resistance and treatment failure (Salomon-Perzyński *et al.*, 2017). Growing data reporting ovarian cancer diversity at a molecular level indicates that a heterogeneous model is required to unravel the molecular biology of HGSOC. Further, the association between the diverse molecular profiles contributing to intra-tumoural heterogeneity and the prognosis for ovarian cancer patients also remains unclear. In addition, there is limited evidence demonstrating variable responses to chemotherapy within a single patient across various tumour sites. Therefore, further studies defining phenotypic signatures are necessary to predict the outcome of patients with advanced ovarian cancer and thus improve treatment strategy.

Gaining a better understanding of tumour biology allows for the development of novel therapeutic targets to improve treatments, such as agents that target DNA repair pathways. Previous work from our laboratory has demonstrated the importance of the AKT pathway in platinum-resistant disease and showed that the inhibition of DNA-PKcs resensitises platinum-resistant ovarian cancer cells to cisplatin treatment (Stronach *et al.*, 2011). The potential therapeutic target, DNA-PKcs, is further investigated in this chapter to assess its ability to enhance tumour response to cisplatin, especially in advanced HGSOC disseminated throughout the abdominal cavity.

Primary cells extracted from ovarian cancer patients are noticeably different from immortalised cell lines from a similar origin (O'Donnell et al., 2014). They provide an invaluable experimental model which is more relevant to the patient's condition. The ability to establish a primary cell culture is crucial, especially for creating a model system that predicts patient response to various drugs. This approach would greatly improve the development and implementation of novel treatment for personalised medicine of cancer patients. Numbers of methods for culturing primary ovarian cancer extracted from ascites and solid tumours have been developed (summarised in Cunnea & Stronach, 2014). Work in this chapter investigates the extent of inter and intra-tumoural heterogeneity in advanced ovarian cancer using extracted primary tumour cells, and the relationship of this heterogeneity to chemotherapy response and clinical outcome.

3.2 Results

3.2.1 Sample characteristics

Any human biological samples were collected in agreement with the terms of informed consent. Tumours from primary and metastatic sites were collected from 27 patients with advanced ovarian cancer from the start of this ITH study. The median age was 60 years (interquartile range (IQR) 50-69 years). Thirteen patients (48%) were > 60 years old. The histopathological results including tumour grade and stage for each patient were obtained 2 weeks after surgery. Eligible patients had a histologically confirmed diagnosis of HGSOc. All patients were FIGO stage III, or above, indicating the advancement of the disease. The majority of patients were stage III (67%).

The intraoperative tumour dissemination pattern in each patient was recorded according to IMO, a validated descriptive system developed especially for ovarian cancer (Fotopoulou *et*

al., 2016). There are three IMO levels which divide the abdomen into lower (level 1), middle (level 2) and upper (level 3) space (Figure 3.1). Each of these three levels is further divided into three fields, constituting 9 IMO fields in total. This sub-classification exists to provide a comprehensive picture of the tumour pattern in the abdomen and accurate staging. The median number of tumour-involved fields based on IMO was five (IQR 4-6). Sixteen patients (59%) had tumour involvement in more than four IMO fields, indicating a multifocal tumour-dissemination pattern. The extent of residual disease was recorded for each patient following surgery, when patients were either described as optimally debulked (the largest remaining tumour mass measuring less than 1 cm in diameter, macroscopically) or sub-optimally debulked (with macroscopic residual disease measuring 1 cm or greater). The majority of patients in this study were operated to tumour free. The clinicopathological summary of patients included in this ITH study is shown in Table 3.1.

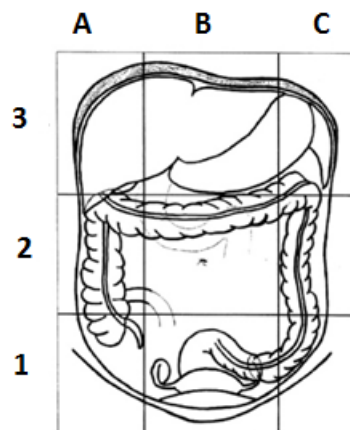


Figure 3. 1: Intraoperative Mapping of Ovarian Cancer (IMO). During primary debulking surgery, tumour samples are taken and mapped according to the IMO

Table 3. 1 Clinicopathological details for all patients in this study

Variable	Patients (N=28)
Median age (IQR)	60 (50 – 69)
FIGO stage at initial diagnosis	
III	18 (67%)
IV	9 (33%)
IMO fields involvement median (IQR)	5 (4 - 6)
Postoperative residual tumour	
<1 cm	25
>1 cm	2
PFS median (IQR) in months	14.5 (10.25 – 25.5)
OS median (IQR) in months	32.5 (21.25 – 41.75)
Median number of deposits collected (IQR)	8 (6 - 10)

3.2.2 Establishment of Short-Term Primary Cultures

In total, 237 primary deposits and 10 ascites samples were included in this study. Isolation and cultures of tumour deposits were successfully carried out in all cases. Figure 3.2 A shows the original image of each deposit of a case in our cohort. The optimal condition for dissociation of viable HGSOC cells for primary culture and the further downstream applications were modified from Langdon et al. and Sueblinvong et al and optimised in our laboratory (Cunnea, unpublished data). Briefly, the samples were mechanically dissociated into 1 mm pieces using crossed scalpels (Langdon, 2004), enzymatically digested for 60 minutes with protease Dispase II to separate the cells (Sueblinvong *et al.*, 2012), and then mechanically disrupted via passage through a 70 µm mesh to eliminate noncellular components. To remove erythrocytes from samples, RBC lysis buffer was used.

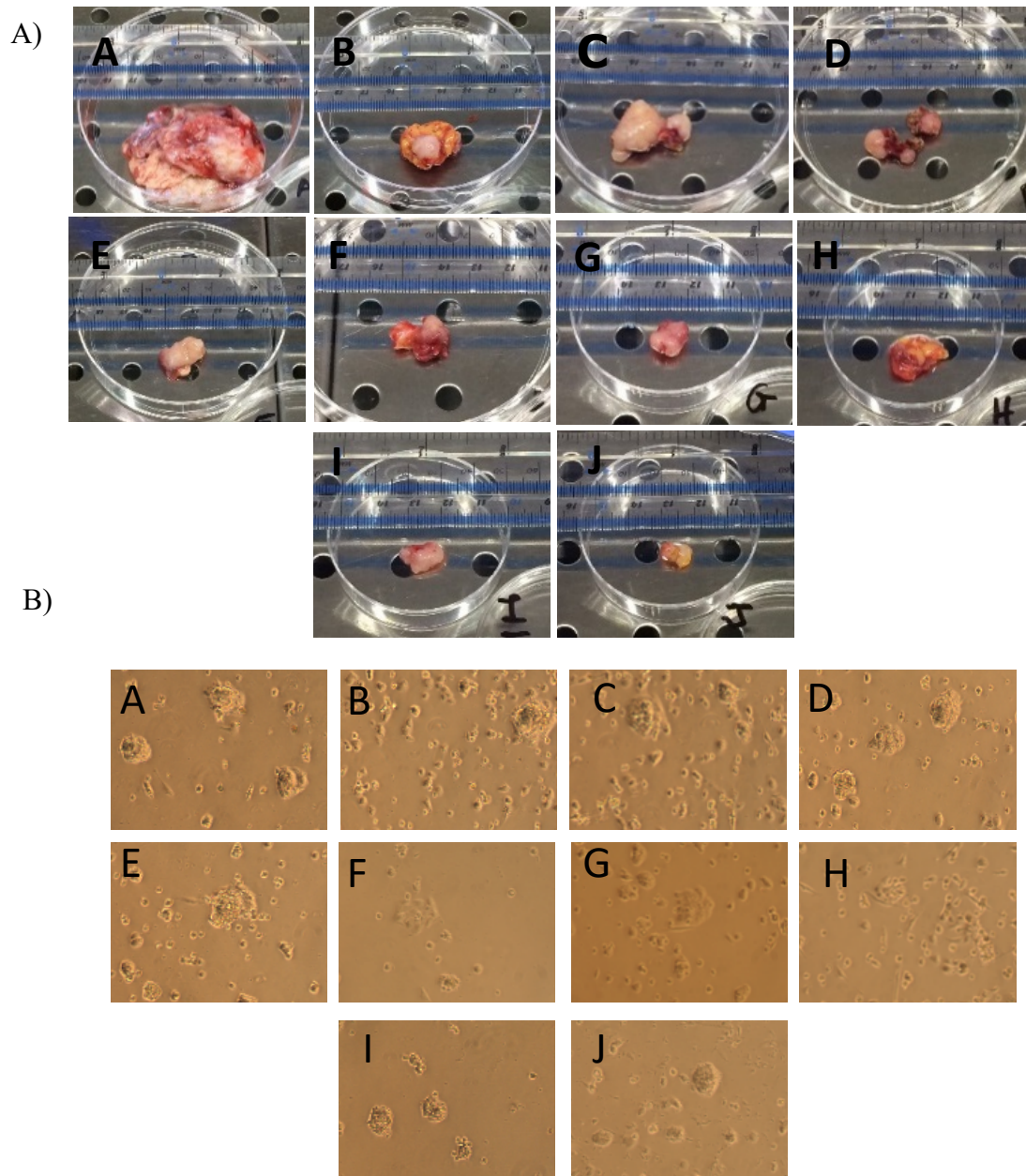


Figure 3. 2: Establishment of primary tumour cell cultures. A) Images of each deposit of case T15 033. B) The resulting primary cell culture from each deposit. A: Primary tumour (ovary), B: Omentum, C: Right Diaphragm, D: Liver Capsule, E: Recto-sigmoid, F: Bladder, G: Abdominal wall, H: Spleen, I: Left Diaphragm, J: Mesenteric Root. Tumour cells, which are the main population of cells in all the samples, typically have a rounded shape and tend to grow in a cobblestone morphology. Sample H contains many fibroblasts, which are recognizable by their elongated cytoplasm. Images were taken with inverted microscope Eclipse TE2000-U (Nikon, Japan) at 20x by Irene Rius Ruiz (MRes Cancer Biology 2014).

Figure 3.2 B shows tumour cells that were extracted from the initial tumour deposits and cultured for 24 hours post isolation. Tumour cells tended to grow in clusters with cobblestone morphology. Fibroblasts with elongated morphology were present in a small number of

samples and were removed by selective trypsinisation. Immune cells such as lymphocytes are the smallest compared to other cells and were easily removed as they tend not to attach to the culture flask. After 48 hours, cells in the culture were detached and split for phenotypic assay (section 3.2.3) and also for the tumour cellularity determination in each culture (section 3.2.6). Lastly, the cells also used for RPPA assays (chapter 4).

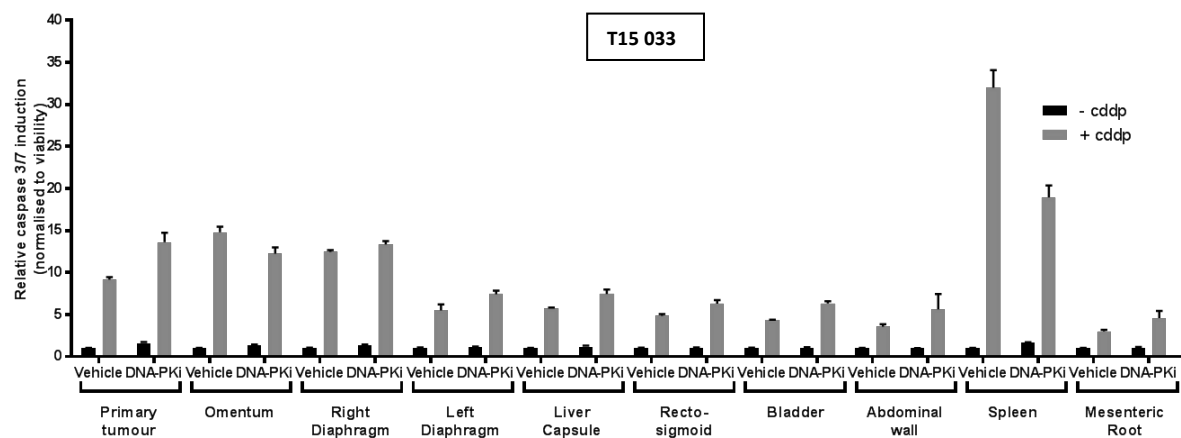
3.2.3 Phenotypic Heterogeneity Present in Multiple Deposits

To investigate the response of each tumour deposit to cisplatin and the DNA-PK inhibitor NU7441, tumour cells harvested from disseminated HGS tumours were cultured and treated in triplicate with either 10 μ M DNA-PK inhibitor or dimethyl sulfoxide (DMSO, vehicle control) for 1 hour followed by addition of 25 μ M cisplatin or media for 24 hours. The cells in each culture are a mixed of tumour and stromal cells, with majority are tumour cells. Tumour cellularity was determined by PAX8 positive staining (detailed explanation about culture cellularity determination is in section 3.2.5-3.2.6).

Figure 3.3 displays the different apoptotic responses from two primary cases (T15033 and T15058) in this study. There was a large variation in apoptotic response to cisplatin in case T15033, from a small four-fold increase (mesenteric root) up to thirty-fourfold increase (spleen). In case T15058, each tumour deposit had a good response to the combination of cisplatin and DNA-PKcs inhibitor (more than a five-fold increase, based on Stronach et al.'s finding), except the para-aortic lymph node cells. This was due to the low population of cells in the sample which was confirmed by tumour cellularity determination (Table 3.5). In this case, two different tumour samples were taken from the right diaphragm, which had a different consistency macroscopically. The first had a more solid (1st sample) and the second a much softer consistency (2nd sample). These samples were cultured and treated separately; the first

sample had a better overall apoptotic response to cisplatin and DNA-PK inhibition compared to the second sample. Additionally, increased apoptosis induction was observed following combination treatment with cisplatin and the DNA-PK inhibitor, thus indicating the potential of DNA-PK inhibition as a therapeutic strategy in treating HGSOC. The wide range of responses to cisplatin with or without the DNA-PK inhibitor across all deposits is indicative of the intra-tumour heterogeneity found in HGSOC in many patients.

A)



B)

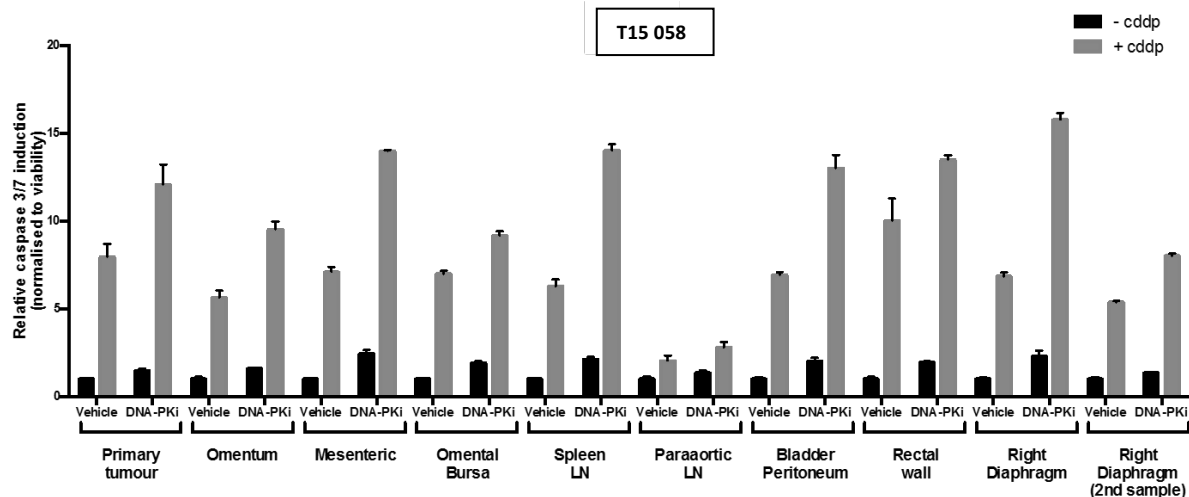


Figure 3. 3: Results of the phenotypic assay for different tumour deposits from case T15 033 (A) and T15 058 (B). Following short term primary cell cultures, cells were treated with cisplatin $\pm$ DNA-PK inhibitor for 24 hours. Apoptotic and viability assays were performed using ApoTox-GloTM Triplex Assay. Error bars indicate the standard deviation for the three replicates measured. - cddp = without cisplatin, + cddp = with cisplatin treatment.

All patients were tracked and monitored for relapse or death. The majority of the patients in this study received platinum-based chemotherapy during their treatment, except one who died in the hospital following surgery due to liver failure. Chemotherapy response was determined by serial clinical examinations, serum CA125 levels and computed tomography (CT) scans at certain intervals and at the completion of chemotherapy unless new onset symptoms appeared and needed immediate evaluation. Figure 3.4 (A and B) shows a summary of the phenotypic assays performed to date in this project. These results clearly demonstrate phenotypic inter- and intra-tumour heterogeneity in response to cisplatin treatment in all patients examined. Amongst 27 patients, 75% have relapsed with the majority of patients relapsing in a sensitive time-frame (13 patients). Seven relapses occurred within a resistant period and only one patient had refractory disease. Eleven patients have died, with a median overall survival of 32 (21–42) months (Table 3.1).

A Student paired t-test was performed, comparing patients' phenotypic apoptotic responses to cisplatin only versus cisplatin + DNA-PK inhibitor to investigate the effect of DNA-PK inhibitor in each patient (Table 3.2). The addition of the DNA-PK inhibitor to the cisplatin treatment significantly increased the apoptotic response in 13 patients. Almost all patients who had resistant and refractory relapse benefitted from the addition of DNA-PK inhibitor with enhanced apoptotic response observed. This interesting finding supports the idea that DNA-PK inhibition could resensitise platinum-resistant ovarian cancer cells to cisplatin treatment.

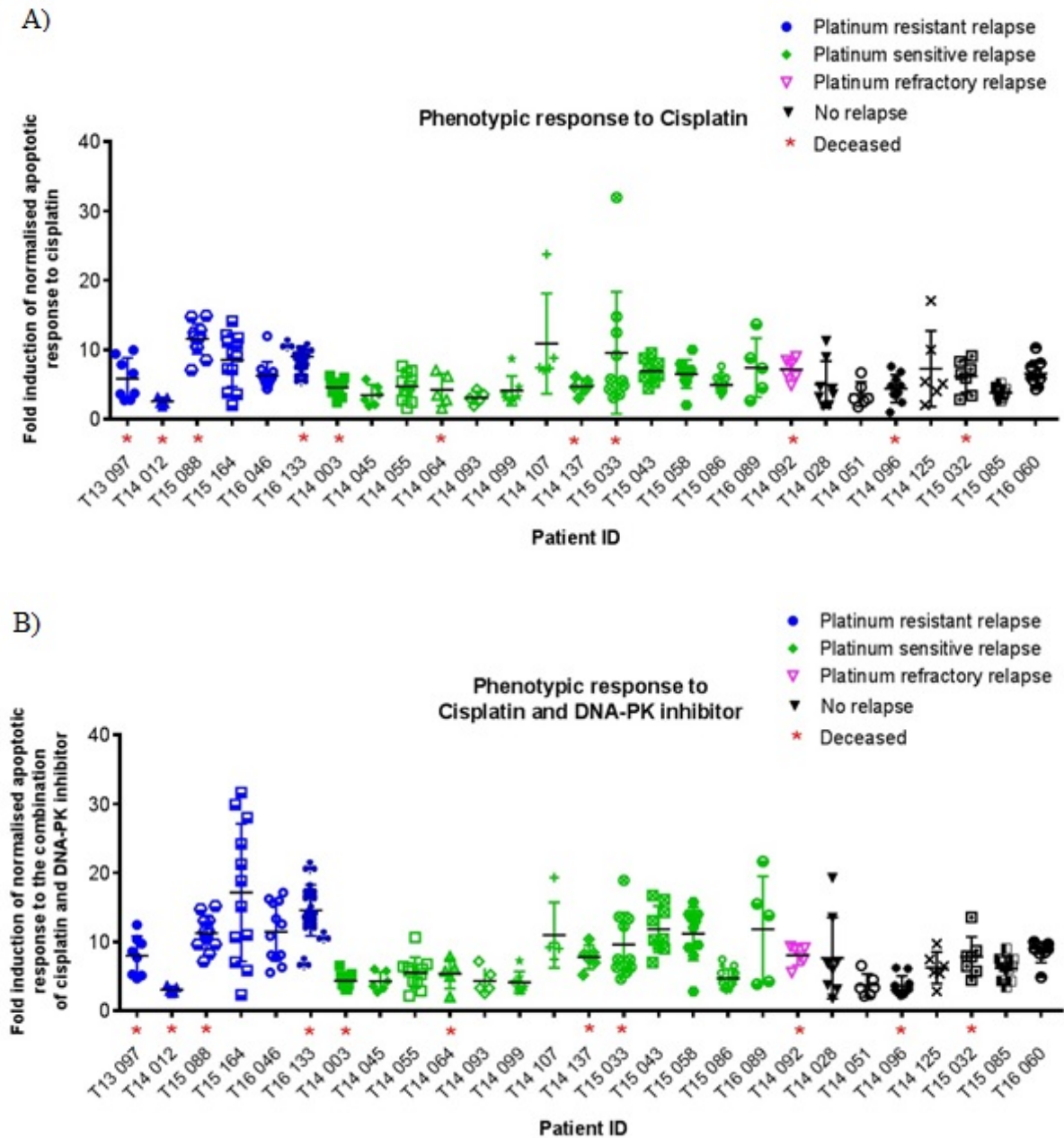


Figure 3. 4: Phenotypic inter- and intra-tumour heterogeneity in response to drug treatment was observed in all patients examined. Tumour cells extracted from disseminated tumour deposits (depicted by a symbol for each tumour) were treated with cisplatin (A) and DNA-PK inhibitor (B) in 2D cell culture for 24 hours and apoptosis/viability assays performed. Data is displayed as fold induction in relative caspase 3/7 activity (apoptosis normalised to viability) compared to vehicle control.

Table 3. 2 Paired Student's t-test of cisplatin vs cisplatin+DNA PK inhibitor

No	Patient ID	P value	Clinical Response to Platinum
1	T13 097	0.002	Platinum resistant relapse
2	T14 012	0.006	
3	T15 088	0.054	
4	T15 164	0.002	
5	T16 046	0.003	
6	T16 133	0.0003	
7	T14 003	0.434	Platinum sensitive relapse
8	T14 045	0.104	
9	T14 055	0.056	
10	T14 064	0.070	
11	T14 093	0.066	
12	T14 099	0.959	
13	T14 107	0.937	
14	T14 137	0.004	
15	T15 033	0.979	
16	T15 043	<0.0001	
17	T15 058	0.0003	
18	T15 086	0.409	
19	T15 089	0.059	
20	T15 092	0.007	Platinum refractory relapse
21	T14 028	0.026	No relapse
22	T14 051	0.432	
23	T14 096	0.023	
24	T14 125	0.616	
25	T14 032	0.144	
26	T15 085	0.0002	
27	T16 060	0.004	

For all analyses, bold text signifies statistical significance (p<0.05)

Figure 3.5 (A and B) shows the distribution of deposits collected based on the IMO location. Most of the samples came from region B1 (lower level abdomen) where the female reproductive organ is located, and region B2 (middle level abdomen) where the omentum is located. About 86% of patients in this study cohort had omental metastasis.

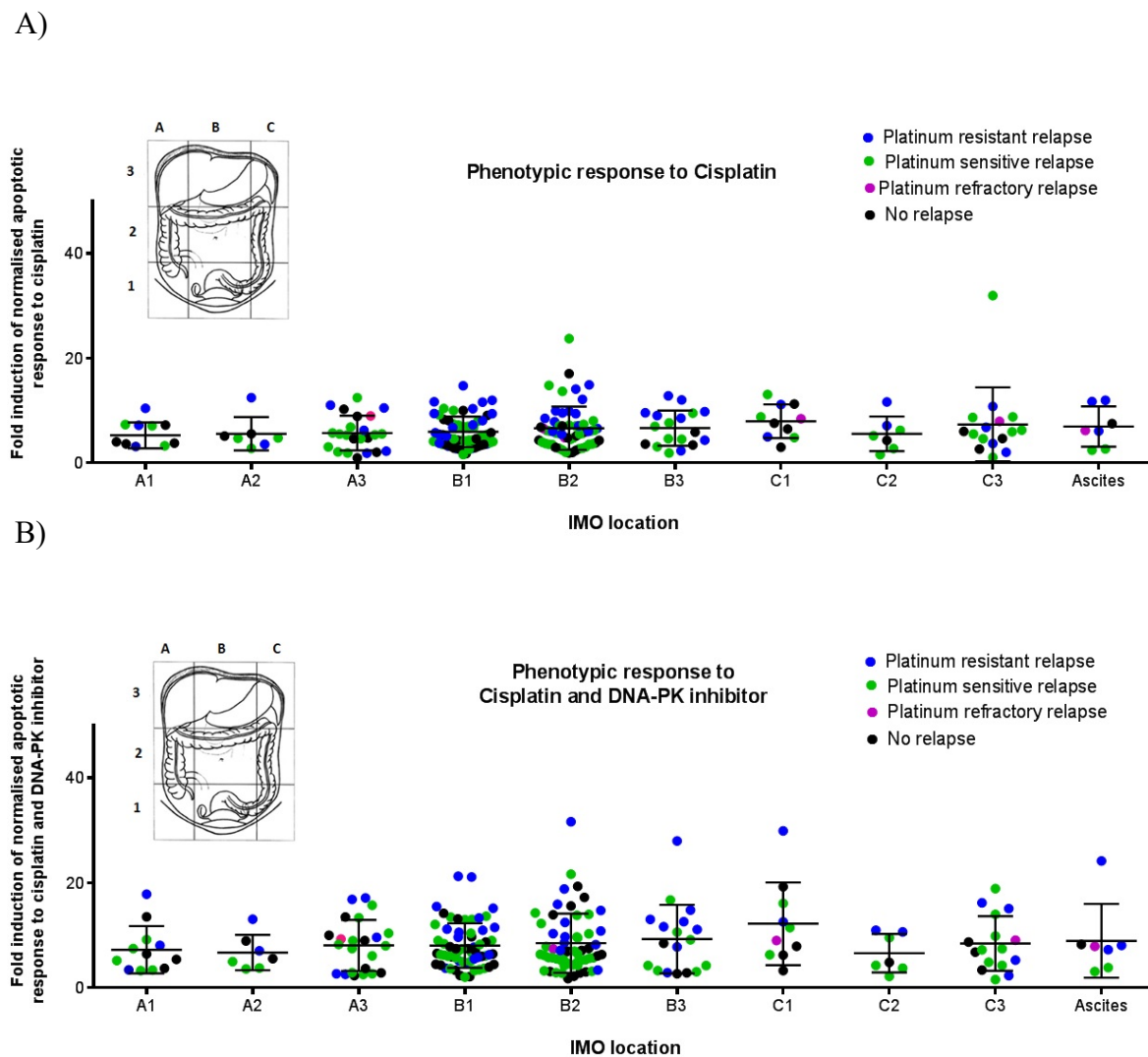


Figure 3. 5: Disseminated HGSOC tumours from different deposits according to IMO displayed differing apoptotic responses to drug treatment. Tumour cells extracted from disseminated tumour deposits (depicted by a symbol for each tumour) were treated with cisplatin (A) and DNA-PK inhibitor (B) in 2D cell culture for 24 hours and apoptosis/viability assays were performed. Data is displayed as fold induction in relative caspase 3/7 activity (apoptosis normalised to viability) compared to vehicle control.

Relapse samples from four patients were collected during this study cohort. However, the phenotypic assays were only performed in two cases due to the small size of the biopsy collected (Figure 3.6 and Appendix Figure A1). One relapse sample set was a sensitive relapse (T14093, Appendix Figure A1), and the other was a resistant relapse. Figure 3.6 shows the phenotypic assay of a primary case (T14137) and its corresponding relapse case which was taken when the patient had a resistant relapse (T17067). The phenotypic assay result for the primary case showed variation in apoptotic responses; all had a four-fold increase, except for the right pelvic sidewall sample. This high apoptotic response to platinum is in concordance with the positive response that most HGSOC patients have at the beginning of the treatment. In contrast, the phenotypic assay of the relapse case showed a more homogenous, low apoptotic response to cisplatin observed over all deposits. All of the relapse cases showed the same pattern (low responses to platinum). In the case of T17067, the addition of DNA-PK inhibitor did nothing to the cultures but in the case of T16111, DNA-PK inhibitor enhanced apoptosis with cisplatin.

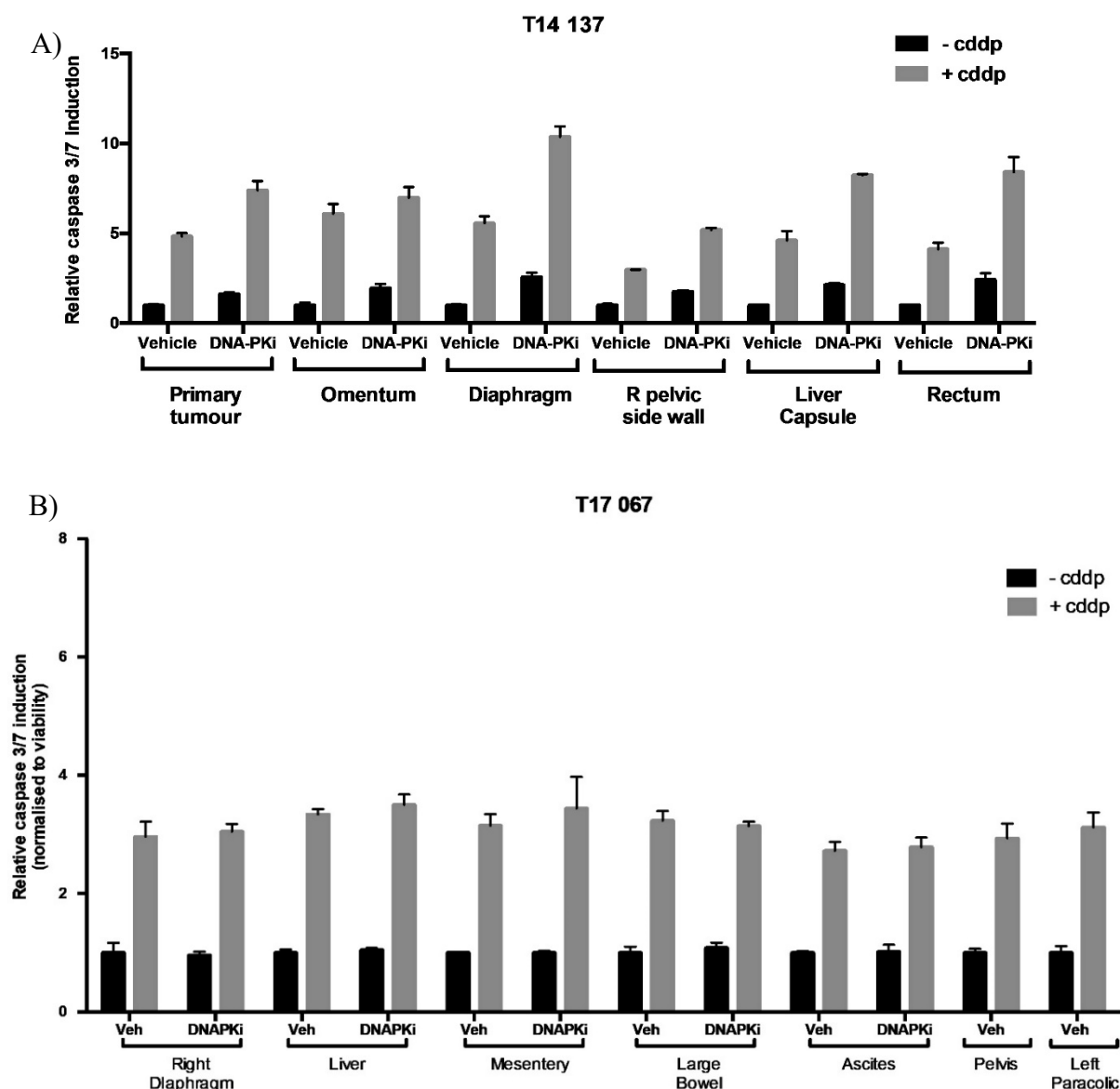


Figure 3. 6: Temporal phenotypic heterogeneity was observed in response to cisplatin treatment. Following short term primary cell cultures, cells were treated with cisplatin (25 μ M) for 24 hours. Apoptotic and viability assays were performed using ApoTox-Glo Triplex Assay. Tumour cells from different metastatic sites displayed differing apoptotic responses to cisplatin in a primary case (A) and corresponding phenotypic assay result from a relapse case (B). - cddp = without cisplatin, + cddp = with cisplatin treatment.

3.2.4 Relationship of Phenotypic Heterogeneity to Clinical Outcome

By categorising patients based on their clinical sensitivity to platinum, assessment was made regarding the accuracy of *in vitro* cisplatin apoptotic response as a biomarker of actual clinical outcome. Figure 3.7 (A) shows the coefficient of variation (CV) based on sensitivity of tumour

deposits from relapsed patients in our study cohort to cisplatin. The CV is the ratio of the standard deviation to the mean, which measures relative variability. In the figure below, it can be observed that a low CV score is more likely to occur in a platinum-resistant relapse, and a high CV score in a platinum-sensitive relapse. However, no significant difference between the two groups was evident due to low patient numbers. The same analysis was carried out on the CV of the combined cisplatin plus DNA-PK inhibitor response and the clinical response to cisplatin (Figure 3.7 (B)). Still, there was no obvious difference between the resistant and sensitive relapse group.

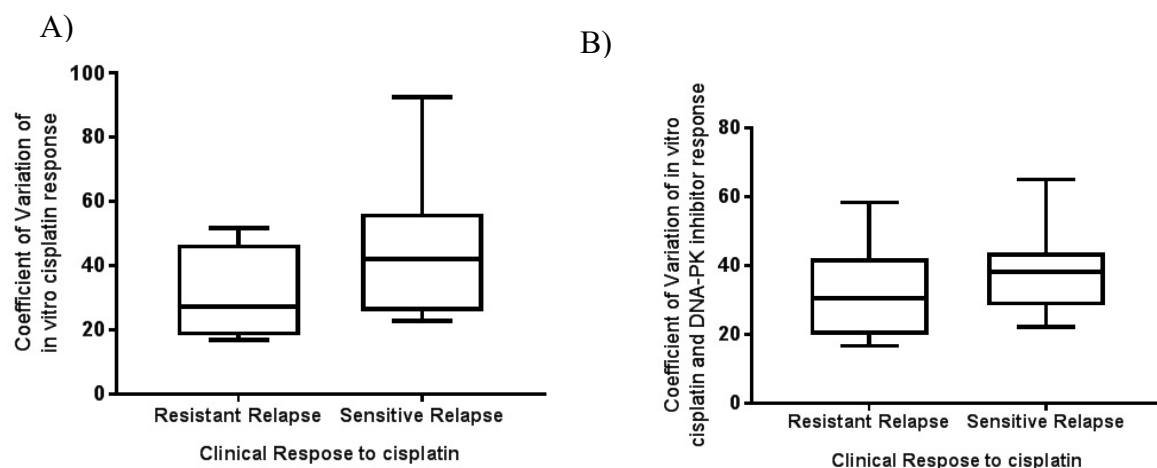


Figure 3. 7: Association between phenotypic tumour response to platinum chemotherapy and type of relapse. There was no statistical relationship between heterogeneity in *ex vivo* cisplatin response (A) and cisplatin+DNA-PK inhibitor response (B) and the relapse status. However, a trend of low CV in response to cisplatin appears in the resistant relapse group and high CV in the sensitive relapse group (A).

Further assessment of how *in vitro* heterogeneity in cisplatin response predicts actual clinical response to platinum was performed using AUC calculations from ROC curve analysis. A ROC area of 0.752 indicates the CV of *in vitro* tumour culture sensitivity's potential ability to predict clinical sensitivity to platinum, with significant p-value ($p=0.024$), as shown in Figure 3.8. In this analysis, platinum sensitivity was defined as no relapse or relapse six months after the

cessation of chemotherapy. Patients who had never received platinum therapy were excluded from analysis.

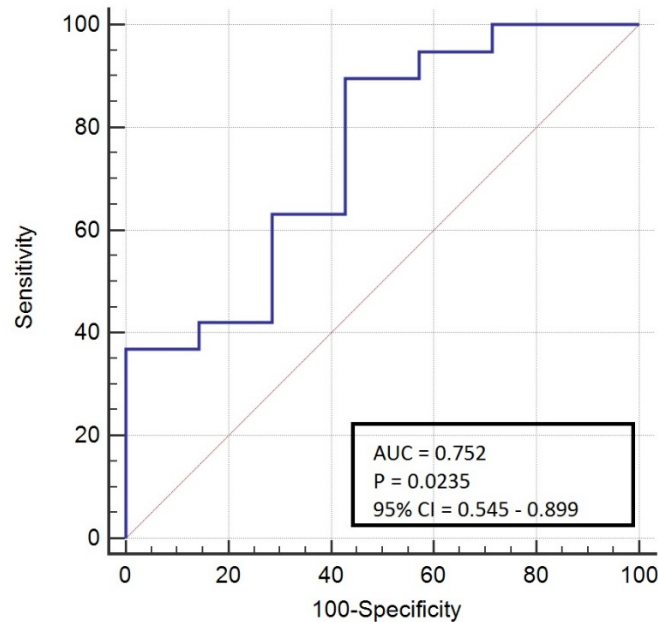


Figure 3. 8: *In vitro* culture sensitivity to cisplatin as a predictor of clinical sensitivity to platinum-based chemotherapy. The analysis was performed using the CV value of tumour cell response to cisplatin and limited to patients who went on to receive platinum-based chemotherapy.

To assess the value of *ex vivo* phenotypic responses as prognostic of survival markers, univariate Cox proportional hazards modelling was carried out, as well as, for the potential confounding variables (Table 3.3). Survival data was calculated from the date of primary debulking surgery. Regarding the PFS, patients who were alive with tumour progression or who died of cancer were considered uncensored, whereas patients who were alive without tumour progression at follow up were considered censored. Regarding the OS, patients who had died at follow-up (from any cause) were uncensored, whereas patients alive at follow-up were censored.

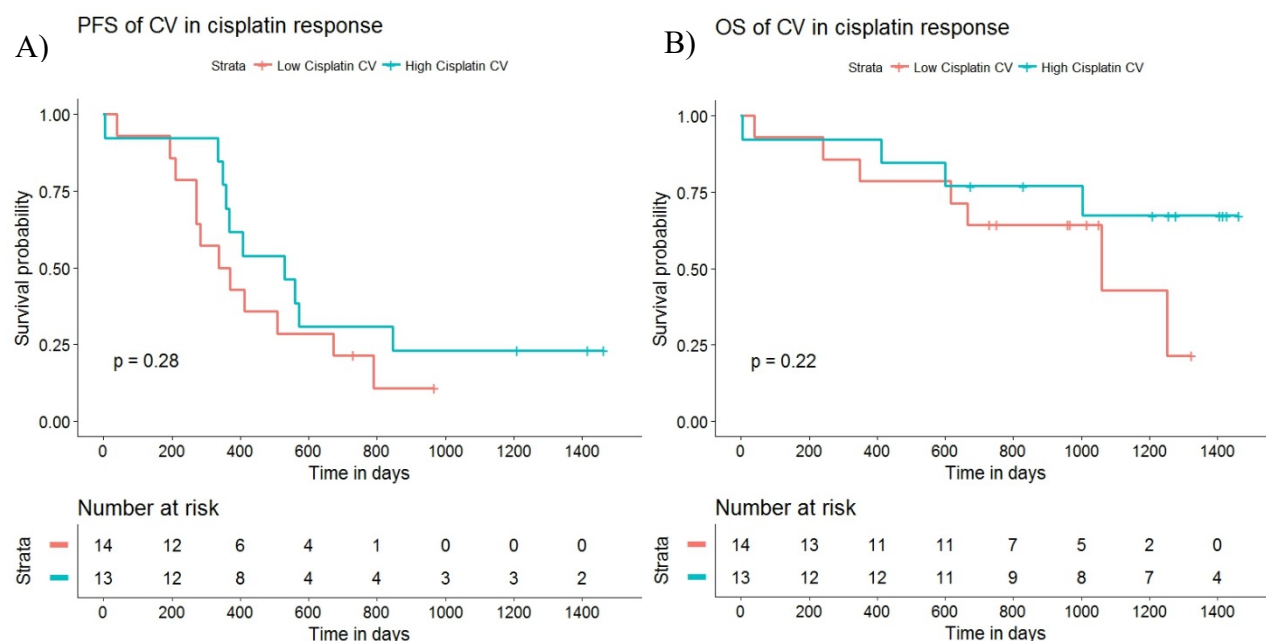


Figure 3. 9: Kaplan-Meier survival curves for patients, stratified by CV of cisplatin response obtained from *in vitro* primary cultures. Univariate analyses for (A) PFS and (B) OS.

Median CV was used as a cut off to divide the patients: low CV and high CV categories for the dichotomous analysis. The median PFS of the low CV group was 370 (271-702) days and 469 (345.5-938) days for patients with a high CV of *in vitro* platinum response. The median OS for the low CV group was 961 (641.5-1057) days and 1108 (554-1410) days for the high CV group. There were no statistically significant differences in PFS between the low and high CV group in this study cohort ($p = 0.28$). However, from Figure 3.9 (A) it is apparent that the high CV group had better PFS than the low CV group, which matched our previous analysis (Figure 3.7 (A)). The same trend appears in the OS curve, where the low CV group has poorer OS compared to the high CV group, although the difference is not statistically significant ($p = 0.22$).

The same analysis was performed to assess the value of heterogeneity in combination therapy (cisplatin and DNA-PK inhibitor) in relation to HGSOC patients' survival. The median PFS of the low CV group was 349 (271-620) days and 545 (339.8-938) days for patients with a high CV in response to *ex vivo* cisplatin and DNA-PK inhibitor. The median OS for the low CV group was 969 (641.5-1057) days and 1020 (554-1347) days for the high CV group. The same trend is observed in Figure 3.10, where the low CV group has a lower PFS compared to the high CV group ($p = 0.18$). It can also be observed that the high CV group has better OS, although it is not statistically significant ($p = 0.31$). In multivariate Cox proportional hazards modelling, none of the variables significantly affected both the PFS and OS in our study cohort as summarised in Table 3.3. These statistically insignificant results could be due to the low number of samples.

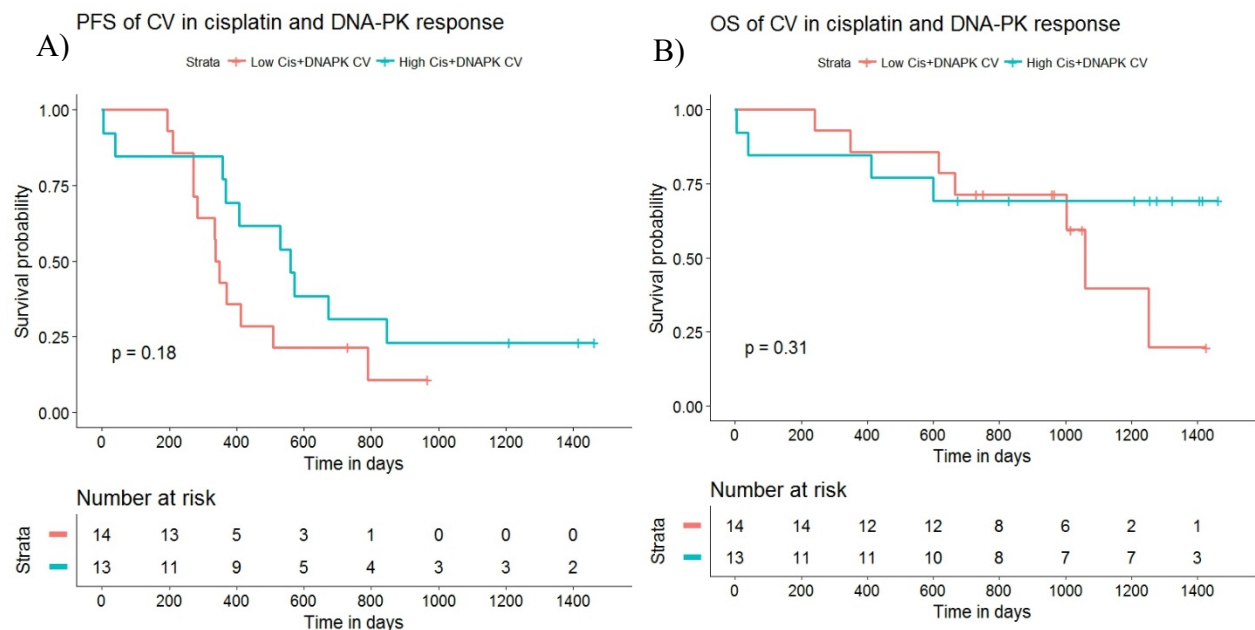


Figure 3. 10: Kaplan-Meier survival curves for patients stratified by CV of cisplatin and DNA-PK response obtained from *in vitro* primary cultures. Univariate analyses for (A) PFS and (B) OS.

Table 3. 3 Univariate analysis of phenotypic responses and clinical factors known to be of prognostic importance in HGSOc

<i>Continuous Univariate analysis</i>				
<i>Clinical outcome</i>	Variable	HR	95%	p-value
<i>PFS</i>	CV of cisplatin response	0.98	0.96 - 1.01	0.158
	CV of cisplatin+DNAPK inhibitor response	0.98	0.95 - 1.01	0.183
<i>OS</i>	Age	1	0.97 - 1.03	0.943
	CV of cisplatin response	0.98	0.95 - 1.02	0.345
	CV of cisplatin+DNAPK inhibitor response	0.94	0.89 - 1.00	0.065
	Age	1	0.95 - 1.04	0.889
<i>Dichotomised Univariate analysis</i>				
<i>Clinical Outcome</i>	Variable	HR	95%	p-value
<i>PFS</i>	CV of cisplatin response (high vs low)	0.63	0.27 - 1.47	0.283
	CV of cisplatin+DNAPK inhibitor response (high vs low)	0.56	0.24 - 1.31	0.18
	Age (> 60 vs ≤60)	0.95	0.41 - 2.20	0.907
	FIGO stage (IV vs III)	1.89	0.75 - 4.79	0.179
<i>OS</i>	CV of cisplatin response (high vs low)	0.46	0.13 - 1.63	0.231
	CV of cisplatin+DNAPK inhibitor response (high vs low)	0.53	0.15 - 1.86	0.319
	Age (> 60 vs ≤60)	0.61	0.18 - 2.09	0.429
	FIGO stage (IV vs III)	1.99	0.57 - 6.96	0.28
<i>Multivariate analysis</i>				
<i>Clinical Outcome</i>	Variable	HR	95%	p-value
<i>PFS</i>	CV of cisplatin response (high vs low)	0.51	0.20 - 1.34	0.175
	Age (> 60 vs ≤60)	1.42	0.54 - 3.73	0.474
	FIGO stage (IV vs III)	2.11	0.82 - 5.47	0.124
<i>OS</i>	CV of cisplatin response (high vs low)	0.43	0.11 - 1.70	0.229
	Age (> 60 vs ≤60)	0.80	0.21 - 3.01	0.745
	FIGO stage (IV vs III)	2.43	0.66 - 8.94	0.182

3.2.5 Optimisation of Immunofluorescent Staining to Determine Tumour Cellularity

In total, eight antibodies were optimised to obtain optimal reproducibility in determining tumour cellularity of each tumour deposit (summarised in Table 3.4). A combination of ovarian tumour cell lines or tumour, fibroblast, and mesothelial cells from ascites were placed onto glass slides by cytopspin and used to optimise the staining of each antibody. The antibodies used were CA125, epithelial cell adhesion molecule (EpCAM), WT1, and PAX8 to stain the tumour cells; cluster differentiation 90 (CD90) and Anti-fibroblast surface protein (1B10) to stain fibroblasts; and calretinin and thrombomodulin to stain mesothelial cells.

Table 3. 4 The different antibodies used in this study

No	Markers	Result	Target
1.	CA125 (Calbiochem CA1004)	Positive staining (cell surface staining)	Tumour cells
2.	PAX8 (Abcam ab189249)	Positive staining (nuclear staining)	Tumour cells
3.	EpCAM (Abcam ab124825)	Positive staining (cytoplasmic staining)	Tumour cells
4.	WT1 (Abcam ab96792)	Positive staining (nuclear and cytoplasmic staining)	Tumour cells
5.	Anti-fibroblast surface protein -1B10 (Abcam ab11333)	Too much background	Fibroblast
6.	CD90 (Calbiochem CP28)	Positive staining (cell membrane staining)	Fibroblast
7.	Thrombomodulin (Abcam ab109189)	Too much background	Mesothelial cells
8.	Calretinin (Dako M7245)	Positive staining (cytoplasmic staining)	Mesothelial cells

CA125 is a high-molecular-weight glycoprotein. We used this marker because it is expressed in 85% ovarian serous adenocarcinoma (Høgdaal *et al.*, 2007). The second marker, WT1, is a

tumour suppressor gene involved in the structural and functional development of the gonads in the female reproductive system. This marker is positively expressed in approximately 90% of primary ovarian carcinomas and more frequently found in the serous subtype of ovarian cancer, it also has been used to differentiate malignancy of ovarian origin from malignancy with other primary sites (Hylander *et al.*, 2006). EpCAM is a transmembrane glycoprotein which is expressed specifically in epithelial and epithelial-derived neoplasm, including ovarian cancer. This marker is overexpressed in almost 70% of ovarian cancer and associated with aggressive behaviour and also decreased survival (Spizzo *et al.*, 2006; Bellone *et al.*, 2009). The final marker examined in the panel of antibodies recommended for ovarian cancer cell identification is PAX8, which is a member of the human paired box genes family (PAX1-PAX9) - a transcription factor essential for embryonic development of Müllerian organs. This marker also found to be highly expressed in 80% of HGSOc patients (Di Palma *et al.*, 2014). Additionally, PAX8 allows the distinction between Müllerian and non-Müllerian origin in the case of an ovarian metastatic malignancy that could derive from other organ (Mhawech-Fauceglia *et al.*, 2012) .

Both anti-fibroblast surface protein 1B10 and CD90 were optimised in this study to identify fibroblast cells. Anti-fibroblast surface protein 1B10 can identify human fibroblasts, tissue macrophages, peripheral monocytes, and fibroblast cell lines (Degraef *et al.*, 2002). CD90 is a cell adhesion molecule which is expressed on fibroblast, neurons, and activated endothelial cells (Kisselbach *et al.*, 2009). The presence of mesothelial cells in the tumour deposits was detected using calretinin or thrombomodulin. Calretinin, which is normally expressed in neurons in the central and peripheral nervous system, has been recognised as a reliable marker of mesothelial cells (Ito, Okamoto and Sasano, 2005). Thrombomodulin, a natural anticoagulant factor, is also expressed in mesothelial cells. Further, one study revealed that the

combined use of calretinin and thrombomodulin has relatively high sensitivity and specificity for distinguishing peritoneal mesothelioma and serous ovarian carcinoma (Takeshima *et al.*, 2008).

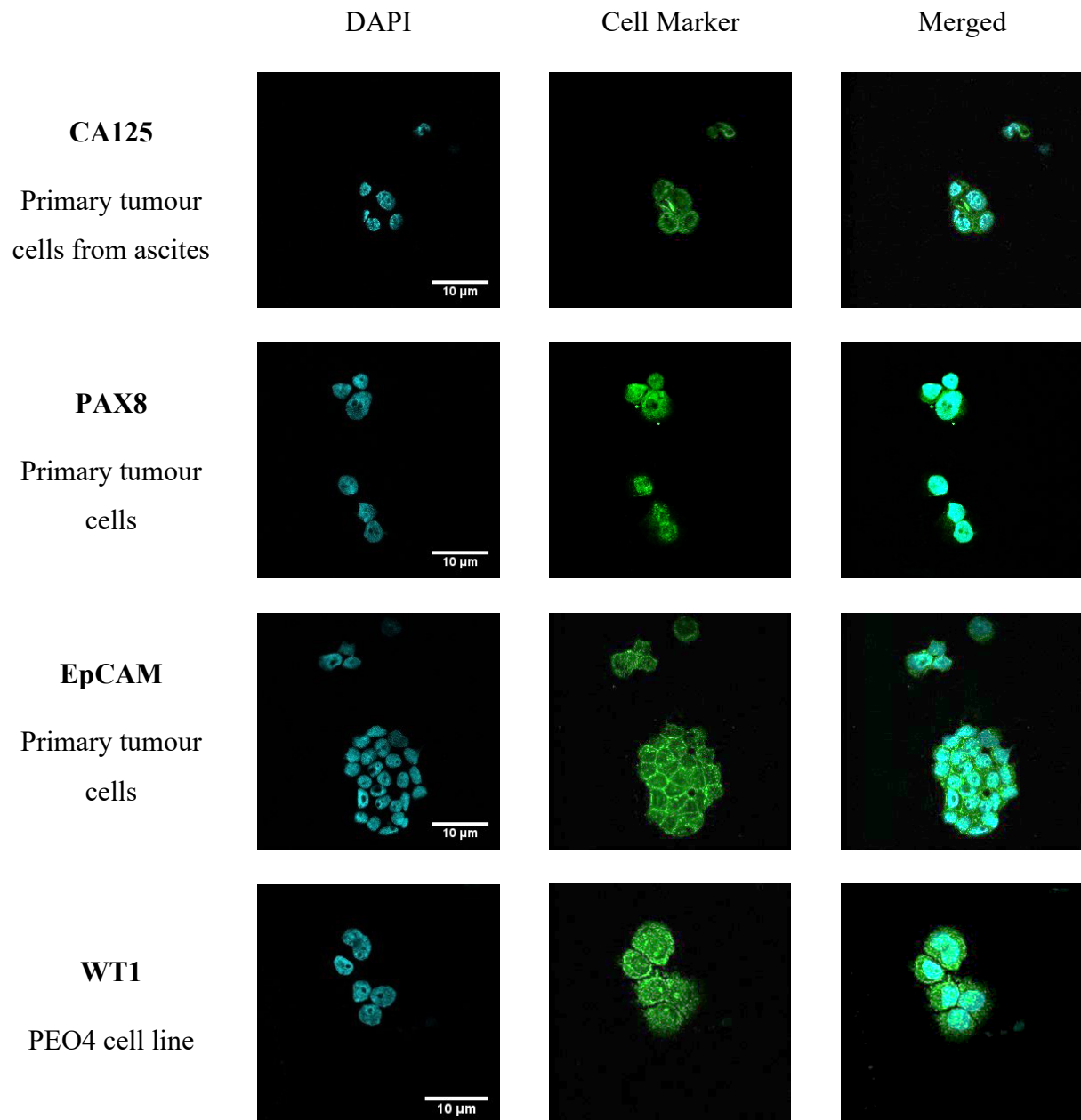


Figure 3. 11: Immunofluorescent staining using different tumour markers. Cells were stained according to the optimum immunofluorescent protocol for each marker. Cellular staining was visualised using the secondary Alexa Fluor 488 (green), and nuclei were detected by DAPI (blue) staining under 63x magnification

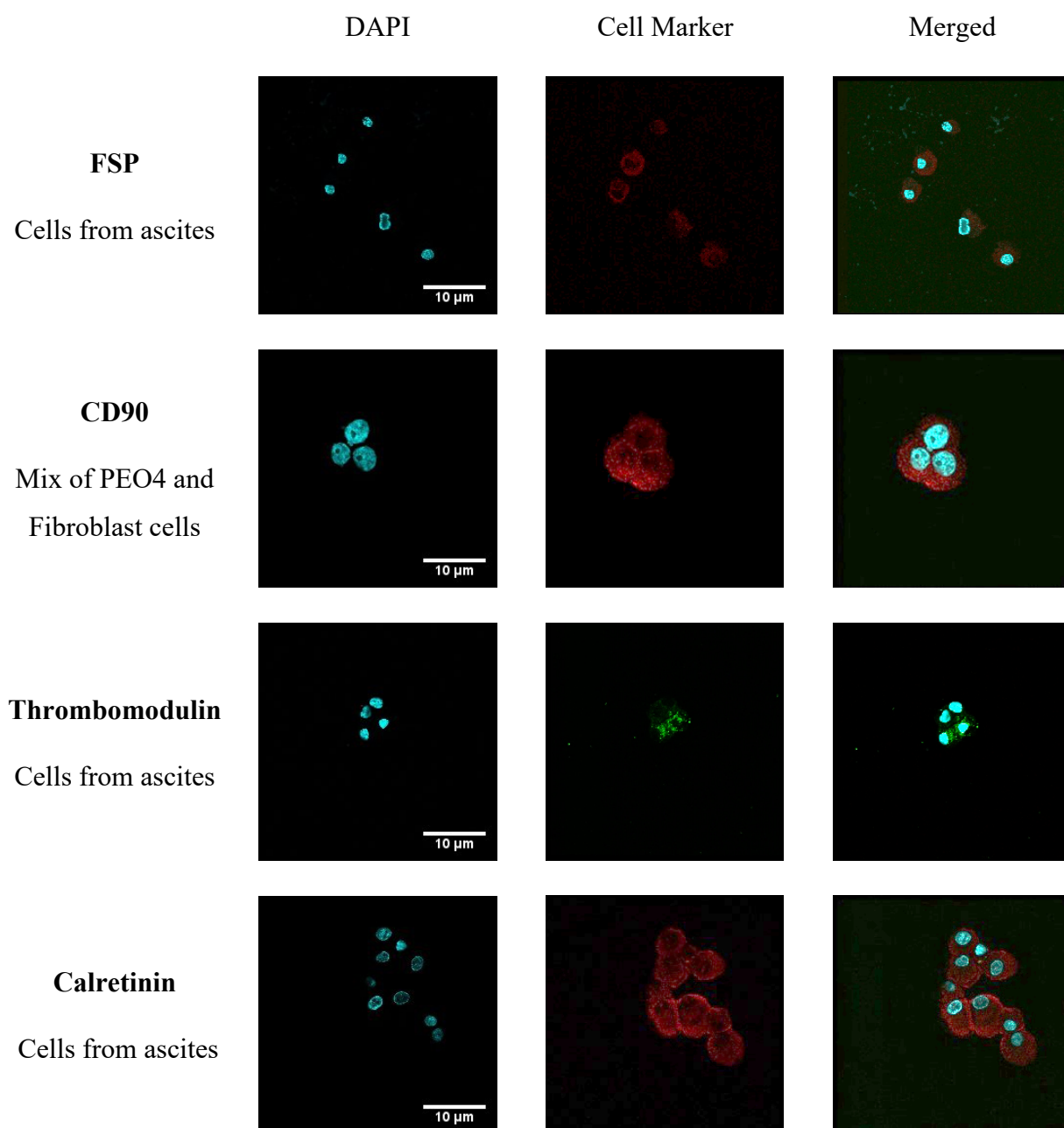


Figure 3. 12: Immunofluorescent staining using different fibroblast and mesothelial markers. Cells were stained according to the optimum immunofluorescent protocol for each marker. Cellular staining was visualised using the secondary Alexa Fluor 488 (green) and Alexa Fluor 546 (red) fluorescent labelled antibodies, and nuclei were detected by DAPI (blue) staining under 63x magnification.

The optimisation experiments consisted of determining the optimum antibody concentration, fixation method, permeabilisation conditions, blocking method, and secondary antibody

concentration. Different protocols to determine each parameter were investigated together with negative controls (stained with secondary antibody only). The optimal protocol was determined as one that produced the strongest specific immunofluorescence signal with the minimum non-specific background fluorescence. Figure 3.11 and 3.12 display representative images of detection of antigens using optimised immunofluorescent staining protocols. Successful staining conditions were achieved for PAX8, CA125, CD90, EpCAM, calretinin and WT1 when staining singly. In contrast, significant background staining was observed in slides stained with anti-fibroblast surface protein and thrombomodulin. Several factors may contribute to the presence of non-specific or background staining, including the types of cells being stained, the concentration of the antibody used for staining, the incubation time and temperature during staining and the type of secondary antibody (fluorochrome) used to detect antibody binding. The dual staining of a tumour-cell marker and stromal-cell marker in different combinations was also optimised to distinguish and quantify the number of tumour cells in our primary cell preparations from tumours in the ITH study. However, the optimal protocol was not achieved to a satisfactory standard, therefore staining was commenced to detect PAX8 tumour-cell markers to estimate tumour cellularity as this marker produced the strongest immunofluorescence signal using DAPI staining of nuclei to exclude unstained non-tumour cells.

3.2.6 Tumour Cellularity Determination for Each Tumour Culture

To assess the specificity of PAX8, an immunising peptide-blocking experiment using PAX8 human recombinant protein was performed. Prior to the staining protocol, the PAX8 antibody was neutralised by incubating with an excess of PAX8 human recombinant protein, so that the PAX8 antibody that is bound to the blocking peptide was no longer available to bind to the

epitope present in the cell. The result of this experiment is displayed in Figure 3.13, which confirmed the specificity of PAX8.

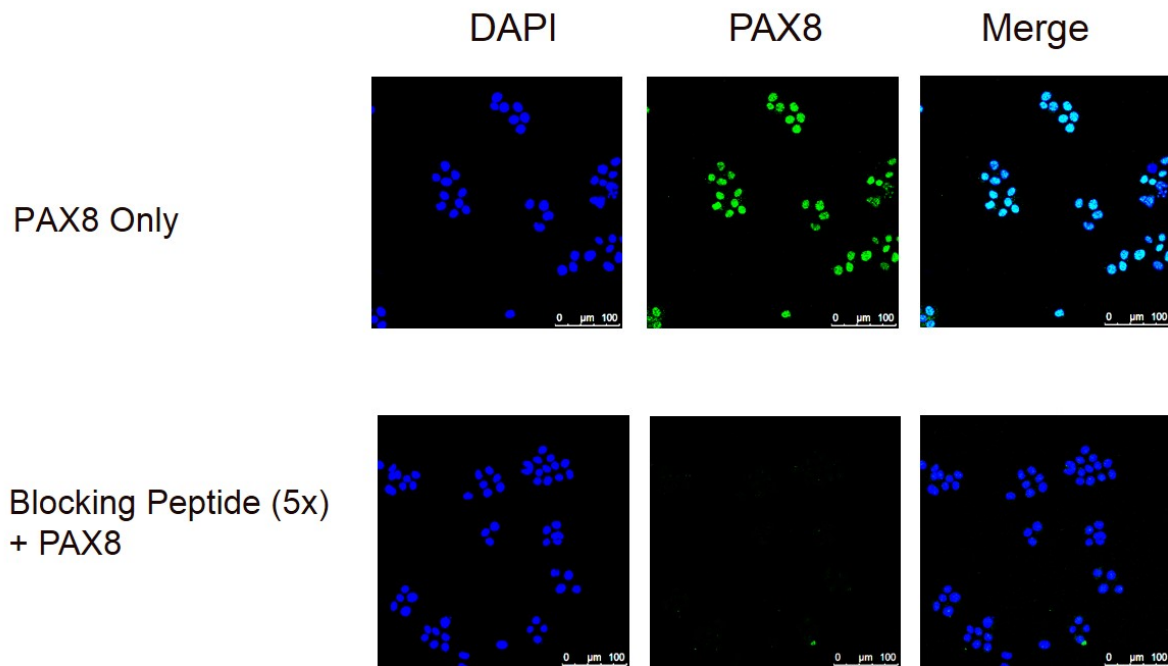


Figure 3. 13: PAX8 expression was blocked following the addition of PAX8 human recombinant protein as blocking peptide. Cellular staining was visualised using the secondary Alexa Fluor 488 (green) fluorescent-labelled antibodies, and nuclei were detected by DAPI (blue) staining under 40x magnification.

Using antibodies against PAX8, all tumour cultures were assessed by staining for PAX8 to quantify the number of tumour cells in the primary cell preparations from tumours in the study. The PEO4 cell line was used as positive control for the PAX8 marker, as well as negative control by incubating with secondary antibody only. Figure 3.14 shows an example of immunofluorescent staining from the T15058 case. Cells were classified as tumour cells if they showed positive nuclear staining for PAX8. A minimum of 10 random microscopy fields of each tumour sample was captured at 40x magnification. The total number of cells was observed by counting all nuclei (DAPI positive), and the number of tumour cells was obtained by manually counting positive cells according to PAX8 staining in each field, according to which the percentage of tumour cells for each tumour deposit was determined.

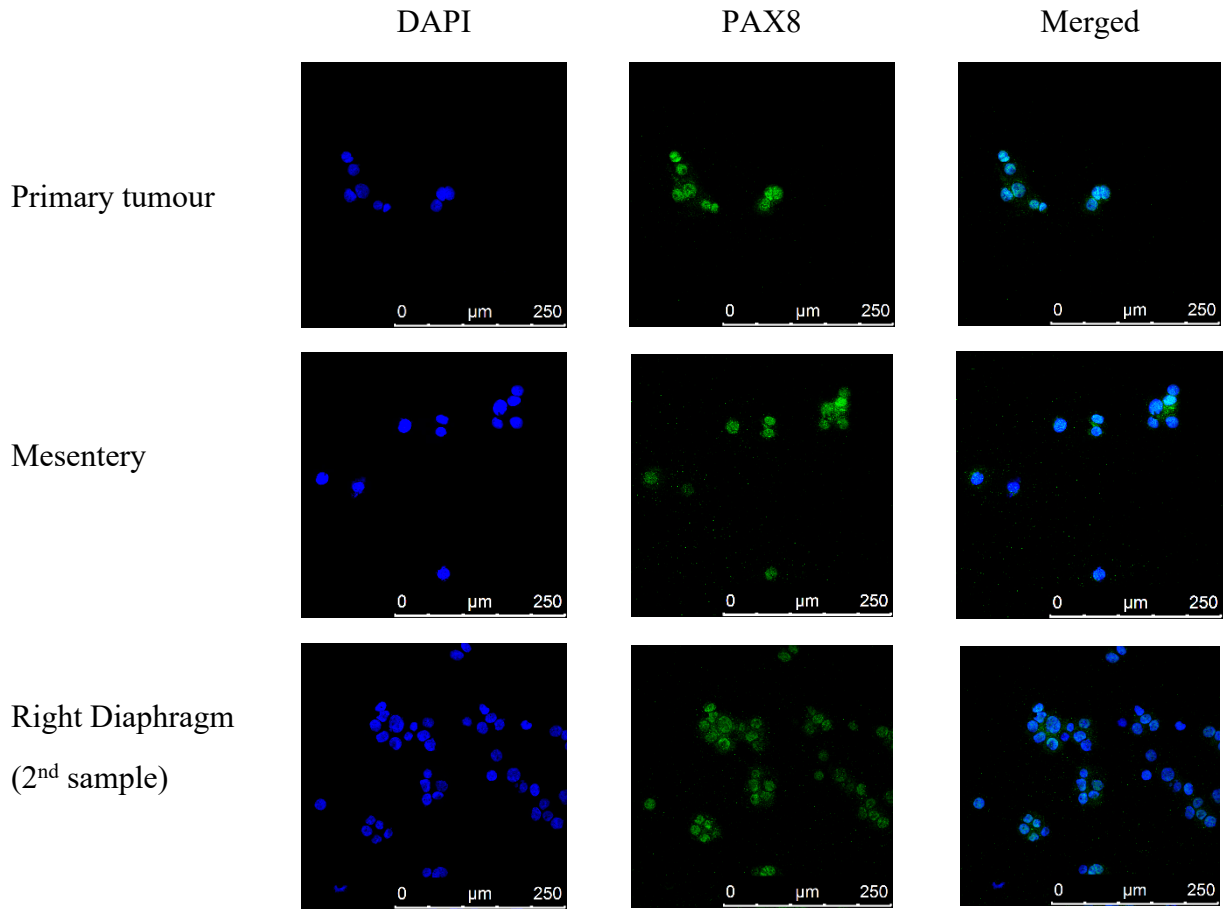


Figure 3. 14: Example of PAX8 positive staining. Cytospin slides were stained for PAX8 positivity to determine tumour cellularity in each tumour deposit. Images were taken at x40 magnification.

Table 3.5 shows an example of the tumour cellularity determination for an individual ITH case. On average, each tumour deposit contained more than 70% tumour cells in each culture, thus confirming that the majority of cells in the phenotypic assays were of tumour origin, so the results from the phenotypic assays could be attributed to tumour cell response to cisplatin and/or DNA-PK inhibition. Appendix Table 1 shows the tumour cellularity determination for all cases in this study cohort.

Table 3. 5 Tumour cellularity determined for T15058

No.	Tumour deposit	Number of cells	Positive cells	Percentage (%)
1.	Primary tumour	121	92	76
2.	Omentum	776	665	86
3.	Mesentery	227	180	79
4.	Omental bursa	294	244	83
5.	Spleen lymph node	900	835	93
6.	Paraaortic lymph node	87	58	66
7.	Bladder peritoneum	166	138	84
8.	Rectal wall	235	189	81
9.	Right diaphragm 1st	311	284	91
10.	Right diaphragm 2nd	637	574	90

3.2.7 Heterogeneity in PAX8 Expression

Spatial intra-tumoural heterogeneity within an individual's cancer is observed in terms of PAX8 antigen expression. To further investigate the heterogeneity of PAX8 expression in our immunofluorescent staining, analysis with CellProfiler (Carpenter *et al.*, 2006) was used to quantify PAX8 intensity staining. This work was performed using a CellProfiler pipeline optimised by Mr Chad Whilding, a microscopist from Imperial College London. Cell cultures which have >60% cellularity were included in this analysis. Figure 3.15 shows an example of heterogeneity in terms of PAX8 intensity staining on one case namely T16060. The top panels are the individual DAPI and PAX8 channel, whilst the bottom panel is the merged channel. In the merged channel, the red circle represents the PAX8 positive staining and the yellow circle represent the PAX8 negative staining. The PAX8 staining intensity of PAX8 positive cells was measured automatically and it was normalised to corresponding DAPI intensity. In this picture, the transverse colon sample has lower intensity staining compared to the liver hilum sample.

A) Transverse colon, PAX8/DAPI intensity: 0.434

B) Liver Hilum, PAX8/DAPI intensity: 0.849

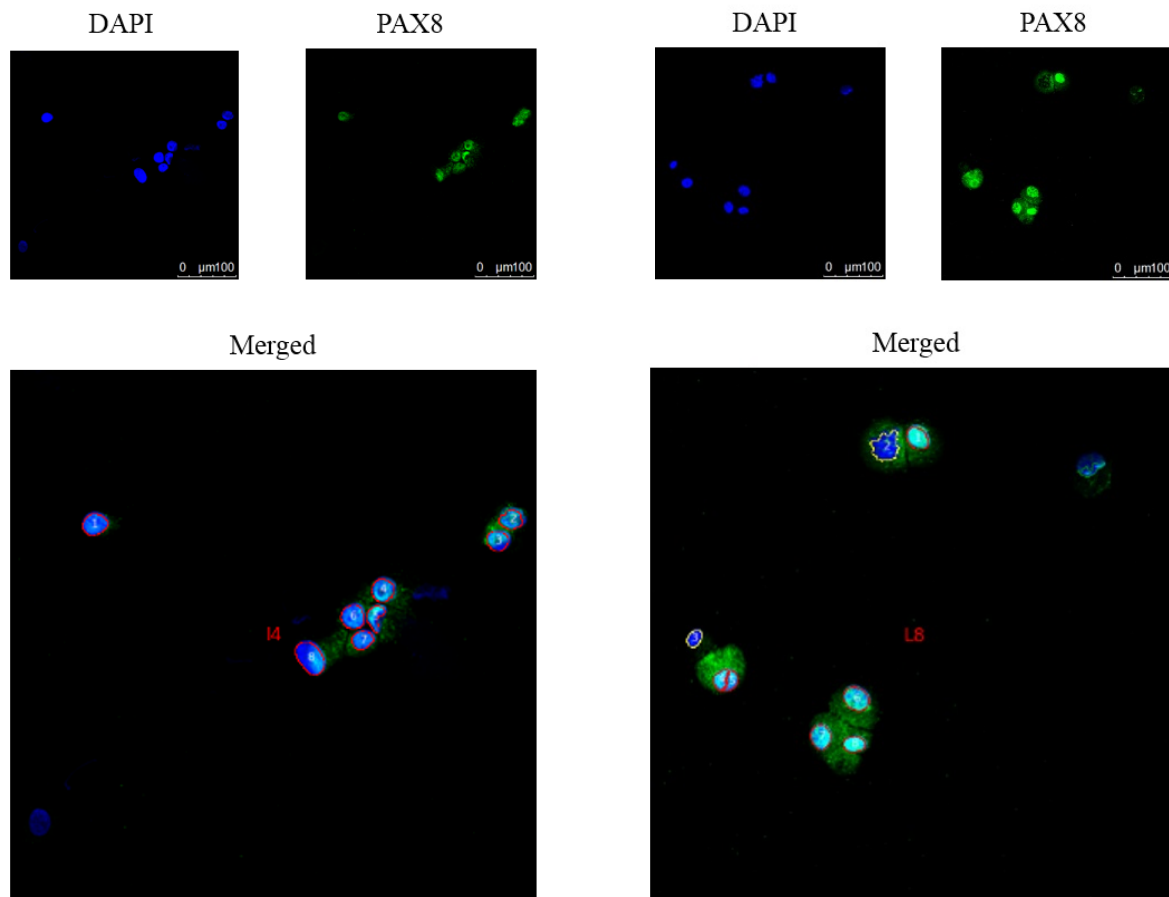


Figure 3. 15: Example of PAX8 intensity measurement using CellProfiler. Images were taken at x40 magnification with the same setting using TCS SP5 confocal microscope (Leica, Germany). The merged panel is the result of CellProfiler analysis marking the negative (yellow circle) or positive (red circle) cell. The number on the merged images represents the number of cells.

A summary of the PAX8 intensity staining measurement is presented in Figure 3.16. In total, there were 14 cases included in this analysis. The remaining 13 cases were excluded due to either the filtered positive cells measuring below 60, or the filtered total cells below 100 after automatic counting using CellProfiler.

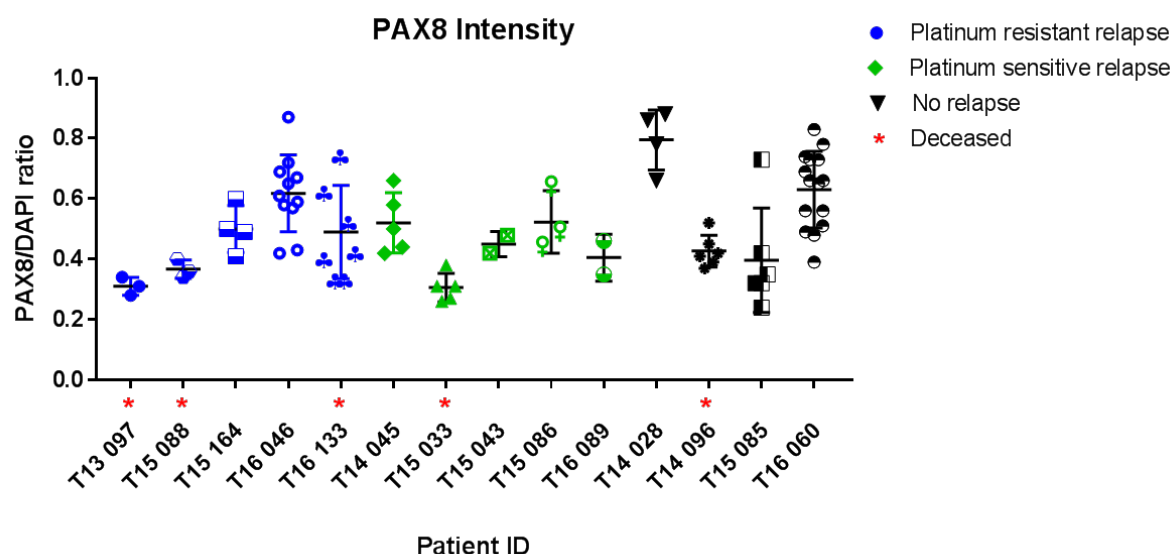


Figure 3. 16: PAX8 intensity heterogeneity was observed in the ITH study cohort. Each dot represents a deposit. PAX8 intensity value in each patient was coloured according to the clinical response to platinum as a pro-apoptotic agent.

3.2.8 Correlation of PAX8 Expression and Phenotypic Response

Previous studies have shown that silencing PAX8 expression in HGSOc cell lines leads to increased apoptosis and reduces cell survival (Di Palma *et al.*, 2014; Rodgers *et al.*, 2016; Ghannam-Shahbari *et al.*, 2018). To investigate the relationship of PAX8 expression to apoptotic induction following platinum treatment in our dataset, a correlation analysis was performed. The results of this analysis are depicted in Figure 3.17 (A), which shows that PAX8 intensity has a weak and non-significant correlation with apoptotic response.

The Project Achilles study found PAX8 is the top gene which important for the proliferation and viability of ovarian cancer cells and is also differentially overexpressed in ovarian cancer cell lines (Cheung *et al.*, 2011). Thus, we investigated whether PAX8 intensity had any correlation with cell viability in our cohort. Figure 3.17 B shows that there was no correlation between PAX8 intensity and cell viability following cisplatin treatment. These negative results

may be attributed to our study's use of primary ovarian cancer cell in comparison with the use of ovarian cancer cell lines the previous study. The role of PAX8 in the primary cancer cells remains unclear to date. Another possible explanation is our selecting of the moderate to high PAX8 expression in our analysis, thus discluding the value of absent or low PAX8 expression in relation to apoptotic response or cell viability.

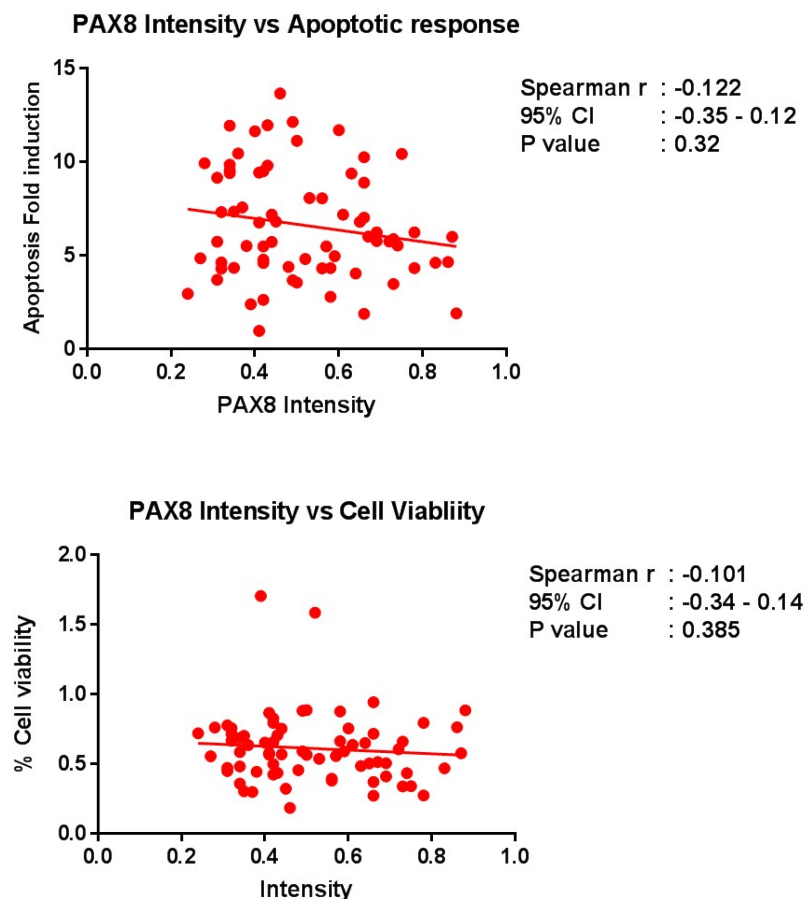


Figure 3. 17: Correlation of PAX8 intensity staining and phenotypic response. Immunofluorescent PAX8 staining and phenotypic assay were performed on different short-term primary cell cultures of the same deposit. Correlation analyses were performed on PAX8 intensity staining with apoptosis response normalised to viability (A) and cell viability (B) following cisplatin treatment.

3.3. Discussion

The ability to generate and exploit the primary culture of ovarian cancer has immense benefit compared to other models, such as immortalised cell lines and animal models. The extracted primary cells are closer to the patient's clinical condition, as well as the progression of the disease. While the use of immortalised cancer cell lines remains important for understanding ovarian cancer biology, it carries the disadvantage of lacking the rich heterogeneity that is observed in HGSOC (O'Donnell et al., 2014). Immortalised cell lines also harbour further genetic alteration due to extended *in vitro* passaging, and, as such, they deviate from their molecular origin. The common animal models are xenografts, generated by injecting ovarian cancer cells into immunocompromised mice. This model lacks the contribution of the full complement immune system for the tumour progression (Bobbs, Cole and Cowden Dahl, 2015). Thus, there is still a need to produce a model that emulates human ovarian cancer to improve understanding of the effect of current chemotherapy and to test novel therapeutic agents pre-clinically.

Several methods have been developed to generate primary cultures both from ascites fluid and solid tumours. A widely used protocol for extracting tumour cells from freshly drained ascites fluid is using centrifugation to create pelleted cells, followed by gradient centrifugation with Histopaque to exclude the RBCs. The cell pellet is then washed with PBS before being suspended in the appropriate culture medium (Langdon, 2004). Another method for ovarian cancer propagation from ascites fluid is achieved by mixing the ascites with culture medium and placing them directly into a culture flask with minimal manipulation (Thériault *et al.*, 2013). Thériault *et al* also compared enzymatic dissociation using collagenase and mechanical tumour-tissue disruption using cell scrapers and found that mechanical disruption was more efficient compared to the other method. A previous study systematically compared mechanical

dissociation of a solid tumour using 70 µm mesh with enzymatic digestion using either collagenase A, hyaluronidase or Dispase II for various time periods followed by mechanical disruption, and concluded that enzymatic digestion using Dispase II was the most effective method (Sueblinvong *et al.*, 2012).

This chapter demonstrates that the method of extracting primary ovarian cancer cells optimised in our laboratory, which incorporate mechanical and enzymatic dissociation successfully results in the generation of an epithelial-rich cell population. We can also generate short-term primary culture and perform phenotypic assays using cisplatin as a standard chemotherapeutic agent and DNA-PK inhibitor as a novel therapeutic agent for ovarian cancer. We use the cells within 48 hours following extraction for this assay, with the aim of minimising the cancer cells' adaptation to the culture environment, thus maintaining a close similarity with *in vivo* tumour biology. The ability to generate primary cell culture from both tumour tissue and ascitic fluid makes possible an important experimental model for developing novel interventions with higher therapeutic applicability.

By collecting several tumour deposits from the same patient, we can explore phenotypic heterogeneity in response to cisplatin and DNA-PK inhibitor treatment. In this study, functional phenotypic inter- and intra-tumour heterogeneity were observed, as well as both spatial and temporal heterogeneity. These results may be partially explained by divergent selective pressures acting on tumour cell subpopulations at various metastatic sites. However, phenotypes are dynamic features of tumour cells and do not result merely from cell-autonomous driving factors (Burrell and Swanton, 2014; Marusyk *et al.*, 2014). Besides the diversity in genetic, epigenetic, transcriptomic, and proteomic levels, differences in extrinsic

factors such as tumour microenvironment may also modulate cancer cell phenotypes and affect cell fitness. As in our primary cell culture, there is a small portion of stromal cells contamination that may contribute to different response to platinum. We observed a homogenous, poor response to platinum therapy in the relapse cases compared to the primary cases, although more samples are clearly needed to confirm this finding. Also, we used a high dose of cisplatin in this study which might kills most of the cancer cells. This dose is not achievable in clinical setting, as 25 μ M of cisplatin for cell line models is equivalent to approximately 3x recommended dose of cisplatin in clinical setting. Thus, the half maximal inhibitory concentration (IC50) for each culture is needed to obtain more objective and applicable result.

We also showed that the addition of the DNA-PK inhibitor can enhance the apoptotic response in patients who later develop platinum-resistant relapse and refractory disease. DNA-PKcs is a serine/threonine protein kinase - a key mediator of non-homologous end joining (NHEJ) DSB repair pathway. This protein has been shown to phosphorylate and activate AKT in response to DNA damage, leading to activation of the downstream pro-survival pathways (Dungl, Maginn and Stronach, 2015). A study from our centre has reported that the DNA-PKcs copy number is frequently amplified in HGSOV and is correlated with overexpression of DNA-PKcs at the genetic level (Cunnea, Curry et al, unpublished data; Curry et al., 2015). Further analysis from this study shows poorer patient outcomes in a highly expressed DNA-PKcs subset using both in-house and publicly available cohorts. Inhibition of DNA-PKcs has been shown to restore cisplatin sensitivity in chemoresistant ovarian cancer cell lines (Stronach et al., 2011; Cunnea, Curry et al, unpublished data). In the previous study, DNA-PKcs inhibition did not enhance the apoptotic response to platinum in already sensitive cells. In the primary tumour cultures derived from this study, the increased apoptotic response is also found in a small subset

of patients who later develop platinum-sensitive phenotypes and in the non-relapse cohort. This may be due to the presence of a resistant subclone in that culture. The comparison between DNA-PK inhibitor only and cisplatin + DNA-PK inhibitor apoptotic response was not performed in our study. This should be done in the future to confirm the utility of DNA-PK inhibitor in improving apoptotic response following cisplatin treatment. The effect of DNA-PK inhibitor in relapse samples were also assessed in this study. In one relapse case, the addition of DNA-PK did not increase the apoptotic level following cisplatin treatment, while in another, it did. Further study investigating apoptotic response following DNA-PK inhibitor treatment in relapse cases is necessary to confirm the benefit of DNA-PK inhibitor in reversing platinum resistance in HGSOC.

Beyond the identification of several ovarian cancer histotypes, heterogeneity exists at the phenotypic and molecular level within tumours of the same histotype. This heterogeneity makes assessing responses to treatment and patient outcomes significantly more complex. It is not yet possible to accurately predict the likelihood of a patient's response to chemotherapy. There have been several studies investigating the correlation between chemoresponse assays and patient outcomes in ovarian cancer, but results have been inconsistent (Gallion et al., 2006; Herzog, Krivak, Fader, & Coleman, 2010; Holloway et al., 2002; Jenks, 2012). The data from this chapter suggest that a tumour demonstrating higher CV in response to cisplatin treatment at presentation is more likely to respond to treatment. This is shown by the tendency towards higher CV in the sensitive-relapse cohort and the ability of CV levels in response to platinum *in vitro* to predict clinical sensitivity, with the AUC from ROC curve of 0.752 indicating potential test accuracy. Lower CV also tends to correlate with better PFS and OS as, displayed using Kaplan-Meier curves. However, these findings are not statistically significant. It is also unclear if this is an effect of small sample size or a true indication of underlying tumour

biology; further investigation using a larger cohort is essential. It is also will be interesting to investigate the extremes CV values and its correlation to chemotherapy response in the future.

In our characterisation of tumour culture staining using a panel of several markers, the optimal conditions were achieved for PAX8, CA125, WT1, EpCAM (tumour markers), CD90 (fibroblast marker), and calretinin (mesothelial marker). PAX8 was chosen as a tumour marker in our study because it produces the strongest and most consistent fluorescent signal. However, conditions for dual staining of PAX8 with CD90 or calretinin were not achieved because PAX8 is a nuclear marker which needs a longer duration of permeabilisation compared to the two other markers which are surface or cytoplasmic markers. PAX8 is a transcription factor involved in the development of thyroid, epididymis, and the Müllerian system (Mittag *et al.*, 2007). In HGSOE, PAX8 is expressed in almost all cases (Nonaka, Chiriboga and Soslow, 2008; Laury *et al.*, 2010, 2011) and is indicated to have a pro-proliferative role by promoting tumour cell growth through transcriptional activation of E2F Transcription Factor 1 (E2F1) expression and upregulation of the RB-E2F1 network (Li *et al.*, 2011). We confirmed the high tumour content in our primary culture using a PAX8 marker in immunofluorescent staining. This result indicates that the majority of cells in the phenotypic assays were tumour cells; thus, the outcomes from the phenotypic assays can be attributed to tumour cell response to cisplatin and/or DNA-PK inhibitor. We also observed the different PAX8 intensity staining across several deposits and across patients, which provides further evidence of inter- and intra-tumoural heterogeneity. The previous study also observed intra-tumoural heterogeneity of tumour markers' (EpCAM and CA125) expressions in its immunofluorescent characterisation panel (O'Donnell *et al.*, 2016). However, the intensity measurements in our study only cover a small subset of samples, since 13 cases were excluded due to the filtered positive cells measuring below 60 cells or filtered total cells measuring below 100 cells, after automatic

counting using CellProfiler. In our analysis, the intensity of PAX8 staining did not correlate with apoptosis or cell viability. A recent study has revealed that PAX8 positively regulates *TP53* expression, and its pro-proliferative property is facilitated by mutant p53 gain of function (GOF) activity (Ghannam-Shahbari *et al.*, 2018). Another study shows that PAX8 inhibits apoptosis by targeting tumour protein 53 induced nuclear protein 1 (tp53inp1) which has a role in cell cycle arrest and in TP53-mediated apoptosis (Di Palma *et al.*, 2013).

This study adds to the current evidence of ITH in HGSOC in terms of phenotypic response to cisplatin chemotherapy and antigen expression. Moreover, it shows that the addition of the DNA-PK inhibitor to cisplatin treatment could enhance the apoptotic response in patients who later develop platinum-resistant disease. The existing two-dimensional cell culture model is an important resource, which outperforms immortalised cell lines in its ability to reproduce *in vivo* tumour biology. It provides a valuable resource for *ex vivo* testing and for investigating tumour-molecular aberrations, which serve as potential drug targets. Future work should focus on the ongoing development of an improved culture model such as three-dimensional organotypic models or *ex vivo* tumour slice cultures in which *ex vivo* assessment could be performed simultaneously in a reproducible and clinically relevant manner because these models contain tumour microenvironment.

Chapter 4

Proteomic Analysis of HGSOC using RPPA

4.1 Introduction

As discussed, the development of resistance to platinum therapy contributes to poor outcomes for HGSOC patients. Understanding drug responses could aid in revealing the mechanisms which lead to this resistance (Bowtell *et al.*, 2015). Investigating cell-signalling proteins in cancer also provides an opportunity to identify potential novel therapeutic targets and find pharmacodynamic markers to predict responses to treatment, thus aiding in the personalisation of therapy (Avril *et al.*, 2017).

One method that can facilitate investigation of signalling events is reverse phase protein array (RPPA). In recent years, RPPA has become a powerful high-throughput and cost-effective molecular analysis platform for targeted proteomics. This platform is particularly useful for specific focus and enquiry such as investigating the effect of a specific drug on signalling cascades, comprehensive evaluation of cancer tissues for molecular classification, or identification of novel targets (Nishizuka and Mills, 2016). The primary advantage of RPPA is that it allows these possibilities by generating proteomics data on large sample sets from clinical materials with limited amounts of biological samples or from experimental models treated previously using drugs or growth factors (Petricoin *et al.*, 2002; Nishizuka and Spurrier, 2008).

Inter and intra-tumoural heterogeneity need to be taken into consideration when assessing proteomics profiles from patient samples. Our work in the previous chapter demonstrated intra-tumoural variation in phenotypic responses to cisplatin in HGSOC. We approached the challenge in this chapter by investigating changes in protein signalling cascades in different

tumour deposits following platinum treatment. The goal of this proteomic study was to assess the level of heterogeneity related to platinum-induced cell signalling at the protein level in our primary cell cultures derived from multiple tumours. We also aimed to assess the difference between platinum-induced protein changes in resistant and sensitive-relapse cases. This information could contribute towards a better understanding of the mechanism of platinum resistance in ovarian cancer. The RPPA Functional Proteomics Unit, MD Anderson, accessed in this project, initially designed arrays for PI3K/Akt signalling pathway analysis. The array has now expanded to contain 290 probes, including receptor tyrosine kinases (RTKs), MAPK cascades, AMPK and TGF β cascades as well as cell cycle, DNA repair, and apoptosis regulator related proteins.

4.2 Results

4.2.1 Effect of Cisplatin on Signalling Cascades in HGSOc *In Vitro*

In the present study, tumour cells were seeded for 48 hours and treated with 25 μ M cisplatin or media for 4 hours, after which the cell lysates were collected. A total of 60 paired samples (case and control) from 14 patients, including three matched primary and relapse cases was submitted to RPPA core facility in MD Anderson centre. However, due to a lower protein concentration in some sample pairs, a number of samples were excluded from further analysis. The subsequent RPPA analysis in the ITH study consisted of 45 paired samples from 14 patients, including one matched primary and relapse case. Normalised log₂ data from the RPPA process was provided by the Functional Proteomics Unit for analysis. Figure 4.1 shows the sample distribution in this study cohort visualised using multidimensional scaling (MDS) analysis. It depicts the samples clustered based on the tumour deposits (samples) location, irrespective of whether the sample was treated with cisplatin or untreated. Based on this

finding, the protein-expression value used to compare different samples in subsequent analyses was the protein-expression differences between cisplatin-treated and untreated control ($\log_2$ cisplatin treated – $\log_2$ control).

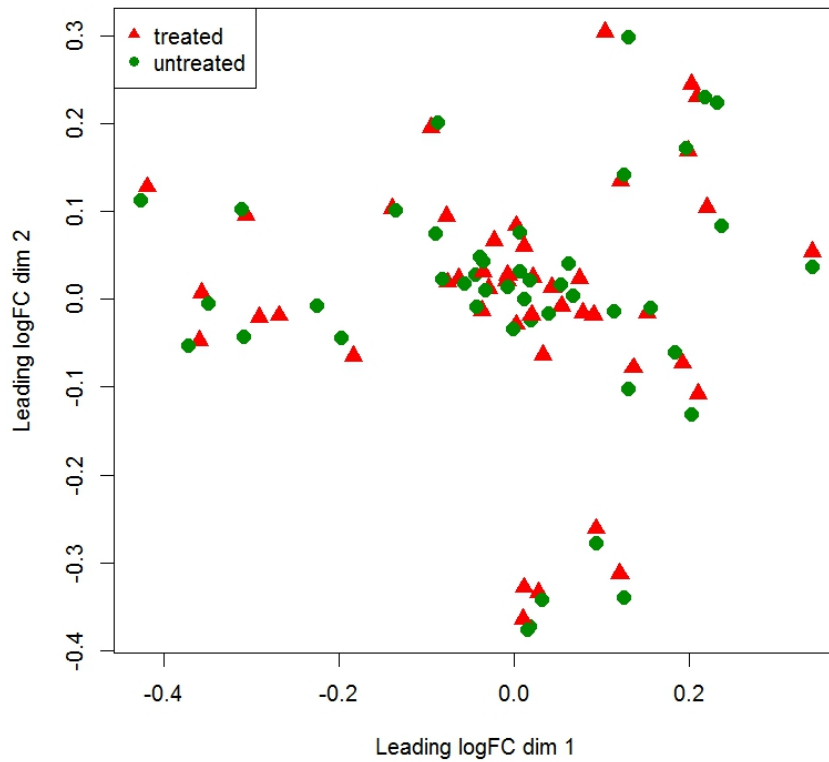


Figure 4. 1: ITH samples in the RPPA dataset were clustered based on their location. Sample distribution was visualised using MDS analysis in R which plot samples on a 2D scatterplot, thus the distances on the plot are similar to the $\log_2$ fold changes between the samples. Red colours represent treated samples, whereas green colours represent untreated controls.

Linear regression was used in order to determine the statistical differences in protein expression using the `lmFit` function in the `limma` package in R. Figure 4.2 shows the 61 differentially expressed proteins across all cisplatin-treated and untreated tumour cells in our RPPA cohort (globally). In this figure, there are 22 up-regulated proteins (Table 4.1) and 39 downregulated proteins (Table 4.2). C-Jun, a stress-activated protein in the *c-Jun* NH₂-terminal protein kinase (JNK)/SAPK family; p38, an important kinase activated by cellular stress that links

inflammatory and environmental stress to transcription factors; and HSP27, a 27-kDa protein which belongs to the small heat shock proteins family (sHsps) were upregulated and activated following cisplatin treatment in this cohort. We found that p38 phosphorylation at Thr180 and Tyr182 was increased greatly, but the total p38 MAPK was slightly decreased. Other stress-related proteins, Chk1 and Chk2, which are essential to cell cycle control and the maintenance of genomic integrity in response to DNA damage, were also found to be activated in this cohort.

Histone, a main component of chromatin, plays an important role in transcription regulation, DNA repair, DNA replication and chromosomal stability. Histone H3 and histone H3 lysine 9 (H3K9me) proteins were found to be upregulated in our cohort. Phosphorylation at Ser140 of H2AX was also increased in this cohort as a result of DNA damage induced by platinum.

This study also confirmed increased Akt activation both at Ser473 and Thr308 following cisplatin treatment. Total Akt was slightly decreased following cisplatin treatment for 4 hours. Another class of serine/threonine kinases, PKC-b-II, was found to have increased phosphorylation at Ser660. This protein is involved in various cellular processes such as oxidative stress-induced apoptosis, insulin signalling, and endothelial cell proliferation. Downregulation of AMPKa-Thr172 was also found, suggesting downregulation of cellular metabolism-signalling pathway after cisplatin treatment.

In this cohort, we found decreased COX IV expression, which is essential for energy production in cells. We also observed the downregulation of phosphorylated focal adhesion kinase (FAK) at Tyr397 and its partner, Src at Tyr527, indicating inhibition of cancer cell viability, clonogenicity, and tumour growth. Hypoxia-induced factor (Hif)-1-alpha showed the

third greatest reduction in expression in our RPPA dataset, indicating downregulation of the survival pathway, which is expected following cisplatin treatment. EMA (MUC1) was also found to be downregulated. The expression of this protein in cancer may function as an anti-adhesion molecule, which is important for metastasis. In addition, AXL, an RTK which is essential for driving epithelial-to-mesenchymal transition (EMT) and tumour progression, was decreased after cisplatin treatment.

Table 4. 1 Up-regulated proteins following cisplatin treatment compared to untreated control in RPPA dataset

Protein probe names	p-value	Adjusted p-value	Fold change
HSP27_pS82-R-V	5.04E-13	7.32E-11	1.46
p38_pT180_Y182-R-V	2.63E-11	2.54E-09	1.46
Chk1_pS296-R-V	3.92E-13	7.32E-11	1.36
Histone-H3-R-V	0.000122	0.001357	1.28
c-Jun_pS73-R-V	1.12E-10	8.11E-09	1.20
H2AX_pS140-M-C	9.62E-06	0.000155	1.16
Akt_pS473-R-V	8.04E-08	2.59E-06	1.15
DM-K9-Histone-H3-R-C	2.60E-05	0.000359	1.13
PKC-b-II_pS660-R-V	3.40E-09	1.97E-07	1.12
Chk2_pT68-R-C	3.33E-07	8.79E-06	1.10
Akt_pT308-R-V	7.58E-08	2.59E-06	1.10
MSI2-R-C	5.68E-06	9.69E-05	1.08
YB1_pS102-R-V	2.53E-05	0.000359	1.07
NDRG1_pT346-R-V	0.000562	0.004249	1.07
MAPK_pT202_Y204-R-V	0.000571	0.004249	1.07
LC3A-B-R-C	0.001972	0.013297	1.07
eIF4E_pS209-R-V	0.003186	0.018856	1.06
ER-a_pS118-R-V	0.002384	0.015715	1.06
Cyclin-E1-M-V	6.28E-05	0.000765	1.06
Bad_pS112-R-V	3.14E-05	0.000414	1.05
MEK1_pS217_S221-R-V	0.000638	0.004511	1.04
p27-Kip-1-R-V	0.004726	0.026359	1.04

Statistics associated with linear regression analysis are the p-value and the adjusted p-value. Fold changes are also shown, where 1 = no change. For all analyses, statistical significance is defined as $p < 0.05$. The table is ranked based on the adjusted p-value. Antibody status: R-Rabbit, M-Mouse, V-validated, C-use with caution.

Table 4. 2 Down-regulated proteins following cisplatin treatment compared to untreated control in RPPA dataset

Protein probe names	p-value	Adjusted p-value	Fold change
Cox-IV-R-V	4.36E-08	1.81E-06	0.84
FAK_pY397-R-V	4.73E-06	9.15E-05	0.87
Hif-1-alpha-M-C	8.13E-09	3.93E-07	0.88
EMA-M-C	0.000485	0.003802	0.89
AMPKa_pT172-R-C	5.21E-06	9.44E-05	0.90
Axl-R-V	0.001378	0.009518	0.90
Stat3_pY705-R-V	2.03E-06	4.53E-05	0.91
eEF2K-R-V	1.36E-07	3.94E-06	0.91
BRD4-R-V	0.003061	0.018492	0.91
eIF4G-R-C	0.003829	0.022208	0.92
SHP-2_pY542-R-C	0.000424	0.003725	0.92
p38-MAPK-R-V	0.000257	0.002487	0.92
CDK1-R-C	0.000117	0.001355	0.92
E-Cadherin-R-V	0.010425	0.049562	0.93
Akt-R-V	4.31E-06	8.92E-05	0.93
S6-M-V	0.006552	0.033928	0.93
NF-kB-p65_pS536-R-C	0.000221	0.002208	0.93
VEGFR-2-R-V	4.00E-07	9.66E-06	0.93
PDGFR-b-R-V	0.000621	0.004502	0.94
DJ1-R-V	0.000143	0.001536	0.94
Gab2-R-V	6.33E-05	0.000765	0.94
b-Catenin-R-V	0.00255	0.016436	0.94
Stat3-R-C	0.000484	0.003802	0.95
Bcl-xL-R-V	0.000441	0.003765	0.95
PLC-gamma2_pY759-R-C	0.004872	0.026658	0.95
PEA-15-R-V	0.009598	0.047176	0.95
Notch1-R-V	0.00017	0.001758	0.95
Mnk1-R-V	2.12E-05	0.000324	0.96
Cyclin-B1-R-V	0.002723	0.017166	0.96
HER3_pY1289-R-C	0.000485	0.003802	0.96
IR-b-R-C	0.005385	0.02892	0.96
Stat5a-R-V	0.000322	0.002919	0.96
4E-BP1_pS65-R-V	0.000301	0.00282	0.96
MEK1-R-V	0.007139	0.036321	0.96
p44-42-MAPK-R-V	0.006143	0.032392	0.96
TAZ-R-V	0.010089	0.048766	0.97
Src_pY527-R-V	0.002914	0.017982	0.97
PRAS40_pT246-R-V	0.007653	0.038263	0.97
COG3-R-V	0.004399	0.025015	0.97

Statistics associated with linear regression analysis are the p value and the adjusted p value. Fold changes are also shown, where 1 = no change. For all analyses, statistical significance is

defined as $p < 0.05$. The table is ranked based on the adjusted p-value. Antibody status: R-Rabbit, M-Mouse, V-validated, C-use with caution.

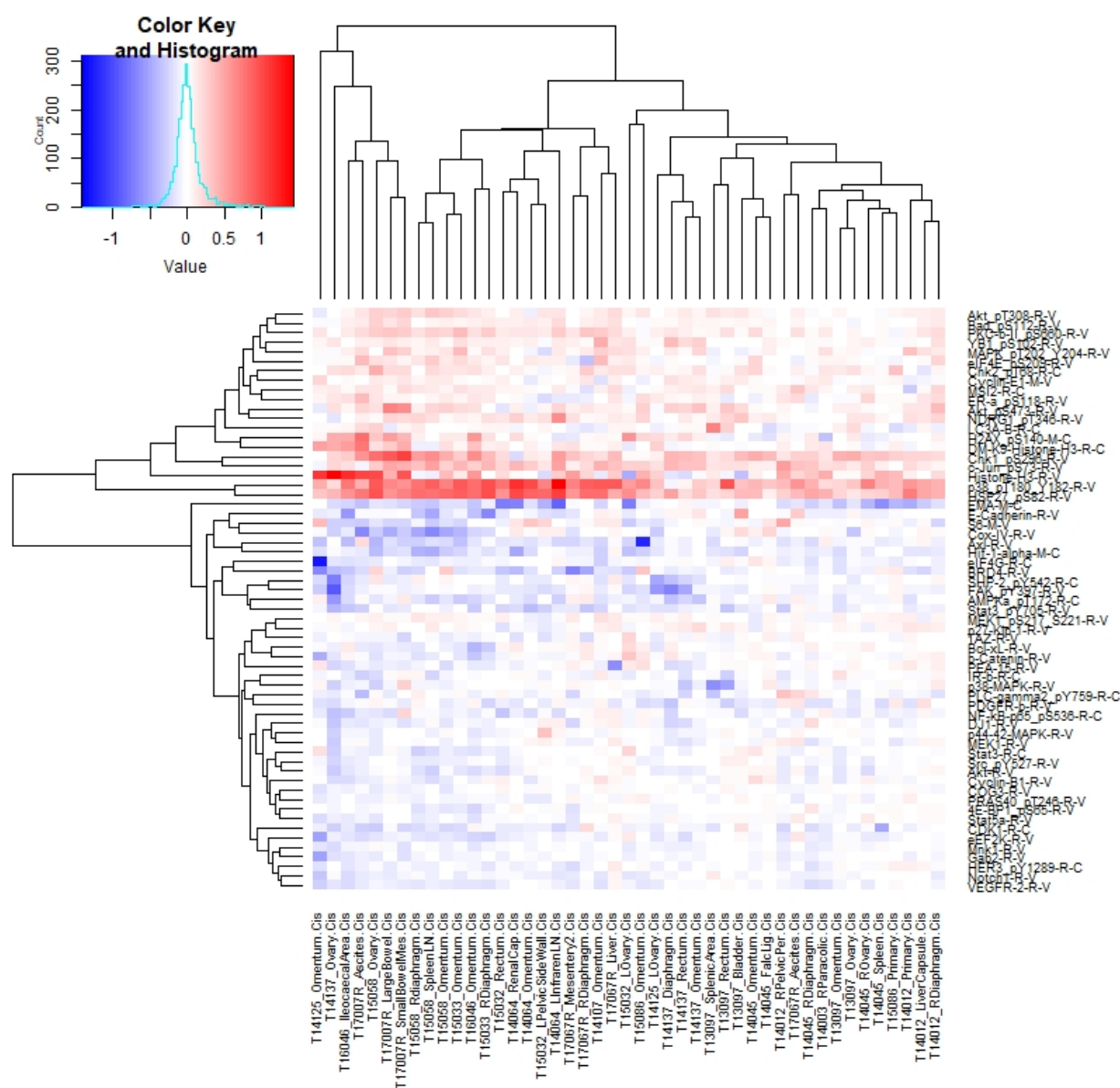


Figure 4. 2: Platinum induced molecular signalling changes between different tumour sites from multiple patients. Primary cultures from each deposit were treated with 25 μ M cisplatin for 4 hours. Protein lysates were collected and sent for RPPA analysis. The row of the heat map shows significantly dysregulated proteins following cisplatin treatment. The red bar indicates upregulation and blue bar indicates downregulation. The column dendrogram indicates unsupervised hierarchical clustering of 45 deposits.

For better understanding the signalling changes following cisplatin treatment, protein-protein interaction and pathway enrichment analysis were performed on the differentially expressed proteins in our RPPA dataset. This analysis was performed using STRING interactome database (Szklarczyk *et al.*, 2015). We considered STRING interactions that were of high confidence (combined STRING score ≥ 0.7) and selected active interaction sources from three out of seven channels: experiments, databases, and co-expression (text-mining, neighbourhood, gene fusion and co-occurrence were unselected). From this analysis, we observed highly connected nodes in our cohort. However, nine nodes did not have any interaction with others (MAP1LCA, YBX1, PARK7, WWTR1, COX4I1, COG3, MUC1, BRD4 and MS12). One hundred and ten pathways were found to differ significantly between cisplatin-treated and untreated cells after multiple testing (Appendix Table 2). Figure 4.3 shows the protein-protein interaction and the ten most significant results from the KEGG pathway enrichment analysis. Among these pathways, ‘pathways in cancer’, ‘PI3K-Akt signalling pathway’, and ‘proteoglycans in cancer’ had the most annotated nodes at 18, 16, and 15 annotations, respectively.

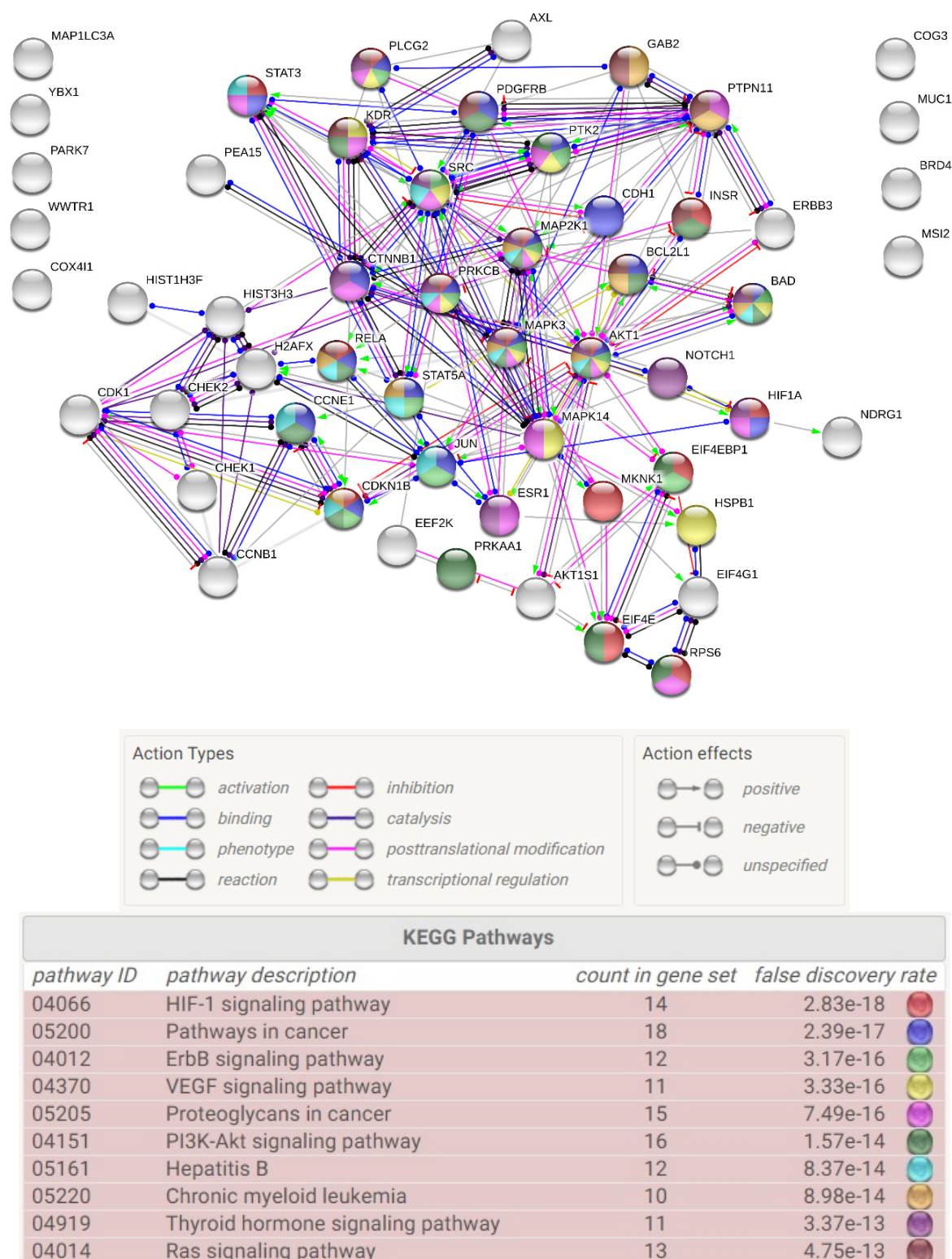


Figure 4. 3: Protein networks from platinum-induced protein expression changes in HGSOc. Protein-protein interaction networks consisting of proteins significantly changed following platinum treatment, were constructed using STRING database. The node colours were based on the colour legend of the top 10 KEGG pathways.

4.2.2 Proteomic Heterogeneity in HGSOC

Linear regression analysis was performed for each case to find significantly altered proteins following cisplatin treatment. Figure 4.4 shows cisplatin-induced protein changes in a matched case, T14137 (primary case) and T17067R (relapse case). This patient initially relapsed in a platinum sensitive time frame; however, the relapse samples were collected when the patient had a resistant relapse phenotype (second relapse). In the primary case, there were eight proteins which were significantly altered across all deposits following platinum treatment after multiple testing (Table 4.3). Conversely, in the relapse case, there was only one protein (p38_pT180_Y182) with an adjusted p-value < 0.05 that was altered following platinum treatment (Table 4.4).

Figure 4.5 shows the cisplatin-induced protein changes in two different case examples. In T15058, there were 21 proteins that remained significantly up or downregulated after multiple testing (Table 4.5). In contrast, in T15086, ATRX, ARID1A, p38_pT180_Y182, Histone-H3, HSP27_pS82 and HES1 proteins had an adjusted p-value of < 0.05 following platinum treatment (Table 4.6). This demonstrates that different signalling changes occurred in each patient's tumours in response to platinum chemotherapy.

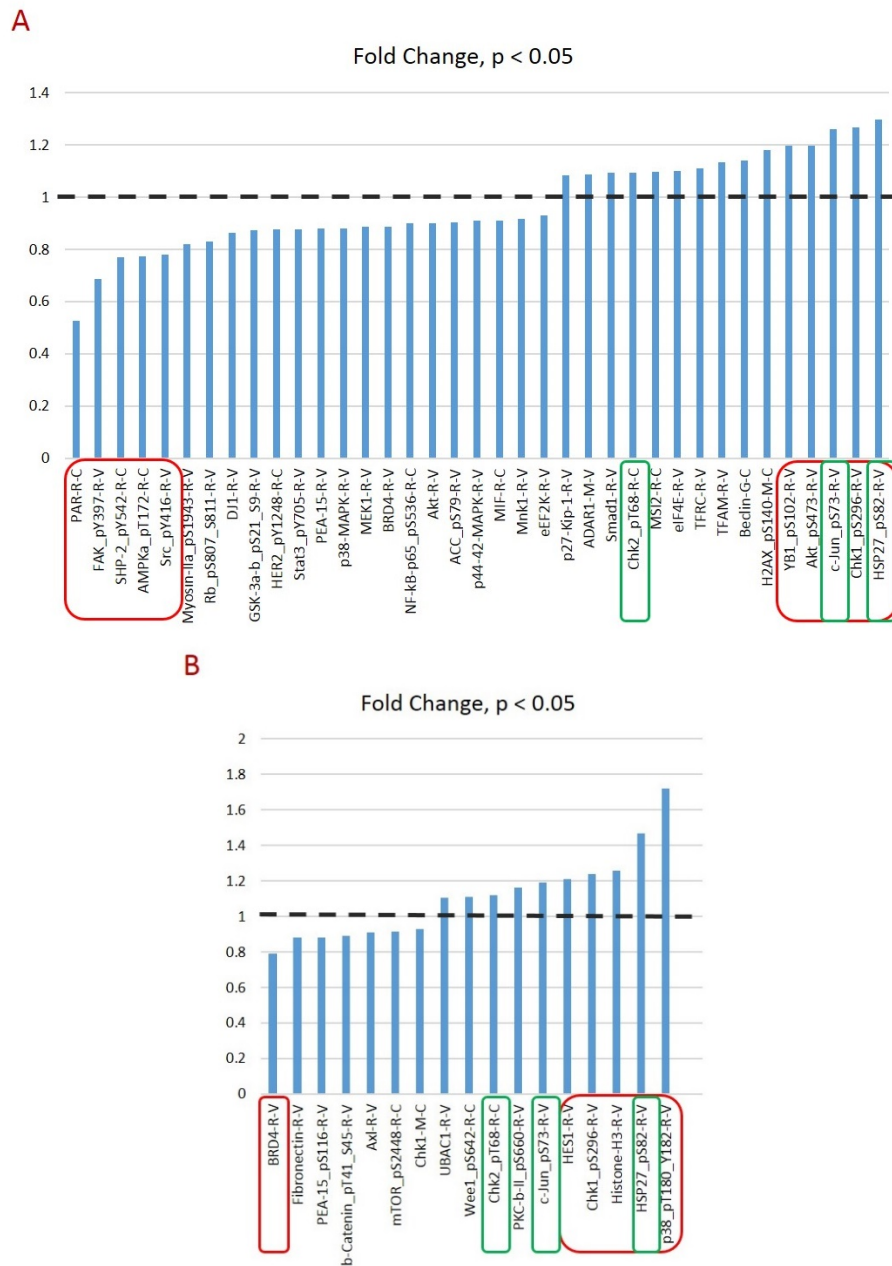


Figure 4. 4: Platinum-based chemotherapy-induced protein expression changes in a matched case. T14137 primary case (A) and T17067 relapse case (B). The red box represents proteins that had dysregulated (increased/decreased) expression of >20% following platinum treatment. The green box represents proteins that significantly changed in both the primary and relapse case. The rest of the proteins were unique to each sample.

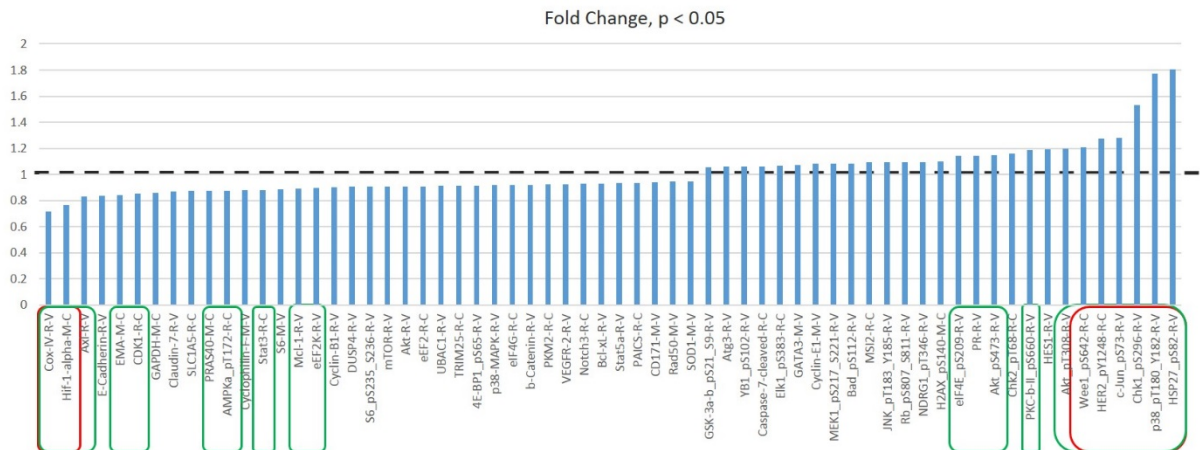
Table 4. 3 Ten most significant differentially expressed proteins following cisplatin treatment compared to untreated control in T14137

Protein probe names	p-value	Adjusted p-value	Fold change
Chk1_pS296-R-V	7.03E-05	0.01109	1.27
c-Jun_pS73-R-V	7.65E-05	0.01109	1.26
HSP27_pS82-R-V	0.000156	0.012149	1.30
PAR-R-C	0.000168	0.012149	0.53
AMPKa_pT172-R-C	0.000223	0.012936	0.77
Akt_pS473-R-V	0.000396	0.01916	1.20
YB1_pS102-R-V	0.000676	0.028026	1.20
Rb_pS807_S811-R-V	0.000891	0.032301	0.83
FAK_pY397-R-V	0.001746	0.050837	0.69
SHP-2_pY542-R-C	0.001837	0.050837	0.77

Table 4. 4 Five most significant differentially expressed proteins following cisplatin treatment compared to untreated control in T17067

Protein probe names	p-value	Adjusted p-value	Fold change
p38_pT180_Y182-R-V	8.14E-05	0.023593	1.72
Chk1_pS296-R-V	0.000686	0.070256	1.24
HES1-R-V	0.000934	0.070256	1.21
HSP27_pS82-R-V	0.000969	0.070256	1.47
Histone-H3-R-V	0.003443	0.199713	1.26

A



B



Figure 4. 5: Platinum-based chemotherapy-induced protein expression changes in two different cases (T15058 (A) and T15086 (B)). The red box represents proteins that had dysregulated (increased/decreased) expression of >20% following platinum treatment. The green box represents proteins that significantly changed in both cases. The rest of the proteins were unique to each sample.

Table 4. 5 Differentially expressed proteins following cisplatin treatment compared to untreated control in T15058

Protein probe names	p-value	Adjusted p-value	Fold change
HSP27_pS82-R-V	3.69E-07	0.000107	1.80
p38_pT180_Y182-R-V	1.16E-06	0.000168	1.77
Chk1_pS296-R-V	2.46E-05	0.002383	1.53
Hif-1-alpha-M-C	4.27E-05	0.003097	0.76
Cox-IV-R-V	0.000124	0.007196	0.71
Akt_pT308-R-V	0.000244	0.011385	1.20
PKC-b-II_pS660-R-V	0.000278	0.011385	1.19
c-Jun_pS73-R-V	0.000314	0.011385	1.28
Axl-R-V	0.000801	0.023404	0.83
CDK1-R-C	0.00083	0.023404	0.85
EMA-M-C	0.000921	0.023404	0.84
Wee1_pS642-R-C	0.000968	0.023404	1.21
HER2_pY1248-R-C	0.001139	0.025402	1.27
PRAS40-M-C	0.001328	0.027517	0.88
PR-R-V	0.001696	0.03156	1.14
Akt_pS473-R-V	0.001781	0.03156	1.15
AMPKa_pT172-R-C	0.00185	0.03156	0.88
eEF2K-R-V	0.002164	0.034642	0.90
Mcl-1-R-V	0.002361	0.034642	0.89
eIF4E_pS209-R-V	0.002389	0.034642	1.14
Stat3-R-C	0.003139	0.04335	0.88

Table 4. 6 Ten most significant differentially expressed proteins following cisplatin treatment compared to untreated control in T15086

Protein probe names	p-value	Adjusted p-value	Fold change
ATRX-R-C	6.24E-06	0.001363	1.52
ARID1A-R-C	9.40E-06	0.001363	1.52
p38_pT180_Y182-R-V	6.45E-05	0.006232	1.44
Histone-H3-R-V	0.000307	0.022277	1.33
HSP27_pS82-R-V	0.000487	0.028225	1.31
HES1-R-V	0.000697	0.033703	1.40
Axl-R-V	0.001424	0.059002	0.64
Caspase-7-cleaved-R-C	0.002748	0.09962	1.25
WIPI1-R-C	0.010056	0.32403	1.40
LDHA-R-C	0.011404	0.330703	1.29

Correlation analysis between each deposit in our cohort was performed to assess whether any sample correlated with another in terms of proteomic profiles. We also investigated whether any specific cluster correlated between each IMO location, or between patients or between primary and relapse cases as shown in Figure 4.6. It can be observed that each deposit strongly correlated with the deposit itself. There was no clustering based on each patient, primary or relapse case, or IMO location.

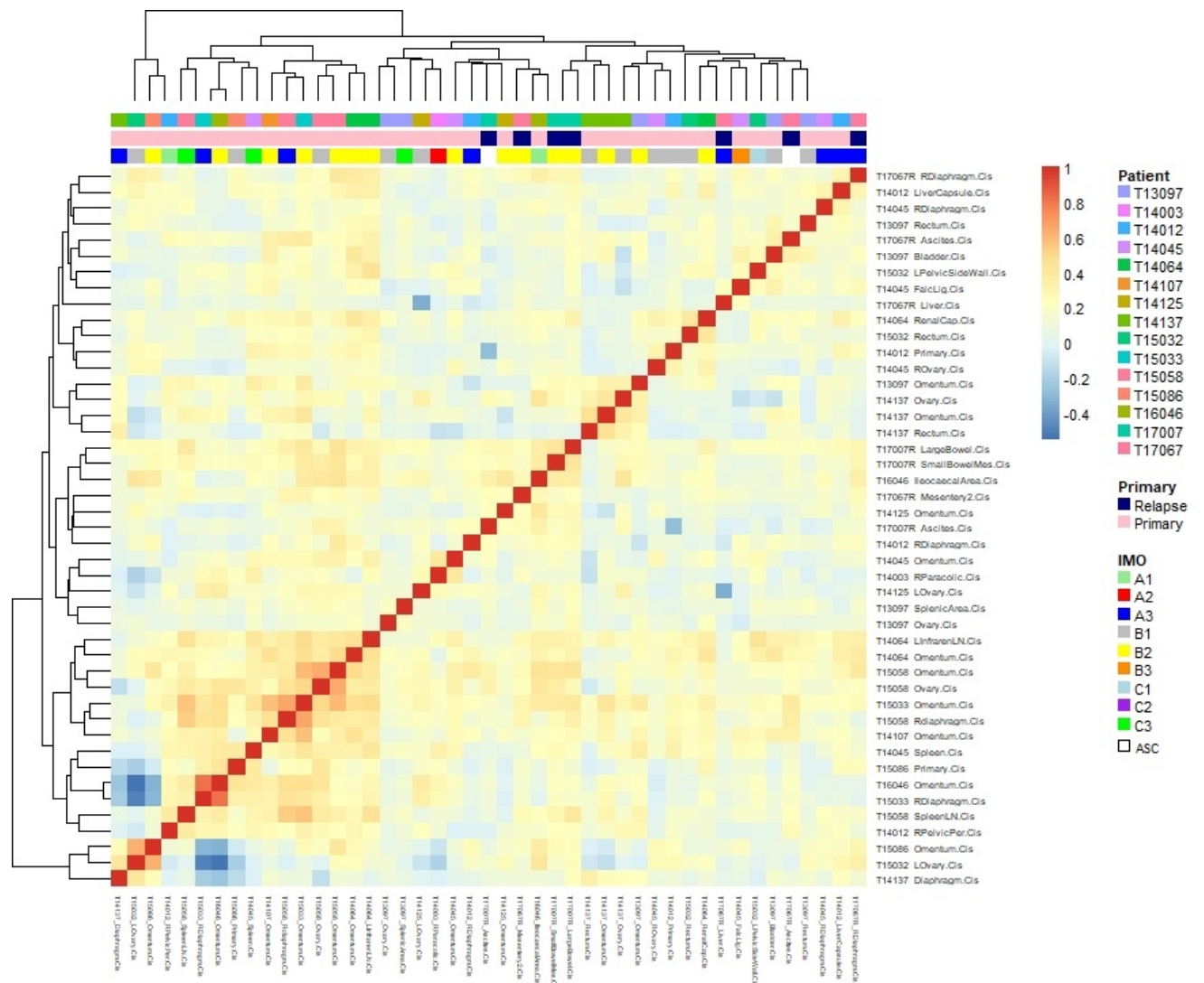


Figure 4. 6: Proteomic intratumor heterogeneity in all tumour deposits. This heatmap was based on the correlation between each deposit. The red colour bar indicates strong correlation, while the blue colour bar indicates weak correlation. The clustering was made based on several different parameters: clustering based on patient, primary or relapse cases, and IMO.

A correlation matrix analysis was also performed on a matched case, T14137 and T17067 (Figure 4.7), to investigate the temporal proteomic heterogeneity in HGSOc. We found the same result as in the previous analysis, in which each sample had the highest correlation with itself. However, it can be observed that there were two clusters on this heatmap, which indicates the similarity in proteomics profile within each cluster, suggesting that these samples came from the same clone.

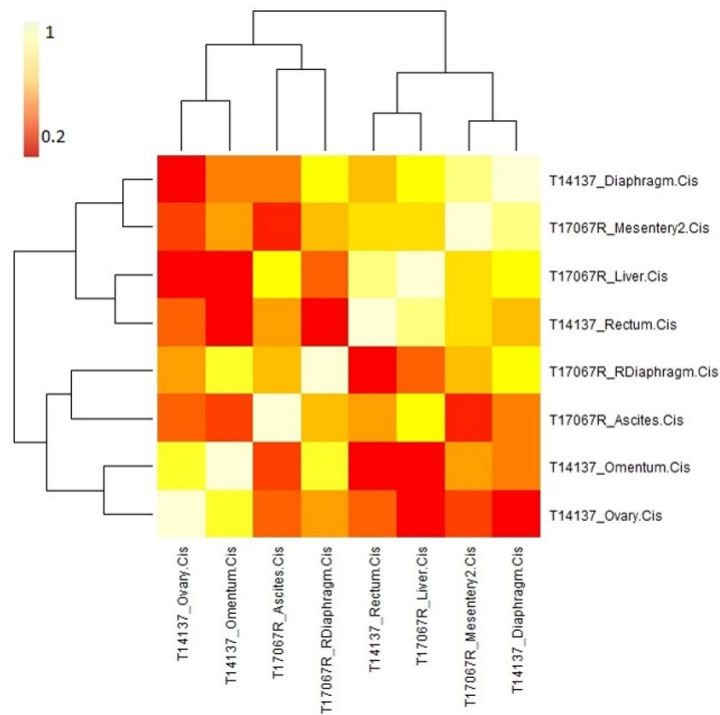


Figure 4. 7: Correlation matrix between primary and relapse cases. This heatmap was based on the correlation between each deposit. The lighter the colour, the higher the correlation between each sample.

To quantify the level of proteomic heterogeneity for each patient, we computed the mean of pair-wise correlation coefficients for all deposits from the same patient (Table 4.7); higher correlation meant lower heterogeneity and vice versa. In this analysis, two patients were excluded due to a limited number of samples. Based on the quantification, patient T15086 had the highest heterogeneity score, while patient T15058 had the lowest. This value is further analysed in section 4.2.3 to determine the association between heterogeneity and clinical features.

Table 4. 7 Heterogeneity score (mean of pair-wise correlation coefficient) in the RPPA dataset

Patient ID	Mean Correlation
T15086	-0.06862
T16046	0.053381
T14125	0.100942
T15032	0.144395
T17067R	0.148676
T14045	0.150424
T13097	0.156099
T14012	0.159879
T17007R	0.297079
T14137	0.335054
T14064	0.407442
T15033	0.415971
T15058	0.450282

4.2.3 Relationship between Proteomic Changes and Clinical Outcome

To investigate the value of a proteomic heterogeneity score as a prognostic indicator of survival, univariate Cox proportional hazards modelling was performed, as well as for the potential confounding factors (Table 4.8). The correlation value was divided into two categories using mean low heterogeneity (high mean correlation) and high heterogeneity (low mean correlation) for the dichotomous analysis. Three patients were excluded from the survival analysis due to a limited number of samples from their cases. The median PFS of the low heterogeneity group was 470.5 (378 - 725) days, and 335 (271 - 349) days for patients with high heterogeneity. The median OS for the low heterogeneity group was 809 (460 - 1050) days, and 961 (666-1211) days for the high heterogeneity group. There were no statistically

significant differences in PFS between the low and high heterogeneity groups in this study cohort (Log rank test, $p = 0.19$). This might be due to low number of patients. However, from Figure 4.8 (A) we can observe that the low heterogeneity group had a greater PFS. In contrast, there was no clear trend regarding heterogeneity and OS (Figure 4.8 B). Multivariate Cox proportional hazards modelling was also performed on this cohort, but none of the factors had any association with either PFS or OS (Table 4.8) because of the small number of patients.

Table 4. 8 Cox proportional hazards analysis of heterogeneity score and clinical factors which are known to be of prognostic importance in HGSOc

<i>Continuous Univariate Analysis</i>				
Clinical Outcome	Variable	HR	95%	p-value
PFS	Heterogeneity score	0.08	0.01 - 5.89	0.247
	Age	1.01	0.96 - 1.07	0.591
OS	Heterogeneity score	20.67	0.08 - 49	0.280
	Age	1.06	0.97 - 1.15	0.228
<i>Dichotomised Univariate Analysis</i>				
Clinical Outcome	Variable	HR	95%	p-value
PFS	Heterogeneity score (high vs low)	2.62	0.58 - 11.72	0.208
	Age (> 60 vs ≤60)	0.82	0.20 - 3.32	0.784
	FIGO stage (IV vs III)	1.77	0.32 - 9.89	0.513
OS	Heterogeneity score (high vs low)	0.52	0.11 - 2.64	0.435
	Age (> 60 vs ≤60)	2.25	0.35 - 14.41	0.392
	FIGO stage (IV vs III)	2.02	0.34 - 12.19	0.443
<i>Multivariate Analysis</i>				
Clinical Outcome	Variable	HR	95%	p-value
PFS	Heterogeneity score (high vs low)	3.94	0.54 - 28.94	0.178
	Age (> 60 vs ≤60)	1.60	0.25 - 10.00	0.617
	FIGO stage (IV vs III)	2.03	0.33 - 12.51	0.446
OS	Heterogeneity score (high vs low)	0.49	0.08 - 3.11	0.446
	Age (> 60 vs ≤60)	1.65	0.22 - 12.33	0.625
	FIGO stage (IV vs III)	2.49	0.35 - 17.92	0.365

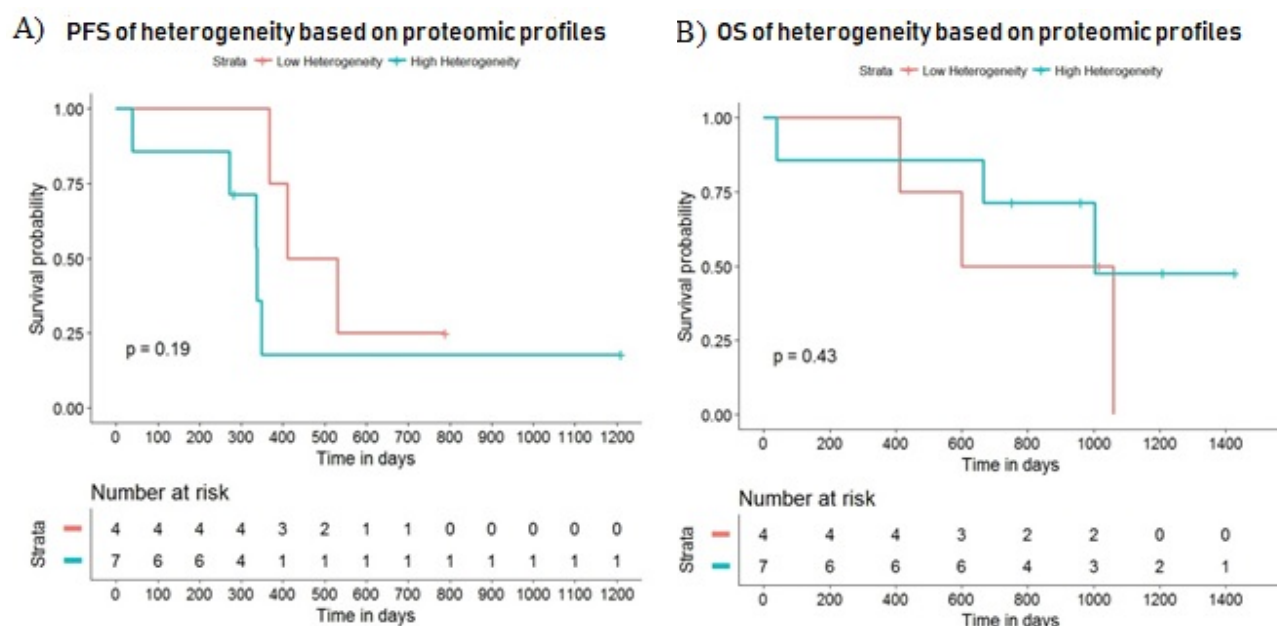


Figure 4. 8: Kaplan-Meier survival curves for patients, stratified by heterogeneity based on their proteomic profiles. Univariate analyses for (A) PFS and (B) OS.

We further investigated which proteins varied according to PFS using linear regression analysis (Table 4.9). There were only two proteins that remained significant after multiple testing: eEFK2K and PR. The levels of eEFK2K and PR expression across each patient were divided into low and high expression according to the median, and Kaplan-Meier survival analyses were performed (Figure 4.9). From these analyses, it can be observed that platinum-induced high eEF2K expression is associated with worse outcome ($p = 0.042$), as is platinum-induced low PR expression ($p = 0.019$). The same analysis was performed to investigate whether either protein had an association with OS following cisplatin treatment; however, neither protein had a significant association following multiple testing (Table 4.10).

Table 4. 9 Platinum-induced protein changes in relation to PFS

Protein Probe Names	p-Value	Adjusted p-Value
eEF2K-R-V	3.63E-05	0.00669
PR-R-V	4.61E-05	0.00669
eEF2-R-C	0.000943	0.058955
Hexokinase-II-R-V	0.000999	0.058955
Stat5a-R-V	0.001016	0.058955
Cox-IV-R-V	0.002559	0.123706
b-Catenin-R-V	0.005525	0.175317
4E-BP1_pS65-R-V	0.005949	0.175317
PRAS40-M-C	0.006845	0.175317
B7-H4-R-C	0.007156	0.175317

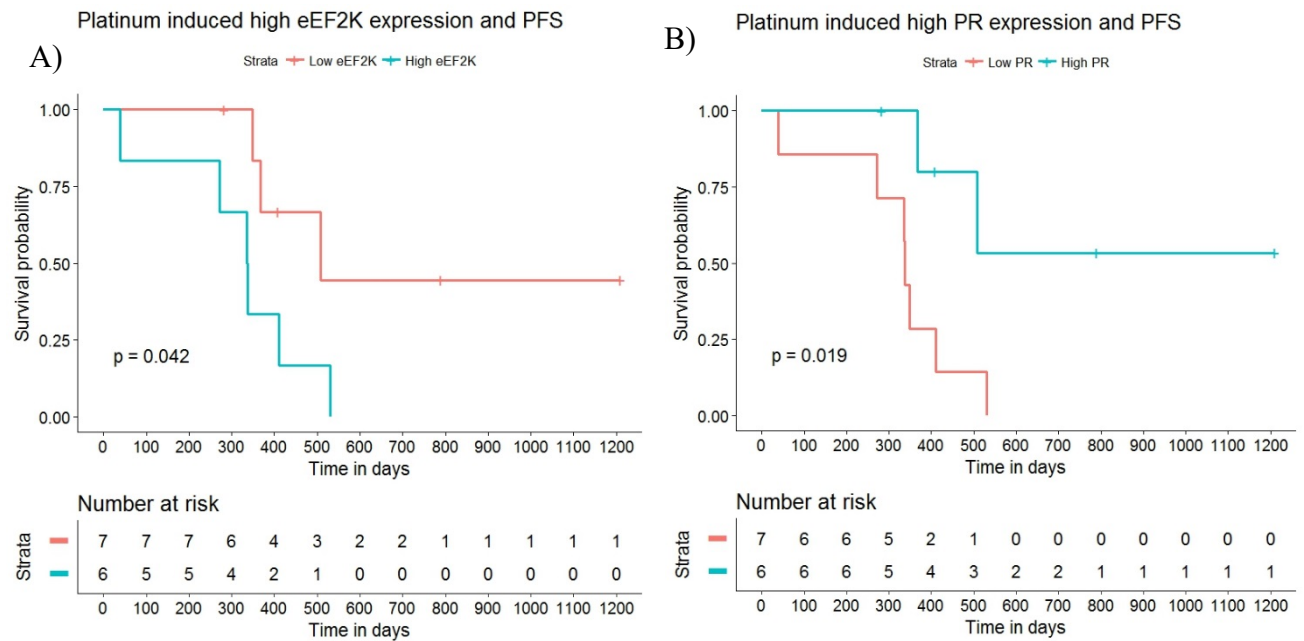


Figure 4. 9: Kaplan-Meier survival curves for patients, stratified by heterogeneity based on proteomic profiles. Univariate analyses for (A) eEF2K and PFS and (B) PR and PFS.

Table 4. 10 Platinum induced proteins changes in relation to OS

Protein Probe Names	p- Value	Adjusted p- Value
CD29-M-V	0.001381	0.400424
B7-H4-R-C	0.003786	0.424682
E2F1-M-V	0.004618	0.424682
eEF2-R-C	0.005858	0.424682
NDRG1_pT346-R-V	0.011462	0.467434
HSP27_pS82-R-V	0.013672	0.467434
FoxM1-R-V	0.018286	0.467434
PR-R-V	0.020158	0.467434
TWIST-M-C	0.021723	0.467434
PDHK1-R-C	0.022031	0.467434

Next, we assessed whether any protein had any association with clinically-resistant relapse. In order to achieve this aim, a comparison was made between samples deemed platinum resistant or sensitive according to the patient's clinical response, both in cisplatin-treated and untreated samples. Twenty proteins that were differentially expressed as a result of this analysis; however, none were significant after false discovery rate (FDR) testing (Table 4.11). In addition, the fold changes observed were < 20%, both for down-regulated and up-regulated proteins, making it doubtful that these differences have biological relevance. In the following table, GSK-3a-b_pS21_S9 showed the highest increase in expression (1.09 fold), whereas Rad51 showed the greatest reduction (0.86 fold). Interestingly, the platinum-induced expression of GSK-3a-b_pS21_S9 in a resistant relapse case (T17067R) was higher than in the primary case (T14137) (Figure 4.10) which had a good apoptotic response to cisplatin according to the phenotypic result shown in Chapter 3 (Figure 3.5). However, this is not statistically significant, and a comparison of GSK-3a-b_pS21_S9 levels between further sensitive and resistant-relapse samples is needed to confirm this finding.

Table 4. 11 Ten most significant platinum-induced protein changes in relation to resistant relapse

Protein Probe Names	p-Value	Adjusted p-Value	Fold change
Rad51-R-V	0.003971	0.444999	0.86
E2F1-M-V	0.006108	0.444999	0.92
YB1_pS102-R-V	0.006605	0.444999	0.93
FoxO3a_pS318_S321-R-C	0.009387	0.444999	1.06
MMP2-R-V	0.009827	0.444999	0.94
D-a-Tubulin-R-V	0.00997	0.444999	0.94
GSK-3a-b_pS21_S9-R-V	0.011235	0.444999	1.09
GSK-3a-b-M-V	0.013516	0.444999	0.87
MIF-R-C	0.013939	0.444999	1.06
MEK1-R-V	0.016481	0.444999	1.06

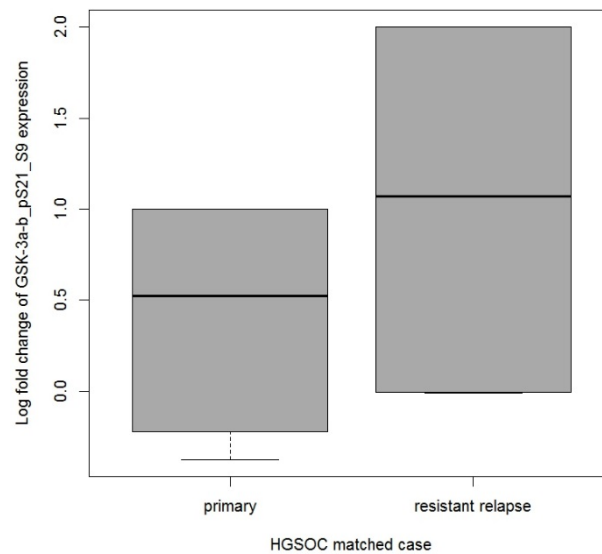


Figure 4. 10: Platinum-induced GSK-3a-b_pS21_S9 expression in a matched case, T14137 (primary case) and T17067R (relapse case). The fold change of platinum-induced GSK-3a-b_pS21_S9 expression in resistant relapse samples was higher than in primary samples, indicating that the expression level of GSK-3a-b_pS21_S9 increased more in platinum-resistant patient samples ($p = 0.188$, 95% CI = 0.40 - 1.02).

4.2.4 Correlation of Proteomic Changes and Phenotypic Response

The key mechanism of cisplatin-induced cancer cell death is apoptosis. Linear regression analysis was performed to investigate platinum-induced proteins changes in relation to corresponding data from phenotypic apoptotic responses to cisplatin treatment (25 μ M) for 24 hours (see Chapter 3) in our global RPPA cohort. Table 4.12 shows the top 10 proteins that varied most significantly according to apoptosis response. Among these proteins, only one protein (DUSP4) remained significant after multiple testing. We further investigated the correlation of DUSP4 and Cdc2_pY15 (the latter had borderline significance, with an adjusted p-value of 0.055068) with apoptosis response using scatter plots (Figure 4.11). In this analysis, DUSP4 protein expression had a moderately positive correlation with apoptotic response to cisplatin treatment (Spearman correlation coefficient = 0.413, p-value = 0.0052). DUSP4 is a member of the dual-specificity phosphatases (DUSPs) family and is also referred to as mitogen-activated protein kinase phosphatase 2 (MKP2). It may promote both survival and apoptosis by dephosphorylating ERK, JNK and p38. We also found that Cdc2_pY15 protein expression had a moderate and significant positive correlation with apoptotic response (Spearman correlation coefficient = 0.386, p-value = 0.0092). Phosphorylation of Cdc2 at Tyr15 results in the inhibition of Cdc2 activity, which mainly regulates the progression from G2- to M-phase.

Table 4. 12 Platinum induced protein changes in relation to apoptotic response to cisplatin treatment

Protein Probe Names	p-Value	Adjusted p-Value
DUSP4-R-V	0.000154	0.044603
Cdc2_pY15-R-C	0.00038	0.055068
eIF4G-R-C	0.000694	0.067043
Notch1-R-V	0.002115	0.153306
eEF2K-R-V	0.004492	0.258392
VEGFR-2-R-V	0.005939	0.258392
YB1_pS102-R-V	0.006356	0.258392
p70-S6K1-R-V	0.007128	0.258392
p90RSK_pT573-R-C	0.008857	0.269045
PR-R-V	0.010014	0.269045

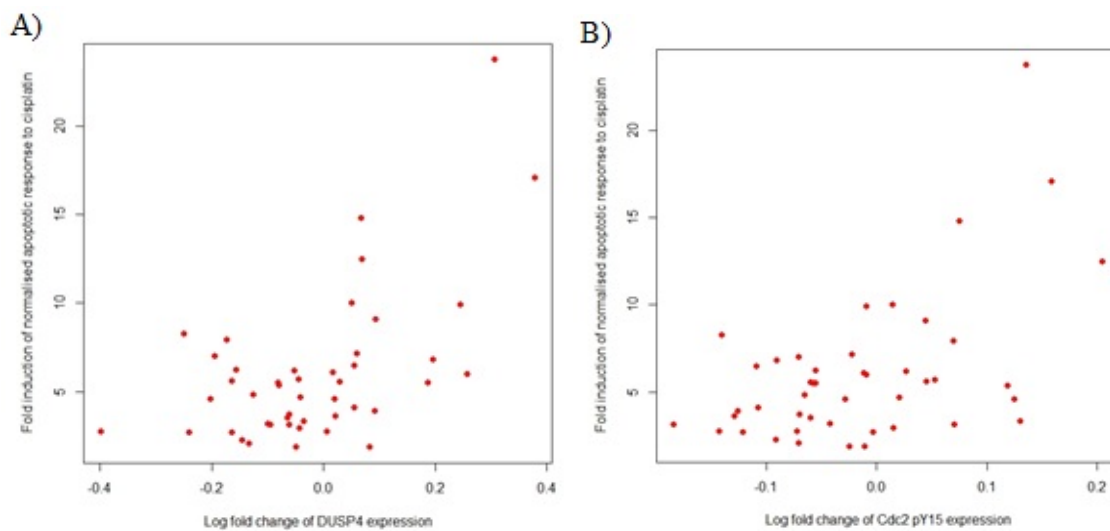


Figure 4. 11: Scatter plots demonstration correlation between platinum-induced protein expression changes and phenotypic apoptotic response following cisplatin treatment. Each dot represents a tumour deposit. A) DUSP4 protein expression has a positive correlation with apoptotic response, Spearman correlation coefficient = 0.413, p-value = 0.0052. B) Cdc2_pY15 protein expression also has a positive correlation with apoptotic response, Spearman correlation coefficient = 0.386, p value = 0.0092.

4.2.5 Validation of RPPA Data in Paired Platinum Sensitive and Resistant HGSOC Cell Lines *In Vitro*

Both p-Chk1 (Ser296) and p-p38 (Thr180/Tyr182) expression levels increased following cisplatin treatment

To validate the result of RPPA analysis, which showed an increase of p-Chk1 (Ser296), p-p38 (Thr180/Tyr182), and p-HSP27 (Ser82) after cisplatin treatment in primary tumour cultures, a pair of known platinum-sensitive and resistant HGSOC cell lines, PEO1 and PEO4 were treated with cisplatin for 2 hours and 4 hours, and the protein lysates were collected according to the RPPA protocol. Western blotting was then performed to demonstrate the protein expression changes in the paired cell lines. The validation experiments in this section were performed in collaboration with an Imperial College Cancer Biology MRes student Linda Han. Cisplatin treatment led to a dramatic increase in Chk1 phosphorylation at Ser296 in both PEO1 and PEO4 cell lines (Figure 4.12 A). Using densitometry analysis with vinculin as a loading control, an increase of p-Chk1 (Ser296) protein expression was observed in PEO1 2 hours and 4 hours after cisplatin treatment and in PEO4 after 2 hours of cisplatin treatment (Figure 4.12 B). However, only a minor change in protein expression in PEO4 was observed after 4 hours of platinum treatment (Figure 4.12 C).

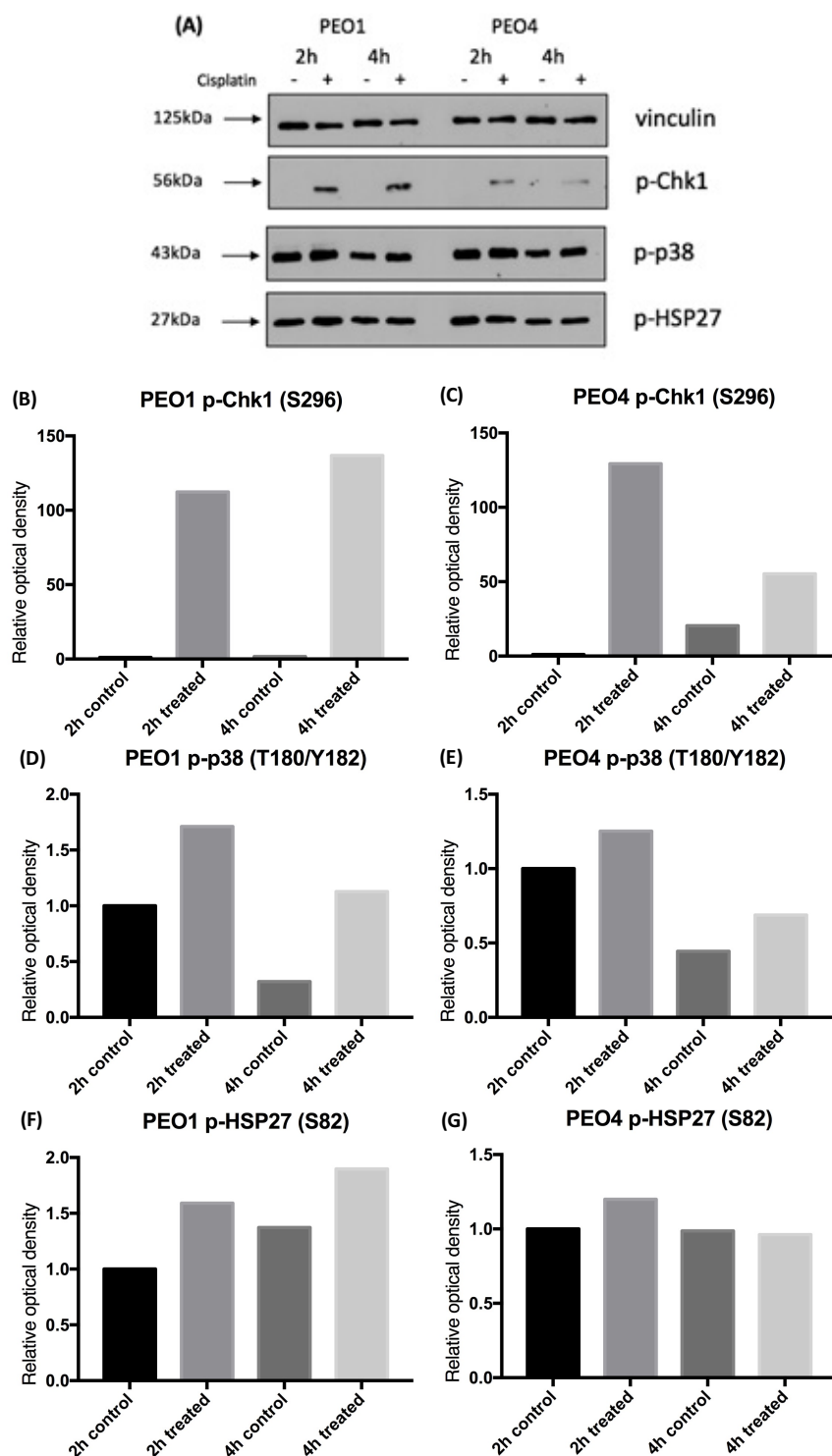


Figure 4. 12: Protein expression changes after platinum treatment. (A) Western blot for vinculin (loading control), p-Chk1, p-p38, and p-HSP27 after 2-hour and 4-hour platinum treatment in the PEO1 and PEO4 cell lines. (B)(D)(F) Relative optical densitometry of vinculin normalised p-Chk1 (S296), p-p38 (T180/Y182) and p-HSP27 (S82) expression in PEO1 cells with or without cisplatin treatment. (C)(E)(G) Relative optical densitometry of vinculin normalised p-Chk1 (S296), p-p38 (T180/Y182) and p-HSP27 (S82) expression in PEO4 cells with or without platinum treatment. Densitometry is representative of the above blots, the experiment was repeated three times.

Figure 4.12A also shows minor increases in phosphorylation of p38 (Thr180/Tyr182) following cisplatin treatment in both cell lines. Measuring the relative optical density of p-p38 with densitometry revealed that the expression levels of this phosphorylated protein were lower after 4 hours compared to 2 hours treatment in both PEO1 and PEO4 (Figure 4.12 D and E). There was only a minor increase in phosphorylated HSP27 observed in PEO1 cell lines (Figure 4.11 F), and almost no change was seen in PEO4 cell lines (Figure 4.12 G). This confirms that the protein expression level of p-Chk1 (Ser296) and p38 (Thr180/Tyr182) were increased in sensitive and resistant HGSOC cell lines after cisplatin treatment, which is consistent with the results from our RPPA analysis.

Effects of Chk1 and p38 inhibition on p-Chk1 and p-p38 expression

To investigate the potential benefit of Chk1 and p38 expression modulation using selective inhibitors, we first assessed the efficacy of inhibitors for these proteins using the paired cell lines PEO1 and PEO4. The doses of both inhibitors used in these preliminary experiments were based on previous studies (Moscova, Marsh and Baxter, 2006; Brill *et al.*, 2017). Each cell line was treated with each inhibitor and cisplatin, and Western blots were performed to check for changes in protein expression levels (Figure 4.13 A and B). The expression of phosphorylated Chk1 protein was significantly higher following cisplatin treatment, compared to that of the untreated cells. Relative optical density shows that the inhibition of Chk1 with prexasertib abolished phosphorylation of Chk1 in both PEO1 and PEO4 cell lines (Figure 4.13 C). In PEO1 cell lines, cisplatin treatment did not result in any significant change to the level of p-p38 protein expression (Figure 4.13 B and D), whereas in PEO4 cell lines, the protein expression of p-p38 following cisplatin treatment was doubled compared to that of untreated cells. According to the densitometry analysis, treatment with the p38 inhibitor SB203580 alone

resulted in the slight decrease of p-p38 expression levels both in PEO1 and PEO4 cells, compared to untreated cells (Figure 4.13 D). Whereas the combination treatment of p38 inhibitor SB203580 with cisplatin leads to small increased p-p38 expression in both cell lines compared to SB203580 single treatment.

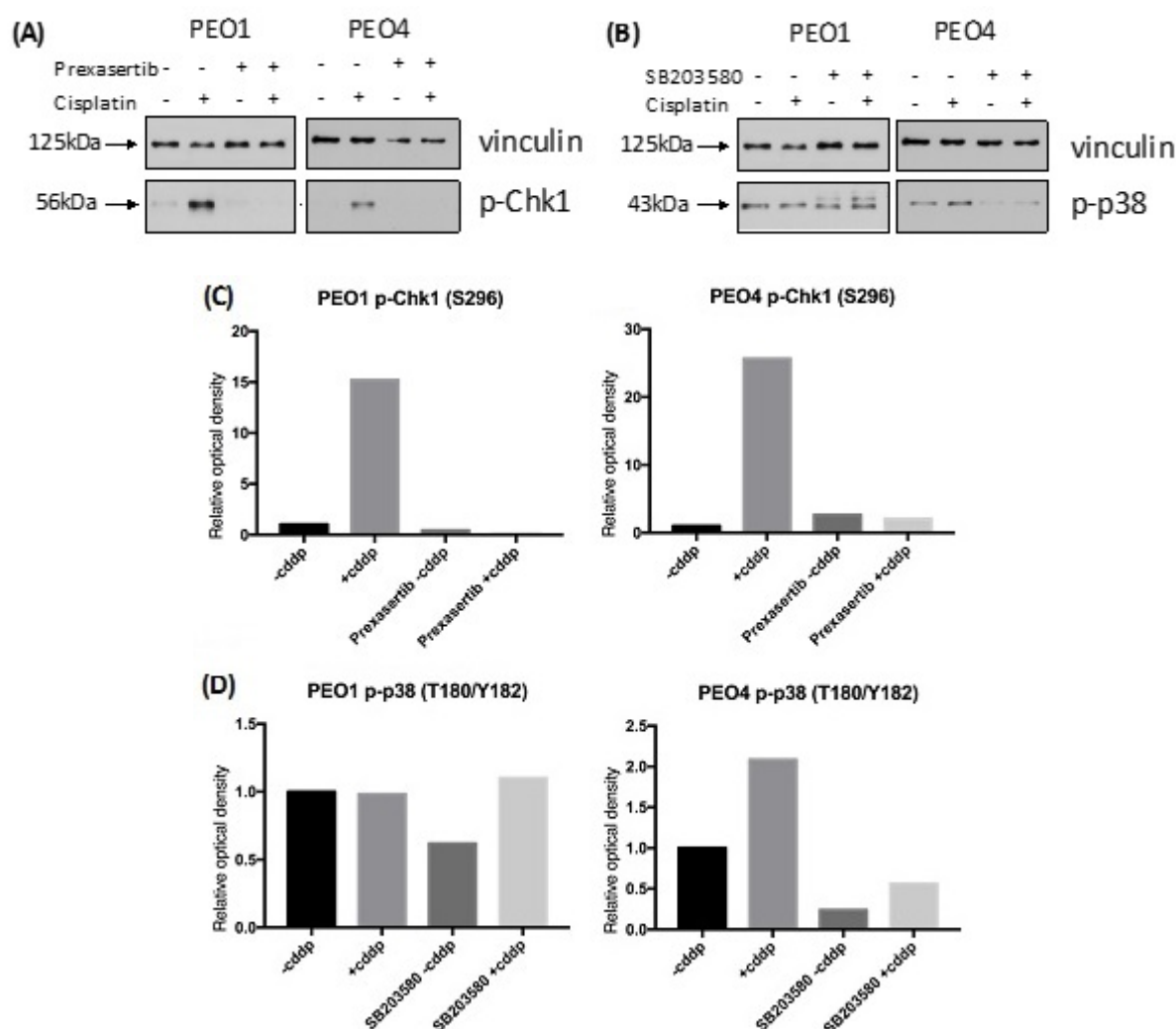


Figure 4. 13: Effects of Chk1 and p38 inhibition on p-Chk1 and p-p38 expression. Both PEO1 and PEO4 cells were treated with a combination of cisplatin (25 μ M) with prexasertib (50nM) and SB203580 (10 μ M) separately for 4 hours. DMSO was used as a control for untreated cells. Western blots for vinculin, p-Chk1 (A) and p-p38 (B) show protein expressions in PEO1 and PEO4 after the 4 hours drug treatment. Relative optical density for p-Chk1 (C) and p-p38 (D) in PEO1 and PEO4 which were calculated relative to the optical density of vinculin (control).

Chk1 inhibitor enhances cisplatin-induced apoptosis in HGSOC cell lines

Next, we investigated whether Chk1 and p38 inhibitors could enhance platinum-induced apoptosis in sensitive HGSOC cell lines or re-sensitise platinum-resistant cell lines to cisplatin. Prexasertib (Chk1 inhibitor) and SB203580 (p38 inhibitor) were used to treat both PEO1 and PEO4 cell lines as a monotherapy or in combination with platinum chemotherapy. In both cell lines, the combination of prexasertib and cisplatin (10 μ M for PEO1 and 25 μ M for PEO4) resulted in a significant increase of apoptosis normalised to viability compared to cells treated with cisplatin alone (student T-test, $p=0.0007$ for PEO1 and $p=0.04$ for PEO4, Figure 4.14 A and B respectively). The combined therapy led to more than a twofold increase in relative apoptosis, which was mainly due to the high percentage of apoptosis rather than decreased cell viability. In contrast, the combination of SB203580 with cisplatin treatment did not enhance the apoptotic response in either of the HGSOC cell lines (Figure 4.14 C and D). This result is consistent with the Western blot result in the previous section, indicating that p38 inhibitor at this dose did not have any effect on PEO1 or PEO4.

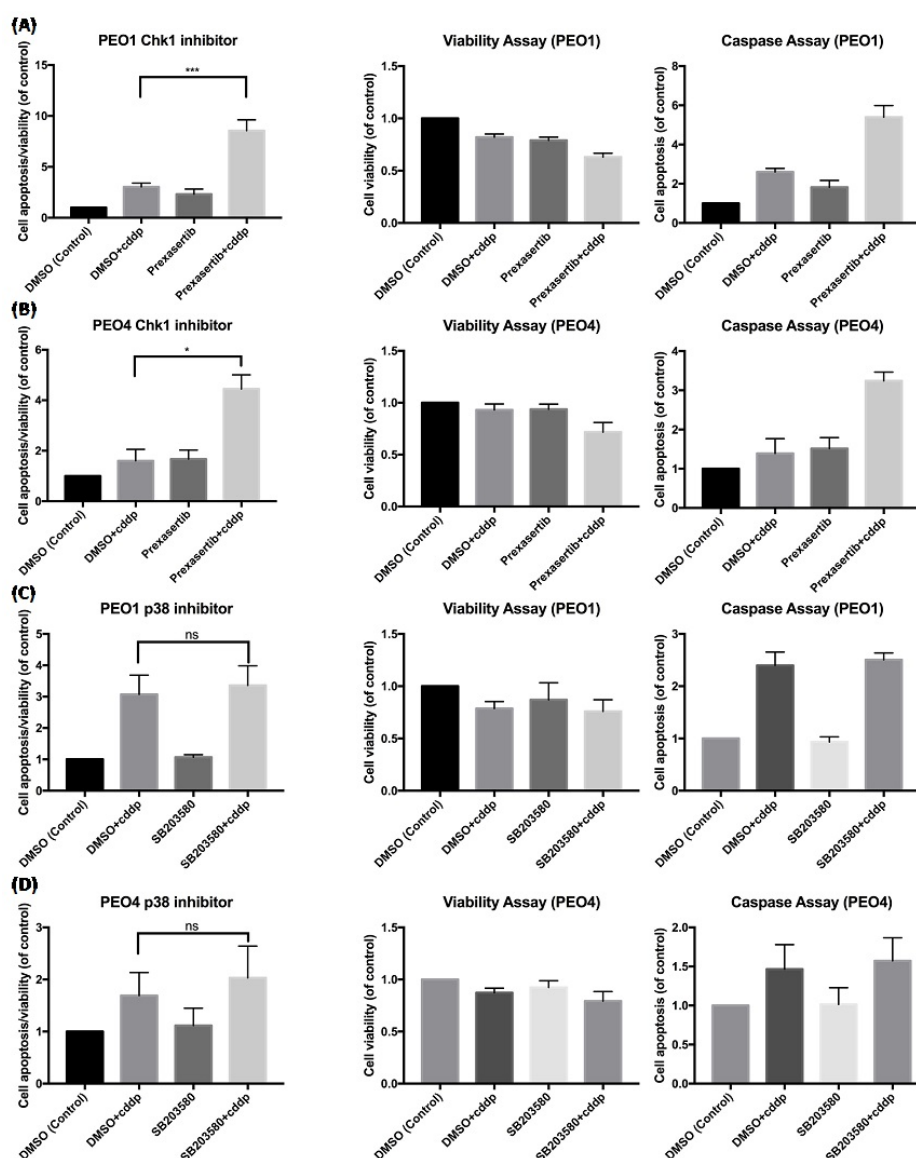


Figure 4. 14: Apoptosis and cell viability in PEO1 and PEO4 cells treated with Chk1 and p38 inhibitors as a single agent or in combination with cisplatin. The combinations of cisplatin with 50nM prexasertib (A and B) and 10 μ M SB203580 (C and D) treatment were performed in both PEO1 and PEO4 cell lines. Cell viability and caspase-3/7 assays were conducted after 24 hours of drug treatment. The error bar represents the standard error (SE) of the mean of the three independent experiments. Student t-tests were performed to determine the significance (***) $p \leq 0.001$, * $p \leq 0.05$, ns $p > 0.05$)

4.3 Discussion

Cis-diaminodichloroplatinum (cisplatin) consists of a central platinum atom, two chlorides and two NH₃ groups in the *cis* position. This drug is one of the main chemotherapy regimens in ovarian cancer; however, drug resistance is commonly observed in many patients treated with

platinum agents. Mechanisms describing the development of cisplatin resistance include the decreased cisplatin accumulation in the cancer cells, increased drug metabolism in the liver, increased cisplatin-DNA adduct repair and modulation of apoptotic pathways (Stewart *et al.*, 2006). Currently, the diversity in platinum response and complete lack of predictive biomarkers means that delivering the best treatment for HGSOC patients remains challenging. This chapter investigated the proteomic signalling changes in tumour cells extracted from HGSOC deposits following *in vitro* cisplatin treatment using reverse phase protein assay proteomics, by analysing the differential protein expression between cisplatin-treated and untreated cells.

Modulation of signal transduction pathway following cisplatin treatment in HGSOC

The formation of cisplatin-DNA adducts following drug uptake into the nucleus modulates several signal transduction pathways that mediate cisplatin's cytotoxicity. There are three major pathways of MAPKs in human cells: p38 kinase, stress-activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK), and extracellular signal-regulated kinase (ERK). The importance of MAPK activation in cell response and the development of resistance to cisplatin has been gradually appreciated in recent years. The MAPK activation is believed to be a major component in deciding the cell's fate in response to platinum. Their role in cisplatin response is complex, as these proteins can induce apoptosis, but also play a role in cell survival. The final outcome depends on a balance of activity between several of these networks and cell types (Brozovic and Osmak, 2007). In this study, we found the induction of p38 MAPK pathway as a cellular response to cisplatin treatment. We confirmed the increased level of phosphorylated p38 after 4 hours of cisplatin treatment by Western blotting, which is consistent with the trend of fold change of p-p38 (Thr180 and Tyr182) in our RPPA dataset. This finding is in line with earlier findings that reported the activation of p38 MAPK following cisplatin treatment (Benhar

et al., 2001; Losa *et al.*, 2003). This pathway has been shown to mediate cell death through its activation. The pro-apoptotic role of p38 includes the phosphorylation and translocation of the Bcl-2 family proteins, leading to the release of cytochrome-c from the mitochondria and the caspase 8 activation induced by transforming growth factor- β (Lenassi and Plemenitaš, 2006). This is further supported by the observation of increased pBad and decreased Bcl-xL in our dataset. Another study demonstrated that the p38 MAPK pathway contributed to cisplatin-induced phosphorylation of histone H3 at Ser10 (Wang and Lippard, 2004). In contrast to the pro-apoptotic role of p38, this modification facilitates the binding of the remodelling complex and repair proteins to the nucleosome, which leads to DNA repair activation and helps cells to survive cisplatin-induced stress. Our finding showed the upregulation of histone H3 upon cisplatin treatment. Unfortunately, its phosphorylation status at Ser10 was not investigated as it is currently not available on the list of RPPA probes. In addition, a previous report showed that inhibition of p38 MAPK resulted in ROS upregulation, JNK pathway activation, and, in turn, sensitised human tumour cells to cisplatin-induced apoptosis (Pereira *et al.*, 2013). The potential use of p38 inhibitor in enhancing cell death in response to cisplatin in our cohort is discussed in following section.

One report demonstrated that p38 MAPK activation induced phosphorylation of HSP27 via MAPKAPK2 pathway, affecting the control of apoptosis (Nakashima *et al.*, 2011). The upregulation of phosphorylated HSP27 was also found in the current cohort. However, this finding did not appear to be validated in the paired platinum-sensitive and resistant HGSOC cell lines, although the fold change of phosphorylated HSP27 at Ser82 was the highest (1.46) among all proteins in the RPPA analysis, and the upregulation of p-HSP27 was observed in all samples analysed. This may be due to the different source of samples utilised (primary cells vs

cell lines). Thus, further research using samples from different HGSOC patients is needed to confirm the upregulation of HSP27 at Ser82 in protein lysate following cisplatin treatment. Overexpression of HSP27 has been observed in ovarian cancer, and its upregulation has been associated with cisplatin resistance via the upregulation of the Akt pathway (Langdon *et al.*, 1995; Yamamoto *et al.*, 2001). The upregulation and activation of Akt was also found in our analysis. This finding was confirmed by the pathway enrichment analysis, which showed the enrichment of the PI3K-Akt signalling pathway. Akt activation has been linked to enhanced cell survival and is also involved in the development of platinum resistance in HGSOC (Winograd-Katz and Levitzki, 2006; Stronach *et al.*, 2011). The phosphorylation of Akt can be catalysed by PKC- β -II, which was also found to be activated and upregulated in our dataset. This finding is in line with a recent study which demonstrated that cisplatin promoted PKC β expression in Hela cells (Li and Zhang, 2017). The same study also suggested that PKC β is involved in cisplatin cytotoxicity through its inhibition of autophagy (Li and Zhang, 2017). Collectively, our data indicates that cisplatin treatment results in mediation and crosstalk between several pathways involved in cell survival and apoptosis.

Several reports have shown that c-Jun phosphorylation may result in determining cell survival or apoptosis, depending on the duration of JNK activation (Mansouri *et al.*, 2003; Li *et al.*, 2005; Yan, An and Kuo, 2016). According to a study, transient JNK activation (1-3 hours) resulted in cell survival and was observed in cisplatin-resistant ovarian cell lines, whereas prolonged JNK activation (up to 12 hours) promoted apoptosis and was observed in the cisplatin-sensitive cell lines (Mansouri *et al.*, 2003). c-Jun phosphorylation at Ser73 was found to be activated after a 4-hour cisplatin administration in our cohort. This result supports the notion that cancer cells are most likely to respond to front-line platinum treatment.

Checkpoint kinases (Chk1 at Ser296 and Chk2 at Thr68) were increased in this RPPA dataset as a result of cisplatin-induced DNA damage. We also confirmed the increased level of phosphorylated Chk1 (Ser296) in both platinum-sensitive and platinum-resistant HGSOC cell lines using Western blotting. Phosphorylation of Chk1 led to checkpoint activation and cell cycle arrest, as well as activating HR repair of DSBs (Bartek and Lukas, 2003), indicating activation of the survival pathway following cisplatin treatment. The activation of this protein is important for protecting the cells in response to DNA damage, particularly in cells lacking functional p53 (a functional G1 checkpoint), including ovarian cancer cells (Massey *et al.*, 2015).

In this study we demonstrated increased phosphorylation of H2AX at Ser40 (also known as γ H2AX) upon cisplatin treatment. This phosphorylated protein is essential for the recruitment of DNA damage-repair proteins in response to DNA double-strand breaks (Kinner *et al.*, 2008). The phosphorylation of H2AX at Ser140 can be mediated by PI3K proteins including DNA-PK, ATM, and ATR (Bonner *et al.*, 2008). The expression level of another histone-modification marker, di-methylation of histone H3 lysine 9 (H3K9me2), was increased in our dataset. This post-translational modification is associated with ‘closed’ chromatin and presents at DNA-hypermethylated promoters of tumour-suppressor genes in cancers; it is also linked with poor prognosis in ovarian cancer (Hua *et al.*, 2014). A recent study on ovarian cancer cell lines demonstrated that dual inhibition of DNA methylation and H3K9 di-methylation by 5-aza-2'-deoxycytidine and G9Ai results in the reduction of H3K9me2 levels and induction of cell death (Liu *et al.*, 2018). Thus, our finding may support the potential combination use of cisplatin and epigenetic modifiers to increase treatment efficacy in ovarian cancer (Meng *et al.*, 2013).

An intra-tumoural hypoxic microenvironment is common in solid tumours. To overcome this condition, tumour cells have to adjust their metabolism and release pro-angiogenic factors through the activation of Hif-1-alpha (Masoud and Li, 2015). Decreased levels of Hif-1-alpha were found in tumour cells treated with cisplatin, indicating suppression of the survival pathway in our cohort. In the future, it will be interesting to confirm the downregulation of Hif-1-alpha in other cohort, for instance using western blotting. COX IV showed the greatest level of downregulation in the HGSOc tumour cells treated with cisplatin. A previous study found that the number of apoptotic cells increased with suppressed COX IV expression, suggesting that a defective cytochrome c oxidase complex could sensitise the cells to apoptotic signals (Li *et al.*, 2006). These results are in agreement with recent studies reporting that HIF-1 α inhibition downregulated COX IV expression and promoted ROS production, and may therefore increase cell death (Hao *et al.*, 2018).

The phosphorylated FAK at Y397 expression was decreased in our cohort, whereas in cholangiocarcinoma cells, treatment with cisplatin did not affect p-FAK (Wandee *et al.*, 2018). A critical cytoplasmic tyrosine kinase, FAK promotes cancer progression and metastasis both via cancer and stromal cells. The inhibition of this protein has been shown to increase chemotherapeutic efficacy in ovarian cancer (Sulzmaier, Jean and Schlaepfer, 2014).

We also identified that DUSP4 has a strong correlation with apoptosis following cisplatin treatment, from correlations with our apoptosis data collected as described in Chapter 3. It has been postulated that DUSP4 can promote both survival and apoptosis by modulating MAPK pathways depending on the tissue and molecular subtypes (Menyhart *et al.*, 2017).

In contrast to our findings, conditional overexpression of DUSP4 reversed cisplatin-mediated apoptosis by selectively dephosphorylating JNK (CadAlbert *et al.*, 2005). Very little is known about the role of DUSP4 in HGSOC; however, one study demonstrated that DUSP4 was down-regulated in serous carcinomas, compared to serous borderline tumours, which could be attributed to increasing stromal invasion (CadAlbert *et al.*, 2005).

This study is the first to our knowledge that investigates platinum-induced signalling changes in primary HGSOC cells extracted from different multi-site tumour deposits in different patients. In general, cisplatin-induced DNA damage modulates several signal transduction pathways including Akt, PKC, c-ABL, p53, and MAPK/JNK/ERK pathways (Wang and Lippard, 2005; Basu and Krishnamurthy, 2010). In this study, we showed that cisplatin activated several pathways that induced apoptosis and cell survival, including Akt, PKC, and MAPKs (JNK and p38 MAPK). We also showed the down-regulation of COX IV and Hif-1-alpha, which are involved in survival pathways and have not previously been linked with cellular responses following cisplatin treatment. Together, this global analysis has provided evidence of the dynamic upregulation and downregulation of apoptotic and survival pathways in HGSOC following cisplatin treatment.

The presence of proteomic heterogeneity in HGSOC

The presence of tumour heterogeneity in cancer pathogenesis has profound consequences regarding the improvement of cancer therapy through the formulation of better strategies to exploit this property (Welch, 2016). Several reports have shown that protein expression varies in HGSOC, both in cell lines and primary samples, which further highlights the proteomic heterogeneity of ovarian cancer (Köbel *et al.*, 2011; Bourgeois *et al.*, 2015;

Coscia *et al.*, 2016). The findings in this chapter provide more evidence of the tumour heterogeneity that exists in HGSOC, especially from a proteomic perspective. We showed that there were differences in protein expression profiles across tumour samples following cisplatin treatment, which suggests that the same chemotherapy may result in different responses for different HGSOC patients (Figure 4.5). There was no clear clustering based on patient, primary or relapse case, or deposit location (Figure 4.6). Our findings were in agreement with a previous study, which observed differences in cell signalling within the primary and metastatic samples of advanced stage EOC and showed that there was no common pattern specific to either of the tissue microenvironments (Sheehan *et al.*, 2005). Another study investigating the molecular heterogeneity in HGSOC using 88 FFPE samples from 13 patients (5–9 samples each), observed that samples from the same patient clustered more closely together compared to samples from different patients (Mittermeyer *et al.*, 2013). However, this result may be explained by the fact that researchers only included samples that originated from the primary tumour. This report is consistent with our MDS analysis result, which depicts the samples are clustered based on their location (deposit), irrespective of whether they were cisplatin-treated or untreated.

This chapter also demonstrated temporal heterogeneity following cisplatin treatment in a matched case. We identified two clusters in a matched case, which may have originated from the same clone. Using a proteomic heterogeneity score generated from our patient cohort data, we could observe that patients with a low heterogeneity score tended to have longer PFS time. This finding is consistent with a recent study that investigated single-cell phenotypic characteristics of HGSOC using a multiparametric mass cytometry (CyTOF) approach (Gonzalez *et al.*, 2018). This study determined tumour diversity using Simpson's

diversity analysis and showed that a higher Simpson's diversity index (greater tumour diversity) was associated with shorter PFS time. Therefore, we suggest that multiple sampling is needed to avoid sampling bias when assessing signalling proteins following specific treatments of HGSOC.

Identification of indicators of survival and drug resistance

In this study, we identified potential biomarkers that could be used to predict the prognosis of HGSOC patients, namely eEF2K and PR. Our findings indicate that platinum-induced high eEF2K expression was associated with worse outcome. eEF2K can negatively modulate protein synthesis by phosphorylating eEF2 or undergoing autophosphorylation to inhibit its function in translation elongation (Fu *et al.*, 2014). This kinase can be activated by AMPK and is inhibited by mTORC1 and ERK/MAP pathways (Erdogan and Ozpolat, 2017). The inhibition of eEF2K activation leads to decreased levels of cyclin D1 and Bcl-xL thus increasing apoptosis. This protein is overexpressed in various cancers, such as breast, colon, pancreatic cancer, and glioblastoma, and also correlates with poor patient survival (Zhang *et al.*, 2011; Tekedereli *et al.*, 2012; Ashour *et al.*, 2014; Xie *et al.*, 2014). A recent study showed that siRNA-mediated knockdown of eEF2K noticeably inhibits ovarian cancer cell lines proliferation, colony formation and invasion (Erdogan and Ozpolat, 2017). The same study also reported that systematic therapy targeting eEF2K by intravenous nanoliposomal eEF2K siRNA repressed SKOV3 tumour growth in xenograft models, suggesting that eEF2K may be a novel potential therapeutic target in ovarian cancer.

We also showed that platinum-induced low PR expression was associated with worse outcome in HGSOC. Consistent with this finding, a previous report indicated that PR expression was

independently related to improved survival in HGSOC (Sieh *et al.*, 2013). Several reports have shown that PR mediates the effects of progestin on apoptosis through several mechanisms, including increased TRAIL-mediated cell death (Syed *et al.*, 2007), p53 induction (Bu *et al.*, 1997), and enhanced FasL expression (Syed and Ho, 2003). Moreover, activation of PR was also shown to induce cell cycle arrest and senescence in ovarian cancer cells (Takahashi *et al.*, 2009).

In addition, we investigated whether any particular protein correlated with OS and resistant relapse, but none had any significant associations after multiple testing in these analyses. This could be due to the heterogeneity of protein expression following cisplatin treatment or to the small sample cohort. Using baseline protein expression data of more than 200 HGSOC patients from the RPPA-TCGA database, a previous study identified 9 protein markers (AR, BID, pTAZ, pEGFR, HSP70, STAT5 α , pPKC α , pMEK1, and EE2) to develop a protein-driven index of ovarian cancer (PROVAR) risk-classification algorithm, and subsequently successfully validated its ability to predict both PFS and OS in an independent validation cohort (Yang *et al.*, 2013). Another study predicted the response of HGSOC patients to platinum-based therapy on normalisation of CA125 by the third cycle of treatment and found that JNK, EGFR, and Smad3 showed significant associations with CA125 normalisation (Carey *et al.*, 2010). Higher expression of JNK also was associated with a shorter PFS. However, subsequent validation in independent sample sets is required to confirm this finding. Using the same RPPA approach as the previous study, a recent study showed that high levels of PDGFR β and VEGFR2 in primary untreated HGSOC tumour samples associated with clinical-resistant relapse, and higher expression of PDGFR β correlated with shorter survival (Avril *et al.*, 2017).

In general, identification of biomarkers that can predict treatment efficacy in HGSOC will offer optimised treatment strategies and improved patient survival.

Targeted therapy to enhance cisplatin-induced apoptosis in HGSOC

Having confirmed the platinum-induced increased expression of p-p38 and p-Chk1, we then wanted to examine whether inhibition of the candidates using specific inhibitors could enhance or restore the platinum sensitivity in HGSOC cell lines. Our results showed that the combination of prexasertib (Chk1 inhibitor) and cisplatin treatment leads to an increased apoptotic response compared to either prexasertib or cisplatin alone in platinum-sensitive and resistant cell lines, suggesting that Chk1 inhibition can enhance or restore platinum sensitivity in platinum-sensitive and resistant HGSOC cell lines. Treatment with prexasertib has been shown to result in DNA damage, cell death, and tumour growth inhibition in xenograft models (King *et al.*, 2015). The potential anti-cancer activity of this drug as a single agent was observed in advanced solid tumours from patients in a phase 1 clinical study (Hong *et al.*, 2016). In a recent phase II clinical trial, prexasertib monotherapy demonstrated its clinical activity and was tolerable in recurrent HGSOC patients without germline BRCA mutations (Lee *et al.*, 2018). Considering its encouraging anti-tumour activity, further development and assessment of Chk1 inhibition as a therapy for treating HGSOC is warranted.

Although treatment with SB203580 showed a reduction of phosphorylated p38 to some extent, it did not show any effect on apoptosis or viability in PEO1 and PEO4 cell lines. This indicates that at the concentration used, SB203580 did not have any impact on platinum sensitivity. Thus, the apoptosis response observed in the combination of SB203580 and cisplatin was likely due

to cisplatin treatment alone. Further experiments should be performed using a concentration range of SB203580 and different time points to determine the optimal conditions for SB203580 to inhibit the growth of ovarian cancer cells. Proteins downstream of p38 should also be checked to confirm the inhibitory effect of SB203580 on p38 MAPK pathway. Our result differs from a previous study by Pereira et al. (2013), which reported that SB203580 treatment (at the same dose as in our experiment, 10 μ M) results in sensitising colon and breast cancer cells to cisplatin-induced apoptosis. Moreover, SB203580 together with cisplatin treatment was shown to potentially reduce tumour size in a mouse model for breast cancer (Pereira *et al.*, 2013). In contrast, recent research has shown that pre-treatment with SB203580 increased resistance to platinum-based treatment in A2780CP (cisplatin-resistant) cells and also increased the number of viable cells in the primary HGSOC cells (Han *et al.*, 2018). It appears that the activation of p38 MAPK plays a dual role in cancer: it can be either pro-apoptotic or pro-survival in different context (Brozovic and Osmak, 2007; Cuadrado and Nebreda, 2010; Igea and Nebreda, 2015; Zou and Blank, 2017). Thus, it remains to be clarified whether p38 inhibition can enhance apoptosis and improve platinum sensitivity in HGSOC.

In conclusion, we have shown several signalling changes induced by platinum in HGSOC, including P13K/Akt and MAPK pathways. This chapter also highlights the proteomic intra-tumoural heterogeneity that exists in advanced EOC and indicates the importance of multiple sampling for analysis to avoid sampling bias. We have also identified eEF2K and PR as potential biomarkers of prognosis following cisplatin treatment. Our results confirm the potential role of prexasertib in enhancing platinum response; however, these findings need to be validated in a larger cohort. Our study had the advantage of using upfront primary tumour cells treated with cisplatin in multiple deposits. Limitations of the current study include the

number of cases and an unequal number of tumour samples per patient. In the time during which this thesis was written, additional samples have been collected for multiple patients and will be analysed. Future work should seek to validate the increased expression of candidate proteins following cisplatin treatment in other ovarian cancer paired cell lines and other HGSOC protein lysates. The heterogeneity score should also be assessed in a larger cohort to investigate and confirm its relationship with patient outcome.

Chapter 5

Genomic Assessment of Intra-tumoural Heterogeneity in HGSOC

5.1 Introduction

HGSOC is characterised by high-frequency somatic copy number variants with ubiquitous TP53 mutations (Ciriello *et al.*, 2013). It has been postulated that genetic heterogeneity may contribute to the development of platinum resistance in HGSOC (Cooke and Brenton, 2011), which can be on single nucleotide variation, copy number variation (CNV), gene expression, and epigenetic levels (Bai *et al.*, 2016). The existence of multiple subclones in a tumour creates the opportunity for evolutionary selection during treatment. A previous study using paired cell lines derived from three cases of HGSOC before and after platinum resistance had developed (PEO1, PEO4 and PEO6; PEA1 and PEA2; and PEO14 and PEO23), concluded that chemotherapy-resistant clones arise from minor clones that exist in a tumour at the time of presentation (Cooke *et al.*, 2010). In addition, there is a negligible accumulation of new mutations during standard treatment in recurrent tumours, even after two rounds of chemotherapy (Castellarin *et al.*, 2013).

Nevertheless, the way in which the degree of genomic intra-tumour heterogeneity influences the treatment outcomes is unknown. Accurately quantifying tumour heterogeneity and reconstructing the evolutionary history of cancer with high somatic copy number alterations (CNAs) is problematic. Recently, however, the Minimum Event Distance for Intra-tumour Copy Number Comparisons (MEDICC) algorithm was developed (Schwarz *et al.*, 2014). This algorithm uses a bioinformatics approach to reconstruct tumour phylogeny by defining the optimum phasing of major and minor alleles from copy number data across both parental alleles. It also allows the quantification of the degree of clonal expansion (CE) on a continuous scale, termed the CE index, which is based on the pairwise distances. A subsequent study showed that a high CE index associated with worse survival outcome in HGSOC (Schwarz *et*

al., 2015). This study also found that subclonal tumour populations were present prior to therapy. The primary aim of this chapter is to investigate whether quantitative measures of genetic heterogeneity using the MEDICC algorithm could predict clinical outcome in HGSOC chemo-naïve patients. The copy number data was pre-processed using Allele-Specific Copy number Analysis of Tumours (ASCAT) pipeline to estimate and correct both tumour cell aneuploidy and non-aberrant cell admixture (Loo *et al.*, 2012).

5.2 Results

5.2.1 Patient-Specific Copy Number Profiles Generated by ASCAT

Genotyping of 143 HGSOC tumour samples from 26 patients was performed, and tumour-specific copy number profiles were generated using ASCAT. The research in this chapter was performed in collaboration with a Cancer Informatics MRes student Enanga Eko who helps to generate the data. The ASCAT algorithm output consists of three figures for each tumour: a sunrise plot, an ASCAT profile, and a raw copy number profile. A tumour deposit always contains some non-aberrant cells, including normal cells, fibroblasts, immune cells and endothelial cells (Witz and Levy-Nissenbaum, 2006). In this platform, the optimal aberrant cell fraction and ploidy of the tumour sample is determined by the sunrise plot. The ASCAT profile is generated using the resulting optimal tumour ploidy and aberrant cell fraction. This profile is the key output of ASCAT analysis, which comprises an estimation of the allele-specific copy numbers across the genome. All gains and losses as well as loss-of-heterozygosity (LOH) are shown in this plot, while the total copy number and the copy number of the minor allele (the allele with the lowest copy number) without rounding to non-negative integers is visualised by the raw copy number profile. Overall, the median of tumour ploidy was 2.24 (2.05 – 3.08), with a median aberrant cell fraction of 62% (37-81%).

Figure 5.1 shows examples of ASCAT profiles and the calculation for a case within our patient cohort. Both of the samples come from the same patient (T15058). The first sample (Figure 5.1 A) is a primary tumour with ploidy $2n$ and the second sample (Figure 5.1 B) is a disseminated tumour in the spleen with ploidy $4n$. Whereas Figure 5.1 C is the germline sample isolated from the buffy coat. First, ASCAT assessed different choices for the tumour ploidy (x-axis) and the aberrant cell fraction (y-axis), visualised in the sunrise plot (left panel of Figure 5.1). The resulting copy-number profile was evaluated for each value plotted. When the copy-number profile matched whole numbers closely, a good match was obtained and annotated by a green cross. The aberrant cell fraction of both samples was 82% and 59%, respectively. The upper-right panel of each Figure 5.1 depicts the raw copy number profile. Finally, ASCAT profiles show the allele-specific copy number across the genome (lower right). In these ASCAT profiles, multiple hemizygous deletions are present, as well as duplications.

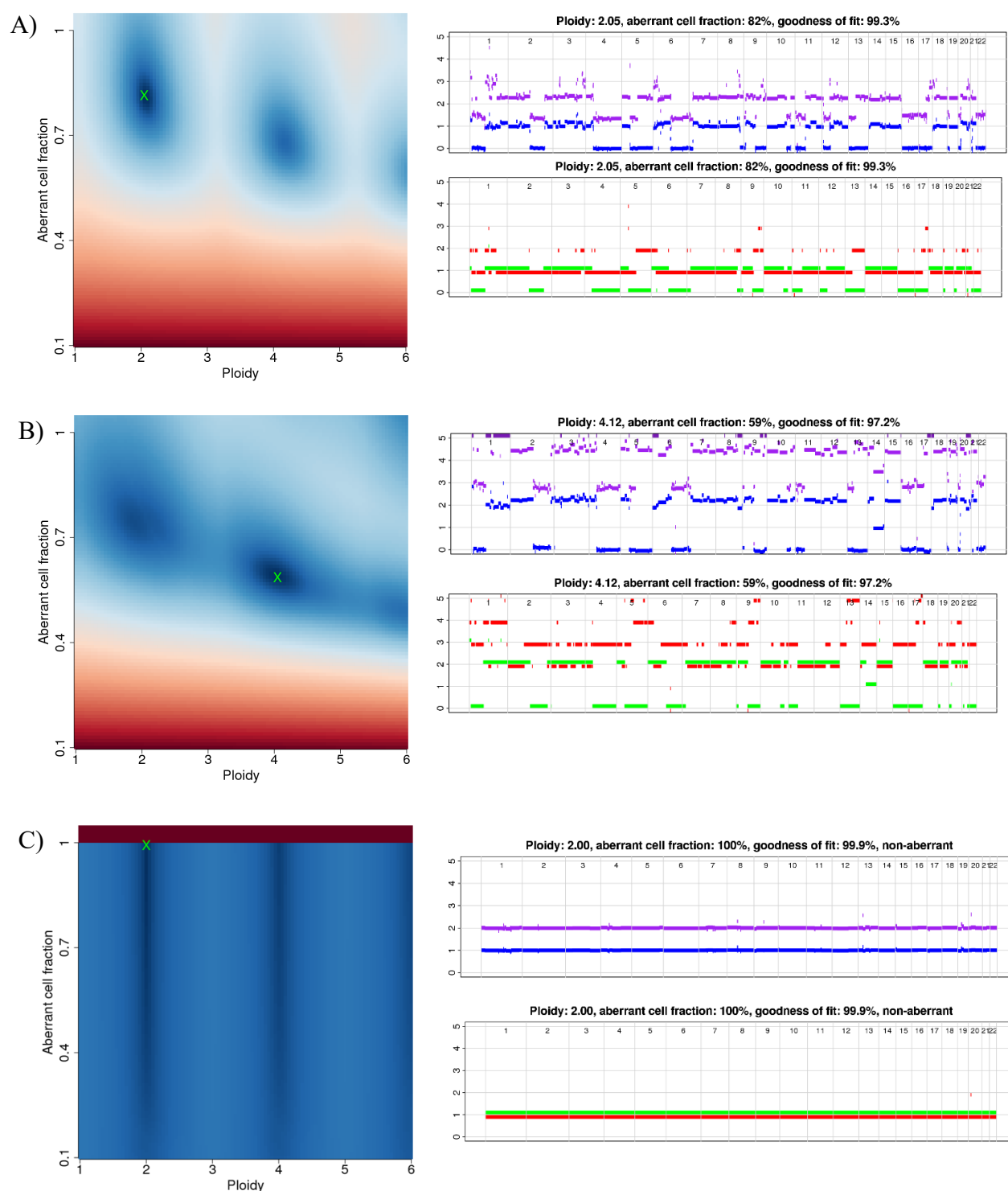


Figure 5. 1: ASCAT profiles of T15058 primary tumour (A), spleen tumour (B), and germline (buffy coat) samples (C). The left panel shows the sunrise plot (fraction of aberrant cells on y-axis and the ploidy of the tumour cells on x-axis). The upper right panel shows the raw copy number profiles. Lower right panel shows ASCAT profiles with their calculation (copy number on the y-axis and the genomic location on the x-axis; green: allele with lowest copy number; red: allele with highest copy number. For illustrative purposes, both lines are slightly shifted so that they do not overlap (red: down; green: up)).

5.2.2 HGSOC Shows Diversity in Clonal Expansion Index

A previous study on breast cancer showed that there are two classes of tumour based on genomic structural variation: monogenomic tumours, which consist of a single major clonal subpopulation with similar genome profiles, and polygenomic tumours which result from the clonal expansion of a minor subpopulation of tumour cells (Navin *et al.*, 2010). This clonal expansion is possibly the result of various selective pressures. Thus the tumours with the greatest fitness advantage are able to survive and grow. In this study, the MEDICC algorithm was used to generate CE indices (CEIs) to investigate the degree of clonal expansion and its potential prognostic value.

The CEIs of 26 HGSOC cases were computed by assessing the degree of local clustering in the phylogenetic tree, and a diverse range of CEIs were observed with a median of 0.90 (IQR 0.74 – 1.00). The median value of the CEIs was used to split patients into two groups: low-CEI and high-CEI (Figure 5.2). Patients with low-CEI, such as T16060, showed the same length of branch and linear emission of samples in the phylogenetic tree (Figure 5.3 A), whereas patients with high-CEI showed various branch lengths and marked local clustering (Figure 5.3 B). The phylogenetic trees in our cohort varied greatly; each tree showed genetic heterogeneity between tumour deposits (Appendix Figure A2-A4). There was no clear relationship between phylogenetic and anatomical distance in our trees. In our cohort, it was observed that the assigned primary tumour sample was less similar to the germline (diploid) sample compared to the metastatic samples in some cases (Appendix Figure A2-A4).

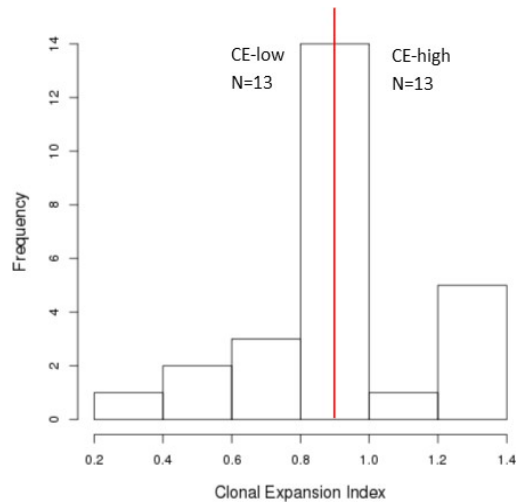


Figure 5. 2: The distribution of CEIs. A median of 0.90 is shown (IQR 0.74–1.00), number of patients=26.

Table 5. 1 Platinum sensitivity status and clonal expansion index (CEI) of patients

Case	Platinum sensitivity status	CEI before relapse	CEI after relapse
T13097	Resistant	0.65	NA
T14003	Sensitive	1.37	NA
T14012	Resistant	0.87	NA
T14028	No relapse	0.91	NA
T14045	Sensitive	0.84	1.027
T14051	No relapse	0.46	NA
T14055	Sensitive	0.94	NA
T14064	Sensitive	0.74	NA
T14092	Refractory	1.01	NA
T14093	Sensitive	0.99	0.89
T14096	No relapse	0.98	NA
T14107	Sensitive	0.59	NA
T14125	No relapse	0.73	NA
T14137	Sensitive	1.23	1.66
T15022	Sensitive	0.86	1.12
T15032	No relapse	1.40	NA
T15033	Sensitive	0.89	NA
T15043	Sensitive	0.88	NA
T15058	Sensitive	0.98	NA
T15085	No relapse	0.88	NA
T15086	Sensitive	1.33	NA
T15088	Resistant	0.55	NA
T15164	Resistant	1.21	NA
T16046	Resistant	0.90	0.65
T16060	No relapse	0.39	NA
T16133	Resistant	0.90	NA

NA : Not Available

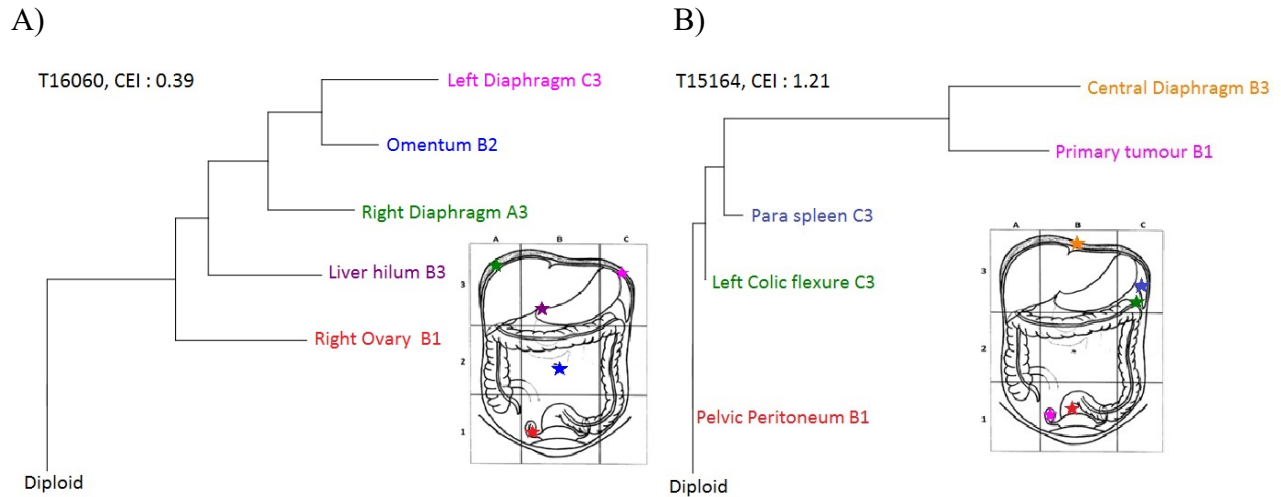
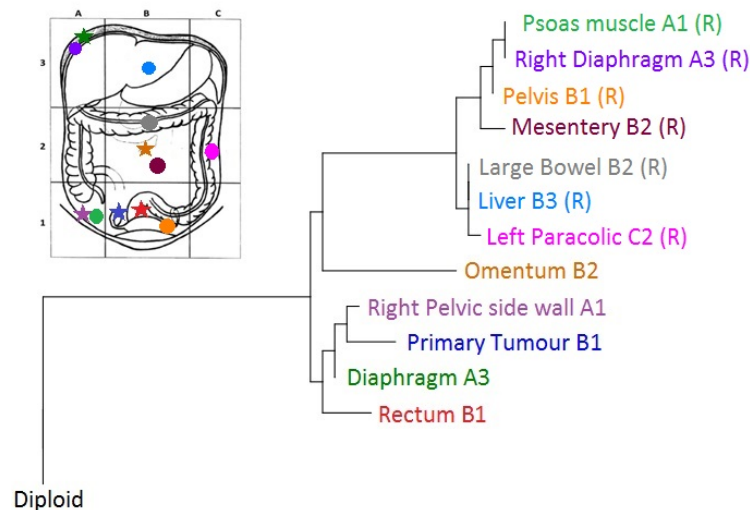


Figure 5. 3: The illustration of clonal expansion using phylogenetic trees. Copy number profiles were used to produce phylogenetic trees using MEDICC pipeline, and each sample deposit was mapped by IMO, and denoted using a coloured star symbol. The low-CEI tree shows similar pattern of branching (A), while the high-CEI tree shows localised clustering (B).

5.2.3 Characterising Spatial and Temporal Genetic Heterogeneity in HGSOC

Samples from five patients were collected at relapse. Four of these patients were classified as platinum-sensitive and one patient as platinum-resistant based on the initial response to chemotherapy. Interestingly, three of the relapse cases had low-CEI before relapse. Figure 5.4 shows the phylogenetic trees for two relapse cases; one with high-CEI (1.23, T14137), and the other with low-CEI (0.86, T15022), before relapse. Both cases have markedly higher CEI after relapse (CE index 1.66 and 1.12, respectively), with multiple samples in strongly diverging subclades. Case T14137 initially relapsed in a platinum-sensitive time frame, but the relapse samples used in the MEDICC analysis were collected following the second, platinum-resistant relapse. However, not all CEIs of relapse cases were increased; we also found that two of the five relapse cases had a decreased CEI after relapse (T14093 and T16046, Appendix Figure A3-A4). This could be due to both of these relapse cases only having one relapse tumour sample and followed the linear branching in the phylogenetic tree.

A) T14137



B) T15022

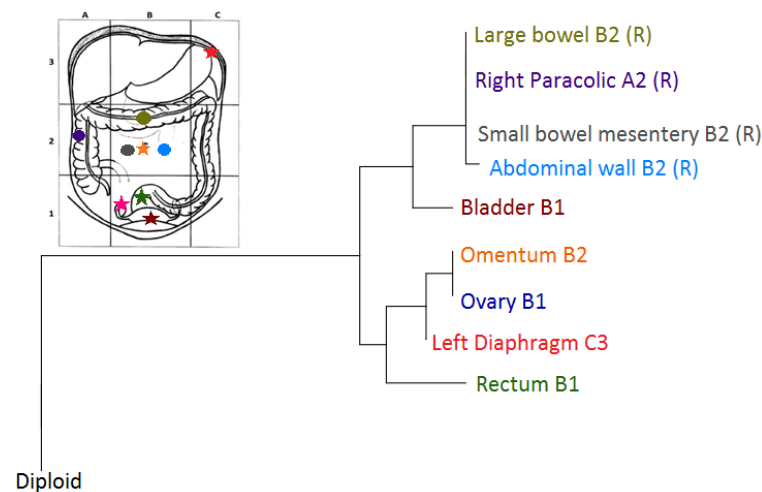


Figure 5. 4: Spatial and temporal genetic heterogeneity is present in copy-number-derived phylogenetic trees of HGSOV. Phylogenetic trees for patients T14137 and T15022 are shown. The relapsed clonal population for each case is indicated with (R). Star symbols in the IMO figure represents the samples taken at primary presentation, while circle symbols denotes relapse samples.

5.2.4 Correlation of Clonal Expansion Index and Outcome

It has been suggested that tumours need to have mutation-endowed fitness to overcome the selection pressure induced by chemotherapy (Greaves and Maley, 2012). Thus, we postulated that polygenomic tumours that have already undergone clonal expansion, like HGSOV, are expected to have a survival advantage during treatment, thus contributing to poor patient

outcome. Initially, we investigated associations between CEIs from primary samples and survival in HGSOc patients via univariate Cox proportional hazards modelling, treating CEI as a continuous variable (Table 5.2). In this analysis, the quantitative CEI had a borderline significant association with PFS with a hazard ratio (HR) of 6.17 (95% CI 1.00 - 38.16; $p = 0.051$), but no significant association with OS. The log-rank test was then performed to test for differences in PFS and OS between the CEI-low and CEI-high groups (Fig. 5.5). There was a trend towards shorter PFS in patients with a high-CEI, although this finding was not statistically significant (median PFS 370 versus 408 days, p -value = 0.27; Fig. 5.5). On the other hand, there was no clear relationship between OS and CEI. As shown in Table 5.2, univariate Cox proportional hazards analysis treating CEI as dichotomous variable, as well as the potential confounding factor for survival were also performed. In our HGSOc cohort, CEI showed no association with either PFS (p -value = 0.283) or OS (p -value = 0.648). However, in the multivariate Cox proportional hazard analysis including patient age and tumour stage, being in the high-CEI group was an independent predictor of PFS, but not of OS (p -value PFS = 0.039; p -value OS = 0.129).

Table 5. 2 Cox proportional hazards analyses of CEI and clinical factors known to be of prognostic importance in HGSOc

Continuous Univariate analysis				
Clinical Outcome	Variable	HR	95%	p-value
PFS	CEI	6.17	1.00 - 38.16	0.051
	Age	1.00	0.97 - 1.03	0.957
OS	CEI	3.14	0.32 - 31.33	0.33
	Age	1.00	0.96- 1.04	0.954
Dichotomised Univariate analysis				
Clinical Outcome	Variable	HR	95%	p-value
PFS	CEI (high vs low)	1.61	0.68 - 3.82	0.283
	Age (> 60 vs ≤60)	1.04	0.44 - 2.46	0.925
	FIGO stage (IV vs III)	2.34	0.91 - 6.00	0.078
OS	CEI (high vs low)	1.32	0.40 - 4.35	0.648
	Age (> 60 vs ≤60)	0.71	0.21 - 2.44	0.587
	FIGO stage (IV vs III)	2.86	0.85 - 9.62	0.089
Multivariate analysis				
Clinical Outcome	Variable	HR	95%	p-value
PFS	CEI (high vs low)	3.36	1.06 - 10.64	0.039
	Age (> 60 vs ≤60)	0.94	0.39 - 2.27	0.894
	FIGO stage (IV vs III)	4.98	1.42 - 17.51	0.012
OS	CEI (high vs low)	3.31	0.71 - 15.54	0.129
	Age (> 60 vs ≤60)	0.66	0.19 - 2.28	0.507
	FIGO stage (IV vs III)	5.96	1.21 - 29.47	0.029

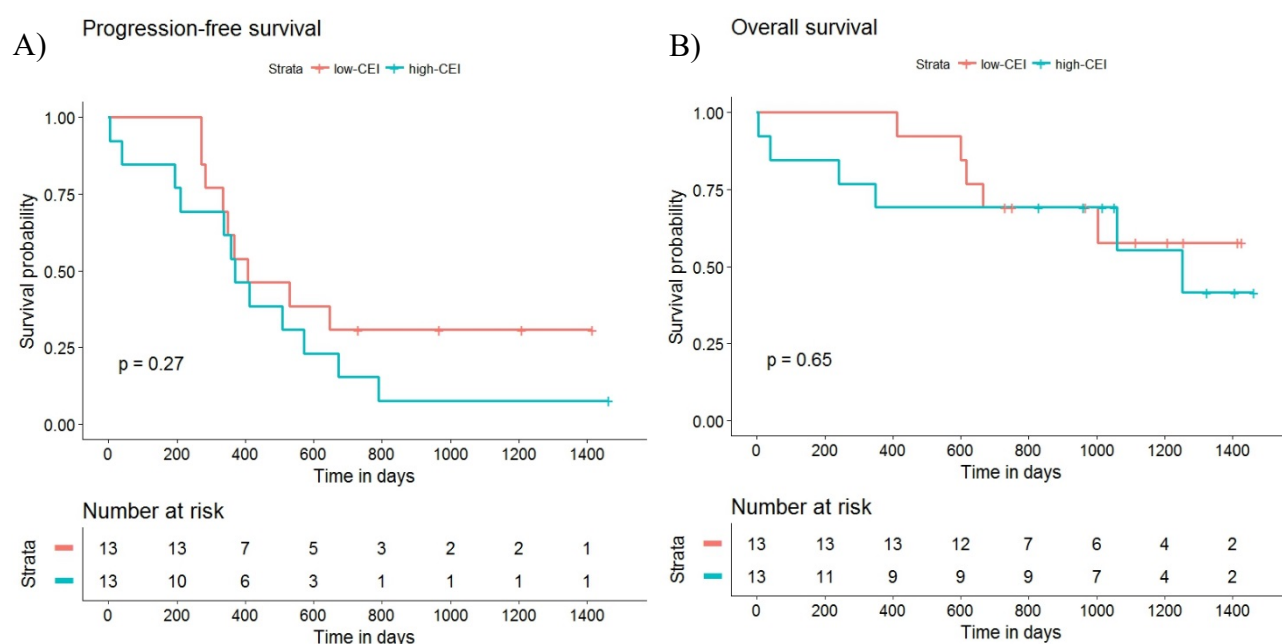


Figure 5. 5: Kaplan-Meier survival curves for patients stratified by CEI. Log-rank analyses for (A) PFS and (B) OS.

5.3 Discussion

Cancer genomes often exhibit several DNA sequence variations, ranging from small changes in genome copy number (gain or loss of a single genome copy) to amplifications, insertions, deletions of chromosomal fragments, and whole-genome duplications (Balmain, Gray and Ponder, 2003). Single nucleotide polymorphism (SNP) arrays are a powerful technology to define genomic aberrations in cancer genomes. However, there are complicating factors in the analysis of SNP array data of cancer samples including tumour aneuploidy (Sansregret and Swanton, 2017) and multiple populations of both tumour and non-tumour cells present in tumour samples (Van Loo *et al.*, 2010). The ASCAT algorithm can analyse SNP array data by estimating both tumour cell aneuploidy and non-aberrant cell admixture. A previous study compared several methods for analysing CNAs on the Illumina platform, such as DNA-Chip Analyzer software (dCHIP), GenoCNA software, ASCAT, OncoSNP software, genome

alteration print (GAP) visualisation, CNVpartition software plug-in for the Genome Studio software, and Partek Genomics Suite software, and found that ASCAT was very stable in estimating CNAs (gain, loss and LOH) and had the best performance compared to the other methods (Oros *et al.*, 2013). In our dataset, we found vast heterogeneity in the ASCAT profiles, with a median tumour ploidy of 2.24 (2.05 – 3.08) and a median aberrant cell fraction of 62% (37 – 81%).

The ASCAT profile was then interpreted using MEDICC, to reveal the phylogeny of tumour deposits in each patient and subsequently quantify the degree of heterogeneity through the CEI (Schwarz *et al.*, 2014). Our phylogenetic trees varied greatly (Figure 5.3, Figure 5.4 and Appendix Figure A2-A4), and each tree shows clear CNV-derived heterogeneity between tumour deposits and between patients. Spreading of HGSOc is thought to occur via physical shedding from the primary tumour onto pelvic structures and into the abdomen, due to a lack of distinct physical barriers between ovarian cancer and the peritoneal cavity (McPherson *et al.*, 2016). In our cohort, there was no clear association observed between phylogenetic and anatomical distance in the trees. This result may be explained by the notion that HGSOc is characterised by frequent dissemination into peritoneal sites, and accommodated by peritoneal fluid, which is rich in cytokines and bioactive lipids (Thibault *et al.*, 2014). Our data also revealed that the assigned primary tumour in some patients' phylogenetic trees was less similar to the diploid (germline) sample than the metastatic deposits. This can be partly explained by the possibility of HGSOc originating from three potential sites: the Fallopian tube, the surfaces of the ovary, or the mesothelium-lined peritoneal cavity (Lengyel, 2010).

Determining the evolutionary pathways of metastases is an important aspect of understanding cancer biology. A number of studies have used the phylogenies of cancer lineages to infer the genetic history of cancer progression, including the origin of metastases. Although we successfully obtained diverse multi-site sampling in this study, there are several ways in which future research could increase the accuracy of assessing metastasis patterns in HGSOC. One suggestion would be to take a number of spatially-distinct biopsies from the primary sample and treat each biopsy as an individual sample in the downstream analysis. A recent study has illustrated that complete characterisation of subclones within a primary tumour is necessary to differentiate parallel metastases (star topology) from a metastatic cascade (metastasis-to-metastasis spread/tree topology) (Hong, Shpak and Townsend, 2015). In the presence of an incomplete phylogeny of the subclones within a primary tumour, a scenario of star topology from a primary tumour is more likely to happen. This hypothesis is supported by recent studies in HGSOC, which found that the majority of patients in their cohorts exhibited monoclonal and single-directional seeding from the ovary to metastatic sites (Schwarz *et al.*, 2015; McPherson *et al.*, 2016). Schwarz and his colleagues address the issue of subclonal spatial heterogeneity by collecting multiple samples from the same metastasis site in each patient. Still, as in our study, they did not take multiple samples from the primary tumour which may affect how precisely the tree reflects the patient's true patient tumour phylogeny. Increasing the level of sequencing within tumour sites using multi-region sampling, although financially and computationally costly, is also recommended in order to gain deeper insight into intra- and inter-tumour comparisons (Turajlic and Swanton, 2016). Nevertheless, we demonstrated the spatial and temporal heterogeneity present in our HGSOC cohort successfully via phylogenetics analysis.

In our temporal analysis, we observed that the relapse samples exhibited less overall divergence and marked local clustering. This finding is in accord with the initial study of CEIs in HGSOC using the MEDICC algorithm, where the phylogenetic clustering correlated with a selection of fitter subclones, as would be expected of relapse samples that were able to overcome the selection pressure applied by chemotherapy (Watkins and Schwarz, 2018). Moreover, a recent study assessing temporal genetic heterogeneity using matched tumour pairs collected before and after platinum therapy confirmed that HGSOC is naturally oligoclonal (Lambrechts *et al.*, 2016). The same study further suggested that relapse tumours are derived from a single clone which escapes platinum treatment.

We found that the high-CEI group was an independent predictor of PFS, which was consistent with the result of the previous study (Schwarz *et al.*, 2015). In comparison to the study by Schwarz and his colleagues, our study only included patients who had no preoperative chemotherapy treatment before sample collection, to reduce bias in the analysis of genetic alterations, whereas their study consisted of patients who had undergone neoadjuvant chemotherapy. Therefore, their findings may be less appropriate for the first-line stratification of patients into the likelihood of treatment response but still helpful to inform interval treatment decisions. Another advantage of our study is that we used a broader range of anatomical tumour sampling than the Schwarz *et al.*; however, their result showed a broader range of CEI (IQR 0.65–1.24 vs 0.74 – 1.00, respectively). These differences may be due to the fact that subjects investigated in the Schwartz *et al.* study had experienced various numbers of rounds of chemotherapy prior to surgery. Moreover, the previous study also had more samples from each patient (with many of the samples collected from the same tumour site); thus, their CEI range is broader than ours. Our median CEI for dividing patients into high-CEI and low-CEI groups

was higher than their median (0.90 and 0.73, respectively). However, ‘high and low-CEI’ are arbitrary terms at present, and the lowest number of samples per case that are required for accurate estimates of CEI has yet to be defined. Thus, it is necessary to perform further analysis in order to determine this benchmark in a larger cohort and differentiate the outcomes in high- and low-CEI groups.

In the present study, we investigated whether inter-patient clonal expansion could predict the outcome in HGSOC through multi-deposit sampling. Considering our strict inclusion criteria, our study is the first to demonstrate how this occurs both prior to and, in some cases, after platinum therapy. Our approach is important to characterise the way in which the natural progression of clonal evolution may be used to inform patient prognoses and appropriate tailoring of patient treatment. A Recent report assessing copy number signatures in HGSOC showed that it had prognostic value in predicting overall survival and the probability of platinum-resistant relapse (Macintyre *et al.*, 2018). The signatures found in that study appear to correlate with mutational processes in HGSOC, for instance, one CN signature is correlated with *NFI* loss and mutated *KRAS*, another CN is highly enriched in cases with *BRCA1/2* mutation. This would be an interesting avenue to investigate whether the same CN signatures are present in our cohort and whether it is also could predict the platinum sensitivity and the outcome of patients.

Chapter 6

Summary and Future work

6.1 Summary

HGSOC is the most lethal form of gynaecological malignancy. The prognosis for patients with this disease is poor, with 20-35% overall 5-year survival, due to the development of resistance to chemotherapy. Tumour heterogeneity is known to contribute to the treatment failure in this cancer. The heterogeneity that exists in HGSOC occurs at several levels including genetic, epigenetic, transcriptomic, proteomic, and cellular phenotype. These differences at multi-omic levels have been detected as cancer progresses through time (temporal heterogeneity) and across space (spatial heterogeneity). Through this study, we have added to the current evidence of intra-tumoural heterogeneity (ITH) in HGSOC with functional data demonstrating heterogeneity in phenotypic apoptotic responses to cisplatin, both temporally and spatially. Considering the existence of intra-tumoural heterogeneity in HGSOC, it is unrealistic to expect a single biopsy or sample to accurately predict the response to a particular cytotoxic agent. Variability in PAX8 antigen expression across disseminated tumours profiled in this study provides additional evidence of inter- and intra-tumoural heterogeneity. From our aim of investigating the clinical value of phenotypic heterogeneity derived from apoptotic responses to platinum chemotherapy, our data suggests that cases with higher coefficient of variation (CV) in response to cisplatin treatment at presentation are more likely to respond to cisplatin treatment. This finding should be validated in a further, larger cohort.

Tumour cell lines are commonly used models for drug development and drug resistance investigation with a number of advantages such as high proliferative capacity, and disadvantages including additional genetic alterations which make them deviate from their cells of origin and loss of heterogeneity (O'Donnell *et al.*, 2014). Thus, the development of improved approaches to generate and utilise primary cultures is needed. This thesis has

demonstrated that primary ovarian cancer cells can be routinely extracted and cultured from solid tumour and ascitic fluid and provided an invaluable resource for the study of HGSOC tumour biology. This experimental model has advantages over commercially available cell lines, including more accurately resembling the patient condition and having the ability to recapitulate the vast heterogeneity present in HGSOC. However, the existing two-dimensional cell-culture model has limitations, most significantly, a lack of the tumour microenvironmental support which has an important role in response to chemotherapy. Thus, future work should focus on improved culture models, for example, three-dimensional organotypic models and tumour slice cultures which incorporates tumour cells and tumour microenvironment including stromal cells and extracellular matrix.

Due to the inevitable progression of platinum resistance in HGSOC, the development of novel therapeutic agents to combat and reverse platinum resistance are necessary. One molecule which is currently under investigation as a therapeutic target is DNA-PKcs due to its significant role in DNA DSBs repair and its role in mediating chemoresistance. Here, we confirmed that the addition of an inhibitor to DNA-PKcs could enhance the apoptotic response to cisplatin in disseminated tumours from patients. Interestingly, the effect of this combined treatment was observed in almost all of the patients who later developed resistant and refractory relapse, supporting the idea that DNA-PK inhibition could resensitise platinum-resistant ovarian cancer cells to cisplatin treatment. However, due to the small number of recurrent tumour samples have been analysed in this study, our investigation regarding the benefit of inhibiting DNA-PKcs in combination with cisplatin treatment in relapse samples is limited. In one relapse case, the addition of DNA-PK did not increase the apoptotic level following cisplatin treatment,

while in another, it did. Further work to confirm the role of a DNA-PK inhibitor in reversing platinum resistance is necessary, particularly in relapse cases.

Understanding the expression and activation states of numerous proteins simultaneously following drug treatment allows for better knowledge of the treatment impact on cancer patients. In this study, proteomic changes in tumour cells extracted from multiple HGSOC deposits following *in vitro* cisplatin treatment were investigated using Reverse Phase Protein Array proteomic profiling. Our data showed that cisplatin activated several pathways that induce apoptosis and survival, including AKT, PKC, and MAPKs (JNK, and p38 MAPK). The PI3K-Akt signalling pathway was found to be a significantly enriched pathway following cisplatin treatment. The overall analysis of proteomic changes provided more evidence of the dynamic regulation of apoptotic and survival pathways in HGSOC following treatment with cisplatin. Future work for validating the platinum-induced protein changes in other cohorts of HGSOC patients are necessary.

Potential proteomic biomarkers of prognosis following cisplatin treatment, eEF2K and PR, were identified in our study, which require further validation in another cohort. Our results suggest that platinum-induced high eEF2K expression is associated with worse PFS, and conversely, so is platinum-induced low PR expression. The study also contributed more evidence of proteomic inter- and intra-tumour heterogeneity in HGSOC and highlighted the importance of multiple sampling in assessing the effect of chemotherapeutic treatment in HGSOC. We were able to quantify the functional proteomic heterogeneity for each patient by computing the mean of pair-wise correlation coefficient for all deposits from a patient;

however, this quantification did not correlated with patients' survival. Further analysis in our study also supports the potential role of prexasertib, a Chk1 inhibitor in enhancing platinum response in ovarian cancer. This drug is currently in clinical trial to investigate its benefit in treating platinum-resistant or refractory recurrent ovarian cancer (NCT03414047) (ClinicalTrials.gov, 2018).

In terms of genetic heterogeneity, we found vast heterogeneity in the copy number profiles generated using ASCAT platform. Using the MEDICC algorithm, we were able to construct phylogenetic trees for each patient. Our results showed considerable variation in these trees, which again highlights the CNV-derived heterogeneity between patients. Our phylogenetic analysis indicated that there was no association between phylogenetic and anatomical distance observed in our cohort so far; and the assigned primary tumour in some trees was less similar to the germline control compared to the metastatic deposits. Future research is needed to increase certainty in assessing the evolutionary pattern, including taking a number of spatially-distinct biopsies from the primary sample and increasing the sequencing coverage. Our temporal analysis in patients with multiple relapse samples showed that the samples exhibited less overall divergence and more clustering, suggesting that the selection of fitter subclones contributes to the recurrence of the disease. Quantification of clonal expansion is warranted in order to gain insight into the functional impact of tumour heterogeneity and patient outcome. Clonal expansion indices were successfully computed in our dataset for each patient. Similar to the previous study by Schwarz *et al.*, the high-CEI group was an independent predictor of PFS. Further expansion of our analysis in a larger cohort to define the cut-off point between low- and high-CEI is required. It would also be interesting to examine CNA associated with platinum-resistant phenotypes and patients' survival in our dataset. The identification of these

CNAs is relevant not only to the investigation of prognostic signatures and affected pathways in tumours, but also for patient stratification and targeted molecular therapies.

6.2 Future Work

Since the writing of this thesis, a further cohort of ITH samples have been collected. A larger cohort of samples is required for a meaningful analysis of the effect of heterogeneity on patient outcome. In this study, we have successfully utilised 2D primary cell culturing and performed phenotypic apoptosis and viability assays within this experimental model. Nonetheless, it is important to consider that adherent cells grown on 2D culture may not reflect tumour biology. A dynamic network of cytokines, growth factors, and adhesion molecules which drives the interactions between cancer and a variety of host cells is needed to aid the growth and spread of cancer cells, and these factors also influence the phenotype of the cancer cells. Therefore, *in vitro* models that mimic the 3D tumour microenvironment (co-cultures of malignant cells with stromal cells) are required to bridge the gap between first-line *in vitro* studies and pre-clinical *in vivo* experiments. Three-dimensional systems aim to provide conditions which are structurally similar to the *in vivo* environment and also facilitate the cell–cell and cell–extracellular matrix communication (Watters *et al.*, 2018). One example of 3D models is organoid culture, a cultivation of cells in a support matrix and customised culture media to recreate the physiological environment for long-term growth of cells (Kaushik, Ponnusamy and Batra, 2018). Recently, a study reported establishment of 56 ovarian cancer 3D organoid lines from 32 patients which showed inter and intra-patient heterogeneity (Kopper *et al.*, 2019). This study also demonstrated the utility of 3D organoid for chemotherapeutic response assessment. The 3D organoid xenografting can be further utilised for *in vivo* drug-sensitivity investigation. Another alternative of an experimental model in *ex vivo* setting is the use of

whole tissue slices (Alkema *et al.*, 2016). This model captures the tumour heterogeneity to a large extent by preserving the complexity of their original microenvironment *in vivo* without extensive manipulation of the tissue. Both the 3D collagen matrix organotypic model and slice culture model are currently under development in our research group.

In this study, we confirmed the intra-tumoural heterogeneity of HGSOC at a phenotypic, proteomic, and genetic level. Measurements of the degree of heterogeneity based on proteomic and genetic profiles in our study also suggest its potential in predicting patients' outcome. A multi-omics analysis combining the functional, proteomic and genetic data generated is the subsequent focus of this ITH study; a fully integrative analysis will provide more comprehensive insights into the biological systems in HGSOC. While every effort was made to have matched functional and omics data for each patient, for some cases it was not possible. Missing values in one of -omics platform or another is a limitation in our dataset, as not every sample or deposit in our cohort had matched functional, proteomic, and genetic data. Moreover, we aim to have at least five samples from different anatomical location per patient to capture the ITH in HGSOC. One possible method to tackle this problem is using the Multi-Omics Factor Analysis (MOFA) platform, a computational method developed for identifying the principal sources of variation in multi-omics data (Argelaguet *et al.*, 2018). MOFA uses the basis of principal component analysis (PCA), treating data matrices as input and subsequently weight matrices. This approach can handle different data types (such as continuous, binary, and discrete data) and also detect outliers. However, this method is only suitable for two-dimensional data, whereas our dataset is comprised of three-dimensional data in which every patient has several tumour deposits. A possible alternative could be to modify MOFA with tensor analysis (Hore *et al.*, 2016). This method decomposes the three-dimensional array, or tensor, into a number of factors that represent major modes of variation in the data set. Another

challenge in the multi-omic analysis of our study is the variability of anatomical sample source. Similar to MOFA, this model can accommodate missing data, such as samples without protein expression data for subsets of samples or deposits, which is a real and prevalent feature of multiple-sampling experiments. Further collaboration incorporating immunomics and metabolomics profiling into our study is currently being undertaken. This exciting collaborative study will enrich the multi-omics analysis of all our ITH data. The analysis will aid in defining a predictive algorithm for overall patients' outcome and the onset of platinum resistance in HGSOc. Currently, a manuscript is in preparation for peer-review publication for submission in autumn 2019.

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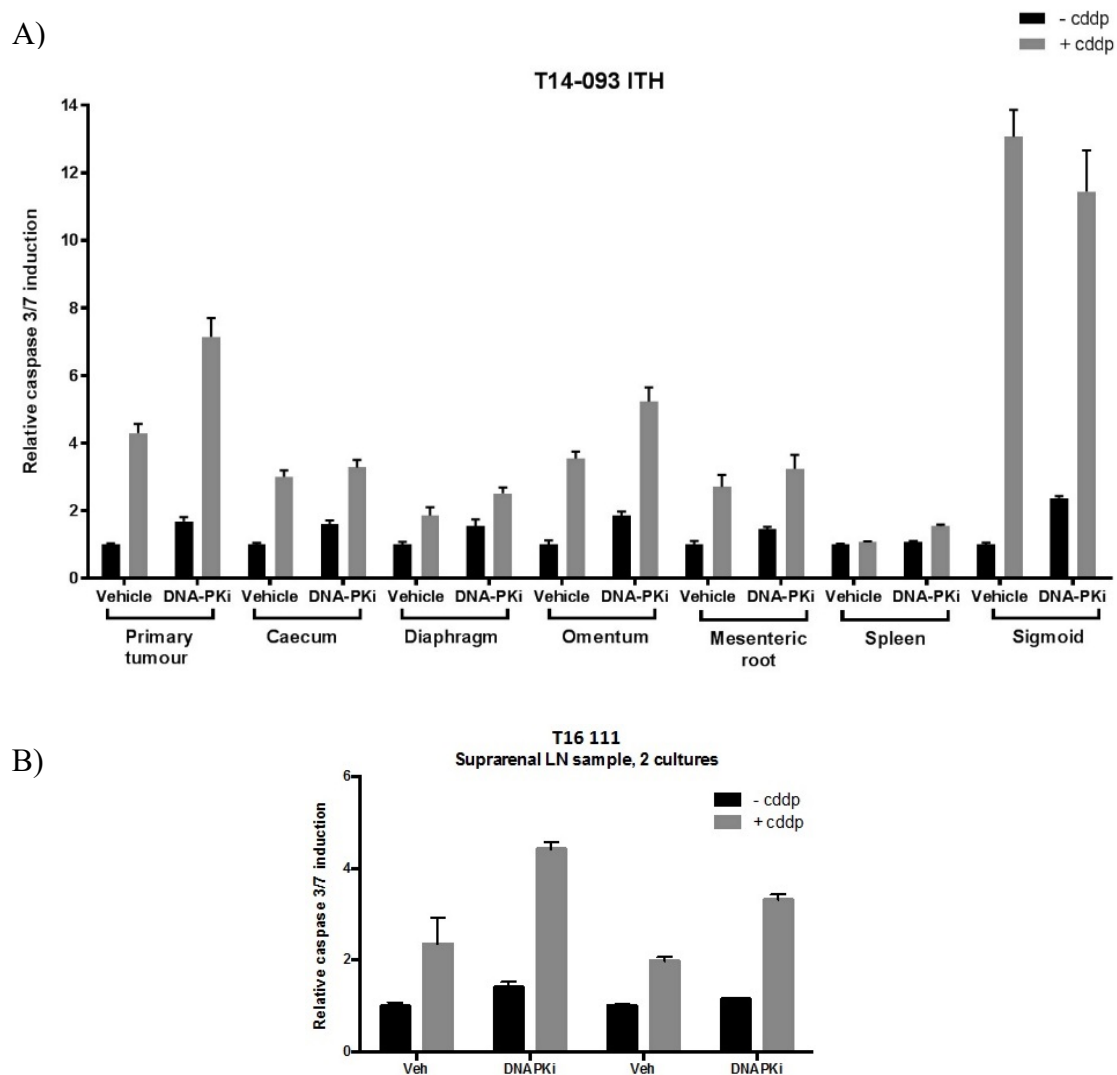
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Appendix



Appendix Figure A1: Temporal phenotypic heterogeneity is observed in response to cisplatin treatment. Following short-term primary cell cultures, cells were treated with cisplatin (25 μ M) for 24 hours. Apoptotic and viability assays were performed using ApoTox-Glo Triplex Assay. Tumour cells from different metastatic sites display differing apoptotic responses to cisplatin in a primary case (A) and corresponding phenotypic assay result from a relapse case (B).

Appendix Table 1. Tumour cellularity determined for ITH cases (1/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T13097	Pelvic tumour/rectum	161	157	98
	Ovarian tumour	237	228	96
	Bladder	312	306	98
	Para aortic lymph node	85	79	93
	Omentum	297	281	95
	Small bowel	299	295	99
	Splenic area	358	350	98
	Omentum 2	474	466	98
T14003	Ovary	193	172	89
	Peritoneum	238	188	79
	Omentum	19	15	79
	Mesenteric nodules	111	87	78
	Right paracolic	107	88	82
	Omentum bursac	112	77	69
	Left colic	117	63	53
	Ascites	121	57	47
T14012	Primary tumour	170	129	76
	Right diaphragm	135	96	71
	Liver capsule	371	309	83
	Right pelvic peritoneum	150	107	71
	Falciform ligament	173	130	75
T14028	Right diaphragm	196	148	76
	Right pelvic wall	104	72	69
	Left pelvic wall	53	28	53
	Left diaphragmatic epicardiac	192	135	70
	Mesentery	104	73	70
	Rectum	115	84	73
	Large bowel	128	97	76
	Omentum	168	128	76
T14045	Right ovary primary tumour	404	344	85
	Omentum	189	153	81
	Spleen nodules	261	195	75
	Right paracolic peritoneum	217	159	73
	Falciform ligament	292	236	81
	Right diaphragm	393	214	54

A minimum of 10 random microscopy fields of each tumour sample was captured at 40x magnification. The total number of cells was observed by counting all nuclei (DAPI positive), and the number of tumour cells was obtained by manually counting positive cells (PAX8 positive), according to which the percentage of tumour cells for each tumour deposit was determined.

Appendix Table 1. Tumour cellularity determined for ITH cases (2/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T14051	Primary tumour	184	169	93
	Left pelvic wall	105	84	80
	Right ovary	133	127	95
	Rectal	120	74	62
	Vaginal wall	111	90	81
	Pouch of Douglas	105	85	81
T14055	Primary ovary	101	63	62
	Left ovary tumour	218	154	71
	Mesenteric root	307	177	58
	Para aortic infra renal lymph node	2132	1482	70
	Rectal wall (rectum)	426	208	48
	Bladder peritoneum	307	165	54
	Omentum	283	186	66
	Left abdominal wall	198	122	62
	Other abdominal wall	139	88	63
	Stomach	227	159	70
	Left paracolic	105	84	80
T14064	Primary	120	114	95
	Omentum	113	106	93
	Renal capsule	137	105	77
	Infra mesenteric	107	81	76
	Left infrarenal lymph node	105	87	83
	Right infrarenal lymph node	32	17	53
T14092	Omentum	101	41	41
	Sigmoid	119	36	30
	Spleen	222	140	63
	Mesenteric root	199	67	34
	Diaphragm	218	158	72
T14093	Primary tumour	298	225	75
	Caecum	350	223	64
	Diaphragm	103	63	62
	Omentum	242	148	61
	Right paracolic	207	158	76
	Mesenteric root	103	62	60
	Duodenum	131	79	60
	Right paracolic	378	247	66
T16111	Suprarenal lymph node 1st	42	35	83
	suprarenal lymph node 2nd	101	61	60

Appendix Table 1. Tumour cellularity determined for ITH cases (3/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T14096	Primary	114	82	72
	Omentum	203	168	83
	Mesenteric tumour	226	155	68
	Upper abdomen	285	176	61
	Liver capsule	118	85	72
	Stomach	264	163	62
	Left pelvic wall	218	180	83
	Right diaphragm	571	412	72
	Small bowel	114	97	85
	Large bowel	104	66	64
T14099	Right primary	177	133	75
	Omentum	162	132	81
	Right diaphragm	119	46	39
	Splenic area	187	82	44
	Upper abdominal	146	80	55
	Right pelvic peritoneum	162	96	59
	Mesenteric	129	74	57
T14107	Primary	88	80	91
	Omentum	106	92	87
	Caecum	168	147	88
	Splenic area	100	93	93
	Right pelvic lymph node	73	38	52
T14125	Right ovary primary tumour	291	245	84
	Tumour left ovary	162	128	79
	Right diaphragm	167	123	74
	Paracolic	228	135	59
	Omentum	435	209	48
	Pelvic wall right abdominal peritoneum	189	126	67
T14137	Primary tumour	159	112	70
	Omentum	166	94	57
	Diaphragm	109	73	67
	Right pelvic wall	125	81	65
	Liver capsule	385	325	84
	Rectum	244	189	77

Appendix Table 1. Tumour cellularity determined for ITH cases (4/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T17067	Right Diaphragm	132	117	89
	Liver	137	118	86
	Mesentery	149	141	95
	Mesentery	100	90	90
	Left Paracolic	108	79	73
	Large Bowel	114	77	68
	Pelvis	118	80	68
	Ascites	144	120	83
T15032	Primary	255	225	88
	Right ovary	309	238	77
	Para-aortic lymph node	179	85	47
	Rectum	131	101	77
	Left pelvic side wall	128	77	60
	Right pelvic side wall	100	60	60
	Vagina	204	110	54
T15033	Primary tumour	182	156	86
	Omentum	234	200	85
	Right diaphragm	274	243	89
	Left diaphragm	126	106	84
	Liver capsule	179	162	91
	Rectal sigmoid	104	88	85
	Bladder	73	39	53
	Abdominal wall	105	74	70
	Spleen	117	94	80
	Mesenteric root	184	148	80
T15043	Primary tumour	97	56	58
	Omentum	85	58	68
	Diaphragm	112	76	68
	Bladder peritoneum	123	104	85
	Left paracolic	103	76	74
	Sigmoid colon	117	95	81
	Posterior gastric wall	112	81	72
	Rectum	111	100	90
	Mesenteric	32	25	78

Appendix Table 1. Tumour cellularity determined for ITH cases (4/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T15085	Primary	104	80	77
	Omentum	137	116	85
	Left diaphragm	111	85	77
	Ileum	100	75	75
	Bladder peritoneum	101	83	82
	Left colic flexure	47	30	64
	Uterus	124	107	86
	Mesenteric	59	32	54
	Caecum	85	72	85
	Rectum	128	121	95
T15086	Primary tumour	319	247	77
	Omentum	307	179	58
	Right diaphragm	109	65	60
	Right renal capsule	101	68	67
	Mesenteric	109	89	82
	Bladder peritoneum	105	56	53
	Sigmoid	110	94	85
	Ileo-cecal area	100	82	82
T15088	Primary	78	57	73
	Omentum	101	74	73
	Right diaphragm	104	84	81
	Uterus	8	1	13
	Left paracolic	150	128	85
	Right paracolic	25	19	76
	Small bowel	107	96	90
	Porta hepatis	107	99	93
	Bladder peritoneum	135	114	84
	Rectum 1	241	218	90
	Rectum 2	112	36	32
	Mesenteric root	103	88	85
	Lesser sac	100	86	86

Appendix Table 1. Tumour cellularity determined for ITH cases (5/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T15164	Primary tumour	124	105	85
	Left colic flexure	322	234	73
	Para spleen	260	126	48
	Bladder peritoneum	183	162	89
	Pelvic peritoneum	292	243	83
	Left paracolic	306	257	84
	Right paracolic	110	70	64
	Large bowel mesentery	104	96	92
	Central diaphragm	109	83	76
	Mesenteric root	417	308	74
	Recto sigmoid	139	123	88
	Ascites	200	174	88
T16046	Primary tumour	101	76	75
	Omentum	246	224	91
	Left colic flexure	342	308	90
	Mesenteric root	106	85	80
	Pancreatic area	103	83	81
	Small bowel mesentery	111	104	94
	Liver capsule	104	96	92
	Pelvic peritoneum	170	143	84
	Bladder peritoneum	158	121	77
	Recto sigmoid	112	93	83
	Ileocaecal area	211	173	82
	Ascites	159	109	69
T16060	Left ovary	102	80	78
	Left ovary	293	267	91
	Right ovary	236	214	91
	Omentum	127	119	94
	Omentum	317	287	91
	Left diaphragm	102	78	76
	Left diaphragm	126	107	85
	Right diaphragm	111	81	73
	Right paracolic	107	103	96
	Left paracolic	193	145	75
	Pelvic peritoneum	130	105	81
	Transverse colon	166	121	73
	Bladder peritoneum	107	88	82
	Spleen	102	81	79
	Liver hilum	272	250	92
	Ascites	108	61	56

Appendix Table 1. Tumour cellularity determined for ITH cases (6/6)

Case	Tumour deposit	Number of cells	Positive cells	%
T16089	Primary Tumour	54	40	74
	Omentum	101	73	72
	Mesentery	104	78	75
	Sub Splenic Area	37	26	70
	Falciform ligament	105	71	68
	Small bowel	55	40	73
	Ascites	27	20	74
T16133	Primary tumour	104	99	95
	Omentum	103	78	76
	Right diaphragm	105	88	84
	Mesenteric nodule	106	92	87
	Bladder peritoneum	174	150	86
	Falciform ligament	112	84	75
	Lesser sac	209	198	95
	Subhepatic	83	72	87
	Right pelvic side wall	107	98	92
	Sigmoid	103	93	90
	Ascites	9	9	100

Appendix Table 2. Pathway enrichment FDR values associated with RPPA data analysis (1/3)

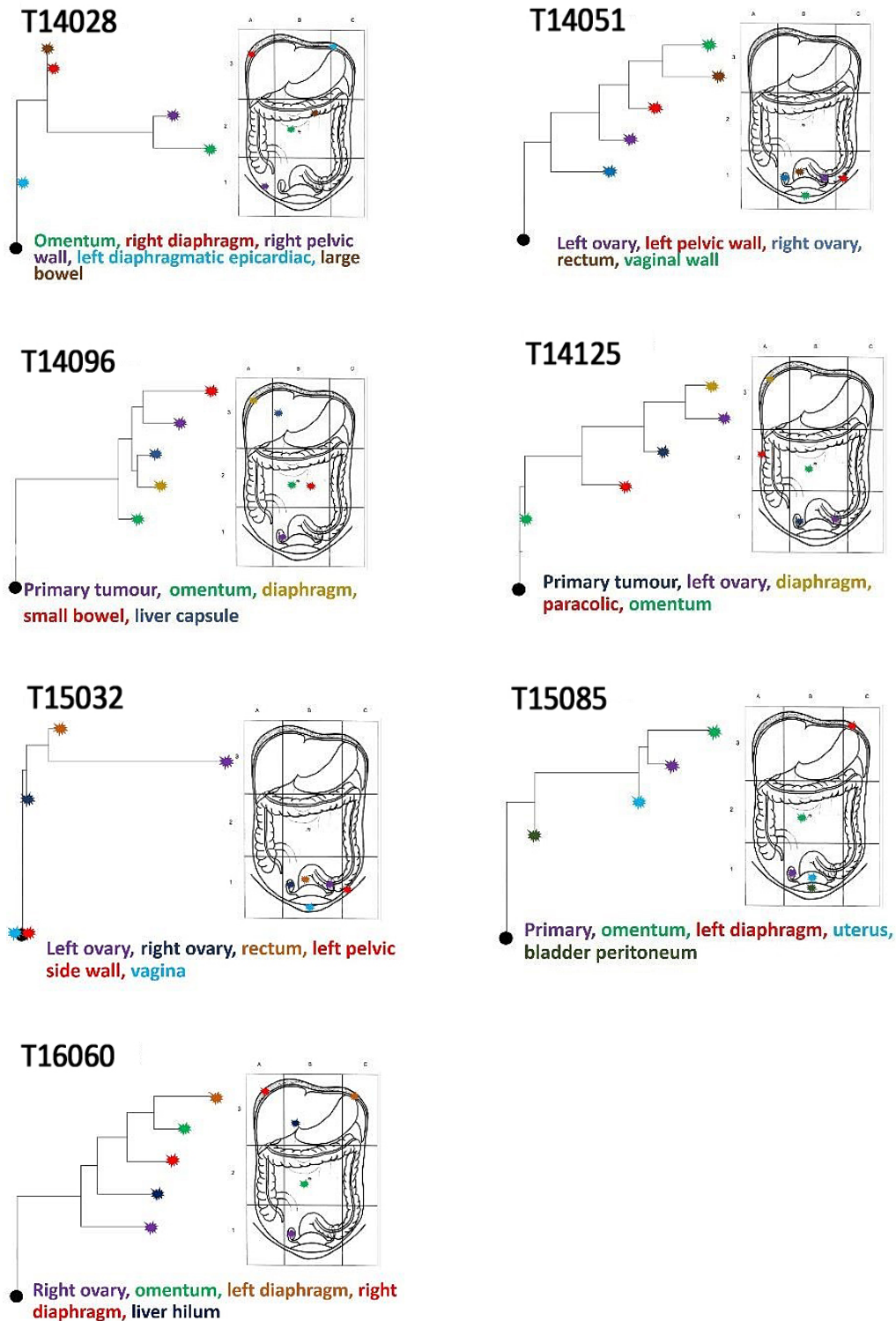
Pathway ID	Pathway description	Observed gene count	FDR
4066	HIF-1 signaling pathway	14	2.83E-18
5200	Pathways in cancer	18	2.39E-17
4012	ErbB signaling pathway	12	3.17E-16
4370	VEGF signaling pathway	11	3.33E-16
5205	Proteoglycans in cancer	15	7.49E-16
4151	PI3K-Akt signaling pathway	16	1.57E-14
5161	Hepatitis B	12	8.37E-14
5220	Chronic myeloid leukemia	10	8.98E-14
4919	Thyroid hormone signaling pathway	11	3.37E-13
4014	Ras signaling pathway	13	4.75E-13
4150	mTOR signaling pathway	9	1.09E-12
4917	Prolactin signaling pathway	9	4.42E-12
5215	Prostate cancer	9	2.87E-11
5221	Acute myeloid leukemia	8	3.63E-11
4910	Insulin signaling pathway	10	3.87E-11
4510	Focal adhesion	11	9.72E-11
4015	Rap1 signaling pathway	11	1.02E-10
4722	Neurotrophin signaling pathway	9	3.33E-10
4068	FoxO signaling pathway	9	4.65E-10
5203	Viral carcinogenesis	10	6.81E-10
5212	Pancreatic cancer	7	3.64E-09
4664	Fc epsilon RI signaling pathway	7	6.14E-09
4662	B cell receptor signaling pathway	7	8.09E-09
4010	MAPK signaling pathway	10	1.39E-08
4380	Osteoclast differentiation	8	1.50E-08
5213	Endometrial cancer	6	4.57E-08
5206	MicroRNAs in cancer	8	4.59E-08
5223	Non-small cell lung cancer	6	6.84E-08
5210	Colorectal cancer	6	9.22E-08
5214	Glioma	6	1.35E-07
5120	Epithelial cell signaling in Helicobacter pylori infection	6	1.76E-07
5145	Toxoplasmosis	7	1.76E-07
5166	HTLV-I infection	9	1.76E-07
5211	Renal cell carcinoma	6	1.76E-07
4062	Chemokine signaling pathway	8	1.98E-07
5218	Melanoma	6	2.39E-07
5169	Epstein-Barr virus infection	8	2.98E-07
5222	Small cell lung cancer	6	7.45E-07
4666	Fc gamma R-mediated phagocytosis	6	8.74E-07
4912	GnRH signaling pathway	6	8.74E-07
4921	Oxytocin signaling pathway	7	1.15E-06

Appendix Table 2. Pathway enrichment FDR values associated with RPPA data analysis (2/3)

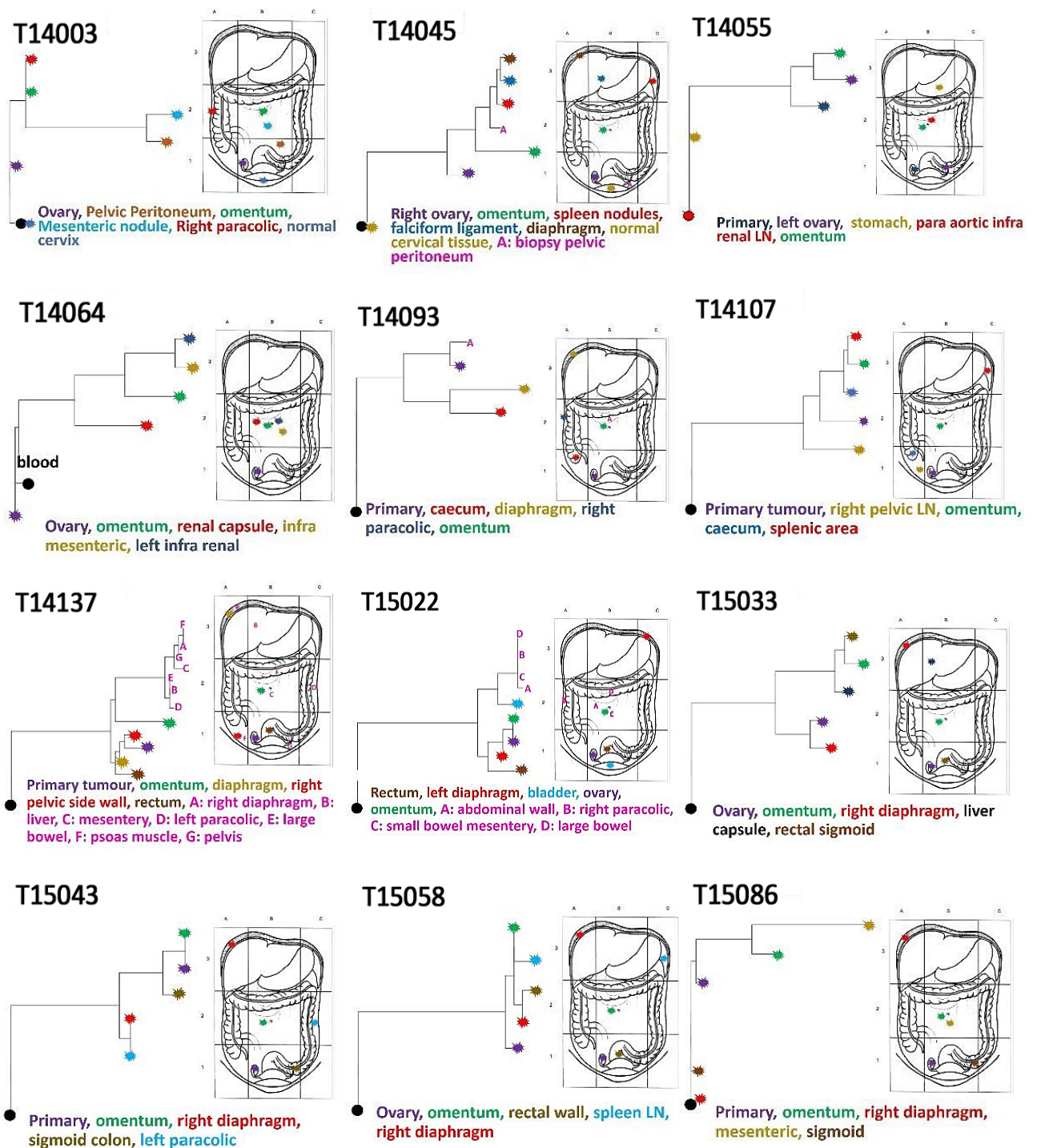
Pathway ID	Pathway description	Observed gene count	FDR
4915	Estrogen signaling pathway	6	1.24E-06
4660	T cell receptor signaling pathway	6	1.66E-06
4620	Toll-like receptor signaling pathway	6	1.90E-06
5164	Influenza A	7	1.90E-06
4668	TNF signaling pathway	6	2.47E-06
4670	Leukocyte transendothelial migration	6	3.53E-06
4152	AMPK signaling pathway	6	4.59E-06
5216	Thyroid cancer	4	4.59E-06
4920	Adipocytokine signaling pathway	5	5.70E-06
5140	Leishmaniasis	5	6.47E-06
5160	Hepatitis C	6	6.64E-06
4520	Adherens junction	5	6.69E-06
5162	Measles	6	6.69E-06
4914	Progesterone-mediated oocyte maturation	5	1.18E-05
4932	Non-alcoholic fatty liver disease (NAFLD)	6	1.38E-05
4540	Gap junction	5	1.45E-05
5202	Transcriptional misregulation in cancer	6	2.51E-05
5142	Chagas disease (American trypanosomiasis)	5	2.88E-05
5152	Tuberculosis	6	2.98E-05
5131	Shigellosis	4	7.82E-05
4110	Cell cycle	5	8.47E-05
4650	Natural killer cell mediated cytotoxicity	5	9.02E-05
4611	Platelet activation	5	9.98E-05
4320	Dorso-ventral axis formation	3	0.000121
4115	p53 signaling pathway	4	0.000129
5034	Alcoholism	5	0.000143
5133	Pertussis	4	0.00015
5100	Bacterial invasion of epithelial cells	4	0.000184
4630	Jak-STAT signaling pathway	5	0.000239
4022	cGMP-PKG signaling pathway	5	0.000258
5132	Salmonella infection	4	0.000266
4210	Apoptosis	4	0.000302
4064	NF-kappa B signaling pathway	4	0.000358
4750	Inflammatory mediator regulation of TRP channels	4	0.000456
4960	Aldosterone-regulated sodium reabsorption	3	0.00046
5020	Prion diseases	3	0.00046
4916	Melanogenesis	4	0.000476
5219	Bladder cancer	3	0.000487
5146	Amoebiasis	4	0.000632
4114	Oocyte meiosis	4	0.000648
4725	Cholinergic synapse	4	0.000688

Appendix Table 2. Pathway enrichment FDR values associated with RPPA data analysis (3/3)

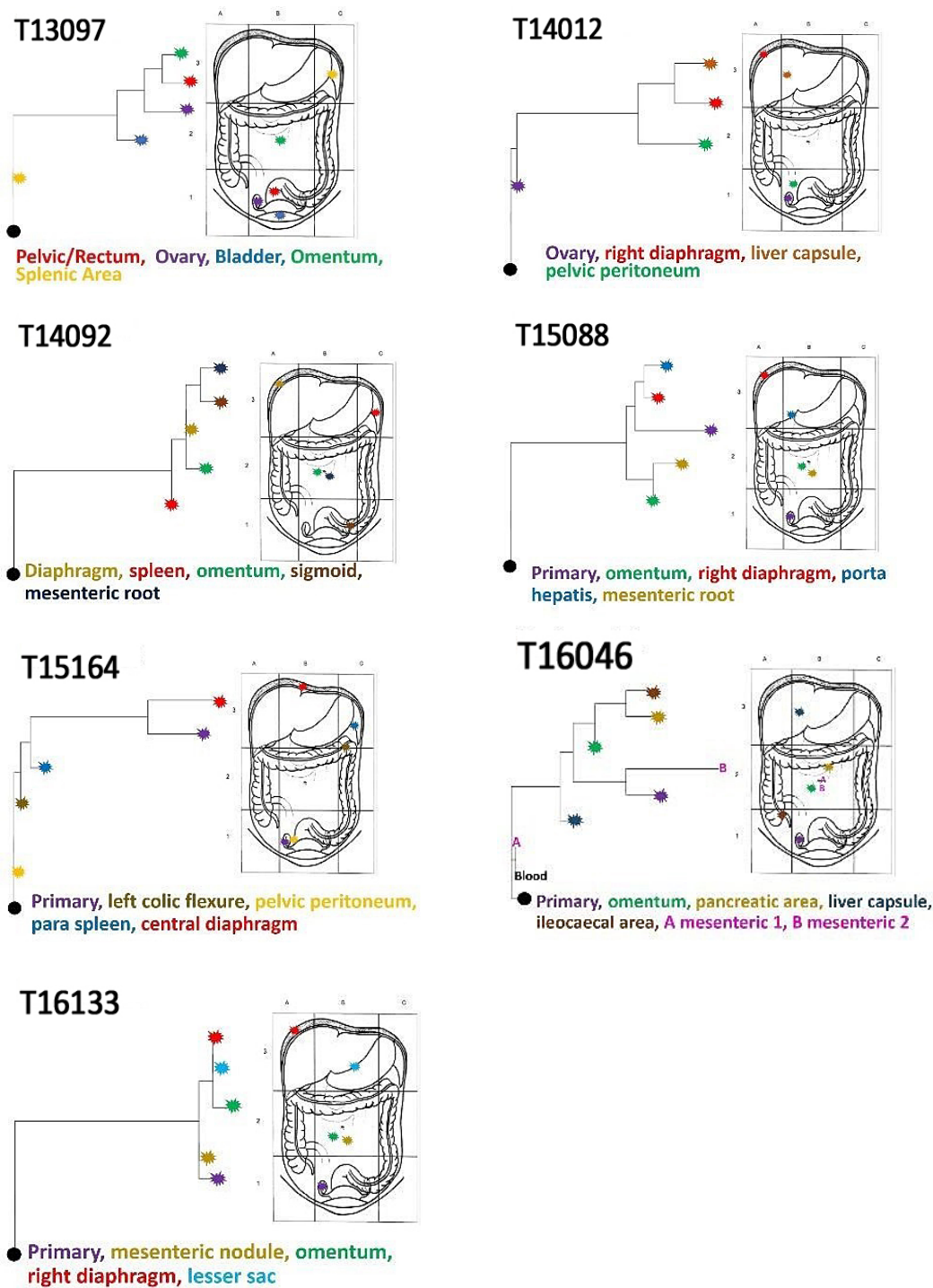
Pathway ID	Pathway description	Observed gene count	FDR
4810	Regulation of actin cytoskeleton	5	0.000824
5014	Amyotrophic lateral sclerosis (ALS)	3	0.00113
4530	Tight junction	4	0.00128
4621	NOD-like receptor signaling pathway	3	0.00154
4730	Long-term depression	3	0.00169
5321	Inflammatory bowel disease (IBD)	3	0.00203
4720	Long-term potentiation	3	0.0021
5322	Systemic lupus erythematosus	3	0.00661
4723	Retrograde endocannabinoid signaling	3	0.00694
4726	Serotonergic synapse	3	0.00985
4270	Vascular smooth muscle contraction	3	0.0113
4728	Dopaminergic synapse	3	0.0138
4973	Carbohydrate digestion and absorption	2	0.0142
4310	Wnt signaling pathway	3	0.0172
4261	Adrenergic signaling in cardiomyocytes	3	0.0191
4930	Type II diabetes mellitus	2	0.0191
4961	Endocrine and other factor-regulated calcium reabsorption	2	0.0197
4390	Hippo signaling pathway	3	0.0204
5030	Cocaine addiction	2	0.0219
3013	RNA transport	3	0.022
5110	Vibrio cholerae infection	2	0.0232
5130	Pathogenic Escherichia coli infection	2	0.0238
5010	Alzheimer s disease	3	0.0267
4020	Calcium signaling pathway	3	0.0308
5168	Herpes simplex infection	3	0.0308
5031	Amphetamine addiction	2	0.0352
4622	RIG-I-like receptor signaling pathway	2	0.0391
4070	Phosphatidylinositol signaling system	2	0.0488



Appendix Figure A2: Phylogenetic trees from HSGOC ITH cohort. Copy-number derived phylogenetic trees produced using MEDICC are mapped into IMO, showing anatomical locations of all tumour samples of patients who did not experience relapse. N=7. The black circle indicates diploid genome.



Appendix Figure A3: Phylogenetic trees from HSGOC ITH cohort. Copy-number derived phylogenetic trees produced using MEDICC are mapped into IMO, showing anatomical locations of all tumour samples of patients who relapsed within a sensitive timeframe (>6 months post-chemotherapy relapse). N=12. Relapse samples are indicated by pink lettering; black circle indicates diploid genome.



Appendix Figure A4: Phylogenetic trees from HSGOC ITH cohort. Copy-number derived phylogenetic trees produced using MEDICC are mapped into IMO, showing anatomical locations of all tumour samples of patients who relapsed within a resistant timeframe (<6 months post-chemotherapy relapse), progressed on chemotherapy, or had refractory disease. N=7. Relapse samples are indicated by pink lettering; black circle indicates diploid genome.