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High Resolution Analysis of the Tumour Microenvironment of High
Grade Serous Ovarian Cancer (HGSOC) using Single Cell
Transcriptomics and Quantitative Histopathological Examination



THE UNIVERSITY
of EDINBURGH

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Submitted for the degree of

Doctor of Philosophy

The University of Edinburgh

2023

Declaration

I declare that the materials presented in this thesis has not been submitted as an exercise for a degree at this or any other university. This thesis is the result of original work carried out by the author, Jessica Lim, at the Cancer Research UK Edinburgh Centre, Institute of Genetics and Cancer, University of Edinburgh. All external resources have been properly acknowledged.

Jessica Lim

Acknowledgements

I wish to express my heartfelt gratitude to my amazing supervisor, Professor Charlie Gourley, for his unwavering support and inspiration, both professionally and personally throughout the past five years. His enthusiasm and strong guidance have been pivotal in my academic journey, with which will always be a part of my career as a surgical gynaecologist. I also would like to thank Professor Simon Herrington for his invaluable insights and expertise which have been instrumental to the development of this work.

I am also very grateful to the team mates at Gourley Laboratory for their utmost willing help and unending assistance. All of you have been extraordinary invisible hands and minds, without which this work would not have materialised. Thank you Robb Hollis, Tom Parry, Mike Churchman, John Thomson, Pam Haig, Clare Bartos and Grant Cameron.

My sincerest appreciations to the Surgical Gynaecology Team at Edinburgh Royal Infirmary (ERI) and Western General Hospital (WGH), the Lothian NRS Bioresource, the FACS Team at IGC and the Pathology Team at WGH. My special thanks to Dr Hung-Chang Chen, a cancer immunologist in Cambridge for his enthusiasm and strong guidance through this project.

I would also like to express my gratitude for my family for their unconditional love and ceaseless support which had provided me with the fortitude and endurance throughout the last few years. Not forgetting my 2-year-old son, Aaron who had brought so much sunshine and hope which helped me through this enduring path.

Lastly, I am forever grateful to the women who had been part of this study, many of whom were no longer here. You had truly made a difference to the lives of other

women through your great contributions to the research world! In a gentle way, you can shake the world. – *Mahatma Gandhi*.

Funding

These projects were funded by the Cancer Research UK as part of the ECAT-linked CRUK Clinical Research Fellowship. I am also grateful for the sponsorship of our laboratory by The Nicola Murray Foundation.

*This work is dedicated to my parents Dr and Mrs Lim Boon Seng;
and also the late Professor Martin Gore CBE,
a great mentor who devoted his life and career
to improving lives of women affected by cancer.*

Abstract

Ovarian cancer (OC) is a highly lethal gynaecologic malignancy with high grade serous ovarian carcinoma (HGSOC) accounting for more than 70% of all OC deaths. Although the prognosis for HGSOC patients has shown some improvement over the last 30 years with an overall five-year survival increasing from 31% to 46%, the disease remains highly recurrent with progressively shorter survival after each relapse. The remitting and relapsing course of this disease arises in part due to the cellular and molecular diversity within the tumour microenvironment (TME) accounting for the highly heterogenous nature of the disease. Bulk gene expression profiling has identified novel subgroups of HGSOC but only interrogates the average signal of cells within a tumour. Single cell RNA-sequencing (scRNA-seq) enables the quantification of gene expression from individual cells, allowing assessment of potential rare subpopulations including chemo-resistant tumour cells.

A major part of this study comprised a thorough methodological optimisation of tumour dissociation using fresh and cryopreserved samples in order to obtain high quality single cell suspensions (>70% viable cells) essential for further scRNA-seq. To investigate the heterogenous landscape of HGSOC, this study utilised droplet-based scRNA-seq to profile ~134,000 cells from multi-site tumour specimens with the representation of a high-resolution analysis of the HGSOC TME and further demonstration of the cellular subclonal phenotypes and their functional importance. Rare cell types were identified such as T cells with unique metallothionein expression profile in treatment resistant samples, and ovarian cancer stem cells (CSCs) which may be targeted in future personalised therapies. Bulk TCGA HGSOC transcriptomic subtypes were found to be driven by the different cellular composition within the HGSOC TME with cancer epithelial cells accounting for the 'Differentiated' subtype,

immune cells for the 'Immunoreactive' subtype, fibroblasts for the 'Mesenchymal' subtype and endothelial cells for the 'Proliferative' subtype.

Quantitative digital histopathological analysis performed here using clinically and molecularly well-annotated Edinburgh cohorts demonstrated the value of using tumour stromal proportion (TSP) and necrosis as a histological feature for helping to stratify and prognosticate the disease. Patients with stroma-rich and lack of necrosis tumours at pre-chemotherapy stage were found to have poorer prognosis. Combined with bulk transcriptomic profiling of these tumours, 'stroma-rich' signature was associated with pro-tumourigenic biological processes including epithelial-mesenchymal-transition (EMT); while 'necrosis' signature was linked with anti-tumourigenic processes such as upregulation of T cell proliferation.

Finally, integration of digital histopathological stratification using TSP with molecular markers from bulk 'stroma-rich' signature and single cell cancer-associated fibroblast (CAF) markers was performed to deconvolute the clinical significance of the stromal compartment using a multi-model characterisation. Patients with TSP of 0.5 or greater had increased expression of 'stroma-rich' and pro-tumourigenic CAF markers. A prognostic multi-gene signature (comprised of 26 genes) was constructed from the intersection of bulk and single cell stromal biomarkers which may aid risk stratification for HGSOC patients and facilitate future personalised therapies.

In summary, these data expanded our knowledge of the heterogenous TME of HGSOC with extensive cross-talk occurring between the diverse cellular and molecular components which should be better interrogated using multi-omics studies.

Lay Summary

Ovarian cancer (OC) is the leading cause of death for women diagnosed with gynaecological cancers. The commonest type of OC is high grade serous ovarian carcinoma (HGSOC) and survival of patients with HGSOC is poor. It is now clear that HGSOC frequently relapses leading ultimately to death due to the highly diverse and changeable cell states within the tumour. Most studies have interrogated the tumour as a whole resulting in averaging the cell signals from the tumour. However, this may result in missing crucial and important signals from rare individual cells including treatment resistant cells.

This study focused on processing the tumours into live single cells and studying their signals. Within the tumours, there are different types and states of cells contributing to the disease development and progression. Specifically, cancer cells and their supporting cells exist in different states driving the cancer. Rare cells including cells that survive chemotherapy and ancestral ('stem') cells were detected here which potentially could be targeted for future improved treatment strategies.

The work here also found that supporting cells called cancer-associated fibroblasts contributed to more aggressive disease and these could be detected on microscopic examination as well as by studying their genetic features by novel RNA sequencing technologies with resolution at single cell level. Additionally, tumours with a higher proportion of dead cells at the pre-treatment stage were found to have better prognosis.

In summary, combining different scientific technologies such as digital microscope assessment and studying the genetic features of tumour at bulk and single cell levels,

provides a better understanding of the diversified nature of the disease. This paves the way for improving treatment strategies for patients.

List of Abbreviations

AFP	alpha fetoprotein
BRCA1	breast cancer 1
BRCA2	breast cancer 2
CA 19-9	Cancer Antigen 19-9
CA-125	Cancer Antigen-125
CAFs	cancer-associated fibroblasts
CCNE1	cyclin E1
CEA	carcinoembryonic antigen
CR	complete response
CSCs	cancer stem cells
CT	computed tomography
CTCs	circulating tumour cells
DEGs	differentially expressed genes
DIF	differentiated
DNA	deoxyribonucleic acid
DSB	double-strand break
EMT	epithelial-mesenchymal-transition
EOC	epithelial ovarian cancer
FFPE	formalin-fixed paraffin-embedded
FIGO	the International Federation of Gynaecology and Obstetrics
FT	fallopian tube
GIT	gastrointestinal tract
H&E	haematoxylin and eosin
hCG	human Chorionic Gonadotropin
HGSOC	high grade serous carcinomas
HR	homologous recombination
HRP	homologous recombination repair proficient
HRR	homologous recombination repair
IDS	interval debulking surgery
IMR	immunoreactive
LGSOC	low grade serous carcinomas
MES	mesenchymal
MHC	histo-compatibility complex class
MMS	multimodal screening
MRI	magnetic resonance imaging
MSCs	mesenchymal stem cells
NAC	neoadjuvant chemotherapy
NC	no change
OC	ovarian cancer
OS	overall survival
OSE	ovarian surface epithelium
PAR	ADP-ribose polymers

PARP	poly(ADP-ribose) polymerase
PD	progressive disease
PDL-1	programmed death ligand-1
PDS	primary debulking surgery
PET	positron emission tomography
PFS	progression free survival
PR	partial response
PRO	proliferative
PTEN	phosphatase and tensin homolog
QC	quality control
RB	retinoblastoma protein
RD	residual disease
RMI	risk malignancy index
ROCA	risk of ovarian cancer algorithm
RRSO	risk-reducing bilateral salphingo-oophorectomy
scRNA-seq	single cell RNA-sequencing
SSB	single-strand break
STIC	serous tubal in situ carcinoma
TAMs	tumor-associated macrophages
TCGA	The Cancer Genome Atlas
tDCs	tumor-associated dendritic cells
TEXs	tumour-derived exosomes
TILs	tumour infiltrating lymphocytes
TME	tumour microenvironment
TP53	tumour protein 53
Tregs	regulatory T cells
TSP	tumour stroma proportion
TSR	tumour stroma ratio
TVS	transvaginal ultrasound scanning
UK	United Kingdom
US	United States
WGS	whole genome sequencing

TABLE OF CONTENTS

DECLARATION.....	i
ACKNOWLEDGEMENTS.....	ii
ABSTRACT.....	v
LAY SUMMARY.....	vii
LIST OF ABBREVIATIONS.....	ix
TABLE OF CONTENTS.....	xi
TABLE OF FIGURES.....	xvi
TABLE OF TABLES.....	xx
1 CHAPTER 1: INTRODUCTION	1
1.1 Ovary anatomy and function.....	2
1.2 Ovarian cancer	2
1.2.1 Classification of epithelial ovarian cancer.....	2
1.2.2 Epidemiology and risk factors.....	6
1.2.3 Clinical presentation	6
1.2.4 Screening and prevention	7
1.2.5 Diagnosis	9
1.2.6 Staging and survival.....	10
1.3 High grade serous ovarian cancer (HGSOC).....	16
1.3.1 Genomics and transcriptomics landscape of HGSOC	16
1.3.2 Molecular subgroups of HGSOC as defined by gene expression	19
1.4 The complex tumour microenvironment (TME) of HGSOC	20
1.4.1 The role of cancer-associated fibroblasts (CAFs) in OC	22
1.4.1.1 Classification of CAFs.....	22
1.4.1.2 Specific mechanisms of CAFs in ovarian cancer progression	26
1.4.1.2.1 Cytokines secretion by CAFs facilitate tumour growth, proliferation, and metastasis.....	26
1.4.1.2.2 Immune-regulatory function of CAFs.....	26
1.4.1.2.3 Tumour cells metabolic regulation by CAFs	28
1.4.2 The role of immune cells in OC	30
1.5 The tumour heterogeneity of HGSOC	32
1.6 HGSOC treatment and chemoresistance	34
1.6.1 First line treatment	34
1.7 Single cell sequencing (SCS).....	39
1.7.1 Single cell RNA-sequencing (scRNA-seq) in OC.....	41
1.7.1.1 Characterisation of tumour heterogeneity through cell mapping, detection of resistant clones and study of tumour-immune microenvironment	41
1.7.1.2 Detection of OC stem cells/ stem-like cells	43
1.7.1.3 Study of circulating tumour cells (CTCs).....	44
1.7.1.4 Study of tumour-derived exosomes (TEXs)	45
1.8 Quantitative histopathological analysis in HGSOC	46

1.8.1 Conventional histological diagnosis.....	46
1.8.2 Role of quantitative histopathological analysis in HGSOC	47
2 CHAPTER 2: OPTIMISATION OF TUMOUR DISSOCIATION INTO SINGLE CELLS FOR RNA-SEQUENCING.....	49
2.1 Introduction	50
2.2 Study Aims	53
2.3 Materials and methods	54
2.3.1 Patients' Samples.....	54
2.3.1.1 Patient Recruitment	54
2.3.1.2 Acquisition, Storage and Transport of Patients' Samples.....	54
2.3.1.3 Sample Processing.....	56
2.3.2 Manual Tumour Dissociation	58
2.3.2.1 Enzymatic Dissociation.....	58
2.3.2.2 Filtration, Enzyme Blocking and Red Blood Cell Lysis	59
2.3.2.3 Cell viability count.....	60
2.3.2.4 FACS staining, sorting and analysis	61
2.3.3 Automated Tumour Dissociation.....	63
2.3.3.1 Enzymatic Dissociation and Filtration	64
2.3.3.2 (Optional) Tumour Cell Isolation	64
2.3.3.3 Dead Cell Removal.....	65
2.3.3.4 Red Blood Cell Lysis.....	66
2.3.3.5 Second Dead Cell Removal.....	66
2.3.3.6 FACS Staining and Analysis	66
2.3.4 Comparison between Manual vs Automated Protocol	66
2.3.5 Optimising Automated Protocol	67
2.4 Results	68
2.4.1 Manual Dissociation	68
2.4.1.1 Determining the optimal dissociation reagent, incubation condition and sample state	68
2.4.1.2 Cell viability for manually dissociated samples after FACS sorting.....	76
2.4.2 Manual vs Automated Dissociation	76
2.4.2.1 Comparing the efficacy of manual vs automated dissociation	76
2.4.3 Optimisation of Automated GentleMACS Dissociation	80
2.4.3.1 Checking the effects of automated dissociation on cells	80
2.4.3.2 Experiments to optimise the automated GentleMACS dissociation protocol..	81
2.4.3.3 Importance of Dead Cell Removal (DCR) Step	84
2.4.3.4 Fresh vs Cryopreserved Samples Cell Viabilities	87
2.4.4 Quality Control Checks.....	89
2.4.4.1 FACS sorting for live cells following dissociation.....	89
2.4.4.2 FACS analysis showing purer live single cells following dead cell removal ...	89
2.4.4.3 FACS sorting and analysis to select for cells of interest using antibody markers	92
2.4.4.3.1 Fluorescence Minus One (FMO) controls	92
2.4.4.3.2 FACS sorting and analysis following manual dissociation	92
2.4.4.3.3 FACS analysis following manual and automated dissociation to check for cells of interest using antibody markers	95

3 CHAPTER 3: SINGLE CELL RNA-SEQUENCING TO ANALYSE THE TUMOUR MICROENVIRONMENT (TME) OF HGSOC	106
3.1 Introduction	106
3.1.1 Single cell RNA-sequencing (scRNA-seq) in OC	106
3.1.2 Evolution of scRNA-sequencing technology	107
3.1.3 Basic principles of scRNA-sequencing	108
3.1.4 Division of work in Chapter 3	114
3.1.5 Literature review: Summary of all relevant published work on scRNA-seq in ovarian cancer (OC)	114
3.2 Study aims	124
3.3 Materials and methods	125
3.3.1 GEM generation and barcoding (Genomics, 2022)	127
3.3.2 Post GEM-RT cleanup and cDNA amplification (Genomics, 2022)	133
3.3.3 3' Gene expression library construction	139
3.3.4 Sequencing	147
3.3.5 Bioinformatics pipeline, software and statistical analysis	148
3.3.5.1 Sequenced data pre-processing	148
3.3.5.2 Quality control	148
3.3.5.3 Normalisation	149
3.3.5.4 Cell clustering	149
3.3.5.5 Cell types and subtypes identification	150
3.3.5.6 Gene set enrichment analysis	151
3.3.5.7 Comparison of cell types/ populations to TCGA HGSOC molecular subtypes	151
3.4 Results	154
3.4.1 Single cell RNA-sequenced libraries and QC checks using cell viability, bioanalyser, 10x CellRanger and R/Seurat	154
3.4.2 Patient Description	167
3.4.3 Cell identification	171
3.4.3.1 Confidence of cell type identification	173
3.4.3.2 Cancer epithelial and ciliated cancer epithelial cells belong to the same cell group (tumour epithelial cells)	175
3.4.4 Cell population by patient and by site	177
3.4.5 Inference of CNVs of HGSOC epithelial cells to determine cancer epithelial cells (aneuploid/ malignant) vs normal epithelial cells	180
3.4.6 Identification of subclusters of cells within the TME of HGSOC	185
3.4.6.1 Cancer epithelial cells subclustering and biological significance	185
3.4.6.2 Fibroblast cells subclusters and their biological significance	190
3.4.6.3 Immune cells subpopulations and their biological significance	195
3.4.7 Detecting cancer stem cells	198
3.4.8 Comparison of cell populations to TCGA HGSOC molecular subtypes	200
3.4.9 Cells uncovered in lower quality datasets	205
3.4.10 Broad cell composition of a low grade serous ovarian cancer (LGSOC) case	208
3.5 Discussion	209
4 CHAPTER 4: ANALYSIS OF HGSOC TUMOUR MICROENVIRONMENT USING QUANTITATIVE HISTOPATHOLOGICAL EXAMINATION	221

4.1	Introduction	221
4.1.1	Quantitative histopathological analysis of HGSOc TME.....	221
4.1.2	Role of tumour and stromal component in modulating TME of HGSOc	222
4.1.2.1	Tumour component.....	222
4.1.2.2	Stromal component.....	223
4.1.2.2.1	Desmoplasia and role of cancer associated fibroblasts (CAFs) in HGSOc	223
4.1.3	Tumour stroma ratio (TSR) or tumour stroma proportion (TSP).....	225
4.2	Study aims	227
4.3	Materials and methods	228
4.3.1	Patient's samples and cohorts.....	228
4.3.2	Pilot cohorts (A and B)	229
4.3.3	Cohort C	236
4.3.4	Clinical data collection.....	238
4.3.5	Molecular subtyping, phosphatase and tensin homolog (PTEN) and retinoblastoma protein (RB) status, and immune cell infiltration data procurement for cohort C	238
4.3.6	Statistical analyses.....	239
4.4	Results	241
4.4.1	Pilot cohorts (A and B) results as proof of principle	241
4.4.1.1	Patient demographics, clinical characteristics and histopathological findings	241
4.4.1.2	Evaluation of the prognostic value of TSP and correlation with clinicopathological features in pilot cohorts.....	246
4.4.1.3	Evaluation of the relationship between TSP and desmoplasia and necrosis using pilot cohorts	248
4.4.1.4	Evaluation of prognostic value of desmoplasia by sites in pilot cohorts.....	250
4.4.2	Cohort C results (Large dataset of 290 patients)	252
4.4.2.1	Patient characteristics.....	252
4.4.2.2	Determination of TSP cut-off to categorise patients into stroma-poor and stroma-rich groups	253
4.4.2.3	Evaluation of the TSP by sites	256
4.4.2.4	Relationship between TSP and clinicopathological features and molecular characteristics	257
4.4.2.5	The prognostic value of TSP and other clinicopathological variables	261
4.4.2.6	Evaluation of the prognostic value of desmoplasia and correlation with clinicopathological features in cohort C	264
4.4.2.7	Evaluation of the prognostic value of necrosis and correlation with clinicopathological features in cohort C	272
4.4.3	Construction and evaluation of prediction models based on TSP	277
4.5	Discussion.....	279
5	CHAPTER 5: INTEGRATION OF BULK AND SINGLE CELL TRANSCRIPTOMIC DATA IN HGSOc	288
5.1	Introduction.....	288
5.1.1	Tumour stroma proportion (TSP) in HGSOc	288
5.1.2	Transcriptomic profiles of stroma-rich and stroma-poor HGSOc tumours stratified by TSP	288

5.1.3 Transcriptomic profiles of necrotic HGSOC tumours	290
5.2 Study aims	292
5.3 Materials and methods	293
5.3.1 Patient's samples	293
5.3.2 Data processing	293
5.3.3 Differential gene expression analysis of Cohort C patients	294
5.3.4 Correlation of stroma-rich gene signature to bulk RNA-seq in TCGA data.....	294
5.3.5 Functional geneset enrichment analysis (GSEA).....	294
5.3.6 Integration of single cell fibroblast subclusters markers and bulk TCGA 'Mesenchymal' markers	295
5.3.7 Predictive prognostic novel gene signature in HGSOC.....	295
5.4 Results.....	296
5.4.1 Transcriptomic profiles of stroma-rich vs stroma-poor tumours from Cohort C ..	296
5.4.2 Stroma-rich gene signature displayed by TCGA HGSOC 'Mesenchymal' subtype	303
5.4.3 CAF subclusters and TCGA 'Mesenchymal' markers were associated with stroma- rich tumours	305
5.4.4 Predictive prognostic novel gene signature in HGSOC.....	310
5.4.5 Transcriptomic profiles of necrotic vs non-necrotic tumours from cohort C	312
5.5 Discussion.....	317
6 CHAPTER 6: DISCUSSION AND CONCLUSION.....	323
7 REFERENCES	332
Appendix 1 100 Gene Symbols Defining TCGA HGSOC Subtype Signatures.....	376
Appendix 2 Work for Publication in Progress.....	381

TABLE OF FIGURES

Figure 1.1 A model of initiation and progression of low-grade (LGSOC) and high-grade (HGSOC) serous carcinomas.....	4
Figure 1.2 Subtypes of EOC, their origins and the associated molecular genetic changes.....	5
Figure 1.3 Relative five survival rate for invasive EOC (Society, 2016).....	14
Figure 1.4 Genomic alteration profile of HGSOC.....	17
Figure 1.5 Pan Cancer Data.....	18
Figure 1.6 OC TME are comprised of cancer cells, stromal and immune cells	21
Figure 1.7 Ovarian cancer-associated fibroblasts.....	25
Figure 1.8 Summary of Genomic Instability in HGSOC resulting in Tumour Heterogeneity.....	34
Figure 1.9 The role of single cell molecular phenotyping in characterisation of tumour heterogeneity and in clinical applications	40
Figure 2.1 Graphical Experimental Design	52
Figure 2.2 Sample Processing.	58
Figure 2.3 Haemocytometer Counting Chamber with Live Cells (unstained) and Dead Cells (stained blue).....	60
Figure 2.4 Comparison of Thermoblock Rotation Speed for Enzymatic Incubation for All Sample Sites.....	72
Figure 2.5 Comparison of % Cell Viability Yield by Tumour Weight.....	73
Figure 2.6 Comparison of Enzyme Efficacies on All Sites	74
Figure 2.7 Control Experiment using Trypsinised OVCAR-8 Cell Lines to Check the Effects of Automated Dissociation on the Cells.....	80
Figure 2.8 % Cell Viability Pre- and Post-DCR.....	85
Figure 2.9 Microscope Images of Cells Following Manual and Automated Dissociations Pre-and Post-DCR.	86
Figure 2.10 Cell Viability of Fresh vs Cryopreserved Samples	88
Figure 2.11 FACS Results of Cryopreserved Ovarian Sample from Experiment M2.6	90-91
Figure 2.12 FACS Results of Cryopreserved Ovarian Sample from Experiment MA1.6.....	91
Figure 2.13 FMO Controls to Determine Thresholds between Background Fluorescence and Positive Cell Populations using Different Antibodies	93
Figure 2.14 FACS Sorting and Analysis for Immune and Non-immune Cells ...	95
Figure 2.15 FACS Results of Cryopreserved Ovarian Sample from Experiment MA1.5.	96
Figure 3.1 Evolution of Single Cell RNA-sequencing Technology.....	107
Figure 3.2 Basic Steps of scRNA-seq and Different Experimental Approaches	111
Figure 3.3 Stepwise Approach to 10x Genomics Chromium Single Cell 3' Protocol.....	113
Figure 3.4 Chromium Chip B Loading	128
Figure 3.5 GEM Appearance.....	132

Figure 3.6 Example of cDNA Bioanalyser trace and Calculation for cDNA Quantification	138
Figure 3.7 Correlation between cDNA Input and Library Complexity	139
Figure 3.8 Example of Library Bioanalyser Trace	146
Figure 3.9 Chromium Single Cell 3' Gene Expression Library	147
Figure 3.10 Comparison of Fresh vs Cryopreserved Samples	158
Figure 3.11 Cell Recovery Post-Sequencing Affected by % Cell Viability and scDNA Library Concentration	159
Figure 3.12 Sequencing Statistics for 17 Libraries of OC Patients	162
Figure 3.13 Mapping Statistics for 17 Libraries of OC Patients	163
Figure 3.14 Distinct Cell Types across 4 HGSOC Patients with 8 Samples	171
Figure 3.15 Z-score Pearson Correlation of Shared Highly Variable Genes (NHGVs = 411) across the Average Transcriptome of Each Cell Type from Every Sample	174
Figure 3.16 Comparison of Cancer Epithelial Cells and Ciliated Cancer Epithelial Cells	176
Figure 3.17 UMAPs of the individual samples of the 4 HGSOC Patients Used for Cell Identification	178
Figure 3.18 Proportions of Cells Making Up Each Sample	179
Figure 3.19 Copy Number Profile in Cancer Epithelial Cells vs Normal Background Myeloid Cells	182
Figure 3.20 Differences between Cancer Epithelial Cells and Normal Epithelial Cells	183
Figure 3.21 CNV Signature Sub-clones within Primary Ovarian and Peritoneal Samples of a Single Patient	184
Figure 3.22 Cancer Epithelial Cell Sub-clusters UMAP	186
Figure 3.23 Gene Expression Heatmap of Top 10 Differentially Expressed Genes (DEGs) for Each Subcluster of Cancer Epithelial Cells	187
Figure 3.24 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of Cancer Epithelial Cells	189
Figure 3.25 Fibroblast Cell Subclusters UMAP	191
Figure 3.26 Gene Expression Heatmap of Top 10 Differentially Expressed Genes (DEGs) for Each Subcluster of Fibroblasts	191
Figure 3.27 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of CAFs using MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer'	193
Figure 3.28 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of CAFs using Gene Ontology 'Biological Processes'	194
Figure 3.29 UMAP of Immune Cells	196
Figure 3.30 Marker Genes Identifying Subpopulations of Immune Cells	197
Figure 3.31 CSC Markers Detected in the Integrated Cancer Epithelial Cells Dataset	198
Figure 3.32 UMAPs of Lower Quality Libraries	207
Figure 3.33 Cellular Make-up of the Duplicate LGSOC Samples from the Same Single Cell Suspension Post-dissociation	208

Figure 4.1 Metastatic nodule.....	234
Figure 4.2 H&E stained section of a HGSOC ovary.....	234
Figure 4.3 Section from an Ovary: Workflow of QuPath Image Analysis.....	235
Figure 4.4 Cohort C Identification.....	237
Figure 4.5 Images Pre- and Post-QuPath Classification.....	244
Figure 4.6 Desmoplasia Detection Using Manual and Automated Methods ...	245
Figure 4.7 Relationship between TSP and Survival Outcomes for Cohorts A and B	247
Figure 4.8 Correlation of TSP with Desmoplasia and Necrosis	249
Figure 4.9 % Desmoplasia and % Necrosis in Stroma-poor and Stroma-rich Groups.....	249
Figure 4.10 Correlation of %Desmoplasia and OS by Sites for Cohorts A and B	251
Figure 4.11 Histopathological and Molecular Landscape of Cohort C.	252
Figure 4.12 Figure Representative images of H&E stained 5 µm sections of HGSOC patients from Cohort C (original magnification 40X).....	255
Figure 4.13 TSP by Sites. More than 2/3 of patients in Cohort C had histological sections from primary sites.....	256
Figure 4.14 Clinicopathological and Molecular Characteristics of Stroma-poor and Stroma-rich Groups	260
Figure 4.15 Kaplan-Meier Curves for the Survival of 287 Patients in Cohort C Stratified by TSP	262
Figure 4.16 Frequency Distribution of Desmoplasia for Cohort C	265
Figure 4.17 Figure Desmoplasia by Sites for Cohort C.....	267
Figure 4.18 Correlation of % Desmoplasia and TSP	271
Figure 4.19 % Desmoplasia in Stroma-poor and Stroma-rich Groups	271
Figure 4.20 Distribution of % Necrosis in Cohort C patients.....	273
Figure 4.21 Kaplan-Meier Curves for the Survival of 287 Patients in Cohort C Stratified by Necrosis.....	274
Figure 4.22 Performance of the TSP Model. Time-dependent AUC was measured for at least 5 years at daily intervals, demonstrating the prediction performance at different time-points for the reference vs TSP model.....	278
Figure 5.1 Differentially Expressed Genes (DEGs) between Stroma-rich and Stroma-poor Tumours of Cohort C1 (training dataset)	299
Figure 5.2 Cohort C1 Stroma-rich Significantly Upregulated Gene Expression Enrichment Analysis	300
Figure 5.3 Levels of Expression of the Upregulated Genes (Top 20) of C1 Stroma-rich Tumours in both C1 and C2 Cohorts	302
Figure 5.4 Levels of Expression of the Downregulated Genes (Top 10) of C1 Stroma-rich Tumours in both C1 and C2 Cohorts	302
Figure 5.5 Expression of Stroma-rich Gene Signature (Top 45 Upregulated Genes) in TCGA tumours.....	304
Figure 5.6 Expression of Fibroblast Subclusters and TCGA ‘Mesenchymal’ Gene Markers in Stroma-poor vs Stroma-rich Tumours of Cohort C1 .	308

Figure 5.7 Expression of Fibroblast Subclusters and TCGA ‘Mesenchymal’ Gene Markers in Stroma-poor vs Stroma-rich Tumours of Cohort C2	309
Figure 5.8 Novel Gene Signature Expression in Cohort C	310
Figure 5.9 The Correlation between Signature Gene Expression and Survival Outcomes of Patients in Cohort C	311
Figure 5.10 Cohort C1 Necrosis Significant Downregulated Gene Expression Enrichment Analysis	314
Figure 5.11 Cohort C1 Necrosis Significant Downregulated Gene Expression Enrichment Analysis	315
Figure 5.12 Levels of Expression of the Downregulated Genes (Top 20) of C1 Necrotic Tumours in both C1 and C2 Cohorts	316

TABLE OF TABLES

Table 1.1 Calculation of RMI (Fotopoulou <i>et al.</i> , 2017).....	10
Table 1.2 Comparison of the revised International Federation of Gynaecology and Obstetrics (FIGO) staging classification for cancer of the ovary, fallopian tube, and peritoneum (2013) and FIGO staging classification of the ovary (1988). (Saida <i>et al.</i> , 2016)	12-13
Table 1.3 Mechanisms of CAFS in promoting OC progression.....	28
Table 1.4 Summary of Immuno-effector Cells and Molecules.....	31
Table 1.5 Immuno-histochemical features of HGSOC vs LGSOC.....	46
Table 2.1 Experiments on 3 Different Sites using Collagenase, Liberase and Neutral Protease	70
Table 2.2 Experiments Comparing 3 Different Enzymes on a Single Site	71
Table 2.3 Comparison of Efficacy of 3 Different Enzymatic Dissociation	75
Table 2.4 Cell Viability Pre- and Post-FACS sorting.....	77
Table 2.5 Comparing the Efficacy of Manual vs Automated GentleMACS Dissociation	78
Table 2.6 Experiments Optimising Automated GentleMACS Dissociation Protocol.....	82
Table 2.7 Quantity of Antibodies and DAPI stain Used for FMOs	92
Table 3.1 Comparison of SMART-Seq2 and 10x Genomics Chromium	110
Table 3.2 Summary of All Relevant Published Papers on single cell RNA-seq in OC.....	110
Table 3.3 10x Genomics Chromium Single Cell 3' v 3 Protocol Steps and Timing.....	125
Table 3.4 Risk of Multiplets with Increasing Cells Loaded	127
Table 3.5 Master Mix	128
Table 3.6 Cell Suspension Volume Calculator. Adapted from (Genomics, 2022b).	130
Table 3.7 Thermal Cycler Settings	133
Table 3.8 Dynabeads Cleanup Mix.....	134
Table 3.9 Elution Solution I	134
Table 3.10 cDNA Amplification Reaction Mix	135
Table 3.11 Thermal Cycler Settings (Genomics, 2022b)	136
Table 3.12 Optimal Number of Cycles (Genomics, 2022b)	136
Table 3.13 Thermal Cycler Settings	140
Table 3.14 Fragmentation Mix	140
Table 3.15 Adaptor Ligation Mix	142
Table 3.16 Thermal Cycler Settings	142
Table 3.17 Sample Index PCR Mix	144
Table 3.18 Thermal Cycler Settings	144
Table 3.19 List of 18 Single Cell Libraries Generated and Sequenced	156
Table 3.20 Summary of Cell Count Metrics by CellRanger (17 Libraries of OC Patients)	160
Table 3.21 QC by CellRanger Algorithm vs Seurat Filtering (17 Libraries of OC Patients)	165

Table 3.22 Patients and Sample Sites Description.....	168
Table 3.23 Canonical Marker Genes Used for Cell Type Identification.....	172
Table 3.24 Proportion of Cell Types (%) Making up Each Sample	178
Table 3.25 Proportions of HGSOC Epithelial Cells Classified as Cancer Epithelial or Normal Cells by CopyKAT	181
Table 3.26 Ratio of CD8 ⁺ T cells to CD4 ⁺ Treg cells in Different Patient Samples	197
Table 3.27 OC CSC Markers (Kenda Suster and Virant-Klun, 2019)	199
Table 3.28 Comparison to TCGA HGSOC Subtypes by Patient Sample ID	202
Table 3.29 Example of Calculation for the Average of RNA Expression of All Cells in Patient 5 OV for All the Genes of the TCGA ‘Differentiated’ Subtype from Verhaak’s List	203
Table 3.30 Comparison to TCGA HGSOC Subtypes by Cell Types	204
Table 3.31 Summary of the Lower Quality Datasets	206
Table 4.1 Cohorts. Characteristics of Cohort A, B and C.	228
Table 4.2 Interobserver Agreement for Classification of Histologic Features in Validation Set.....	232
Table 4.3 Table Summary of Patients Demographics and Clinical Characteristics.....	242
Table 4.4 Table Histopathological Findings of Cohort A	242
Table 4.5 Table Histopathological Findings of Cohort B	243
Table 4.6 Table Determining Cut-off for TSP (Stroma-poor and stroma-rich groups)	254
Table 4.7 Table Patient Clinicopathological Features and Molecular Characteristics (Grouped by tumour stroma proportion)	258
Table 4.8 Cox Univariate and Multivariate Analysis for OS and PFS for Cohort C patients.	263
Table 4.9 Patient Clinicopathological Features and Molecular Characteristics (Grouped by proportions of desmoplasia).	268
Table 4.10 Patient Clinicopathological Features and Molecular Characteristics (Grouped by presence and absence of necrosis).....	275
Table 4.11 Table Discrimination Performance of the Reference and TSP Models in the Discovery and Validation Cohorts.....	277
Table 5.1 Top 20 Upregulated Genes by Stroma-rich Tumours of Cohort C1.	297
Table 5.2 Top 10 Downregulated Genes by Stroma-rich Tumours of Cohort C1	298
Table 5.3 Statistical Comparison of the Expression Levels of Marker Genes for the Different CAF Subclusters and TCGA ‘Mesenchymal’ Subtype between Stroma-rich and Stroma-poor Tumours	305
Table 5.4 List of Marker Genes for the Fibroblast Subclusters and TCGA HGSOC ‘Mesenchymal’ Subtype.....	306
Table 5.5 Top 20 Downregulated Genes by Necrotic Tumours of Cohort C1 .	313

CHAPTER 1: INTRODUCTION

1 CHAPTER 1: INTRODUCTION

1.1 Ovary anatomy and function

The ovaries are the primary female reproductive organs located in the ovarian fossae, which are shallow depressions in the lateral walls of the pelvic cavity (U. S. National Institutes of Health, 2022). They are attached to the pelvic side walls by the infundibulopelvic ligaments which contain the ovarian arteries originating from the abdominal aorta. The ovary starts developing at around 5 weeks of gestation from the gonadal ridge. There are 4 main components in a developed ovary with each arising from different embryological origins: 1) surface epithelium arises from coelomic epithelium, 2) ovarian stroma formed by subcoelomic mesoderm, 3) germ cells from the yolk sac endoderm, and 4) sex cords (pre-granulosa cells) in the superficial ovarian cortex formed by invaginations of coelomic epithelium (Cox & Takov, 2022). The main functions of the ovaries are: 1) secretion of female sex hormones (primarily oestrogen and progesterone), and 2) production and maturation of ovarian follicles into ova.

1.2 Ovarian cancer

1.2.1 Classification of epithelial ovarian cancer

Epithelial ovarian cancer (EOC) makes up about 90% of all ovarian cancers and the remaining are comprised of rarer types including germ cell tumours, sex cord stromal tumours, small cell carcinomas, neuroendocrine carcinomas and sarcomas (La Vecchia, 2017).

EOC is comprised of different types of malignant ovarian tumours with distinct molecular and histological characteristics. In 2014, the WHO classification has been

updated and this informs most of the current clinical guidelines of EOC (Meinhold-Heerlein et al., 2016). There are at least eight subtypes of EOC including low grade serous carcinomas (LGSOC), endometrioid carcinomas, clear cell carcinomas, mucinous carcinomas, malignant Brenner tumours, high grade serous carcinomas (HGSOC), carcinosarcomas and undifferentiated carcinomas (Meinhold-Heerlein et al., 2015). Kurman and Shih's dualistic classification model of EOC (Type I and II tumours) having previously received some acceptance now seems an oversimplification. Based on this model, Type I tumours are subdivided into three groups comprised of i) LGSOC; ii) endometriosis-related tumours including endometrioid, clear cell and what were previously known as seromucinous carcinomas; and iii) mucinous carcinomas and malignant Brenner tumours (Kurman & Shih, 2016). Each histologic subtype display specific mutations with *KRAS*, *BRAF*, *ERBB2* mutations occurring mainly in LGSOC; somatic mutations of *ARID1A*, *CTNNB1*, *PTEN*, *PIK3CA* and MMR deficiency occurring in endometrioid carcinomas; *ARID1A*, *PTEN*, *PIK3CA* mutations in clear cell carcinomas; *ARID1A* mutation in seromucinous carcinomas; *KRAS* mutations and *HER2* amplification in mucinous carcinomas and *KRAS* and *PIK3CA* mutations in malignant Brenner tumours (Kurman & Shih, 2011, 2016; Meinhold-Heerlein et al., 2016). Meanwhile, Type II tumours are comprised of HGSOC, carcinosarcoma and undifferentiated carcinomas. They are highly aggressive with common *TP53* mutation and often present at an advanced stage (Kurman & Shih, 2011, 2016). From large genomic studies, it is now clear that Type I tumours are not homogenous and may have an aggressive course of disease if presenting at later stages (Salazar et al., 2018).

HGSOC is the most common subtype of EOC, constituting about 70% of ovarian cancer diagnoses (Prat, 2012). Although the prognosis for HGSOC patients is poor, it

has shown some improvement over the last 30 years with an overall five-year survival increasing from 31% to 46% (Ross et al.; The Cancer Genome Atlas Research, 2011). The origin of HGSOC was previously thought to be the ovarian surface epithelium or cortical inclusion cysts. However, recent data suggest at least 50% of HGSOC cases originate from the serous tubal in situ carcinoma (STIC) lesions in the distal fallopian tube (Crum et al., 2007; Ducie et al., 2017; Kuhn et al., 2012).

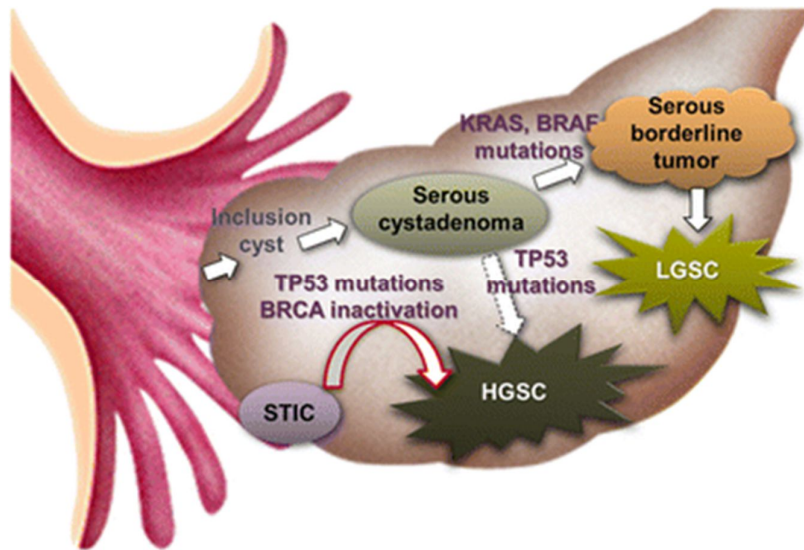


Figure 1.1 A model of initiation and progression of low-grade (LGSOC) and high-grade (HGSOC) serous carcinomas. HGSOC are considered to be *de novo* cancers promoted by *TP53* and *BRCA* abnormalities. LGSOC are considered to arise from serous borderline tumours in the stepwise process and are promoted by *KRAS* and *BRAF* mutations in some cases. Figure from (Saida et al., 2016).

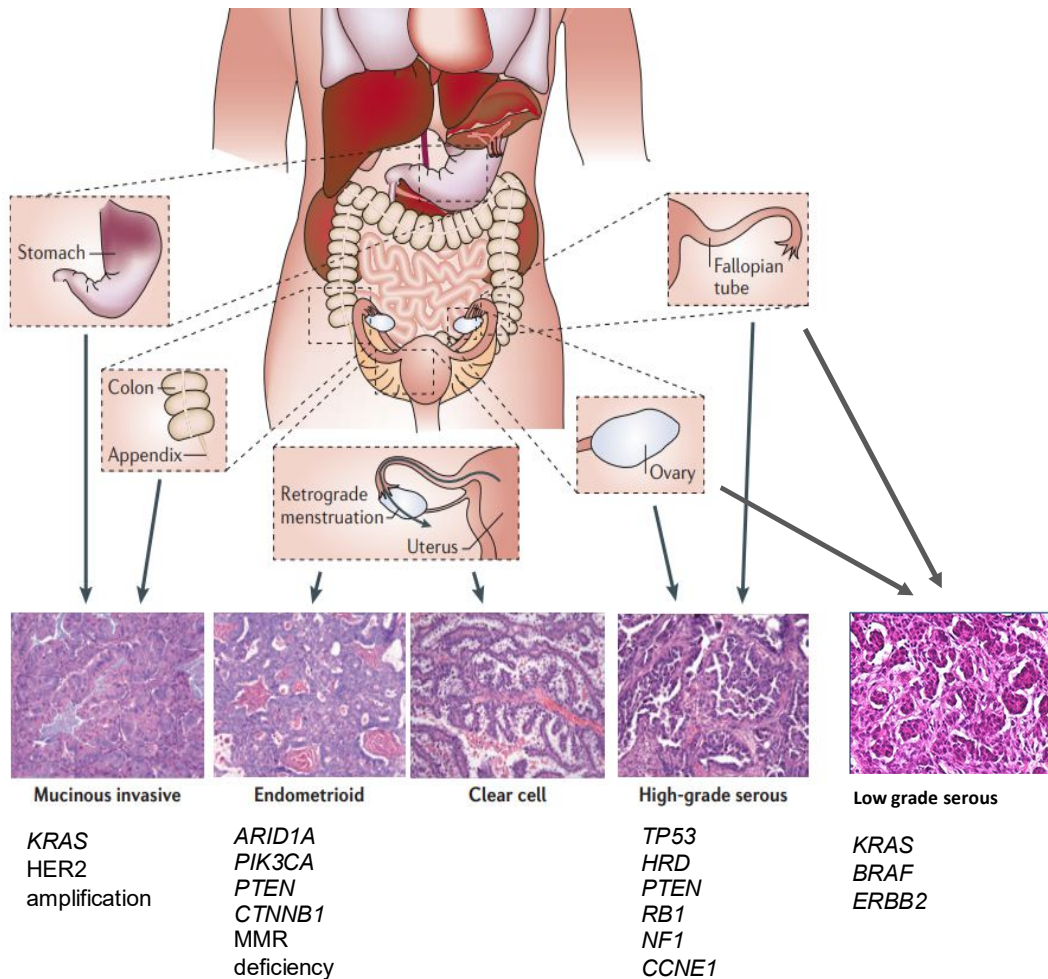


Figure 1.2 Subtypes of EOC, their origins and the associated molecular genetic changes. Figure adapted from (Vaughan et al., 2011). Mucinous EOC (MOC) is unique with uncertain origin, where previously non-gynaecological metastases could now be discriminated from primary MOC. However, it remains unclear whether the ovarian surface epithelium is the cell of origin for MOC (Cheasley et al., 2019). Endometrioid and clear cell EOC are mainly derived from endometriosis with retrograde menstruation from the endometrium. HGSOC and LGSOC are thought to originate from ovarian surface epithelium and distal fallopian tube. HGSOC is almost universally associated with *TP53* mutation and 50% of cases have homologous recombinant defects. Other significant mutations occurring in HGSOC include *PTEN*, *RB1*, *NF1*, *CCNE1* (Patch et al., 2015; The Cancer Genome Atlas Research, 2011).

1.2.2 Epidemiology and risk factors

Ovarian cancer (OC) is the most lethal of all gynaecological malignancies with nearly 300,000 new diagnoses in 2018 and 180,000 deaths per year worldwide (Bray et al., 2018). In the United Kingdom, OC is the sixth most common cancer in women with 7,400 cases diagnosed each year (Cancer Research UK Cancer Statistics). The incidence of OC differs throughout the world due to varying risk factors in different regions. In terms of prevalence by ethnicity, OC affects non-Hispanic white women the most (12.0 per 100,000), followed by Hispanic (10.3 per 100,000), non-Hispanic black (9.4 per 100,000), and Asian/Pacific Islander women (9.2 per 100,000) (Torre et al., 2018). The lifetime risk of developing OC is approximately 2.7%, in the absence of protective factors (Sopik et al., 2015).

There are many risk factors that contribute to OC (see section 1.2.4). This is a predominantly post-menopausal disease with an increased incidence in women aged over 65 and median age of diagnosis between 50 to 79 years old (Arora et al., 2018; Chan et al., 2006; Zheng et al., 2018). Family history of breast or ovarian cancer plays an important contributing role to the risk of developing OC.

1.2.3 Clinical presentation

HGSOC predominantly affects older, postmenopausal women with peak incidence between ages of 60 to 64 years old (Ince et al., 2015). In the early stages of the disease, patients are either asymptomatic or may have a few vague symptoms such as non-specific abdominal bloating or pain and fatigue. More than 70% of the newly diagnosed cases are often at an advanced stage (FIGO Stage III or IV). Symptoms associated with the advanced stages of ovarian cancer are reflective of peritoneal and

extra-peritoneal spread and are more suggestive when present for >1 year and occurring >12 times per month. These include abdominal distension, bloating, early satiety, urinary urgency and/or frequency, unexplained weight loss, vaginal bleeding, respiratory symptoms from pleural effusions, palpable masses (from abdominal or enlarged lymph nodes in the inguinal, supraclavicular or axillary regions) and changes in bowel habits (constipation or diarrhoea) (Ledermann et al., 2013).

1.2.4 Screening and prevention

Factors protective against ovarian cancer are combined oral contraceptive pill, pregnancy, sterilization/tubal ligation, bilateral salpingectomy and/or salphingo-oophorectomy and hysterectomy (Ely & Truong, 2017).

Risk factors associated with ovarian cancer are nulliparity or first birth after age 35 years, early menarche, late menopause, family history of inherited *BRCA1/2* mutations or mismatch repair genes (Lynch Syndrome). There is a strong family history association in the development of ovarian cancer with more than twofold increased risk if there is an affected first-degree relative. However, only 14% of EOC cases have known genetic mutation (e.g. *BRCA1/2* genes). Women with *BRCA1/2* mutation have a lifetime risk of around 85% of getting breast cancer. In terms of ovarian cancer, *BRCA1* mutation confers an increased lifetime risk of 15-45% and *BRCA2* mutation an increased risk of 10-20%. Currently, women with cancer (breast, non-mucinous ovarian cancers) or family history of breast/ovarian cancers should be offered genetics counselling and testing (Ledermann et al., 2013).

Women with inherited *BRCA1/2* mutations are recommended to consider risk-reducing bilateral salphingo-oophorectomy (RRSO) as prospective multi-centre cohort

studies have shown that RRSO lowers risk of EOC, breast cancer and lowers breast cancer-/ovarian cancer-specific mortality (Gasparri et al., 2019). However, the residual risk of developing primary peritoneal cancer remains (4%) (Domchek et al., 2010; Finch et al., 2006). As STIC lesions have been demonstrated to contribute to the development of EOC, there are ongoing studies examining the prophylactic role of bilateral salpingectomy in low risk women (Sieh et al., 2013).

Since its discovery by Bob Bast and colleagues in 1981, Cancer Antigen-125 (CA-125), a glycoprotein expressed by cells undergoing Mullerian-type epithelium metaplasia, has been the most extensively used biomarker for detection and monitoring of EOC (Bast et al., 1981). CA-125 has limited specificity and sensitivity for OC. It may be elevated in benign conditions (e.g. pregnancy, endometriosis, pelvic inflammatory disease, ovarian cysts, liver cirrhosis, diabetes mellitus) and other cancers (e.g. endometrial, lung, breast and gastrointestinal cancer) (Bagan et al., 2008; Bast et al., 1998).

Normal values of CA-125 ranges from 0 to 35 U/ml (Nossov et al., 2008). Approximately 85-90% of patients with advanced EOC have an increased level of CA-125 but this is only observed in about 50% of cases in early stage disease (Gupta & Lis, 2010; Ledermann et al., 2013; Nossov et al., 2008). Nevertheless, CA-125 has been shown to predict progression free interval and overall survival (OS) in women receiving primary therapy for advanced ovarian cancer (Pignata et al., 2011). The UK Familial Ovarian Cancer Screening Study (UKFOCCS) acknowledged that each woman has a specific CA-125 baseline and significant increases above the baseline may identify cancers earlier than standard 6-12 monthly CA-125 > 35 U/ml. They have suggested using a risk of ovarian cancer algorithm (ROCA) followed up 3 monthly in high risk women who declined RRSO (Skates et al., 2017).

There is currently no role for screening in low risk women and evidence for screening high risk women has yet to be established. The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) trial set out to study the clinical utility of CA-125 as a screening tool for ovarian cancer. The study randomised 202,638 postmenopausal women to observation alone, annual multimodal screening (MMS) with serial CA-125 followed by transvaginal ultrasound scanning (TVS) for abnormal results or serial TVS alone. They concluded that screening by CA-125 does not offer significant reduction in mortality (15%) when compared to TVS alone (11%). However, longer term follow-up (7-14 years) with MMS could see a higher reduction in mortality (Jacobs et al., 2016).

1.2.5 Diagnosis

Women with symptoms suspicious of ovarian cancer should have CA-125 and pelvic TVS. Additional tumour markers such as carcinoembryonic antigen (CEA) and CA 19-9 levels are measured when the ovarian mass is suspected to be primary mucinous ovarian tumour or to arise from gastrointestinal tract (GIT). When CA-125/CEA ratio is ≤ 25 , this may suggest peritoneal carcinomatosis from GIT, hence gastroscopy and/or colposcopy should be considered. In women younger than 40 years old, tumour markers including alpha fetoprotein (AFP), hCG (human Chorionic Gonadotropin) and inhibin should be taken to identify non-EOC.

Referral to a specialist gynaecological oncology team can be aided by a risk malignancy index (RMI) score of ≥ 250 . The RMI score is calculated based on menopausal status, serum CA-125 levels and ultrasound evaluation.

$$\text{RMI} = \text{U} \times \text{M} \times \text{CA-125 levels}$$

Table 1.1 Calculation of RMI (Fotopoulou et al., 2017).

Ultrasound criteria (U score)		Menopausal status (M score)	
Multilocular cyst	1	Pre-menopausal	1
Solid areas	1	Post-menopausal	3
Bilateral lesions	1		
Ascites	1		
Intra-abdominal metastases	1		
Score 0-1: U = 1		CA-125 (U/ml)	
Score 2-5: U = 3		(the actual value is used)	

In women with presumed ovarian cancer, radiological staging should be performed next, including computed tomography (CT) scan of the chest, abdomen and pelvis to evaluate the extent of the disease and to aid pre-operative planning. Cytological examination should be performed in the presence of a pleural effusion in order to determine whether FIGO Stage IVA disease is present. Magnetic resonance imaging (MRI) and positron emission tomography (PET) scans are not routinely offered (Ledermann et al., 2013).

1.2.6 Staging and survival

The FIGO Committee on Gynecologic Oncology has revised the staging system for ovarian cancer in 2013 replacing the 1988 version. This was based on the concept

that HGSOC may originate from STIC lesions of the distal fallopian tube. The staging criteria for ovary, fallopian tube and peritoneum have been unified. FIGO stage I has now been redefined to encompass tumour confined to the ovaries, fallopian tubes or peritoneal washings. Stage II is confined to pelvic cavity and Stage III with tumour spread to peritoneum outside the pelvis and/or retroperitoneal lymph nodes. Stage IV is defined as distant metastases excluding peritoneum or parenchymal metastases within liver/ spleen or pleural effusion with positive cytology (Saida et al., 2016; Zeppernick & Meinhold-Heerlein, 2014).

Table 1.2 Comparison of the revised International Federation of Gynaecology and Obstetrics (FIGO) staging classification for cancer of the ovary, fallopian tube, and peritoneum (2013) and FIGO staging classification of the ovary (1988). (Saida et al., 2016)

FIGO (2013)	FIGO (Ovary, 1988)
I Tumour confined to ovaries or fallopian tube(s)	I Tumour limited to ovaries
IA Tumour limited to 1 ovary (capsule intact) or fallopian tube	IA Tumour limited to 1 ovary
IB Tumour limited to both ovaries or fallopian tubes	IB Tumour limited to both ovaries
IC Tumour limited to 1 or both ovaries or fallopian tubes , with any of the following	IC Tumour limited to 1 or both ovaries with any of the following: capsule ruptured, tumour on ovarian surface; malignant cells in ascites
IC1 Surgical spill	IC1/2 Malignant cells in peritoneal washings /ascites
IC2 Capsule rupture before surgery or tumour on ovarian or fallopian tube surface	IC(a/b) Capsule ruptured before surgery/surgical spill
IC3 Malignant cells in ascites	
II Tumour involves 1 or both ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer	II Tumour involves 1 or both ovaries with pelvic extension
IIA Extension and/or implants on uterus and/ or fallopian tubes and/or ovaries	IIA Extension and/or implants on uterus and/or tube(s)
IIB Extension to other pelvic intra-peritoneal tissues	IIB Extension to other pelvic tissues
III Tumour with spread to peritoneum outside the pelvis and/or metastasis to retroperitoneal lymph nodes	III Tumour with peritoneal metastases outside pelvis and/or regional lymph node metastasis
IIIA1 Positive retroperitoneal lymph nodes only	IIIA Microscopic peritoneal metastasis beyond pelvis

IIIA1(i) Metastasis $\leq$ 10 mm	
IIIA1(ii) Metastasis $\geq$ 10 mm	
IIIA2 Microscopic extrapelvic peritoneal involvement	
IIIB Macroscopic peritoneal metastasis beyond pelvis < 2 cm	IIIB Macroscopic peritoneal metastasis beyond pelvis < 2 cm
IIIC Macroscopic peritoneal metastasis beyond pelvis > 2 cm	IIIC Peritoneal metastasis beyond pelvis > 2 cm and/or regional lymph node metastasis
IV Distant metastasis excluding peritoneal metastases	IV Distant metastasis (excludes peritoneal metastasis)
IVA Pleural effusion with positive cytology	
IVB Parenchymal metastases and metastases to extra-abdominal organs	
Key changes indicated in bold	

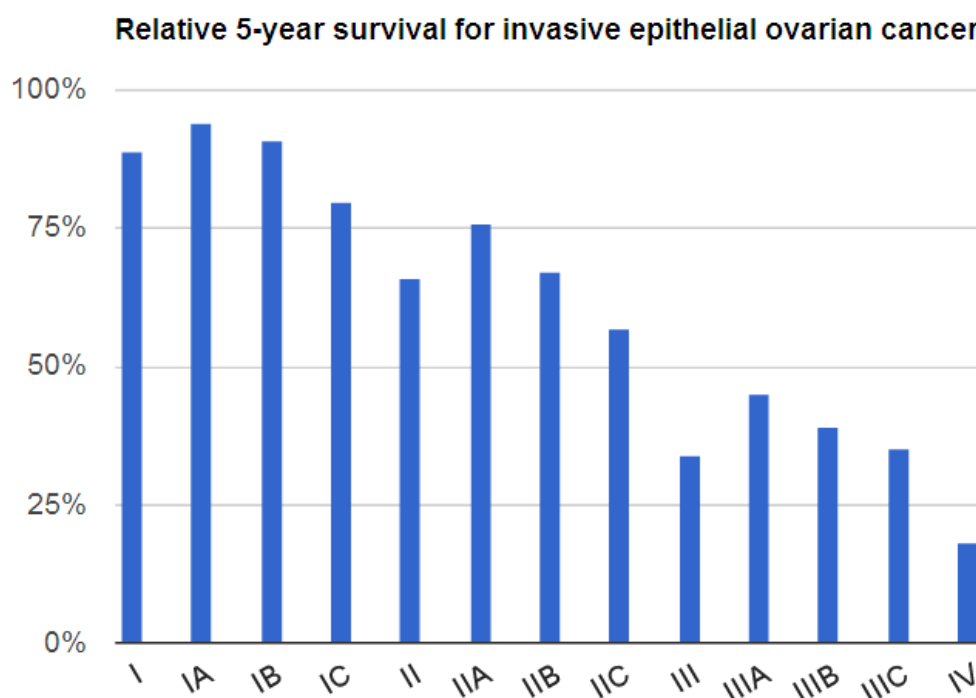


Figure 1.3 Relative five survival rate for invasive EOC. Figure from (Society, 2016).

Genetic mutations in HGSOC and molecular subtypes defined by gene expression have been found to influence survival and response to treatment. HGSOC patients with germline *BRCA1* or *BRCA2* mutations have superior survival to *BRCA1/2* wild-type cases. In the case of *BRCA1*, this advantage ceases by ten years but not for *BRCA2* (the latter patients have the best prognosis) (Alsop et al., 2012; Bolton et al., 2012; Candido-dos-Reis et al., 2015; Cunningham et al., 2014; McLaughlin et al., 2013). *BRCA1/2* are responsible for DNA double-strand break (DSB) repair by homologous recombination and cells deficient in *BRCA1* or *BRCA2* are more susceptible to damage by platinum chemotherapy and poly-ADP ribose polymerase (PARP) inhibitors. PARP is required for DNA single-strand break (SSB) repair. PARP inhibitor suppresses PARP's SSB repair function and results in unrepaired DNA,

hence DSB formation. Cells with *BRCA1* or *BRCA2* mutations fail to repair this DSB using homologous repair, instead using error-prone pathways such as non-homologous end joining which results in accumulation of further errors with consequent cell death (Sunada et al., 2018). More recently, it is now believed that the main mechanism of action of PARP inhibitors is through the trapping of PARP1 on the DNA. PARP1 specifically acts through PARylation which is the synthesis of ADP-ribose polymers (PAR) that covalently modify host proteins (Karlberg et al., 2013). In normal cells, PARP1 binds to damaged DNA and stimulates the synthesis of PAR that recruits host DNA repair proteins resulting in DNA damage repair and maintaining cell viability (Ogata et al., 1981; Satoh & Lindahl, 1992). In the presence of PARP inhibitors, PARP1 binds to DNA damage sites and remained trapped onto the chromatin, which in turn inhibits PARylation leading to replication fork collapse and DNA DSBs (Pommier et al., 2016). In cells with functional homologous recombination (HR), this stalled replication fork can be resolved through the HR pathway. However, in *BRCA* mutants with HR-defective cells, the replication fork stalling cannot be resolved resulting in replication fork collapses and DSB accumulation. This combined HRD and PARP inhibition inducing cytotoxicity specifically in tumour cells but not in non-cancer cells (the latter have functional HR) is known as synthetic lethality (Lord & Ashworth, 2017).

HGSOC patients with other mutations such as *CCNE1* amplification and *PTEN* amplification also have poor outcome with frequent resistance to primary treatment (Bell et al., 2011). In terms of molecular subtypes defined by gene expression, C1 mesenchymal subtype with strong stromal reaction has poorer survival outcomes. Meanwhile, it has become increasingly apparent that C2 immunoreactive and “Immune” subtypes have better survival outcomes which is linked to the accumulation

of intra-tumoural CD8⁺ T cells and lower proportion of inflammatory cells, immune suppressive T-regulatory cells or macrophages (Bell et al., 2011; Tothill et al., 2008; Lin Zhang, Jose R. Conejo-Garcia, et al., 2003).

1.3 High grade serous ovarian cancer (HGSOC)

1.3.1 Genomics and transcriptomics landscape of HGSOC

Recent genomics and transcriptomics studies of HGSOC have resulted in better understanding of the tumour's global biology, revealing different gene sequence and expression characteristics.

HGSOC is primarily driven by loss of p53 function and complex copy number alterations rather than recurrent oncogenic activation (Figures 1.4 and 1.5) (Ciriello et al., 2013a). *TP53* mutation is almost ubiquitous (96% of HGSOC) and this has been confirmed by the Cancer Genome Atlas (TCGA) study on the integrated genomic analyses of 489 HGSOC tumours (using DNA sequencing, methylation, copy number and gene expression analysis) (Bell et al., 2011). Approximately 50% of HGSOC cases display homologous recombination (HR) pathway defects (causing chromosomal instability and resulting in increased sensitivity to platinum chemotherapy and PARP inhibitors) (Bell et al., 2011; Hollis & Gourley, 2016; Tothill et al., 2008). Genomic changes in the HR genes include germline and somatic mutation of *BRCA1/2* (20%), *BRCA1* promoter hypermethylation (11%), amplification or mutation of *EMSY* (6%), hypermethylation of *RAD51C* (< 5%), mutation of *ATM* or *ATR* (3%) and Fanconi anaemia genes (5%) (Bell et al., 2011; Cheaib et al., 2015). Cyclin E amplification is found in 14% of HGSOC and is mutually exclusive with *BRCA1/2* mutation (Bell et al., 2011; Robert L. Hollis et al., 2022). Beyond HR defects,

whole genome sequencing has detected large scale structural variants in *RB1*, *PTEN* and *NF1*, which contrary to previous conception, these are not mutually exclusive and have been found to co-occur with each other and homologous recombination repair (HRR) gene perturbations (Garsed et al., 2022; Robert L. Hollis et al., 2022; Patch et al., 2015).

Other deregulated pathways include the PI3K/RAS, MAPK, FOXM1 and NOTCH signalling pathway which could provide opportunity for targeted treatments (The Cancer Genome Atlas Research, 2011).

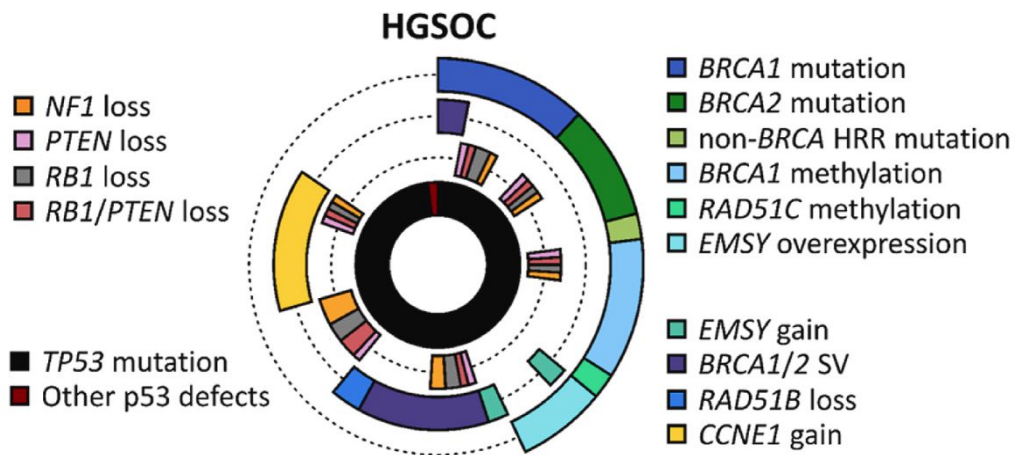


Figure 1.4 Genomic alteration profile of HGSOC. Figure from (Bell et al., 2011; Garsed et al., 2022; Hollis, 2023; Hollis et al., 2019; R. L. Hollis et al., 2022; Norquist et al., 2016; Patch et al., 2015).

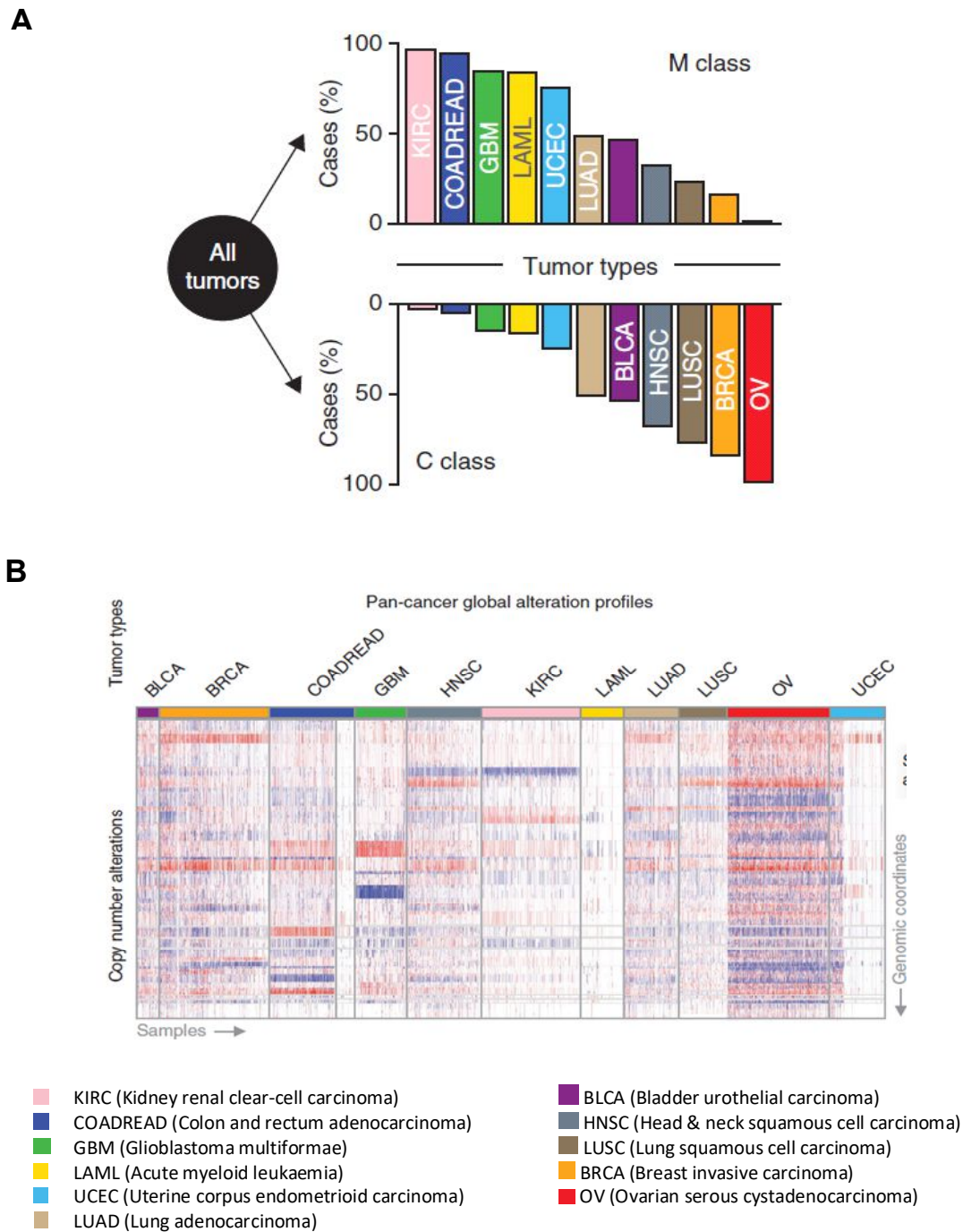


Figure 1.5 Pan Cancer Data. (A) Multiple tumour types classed into two classes – M (recurrent mutations) or C (recurrent copy number alterations). HGSOC (OV) is classified as C class. (B) Recurrent regions of copy number change (loses in blue, gains in red). Figure from (Ciriello et al., 2013b).

1.3.2 Molecular subgroups of HGSOC as defined by gene expression

Bulk gene expression profiling has identified different molecular subgroups of HGSOC in efforts to prognosticate these subtypes according to transcriptomic signatures. Landmark findings included the Tothill and TCGA studies which identified four HGSOC subgroups. Tothill et al. termed the subtypes as 1) C1, a poor prognosis subgroup displaying strong activated stromal response with extensive desmoplasia; 2) C2, an immunoreactive subtype characterised by increased immune cells infiltration and better survival outcomes concordant with other studies of favourable prognosis in high TILs ovarian tumours (Eiichi Sato et al., 2005; Lin Zhang, Jose R. Conejo-Garcia, et al., 2003); 3) C4, a low stromal expression subtype with high levels of circulating CA-125; and 4) C5, with increased expression of N/P-cadherins and reduced TILs (Tothill et al., 2008). TCGA described the subtypes as 1) mesenchymal (MES) with increased stromal markers; 2) immunoreactive (IMR) with increased chemokine expression related to immune response; 3) differentiated (DIF), a subtype with overexpression of OC tumour markers; and 4) proliferative (PRO) with increased proliferation marker expression, but did not report significant survival differences between the transcriptional subtypes (Bell et al., 2011). So far, patients are not clinically subclassified and treatment options are not stratified according to the subgroups. However, further maturation of high-throughput molecular analysis combined with clinical data could prove invaluable towards a more targeted treatment for patients.

A recent multi-omic study using matched genomic and transcriptomic characterisation of large HGSOC cohort has shed further light on the clinical significance of particular HGSOC molecular subgroups (R. L. Hollis et al., 2022). This work identified positive molecular prognostic factors for improved survival outcomes including *BRCA2m* and *EMSY* overexpression (with higher 1st and 2nd line chemotherapy response); C2/IMR

subtype (with higher TILs and *BRCA1/2*m in which immune-checkpoint and PARP inhibitors are more likely to be effective); and aberrant HRR genes (particularly with co-occurrence of retinoblastoma-1 (RB1) gene loss conferring further favourable survival in patients with HRR-aberrant genes). Meanwhile, negative factors for poorer survival outcomes included *CCNE1* gene amplification, and C5/PRO subtype (with low TILs and mainly *BRCA1/2* wild-type in which PARP inhibitors are less likely to be effective) (R. L. Hollis et al., 2022).

1.4 The complex tumour microenvironment (TME) of HGSOC

The TME of HGSOC plays an important role in disease progression, evolution and response to treatment. The TME is comprised of tumour cells and stroma consisting of blood vessels, lymphoid vessels, nerves, fibroblasts and extracellular matrix protein, adipocytes, mesenchymal stem cells (MSCs), endothelial cells, pericytes and immune cells (Ferraresi et al., 2020; Hanahan & Weinberg, 2000). These cells interact intricately and produce signals leading to treatment resistance and disease recurrence (Figure 1.6).

It is believed that in the early phase of carcinogenesis inflammatory mediators and immune cells interact to coordinate 'elimination' of early tumour cells. This is followed by 'equilibrium' whereby the host system and any surviving tumour cells co-exist. Finally, the tumour cells that survived the equilibrium phase proliferate within the immunosuppressive TME and switched to 'escape' phase (Dunn et al., 2004).

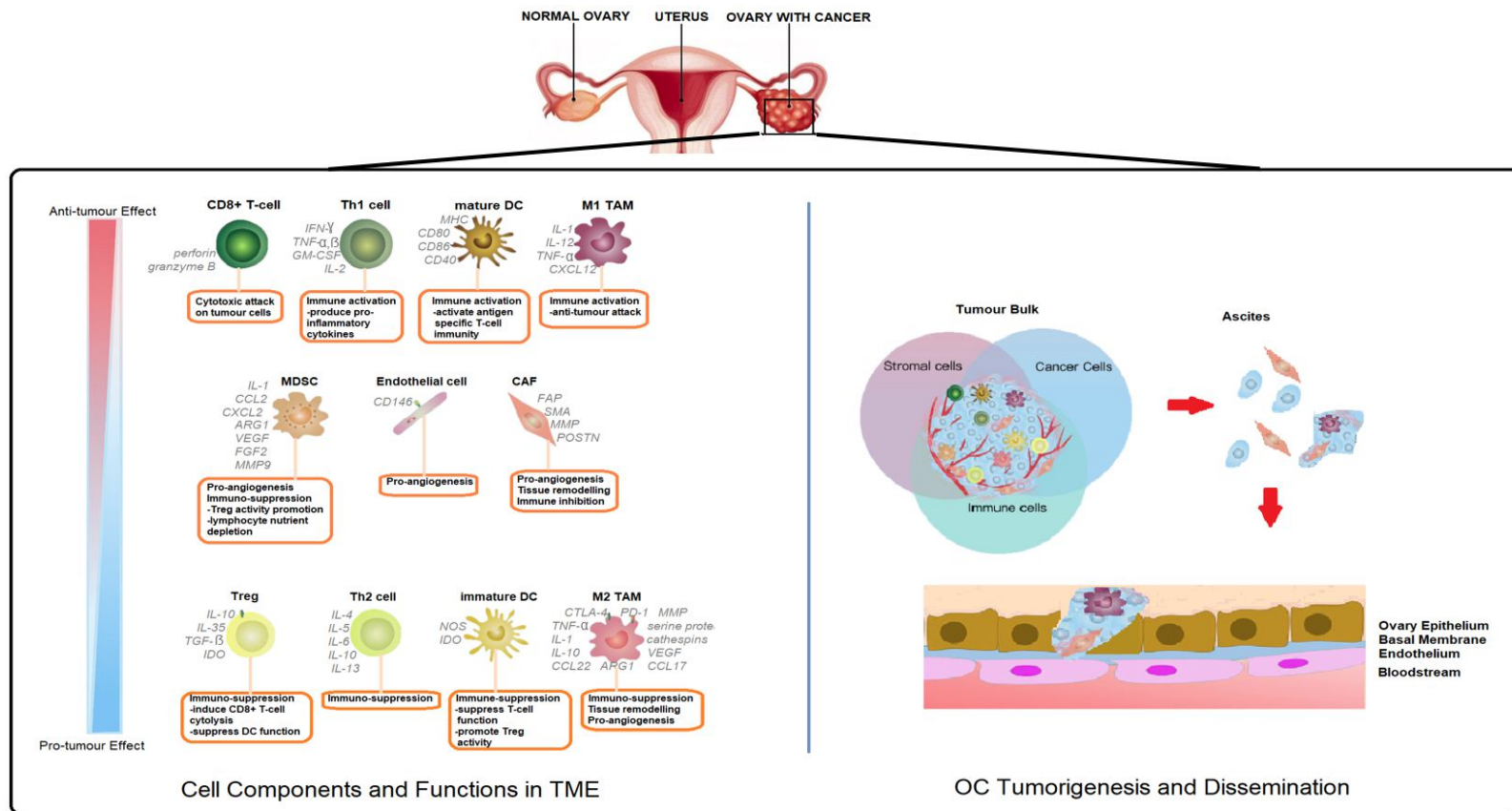


Figure 1.6 OC TME are comprised of cancer cells, stromal and immune cells. OC tumourigenesis and dissemination occur due to a dynamic crosstalk between these cells acting *via* the secretion of molecules (cytokines, chemokines) which bind to receptors on the cells (e.g. histo-compatibility complex class, MHC, programmed cell-death protein 1, PD-1). Different cell components produce anti- or pro-tumourigenic effects and may be re-polarised leading to regression or progression of the disease. Figure adapted from (Ferraresi et al., 2020; Yang et al., 2020)

1.4.1 The role of cancer-associated fibroblasts (CAFs) in OC

In 1889, the English surgeon Stephen Paget proposed the “seed and soil” hypothesis through post-mortem examination of 735 breast cancer patients and noted that the distribution of metastases was not due to chance but dependent on the crosstalk between tumour cells (the “seeds”) and the TME (“the soil”) (Paget, 1989). A host of stromal cells, particularly the CAFs play an important role within the TME by contributing to the growth of the primary tumour and metastatic dissemination through dynamic interaction with the tumour cells either directly or via paracrine signalling (Joyce & Pollard, 2009).

1.4.1.1 Classification of CAFs

CAFs are vastly heterogenous and can be classified according to their origins or cellular states (Desbois & Wang, 2021). Based on origins, subsets of CAFs include reprogrammed resident fibroblasts, mesenchymal stem cells from bone marrow or adipose tissue, and mesenchymal cells trans-differentiated from endothelial cells, epithelial cells, pericytes and adipocytes. Based on cellular states, CAFs exist in either a quiescent form which mainly represent normal fibroblasts or an activated state (also known as myofibroblasts). Quiescent CAFs can be activated by different pathways including JAK/STAT signalling pathway, the focal adhesion kinase (FAK) pathway, the Hedgehog (Hh) signalling pathway, and the platelet-derived growth factor signalling (PDGF) pathway (Zhang et al., 2022). In the activated form, these CAFs have enhanced proliferative and metabolic activity which are designed for ‘wound-healing’ in normal tissues but in the presence of tumour cells, they are recruited by the tumour cells to promote the cancer development (Di Carlo & Peduto, 2018). The common

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marker for quiescent CAFs is vimentin (VIM) (Desbois & Wang, 2021). For the activated CAFs, many markers have been utilised in their identification including α -smooth muscle actin (α -SMA) or also known as ACTA2, fibroblast activated protein (FAP), low caveolin1 (CAV1), CD10, G protein-coupled receptor 77 (GPR77), fibroblast specific protein 1 (FSP-1), periostin (POSTN), platelet-derived growth factor receptor α or β (PDGFR- α /PDGFR- β), podoplanin (PDPN), and integrin β 1 (CD29) (Kanzaki & Pietras, 2020; Mhaidly & Mechta-Grigoriou, 2020). The CAFs display varying levels but also overlapping expression profiles of these markers, suggesting that a heterogenous population of CAFs exists within the TME characterised by distinct phenotypes and functions (Zhang et al., 2022).

In ovarian cancer, the concomitant analysis of 6 CAF markers including FAP, SMA and CD29 has revealed 4 different subpopulations of CAFs (CAF-S1 to CAF-S4) (Givel et al., 2018). Functionally CAF-S2 (FAP⁻ CD29^{low} SMA⁻) and CAF-S3 (FAP⁻ CD29^{med} SMA⁻) resemble normal fibroblasts detected in healthy tissue, while CAF-S1 (FAP^{high} CD29^{med-high} SMA^{high}) and CAF-S4 (FAP⁻ SMA^{high} CD29^{high}) fibroblast cells are restricted to cancer and metastatic lymph nodes (Givel et al., 2018). The mesenchymal transcriptomic HGSOC subtype has been shown to have higher concentrations of CAF-S1 fibroblasts, which are characterised by ECM, adhesion, and wound-healing signatures and exhibit immuno-suppressive functions by enhancing the recruitment, differentiation, and activation of CD25⁺FOXP3⁺ Treg cells (Givel et al., 2018). CAF-S4 is defined by a perivascular contractile signature and promotes metastasis (Givel et al., 2018). Through bulk and single cell studies on CAFs, the existence of CAF-S1 (ECM-rich) and CAF-S4 (contractile) fibroblasts have been confirmed in other cancers such as pancreatic cancer (Dominguez et al., 2020; Elyada et al., 2019), colorectal

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cancer (H. Li et al., 2017), breast cancer (Bartoschek et al., 2018) and lung cancer (Lambrechts et al., 2018).

Single cell sequencing analysis of malignant ascites and ovarian cancer tumours has further confirmed the existence of distinct subsets of CAF-S1, referred to as inflammatory CAFs (iCAFs) and myofibroblasts CAFs (myCAFs) (Desbois & Wang, 2021; Hornburg et al., 2021; Izar et al., 2020). iCAFs characterised by immune-suppressive function by secreting inflammatory cytokines, such as IL-6, are located distal to tumour edges. Meanwhile, myCAFs are found adjacent to tumour cells and support tumourigenesis by producing dense matrices (Hornburg et al., 2021; Izar et al., 2020).

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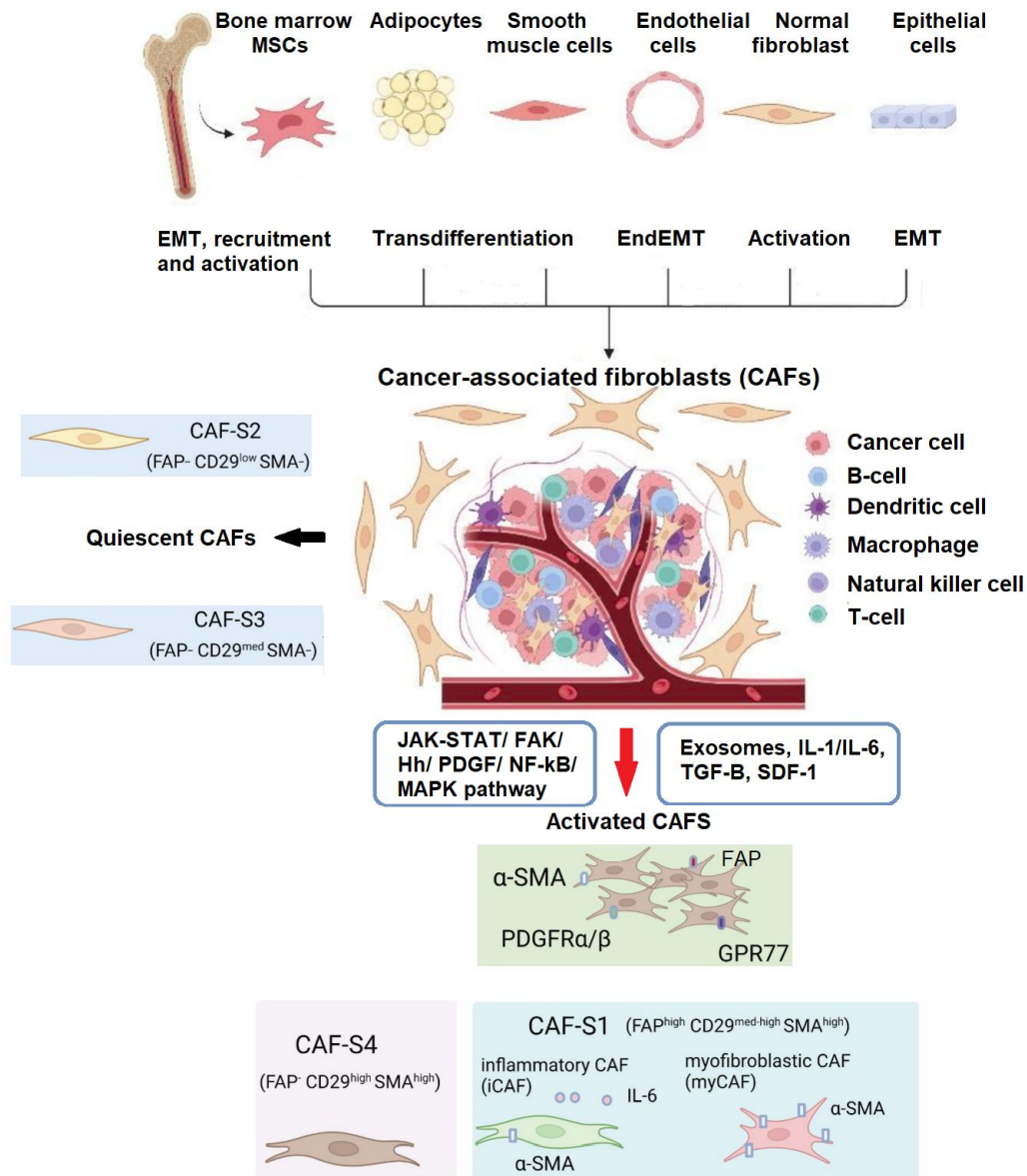


Figure 1.7 Ovarian cancer-associated fibroblasts. CAFs originate from several cellular sources such as normal fibroblasts, epithelial cells (through EMT), smooth muscle cells (through trans-differentiation), adipocytes (through trans-differentiation), endothelial cells (EndMT), and bone marrow MSCs (through EMT, recruitment, and activation). Resting CAFs can be transformed into activated CAFs via different pathways, exosomes and inflammatory cytokines. Four different subtypes of CAFs have been identified in OC: CAF-S2 and CAF-S3 resemble quiescent CAFs, while CAF-S1 and CAF-S4 are activated with 2 further distinct subsets identified within CAF-S1 (iCAF and myCAF). EMT, epithelial-to-mesenchymal transition; EndMT, endothelial-to-mesenchymal transition; MSCs, mesenchymal stem cells; iCAF, inflammatory CAF; myCAF, myofibroblasts CAF. Figure adapted from (Zhang *et al.*, 2022).

1.4.1.2 Specific mechanisms of CAFs in ovarian cancer progression

CAFs modulate OC progression through 3 main mechanisms: 1) secretion of cytokines *via* paracrine signalling hence directly stimulates tumour growth, proliferation, differentiation, invasion, and metastasis of surrounding tumour cells.; 2) regulation of immune response by exerting an overall immune-suppressive effect; and 3) establishing a metabolic crosstalk with the tumour cells and surrounding TME (Zhang et al., 2022).

1.4.1.2.1 Cytokines secretion by CAFs facilitate tumour growth, proliferation, and metastasis

Cytokines released by CAFs *via* paracrine modes play an important role in regulating the cancer-stromal interaction which helps to stimulate tumour initiation and immune evasion (Flavell et al., 2010). Myofibroblasts secrete TGF- β which induces further expression of pro-tumorigenic molecules by CAFs (such as CXCL12, IL-6, and VEGF-A); and TGF- β signalling-related genes derived from CAFs (including *POSTN*, *COL11A1*, *CTHRC1*, and *VCAN*). CAFs-derived HGF has been shown to promote OC proliferation in cell lines SK-OV-3 and HO-8910 by activating the c-MET/PI3K/Akt and GRP78 signalling pathways; and also reduce paclitaxel efficacy (Deying et al., 2017).

1.4.1.2.2 Immune-regulatory function of CAFs

It has been demonstrated that presence of tumour-infiltrating lymphocytes (TILs) are associated with improved clinical outcomes in OC (Desbois et al., 2020; Goode et al., 2017; J. Li et al., 2017; L. Zhang et al., 2003). Translational analysis of tumours from

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the ICON7 phase III clinical trial combined transcriptomic and digital pathology analysis and in doing so defined 3 tumour-immune phenotypes with varying levels of TILs: 1) immune-inflamed/hot tumours where TILs are present in both tumour epithelium, and tumour stroma; 2) immune-excluded tumours where TILs are present in the tumour stroma but excluded from the tumour epithelium; and 3) immune-desert/cold tumours with low or absent TILs both in the tumour epithelium, and stroma (Desbois et al., 2020).

CAFs play a host of important roles within the tumour-immune microenvironment. In the immune-inflamed tumours, studies have observed an enrichment for iCAF (a subset of CAF-S1) with increased expression of IL-6, CXCL12, CXCL1, CXCL2, CCL2, CCL5, or LIF which could exert anti-tumorigenic functions by recruiting cytotoxic immune cells or pro-tumorigenic functions via the recruitment of immune-suppressive cells such as Tregs or myeloid-derived suppressive cells (MDSCs) (Desbois & Wang, 2021; Hornburg et al., 2021). In the immune-excluded ovarian tumours, high expression of TGF- β and fibroblast TGF- β response signature (F-TBRS), upregulation of ECM-related genes, and immune-suppressive cytokines show that CAFs promote T cell exclusion (Desbois et al., 2020). CAFs also promote dysfunctional vascularisation through pro-angiogenic factors VEGF and PDGF which reflect the high expression of angiogenic signatures within immune-excluded tumours (Ferrara, 2010). In immune-desert OC, the MYC and WNT/ β -catenin signalling pathways are activated resulting in immune exclusion. CAFs express PS1 which suppresses CD8⁺ T cells and dendritic cells infiltration *via* the WNT/ β -catenin pathway (Zhang et al., 2020).

1.4.1.2.3 Tumour cells metabolic regulation by CAFs

CAFs regulate the metabolic processes within OC through the secretion of cytokines, metabolites, TGF- β and exosomes (Coller, 2019). CAFs contribute to tumourigenesis by providing energy through tricarboxylic acid cycle (TCA) (Desbois & Wang, 2021).

Table 1.3 Mechanisms of CAFs in promoting OC progression.

Mechanisms	Mediators	Actions	References
Cytokines secretion to facilitate tumour growth, proliferation and metastasis	CTHRC1	Activates EGFR, ERK1/2, AKT signalling pathway.	(Mei et al., 2020)
	COL11A1	Activates ERK pathway, TGF- β 1-MMP3 axis.	(Y.-H. Wu et al., 2021; Wu et al., 2014)
	VCAN	Activates NF- κ B pathway, upregulates expression of CD44, MMP-9, and the hyaluronan-mediated motility receptor.	(Yeung et al., 2013)
	HGF	Activates c-MET/PI3K/Akt and GRP78 signalling pathways.	(Deying et al., 2017)
	FGF-1	Activates FGF-1/4 signalling, MAPK/ERK pathway, upregulates EMT-related genes.	(Sun et al., 2017)
Immune suppression	TGF- β	Inhibits effector immune cells.	(Flavell et al., 2010)
	PS-1	Regulates innate and adaptive immune cells <i>via</i> the WNT/ β -catenin pathway.	(Zhang et al., 2020)

	CXCL12	CXCR4 dependent pathway, prevents effector T cells from making contact with cancer cells, and enhances recruitment of Tregs.	(Feig et al., 2013)
	IL-33	Modifies the immune microenvironment, and drives type 2 immunity.	(Shani et al., 2020)
	IL-6	Activates STAT3 pathway to protect cancer cells from immune attack.	(Bretz et al., 2013)
Tumour cells metabolic regulation	p38αMAPK	Promotes glycolysis to supply tumour cells with energy for growth.	(Curtis et al., 2019)
	CXCL14	Promotes glycolysis and tumour cell growth.	(Zhao et al., 2017)
	ROS	Induces autophagy, mitosis, and glycolysis <i>via</i> NF-κB activation, with upregulation of MCT4 and loss of CAV1 expression.	(Capparelli et al., 2012)

AKT, protein kinase; CAV1, caveolin-1; CTHRC1, collagen triple helix repeat containing-1; COL11A1, collagen type XI alpha 1 chain; CXCL12, C-X-C chemokine ligand 12; CXCL14, C-X-C chemokine ligand 14; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; EMT, epithelial–mesenchymal transition; FGF-1, fibroblast growth factor-1; GRP78, glucose-regulating protein 78; HGF, hepatocyte growth factor; IL-6, interleukin-6; IL-33, interleukin-33; MAPK, mitogen-activated protein kinases; MMP3, matrix metalloproteinase-3; MCT4, monocarboxylate transporters 4; PI3K, phosphatidylinositol 3-kinase; PS-1, presenilin-1; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-beta; VCAN, versican. Table adapted from (Zhang et al., 2022).

1.4.2 The role of immune cells in OC

Tumour-reactive TILs (tumour-infiltrating lymphocytes) found in HGSOC have been associated with increased IFN- γ , IL-2 and lymphocyte-attracting chemokines which all confer better overall survival (OS) and progression free survival (PFS) (Lin Zhang, Jose R Conejo-Garcia, et al., 2003). TILs are common in *BRCA1* mutant tumours but less so in *BRCA2* mutant tumours. There are immune-suppressive factors which often suppress TILs such as CD4⁺CD25⁺FOXP3⁺ Treg cells, myeloid cells, M2 tumour associated macrophages (TAMs), myeloid derived suppressor cells; immune-suppressive cytokines and immune checkpoint molecules (Bowtell et al., 2015). TILs have been found to express PD-1 and its activating ligand PD-L1 is found on tumour cells, B cells, macrophages, dendritic cells and T cells themselves. PD-L1 on tumour cells binds to PD-1 on effector T cells and this inactivates the lytic activity of the T cells. Immunotherapy using nivolumab (a fully human immunoglobulin G4 anti-PD-1 receptor-blocking monoclonal antibody) binds to PD-1 and inhibits the engagement with PD-L1 allowing CD8⁺ T cells to work. This was examined in a phase II trial by Hamanishi *et al.* where 3 out of 20 patients (15%) with platinum-resistant HGSOC treated with nivolumab showed complete response, while 45% of their patients had stable disease (Hamanishi et al., 2015). A number of clinical trials of immune checkpoint inhibitors have subsequently been performed in HGSOC (Konstantinopoulos et al., 2019; Lampert et al., 2020; Moore & Pignata, 2019).

The results have been generally disappointing probably attributed to a relatively low mutational burden in comparison to other tumours, lack of (neo-)antigens presentation and a TME with high immunosuppressive factors including Treg cells and tumour-associated macrophages (Bronger, 2021). The little success thus far does not mean that immunotherapy does not have a role in HGSOC, just that the most suitable

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strategy has not yet been identified. A better understanding of the immunosuppressive factors affecting TIL density in HGSOC and a well-defined HGSOC 'immunoscore' may help in predicting the response of HGSOC towards immunotherapy. The development of a HGSOC immunoscore will require extensive multi-site tumour sampling linked with multiplex immunohistochemistry to define important immune prognostic features such as immune cell type, functions, location in the tumour and density (Bowtell et al., 2015).

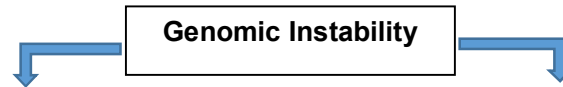
Table 1.4 Summary of Immuno-effector Cells and Molecules.

Immuno-reactive	Immuno-suppressive
TILs (CD8 ⁺ , CD4 ⁺ , CD20 ⁺) IFN- γ , IL-2, lymphocyte-attracting chemokines	Treg, myeloid cells, macrophages (M2), platelet, myeloid derived suppressor cells Immuno-suppressive cytokines (IL-6/IL-8/IL-10) CCR1 and CXCR2 ligands (neutrophils), CCL2, CCL5, CXCL12, CXCL1, MIF (macrophages) Immune checkpoint molecules (PD-L1/2) CAF (secrete pro-tumourigenic factors PDGF, IGF, TGF- β , VEGF)

(Bowtell et al., 2015)

1.5 The tumour heterogeneity of HGSOC

Large-scale genomic analyses using next-generation sequencing technology over the last five years have demonstrated that spatial and temporal tumour heterogeneity exists within HGSOC which could account for the high degree of chemoresistance (Bashashati et al., 2013; De Mattos-Arruda et al., 2014; J.-Y. Lee et al., 2015). It is apparent that HGSOC has a high degree of genomic instability caused mainly by DNA repair defects and during the course of the disease further DNA alterations may lead to the generation of genetically distinct subclones within the same tumour. A tumour with high degree of genomic instability could result in greater chemoresistance due to subclones acquiring the ability to survive and expand under the selective pressures of chemotherapy. This has been observed in cases where reversion of germline *BRCA1/2* mutation have been detected in metastatic sites at relapse (Patch et al., 2015). Conversely, a high degree of genomic instability could also pose a selective disadvantage towards clonal expansion of chemoresistant cells through deleterious genomic alterations (Aleksander Salomon-Perzyński et al., 2017). This is consistent with clinical observations where highly unstable HR-deficient HGSOC responds better to platinum chemotherapy compared to HR-intact cases (Pennington et al., 2014).



Clonal Evolution (CE) of HGSOC	Clonal Evolution (CE) of HGSOC
Pre-treatment	During Treatment
• HGSOC is composed of multiple distinct subclones evolving from single ancestral clone. (Bashashati et al., 2013; Hoogstraat et al., 2014) • CE is caused by an interplay between dominant clonal mutation (<i>TP53</i>) which is acquired early and subclonal mutations (e.g. <i>PIK3CA</i>, <i>CTNNB1</i>, <i>PDGFR</i>, <i>NF1</i>) acquired along tumorigenesis. (Bashashati et al., 2013) • In untreated HGSOC, clonal expansion is driven by a combination of genetic, epigenetic, transcriptomic, proteomic factors along with selective pressures imposed by the highly heterogeneous tumour microenvironment (TME). (Burrell & Swanton, 2014) • The dominant and minor subclones interact within the complex “ecosystem” supported by the TME resulting in cancer growth. This has been reflected in recent studies using 3D model of HGSOC cell lines. (Myungjin Lee et al., 2013; Zietarska et al., 2007) • ITH is caused by a combination of CE, tissue hierarchies, rare cancer cells and dynamic cell states. (Rantalainen, 2017)	• Comparing paired samples pre- and post-chemotherapy using WES (whole exome sequencing) and SNP (single nucleotide polymorphism) profiling has shown substantial heterogeneity between primary and recurrent tumour. (Lambrechts et al., 2016) Some subclonal mutations may be increased or decreased between primary and relapsed tumours. • Clonal architecture of recurrent tumours are more homogenous compared to matched primary tumours suggesting that during treatment most of the somatic mutations were eliminated by chemotherapy. (Beltrame et al., 2015) • CE continues to occur during the course of treatment and clonal expansion varies widely between patients confirming that HGSOC exhibits patient-specific intratumoural heterogeneity (ITH). (Bashashati et al., 2013; Hoogstraat et al., 2014) • Patients with greater clonal expansion (i.e. highly heterogeneous polygenomic tumours) have shorter PFS and OS. • Studies consistently shown that recurrent tumours are caused by inherently chemo-resistant subclones pre-existing at diagnosis which expand under the selective pressures of chemotherapy. (Beltrame et al., 2015; Lambrechts et al., 2016; Mota et al., 2015; Schwarz et al., 2015) • Variations in local TME could cause further phenotypic changes in cancer cells (i.e. undergo <i>de novo</i> mutation) resulting in CE.

Figure 1.8 Summary of Genomic Instability in HGSOC resulting in Tumour Heterogeneity (A. Salomon-Perzyński et al., 2017).

1.6 HGSOC treatment and chemoresistance

1.6.1 First line treatment

Generally, the standard treatment involves cytoreductive debulking surgery and first line platinum-containing chemotherapy (Ledermann et al., 2013). Patients with advanced HGSOC should be managed in a tertiary centre with the aim of complete cytoreduction and zero macroscopic residual disease by intestinal resection, peritoneal stripping, diaphragmatic resection, removal of bulky para-aortic lymph nodes and splenectomy (Vergote et al., 2010). This increases OS and PFS. Du Bois et al in a meta-analysis of 3120 patients have demonstrated that residual disease is a stronger determinant of prognosis than FIGO stage; where completely debulked stage IIIC HGSOC performed better than suboptimally debulked stage IIB-IIIB disease (du Bois et al., 2009). Recent evidence has suggested that 3 cycles of platinum-based neoadjuvant chemotherapy (NACT) followed by interval debulking surgery (IDS) is not inferior to primary debulking surgery (PDS) followed by adjuvant chemotherapy (ACT) (Cornelis et al., 2012; Fagö-Olsen et al., 2014; Kehoe et al., 2013; Vergote et al., 2010). This is a particularly useful strategy in patients with poor performance status at presentation, low albumin and extensive tumour dissemination. By giving upfront chemotherapy, the surgical morbidity could be reduced and increases the chance of optimally debulking the tumour (Vergote et al., 2010). However, some of the problems associated with NACT-IDS relate to masking the peri-operative visual assessment of

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tumour dissemination and possible platinum resistance (Chi et al., 2012; Hynninen et al., 2013; Matsuo et al., 2010).

The standard chemotherapy regime is comprised of paclitaxel 175 mg/m² and carboplatin AUC 5-6, both given intravenously every 3 weeks (Ozols et al., 2003). Despite a good initial response in about 80% of patients, the rate of disease relapse is high with 25% of patients developing platinum resistance within 6 months and eventually most will become chemo-resistant (Colombo, 2013). The ICON7 trial has shown that addition of anti-angiogenic therapy (bevacizumab) to conventional first line platinum chemotherapy in patients with advanced HGSOc increases PFS but not OS (Perren et al., 2011). Subsequent results from the final analysis of mature OS data from ICON7 did not show benefit in the whole population but improved OS in patients at high risk of disease progression (Stage IV or suboptimal debulking) (Oza et al., 2015).

1.6.2 Treatment for recurrent HGSOc

The management for recurrent cases encompasses secondary cytoreductive surgery (SCS) and/or systemic chemotherapy. The Descriptive Evaluation of preoperative Selection KriTeria for OPerability in recurrent OVARian cancer (DESKTOP OVAR) trials have been instrumental in guiding decisions for SCS. The DESKTOP III trial enrolled 407 patients with first relapse after 6+ months platinum-free interval (TFIp) and positive AGO score (a predictive score to assess the feasibility of optimal SCS), and randomised the patients to either a combination of SCS and chemotherapy or chemotherapy alone. Recent mature data from this trial demonstrated a significant

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increase in OS, PFS and time to first subsequent therapy (TFST) in these patients, with greatest benefit exclusively seen in completely resected cases (Bois *et al.*, 2020).

Patients should be selected for SCS if they have a high chance of complete resection guided by the following predictive factors: TFIp of > 6 months, positive AGO score (good performance status, complete resection at primary surgery and absence of large amount of ascites), feasibility of complete resection on imaging, absence of contraindications to surgery (e.g. comorbidities, previous severe complications of surgery), solitary rather than multiple site recurrence and absence of carcinomatosis (Bommert *et al.*, 2018; Chi *et al.*, 2006; Harter *et al.*, 2011). However, it should be noted that these are positive predictors and AGO score has a low negative predictive value (Janco, Kumar, Weaver, McGree, & Cliby, 2016). Hence, these predictors should not be used to exclude patients from surgery (Colombo *et al.*, 2019).

Medical treatment is one of the key therapies in relapsed HGSOc. Consensus agreed that platinum response should be therapy-oriented (Colombo *et al.*, 2019). Platinum resistant patients who progressed during therapy or have early symptomatic relapse soon after completing the last platinum-based chemotherapy (PBC) should be treated with sequential non-platinum therapy such as pegylated liposomal doxorubicin (PLD), weekly paclitaxel or topotecan with addition of bevacizumab if indicated. These therapeutic agents have been shown to improve PFS, reduce ascites and generally improve symptoms (Poveda *et al.*, 2015; Pujade-Lauraine *et al.*, 2014). Platinum sensitive patients should be re-challenged with platinum-based combination chemotherapy. The addition of bevacizumab results in a progression free survival advantage as evidenced by GOG-0213 and OCEANS trials (Aghajanian *et al.*, 2015; Coleman *et al.*, 2017) but currently in the UK, this has not been approved by NICE

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(National Institute for Health and Care Excellence) or SMC (Scottish Medicines Consortium).

1.6.3 Targeted therapies

Potential therapeutic targets (or vulnerabilities) for HGSOC include homologous recombination deficiency (HRD), angiogenesis, tumour-intrinsic signalling pathways, immune signalling and hormone receptors (Radu et al., 2021). The current two main licensed targeted therapies in HGSOC are PARP inhibitors and the anti-angiogenic agent bevacizumab based on recommendations from several major clinical trials including GOG0218, PRIMA, SOLO1, ICON7 and PAOLA-1, amongst others (Burger et al., 2011; González-Martín et al., 2019; Moore et al., 2018; Perren et al., 2011; Ray-Coquard et al., 2019).

PARP inhibitor (PARPi) blocks the repair of DNA damage specifically through the trapping of PARP1 onto the DNA which arrests the growth of the OC that have pathogenic germline *BRCA1/2* mutations (Pommier et al., 2016; Radu et al., 2021) (see section 1.2.6). PARP protein plays essential roles in cellular processes by regulating transcription, apoptosis and DNA damage response. PARP1 is activated by DNA damage and promotes the repair of DNA single-strand breaks by base excision repair and recruiting other repair proteins (Rose et al., 2020). In presence of PARP inhibitor, PARP1 is trapped onto the chromatin causing replication fork collapse and DNA double strand breaks (DSBs) (Pommier et al., 2016). *BRCA* mutant and HRD cells are unable to complete homologous recombination mediated repair. PARP inhibition of these cells result in accumulation of DSBs resulting in genomic instability and eventual cell death (Saito & Miki, 2011). Current guidelines from the ASCO

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(American Society of Clinical Oncology) recommends maintenance PARPi for newly diagnosed advanced EOC with complete or partial response to first line platinum-based chemotherapy (in which the highest benefit will be for *BRCA* mutant patients and may be offered for non-*BRCA* mutant or HRD negative) (Tew et al., 2022). In the United States, for recurrent OC, PARPi may be offered to *BRCA* mutant patients but the license for PARPi in *BRCA* wild type patients has been withdrawn (not the case in Europe) (Tew et al., 2022).

Angiogenesis (formation of new blood vessels) plays an important role in cancer progression and involves a complex process mediated by cytokines and molecular factors. Notably, vascular endothelial growth factor (VEGF) is a major driver of angiogenesis through signalling pathways involving intracellular tyrosine kinases (Wada et al., 2005). To date, the most frequently tested anti-angiogenic agent is bevacizumab (a humanised monoclonal antibody inhibiting VEGF-A from binding to its receptors, VEGF1/2) which is licensed for use as first line treatment for advanced OC in addition to platinum-based chemotherapy and also recurrent OC: evident from 3 key studies (OCEANS, GOG0128, AURELIA) (Aghajanian et al., 2012; Burger et al., 2011; Pujade-Lauraine et al., 2014).

Three anti-angiogenic small molecule tyrosine kinase inhibitors have been investigated in randomised trials demonstrating benefits for increased PFS: intedanib, pazopanib and cediranib (Aghajanian et al., 2012; Burger et al., 2011; Pujade-Lauraine et al., 2014).

Other targeted therapies that are being investigated in pre-clinical or early phase trials with emerging potential benefits include folate receptor inhibitors (Naumann et al., 2013), IGFR inhibitors (Lim & Ledger, 2016), Wee1-like protein kinase (WEE1)

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inhibitor (Zhang et al., 2017), and immunotherapies (such as immune-checkpoint inhibitors against programmed death 1, PD-1 and cytotoxic T lymphocyte-associated protein 4, CTLA-4; and antibody drug conjugates targeting antigens overexpressed on cancer cells as a way of delivering a cytotoxic payload) (Hamanishi et al., 2007; Hodi et al., 2003; Stewart & Cristea, 2019).

With better understanding of the complex genomic of HGSOC and evolution of tumour subclones, it is hoped that treatment could be better targeted and stratified, resulting in improved survival of patients.

1.7 Single cell sequencing (SCS)

Gene expression so far has mainly focused on the analysis of bulk tumour samples. This may overlook subclones with lower prevalence. Single cell sequencing (SCS) which uses more precise strategies presents a novel alternative to bulk sequencing. SCS refers to single cell genome or transcriptome sequencing in order to unravel the genomic, transcriptomic or other multi-omics information at single cellular level which could detect cell population differences and evolutionary relationships (Tang et al., 2019). In comparison to bulk sequencing, SCS can help to reveal low prevalent subclones (e.g. with chemoresistant signatures or cancer stem cell-like phenotypes) and provide a full insight into the temporal and spatial subclonal composition of human cancer.

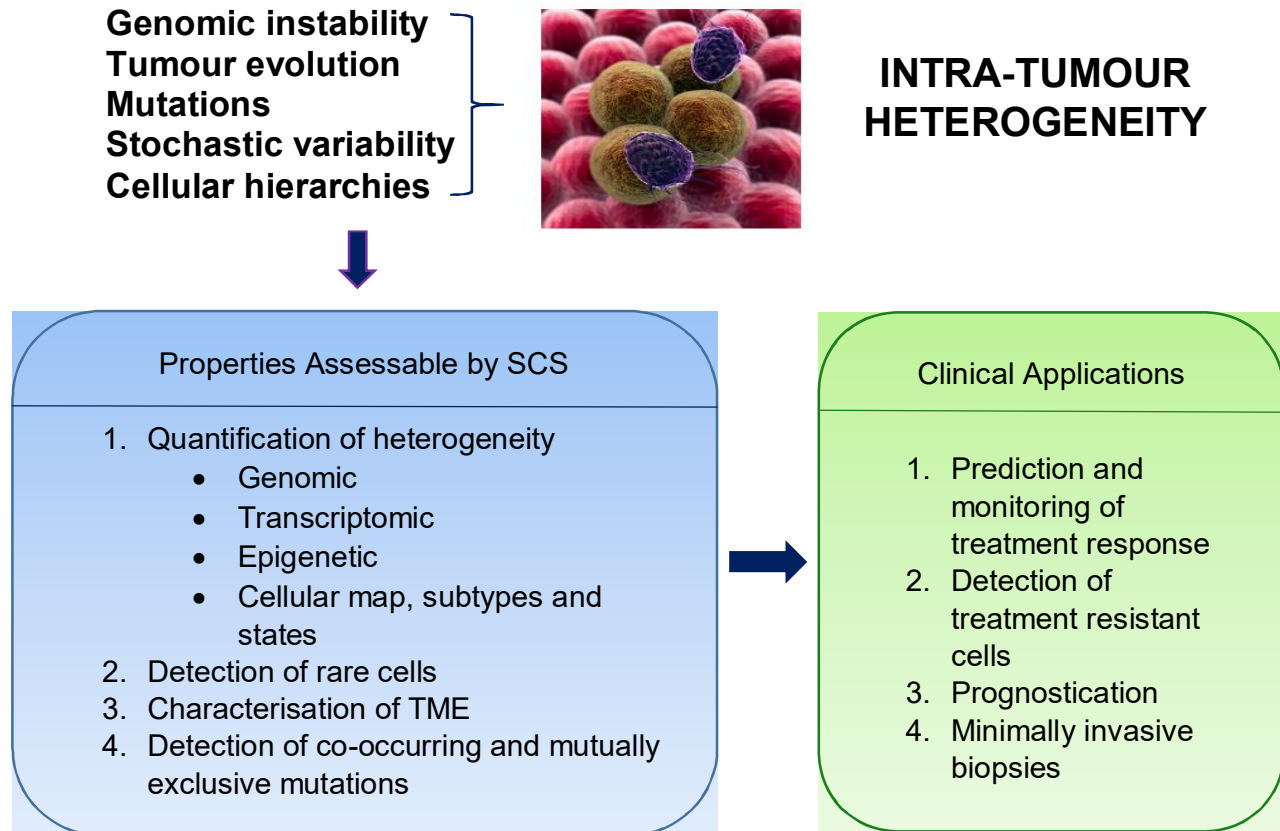


Figure 1.9 The role of single cell molecular phenotyping in characterisation of tumour heterogeneity and in clinical applications. Multiple molecular mechanisms and environmental factors lead to intra-tumour heterogeneity. Single cell molecular phenotyping enables characterization of many aspects of intra-tumour heterogeneity and has the potential to be applied to generate clinically relevant information. Figure adapted from (Rantalainen, 2017).

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Generally, SCS involves the amplification of genomic DNA or complementary DNA (for RNA-seq) prior to generation of sequencing libraries. Thus far, single cell DNA-sequencing (scDNA-seq) has been proven to be more challenging than single cell RNA-sequencing (scRNA-seq) due to very minute biological material available (each cell only has two copies of DNA compared to multiple RNA molecules). scRNA-seq has more established techniques and studies a larger number of cells compared to that of scDNA-seq. (Rantalainen, 2017) Based on the aforementioned reasons, this study has opted to use scRNA-seq to study the intra-tumoural heterogeneity of HGSOC.

1.7.1 Single cell RNA-sequencing (scRNA-seq) in OC

Single cell transcriptomics studies have been utilised in different cancers and early notable research arose in the fields of brain cancer (particularly glioblastoma), breast cancer and melanoma. (Aceto et al., 2014; Patel et al., 2014; Ramsköld et al., 2012) It is gaining popularity in OC and the main applications are as follows:

1.7.1.1 Characterisation of tumour heterogeneity through cell mapping, detection of resistant clones and study of tumour-immune microenvironment

A major cause of treatment failure and resistance resulting in poor prognosis in OC is attributable to the vast intra- and inter-tumour heterogeneity of the disease. This heterogeneity varies between different patients which adds further complexity to generalising treatment as different stages, genetic lesions or gene expression profiles are associated with varying survival outcomes and treatment response (Talukdar et al., 2021). Recent emerging evidence from scRNA-seq studies suggests that

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traditional bulk molecular subgrouping of HGSOC may mask the subpopulations of cells within the cancer TME responsible for the disease development, progression and recurrence. (Geistlinger et al., 2020; Izar et al., 2020)

scRNA-seq is a powerful tool that can be utilised to investigate the evolution of therapy resistance in OC. It remains challenging to elucidate the actual mechanism of chemoresistance, whereby there is an interplay of adaptive (with pre-existing resistant clones evolving during treatment) and acquired resistance (therapeutic agents giving rise to new mutations resulting in iatrogenic resistance). Almendro *et al.* demonstrated that epithelial tumour cells may convert to mesenchymal phenotype during chemotherapy suggesting that EMT and cell plasticity play a role in chemoresistance. (Almendro et al., 2014)

The study of the tumour-immune microenvironment (TIM) may be helpful in establishing how to overcome resistance to currently available immunotherapeutic strategies. (Desbois et al., 2020; Hamanishi et al., 2015; Konstantinopoulos et al., 2019; Lampert et al., 2020; Moore & Pignata, 2019) scRNA-seq will allow the fine dissection of the TIM in OC and shed further light into the anti-tumour activity of the immune cells including TILs (which has been shown to confer favourable outcomes, notably CD3⁺ and CD8⁺ T cells) (Gregory M. Chen et al., 2018; Gourley et al., 2014a; R. L. Hollis et al., 2022); and pro-tumourigenic immune cells such as tumour-associated macrophages (TAMs), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumour-associated dendritic cells (tDCs) (which lead to immune evasion and tumour progression) (Cai & Jin, 2017).

1.7.1.2 Detection of OC stem cells/ stem-like cells

scRNA-seq is instrumental in the detection of cancer stem cells (CSCs) which play a significant role in driving the progression and relapse of OC. CSCs are a population of cells that have undergone genetic mutations allowing them to gain stem-like properties, where they are able to reproduce the original phenotype of the tumour and capable of self-renewal, both features crucial for tumour proliferation, differentiation, recurrence, metastasis and chemo-resistance (Al-Alem et al., 2019; Nakayama et al., 2006; Wilczyński et al., 2022). The concept of CSCs were first raised in 1858, when Virchow proposed that cancers might originate from immature cells and later in 1875, Cohnheim described embryonic-like cells which might be activated later in adult life and developed into cancer (Cohnheim & Beneke, 1914; Sell, 2004; Virchow, 1902). CSCs may be present in two different subpopulations: proliferative (chemo-resistant but could be killed by high doses of drugs) and quiescent (capable of surviving even high doses of chemotherapeutic drugs, hence promoting tumour relapse) (Batlle & Clevers, 2017; Lee et al., 2019; Wang, 2019). CSCs are commonly quiescent and have a slow cycling rate, hence posing a major challenge in targeting them through standard chemotherapy (Chen, Dong, Haiech, Kilhoffer & Zeniou, 2016; Nassar & Blanpain, 2016). The origin of CSCs may be through differentiation of progenitor, normal stem cells or normal cancer cells which acquire stemness *via* EMT process (Mani et al., 2008; Marcucci et al., 2019). Recently, CSCs have been found to exhibit outstanding plasticity, capable of switching between stem and non-stem like states regulated by stressors (hypoxia, pH, drugs, immunological response), stress-triggered epigenetic changes (i.e. histone and non-coding RNA modifications) and activation of “stemness” signalling pathways (i.e. wingless-related integration site - Wnt,

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Hedgehog, neurogenic locus notch homolog protein – NOTCH) (Berabez et al., 2019; Dumont et al., 2008; Marcucci et al., 2014; Takebe et al., 2015).

Additionally, CSCs constitute a very small proportion of the tumour cells with lack of reliable markers for identification, making scRNA-seq the preferred technology for examining such rare cell populations. Winterhoff and colleagues detected CSCs (comprising of 1-2% of all the OC cells analysed in their study) which were mainly within the stromal compartment (Winterhoff et al., 2017). Numerous markers have been described to identify ovarian CSCs including glycoprotein CD44, receptor tyrosine kinase CD117, surface protein CD133, heat stable antigen CD24, MyD88 protein, EpCAM, Aldehyde Dehydrogenases ALDH, multi-positive cell surface markers (CD44⁺/CD133⁺/ALDH1A1⁺, CD44⁺/CD24⁺/EpCAM⁺, CD44⁺/MyD88⁺, CD44⁺/CD117⁺), MYC, SOX2, ROR1 and ABCB1, amongst others (Chen et al., 2021; Liu et al., 2013; Motohara et al., 2011; Steffensen et al., 2011; Takebe et al., 2015; Wilczyński et al., 2022).

1.7.1.3 Study of circulating tumour cells (CTCs)

CTCs are involved in cancer metastasis through shedding of cancer cells into the bloodstream and metastatic seeding of CTCs at distant sites (Fidler, 2003). In OC, the prognostic impact of CTCs have been confirmed in several studies which helped in the identification of potential blood-based biomarkers for specific biological therapies (Abu-Rustum et al., 1999; Aktas et al., 2011; Kuhlmann et al., 2014; Poveda et al., 2011; Zeng et al., 2017; Zhou et al., 2015). Single cell analysis of CTCs allows the precise study of the highly heterogeneous cell populations which enables the investigation of the evolution of CTCs throughout treatment providing a real time

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monitoring using 'liquid biopsy' (Aktas et al., 2009; Barrière et al., 2012; Giordano et al., 2012; Kasimir-Bauer et al., 2012).

scRNA-seq has been used to study the role of CTCs in several cancers including melanoma, breast cancer and pancreatic cancer (Chen et al., 2016; Papadaki & Agelaki, 2022; Ramsköld et al., 2012). The study of CTCs in OC using scRNA-seq is still in its infancy. Blassl and colleagues have established a robust workflow for the gene expression profiling of single OC CTCs which led to the detection of single ovarian CTCs expressing stem cell and EMT markers. (Blassl et al., 2016) Future research in this area will add knowledge to the disease dissemination and recurrence.

1.7.1.4 Study of tumour-derived exosomes (TEXs)

TEXs are membrane-bound functional biomolecules (approximately 30-150 nm in diameter) produced and secreted by tumour cells (Balaj et al., 2011; Choi et al., 2013; Ting et al., 2014). They are involved in intercellular communication between tumour and healthy cells mediating exchange of protein and genetic material between these cells (Talukdar et al., 2021; Théry et al., 2009). TEXs are a vital component of the TME and promote tumour development through primary tumour growth, angiogenesis, immune evasion and metastasis (Bai et al., 2022). Single exosome RNA-sequencing can be used to profile exosomal mRNAs in patients undergoing chemo- or immunotherapy to study changes in the immune signalling pathway and detect differential expression of gene signatures between responders and non-responders (Hurley et al., 2015; Talukdar et al., 2021).

1.8 Quantitative histopathological analysis in HGSOC

1.8.1 Conventional histological diagnosis

Epithelial ovarian cancer (EOC) is diagnosed histologically based on biomarkers where 96% of cases express CK7, with low CK20 and CDX2 immunostaining (Groisman et al., 2004). Colorectal cancers can metastasize to the ovaries (2-13% of patients) and these may be mistaken for primary ovarian carcinomas. In order to distinguish them, colorectal cancers have been found to express high levels of CK20 and CDX2, but low CK7 (Groisman et al., 2004).

Serous EOC is characterised by positive staining for nuclear Wilms Tumour-1 (WT-1) (Kriplani & Patel, 2013).

Table 1.5 Immuno-histochemical features of HGSOC vs LGSOC.

	HGSOC	LGSOC
p53 (pattern of expression)	Diffuse	Negative, focal or patchy
p16 (pattern of expression)	Diffuse	Negative, focal or patchy
Ki-67 (proliferation index)	Moderately elevated to high	Low

(R. Vang et al., 2009)

p53, p16 and Ki-67 immunostaining are used to differentiate high grade serous from low grade. HGSOC displays either diffused p53 staining (representing missense mutations) or complete absent p53 staining but diffuse p16 staining (null mutations); and moderate to high levels of Ki-67 staining. By comparison, LGSOC normally has negative or low p53 and p16 staining (reflective of wild type p53) and low Ki-67

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staining. Other markers such as ER, PR, PAX-8 and CA-125 are positive in both HGSOC and LGSOC (Russell Vang et al., 2009).

1.8.2 Role of quantitative histopathological analysis in HGSOC

Genomic studies have been instrumental in providing deep insight into the highly heterogeneous nature of HGSOC. However, in terms of application in clinical decision-making, only homologous recombination deficiency testing has been widely implemented (Azzalini et al., 2021). Comprehensive histopathological analysis through combination of morphological and architectural characterisation with objective quantification using artificial intelligence is gaining traction in efforts to use alternative technology to stratify and prognosticate OC.

Several studies have utilised histopathology to subgroup HGSOC and linking these morphological features to specific genotype. In a study by Soslow et al., a subgroup of HGSOC with 'SET' (Solid, pseudoEndometrioid, and Transitional cell carcinoma-like morphology) features was described in which there was a high association with *BRCA1* mutation and characterised by high mitotic index, more TILs and necrosis (Soslow et al., 2012). More recently, Azzalini et al. used the SET features to prognosticate a cohort of patients treated with PDS or NACT-IDS and found that the SET features group had longer OS and PFS (Azzalini et al., 2021). Another group identified specific morphologic features linked to *BRCA1* mutation (Fujiwara et al., 2012). Bromley et al. found significantly different morphological features in advanced HGSOC patients treated with NACT and PDS, but could not link specific features to chemotherapy response (Bromley et al., 2012).

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The development of Chemotherapy Response Score (CRS) is a landmark in histopathological scoring of OC. Its implementation in clinical practice to measure response to NACT has been recommended by the International Collaboration on Cancer Reporting (Böhm et al., 2015). The CRS system was based on the analysis of surgically removed omental masses in NACT-IDS cases. It has been validated that the CRS system is reproducible and a CRS3 score (complete or near-complete response characterised by lack of residual tumour cells) is observed in ~30-40% of HGSOc patients after NACT with improved prognosis (Böhm et al., 2015; Ivantsov, 2018).

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CHAPTER 2: OPTIMISATION OF TUMOUR DISSOCIATION INTO SINGLE CELLS FOR RNA-SEQUENCING

2 CHAPTER 2: OPTIMISATION OF TUMOUR DISSOCIATION INTO SINGLE CELLS FOR RNA-SEQUENCING

2.1 Introduction

Single cell RNA-sequencing (scRNA-seq) in ovarian cancer (OC) offers a high-resolution molecular phenotyping of individual cancer cells and supporting cells from the tumour microenvironment. This provides a new frontier of precision medicine and a greater insight into the spatial and temporal heterogeneity of the disease at single cell level, previously not afforded by bulk transcriptomic studies which rely on aggregate molecular signals across the tumour.

Single cell transcriptomic analysis relies on high quality data generated from single cells following sample processing in a multi-step process from tissue harvesting through to single cell capture for downstream sequencing and analysis. One of the main challenges is to obtain a high cell viability following tumour dissociation, ensuring high quality of mRNA being analysed. Single cell suspensions with a low number of viable cells are likely to produce poor quality data that may not be suitable for the desired analysis, and may result in the introduction of artefacts from cell stress signals due to high mitochondrial content (Rodriguez de la Fuente et al., 2021). This may impact on the study of the actual biology of the tumour and lead to misinterpretation of the data.

scRNA-seq studies in OC have utilised fresh or cryopreserved human tissues and ascites, xenograft models and cells from 2D or 3D culture. The isolation of viable individual cells remains the most critical and challenging part of the process. We have opted to optimise the scRNA-seq dissociation protocol for patient materials obtained from the operating theatre. Careful consideration must be given to ensure a successful

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downstream scRNA-seq output including optimal tissue collection, transport, storage and processing. During tissue harvesting, steps should be taken to minimise vascular and mechanical damage and avoidance of thermal injury if possible. Further ischaemic or hypoxic decay should be reduced with proper tissue transport and storage conditions. Different fresh and cryopreserved tissue dissociation protocols have been reported for OC studies (Hu et al., 2020; A. J. Shih et al., 2018). The optimal dissociation protocol should balance the adequate cell release from the tumour tissue while maintaining intact or high cellular viability during mechanical and enzymatic digestion.

Here, I describe a single cell study of serous OC in our lab (in collaboration with Dr Thomas Parry, PhD Research Fellow at CRUK Scotland Centre University of Edinburgh and others at the Institute for Genetics and Cancer) by setting out the experimental design (led by myself; see steps outlined below) and subsequent single cell transcriptomic data analysis in Chapter 3 (led by Dr Thomas Parry).

The general steps involved 1a) tumour harvesting and sample selection for visible gross tumour; 1b) enzymatic tumour dissociation into single cell suspensions and sample cryopreservation and fixation in formalin/paraffin for future studies; 2) dead cell removal, red blood cell lysis; 3a) cell counting and viability; 3b) quality control checks of cell viability and cellular content; 4a) single cell library preparation and quality control check; 4b) single cell RNA-sequencing (Figure 2.1).

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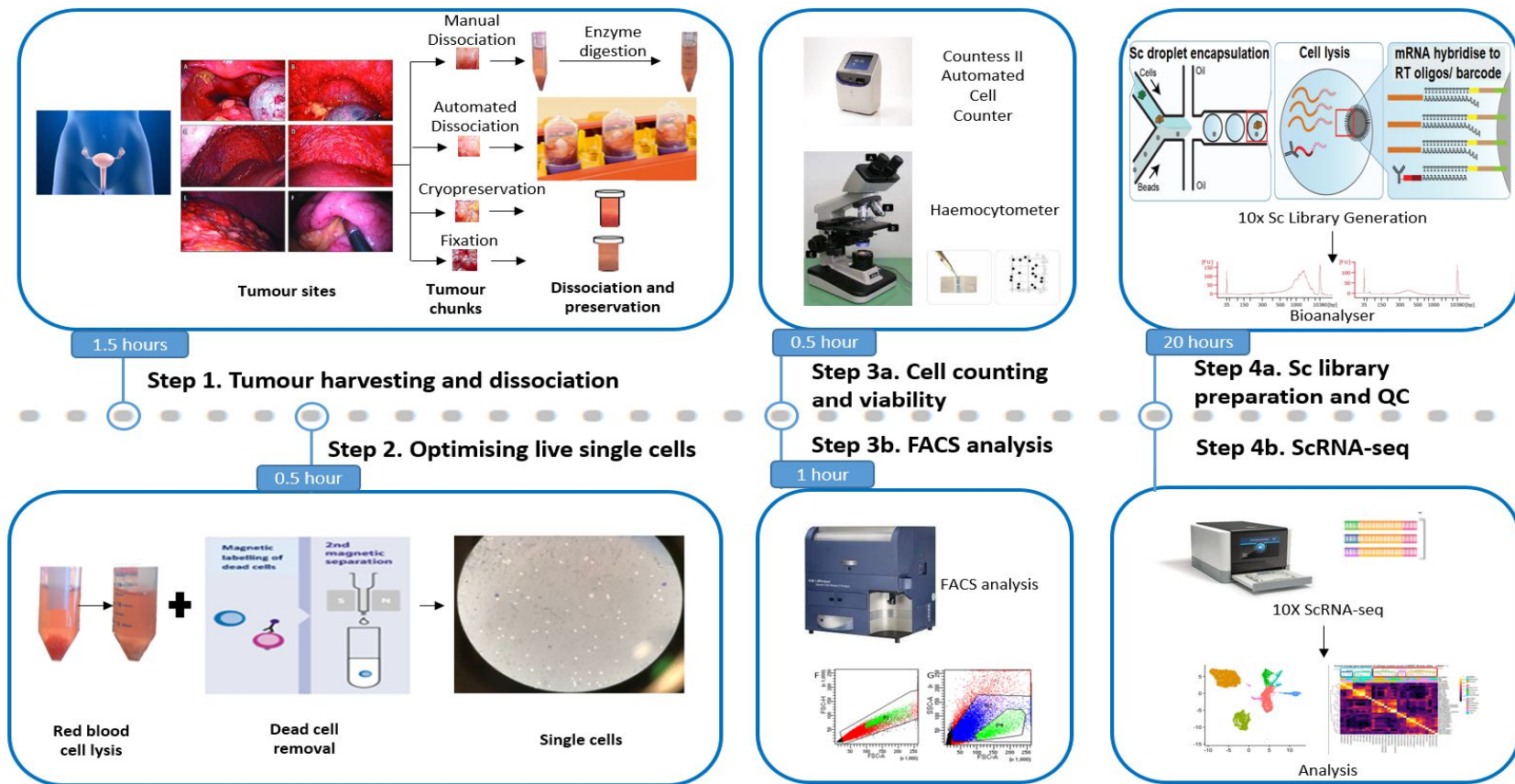


Figure 2.1 Graphical Experimental Design. Schematic diagram illustrating the general steps (1-4) involved in sample preparation for scRNA-seq from tumour harvesting, optimisation of tumour dissociation, quality control checks through to single cell library preparation and data analysis. Figure adapted from (Biosciences, 2022; Biotec, 2021; Genomics, 2022a; Scientific, 2022).

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2.2 Study Aims

This study aims to:

1. Prospectively identify and recruit participants diagnosed with serous OC treated at the Edinburgh Cancer Centre
2. Harvest tumour samples from multiple sites from these recruited participants during laparoscopic biopsies, primary debulking surgeries and interval debulking surgeries
3. Determine the best cryopreservation method for tumour samples in order to preserve the integrity of the mRNA for subsequent scRNA-seq study
4. Optimise the logistics for single cell study from sample collection, transport, laboratory processing to sample storage
5. Optimise tumour dissociation using manual and automated protocols in order to obtain single cell suspensions with a high cell viability for subsequent downstream processing and analysis
6. Perform quality control checks of cell viability and cellular content

2.3 Materials and methods

2.3.1 Patients' Samples

2.3.1.1 Patient Recruitment

Patients who had been assessed to have Stage III-IV of HGSOC were identified through the Gynaecology multi-disciplinary meeting (MDM) where their clinical case, CT scans, other radiological images and blood tumour markers were examined and discussed. Prior to recruitment, patients were counselled, given a 'Participant information sheet for the collection of tissue, biospecimens and data for research (Gynae Version 1.1)' and consented (using form QF-TGU-A-CONSNTGY with ethical approval obtained from the Lothian Local Research Ethics Committee -Reference SR865/15/ES/0094). For pre-chemotherapy cases without prior biopsy results, patients with a high level of cancer antigen 125 (CA-125 > 500) and disseminated disease on abdominal/pelvic CT scans who were undergoing primary debulking surgeries were selected. Another patient group of interest were those with likely advanced stage HGSOC who were undergoing laparoscopic biopsies. These patients were selected, consented and biopsy samples collected. Cases were then followed through their treatment journey. When they returned for interval debulking surgeries following three cycles of neo-adjuvant chemotherapy, further tumour samples were collected where possible.

2.3.1.2 Acquisition, Storage and Transport of Patients' Samples

Prior to any sample acquisition, clear selection criteria and processes were agreed with the Pathology Department and Medical/Surgical Gynaecological Department

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of Western General Hospital (WGH) and Edinburgh Royal Infirmary (ERI). To address spatial heterogeneity, tumour materials were obtained from multiple sites (including primary ovarian masses, omental and metastatic nodules). These debulking surgeries were commonly complex procedures lasting an average of 4-5 hours. Time between tissue harvesting and processing was minimised. Additionally, several important criteria for tissue selection were set to ensure good quality samples were obtained. These included selecting for samples with visible gross tumour, collecting adequate sample size for fresh processing and cryopreservation, ensuring the specimen had minimal hypoxic stress or damage by selecting for tumour samples with less diathermy cutting and preferentially harvested through scalpel excision/ suture ligation and avoiding clear necrotic regions.

Once a tumour site had been collected, the appropriate sample size (approximately 3 x 3 cm) was excised from the whole specimen. The remaining specimens were sent to the pathology laboratory for routine diagnostic histopathological analysis. The fresh sample obtained for our study was placed in magnetic-activated cell sorting (MACS) tissue storage solution in a 50 ml Falcon tube and stored in an ice box to preserve tissue freshness prior to harvesting (Figure 2.2). When surgeries lasted beyond 5 hours, the initial samples collected were transported to our laboratory to be processed first, meanwhile further sample sites would be collected using the same standardised protocol by a tissue collector and processed later. The average time for transportation of samples from the operating theatre to the laboratory was 60 minutes. For some cases, on site histological diagnosis was carried out using intra-operative frozen sections (IFS) which aided the rapid identification of HGSOC cases. For cases without IFS, samples were dissociated (or cryopreserved) and single-cell libraries prepared as

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normal (subject to quality control below), with these libraries only then sent for sequencing when subsequent formal HGSOC diagnosis was complete.

In the early stages, single cell suspensions with cell viability of > 60% and confirmed malignant cells within the samples by FACS staining for EPCAM⁺ cells were considered to pass quality control. Later, through rigorous optimisation protocols, we discovered that the optimal selection criteria for high quality single cell suspensions for downstream processing should include samples with higher cell viability of > 70% and adequate cDNA library concentrations which through optimisation was determined at > 10 ng/μl consistent with recommendations by 10x Genomics (Genomics, 2022a).

2.3.1.3 Sample Processing

Upon arrival in the laboratory, tumour specimens from each site were divided into 4 sections where two parts were processed through manual and automated dissociation, one part was preserved in cryopreservation media and another part was fixed in formalin, dehydrated in 70% ethanol and embedded in paraffin (Figure 2.2). As the study progressed, several key learning points on selecting for certain tumour regions to enhance cell viability were noted. Tumour sections used for dissociation were selected from parts with minimal or absent necrosis. For omental samples, sections were selected from areas with minimal fibro-adipose tissues as adipocytes were noticed to be more fragile and were less likely to survive the dissociation (Table 2.5).

As the timing of sample acquisition was variable due to the prolonged hours of debulking surgeries, this could pose potential difficulty for the samples to be processed

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fresh during working hours. To overcome this problem, a cryopreservation method was optimised to preserve the tumour samples at -80°C to allow more time for processing while maintaining cellular viability and the integrity of mRNAs. Two cryopreservation media were compared for their efficacy in maintaining cellular viability – 1) 10% dimethyl sulfoxide (DMSO), 10% fetal calf serum (FCS), 80% Dulbecco's Modified Eagle Medium (DMEM); and 2) 20% DMSO, 40% FCS, 40% Roswell Park Memorial Institute (RPMI) 1640. The tumour specimen (~2g) were placed in a cryovial in the cryopreservation media and stored in a cryobox (to allow for slow freezing of $-1^{\circ}\text{C}/\text{min}$) at -80°C . On recovery of the specimen, the cryovials were placed directly from -80°C storage into 37°C thermal water baths while monitoring the specimen carefully for immediate processing when fully thawed.

To prepare the formalin-fixed, paraffin-embedded tissue (FFPE) sections, the freshly dissected tissues (< 3 mm thick) were fixed with 10% formalin for 48 hours at room temperature. The formalin-fixed tissues were then passed onto the pathology services at IGMM/WGH for further processing to produce 5 μm thick FFPE sections. We started processing the tumour samples fresh using in-house manual dissociation protocol as outlined in 2.3.2. After dissociation, the single cell suspensions were then passed through fluorescence-activated cell sorting (FACS) to select for 4',6-diamidino-2-phenylindole (DAPI) negative live cells of interest (immune and non-immune cells). These cells were then processed through single cell library generation.

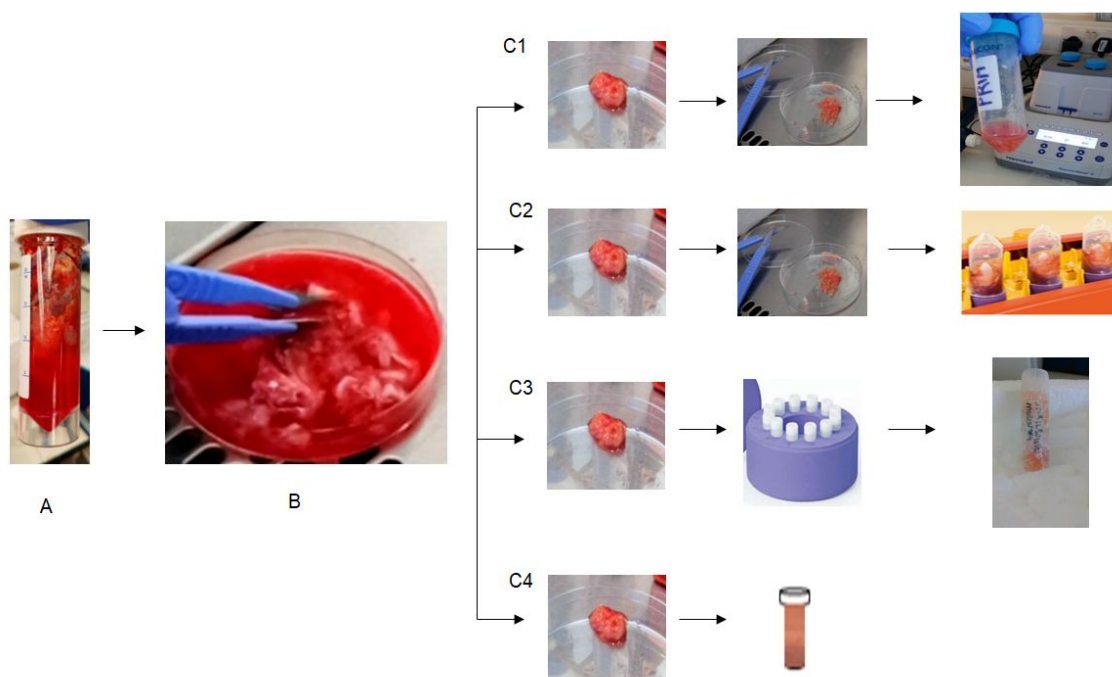


Figure 2.2 Sample Processing. (A) From operating theatre, the specimen was placed in MACS tissue storage solution in a 50ml Falcon tube. (B) Specimen divided into 4 parts. (C1) Sample cut into 1-2 g piece and manually dissociated. (C2) Sample cut into 1-2 g piece for automated dissociation. (C3) Sample placed in cryovials in cryo-media and stored at -80°C. (C4) Sample fixed in FFPE for future validation.

2.3.2 Manual Tumour Dissociation

2.3.2.1 Enzymatic Dissociation

The protocol developed for our in-house manual dissociation is outlined below.

1. Rinse the tissue sample in cold phosphate-buffered saline (PBS) in a petri dish.
2. Prepare the collagenase P (1 mg/ml) (Roche) – DNase I (10 U/μl) (Roche) solution.
3. Prepare the liberase DL (0.26 U/ml) (Roche)/TL (0.5 U/ml) (Sigma) – DNase I (5 U/ml) (Roche) solution.

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4. Prepare the neutral protease (1 U/g of tissue) (Roche) solution.
5. Mince the tissue (1 g – 2 g) in cold PBS with two blades until turbid (approximately 10 minutes). Then, transfer to 50 ml Falcon tube.
6. Incubate the tumour samples for 10 minutes at 400rpm and 37°C using Eppendorf ThermoMixer thermoblock. Compare with incubation at 100 rpm.

The efficacy of dissociation using three different enzymes (collagenase, liberase and neutral protease) were compared. The speed of incubation on the thermoblock were also compared between 100rpm and 400rpm.

2.3.2.2 Filtration, Enzyme Blocking and Red Blood Cell Lysis

Following mechanical and enzymatic manual dissociation, process the sample through the steps below.

1. Vortex for 10 seconds and pipette up and down with 25 ml/10 ml/5 ml pipettes serially.
2. Filter suspension through 70 µm strainer to remove residual clumps. Repeat if strainer becomes blocked.
3. Add 30 ml of 2% Fetal calf serum-phosphate-buffered saline (FCS-PBS) and centrifuge for 6 minutes at 580 xg at 4°C.
4. The pellet usually contains red blood cells (RBCs). Proceed to lysis by resuspending pellet in 3 ml of RBC lysis buffer. Leave on ice for 10 minutes. Then, add in 30 ml of 2% Bovine serum albumin- phosphate-buffered saline (BSA-PBS) and centrifuge again. Resuspend in 1 ml of blocking buffer (900 µl PBS + 100 µl human serum) and leave suspension on ice for further 30 minutes. In the meantime, measure the cell viability and concentration.

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2.3.2.3 Cell viability count

To count the number and percentage of live cells, the sample was mixed 1:1 with trypan blue. The mixture was then mounted onto Countess slides and the cell viability measured on the Countess II Automated Cell Counter. The cell counts were also checked manually using a haemocytometer. Cells stained blue were dead cells which were distinguished from live cells where intact membrane excluded the blue dye from permeating the cells.

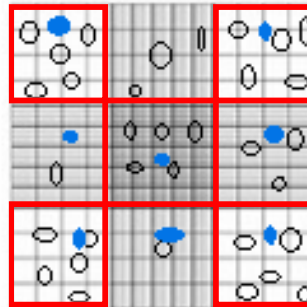


Figure 2.3 Haemocytometer Counting Chamber with Live Cells (unstained) and Dead Cells (stained blue). Five squares containing cells (marked with red squares) were selected for the cell count.

Formula

Total live cells in 5 squares = 50

Total dead cells in 5 squares = 5

1. % of live cells = $50/55 = 90.91\%$
2. Average number of live cells per square = $50/5 = 10$ cells/square
3. Dilution factor = Final volume/Volume of cell suspension (Vc)
$$= (100 \mu\text{l trypan blue} + 100 \mu\text{l Vc})/100 \mu\text{l}$$
$$= 2$$

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$$\begin{aligned} 4. \text{ Concentration of live cells} &= \text{Avg no. of live cells/ square} \times \text{Dilution factor} \times 10^4 \\ &= 10 \times 2 \times 10^4 \\ &= 2.0 \times 10^5 \text{ cells/ml} \end{aligned}$$

2.3.2.4 FACS staining, sorting and analysis

Following tumour sample dissociation, the single cell suspensions are stained with antibodies and DAPI and passed through FACS to enrich for the cell population of interest. FACS is a fast and efficient cell sorting method which can enrich for a specific cell type using fluorescent labels to attach to antibody that recognises a target feature on or in the cell. In this study, anti-CD45, anti-EpCAM (epithelial cellular adhesion molecule), anti-CD34 and anti- α -SMA antibody from Abcam were utilised to select for immune and non-immune cells (including tumour, endothelial and stromal cells). CD45 antigen (leukocyte common antigen) is a glycoprotein expressed on almost all haematopoietic cells except mature erythrocytes and platelets. EpCAM is transmembrane glycoprotein exclusively expressed by epithelial-derived cancers. (Schnell et al., 2013). CD34 is a glycoprotein expressed on early lymphohaematopoietic stem cells and endothelial cells. In human ovary, CD34 identifies vascular endothelial cells (Dath et al., 2011). Alpha-smooth muscle actin (α -SMA) is commonly used as a marker of myofibroblasts and stains positively in stroma of epithelial tumours (Czernobilsky et al., 1989).

The steps for FACS staining, sorting and analysis are as follows.

- 1) Incubate 100 μ l of the suspension (prepared above) with 5 μ l of anti-CD45 [clone EP322Y] (ab40763), 5 μ l of EpCAM antibody [clone VU-1D9]

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(ab187372), 5 μ l of anti-CD34 [clone QBEND-10] (ab8536) and 5 μ l of α -SMA antibody [clone 1A4] (ab7817) for 1 hour at 4°C in the dark.

- 2) Prepare controls by adding 1 drop of compensation beads (Thermo Fisher Scientific catalog #01-3333-42) with anti-CD45, EpCAM antibody, anti-CD34 and α -SMA antibody into 4 eppendorf tubes respectively.
- 3) After antibodies incubation, centrifuge the suspension above along with 400 μ l of staining buffer for 5 minutes at 500 xg at 4°C. Then resuspend them in 500 μ l of staining buffer and keep the cells in the dark on ice.
- 4) Send the suspension for FACS sorting and analysis using BD FACSAria (BD Biosciences) and BD FACSDiva software.
- 5) Immediately prior to FACS sorting, add 1 μ l of DAPI to 100 μ l of cells.
- 6) The flow sorter should deposit as little volume as possible (< 0.5 μ l otherwise the efficiency of reverse transcription will be affected). Adjust flow rate to a minimum.
- 7) Immediately following sorting of the suspension (now containing live single cells CD45⁺ and CD45⁻/EpCAM⁺ and CD34⁺/ α -SMA⁺ after sorting), proceed to scRNA-seq.

2.3.3 Automated Tumour Dissociation

As part of the optimisation, we trialled an automated dissociation protocol using the gentleMACS dissociator on fresh and cryopreserved samples. Once tissue samples were dissociated, we used the Miltenyi tumour cell isolation kit containing enzyme cocktails which selected for the non-tumour cells and depleted them using magnetic separation method. The isolated tumour cells were collected as effluent once the single cell suspension was passed through the magnetic column with the enzyme cocktails. The presence of tumour cells were checked using FACS analysis where the isolated tumour cell suspension identified as a high pure population of EPCAM⁺/CD45⁻ (Results section 2.5.6.3.3). Subsequently, as our research questions evolved to interrogate both the tumour and non-tumour microenvironment of HGSOC, our samples were processed through automated dissociation without tumour cell isolation. Following dissociation, RBC lysis was performed to remove erythrocytes whose presence might result in reduced single cell purity due to cell clumping. Dead cell removal (DCR) was also performed to increase the live cell yield above 70%. DCR was performed using Miltenyi magnetic microbeads which labelled cell debris, dead cells and dying cells. The magnetically labelled material was removed by magnetic separation and a purer, higher yield of viable cells was obtained.

The steps of gentleMACS dissociation, optional tumour cell isolation, filtration and RBC lysis and dead cell removal are as follows.

2.3.3.1 Enzymatic Dissociation and Filtration

- 1) Prepare enzyme mix by adding 4.7 ml of RPMI-1640 or DMEM (without L-glutamine), 200 µl of Enzyme H, 100 µl of Enzyme R and 25 µl Enzyme A into a gentleMACS C tube.
- 2) Cut 1 g of tumour into small pieces of 2-4 mm.
- 3) Transfer the tissue pieces into the gentleMACS C tube containing the enzyme mix.
- 4) Attach the C tube onto the gentleMACS Octodissociator sleeve.
- 5) Run the gentleMACS Octodissociator programme 37C_h_TDK_2.
- 6) Upon completion of the incubation, perform a short centrifugation step to collect the cells at the bottom of the tube.
- 7) Resuspend the sample and apply the cell suspension to a MACS SmartStrainer (70 µm) placed on a 50 ml tube.
- 8) Wash cell MACS SmartStrainer (70 µm) with 20 ml of RPMI-1640 or DMEM.
- 9) Centrifuge cell suspension at 300 xg for 7 minutes. Resuspend cell pellet in 1 ml of RPMI or DMEM.
- 10) Determine cell number and viability using Countess II (Refer to 2.3.2.3).

2.3.3.2 (Optional) Tumour Cell Isolation

- 1) Centrifuge cell suspension at 300 xg for 10 minutes at 4°C. Aspirate supernatant completely. Resuspend cell pellet up to 2×10^6 tumour cells/ up to 1×10^7 total cells in 60 µl buffer (0.5% BSA in PBS).

[Type here]

*For more cells, scale up all reagent and buffer. E.g. 4×10^6 tumour cells/
 2×10^7 total cells, double up volume of reagent and buffer.

- 2) Add 20 μ l cocktail A and 20 μ l of cocktail B. Mix well and incubate for 15 minutes in the refrigerator (2-8°C).
- 3) Add buffer up to a total volume of 500 μ l.
- 4) Magnetic separation using LS columns. Prepare column by rinsing with 3 ml of buffer. Apply cell suspension onto column. Collect flow-through (enriched tumour cells). Wash column with 2 x 1 ml of buffer.
- 5) Determine cell number and viability using Countess II (Refer to 2.4.2.3).

2.3.3.3 Dead Cell Removal

- 1) Prepare binding buffer. Per 1×10^7 total cells, dilute 0.25 mL of 20x Binding Buffer (BB) Stock Solution with 4.75 ml of sterile, double distilled water.
- 2) Centrifuge cells at 300 xg for 7 minutes at 4°C. Remove supernatant completely. Resuspend cells in DCR microbeads (100 μ l DCR microbeads per 1×10^7 total cells). For fewer cells, use the same volume of DCR microbeads. Mix well and incubate for 15 minutes at room temperature (20-25°C).
- 3) Place LS column in magnetic field and rinse with 3 ml of BB. Apply cell suspension in 1000 μ l of 1x BB onto column. Rinse with 4 x 3 ml of BB. Collect effluent as live cell fraction.

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2.3.3.4 Red Blood Cell Lysis

- 1) Perform RBC lysis by incubating 1 ml of cell suspension in 10 ml of RBC lysis buffer for 2 minutes at room temperature. Centrifuge cell suspension at 300xg for 10 minutes. Resuspend cell pellet in 1 ml of RPMI-1640 or DMEM.
- 2) Determine cell number and viability using Countess II (Refer to 2.3.2.3).

2.3.3.5 Second Dead Cell Removal

If cell viability is < 70%, perform second DCR and then determine cell number and viability using Countess II.

2.3.3.6 FACS Staining and Analysis

Approximately 100 µl of the single cell suspension was incubated with CD45/EpCAM/CD34/ α -SMA antibodies and analysed by FACS to check for purity of tumour, immune and stromal cell populations as a quality control step. If the sample passed first quality control check by yielding > 70% viable cells and a satisfactory percentage of tumour population, the remaining single cell suspension was processed further for scRNA-seq.

2.3.4 Comparison between Manual vs Automated Protocol

Experiments comparing the efficacy of manual and automated dissociation methods in yielding a high cell viability were performed. These were carried out initially on fresh

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and cryopreserved primary ovarian samples pre-and post-chemotherapy followed by omental samples.

2.3.5 Optimising Automated Protocol

Following automated tumour dissociation, different sequences of RBC lysis and DCR were trialled including RBC lysis with 2 separate steps of DCRs or DCR followed by RBC lysis and further DCR if cell viability was less than 70%. During the optimisation of the automated protocol, OVCAR-8 cells were used as a control to assess potential damaging effects of the dissociation process on viable cells.

2.4 Results

2.4.1 Manual Dissociation

2.4.1.1 Determining the optimal dissociation reagent, incubation condition and sample state

The first set of manual dissociations were carried out on samples from 3 different sites (ovary, omentum, metastatic nodule) using collagenase, liberase and neutral protease in 6 separate experiments (Table 2.1). The samples were incubated in the enzymes for 10 minutes at 37° C on Eppendorf ThermoMixer thermoblock at different rotational speeds. The thermoblock rotation speed of 100 rpm performed better compared to 400 rpm when collagenase enzyme was used to dissociate the 3 different sites (mean live cell yield of 58.6% vs 18.7% from all sites respectively) (Table 2.1 and Figure 2.4). Larger tumour chunks yielded a high number of live cells (2 g of tumour resulted in 10^6 - 10^7 cells/ml compared to 1 g of tumour with 10^5 - 10^6 cells/ml). However, larger tumour size did not result in higher percentage of viable cells (median cell viability for 2 g tumour 21.0% vs median cell viability for 1 g tumour 48.5%) (Table 2.1 and Figure 2.5). As downstream processing during scRNA-seq only required a maximum of 25,000 cells per site/library, 1 g of tumour was more than adequate.

The second set of manual dissociations were carried out on single sites each time but comparing 3 different enzyme efficacies simultaneously (Table 2.2). Similar trends of higher cell viabilities were noted with thermoblock rotation speed of 100 rpm compared to 400 rpm (Table 2.2 and Figure 2.4). Similar observation was seen with bigger chunks of tumour tissues (2 g compared to 1 g) producing a higher number of live cells (2 g tumour yielding 10^6 - 10^7 cells/ml vs 1 g tumour yielding 10^5 - 10^6 cells/ml) but not

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necessarily a higher percentage of viable cells (median cell viability for 2 g tumour 34.0% vs median cell viability for 1 g tumour 42.7%) (Table 2.2 and Figure 2.5).

When comparing different enzyme efficacies at 100 rpm thermoblock rotational speed during incubation, the primary ovarian samples had higher cell viability using neutral protease to dissociate the tissues (median cell viability 55.0-59.5% compared to liberase 43.0-45.9% and collagenase 30.1-47.0%). The omental samples had higher cell viabilities when dissociated using collagenase (median cell viability 57.6-59.0%) or liberase (median cell viability 51.0-52.3%) compared to neutral protease (median cell viability 24.5%). Meanwhile, metastatic nodules achieved higher cell viability with liberase enzymatic dissociation (median cell viability 17.0-60.0%) compared to collagenase and neutral protease (median cell viability 22.0-26.4% and 14.6-20.0% respectively) (Figure 2.6). Nevertheless, when statistical Kruskal-Wallis test was performed to compare the enzymatic dissociation for different sites using the 3 enzymes, only the omental sample returned statistically significant P value. The lack of statistical significance for the other sites when dissociating with different enzymes could be due to the small sample sizes involved.

At 100 rpm incubation, cryopreserved and freshly dissociated tissues yielded comparable cell viabilities (highest cell viability for primary ovarian 65% vs 68.4%; metastatic nodules 63% vs 65.5%; and omentum 62% vs 61.7%) (Table 2.3).

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Table 2.1 Experiments on 3 Different Sites using Collagenase, Liberase and Neutral Protease

Experiment	Sample Site	Sample State	Surgery	Collagenase (%/ No. of live cells x 10 ⁵ /ml)	Liberase (%/ No. of live cells x 10 ⁵ /ml)	Neutral (%/ No. of live cells x 10 ⁵ /ml)	Thermo- block Rotation Speed (rpm)	Weight of Tumour (g)
M1.1	Ovary	Fresh	Pre- chemo	25.0% (123.0)	NA	NA	400	2
	Omentum			14.0% (37.4)	NA	NA	400	2
	Peritoneal nodule			17% (78.5)	NA	NA	400	2
M1.2	Ovary	Fresh	Pre- chemo	65.3% (4.3)	NA	NA	100	1
	Omentum			57.6% (5.4)	NA	NA	100	1
	Small bowel nodule			52.9% (1.2)	NA	NA	100	1
M1.3	Ovary	Fresh	Post- chemo (1 cycle)	6.6% (9.6)	NA	NA	100	1
	Omentum			57.2% (10.7)	NA	NA	100	1
	Small bowel nodule			7.3% (32.5)	NA	NA	100	1
M1.4	Ovary	Fresh	Pre- chemo	NA	38.5% (217.5)	NA	100	2
	Omentum			NA	53.5% (400.0)	NA	100	2
	Peritoneal nodule			NA	15.5% (58.3)	NA	100	2
M1.5	Ovary	Cryo- preserve d	Post- chemo (1 cycle)	NA	35.4% (14.4)	NA	100	1
	Omentum			NA	50.0% (20.0)	NA	100	1
	Peritoneal nodule			NA	47.0% (27.0)	NA	100	1
M1.6	Ovary	Fresh	Pre- chemo	NA	NA	59.5% (3.9)	100	1
	Omentum			NA	NA	14.0% (0.9)	100	1
	Peritoneal nodule			NA	NA	14.5% (0.8)	100	1

Table 2.2 Experiments Comparing 3 Different Enzymes on a Single Site

Experiment	Sample Site	Sample State	Surgery	Collagenase (%/ No. of live cells x 10 ⁵ /ml)	Liberase (%/ No. of live cells x 10 ⁵ /ml)	Neutral Protease (%/ No. of live cells x 10 ⁵ /ml)	No enzyme (%/ No. of live cells x 10 ⁵ /ml)	Thermoblock rotation speed (rpm)	Weight of tumour (g)
Primary Ovarian Samples									
M2.1	Ovary	Fresh	Pre-chemo	35.5% (2.8)	46.5% (3.7)	48.0% (4.0)	NA	400	1
M2.2	Ovary	Fresh	Pre-chemo	64.0% (4.1)	54.7% (3.3)	68.4% (5.0)	NA	100	1
M2.3	Ovary	Fresh	Post-chemo (3 cycles)	30.0% (1.5)	43.0% (2.4)	50.3% (3.4)	NA	100	1
M2.4	Ovary	Cryo-preserved	Pre-chemo	11.0% (145.0)	39.5% (715.0)	28.5% (21.5)	16.5% (310.0)	400	2
M2.5	Ovary	Cryo-preserved	Pre-chemo	32.0% (2.2)	61.0% (4.8)	65.0% (5.0)	NA	100	1
M2.6	Ovary	Cryo-preserved	Post-chemo (1 cycle)	28.3% (1.1)	41.3% (1.3)	45.0% (2.0)	3.3% (2.4)	100	1
Omental Samples									
M2.7	Omentum	Fresh	Pre-chemo	36.7% (2.4)	35.5% (2.0)	30.0% (1.9)	NA	400	1
M2.8	Omentum	Fresh	Pre-chemo	61.7% (3.2)	51.0% (2.3)	35.0% (1.9)	NA	100	1
M2.9	Omentum	Cryo-preserved	Pre-chemo	56.0% (53.1)	61.0% (60.0)	15.0% (12.3)	NA	100	1
M2.10	Omentum	Cryo-preserved	Pre-chemo	NA	NA	34.0% (77.7)	NA	100	1
M2.11	Omentum	Cryo-preserved	Post-chemo (3 cycles)	62.0% (488.0)	50.0% (300.0)	NA	NA	100	2
Metastatic Nodule Samples									
M2.12	Peritoneal nodule	Fresh	Pre-chemo	42.7% (1.8)	65.0% (2.9)	25.0% (0.5)	NA	100	1
M2.13	Peritoneal nodule	Fresh	Post-chemo (3 cycles)	10.0% (0.4)	17.0% (0.8)	14.6% (0.5)	NA	100	1
M2.14	Peritoneal nodule	Cryo-preserved	Pre-chemo	22.0% (0.9)	60.0% (1.9)	20.0% (0.7)	NA	100	1
M2.15	Peritoneal nodule	Cryo-preserved	Post-chemo (1 cycle)	NA	63.0% (8.0)	NA	NA	100	1

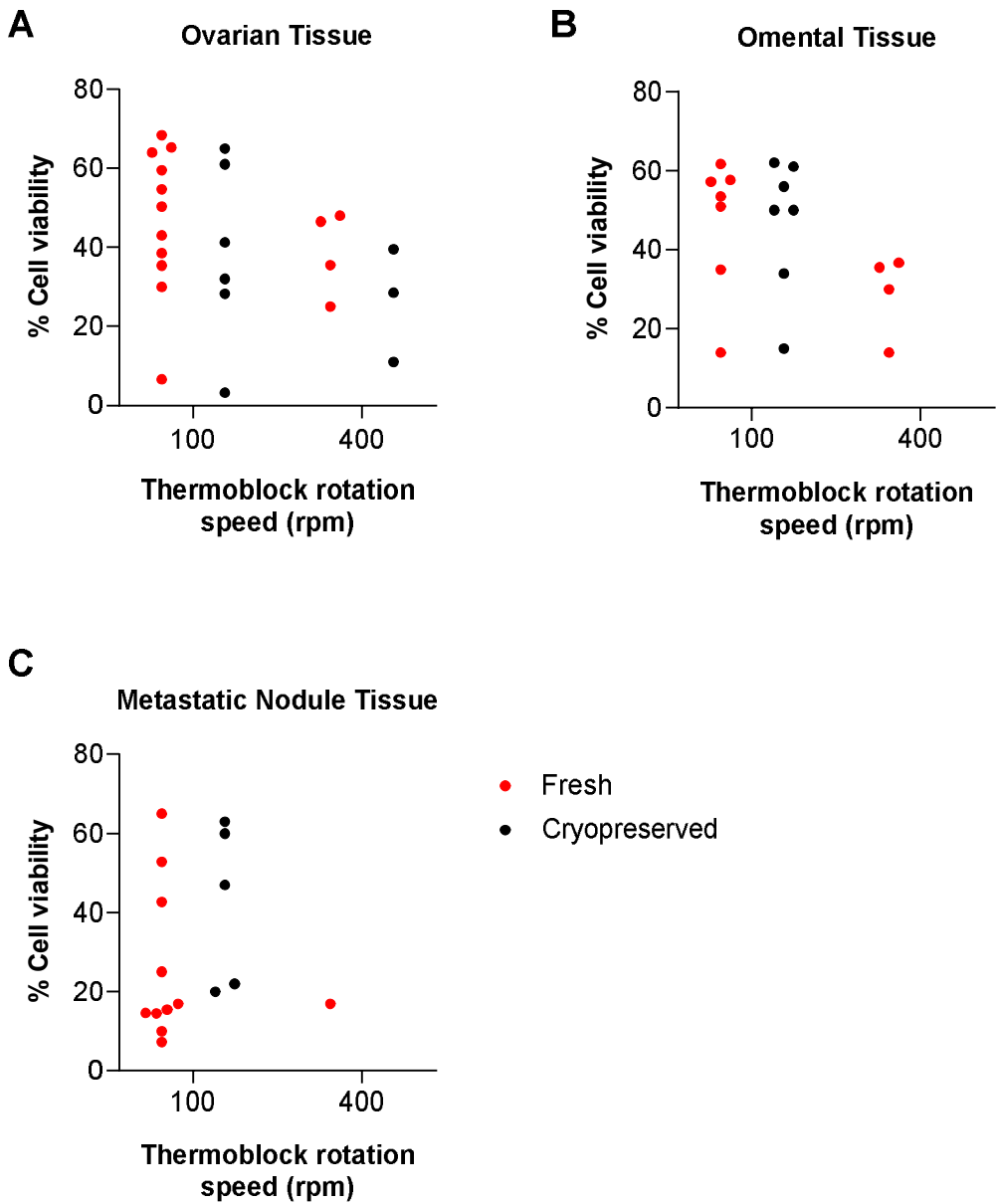


Figure 2.4 Comparison of Thermoblock Rotation Speed for Enzymatic Incubation for All Sample Sites. (A-C) From both sets of experiments, enzymatic incubation at 100 rpm yielded higher live cells compared to 400 rpm regardless of fresh or cryopreserved sample states.

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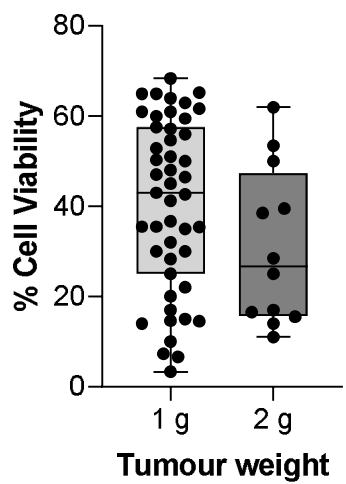


Figure 2.5 Comparison of % Cell Viability Yield by Tumour Weight.

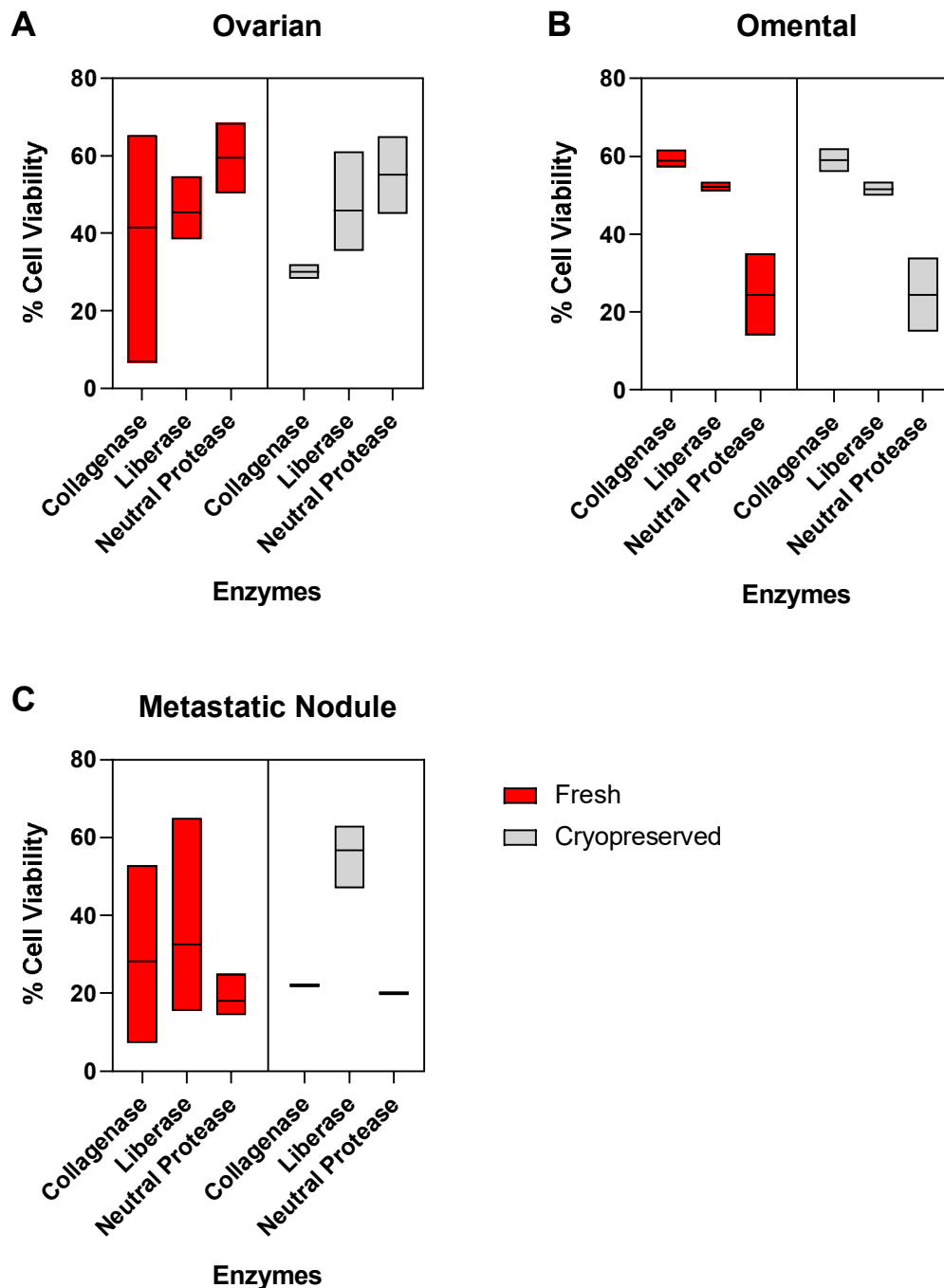


Figure 2.6 Comparison of Enzyme Efficacies on All Sites. (A) For primary ovarian tissues (number of samples tested, N=12), dissociation with neutral protease yielded the highest cell viability (median 55.0-59.5%). (B) For omental tissues (number of samples tested, N = 11), collagenase yielded the highest cell viability (median 57.6-59.0%). (C) For metastatic nodules (number of samples tested, N = 10), liberase was most efficacious in producing higher cell viability (median 17.0-60.0%) compared to collagenase and neutral protease.

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Table 2.3 Comparison of Efficacy of 3 Different Enzymatic Dissociation

Sample Site	Sample State	Median and Range of Cell Viability (%)			P value	Highest Cell Viability (%)
		Collagenase	Liberase	Neutral Protease		
Ovary	Fresh	47.0 (6.6-65.3)	43.0 (38.5-54.7)	59.5 (50.3-68.4)	0.1429	68.4
	Cryopreserved	30.1 (28.3-32.0)	45.9 (35.4-61.0)	55.0 (45.0-65.0)		65.0
Omentum	Fresh	57.6 (57.2-61.7)	52.3 (51.0-53.5)	24.5 (14.0-35.0)	<0.0001	61.7
	Cryopreserved	59.0 (56.0-62.0)	51.0 (50.0-61.0)	24.5 (15.0-34.0)		62.0
Metastatic Nodule	Fresh	26.4 (7.3-52.9)	17.0 (15.5-65.5)	14.6 (14.5-25.0)	0.1641	65.5
	Cryopreserved	22.0 (NA)	60.0 (47.0-63.0)	20.0 (NA)		63.0

*P values calculated using Mann-Whitney U test to compare cell viability of fresh and cryopreserved samples dissociated using different enzymes.

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2.4.1.2 Cell viability for manually dissociated samples after FACS sorting

We found that FACS sorting for live immune and non-immune cells of interest could reduce the cell viability of the single cell suspensions following manual dissociation protocol (Table 2.4).

2.4.2 Manual vs Automated Dissociation

2.4.2.1 Comparing the efficacy of manual vs automated dissociation

The in-house manual dissociation protocol had consistently resulted in < 70% of live cells after optimisation of the enzymatic dissociation process with the use of different enzymes, incubation speed and also the size of the tumour tissue. This was less than the recommended cell viability of $\geq 70\%$ for a good quality single cell suspension as input into the 10x Genomics single cell RNA sequencing platform. (Genomics, 2022a)

The presence of large proportion of non-viable cells may impact on the quality of transcriptomics data obtained due to reduced number of actual cell recovery, increased mitochondrial transcripts and significantly reducing the genes detection per cell. Therefore, to increase our cell viability, we proceeded to investigate the efficacy of manual and automated GentleMACS dissociation protocols. Adjacent sample tissues from primary ovarian and omental sites were divided into 1 g tissue portions each and processed through both dissociation methods and their cell viabilities documented post dissociation. From this series of 14 experiments, automated protocol produced a higher live cell yield after dissociation, pre- and post-dead cell removal (maximum cell viabilities pre- and post-DCR – 68% and 90% for ovary; 59% and 88% for omentum) compared to manual protocol (maximum cell viabilities pre- and post-DCR – 52% and 66% for ovary; 52% and 55% for omentum) (Table 2.5).

Table 2.4 Cell Viability Pre- and Post-FACS sorting

Experiment	Sample Site	Sample State	Surgery	Post Dissociation (%/ No. of live cells x 10 ⁵ / ml)	Post FACS sorting (%/ No. of live cells x 10 ⁵ / ml)
M1.4	Ovary	Fresh	Pre- chemo	38.5% (217.5)	7.0% (0.1)
	Omentum			53.5% (400.0)	0.0%
	Peritoneal Nodule			15.5% (58.3)	0.0%
M1.5	Ovary	Cryopreserved	Post- chemo (1 cycle)	35.4% (14.4)	61.0% (7.3)
	Omentum			50.0% (20.0)	63.0% (8.0)
	Peritoneal Nodule(E05)			47.0% (27.0)	61.0% (5.7)
M1.6	Ovary (E09A)	Fresh	Pre- chemo	59.5% (3.9)	54.0% (0.8)
	Omentum			14.0% (0.85)	0.0%
	Peritoneal Nodule			14.5% (0.8)	0.0%
M2.6	Ovary	Cryopreserved	Post- chemo (1 cycle)	28.3% (1.1)	76.0% (1.5)
				41.3% (1.3)	27.0% (0.2)
				45.0% (2.0)	61.0% (1.6)
				3.3% (2.4)	10.5% (0.8)
M2.11	Omentum	Cryopreserved	Post- chemo (3 cycles)	62.0% (488.0)	61.0% (2.6)
				50.0% (300.0)	40.0% (1.0)

Table 2.5 Comparing the Efficacy of Manual vs Automated GentleMACS Dissociation

Experi- ment	Sample Site	Sample State	Surgery	Manual Protocol (%/ No. of live cells x 10 ⁵ /ml)		Automated Protocol (%/ No. of live cells x 10 ⁵ /ml)	
				Pre-dead cell removal	Post-dead cell removal	Pre-dead cell removal	Post-dead cell removal
Ovary							
MA1.1	Ovary	Fresh	Pre- chemo	NA	NA	18.0% (2.8)	66.0 % (1.1)
		Cryo		NA	NA	18.0% (140.0)	86.0% (50.0)
MA1.2	Ovary	Fresh	Pre- chemo	4.0-39.0% (NP) (15.5-49.8)	26.0- 42.0% (10.3- 18.9)	7.0-60.0% (26.0-29.7)	46.0- 67.0% (11.0- 25.4)
MA1.3	Ovary	Fresh	Pre- chemo	37.0% (NP) (102.0)	66.0% (52.4)	68.0% (194.0)	90.0% (20.8)
MA1.4	Ovary	Cryopreserved	Pre- chemo	4.0% (NP) (14.3)	1.5% (2.93)	13.0% (66.4)	3.0% (10.9)
MA1.5	Ovary	Cryopreserved	Pre- chemo	52.0% (NP) (18.7)	36.0% (1.41)	8.0% (14.1)	45.0% (4.0)
MA1.6	Ovary	Cryopreserved	Pre- chemo	38.0% (NP) (12.7)	54.0% (1.3)	49.0% (39.8)	79.0% (3.6)
MA1.7	Ovary	Cryopreserved	Pre- chemo	NA	NA	65.0% (202)	73.0- 90.0% (12.2- 17.3)
Maximum live cell yield pre-DCR				52.0%		68.0%	
Maximum live cell yield post-DCR				66.0%		90.0%	
Omentum							
MA1.8	Omentum *	Fresh	Pre- chemo	NA	NA	49.0% (462.0)	48.0% (394.0)
MA1.9	Omentum **	Fresh	Pre- chemo	1.0% (C) (3.9)	2.0% (0.5)	4.0% (10.3)	9.0% (3.5)
MA1.10	Omentum	Fresh	Pre- chemo	52.0% (C) (14.5)	55.0% (3.2)	59.0% (22.7)	88.0% (5.6)

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MA1.1 1	Omentum *	Cryopreserved	Pre- chemo	5.0% (C) (5.2)	20.0% (1.0)	7.0-9.0% (29.2-40.2)	43.0- 47.0% (2.0-2.5)
MA1.1 2	Omentum	Cryopreserved	Pre- chemo	NA	NA	6.0-8.0% (43.2-47.8)	86.0% (37.0)
MA1.1 3	Omentum	Cryopreserved	Pre- chemo	NA	NA	18.0% (140.0)	87.0% (50.0)
Maximum live cell yield pre-DCR				52.0%		59.0%	
Maximum live cell yield post-DCR				55.0%		88%	

NP=neutral protease, C= collagenase, DCR=dead cell removal

*Moderate fibroadipose omental sample

**Highly fibroadipose omental sample

2.4.3 Optimisation of Automated GentleMACS Dissociation

2.4.3.1 Checking the effects of automated dissociation on cells

A control experiment (performed on 3 separate occasions) of automated dissociation using trypsinised cells from the OVCAR-8 cell line demonstrated that the automated dissociation protocol did not have a significant damaging effect on cell. A sample of the OVCAR-8 cells was processed through the dissociation and the control without any dissociation. Cell viabilities for these two samples were comparable (mean live cells pre-DCR 95.5% and post-DCR 98.5% for automated dissociation; pre-DCR 94.5% and post-DCR 95.5% for non-dissociated cells) (Figure 2.7).

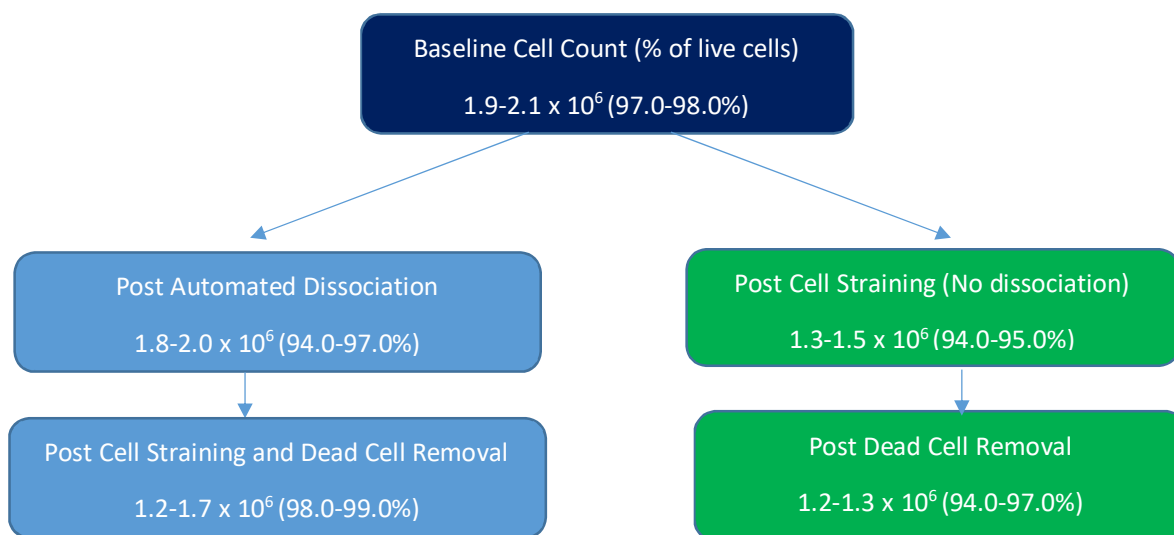


Figure 2.7 Control Experiment using Trypsinised OVCAR-8 Cell Lines to Check the Effects of Automated Dissociation on the Cells.

2.4.3.2 Experiments to optimise the automated GentleMACS dissociation protocol

From the above experiments, we decided to proceed with the automated GentleMACS dissociation protocol as it was shown to produce higher cell viabilities when compared to our in-house manual method. We went on to optimise the automated dissociation to adapt to the nature of our tissue samples mainly focusing on the primary ovarian and omental sites. Through these optimisation experiments, we discovered that specific to our samples, automated tumour dissociation followed by DCR, RBC lysis and second DCR (especially if cell viability <70%) was more effective in producing a higher live cell yield compared to dissociation-RBC lysis-2nd DCRs (Table 2.6). Following tumour dissociation, samples should not be left sitting on ice for prolonged periods of time as this may result in reduced cell viability (experiment A1.2, Table 2.6) but rather if possible further processing should be carried out simultaneously for multiple samples to avoid hypoxic stresses. Cryopreservation in 10% DMSO, 10% FCS, 80% DMEM was more effective in maintaining cell viability compared to 20% DMSO, 40% FCS, 40% RPMI media. Sample selection should ideally avoid tissues which were excised using diathermy as this could cause more thermal damages resulting in reduced cell viability after dissociation processes (experiment A4.2, Table 2.6).

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Table 2.6 Experiments Optimising Automated GentleMACS Dissociation Protocol

Experiment	Sample Site	Sample State	Surgery	Process	No. of live cells x 10 ⁵ / ml	% of Live Cells	Comments
Experiments A1.1-A3.4 (except for A1.3) are from the same patient and adjacent sites for the ovarian and omental samples respectively							
A1.1	Ovary	Fresh	Pre-chemo	Tumour dissociation	481.0	67%	TD-DCR-RBC lysis-DCR yielded high cell viability
				1st DCR	176.0	44%	
				1st wash	195.0	44%	
				2nd wash	119.0	47%	
				RBC lysis	83.9	64%	
				2nd DCR	60.8	90%	
A1.2	Ovary (Part of sample A1.1 after tumour dissociation)	Fresh	Pre-chemo	Tumour dissociation (Sit in ice for 4 hours after tumour dissociation)	481.0	67%	Sample should be processed immediately after tumour dissociation to avoid further hypoxic damage
				1st DCR	100.0	38%	
				RBC lysis and 2nd DCR	78.0	75%	
A1.3	OVCAR-8 cell line	Trypsinised	NA	Baseline cell count	16.0	97%	Control to show that automated dissociation process does not impact on cell viability
				Tumour dissociation	15.0	97%	
				DCR	14.0	99%	
A1.4	Omentum	Fresh	Pre-chemo	Tumour dissociation	462.0	49%	Lack of RBC lysis may result in reduced cell viability
				1st DCR	394.0	36%	
				1st wash	389.0	36%	
				2nd wash	300.0	35%	
				RBC lysis	NA	NA	

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				(Not done as sample was not red looking)			
				2nd DCR	10.0	1%	
A2.1	Ovary	Cryopreserved in 20% DMSO, 40% FCS, 40% RPMI	Pre-chemo	Tumour dissociation and RBC lysis	552.0	73%	TD-RBC lysis-2DCRs may be less effective compared to TD-DCR-RBC lysis-DCR
				1st DCR	324.0	61%	
				2nd DCR	29.6	61%	
A2.2	Ovary	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre-chemo	Tumour dissociation and RBC lysis	46.7	39%	Cryopreservation in 10% DMSO may be more effective in maintaining cell viability
				1st DCR	32.6	71%	
				2nd DCR	1.4	75%	
A2.3	Omentum	Cryopreserved in 20% DMSO, 40% FCS, 40% RPMI	Pre-chemo	Tumour dissociation and RBC lysis	10.9	40%	
				1st DCR	1.7	57%	
				2nd DCR	2.1	56%	
A2.4	Omentum	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre-chemo	Tumour dissociation and RBC lysis	4.2	4%	Cryopreservation in 10% DMSO may be more effective in maintaining cell viability
				1st DCR	1.4	61%	
				2nd DCR	1.0	65%	
A3.1	Ovary	Cryopreserved in 20% DMSO, 40% FCS, 40% RPMI	Pre-chemo	Tumour dissociation	93.7	23%	TD-DCR-RBC lysis-DCR yielded higher viable cells compared to TD-RBC lysis-2DCRs
				1st DCR	127.0	61%	
				RBC lysis	114.0	62%	
				2nd DCR	15.3	74%	
A3.2	Ovary	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre-chemo	Tumour dissociation	108.0	48%	Cryopreservation in 10% DMSO may be more effective in maintaining cell viability
				1st DCR	123.0	80%	
				RBC lysis	105.0	82%	
				2nd DCR	18.2	87%	

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A3.3	Omentum	Cryopreserved in 20% DMSO, 40% FCS, 40% RPMI	Pre- chemo	Tumour dissociation	14.0	35%	TD-DCR-RBC lysis-DCR yielded higher viable cells compared to TD- RBC lysis- 2DCRs
				1 st DCR	11.2	60%	
				RBC lysis	10.0	65%	
				2 nd DCR	6.3	70%	
A3.4	Omentum	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre- chemo	Tumour dissociation	27.8	40%	Cryopreservation in 10% DMSO may be more effective in maintaining cell viability
				1 st DCR	20.0	68%	
				RBC lysis	18.3	69%	
				2 nd DCR	8.5	75%	
Experiment A4.1 and A4.2 are from 2 different patients							
A4.1	Ovary	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre- chemo	Tumour dissociation	53.8	73%	TD-DCR-RBC lysis-DCR is effective at yielding high cell viability
				1st DCR	75.8	87%	
				RBC lysis	11.3	84%	
				2nd DCR	8.1	96%	
A4.2	Ovary	Cryopreserved in 10% DMSO, 10% FCS, 80% DMEM	Pre- chemo	Tumour dissociation	17.0	17%	Sample was a biopsy excised using diathermy. Poor viability likely due to thermal damage
				1st DCR	2.7	18%	
				RBC lysis	0.9	26%	
				2nd DCR	0.5	30%	

2.4.3.3 Importance of Dead Cell Removal (DCR) Step

DCR step was important in improving the final cell viability of the single cell suspensions after tumour dissociation. This was demonstrated in experiments MA1.1-MA1.14 and A1.1-A4.2 where the cell viabilities improved significantly post-DCR (median cell viability pre-DCR 37.5% and post-DCR 63%, Wilcoxon matched-pairs signed rank test P-value <0.0001). When automated dissociation experiments were assessed alone pre-and post-DCR, the cell viabilities improved further (median cell viability pre-DCR 39% and post-DCR 74%, Wilcoxon matched-pairs signed rank test

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P-value <0.0001) (Figure 2.8). Furthermore, Automated dissociation followed by DCR produced a purer single cell suspension as examined by microscopy (Figure 2.9).

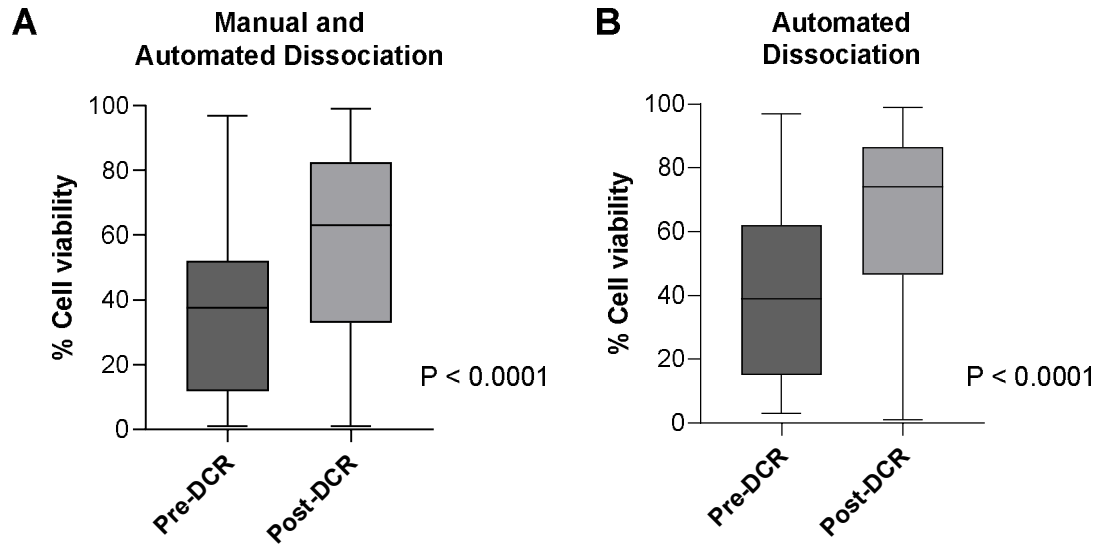


Figure 2.8 % Cell Viability Pre- and Post-DCR. (A) Both manual and automated dissociation cell viabilities pre- and post-DCR. (B) Only automated dissociated cell viabilities pre- and post-DCR.

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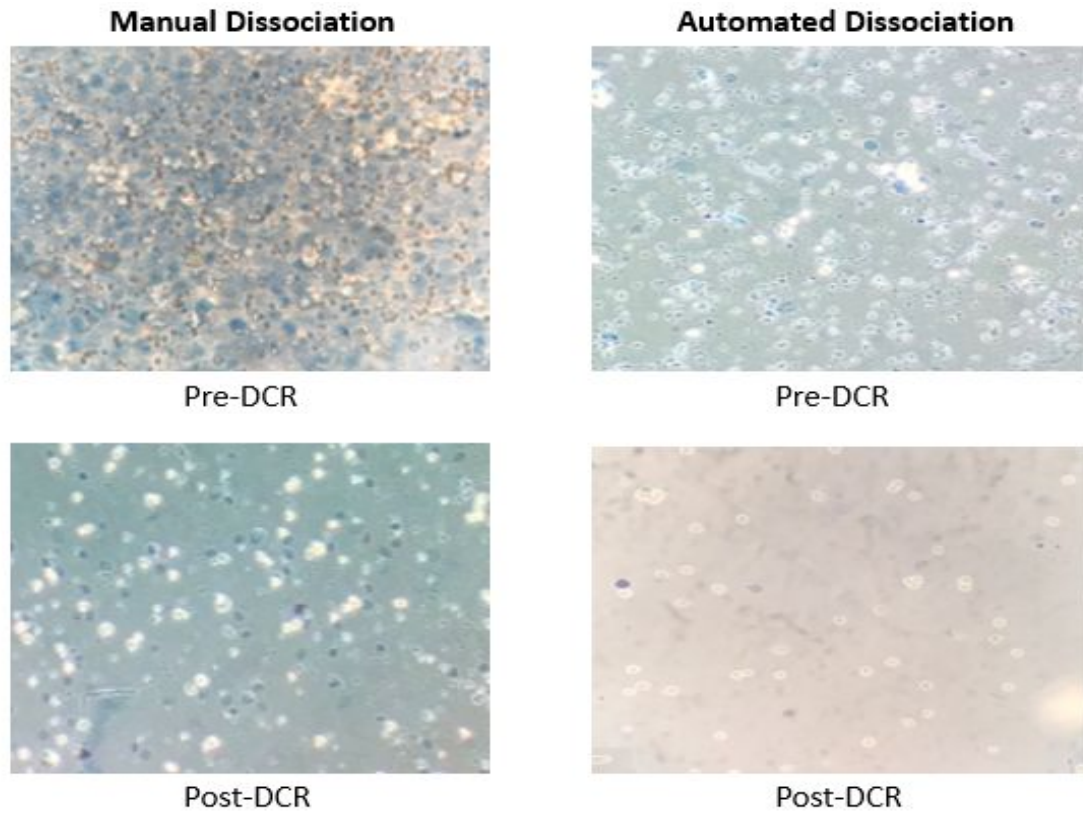


Figure 2.4 Microscope Images of Cells Following Manual and Automated Dissociations Pre-and Post-DCR. Automated dissociation followed by DCR produced a purer single cell suspension evident from bottom right image.

2.4.3.4 Fresh vs Cryopreserved Samples Cell Viabilities

After manual or automated dissociation, pre- and post-DCR, cell viabilities for fresh and cryopreserved samples were not significantly different from each other. For manual dissociation, pre-DCR median cell viabilities were 37.0% for fresh and 38.2% for cryopreserved, Mann-Whitney U test P value 0.5845; and post-DCR median cell viabilities were 34% for fresh vs 28% for cryopreserved, P value 0.7300. For automated dissociation, pre-DCR median cell viabilities were 49.0% for fresh and 29.0% for cryopreserved, Mann-Whitney U test P value 0.3873; and post-DCR median cell viabilities were 85.0% for fresh vs 82.3% for cryopreserved, P value 0.2360 (Figure 2.10). Nevertheless, the directionality of cell viability favoured automated dissociation compared to a manual protocol as demonstrated in 2.4.2.

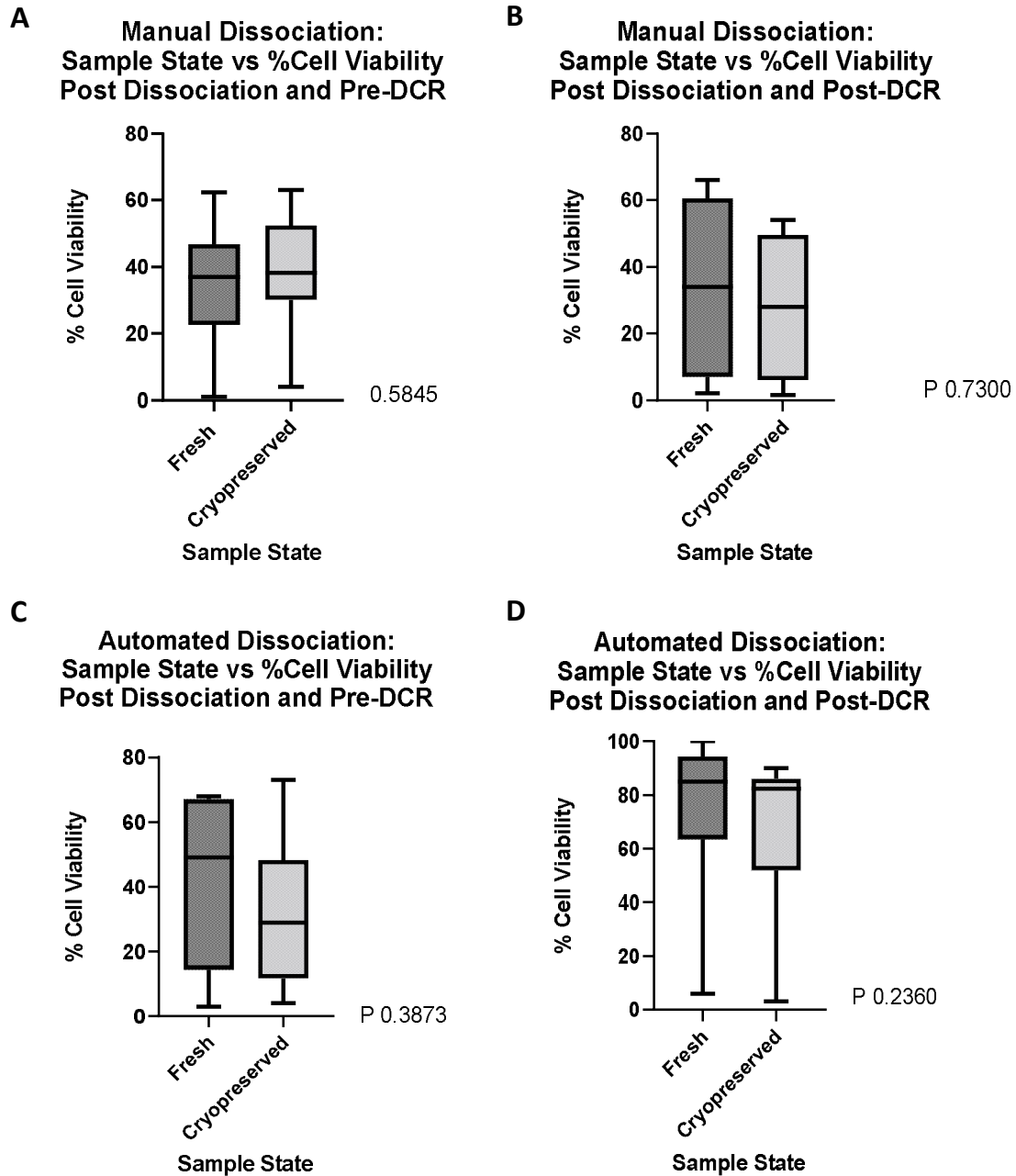


Figure 2.10 Cell Viability of Fresh vs Cryopreserved Samples. (A) and (B) Cell viabilities following manual dissociation, pre- and post-DCR (median cell viabilities for fresh samples 37% and 34% vs cryopreserved samples 38.2% and 28%, Mann-Whitney U test P values 0.5845 and 0.7300 respectively). (C) and (D) Cell viabilities following automated dissociation, pre- and post-DCR (median cell viabilities for fresh samples 49% and 85% vs cryopreserved samples 29% and 82.3%, Mann-Whitney U test P values 0.3873 and 0.2360 respectively).

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2.4.4 Quality Control Checks

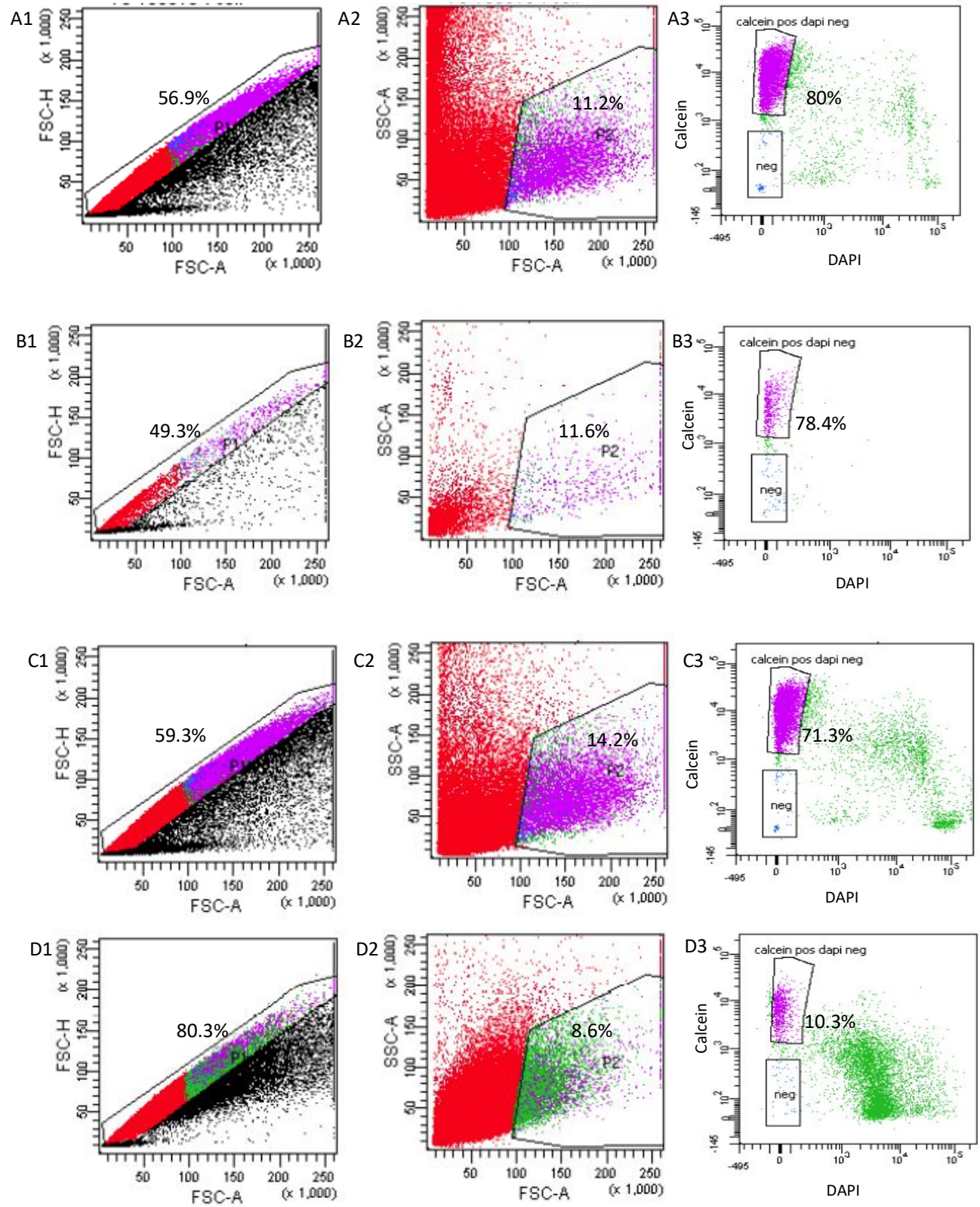
2.4.4.1 FACS sorting for live cells following dissociation

As part of the quality control check following manual dissociation, alongside measuring cell viability using the automated cell counter Countess II, samples were stained using DAPI and/or calcein to select for live singlets (Figure 2.11).

2.4.4.2 FACS analysis showing purer live single cells following dead cell removal

Quality control checks were also performed using FACS analyses to show that live single cell populations were higher following dead cell removal other than measuring cell viabilities using Countess II (Figure 2.12).

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Figure 2.11 FACS Results of Cryopreserved Ovarian Sample from Experiment M2.6.

Sample (A) was dissociated with collagenase enzyme, (B) with liberase enzyme, (C) with neutral protease enzyme and (D) without any enzyme. (A1, B1, C1, D1) Forward scatter density plot identifies P1 as single cells and excludes doublets (black dots). (A2, B2, C2, D2) Forward vs side scatter density plot identifies P2 as single cells of interest and excludes debris (red dots where these are smaller in size). (A3, B3, C3, D3) DAPI vs calcein plot identifies live cells as DAPI/calcein⁺ (10.3%-80% are the live fraction of P2).

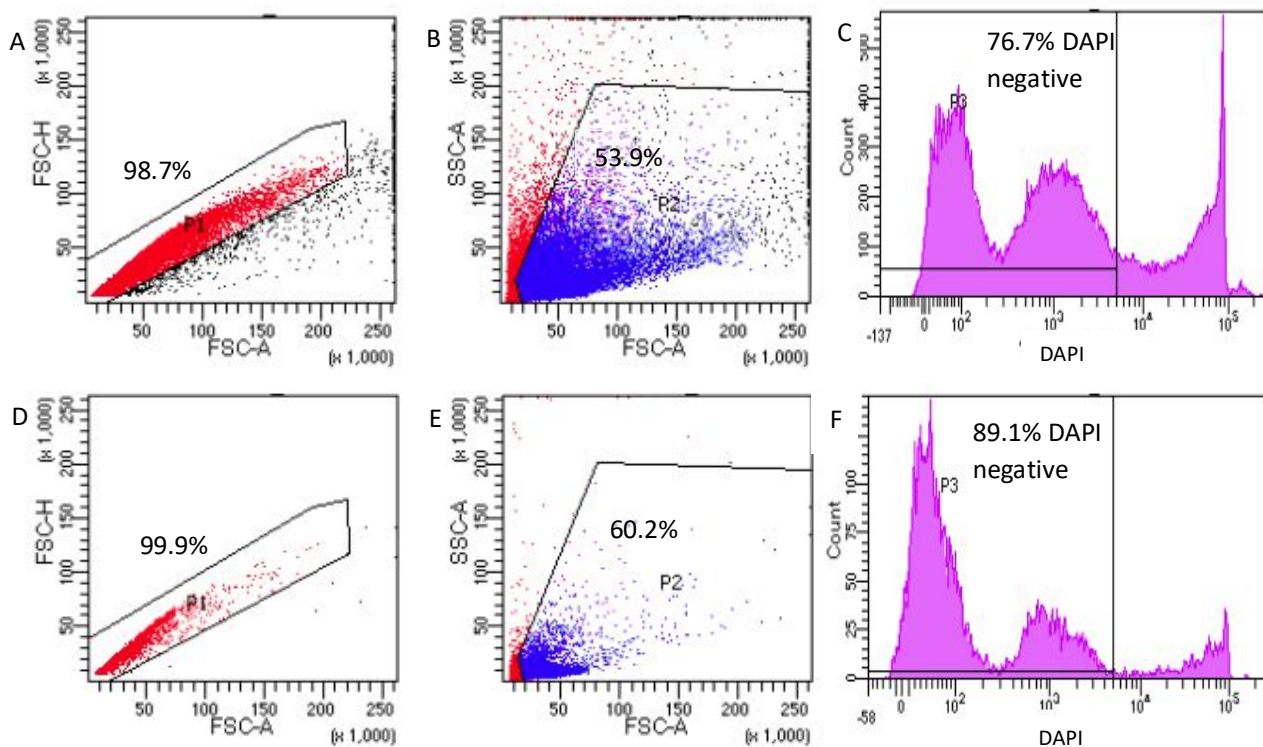


Figure 2.12 FACS Results of Cryopreserved Ovarian Sample from Experiment MA1.6.

(A-C) Sample after automated tumour dissociation. Live single cells were identified with doublets and debris excluded. (D-F) Sample after dead cell removal. This sample was now purer compared to prior to dead cell removal with P1 in (D) containing 99.9% of singlets and less debris (red dots) in (E). (F) has a smaller DAPI positive peak, hence more dead cells eliminated.

2.4.4.3 FACS sorting and analysis to select for cells of interest using antibody markers

2.4.4.3.1 Fluorescence Minus One (FMO) controls

FMO controls were performed at the beginning of the experiments when using antibody markers to identify cell population of interest (Table 2.7); these data were used to determine the cut-off point between background fluorescence and positive cell populations in multi-colour immunofluorescent flow cytometry (Figure 2.13).

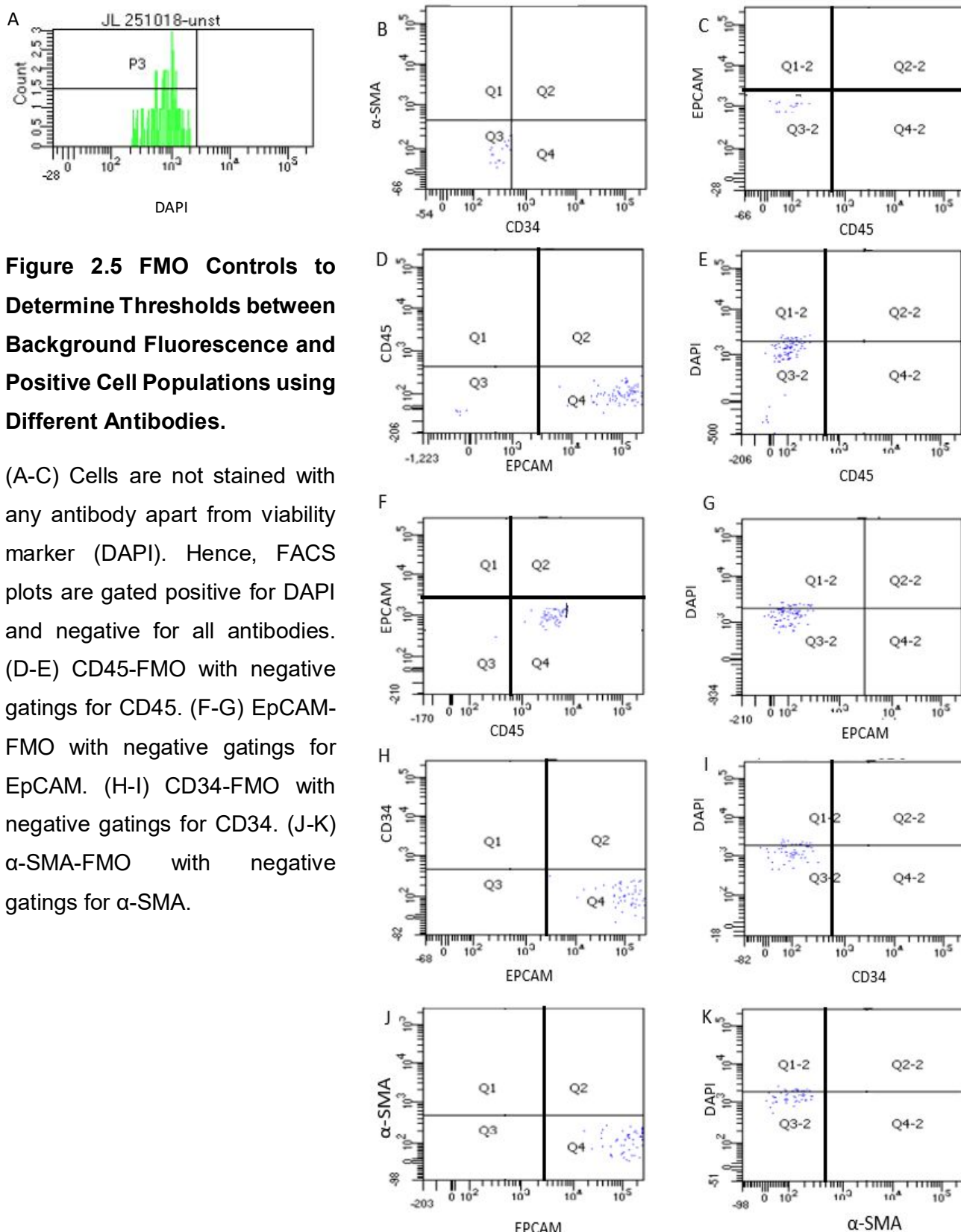
Table 2.7 Quantity of Antibodies and DAPI stain Used for FMOs

FMO	CD45 (µl)	EpCAM (µl)	CD34 (µl)	α-SMA (µl)	DAPI (µl)
Unstained cells	-	-	-	-	1
CD45-FMO	-	2	2	0.5	1
EpCAM-FMO	2	-	2	0.5	1
CD34-FMO	2	2	-	0.5	1
α-SMA-FMO	2	2	2	-	1

2.4.4.3.2 FACS sorting and analysis following manual dissociation

After manual dissociation, FACS sorting was used to select for live immune and non-immune cells (including tumour, stromal and endothelial cells) (see Figure 2.14 for gating strategies). These cells were then pooled together and input into the single cell libraries.

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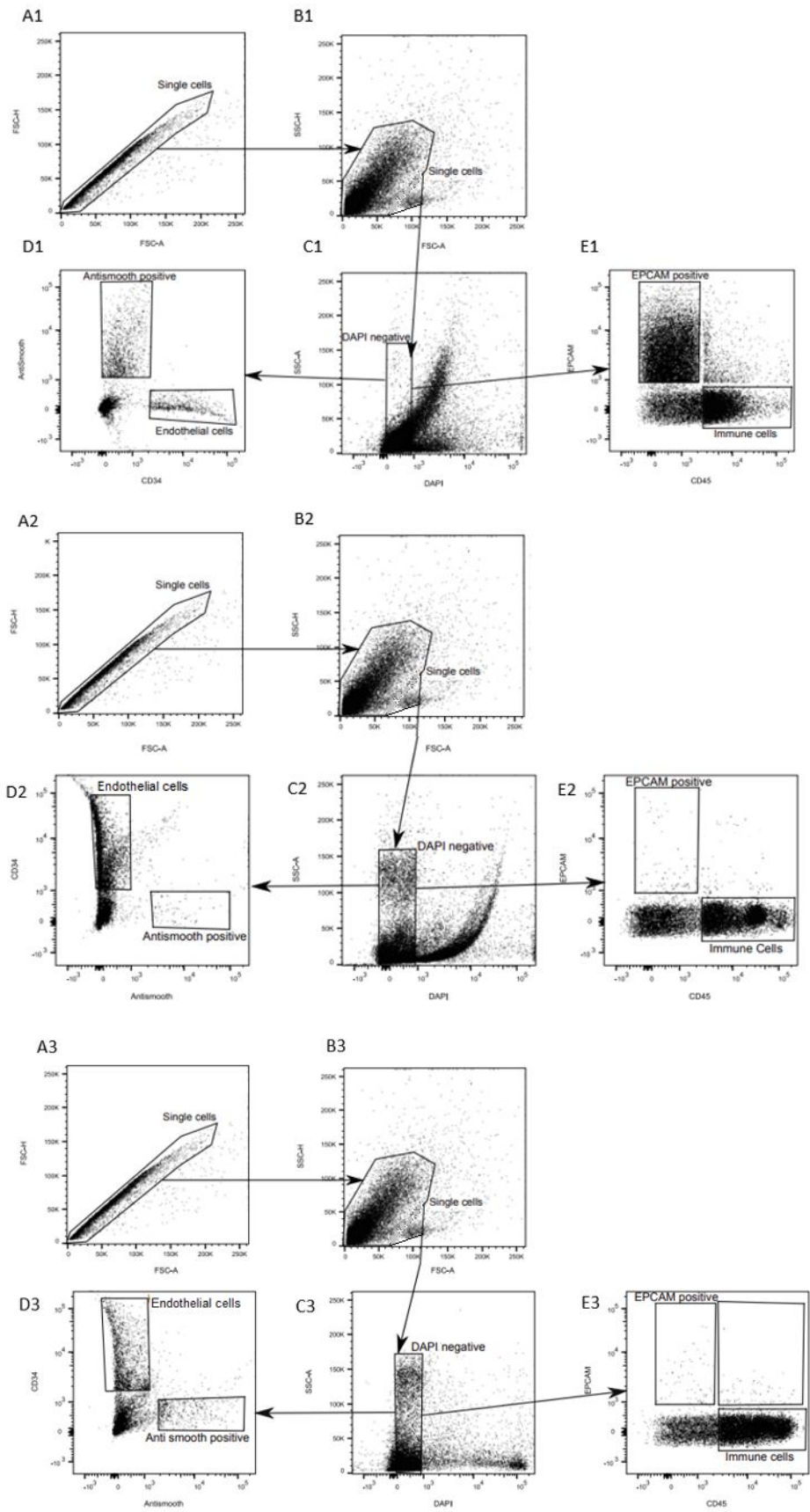


Figure 2.6 FACS Sorting and Analysis for Immune and Non-immune Cells. (A1-E1) Primary ovarian sample. (A2-E2) Omental sample. (A3-E3) Metastatic nodule. (A1-3) FSC-A vs FSC-H density plots identify the single cells of interest and excludes doublets. (B1-3) Forward and side scatter density plots identify the single cell population of interest and excludes debris. (C1-3) DAPI vs side scatter plots identify the DAPI negative cells to exclude dead cells (DAPI positive). (D1-3) CD34 vs α -SMA plots identify endothelial cells (CD34+/ α -SMA-) and fibroblasts (CD34-/ α -SMA+). (E1-3) CD45 vs EpCAM plots identify tumour cells (CD45-/EpCAM+) and immune cells (CD45+/EpCAM-) where (E1) from primary ovarian sample has the most abundant epithelial tumour cells compared to (E2) omental and (E3) metastatic nodule samples.

2.4.4.3.3 FACS analysis following manual and automated dissociation to check for cells of interest using antibody markers

It was important that the tissue samples that were processed contained tumour cells in order to investigate the HGSOc tumour microenvironment. This study had demonstrated that following enzymatic dissociation, FACS sorting to select for the cell populations of interest particularly tumour epithelial cells resulted in further reduction of the cell viability. Therefore, to overcome this problem, following manual and automated dissociations, the suspensions were passed through the tumour cell isolation kit and then incubated with antibodies (including anti-EpCAM/anti-CD45/anti-CD34/anti- α -SMA) and FACS analysed to retrieve the tumour epithelial population using antibody markers. Following both dissociation protocols, this QC check showed that cancer epithelial cells were present. However, the proportion of the tumour population after manual dissociation was smaller due to the lower number of live cells at the end of the processing compared to automated dissociation (Figure 2.15). Since at the end of both manual and automated processing there were tumour cells present, it was decided that FACS analysis was sufficient as a QC step rather than a cell selection tool. This allowed maintenance of higher viability cells prior to library

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preparation. FACS sorting has been shown from the above experiments (section 2.4.1.2, Table 2.4) to further deplete the live singlets which subsequently compromises the single cell library quality and downstream analysis.

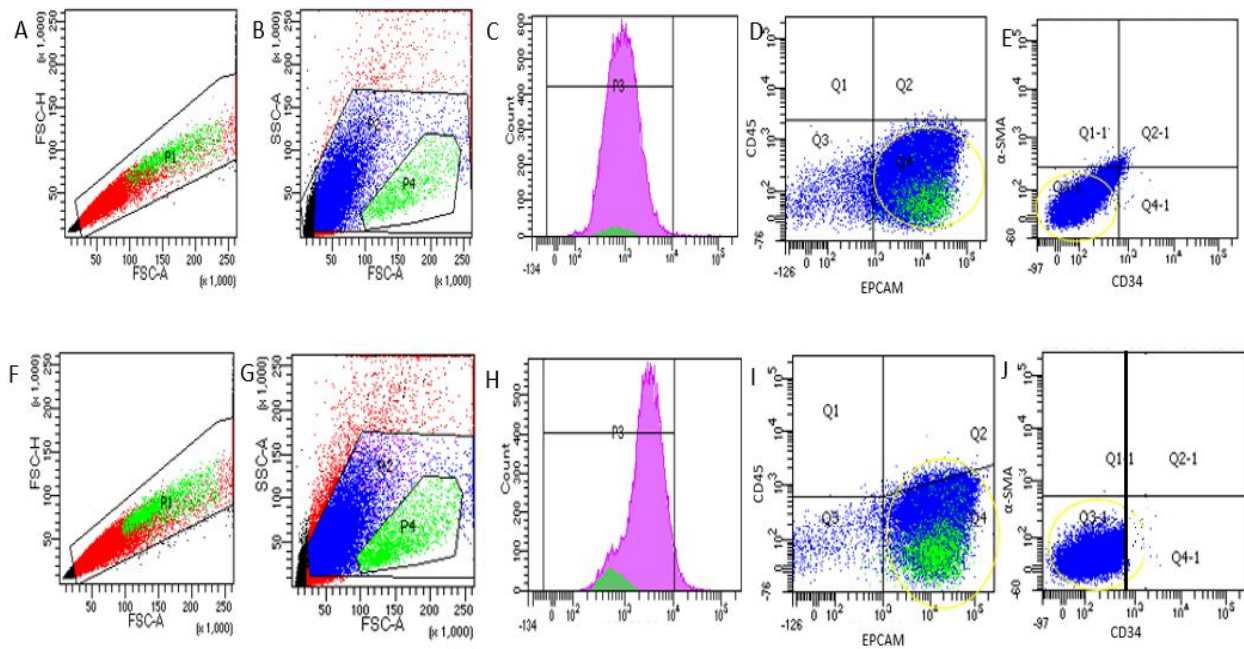


Figure 2.7 FACS Results of Cryopreserved Ovarian Sample from Experiment MA1.5. (A-E) This sample was processed through automated dissociation, dead cell removal and tumour cell isolation. At the end of these steps, Countess II measured a total of $3.46 \times 10^5/\text{ml}$ live cells. (F-J) This sample was from the same site and was processed using manual dissociation, followed by dead cell removal and tumour cell isolation. The live cells yield for this sample was $2.93 \times 10^5/\text{ml}$. (A-C) and (F-H) density plots identified live single cells. (D-E) and (I-J) show the live population of tumour cells for both samples identified by EPCAM⁺/CD45⁻ and CD34⁻/α-SMA⁻ respectively.

2.5 Discussion

Single cell RNA-sequencing is a powerful tool that allows for quantitative and qualitative analysis of the cellular composition of HGSOC to reveal the intra- and inter-tumour heterogeneity (Xu et al., 2022). Understanding the HGSOC transcriptomic landscape at single cellular level enables the fine molecular dissection of the cancer at an in-depth level which is not previously possible through bulk sequencing. This may potentially reveal specific biomarkers and signalling pathways responsible for the disease development and progression, thus allowing for directed targeted therapies tailored to individual patients. Single cell transcriptomic studies require a highly viable single cell suspension as a pre-requisite in order to minimise artefacts which could be introduced through dead cells with hypoxic stresses, as these cells could cause the mitochondrial oxidative genes to be read obscuring the actual biology of the tumours. Through extensive experiments, the protocol for processing fresh and cryopreserved tissue samples of ovarian cancer from multiple anatomical sites have been optimised in this study.

To start, working with human tissues requires a good understanding of the best way to harvest and handle the tissues once obtained. Each type of tissue is unique and tissue handling should be tailored specific to the nature and origin of the tissue. In terms of tissue harvesting, it will be ideal if the surgeon dissects the tissue of interest using methods which have minimal impact on tissue hypoxia such as scalpel excision rather than diathermy, ligating blood vessels close to the tissue site using suture instead of thermal instruments where possible, facilitate handing over the tissue once excised to the tissue operator for further processing to minimise tissue resting ischaemia, and providing adequate sample size for the single cell study purposes whilst saving enough tissue for diagnostic pathology and other research if applicable.

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With regard to tissue handling, ovarian tissue preferably should be taken from the cortex of the ovary and avoiding the tough capsule which had been shown to result in lower number of viable cells despite different enzymatic, manual or automated dissociative methods. For the omental tissue, fibro-fatty adipose tissues were found to be more fragile and often did not withstand the dissociation process. This observation was also noted in other studies supporting that the mature adipocytes were too large and fragile to withstand traditional single cell approaches, resulting in these groups focusing on the non-adipocyte stromal-vascular fraction or developed alternative strategy involving single nucleus sequencing (Emont et al., 2021; Sun et al., 2020; Vijay et al., 2020). The metastatic nodules were commonly smaller in size compared to ovarian or omental samples and required minimal or no mincing at all prior to dissociation. Tumour sections should be selected from parts with minimal or absent necrosis as these areas naturally have higher composition of dead cells. A feature of necrotic areas is the darker, softer and 'mash-like' consistency, usually located in the inner parts of the tumour mass (Sun et al., 2020). The avoidance of necrotic areas is a common practice in tumour dissociation for single cell -omic analyses in studies of other cancers such as breast cancer and glioblastoma to eliminate bias from necrotic contamination (Jacob et al., 2020; Sun et al., 2020).

Working out the logistics for the transport of the samples from the operating theatre to the laboratory is highly important to minimise transfer time between harvest to tissue processing. Prior agreement with the pathology department was crucial as it allowed samples to be transported directly from operating theatre to the laboratory. Due to complex debulking surgeries with prolonged hours, a system was devised in the event of delays in obtaining subsequent tissue sites, a separate tissue collector was available to collect and deliver the samples using the same handling protocol whilst

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the primary samples were transported to the laboratory for immediate processing. From the laboratory perspective, it was crucial to ensure that all the required reagents/equipment were prepared. The Category 2 blood room, FACS facilities and 10x Chromium machine were booked in advance for sample processing once these arrived at the laboratory. The occasional malfunction of the FACS machine was not allowed to delay or prevent the first step of the single cell library generation after the single cell suspensions were obtained as this could impact on the viability of the cells post dissociation. A small proportion of the single cell suspension was cryopreserved and FACS analysed at a later stage to confirm the presence of the cells of interest. Additionally, this optimisation study demonstrated that cell sorting by flow cytometry was not necessary and could in fact lead to loss of fragile cells. Passing the fragile single cells through the high pressured FACS stream potentially resulted in lysing the cells further causing cell death. A human liver dissociation study observed similar effect of cell loss caused by cell sorting (using density gradient, column purification or flow cytometry) which enriched for more resilient cells (MacParland et al., 2018). FACS staining and analysis were performed without the presence of a qualified FACS technician once these skills were mastered. This proved to be crucial as samples often arrived to the laboratory after working hours. It remained a challenge to process all the samples fresh on every occasion due to the time-consuming dissociation protocols which on average required approximately 3.5-4 hours. Collection of samples for surgeries performed in the afternoon posed difficulty for same day processing. An optimised cryopreservation method was developed to overcome this issue. From this optimisation study, the cell viabilities for fresh and cryopreserved samples were comparable following automated dissociation, pre- and post-DCR (median cell viabilities for fresh samples 49% and 85% vs cryopreserved samples 29% and 82.3%,

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Mann-Whitney U test P values 0.3873 and 0.2360 respectively). Once cryopreserved, there were no major difference in cell viability if the samples were processed the next day or even after a period of four weeks. For example, experiments MA1.13 and MA1.14 where the sample from adjacent omental sites of the same patient was cryopreserved and processed the next day and the other one month later, the final single cell suspension viability were 86% and 87% respectively. However, cryopreservation affects the complexity of the single cell library generated with lower number of cells and total genes recovered post-sequencing (see Chapter 3, section 3.5.9). Chapter 3 will further illustrate the effect of cryopreservation in increasing cell stress signatures (such as mitochondrial, ribosomal and apoptotic genes) compared to fresh tissues (section 3.5.9). Despite this, cryopreserved tissues were capable of displaying gene expression of cell types correlating to that uncovered from freshly analysed tissues (section 3.5.3.1). Similar observation were noted in studies of fresh and cryopreserved tissues (up to 6 weeks of cryopreservation) from different cancers including breast, prostate and melanoma (S. Z. Wu et al., 2021).

Comparison of the manual and automated dissociation methods described in this study demonstrated that the automated protocol consistently resulted in higher single cell suspension viabilities for both fresh and cryopreserved samples, hence the automated method was opted moving forward. Dead cell removal (DCR) was an essential step in cleaning up the debris and non-viable cells after dissociation. Performing DCR twice may be necessary especially for samples with less than 70% viable cells after 1st DCR step. Rigorous data from 10x Genomics and several studies have underscored the importance of DCR in removing non-viable cells and the recommended good practice of achieving a cell viability of >70% prior to scRNA-seq in order to increase the library complexity, improve fraction of reads in cells and

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accuracy in target cell count, and decrease mitochondrial transcripts (Genomics, 2022a; Newmeyer & Ferguson-Miller, 2003; Wang, 2001). All these metrics are vital to allow for the detection of the true biology of the disease. Red blood cell (RBC) lysis was commonly required even if the samples were not visibly red on gross inspection. In experiments where the samples were not visibly red hence did not undergo RBC lysis, the viability of the single cell suspensions was significantly compromised despite DCR due to RBC causing clumping of cells.

Analysing the in-house manual protocol, the enzymatic dissociation time may benefit from longer periods by increasing the 10 minutes incubation to at least 30 minutes-1 hour. This would allow more time for the enzymes to break down the tissue samples as presented in the automated GentleMACS protocol and other dissociation studies of human ovarian tissues for single cell RNA-seq (Biotec, 2021; Fan et al., 2019; Karst & Drapkin, 2012; Melissa Javellana, 2022; O'Flanagan et al., 2019; Olalekan et al., 2021; Wagner et al., 2020). When the different enzymes were compared, neutral protease was best for ovarian dissociation, collagenase for omentum and liberase for metastatic nodules. This supports our hypothesis that the tissues differ from each other in terms of their consistency and nature depending on the site of origin necessitating different enzymes for dissociation. Olalekan et al. in their study successfully dissociated fresh omentum using collagenase and hyaluronidase (through 0.5-2 hour enzymatic incubation) and produced a comparable transcriptomic read-outs to samples from this study (~1000 median genes per cell) which were processed via automated protocol (see batch 2 and 3 libraries in Chapter 3, Table 3.4) (Olalekan et al., 2021).

The automated GentleMACS protocol used the human tumour dissociation kit, TDK (Miltenyi Biotec) that contained a mixture of enzymes H, R and A which perhaps

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suggested that different enzymes should be combined together to maximise their digestive efficacy when faced with differing tissue types. Several other studies of ovarian single cell RNA-sequencing have utilised the human TDK (Miltenyi Biotec) with/ or without Miltenyi's GentleMACS dissociator with a high success in achieving transcriptomic read-outs for their investigation of interest including the dissociation of fallopian tube tissues, benign and malignant ovarian tumours (Geistlinger et al., 2020; Hu et al., 2020; A. J. Shih et al., 2018). For example, the study by Shih et al. reported a yield of >85% live cells when dissociating cryopreserved ovarian tumours using the automated GentleMACs protocol (which was comparable to the cryopreserved cell viability yield here) but only a total of 2911 single cells were captured using the BioRad droplet digital SEQ Single-cell Isolator platform (in comparison to ~134 306 cells profiled in this study) (A. J. Shih et al., 2018). Additionally, in terms of transcriptomic read-outs, compared to study by Shih et al. this study was able to further define subclusters of cells within major cell types of the HGSOC TME with the larger number of cells and more complex libraries (greater fraction of reads per cell/deeper sequencing depth) uncovered here, most likely attributed to the inclusion of freshly analysed HGSOC tissues and further optimised automated GentleMACS protocol described above.

Due to the consistent high cell viability after dissociation, the automated (adapted and optimised GentleMACS) protocol was adopted in the tumour dissociation here. The automated method was also more efficient as up to 8 samples could be processed simultaneously and more homogenised dissociation could be performed due to intermittent blending action of the GentleMACS dissociator whilst the samples were incubated at 37°C rotor. In contrast, the manual method lacked this built-in tissue blending rotor whilst the tissues were incubating in the digestive enzymes. Additionally,

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it was important to perform straining of the dissociated suspension using the correct size strainer (i.e. 70 μm) which was a common step in both manual and automated protocol. This helped to remove large residual clumps of debris or cells which may hinder the dead cell removal step due to blockage of the magnetic filtration columns. Throughout the handling of the samples, the suspensions were gently pipetted in each step to avoid mechanical shredding of the cells which may result in reduced cell viability.

Essentially, cellular hypoxia and ischaemia were the prime concerns during the optimisation of the tissue dissociation into single cell suspensions. To minimise this, optimal logistical steps for tissue harvest, transport, storage and processing were devised to reduce both the resting tissue ischaemia and iatrogenic sources of cellular hypoxia. Working with human samples remained a challenge and limitation for this study as they could be unpredictable in nature attributed to various factors such as surgeon dependency during tissue harvest, patient heterogeneity and operator dependency during tissue processing. However, these were minimised with rigorous protocols set up from tissue harvest through to processing including the use of automated dissociation to reduce operator handling bias. Another limitation of this study was the inability to perform a large number of experiments due to the nature of collecting live human tissues. Hence, the power to detect significant differences may be limited including the lack of possibility to study the impact of the dissociation methods on samples from multiple patients being performed simultaneously. Nevertheless, when compared to other published literature which utilised the GentleMACS methodology in dissociating ovarian tumours for scRNA-seq investigations (Geistlinger et al., 2020; Hu et al., 2020; A. J. Shih et al., 2018; Wagner et al., 2020), this study offers additional advantage whereby subtle but highly vital

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steps of improving cell viabilities and quality of live single cell suspensions were described in detail ranging from tissue harvesting, handling to processing tailored to specific tissues according to their site of origin.

A study by Flanagan et al. comparing the tumour dissociation of breast and ovarian cancer using cold active protease (CAP) and collagenase found that the stress response pathways were minimised through CAP dissociation (O'Flanagan et al., 2019). Future studies should seek to analyse the transcriptomic read-outs from the adapted automated protocol above and further optimised manual enzymatic method (particularly with trials of increased duration of incubation) to investigate the effects of these dissociation on the actual transcriptomes of the cells.

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CHAPTER 3: SINGLE CELL RNA-SEQUENCING TO ANALYSE THE TUMOUR MICROENVIRONMENT (TME) OF HGSOC

3 CHAPTER 3: SINGLE CELL RNA-SEQUENCING TO ANALYSE THE TUMOUR MICROENVIRONMENT (TME) OF HGSOE

3.1 Introduction

3.1.1 Single cell RNA-sequencing (scRNA-seq) in OC

ScRNA-seq performs transcriptome-wide RNA analysis on individual cells within the TME (Talukdar et al., 2021). Bulk RNA sequencing measures the average or aggregate RNA signals of thousands of cells in the TME, hence overlooking the molecular diversity at individual cellular level. OC is a highly heterogeneous disease caused by intratumour evolution, cellular plasticity, profound chromosomal instability and multiple sources of stochastic variability (J.-Y. Lee et al., 2015) leading to frequent relapses, treatment resistance and eventual death in patients with widespread disease. ScRNA-seq in OC offers a powerful tool to dissect the tumour and characterise individual cells molecularly enabling the detection of rare subpopulations of cells with clonal diversity accounting for disease relapse and treatment resistance.

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3.1.2 Evolution of scRNA-sequencing technology

There are many platforms for single cell RNA-sequencing. Tang et al. published the first transcriptomes generated using mRNA-seq of a single cell which was shortly after the first RNA-seq was performed on bulk samples (Tang et al., 2009). Since then, the technique has evolved over the last decade to encompass higher throughput involving studies of over a million cells in parallel (Jovic et al., 2022; Nagasawa et al., 2021).

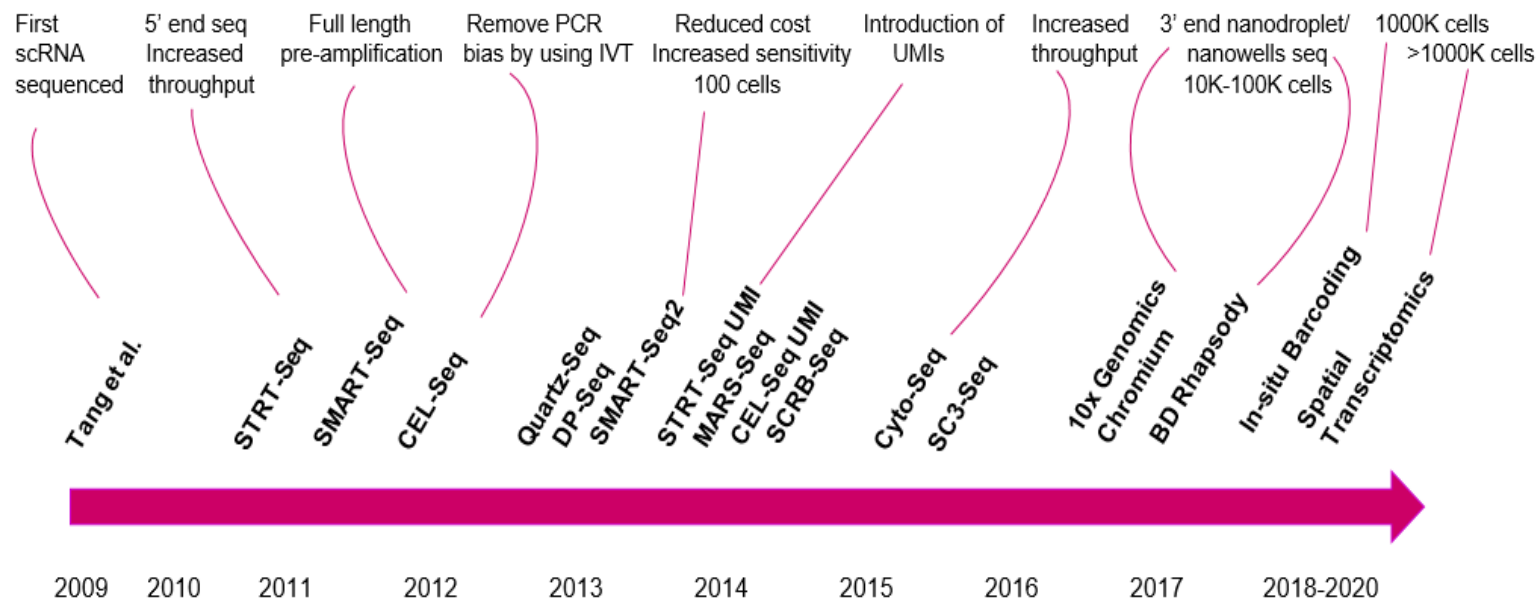


Figure 3.1 Evolution of Single Cell RNA-sequencing Technology (Jovic et al., 2022).

3.1.3 Basic principles of scRNA-sequencing

The basic principles involved in scRNA-seq are as follows (Figure 3.2).

1. Tissue dissociation into single cells
2. Single cell capture and lysis
3. Reverse transcription of mRNA which are selected by polyT priming to generate cDNA
4. cDNA amplification by polymerase chain reaction (PCR) or in vitro transcription (IVT)
5. Generation of sequencing library using amplified cDNA

Following capture and lysis of single cells, scRNA-seq is performed by reverse transcription (RT), cDNA amplification and sequencing of prepared library. In scRNA-seq, most published RT use polyT priming method, except for designed primer-based RNA-sequencing, DP-Seq (Bhargava et al., 2013; Kolodziejczyk et al., 2015). Second strand cDNA synthesis can be performed using two methods; either polyA tailing (Tang protocol, CELL-Seq and QuartzSeq) or template switching (“switching mechanism at 5’ end of RNA template” in SMART-Seq, 10x Genomics Chromium and single-cell tagged reverse transcription in STRT-Seq) (Kolodziejczyk et al., 2015). The main advantage of template switching is the coverage of full-length transcripts with reduction of 3’ coverage biases secondary to incomplete RT. SMART-Seq2 is an improved version of SMART-Seq with better optimised PCR. Template switching of full-length cDNA allows for study of alternative splicing, novel exon detection and allele specific expression (Kolodziejczyk et al., 2015).

After RT, cDNA amplification can be done by either PCR (used in SmartSeq, STRT, the Tang protocol, 10x Genomics Chromium, SC3-seq) (Islam et al., 2012; Nakamura et al., 2015; Picelli et al., 2013; Tang et al., 2009) or *in vitro* transcription (employed in

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CEL-seq and MARS-Seq) (Hashimshony et al., 2012; Jaitin et al., 2014). PCR is a non-linear amplification process, whereas IVT depends on linear amplification, but requires an additional round of RT which adds 3' coverage biases (Kolodziejczyk et al., 2015). Following cDNA library generation, sequencing can be performed on all commercial sequencing platforms with Illumina being the most commonly used by most protocols (Kolodziejczyk et al., 2015).

The introduction of unique molecular identifiers (UMIs) has improved the quantitative capability of scRNA-seq where each individual mRNA molecule within a cell is barcoded during RT (Fu et al., 2011). The number of UMIs associated with all tags that map to the transcript allow quantification of the number of copies of a transcript in a given cell lysate, hence the number of UMIs is considered as a direct representation of gene expression level (Islam et al., 2014). UMIs are utilised in the 10x Genomics Chromium method, STRT-Seq, CEL-Seq, MARS-Seq and CytoSeq protocols (Kolodziejczyk et al., 2015).

The two frequently used scRNA-seq platforms are SMART-Seq2 and 10x Genomics Chromium (See et al., 2018; Svensson et al., 2018) (see Table 3.1). SMART-Seq2 is highly reliable being one of the most successful methods in detecting single cell gene expression. Meanwhile, 10x Genomics is the cheaper alternative and the most commonly used platform.

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Table 3.1 Comparison of SMART-Seq2 and 10x Genomics Chromium

Protocol	Cost per cell US\$	Number of cells	Characteristics	Library
SMART-Seq2	11	96-384	Full length capture	Nextera
10x Genomics Chromium	12 for 1000 cells per run	100-100 000	3'-tag method	10x Genomics

(Baran-Gale et al., 2018)

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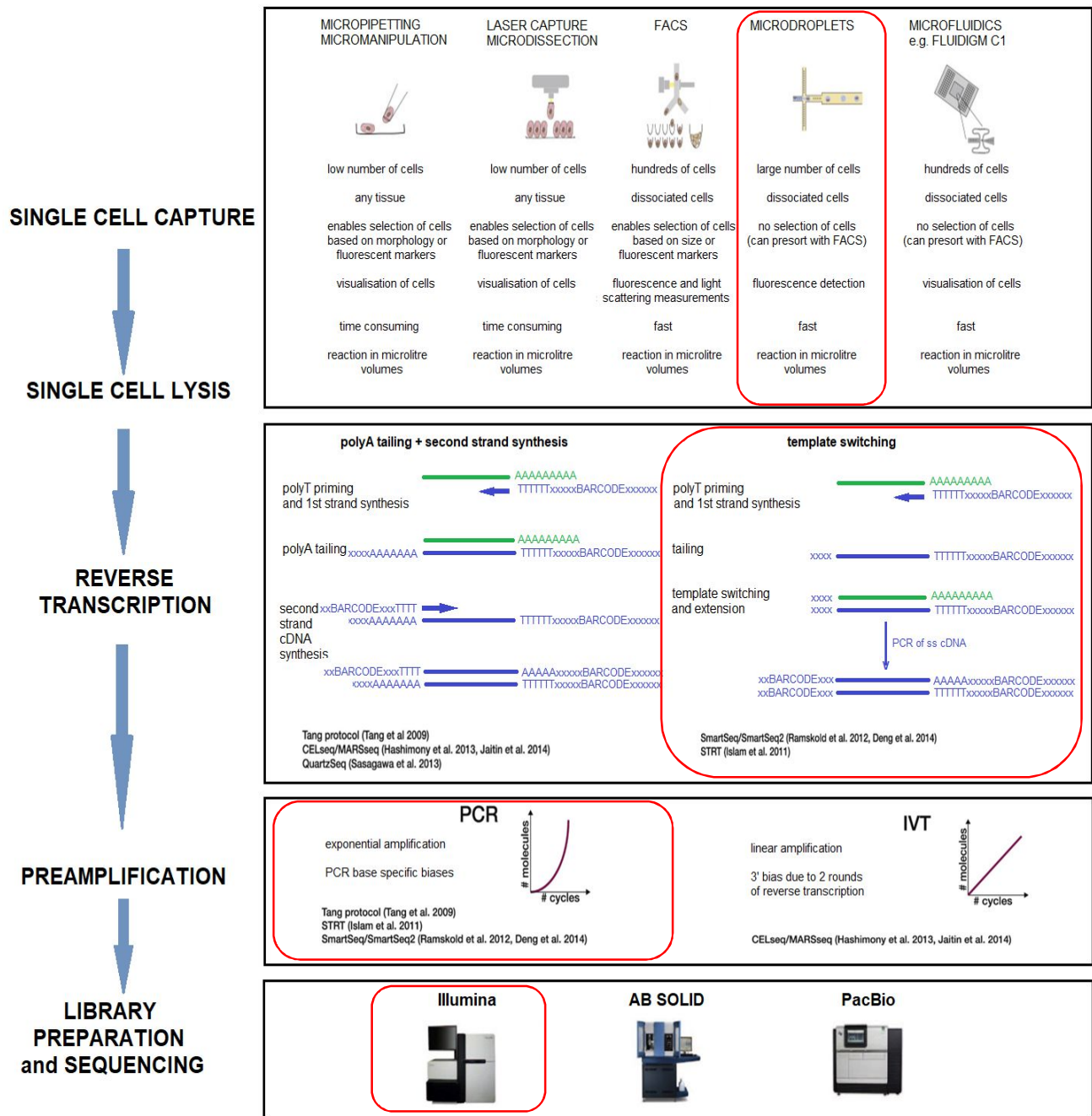


Figure 3.2 Basic Steps of scRNA-seq and Different Experimental Approaches. The principles of 10x Genomics Chromium and sequencing platform used in this project are reflected in red circles. Adapted from The Technology and Biology of scRNA-seq. Figure adapted from (Kolodziejczyk et al., 2015).

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This study has opted for the commercial 10x Genomics Chromium system which offers high-throughput profiling of 3' ends of RNAs of single cells with high capture efficiency at a lower cost. This high-throughput processing method is one of the most successful commercial platforms available which enables the analysis of rare cell types in a heterogeneous biological space. (Gao et al., 2020; Hwang et al., 2018)

Once the single cell suspension is obtained (with $\geq 70\%$ viable cells), the suspension is further processed using this nanodroplet-based microfluidics technique to capture and lyse the single cells. The 10x Genomics Chromium system relies on rapid droplet-based encapsulation of single cells using a gel bead in emulsion (GEM) approach. Each gel bead is labelled with oligonucleotides that consist of a 16 bp unique barcode, a 12 bp UMI, sequencing primers and an anchored 30 bp oligo-dT (Zheng et al., 2017). Within the GEM, the primers hybridise to the mature poly(A) tail of mRNAs released from the lysed single cells. The RT enzymes perform reverse transcription (RT) to form first strand complementary DNA (cDNA) which contains a UMI, shared barcode per GEM and a template switch oligo at the 5' end. The downstream processing involves breaking of the GEM, followed by polymerase chain reaction (PCR) amplification of barcoded cDNA, enzymatic fragmentation and library construction using adapter and sample indices. Read1 captures the 16 bp 10x Barcode and 12 bp UMI while Read2 contains the cDNA insert used to sequence the cDNA fragment. Index reads, I5 contain the cell barcodes and I7 contain the sample indices. The generated library is compatible with next-generation short-read sequencing and this platform is able to detect approximately 500-1500 genes per primary cell (Zheng et al., 2017) (see Figure 3.3).

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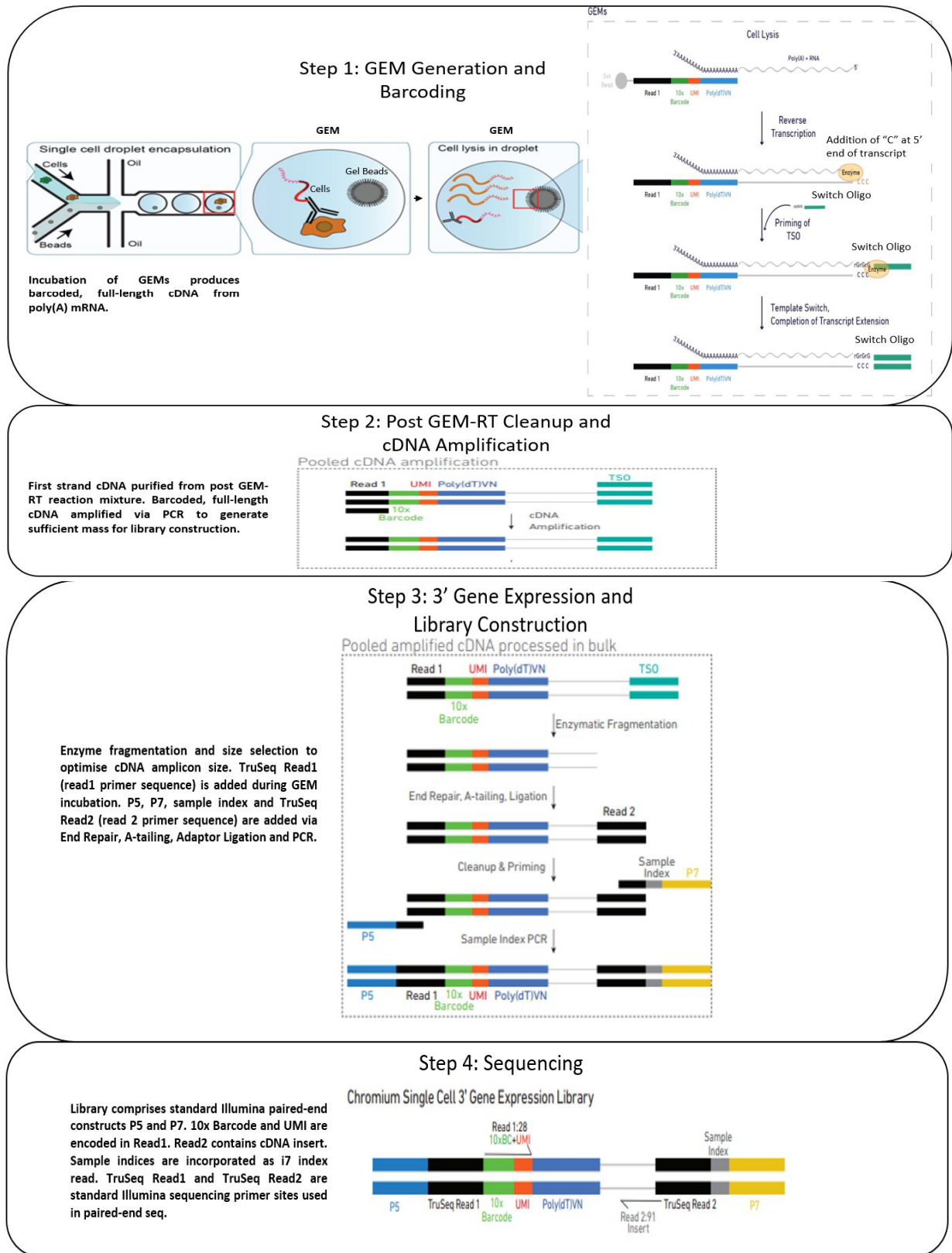


Figure 3.3 Stepwise Approach to 10x Genomics Chromium Single Cell 3' Protocol. Figure adapted from (Genomics, 2022b).

3.1.4 Division of work in Chapter 3

I led the recruitment of patients for this single cell study, the sample acquisition directly from the operating theatre through to optimisation of the tumour dissociation into single cells for RNA-sequencing, the library preparation, subsequent data conceptualisation and data interpretation in this Chapter. The work in sections (3.3.5.1, 3.3.5.2, 3.3.5.3, 3.3.5.4, 3.3.5.5 and 3.3.5.8) was led by Dr Thomas Parry which mainly covered the bioinformatics analysis.

3.1.5 Literature review: Summary of all relevant published work on scRNA-seq in ovarian cancer (OC)

In order to reflect and compare our scRNA-seq study of 8 HGSOC patients, an extensive search and literature review on all the relevant published work related to single cell transcriptomic studies of ovarian cancer was performed. This was done using the following terms on PubMed: 'single cell RNA sequencing', 'single cell transcriptomics', 'ovarian cancer', heterogeneity, 'high grade serous epithelial ovarian cancer' and 'tumour microenvironment'. A total of 15 most relevant studies were examined, summarised and comparison made to our work (see Table 3.2).

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Table 3.2 Summary of All Relevant Published Papers on scRNA-seq in OC.

No.	Paper Title (Citation)	Methodology					Summary of Key Findings	Comments
		Patient and sample details	Dissociation and sequencing platform	No. of cells	Bioinformatics Analysis	Additional studies		
1	Single cell sequencing reveals heterogeneity within ovarian cancer epithelium and cancer associated stromal cells (Winterhoff et al., 2017)	Fresh ovarian sample from 1 HGSOC patient (PDS)	Tumor Dissociation Kit, gentleMACs Dissociator (Miltenyi Biotec, Inc) and Fluidigm C1 chips.	66	Unsupervised hierarchical and K-means clustering using Cluster 3.0 and visualized using Treeview. Principle component analysis (PCA) using R. Geneset enrichment analysis using MSigDB. Comparison to TCGA molecular subtypes using R.	IHC on patient samples.	✓ 2 major cell types characterized by epithelial and stromal gene expression patterns. ✓ Epithelial group characterized by proliferative genes (oxidative phosphorylation and MYC activity). Stromal group increased expression of extracellular matrix, epithelial-mesenchymal-transition and cancer stem cells. ✓ No chemo-resistant genes. ✓ TCGA molecular subtype comparison classified most stromal as mesenchymal and epithelial cells as proliferative.	✓ First scRNA-seq paper on HGSOC.
2	Identification of grade and origin specific cell populations in serous epithelial ovarian cancer by single cell RNA-seq (A. J. Shih et al., 2018)	Cryopreserved samples from 9 patients, multi-sites and differing grades (high grade PDS/	Tumor Dissociation Kit, gentleMACs Dissociator (Miltenyi Biotec, Inc) and BioRad droplet digital SEQ.	2911	R software package Seurat	NA	✓ 16 cell populations with specific cells correlated to high grade tumors, low grade tumors, benign and one population unique to a patient with a breast cancer relapse. ✓ Shift of epithelial to leucocytes from primary to metastatic site. ✓ Comparison to TCGA molecular subtype with myeloid lineage cells defines immunoresponsive subtype, tumour fibroblast and stromal cells define	✓ Differentiation of benign vs malignant cell types within ovarian tumours.

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		IDS, low grade and benign)					mesenchymal and epithelial cells define differentiated.	
3	Single-cell sequencing in ovarian cancer: a new frontier in precision medicine (Winterhoff et al., 2019)	Review paper of scRNA-sequencing techniques, methodology of single cell isolation and sequencing, sequencing data analysis and potential applications in ovarian cancer.					✓ scRNA-seq application to OC includes identification of cell types, subtypes and molecular subtyping according to TCGA subtypes, immune microenvironment study, evolution of therapy resistance, investigation of OC stem cells, circulating tumour cells and metastatic dissemination. ✓ Molecular sub-typing may need to be done at single-cell level to be more prognostic. ✓ Single-exosome RNA-seq to study immune responses to immunotherapy.	✓ Review paper on scRNA-seq application in OC.
4	Probabilistic cell-type assignment of single-cell RNA-seq for tumor microenvironment profiling (A. W. Zhang et al., 2019)	Fresh samples from 2 ovarian sites of a HGSOE patient (PDS)	Protease at low temperature and MACs separator (Mitenyi Biotec, Inc) and 10x Genomics 5' gene kit.	5233	Developed CellAssign to annotate cells.	Also studied TME of follicular lymphoma	✓ CellAssign, a probabilistic model that leverages prior knowledge of cell-type marker genes to annotate single-cell RNA sequencing data into cell types. ✓ Epithelial cells displayed EMT signatures, immune signature with increased HLA and hypoxia (upregulated apoptosis and glycolysis).	✓ Methods paper on assignment of cell type
5	A single-cell landscape of high-grade serous ovarian cancer (Izar et al., 2020)	Fresh samples with 22 ascites from 11 HGSOE patients and 2	Ascites and tumour single cell isolation using own lab method. 10x Genomics 3' gene and	35957	Dimension reduction and clustering using tSNE and DBscan. InferCNV for copy number alterations (CAN) and TCGA subtype	Patient-derived and established OC cell line cultures for drug sensitivity testing.	✓ Ascites cells have immunomodulatory fibroblast sub-populations and dichotomous macrophage (M1/M2). ✓ Immunoreactive and mesenchymal TCGA subtypes reflect abundance of immune infiltrates and fibroblasts, rather than distinct subset of malignant cells.	✓ Insight into CAFs.

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		ovarian HGSOC tumours, combination of PDS/IDS/relapsed cases.	SMART-seq2 platforms.		classification using NMF clustering.		✓ Malignant cell variability could be explained by heterogenous CNA patterns or stemness. Inflammatory signature present in malignant cells. ✓ JAK/STAT-pathway inhibition (expressed in malignant cells and CAFs) had potent anti-tumour activity in primary cultures and PDX models.	
6	The Repertoire of Serous Ovarian Cancer Non-genetic Heterogeneity Revealed by Single-Cell Sequencing of Normal Fallopian Tube Epithelial Cells (Hu et al., 2020)	Combination of fresh and cryopreserved samples from 5 ovarian cancer patients and 5 endometrial cancer patients.	Tumor Dissociation Kit, gentleMACs Dissociator (Miltenyi Biotec, Inc) and SMART-seq2 platform.	~6000	R software packages for clustering and pseudotime analysis. Gene Ontology and pathway enrichment analyses. Deconvolution studies by CIBERSORT. CNVs analysis using HoneyBADGER.	Primary cell culture and organoid culture. IHC, bulk RNA-seq	✓ 5 Fallopian tube epithelial cell sub-types identified including cell cycle, EMT, differentiated, KRT17 and ciliated clusters. ✓ Culturing substantially alters single cell transcriptomes. ✓ Deconvolution identified EMT rich tumours linked to mesenchymal subtype, correlating with poor prognosis. Ciliated FTE cell type enriched in low grade tumours compared to high grade ones.	✓ Fallopian tube epithelial cell signatures are reflected in HGSOC subtypes.
7	Multimic Analysis of Subtype Evolution and Heterogeneity in High-Grade Serous Ovarian Carcinoma (Geistlinger et al., 2020)	Fresh ovarian samples from 5 HGSOC PDS patients.	Tumor Dissociation Kit, gentleMACs Dissociator (Miltenyi Biotec, Inc) and 10x Genomics 3' gene kit.	42000	R software packages for clustering, inferCNV for copy number analysis. Molecular subtype classification using consensusOV.	Bulk RNA-seq and single cell data from 1 fresh HGSOC from Winterhoff et al, 2017.	✓ Chronology of HGSOC subtype evolution studied using absolute copy number analysis, gene expression in TCGA and single cell analysis of 6 HGSOC tumours. ✓ Wide heterogeneity in tumour cell type composition drives bulk subtypes but within tumour epithelial subtypes there is a lack of intrinsic subtypes. ✓ Suggests the dismissal of HGSOC transcriptomic subtypes, instead replace with model of mixtures of subclones,	✓ Benchmark study highlighting bulk HGSOC subtypes likely due to heterogeneous cell types

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							somatic aberrations, immune and stromal cell proportions correlating with tumour stage and tissue origin.	within HGSOC tumour.
8	Single-cell EMT-related transcriptional analysis revealed intra-cluster heterogeneity of tumor cell clusters in epithelial ovarian cancer ascites (Kan et al., 2020)	Fresh ascites from 4 epithelial OC patients.	Ascites dissociation with TrypLE Express Enzyme and own lab protocol. Single-cell qPCR using OpenArray plates.	147	Unsupervised clustering using GAP statistics. Stromal and tumour scoring using ESTIMATE package.	Short-term culture of DTCCs (disseminated tumour cell clusters) and DSCs (disseminated single cells).	✓ Applying single-cell epithelial-to-mesenchymal transition (EMT)-related transcriptional analysis in 120 DSCs and 195 intra-cluster cells from 27 DTCCs, we demonstrated that DTCCs were heterogeneous cellular units comprised of epithelial tumor cells, leukocytes, and cancer-associated fibroblasts (CAFs). ✓ CAFs induced EMT of tumor cells via TGF-β signaling within the DTCC microenvironment. ✓ EMT causes chemoresistance and metastasis of DTCC tumour cells.	
9	Characterizing the tumor microenvironment of metastatic ovarian cancer by single-cell transcriptomics (Olalekan et al., 2021)	Fresh omentum from 6 OC patients (PDS/IDS).	Omental dissociation using collagenase V and Drop-seq platform.	9885	R package Seurat for cell clustering and CellAtlas for cell assignment. TCGA subtype correlation using Modular scores (Tirosh et al., 2016).	IHC tumour staining.	✓ 9 major cell types, including cancer, stromal, and immune cells (high T cell infiltration, high Tinf and low T cell infiltration, low Tinf). ✓ TOX-expressing resident memory CD8⁺ T (CD8⁺ Trm) and granulysin-expressing CD4⁺ T cell clusters are enriched in the high Tinf group. ✓ Presence of plasmablast and plasma B cell clusters, and NR1H2⁺ IRF8⁺ and CD274⁺ macrophage clusters, suggesting an anti-tumor response in the high Tinf group.	✓ Primarily study of immune TME of OC.
10	Single-cell transcriptomes reveal heterogeneity of high-grade	Fresh ovarian and omental samples from 2	Dissociation method not available. scRNA-seq using BD	13571	R package Seurat for cell clustering. Gene Ontology and KEGG enrichment analyses. InferCNV for CNV analysis.	Established cell lines culture and IHC tumour staining.	✓ 6 cell types (epithelial cells, fibroblast cells, T cells, B cells, macrophages, and endothelial cells). ✓ A subset of aggressive epithelial cells with hyperproliferative and drug resistant	✓ Tumour and stromal cells interaction.

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	serous ovarian carcinoma (Qian Hao et al., 2021)	HGSOC patients (PDS).	Rhapsody platform.		Pseudotime, SCENIC, CellPhone DB analyses.		potentials. Ciliated markers also identified in cancer epithelial cells. ✓ Single-cell regulatory network inference and clustering (SCENIC) analysis reveals JUN signaling pathway as a potential therapeutic target for treatment of ovarian cancer, which is validated using the JUN/AP-1 inhibitor T-5224. ✓ Epithelial-fibroblast cell communication atlas identifies important receptor-ligand complexes in OC.	
11	Identification of Specific Cell Subpopulations and Marker Genes in Ovarian Cancer Using Single-Cell RNA Sequencing (Li et al., 2021)	Data from Gene Expression Omnibus of 66 HGSOC cells were employed.		66	R package Seurat for cell clustering. Gene Ontology and KEGG enrichment analyses. Survival analyses performed.	NA	✓ A total of 1,124 differentially expressed genes were identified in ovarian cancer samples. These differentially expressed genes were enriched in several pathways associated with cancer, such as metabolic pathways, pathways in cancer, and PI3K-Akt signaling pathway. ✓ Marker genes, <i>STAT1</i>, <i>ANP32E</i>, <i>GPRC5A</i>, and <i>EGFL6</i>, were highly expressed in ovarian cancer, while <i>PMP22</i>, <i>FBXO21</i>, and <i>CYB5R3</i> were lowly expressed in ovarian cancer.	
12	Single-cell RNA-seq highlights a specific carcinoembryonic cluster in ovarian cancer (Zhao et al., 2021)	Fresh ovarian samples from OC and normal ovary and 9 th -week embryo.	Tumor Dissociation Kit and incubated at 30rpm. Further trypsinised. Embryonic tissue same digestion buffer but different	16027	R package Seurat for cell clustering. Pseudotime analysis and Gene Ontology and KEGG enrichment analyses. SCENIC, CellPhone DB analyses.	Established cell line cultures, bulk RNA extraction, Western blot.	✓ 8 cell types identified with some clusters present in embryo and tumours. ✓ A subtype of malignant epithelial cluster, PEG10+ was associated with poor survival and was an intermediate stage of embryo to tumour. ✓ PEG10 was a CSC marker and might influence CSC self-renewal and promote cisplatin resistance via NOTCH pathway. ✓ Utilising specific gene profiles of PEG10+ EME based on public data sets, four phenotypes with different survival and	✓ First study of reflecting adult and embryonic cell types at single cellular level.

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			incubation times. 10x Genomics 3' gene kit.				clinical response to anti-PD-1/PD-L1 immunotherapy were identified.	
13	Single cell-derived spheroids capture the self-renewing subpopulations of metastatic ovarian cancer (Velletri et al., 2022)	Fresh ascites from HGSOC PDS patients.	Ascites processing using lab protocol and 10x Genomics 3' gene kit.	8777	CellRanger, R packages (edgeR) and Ingenuity Pathway Analysis to identify impact of genes.	Single cell metastatic ovarian cancer spheroids (sMOCS) from HGSOC ascites. Bulk RNA-seq. Drug sensitivity testing on sMOCS vs 2D cultures.	✓ sMOCS compared to fresh HGSOC ascites and 2D culture retain and amplify, differently from 2D cultures, specific cell subpopulations from metastatic samples ✓ These reveal undetected cancer stem-related functional specificities.	✓ Spheroids are better than 2D cultures as experimental models.
14	Single-cell RNA-seq recognized the initiator of epithelial ovarian cancer recurrence (Kan et al., 2022)	Fresh ovarian and peritoneal samples from 8 epithelial OC patients (PDS/relapsed)	Tumor Dissociation Kit (Miltenyi Biotec, Inc) and Drop-seq	13369	R packages	Bulk RNA seq and IHC staining of archival samples.	✓ Primary tumours have subsets of cells where CYR61+ "stress" cells are identified as relapse-initiators and drug resistance. ✓ RGS5+ CAFs support tumour metastasis. ✓ CYR61/RGS5 scores correlates significantly with relapse-free survival of EOC patients and can predict recurrence.	✓ Tumour-stromal crosstalk with novel tumour and CAF markers.
15	Ovarian cancer mutational processes drive site-specific immune evasion	Fresh ascites, tumour tissue from primary and	Tumor Dissociation Kit (Miltenyi Biotec, Inc), cell sorting and 10x	NA	R packages	Whole-genome sequencing, digital histopathology and multiplexed immunofluorescence.	✓ Homologous recombination-deficient HRD-Dup (BRCA1 mutant-like) and HRD-DeI (BRCA2 mutant-like) tumours harboured inflammatory signalling and ongoing immunoediting, reflected in loss of HLA diversity and tumour infiltration	✓ Multi-omic cellular phenotyping of HGSOC allowing

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	(Vázquez-García et al., 2022)	metastatic sites from 42 patients (laparoscopy and PDS).	Genomics 3' gene kit.				with highly differentiated dysfunctional CD8⁺ T cells. ✓ Foldback-inversion-bearing tumours exhibited elevated immunosuppressive TGFβ signalling and immune exclusion, with predominantly naive/stem-like and memory T cells. ✓ Phenotypic divergence specific to anatomical sites and contribute to immune resistance in HGSOC. ✓ Relative paucity of immune cells in adnexal sites.	for development of personalised immunotherapies.

3.1.5 Heterogeneous HGSOc cell populations are likely to account for the bulk HGSOc transcriptomic subtypes

The Cancer Genome Atlas (TCGA) analysed 489 cases of HGSOc using copy number, mRNA expression, exonic sequencing of more than 18 500 genes and methylation arrays (Bell et al., 2011). Based on marker gene expression, this research classified HGSOc into 4 subtypes termed as “Differentiated” (DIF), “Immunoreactive” (IMR), “Mesenchymal” (MES) and “Proliferative” (PRO). The TCGA demonstrated that the DIF subtype had increased ovarian tumour marker expression, the IMR subtype had increased chemokine expression, the MES subtype had expression markers suggestive of increased stromal components and the PRO subtype harboured increased proliferation marker expression, but did not report differences in survival outcomes between these molecular subtypes. Expanding on the description of the 4 subtypes defined in TCGA, Verhaak et al. developed a classifier based on single-sample gene set enrichment analysis (GSEA) using the full catalog of TCGA genomic and clinical data (Verhaak et al., 2013). They integrated subtype and prognostic classifiers defined in a set of 489 expression profiles into a prognostic framework termed “Classification of Ovarian Cancer” (CLOVAR) and tested its accuracy in predicting outcome on a dataset of 879 publicly available expression profiles of HGSOc. They found that the IMR subtype had increased infiltrating T lymphocytes. Meanwhile, the MES subtypes had increased desmoplasia associated with infiltrating stromal cells and this group was found to have the worst survival outcomes compared to other subtypes (with a median overall survival of 23 months vs 46 months for the other cases). Recent single cell studies have indicated that these bulk HGSOc transcriptomic subtypes are driven by the widely heterogenous cell populations with

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the TME of HGSOC (Geistlinger et al., 2020; Izar et al., 2020; A. J. Shih et al., 2018; Winterhoff et al., 2019).

3.2 Study aims

In this chapter, selected single cell suspensions which passed cell viability and FACS cellular content analyses were sequenced and their transcriptomics analysed in depth.

This study specifically aims to:

1. Explore the stepwise approach to single cell library generation and library QC checks
2. Discuss the sequencing technicalities
3. Explore the data analytical aspect of HGSOC single cell libraries
4. Characterise the composition of the tumour microenvironment of HGSOC at single cellular level
5. Perform comparison of the HGSOC TME composition at multiple sites
6. Investigate the effects of chemotherapy on TME of HGSOC
7. Identify subclusters of cells within the HGSOC TME and examine the potential clinical and biological significance of these subclusters
8. Identify novel markers for OC proliferation and migration
9. Investigate the copy number variation (CNV) pattern of tumour cells in HGSOC through computationally inferred CNVs in single cells
10. Compare the single cell composition of HGSOC to bulk TCGA molecular subtypes

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3.3 Materials and methods

The scRNA-seq library preparation using the 10x Genomics Chromium Single Cell 3' protocol is broadly divided into 3 steps involving gel beads-in-emulsion (GEM) generation and barcoding, post GEM-RT cleanup and cDNA amplification and 3' gene expression library construction. The GemCode Technology samples a pool of ~3 500K 10x Barcodes to separately index each cell's transcriptome. Thousands of cells are partitioned into nanoliter-scale GEMs, where all generated cDNA share a common 10x Barcode. Single cell libraries are generated and sequenced from the cDNA and 10x Barcodes are used to associate individual reads back to the individual cells.(Genomics, 2020)

Table 3.3 10x Genomics Chromium Single Cell 3' v 3 Protocol Steps and Timing

Day	Steps	Timing	Stop and store
4 h	Cell Preparation		
	Step 1 – GEM Generation and Barcoding (Section 3.3.1)		
	1.1 Prepare Reaction Mix	20 min	
	1.2 Load Chromium Single Cell B Chip	10 min	
	1.3 Run the Chromium Controller	8.5 min	
	1.4 Transfer GEMs	3 min	
	1.5 GEM-RT incubation	55 min	 4°C ≤ 72 h or -20°C ≤ 1 week

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6 h	Step 2 – Post GEM-RT Cleanup and cDNA Amplification (Section 3.3.2)		
	2.1 Post GEM-RT Cleanup – Dynabead	45 min	
	2.2 cDNA Amplification	40 min	 4°C ≤ 72 h or -20°C ≤ 1 week
	2.3 cDNA Cleanup – SPRIselect	20 min	 4°C ≤ 72 h or -20°C ≤ 1 week
	2.4 cDNA QC and Quantification	50 min	
	Step 3 – 3' Gene Expression Library Construction (Section 3.3.3)		
	3.1 Fragmentation, End Repair	50 min	
	3.2 Post Fragmentation, End Repair and A-tailing Double Sided Size Selection – SPRIselect	30 min	
	3.3 Adaptor Ligation	25 min	
	3.4 Post Ligation Cleanup – SPRIselect	20 min	
8 h	3.5 Sample Index PCR	40 min	 4°C ≤ 72 h or
	3.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect	30 min	 4°C ≤ 72 h or -20°C long term
	3.7 Post Library Construction QC	50 min	



Experiment can be halted and samples stored as specified. (*Genomics, 2022b*)

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3.3.1 GEM generation and barcoding (Genomics, 2022)

Cell concentration loaded onto the Chromium chip for GEM generation should balance the risk of multiplets and obtaining the maximum cell recovery. With increasing cells being loaded, the rate of multiplets increases between 0.4-0.8% while the cell recovery rate remains 1.6x of the loaded cells (see Table 3.4). 10x Genomics recommended the optimal input cell concentration of 700-1200 cells/ μ l and a maximal loading of 25 000 cells per reaction.

Table 3.4 Risk of Multiplets with Increasing Cells Loaded

Multiplet Rate (%)	No. of Cells Loaded	No. of Cells Recovered	Recovery Rate
~0.4	~800	~500	1.6
~0.8	~1 600	~1 000	1.6
~1.6	~3 200	~2 000	1.6
~2.3	~4 800	~3 000	1.6
~3.1	~6 400	~4 000	1.6
~3.9	~8 000	~5 000	1.6
~4.6	~9 600	~6 000	1.6
~5.4	~11 200	~7 000	1.6
~6.1	~12 800	~8 000	1.6
~6.9	~14 400	~9 000	1.6
~7.6	~16 000	~10 000	1.6

(Genomics, 2022b)

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The steps for GEM generation and barcoding are as follows: (Genomics, 2022b)

1.1 Prepare Master Mix

- a. Prepare this mix on ice and pipette mix 15x and centrifuge briefly.

Table 3.5 Master Mix

Master Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(μ l)	(μ l)	(μ l)
RT Reagent	20.0	88.0	176.0
Template Switch Oligo	3.1	13.9	27.7
Reducing Agent B	2.0	8.7	17.3
RT Enzyme C	8.3	36.6	73.1
Total	33.4	147.1	294.2

(Genomics, 2022b)

- b. Prepare a PCR 8-tube strip on ice and add 33.4 μ l of Master Mix into each tube.

1.2 Load Chromium Chip B

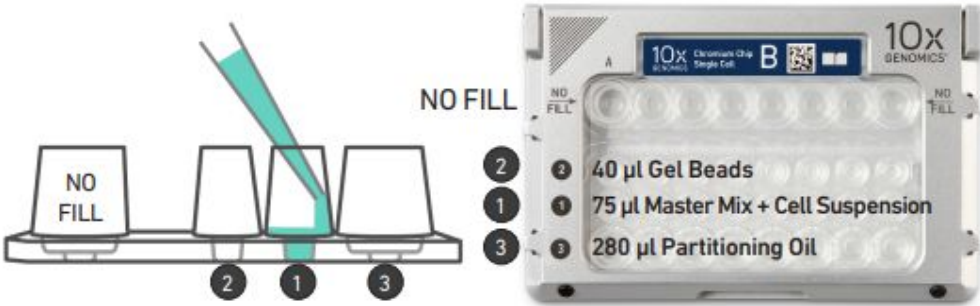


Figure 3.4 Chromium Chip B Loading. Figure from (Genomics, 2022b)

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- a. Place Chromium Chip B in a Chip Holder. While loading the chip, raising and depressing the pipette plunger should each take ~5 sec. Dispense against side of the well and avoid contact with bottom of the well. Bubble formation is normal and does not affect performance.
- b. Dispense 50% Glycerol Solution into unused chip wells
 - i. 75 μ l to unused wells in row 1
 - ii. 40 μ l to unused wells in row 2
 - iii. 280 μ l to unused wells in row 3
- c. Prepare Master Mix and Cell Suspension

Use the Cell Suspension Volume Calculator Table (see Table 3.3) to guide the amount of nuclease-free water and corresponding volume of single cell suspension to be added to Master Mix to make up a total of 80 μ l in each tube. Before adding to Master Mix, gently pipette mix the single cell suspension.

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Table 3.6 Cell Suspension Volume Calculator. Adapted from (Genomics, 2022b).

Cell Suspension Volume Calculator Table (for step 1.2)											
Cell Stock Concentration (Cells/ μ l)	Volume of Cell Suspension Stock per reaction (μ l) Volume of Nuclease-free Water per reaction (μ l)										
	Targeted Cell Recovery										
	500	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000
100	8.0 38.6	16.0 30.6	32.0 14.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
200	4.0 42.6	8.0 38.6	16.0 30.6	24.0 22.6	32.0 14.6	40.0 6.6	n/a	n/a	n/a	n/a	n/a
300	2.7 43.9	5.3 41.3	10.7 35.9	16.0 30.6	21.3 25.3	26.7 19.9	32.0 14.6	37.3 9.3	42.7 3.9	n/a	n/a
400	2.0 44.6	4.0 42.6	8.0 38.6	12.0 34.6	16.0 30.6	20.0 26.6	24.0 22.6	28.0 18.6	32.0 14.6	36.0 10.6	40.0 6.6
500	1.6 45.0	3.2 43.4	6.4 40.2	9.6 37.0	12.8 33.8	16.0 30.6	19.2 27.4	22.4 24.2	25.6 21.0	28.8 17.8	32.0 14.6
600	1.3 45.3	2.7 43.9	5.3 41.3	8.0 38.6	10.7 35.9	13.3 33.3	16.0 30.6	18.7 27.9	21.3 25.3	24.0 22.6	26.7 19.9
700	1.1 45.5	2.3 44.3	4.6 42.0	6.9 39.7	9.1 37.5	11.4 35.2	13.7 32.9	16.0 30.6	18.3 28.3	20.6 26.0	22.9 23.7
800	1.0 45.6	2.0 44.6	4.0 42.6	6.0 40.6	8.0 38.6	10.0 36.6	12.0 34.6	14.0 32.6	16.0 30.6	18.0 28.6	20.0 26.6
900	0.9 45.7	1.8 44.8	3.6 43.0	5.3 41.3	7.1 39.5	8.9 37.7	10.7 35.9	12.4 34.2	14.2 32.4	16.0 30.6	17.8 28.8
1000	0.8 45.8	1.6 45.0	3.2 43.4	4.8 41.8	6.4 40.2	8.0 38.6	9.6 37.0	11.2 35.4	12.8 33.8	14.4 32.2	16.0 30.6
1100	0.7 45.9	1.5 45.1	2.9 43.7	4.4 42.2	5.8 40.8	7.3 39.3	8.7 37.9	10.2 36.4	11.6 35.0	13.1 33.5	14.5 32.1
1200	0.7 45.9	1.3 45.3	2.7 43.9	4.0 42.6	5.3 41.3	6.7 39.9	8.0 38.6	9.3 37.3	10.7 35.9	12.0 34.6	13.3 33.3
1300	0.6 46.0	1.2 45.4	2.5 44.1	3.7 42.9	4.9 41.7	6.2 40.4	7.4 39.2	8.6 38.0	9.8 36.8	11.1 35.5	12.3 34.3
1400	0.6 46.0	1.1 45.5	2.3 44.3	3.4 43.2	4.6 42.0	5.7 40.9	6.9 39.7	8.0 38.6	9.1 37.5	10.3 36.3	11.4 35.2
1500	0.5 46.1	1.1 45.5	2.1 44.5	3.2 43.4	4.3 42.3	5.3 41.3	6.4 40.2	7.5 39.1	8.5 38.1	9.6 37.0	10.7 35.9
1600	0.5 46.1	1.0 45.6	2.0 44.6	3.0 43.6	4.0 42.6	5.0 41.6	6.0 40.6	7.0 39.6	8.0 38.6	9.0 37.6	10.0 36.6
1700	0.5 46.1	0.9 45.7	1.9 44.7	2.8 43.8	3.8 42.8	4.7 41.9	5.6 41.0	6.6 40.0	7.5 39.1	8.5 38.1	9.4 37.2
1800	0.4 46.2	0.9 45.7	1.8 44.8	2.7 43.9	3.6 43.0	4.4 42.2	5.3 41.3	6.2 40.4	7.1 39.5	8.0 38.6	8.9 37.7
1900	0.4 46.2	0.8 45.8	1.7 44.9	2.5 44.1	3.4 43.2	4.2 42.4	5.1 41.5	5.9 40.7	6.7 39.9	7.6 39.0	8.4 38.2
2000	0.4 46.2	0.8 45.8	1.6 45.0	2.4 44.2	3.2 43.4	4.0 42.6	4.8 41.8	5.6 41.0	6.4 40.2	7.2 39.4	8.0 38.6

Grey boxes: Volumes that would exceed the allowable water volume in each reaction

Yellow boxes: Indicate a low transfer volume that may result in higher cell load variability

Blue boxes: Optimal range of cell stock concentration to maximize the likelihood of achieving the desired cell recovery target

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d. Load Row 1

Using the same pipette tip, gently pipette mix and dispense 75 μ l Master Mix and Cell Suspension against the side of each well in row 1.

e. Prepare Gel Beads

Place the Gel Bead strip onto a 10x Vortex Adapter and vortex for 30 sec. Remove the Gel Bead strip and flick in a sharp, downward motion to ensure maximum recovery. Confirm the absence of bubbles at the bottom of the tubes and ensure that liquid levels look even.

f. Load Row 2

Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 40 μ l Gel Beads and dispense against the side of each well in row 2.

g. Load Row 3

Dispense 280 μ l Partitioning Oil against the side of each well in row 3.

h. Attach 10x Gasket

1.3 Run the Chromium Controller

At the end of the GEM formation (~8.5 min), immediately proceed to next step.

1.4 Transfer GEMs

a. Place a tube strip on ice.

b. Open the chip holder by folding the lid back to expose the wells at 45 degrees.

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- c. Check volume in rows 1-3. Abnormally high volume in any well indicates a clog.
- d. Slowly aspirate 100 μ l GEMs from the lowest points of the Recovery Wells in the top row without creating a seal between the tips and the bottom of the wells.
- e. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels.

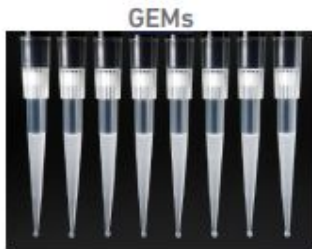


Figure 3.5 GEM Appearance (Genomics, 2022b).

- f. Dispense GEMs into tube strip on ice over 20 sec with pipette tips against sidewalls of the tubes.

1.5 GEM-RT incubation

- a. Incubate in a thermal cycler with the highest reaction volume setting.

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Table 3.7 Thermal Cycler Settings

Lid Temperature	Reaction Volume	Run Time
53°C	125 µl	~55 min
Step	Temperature	Time
1	53°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

(Genomics, 2022b)

- b. Store at 4°C for up to 72 hours or at -20°C for up to a week if could not proceed to next step. This is the point where the samples could be stored for future processing (i.e. after run-through processing from sample harvest to single cell suspension and GEM generation).

3.3.2 Post GEM-RT cleanup and cDNA amplification (Genomics, 2022)

For this part, the steps are as follows:

1.1 Post GEM-RT Cleanup – Dynabeads

- a. Equilibrate the samples post GEM-RT incubation to room temperature (if kept at low temperatures) and add 125 µl of Recovery Agent to each sample. Wait 2 min.
- b. Slowly discard 125 µl of Recovery Agent/ Partitioning Oil (pink) from the bottom of the tube. Do not aspirate any clear aqueous sample.
- c. Prepare Dynabeads Cleanup Mix.

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Table 3.8 Dynabeads Cleanup Mix

Dynabeads Cleanup Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(μ l)	(μ l)	(μ l)
Cleanup Buffer	182	801	1602
Dynabeads MyOne SILANE	8	35	70
Reducing Agent B	5	22	44
Nuclease-free water	5	22	44
Total	200	880	1760

(Genomics, 2022b)

- d. Vortex and add 200 μ l Dynabeads Cleanup Mix to each sample. Pipette mix 10x (setting pipette to 200 μ l). Incubate 10 min at rtp. Pipette mix again at 5 min after start of incubation to resuspend settled beads.
- e. Prepare Elution Solution I. Vortex and centrifuge briefly.

Table 3.9 Elution Solution I

Elution Solution I	1X	10X
Add reagents in the order listed	(μ l)	(μ l)
Buffer EB	98	980
10% Tween 20	1	10
Reducing Agent B	1	10
Total	100	1000

(Genomics, 2022b)

- f. At the end of 10 min incubation, place on a 10x Magnetic Separator•High position (magnet•High) until the solution clears. A white interface

[Type here]

between the aqueous phase and Recovery Agent is normal. Remove the supernatant.

- g. Add 300 μ l 80% ethanol to the pellet while on the magnet. Wait 30 sec. Remove the ethanol.
- h. Add 200 μ l 80% ethanol to pellet. Wait 30 sec. Remove the ethanol. Centrifuge briefly.
- i. Place on the magnet•Low. Remove remaining ethanol. Air dry for 1 min.
- j. Remove from the magnet. Immediately add 35.5 μ l Elution Solution I.
- k. Pipette mix (pipette set to 30 μ l) without introducing bubbles.
- l. Incubate 2 min at room temperature. Place on the magnet•Low until the solution clears.
- m. Transfer 35 μ l sample to a new tube strip.

1.2 cDNA Amplification

- a. Prepare cDNA Amplification Mix on ice and vortex and centrifuge briefly.

Table 3.10 cDNA Amplification Reaction Mix

cDNA Amplification Reaction Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(μ l)	(μ l)	(μ l)
Amp Mix	50	220	440
cDNA Primers	15	66	132
Total	65	286	572

(*Genomics, 2022b*)

- b. Add 65 μ l cDNA Amplification Reaction Mix to 35 μ l sample.
- c. Pipette mix 15x (set pipette to 90 μ l). Centrifuge briefly.
- d. Incubate in thermal cycler using the protocol below.

[Type here]

Table 3.11 Thermal Cycler Settings (Genomics, 2022b)

Lid Temperature	Reaction Volume	Run Time
105 ⁰ C	100 µl	~35-40 min
Step	Temperature	Time
1	98 ⁰ C	00:03:00
2	98 ⁰ C	00:00:15
3	63 ⁰ C	00:00:20
4	72 ⁰ C	00:01:00
5	Go to Step 2, see table 3.11 for total # of cycles	
6	72 ⁰ C	00:01:00
7	4 ⁰ C	Hold

Table 3.12 Optimal Number of Cycles (Genomics, 2022b)

Targeted Cell Number	Total Cycles
<500	13
500-6 000	12
>6 000	11

The optimal number of cycles is a trade-off between generating sufficient final mass for library construction and minimising PCR amplification artifacts. The number of cDNA cycles should also be reduced if large number of cells are sampled.

*In this study, normally 11 cycles of PCR amplification are sufficient to obtain adequate cDNA mass for downstream library construction.

- e. Store at 4°C for up to 72 h or at -20°C for ≤1 week, or proceed to the next step.

1.3 cDNA Cleanup-SPRIselect

- a. Vortex to resuspend the SPRIselect reagent. Add 60 μ l SPRIselect reagent

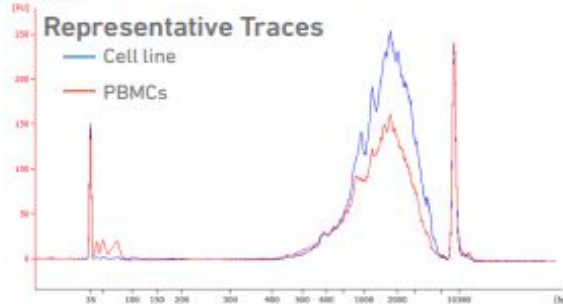
(0.6X) to each sample and pipette mix 15x (pipette set to 150 μ l).
- b. Incubate 5 min at room temperature.
- c. Place on the magnet•High until the solution clears.
- d. Remove the supernatant.
- e. Add 200 μ l 80% ethanol to the pellet. Wait 30 sec.
- f. Remove the ethanol.
- g. Repeat steps e and f for a total of 2 washes.
- h. Centrifuge briefly and place on the magnet•Low.
- i. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
- j. Remove from the magnet. Add 40.5 μ l Buffer EB. Pipette mix 15x.
- k. Incubate 2 min at room temperature.
- l. Place the tube strip on the magnet•High until the solution clears.
- m. Transfer 40 μ l sample to a new tube strip.
- n. Store at 4°C for up to 72 h or at –20°C for up to 4 weeks, or proceed to the next step.

[Type here]

1.4 cDNA QC and Quantification

- a. Run 1 µl of sample (Dilution Factor 1:10) on an Agilent Bioanalyzer High Sensitivity chip.

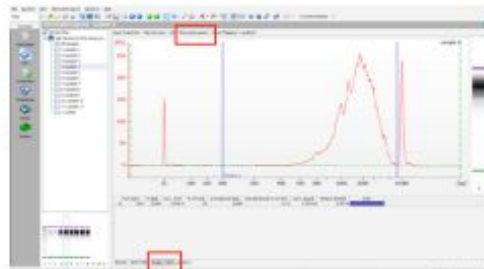
For input cells with low RNA content (<1pg total RNA/cell), 1 µl undiluted product may be run. Lower molecular weight product (35 – 150 bp) may be present. This is normal and does not affect sequencing or application performance.



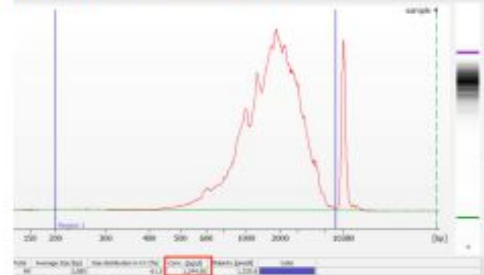
EXAMPLE CALCULATION

i. Select Region

Under the "Electropherogram" view choose the "Region Table". Manually select the region of ~200 – ~9000 bp



ii. Note Concentration [pg/µl]



iii. Calculate

Multiply the cDNA concentration [pg/µl] reported via the Agilent 2100 Expert Software by the elution volume (40 µl) of the Post cDNA Amplification Reaction Clean Up sample (taking any dilution factors into account) and then divide by 1000 to obtain the total cDNA yield in ng.

Example Calculation of cDNA Total Yield

Concentration: 1244.06 pg/µl

Elution Volume: 40

Dilution Factor: 10

Total cDNA Yield

$$= \frac{\text{Conc'n (pg/}\mu\text{l}) \times \text{Elution Volume (}\mu\text{l}) \times \text{Dilution Factor}}{1000 \text{ (pg/ng)}}$$

$$= \frac{1244.06 \text{ (pg/}\mu\text{l}) \times 40 \text{ (}\mu\text{l}) \times 10}{1000 \text{ (pg/ng)}} = 497.62 \text{ ng}$$



Carry forward **ONLY 25%** of total cDNA yield into 3' Gene Expression Library Construction (step 3)

$$= 0.25 \times \text{Total cDNA yield}$$

$$= 0.25 \times 497.62 = 124.4 \text{ ng}$$

Refer to step 3.5 for appropriate number of Sample Index PCR cycles based on carry forward cDNA yield/input cDNA.

Figure 3.6 Example of cDNA Bioanalyser trace and Calculation for cDNA Quantification. Figure from (Genomics, 2022b).

[Type here]

3.3.3 3' Gene expression library construction

From the total cDNA (40 μ l) obtained in step 1.3 of 3.3.2, 10 μ l (25%) is taken to generate a single cell 3' Gene Expression library. The remaining 30 μ l of the cDNA may be stored at 4°C for up to 72 h or at -20°C for longer-term storage (up to 4 weeks).

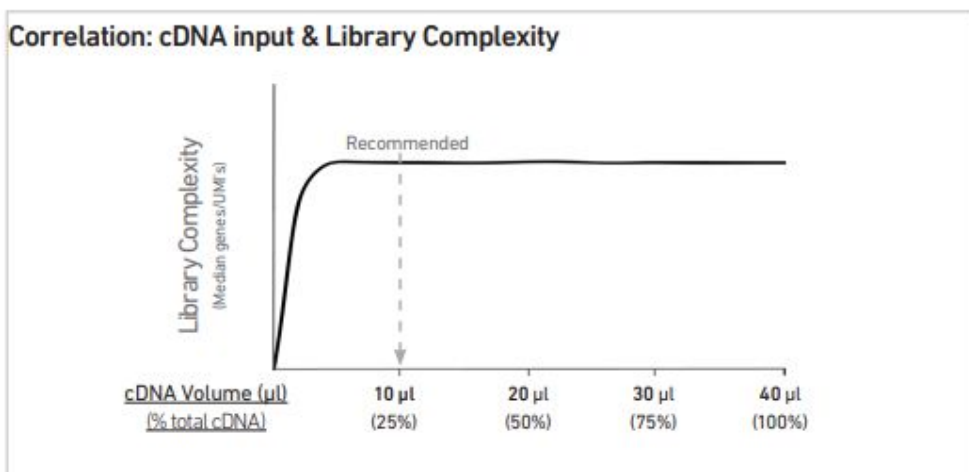


Figure 3.7 Correlation between cDNA Input and Library Complexity. The threshold for cDNA input to generate the optimal library complexity is at 25% of the total cDNA. Beyond this level, a higher proportion of cDNA input will result in comparable complexity of library as a 25% input of the total cDNA. Figure from (Genomics, 2022b).

[Type here]

1.1 Fragmentation, End Repair and A-tailing

- a. Prepare a thermal cycler incubation as follows.

Table 3.13 Thermal Cycler Settings

Lid Temperature	Reaction Volume	Run Time
65°C	50 µl	~35 min
Step	Temperature	Time
Pre-cool block	4°C	Hold
Fragmentation	32°C	00:05:00
End Repair & A-tailing	65°C	00:30:00
Hold	4°C	Hold

(Genomics, 2022b)

- b. Vortex fragmentation buffer and ensure there is no precipitate.
- c. On ice, prepare the Fragmentation mix. Pipette and centrifuge the mix briefly.

Table 3.14 Fragmentation Mix

Fragmentation Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(µl)	(µl)	(µl)
Fragmentation Buffer	5	22	44
Fragmentation Enzyme	10	44	88
Total	15	66	132

(Genomics, 2022b)

- d. Transfer ONLY 10 µl purified cDNA sample from step 1.3 of 3.4.2 to a tube strip.
- e. Add 25 µl Buffer EB and 15 µl Fragmentation Mix to each sample.

[Type here]

- f. Pipette mix 15x (set pipette to 35 µl) on ice. Centrifuge briefly.
- g. Transfer into the pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the protocol.

1.2 Post Fragmentation, End Repair and A-tailing Double Sided Size Selection – SPRIselect

- a. Vortex to resuspend SPRIselect reagent. Add 30 µl SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 75 µl).
- b. Incubate 5 min at room temperature.
- c. Place on the magnet•High until the solution clears. DO NOT discard supernatant.
- d. Transfer 75 µl supernatant to a new tube strip.
- e. Vortex to resuspend SPRIselect reagent. Add 10 µl SPRIselect reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 80 µl).
- f. Incubate 5 min at room temperature.
- g. Place on the magnet•High until the solution clears.
- h. Remove 80 µl supernatant. DO NOT discard any beads.
- i. Add 125 µl 80% ethanol to the pellet. Wait 30 sec.
- j. Remove the ethanol.
- k. Repeat steps i and j for a total of 2 washes.
- l. Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.
- m. Remove from the magnet. Add 50.5 µl Buffer EB to each sample. Pipette mix 15x.

[Type here]

- n. Incubate 2 min at room temperature.
- o. Place on the magnet•High until the solution clears.
- p. Transfer 50 µl sample to a new tube strip

1.3 Adaptor Ligation

- a. Prepare Adaptor Ligation Mix, pipette and centrifuge briefly.

Table 3.15 Adaptor Ligation Mix

Adaptor Ligation Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(µl)	(µl)	(µl)
Ligation Buffer	20	88	176
DNA Ligase	10	44	88
Adaptor Oligos	20	88	176
Total	50	220	440

(Genomics, 2022b)

- b. Add 50 µl Adaptor Ligation Mix to 50 µl sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.
- c. Incubate in a thermal cycler with the following protocol.

Table 3.16 Thermal Cycler Settings

Lid Temperature	Reaction Volume	Run Time
30°C	100 µl	15 min
Step	Temperature	Time
1	20°C	00:15:00
2	4°C	Hold

(Genomics, 2022b)

[Type here]

Post Ligation Cleanup – SPRIselect

- a. Vortex to resuspend SPRIselect Reagent. Add 80 μ l SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 μ l).
- b. Incubate 5 min at room temperature.
- c. Place on the magnet•High until the solution clears.
- d. Remove the supernatant.
- e. Add 200 μ l 80% ethanol to the pellet. Wait 30 sec.
- f. Remove the ethanol.
- g. Repeat steps e and f for a total of 2 washes.
- h. Centrifuge briefly. Place on the magnet•Low.
- i. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
- j. Remove from the magnet. Add 30.5 μ l Buffer EB. Pipette mix 15x.
- k. Incubate 2 min at room temperature.
- l. Place on the magnet•Low until the solution clears.
- m. Transfer 30 μ l sample to a new tube strip.

1.5 Sample Index PCR

It is important to ensure that no sample indices overlap in a multi-plexed sequencing run. Therefore, record the 10x Sample Index name (PN-220103 Chromium i7 Sample Index Plate well ID) used accurately for each sample.

- a. Prepare Sample Index PCR Mix.

[Type here]

Table 3.17 Sample Index PCR Mix

Sample Index PCR Mix	1X	4X +10%	8X +10%
Add reagents in the order listed	(μ l)	(μ l)	(μ l)
Amp Mix	50	220	440
SI Primer	10	44	88
Total	60	264	528

(*Genomics, 2022b*)

- b. Add 60 μ l Sample Index PCR Mix to 30 μ l sample.
- c. Add 10 μ l of an individual Chromium i7 Sample Index to each well and record the well ID used. Pipette mix 5x (pipette set to 90 μ l). Centrifuge briefly.
- d. Incubate in a thermal cycler with the following protocol.

Table 3.18 Thermal Cycler Settings

Lid Temperature	Reaction Volume	Run Time
105	100	~25-40 min
Step	Temperature	Time
1	98 ⁰ C	00:00:45
2	98 ⁰ C	00:00:20
3	54 ⁰ C	00:00:30
4	72 ⁰ C	00:00:20
5	Go to Step 2, see below for # of cycles	
6	72 ⁰ C	00:01:00
7	4 ⁰ C	Hold

(*Genomics, 2022b*)

[Type here]

Recommended cycle numbers

cDNA Input (ng)	Total Cycles
0.25-25	14-16
25-150	12-14
150-500	10-12
500-1 000	8-10
1 000-1 500	6-8
>1 500	5

The total cycles should be optimised based on 25% carry forward cDNA yield/ input calculated during Post cDNA Amplification QC & Quantification step.

1.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

- Vortex to resuspend the SPRIselect reagent. Add 60 µl SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to 150 µl).
- Incubate 5 min at room temperature.
- Place the magnet•High until the solution clears. DO NOT discard supernatant.
- Transfer 150 µl supernatant to a new tube strip.
- Vortex to resuspend the SPRIselect reagent. Add 20 µl SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 µl).
- Incubate 5 min at room temperature.
- Place the magnet•High until the solution clears.
- Remove 165 µl supernatant. DO NOT discard any beads.
- With the tube still in the magnet, add 200 µl 80% ethanol to the pellet. Wait 30 sec. Remove the ethanol.
- Repeat step i for a total of 2 washes.

[Type here]

- k. Centrifuge briefly. Place on the magnet•Low. Remove remaining ethanol.
- l. Remove from the magnet. Add 35.5 µl Buffer EB. Pipette mix 15x.
- m. Incubate 2 min at room temperature.
- n. Place on the magnet•Low until the solution clears.
- o. Transfer 35 µl to a new tube strip.
- p. Store at 4°C for up to 72 h or at –20°C for long-term storage.

1.7 Post Library Construction QC

Using Agilent Bioanalyzer High Sensitivity chip, perform QC for library concentration with 1 µl of sample at 1:10 dilution. The expected single cell DNA library fragments range from 300-1000 bp, with the peak of fragments between 400-600 bp (Genomics, 2022b).

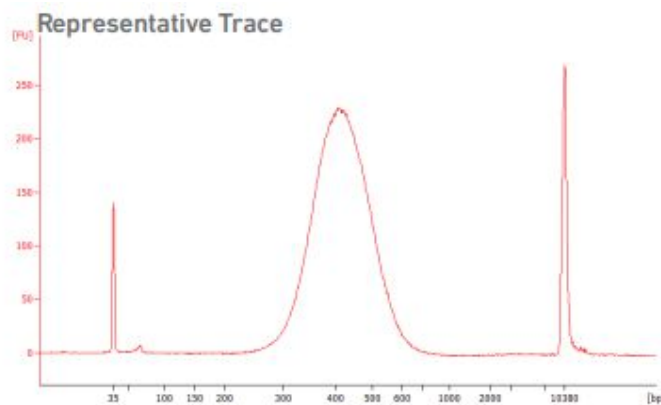


Figure 3.8 Example of Library Bioanalyser Trace. Use the average fragment size as the insert size for library quantification. Figure from (Genomics, 2022b).

[Type here]

3.3.4 Sequencing

Once the Single Cell 3' Gene Expression libraries are generated, they are comprised of standard Illumina paired-end constructs which begin and end with P5 and P7. TruSeq Read1 and TruSeq Read2 are standard Illumina sequencing primer sites used in paired-end sequencing. The 16 bp 10x Barcode and 12 bp UMI are encoded in TruSeq Read1 (see Figure 3.9). TruSeq Read2 contains cDNA insert with sample index incorporated as i7 index read. After sequencing, these libraries produce a standard Illumina BCL data output folder (Genomics, 2022).

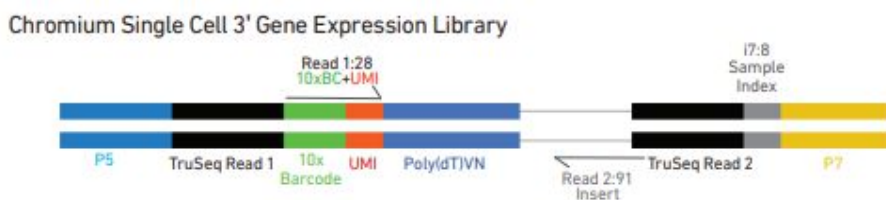


Figure 3.9 Chromium Single Cell 3' Gene Expression Library. Figure from (Genomics, 2022b).

The generated libraries were sent to Edinburgh Genomics for sequencing using NovaSeq platform. The libraries were pooled together and sequenced either using S1 or S2 flow cell lanes which yielded 750M and 1750M reads per sequencing run. A total of 4 or 8 libraries could be pooled for an S1 or S2 run respectively, yielding between 187.5M to 218.75M reads per library.

3.3.5 Bioinformatics pipeline, software and statistical analysis

3.3.5.1 Sequenced data pre-processing

The raw sequencing data processed by Edinburgh Genomics were downloaded into the Gourley group database. The pre-processing was performed using CellRanger version 3.1.0. The data was aligned to the Homo sapiens reference genome build GRCh38 using the CellRanger 10x 'count' function. The output from the aligned data were reported in the form of html files (containing the alignments' summary), and folders with raw and filtered information (containing the cells/ barcodes, genes/ features and count matrices associated with the alignment). The filtered data with only 'actual' single cells (excluding empty droplets/ ambient mRNA or multiplets) were used for downstream data analysis.

3.3.5.2 Quality control

The filtered data was analysed using the *Seurat* R package (version 3). (Stuart et al., 2019) This QC stage involved: 1) filtration of low quality cells through high mitochondrial expression where cells were removed if the total read count, number of genes or % mitochondrial expression were greater than 3 median absolute deviations (Hippen et al., 2021); and 2) regressing out cell cycle variation (uninteresting variation in scRNA-seq data) by using the Seurat function 'CellCycleScoring' (containing gene lists of cell cycle G2/M and S phase marker genes where cells expressing signals associated with cell cycle stage were removed) (Tirosh et al., 2016).

3.3.5.3 Normalisation

Batch effects or systematic differences that normally arise from differences in cDNA capture or PCR amplification efficiency across cells (due to difficulty in achieving consistent library preparation with minimal starting material) and sequencing depth can be removed through normalisation. (Amezquita et al., 2020) Here the 'SCTransform' normalisation method was used to normalise and perform variance stabilisation of molecular count of all genes in the cells. (Hafemeister & Satija, 2019)

3.3.5.4 Cell clustering

The *Seurat version 3* graph-based clustering approach was employed here for the cell clustering. (Hafemeister & Satija, 2019; Xu & Su, 2015) Firstly, the 3 000 most variable genes were identified using 'SCTransform' after normalisation. Next, to identify the dimensionality of the dataset, principle component analysis (PCA) was performed on these 3 000 variable genes with which the first 30 principle components (PCs) were selected for the graph-based clustering. Using these 30 PCs a K-nearest neighbour (KNN) graph was constructed and cells clustered by an algorithm (the Louvain modularity optimisation). (Hafemeister & Satija, 2019) This algorithm was based on a measure of the density of connections of cells within their own cluster against the connections with cells outside their cluster; with the overall objective to iteratively group similar cells together. These cell clusters were then visualised using the Uniform Manifold Approximation and Projection (UMAP) plots.

3.3.5.5 Cell types and subtypes identification

After cell clustering, the 'FindAllMarkers' function in the Seurat R package was used to identify positive and negative markers of each cell cluster in order to define/ label the clusters, implementing the Wilcoxon Rank Sum test to determine statistically significant differentially expressed genes (DEGs) within each cluster. (Hafemeister & Satija, 2019) This automated process adjusted for multiple testings using Bonferroni's correction and returned a list of DEGs with P-values and average Log₂ Fold Change values relative to other clusters.

In this study, a gene was considered significantly differentially expressed within a cluster in comparison to other clusters when the P-value was <0.05 and Log₂ Fold Change value was ≥ 0.5 (specifying a magnitude of expression); and the specific gene must be expressed in at least $\geq 25\%$ of all the cells within that cluster. This threshold for defining DEGs in this study was stricter compared to the threshold applied within the Seurat default parameters and also in other scRNA-seq studies which set a Log₂ Fold Change value of 0.25 and a DEG expression in at least 10% of all cells within a cluster (P-values < 0.05). (Hafemeister & Satija, 2019; H  rault et al., 2021; Vieth et al., 2019) This stricter threshold was selected in order to improve the identification of the cell types by limiting the chance of repetition of canonical markers in multiple clusters.

The cell type identification was performed using canonical markers from the CellMarker database (X. Zhang et al., 2019) and the Human Protein Atlas (Peter J. Thul et al., 2017).

[Type here]

3.3.5.6 Gene set enrichment analysis

Apart from identifying the cell types using canonical markers, gene set enrichment analysis (GSEA) was performed to assign biological significance to the cell clusters and subclusters. The R package clusterProfiler (G. Yu et al., 2012) was utilised to perform the GSEA and mapping of the significant DEGs to MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer' and Gene Ontology 'Biological Processes'. (Liberzon et al., 2015; Mi et al., 2018)

3.3.5.7 Comparison of cell types/ populations to TCGA HGSOC molecular subtypes

Verhaak et al. provided a list of 4 sets of gene symbols (100 total gene symbols), with each set of gene signature associated with a HGSOC subtype (Verhaak et al., 2013). The *consensusOV*, (G. M. Chen et al., 2018) a consensus classifier that assessed 3 major subtype classifiers (Tothill/ Helland, TCGA/ Verhaak, Konecny) by meta-analysis of publicly available expression data in an attempt to provide a standardised HGSOC classification scheme for clinical application has used the 100 gene symbols provided by Verhaak et al (Bell et al., 2011; Helland et al., 2011; Konecny et al., 2014; Tothill et al., 2008). Here, to perform the cell population comparison against the TCGA transcriptomic subtypes, the well-accepted Verhaak 100 gene symbols was used.

The mRNA expression values of all the single cell dataset in this study were normalised using standard log normalisation. Firstly, the comparison against the TCGA subtypes were performed by patient sample ID for the 8 single cell dataset (Patient 5 OV, Patient 6 PE, Patient 7 OV, Patient 7 PE, Patient 9 OV, Patient 9 OM,

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Patient 9 PE and Patient 9 SBN). Using gene sets defining the 4 TCGA HGSOC subtypes provided by Verhaak et al., the average expression of all the cells in each patient dataset were compared against a threshold value. The threshold of classification was defined as two standard deviations (2SDs) above the mean of the combined group comparisons which were all the patient datasets (a method adopted from Shih et al. which investigated scRNA-seq of differing grades of ovarian tumour including HGSOC, LGSOC and benign ovarian tumours). (A. J. Shih et al., 2018)

As for the cell populations comparison to TCGA HGSOC subtypes, all cells from the primary ovarian samples (P5: OV, P7: OV and P9: OV) were merged together as these samples contained all the cell types. Similar process of comparison was performed where the average expression of all the cells for each cell population/ type were compared against a threshold value which was 2SDs above the mean of the combined expression of all the cell populations for each gene set of the TCGA HGSOC subtype.

3.3.5.8 Inference of copy number variation (CNV)

The R package CopyKAT was used to predict whether the epithelial cells in the HGSOC datasets were aneuploid (malignant). (R. Gao et al., 2021) Briefly, CopyKAT utilises Bayesian segmentation analysis to obtain a genome-wide copy number profile of each cell at 5Mb resolution and compare these profiles for different cell types. For each dataset containing epithelial cells, a genome-wide copy number profile of select 'normal' cells (i.e. myeloid) was constructed to produce a 'normal background' cell profile. This process was repeated for the epithelial cells. The copy number profiles between the epithelial cells and 'normal' cells were then overlaid to estimate if there

[Type here]

were evidence of chromosomal amplification or deletion within the epithelial cells in order to predict if the epithelial cells were aneuploid.

3.4 Results

3.4.1 Single cell RNA-sequenced libraries and QC checks using cell viability, bioanalyser, 10x CellRanger and R/Seurat

A total of 18 libraries (13 tumour and 2 blood samples from 8 HGSOC patients, 2 LGSOC tumour samples, 1 control cell line) were generated from the single cell suspensions produced in Chapter 2 (see Table 3.19). The first batch (experiments E05-E14) was performed using cryopreserved samples. Subsequent samples (batch two, experiments E25-28, and three, experiment E29), were performed using fresh material (see Table 3.19). Although cell viabilities of >70% were achieved following the adoption of automated dissociation protocol and DCR on cryopreserved samples, this study opted to process subsequent samples fresh for several reasons. Fresh samples have higher yield of % cell recovery and total genes post-sequencing compared to cryopreserved samples (median % cell recovery of 79.51% vs 13.40% and median total genes of 24 390 vs 16 803 per cell, Mann-Whitney U test P values of 0.0003 and 0.002) (see Figure 3.10).

There are multiple factors which may affect the library complexities obtained post-sequencing. One of the main contributing factors is cell viability. Apart from this, other factors include the number of cells inserted into each library balanced against multiplet risk, integrity of cells prior to library generation which may affect the cell survival after the addition of 10x lysis reagent, intrinsic mRNA content, mRNA integrity, cDNA concentration following PCR amplification which is directly influenced by the mRNA integrity during GEM formation and quality of library generated. From the experiments conducted, increasing % of cell viability increased total genes and cell recovery post-sequencing (moderate correlation with Spearman rho values of approximately 0.5, P

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values 0.03) (see Figure 3.11). Concentration of cDNA of each library (obtained through Bioanalyser analysis) was strongly correlated with the cell recovery post-sequencing (Spearman's rho value of 0.74, P value 0.0004) (see Figure 3.11).

[Type here]

Table 3.19 List of 18 Single Cell Libraries Generated and Sequenced

Experiment	Patient ID	Sample Site	Sample State, Surgery, Dissociation	Cell input (No. of cells)	% Cell Viability	Live cells pre-sequencing (No. of cells)	Qubit post cDNA amplification (normalised to 11 cycles) [mg/μl]	Bioanalyser results			^Cell recovery post-sequencing (No. of cells)	% Cell recovery	Median Genes/Cell	Total Genes
								cDNA length (bp)	[cDNA] (ng/μl)	[cDNA] [Nm]				
E05+	1	Small bowel nodule (Met)	Cryo, IDS, M	12000	63	7560	0.4	438	1.0	3.4	786	10.4	843	16680
E09A+	2	Ovary (Pri)	Cryo, PDS, M	18000	54	9720	1.1	416	19.6	71.4	2042	21.0	369	17176
E09B*	2	Ovary (Pri)	Cryo, PDS, A	18000	73	13140	1.0	497	0.2	0.7	1300	9.9	266	14765
E11*	3	Ovary (Pri)	Cryo, PDS, A	8700	90	7830	0.6	454	10.7	35.8	505	6.5	910	15036
E14*	4	Ovary (Pri)	Cryo, PDS, A	18000	84	15120	1.7	406	11.4	42.7	2480	16.4	459	16926
Control	NA	RPE1 cell line	Fresh, NA, NA	18000	58	10440	4.6	447	19.2	64.9	2996	28.7	498	14182
E21	5	Ovary (Pri)	Cryo, PDS, A	16000	83	13280	5.3	388	38.3	149.6	5094	38.4	1520	24282
E25	6	Peritoneal nodule (Met)	Fresh, IDS, A	18000	81	14580	13.1	388	40.3	157.3	12918	88.6	901	24282
E25	6	PBMC (Blood)	Fresh, IDS, NA	18000	91	16380	2.5	455	22.0	73.3	5957	36.4	1002	20960
E27	7	Ovary (Pri)	Fresh, PDS, A	21000	95	19950	10.0	441	28.6	97.9	15862	79.5	1022	24390

[Type here]

E27	7	Peritoneal nodule (Met)	Fresh, PDS, A	21000	96	20160	33.6	424	39.9	142.4	13539	67.2	1087	22999
E27	7	PBMC (Blood)	Fresh, PDS, A	21000	93	19530	8.8	479	26.8	84.9	18575	95.1	1164	21592
E28~	8	Ovary1 (Pri)	Fresh, PDS, A	25000	74	18500	11.0	518	38.3	112.0	7245	39.2	1010	24171
E28~	8	Ovary2 (Pri)	Fresh, PDS, A	25000	74	18500	7.5	439	43.3	149.6	13398	72.4	1057	25700
E29	9	Ovary (Pri)	Fresh, PDS, A	25000	94	23500	5.5	517	47.0	137.7	21891	93.2	994	24465
E29	9	Hemi-diaphragm peritoneal nodule (Met)	Fresh, PDS, A	25000	98	24500	11.0	526	25.8	74.2	13879	56.7	844	25710
E29	9	Omentum (Met)	Fresh, PDS, A	25000	82	20500	9.3	460	38.6	127.2	16828	82.1	1120	24907
E29	9	Small bowel nodule (Met)	Fresh, PDS, A	25000	100	25000	21.0	564	34.3	92.0	19942	79.8	688	24465

E09A and E09B were from the same patient and adjacent primary sites.

^CellRanger estimate

+Dissociated using manual protocol without DCR as processed earlier before we started using DCR kits.

*Dissociated using automated protocol with tumour cell isolation and DCR performed.

~Low grade serous ovarian cancer (LGSOC) sample from the same site. Two separate libraries were generated using the same single cell suspension from this sample. E28 was used as a control sample to check that the bioinformatics analytical method produced consistent results with similar cell populations uncovered.

Cryo, Cryopreserved sample.

IDS, Interval debulking surgery (surgery post three cycles of chemotherapy).

PDS, Primary debulking surgery (surgery pre-chemotherapy).

M, Manual dissociation; A, Automated dissociation.

Pri, Primary site; Met, Metastatic site.

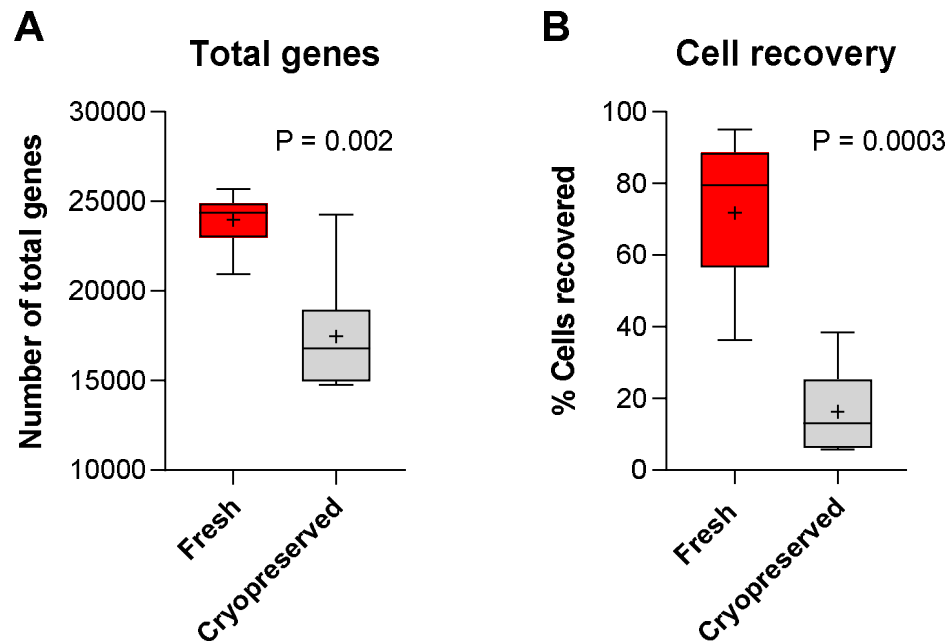


Figure 3.10 Comparison of Fresh vs Cryopreserved Samples. Fresh samples yielded higher total genes per cell and %cell recovery post-sequencing compared to cryopreserved samples (significant P values using Mann-Whitney U test).

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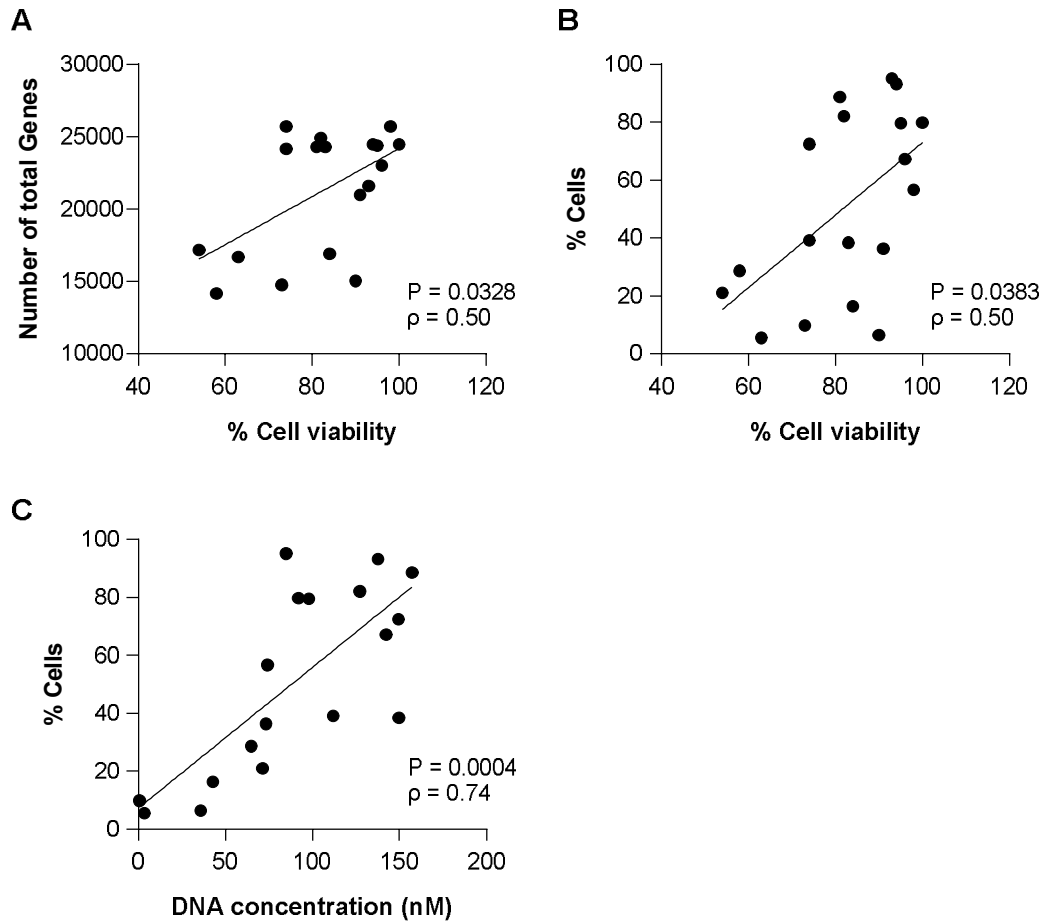


Figure 3.11 Cell Recovery Post-Sequencing Affected by %Cell Viability and ScDNA Library Concentration. (A) and (B) % Cell viability moderately correlated to total genes (present in the sequenced transcripts) and cell recovery post-sequencing. (C) ScDNA library concentration strongly correlated to cell recovery post-sequencing. Correlation calculated using Spearman's correlation with rho, ρ and P values illustrated above.

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As first pass QC of the sequenced data, the raw data were filtered through 10x Cell Ranger that performed alignment, filtering, barcode and UMI counting to provide an estimate of the cell number, library depth and complexity. Using the cell count metrics of Cell Ranger, the library quality from the 3 batches were compared which reflected an overall improvement in library sizes, depth and complexity. The first batch compared to the second and third batches of this study clearly was inferior in terms of average cell recovery (in order of approximately 10K less cells) and shallower read depth with smaller mean transcriptomic size (see Table 3.20).

Table 3.20 Summary of Cell Count Metrics by Cell Ranger (17 Libraries of OC Patients)

Batch	Experiments	Patient ID	Mean % Cell Viability	Total No. of Cells	Mean No. of Cells	Mean Fraction Reads in Cells (%)	Mean Reads/Cell	Median Genes/Cell	Median UMI Counts/Cell	Mean Transcriptome Size (Avg No. of Genes)
1	E05-E14	1-4	72.8	7113	1423	62.8	6441*	569	1167	16117
2	E21-E28	5-8	85.9	92588	11574	77.8	27244	1095	2556	23547
3	E29	9	93.5	72540	18135	68.5	14804	912	1649	24887

*Mean reads/ cell of libraries E09-E14 excluding E05 (this sample was our first ever sequenced library which was sent for sequencing with glioblastoma samples as we only had one sample then and E05 had grossly inflated read depth and sequencing saturation).

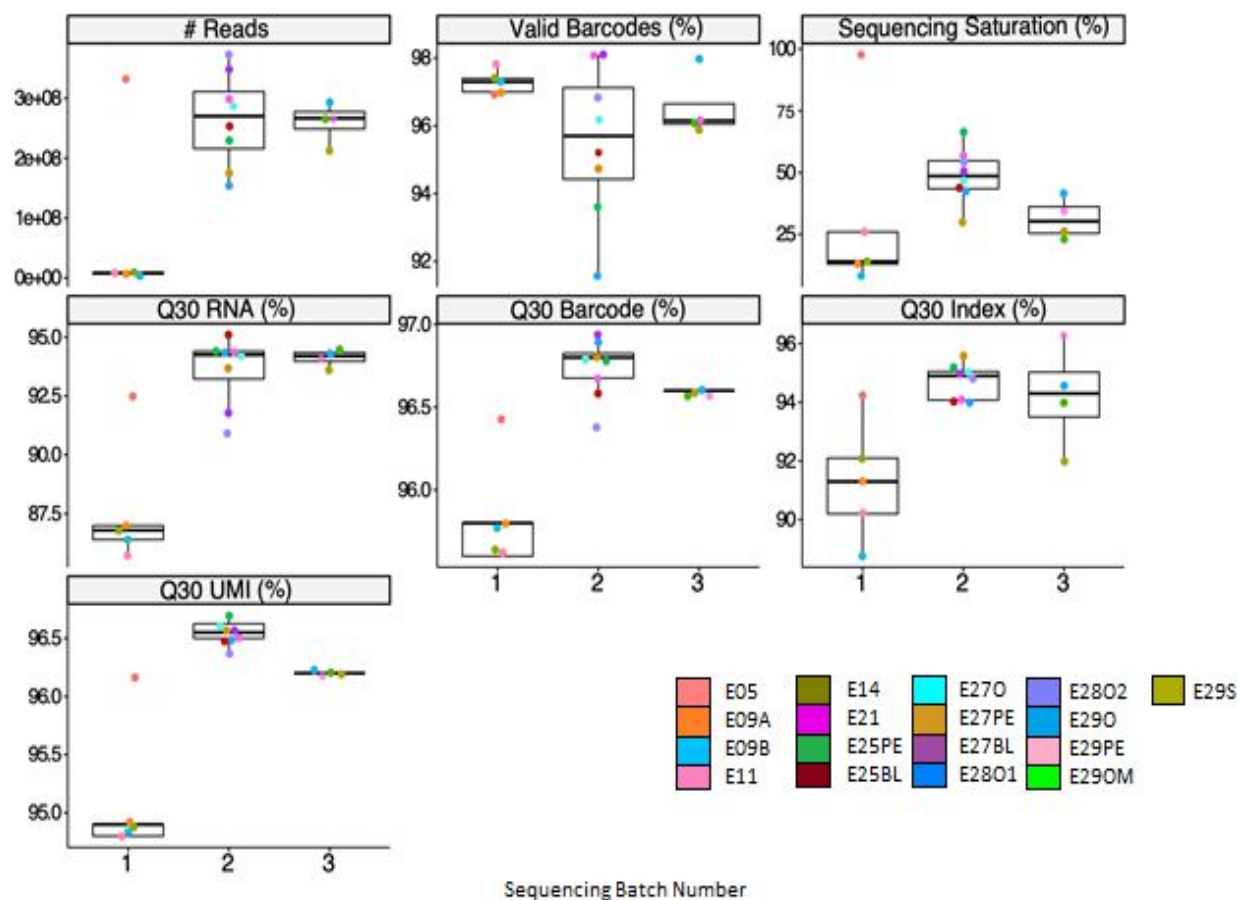
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From the Sequencing Statistical analysis performed by CellRanger, in comparison to batch 1, batches 2 and 3 displayed higher number of Reads and Q30 Bases in RNA Read/Barcode/Sample Index/UMI (where Q-score ≥ 30 represents high quality bases) (see Figure 3.26). Most importantly, the later batches 2 and 3 had greater sequencing saturations (a function of library complexity and sequencing depth) illustrating higher confidently mapped, valid cell-barcode and UMI reads. Note, E05 remained an outlier where its performance was generally comparable to the higher quality samples in batches 2 and 3 (see Figure 3.12 and Table 3.20).

From the Mapping Statistics, generally there is greater exonic mapped reads than intronic (for batches 1 and 2) and almost similar exonic and intronic mapped reads for batch 3 (see Figure 3.13). A higher intronic mapping rate for batch 3 compared to the other two batches could be explained by the priming of internal poly(A) tract in the mRNA instead of the poly(A) tail, resulting in a sense read pair located internally rather than at the end of the transcript. This mechanism (although could result in reads on exons or introns) leads to greater reads occurring on introns as there are ~ 21.2 times as many poly(A) stretches in introns compared to exons in the human genome. A high fraction of intronic reads is considered when $\sim 50\text{-}70\%$ of the reads are mapped to the introns rather than exons. While intronic inclusion increases assay sensitivity and detection of genes that have no exonic UMIs in any cell, the level of intronic mapped reads in our batches are not considered high enough to increase gene length bias as it is still $< 50\%$ of the mapped reads (Genomics, 2021). Another explanation for the higher intronic mapping rate for batch 3 could also be due to the presence of more intronic reads originating from immature transcripts either from full-length pre-mRNA molecules or nascent transcripts where the RNA polymerase has not yet attached to

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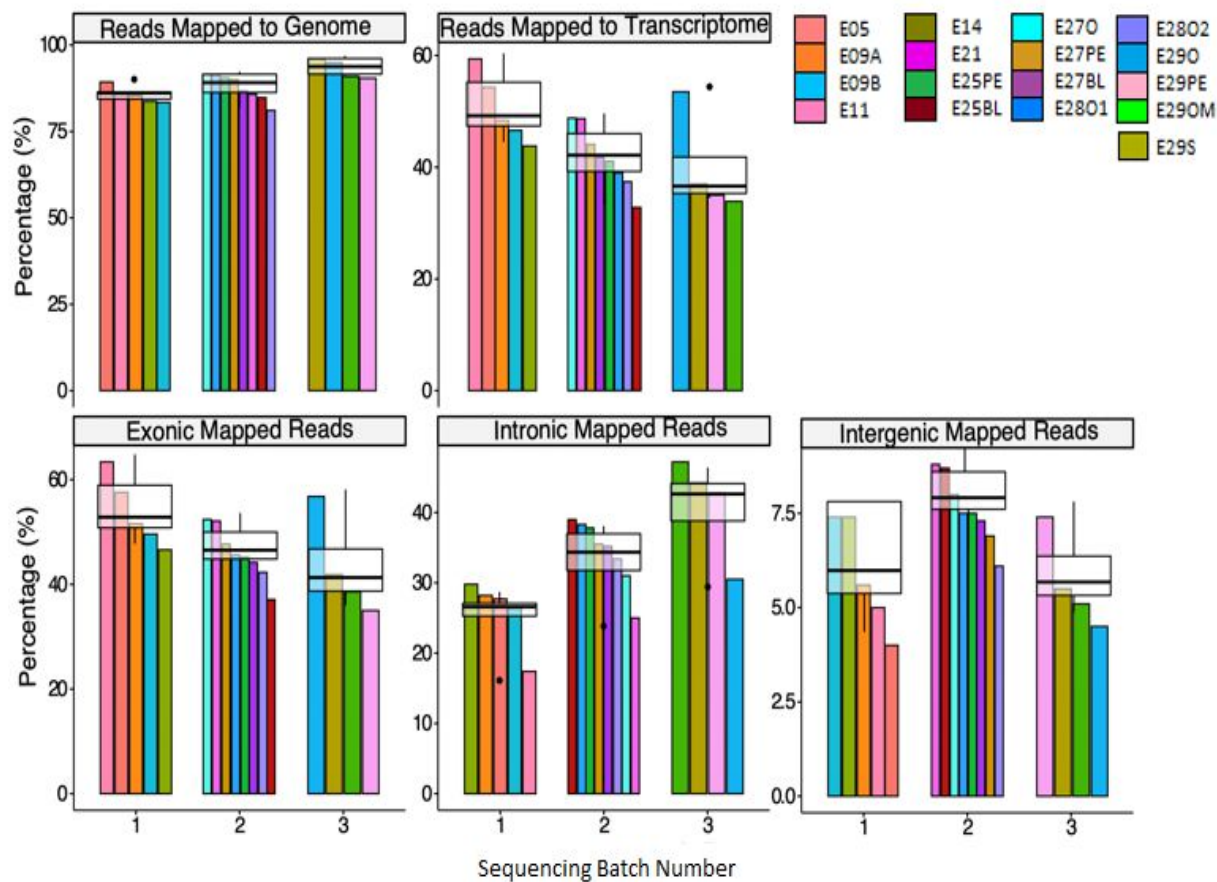
the 3' of the gene. This may well reflect the lower reads mapped to transcriptome for batch 3 (Lee et al., 2020).



PE = Peritoneal nodule, BL = Blood (PBMC), O = Ovary, OM = Omentum, S = Small bowel nodule

Figure 3.12 Sequencing Statistics for 17 Libraries of OC Patients. Samples are divided according to their sequencing batch with E05-E14 in batch 1, E21-E28 in batch 2 and E29 in batch 3.

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PE = Peritoneal nodule, BL = Blood (PBMC), O = Ovary, OM = Omentum, S = Small bowel nodule

Figure 3.13 Mapping Statistics for 17 Libraries of OC Patients. Samples are divided according to their sequencing batch with E05-E14 in batch 1, E21-E28 in batch 2 and E29 in batch 3.

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As part of more stringent QC check, the raw data were filtered through both 10x CellRanger and *Seurat* v3 R package before retaining the 'good quality cells' for analysis. Table 3.21 illustrates one of the metrics used by CellRanger (i.e. 'fraction reads in cells') to partition barcodes as cells versus background to produce a cell estimate and further filtering by R/Seurat predominantly based on mitochondrial gene expression. According to 10x, a low 'fraction reads in cells' <70% typically suggests that there is high ambient RNA (background) within the sample (Genomics, 2022b).

% Cell retention post-filtering is the proportion of cells that were retained for downstream bioinformatics analysis whereby the 'low quality cells' were filtered if they were outwith +/- 3 Median Absolute Deviations (MADs) of their total read or feature counts or percentage of mitochondrial expression. For the 17 libraries of OC patients in this study (excluding the cell line control), % of cell retention post-filtering (by R/Seurat) did not correlate significantly to the % fraction reads in cells (Spearman's rho -0.128, P value 0.625) (Table 3.21). There was a relatively high percentage of cell retention post-filtering by R/Seurat (median cell retention 80.5%). This may be explained by the fact that the cell-calling heuristic by 10x CellRanger did not strictly apply due to the fact that there may be higher variation in mRNA content than expected in samples with low 'fraction reads in cells' (i.e. these samples having more cells with lower mRNA content).

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Table 3.21 QC by CellRanger Algorithm vs Seurat Filtering (17 Libraries of OC Patients)

Experiment	Sample Site	Sample State, Surgery, Dissociation	Fraction Reads in Cells (%)	Cell recovery post-sequencing		Cell Retention Post- filtering (%) by R/Seurat
				CellRanger Estimate	Seurat Cell Number (Filtered)	
E05+	Small bowel nodule (Met)	Cryo, IDS, M	70	786	634	80.7
E09A+	Ovary (Pri)	Cryo, PDS, M	71	2042	1595	78.1
E09B*	Ovary (Pri)	Cryo, PDS, A	58	1300	741	57.0
E11*	Ovary (Pri)	Cryo, PDS, A	40	505	424	84.0
E14*	Ovary (Pri)	Cryo, PDS, A	75	2480	2173	87.6
E21	Ovary (Pri)	Cryo, PDS, A	77	5094	3509	68.9
E25	Peritoneal nodule (Met)	Fresh, IDS, A	81	12918	6732	52.1
E25	PBMC (Blood)	Fresh, IDS, NA	86	5957	4430	74.4
E27	Ovary (Pri)	Fresh, PDS, A	83	15862	12333	77.8

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E27	Peritoneal nodule (Met)	Fresh, PDS, A	76	13539	12303	90.9
E27	PBMC (Blood)	Fresh, PDS, A	87	18575	14959	80.5
E28~	Ovary1 (Pri)	Fresh, PDS, A	62	7245	6268	86.5
E28~	Ovary2 (Pri)	Fresh, PDS, A	70	13398	11911	88.9
E29	Ovary (Pri)	Fresh, PDS, A	74	21891	19578	89.4
E29	Hemi-diaphragm peritoneal nodule (Met)	Fresh, PDS, A	70	13879	10486	75.6
E29	Omentum (Met)	Fresh, PDS, A	64	16828	14052	83.5
E29	Small bowel nodule (Met)	Fresh, PDS, A	66	19942	12178	61.1

E09A and E09B were from the same patient and adjacent primary sites.

+Dissociated using manual protocol without DCR as processed earlier before we started using DCR kits.

*Dissociated using automated protocol with tumour cell isolation and DCR performed.

~Low grade serous ovarian cancer (LGSOC) sample from the same site.

Cryo, Cryopreserved sample; IDS, Interval debulking surgery (surgery post three cycles of chemotherapy).

PDS, Primary debulking surgery (surgery pre-chemotherapy).

M, Manual dissociation; A, Automated dissociation.

Pri, Primary site; Met, Metastatic site.

3.4.2 Patient Description

A total of 134 306 cells were profiled by single cell RNA-sequencing on 9 OC patients with 17 samples. 8 patients were diagnosed with advanced HGSOC and 1 patient had LGSOC. 5 patients including the LGSOC patient had a sample from primary site and underwent primary debulking surgery (PDS) and 2 HGSOC patients had samples from primary and metastatic sites. Further 2 patients had samples from metastatic sites and underwent interval debulking surgery (IDS) after 3 cycles of neo-adjuvant chemotherapy (NACT). Out of these 9 patients, solid tumour samples (N = 8) from 4 advanced HGSOC patients (1 patient with PDS primary site, 1 with IDS metastatic site, 2 with both primary and metastatic sites from PDS) produced a total of 91 171 cells which were most informative in the analysis of the TME of the cancer. Of these 4 patients, 2 of the PDS patients had >2cm residual disease (Patient 5 and 7) at the end of the debulking surgery while the other 2 had <2cm residual disease (Patient 6 was IDS patient with good chemotherapy response, while Patient 9 had a successful PDS).

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Table 3.22 Patients and Sample Sites Description

Experiment	Patient ID	Age	Race	Histologic Type	FIGO Stage	Neo-adjutant therapy	Sample Site	Sample State, Surgery, Dissociation	No. of Genes Post-filtering	No. of Cells	Comments
E05	1	57	White	HGSOC	IVB	Yes	Small bowel nodule (Met)	Cryo, IDS, M	18936	634	
E09A	2	64	White	HGSOC	IIIC	No	Ovary (Pri)	Cryo, PDS, M	9967	1595	
E09B	2						Ovary (Pri)	Cryo, PDS, A	6259	741	
E11	3	67	White	HGSOC	IIIC	No	Ovary (Pri)	Cryo, PDS, A	8320	424	
E14	4	59	White	HGSOC	IIIA2	No	Ovary (Pri)	Cryo, PDS, A	10954	2173	
E21	5	56	White	HGSOC	IIIC	No	Ovary (Pri)	Cryo, PDS, A	19148	3509	> 2cm RD
E25	6 (4)	59	White	HGSOC	IIIA2	Yes	Peritoneal nodule	Fresh, IDS, A	17571	6732	< 2cm RD, good

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							(Met) near small bowel				chemo response
E27	7	67	White	HGSOC	IIIC	No	Ovary (Pri)	Fresh, PDS, A	18566	12333	> 2cm RD
E27	7						Peritoneal nodule (Met) from left paracolic gutter	Fresh, PDS, A	17530	12303	
E28	8	24	White	LGSOC	IIIB	No	Ovary1 (Pri)	Fresh, PDS, A	17438	6268	LGSOC
E28	8						Ovary2 (Pri)	Fresh, PDS, A	19427	11911	
E29	9	50	White	HGSOC	IIIC	No	Ovary (Pri)	Fresh, PDS, A	18652	19578	< 2cm RD

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E29	9						Hemi-diaphragm peritoneal nodule (Met)	Fresh, PDS, A	18714	10486	
E29	9		White				Omentum (Met)	Fresh, PDS, A	18893	14052	
E29	9		White				Small bowel nodule (Met)	Fresh, PDS, A	17791	12178	

Cryo, Cryopreserved sample; IDS, Interval debulking surgery (surgery post three cycles of chemotherapy).

PDS, Primary debulking surgery (surgery pre-chemotherapy).

M, Manual dissociation; A, Automated dissociation.

Pri, Primary site; Met, Metastatic site.

3.4.3 Cell identification

Pooled scRNA-seq data from 8 good quality samples of the 4 HGSOC patients (5, 6, 7, and 9) identified distinct cell types within the tumour microenvironment that correlated with known cellular gene expression patterns. The cell types are broadly divided into 3 major groups defined by expression of canonical marker genes (Andrew J. Shih et al., 2018) (see Figure 3.14):

1. Tumour – cancer epithelial cells (*EPCAM*, *MUC16*, *WFDC2*, *CD24*, cytokerin-encoding genes) and ciliated cancer epithelial cells (*TTPP3*, *PIFO*, *FOXJ*);
2. Stroma – fibroblasts (*VIM*, *CD81*, *ENG*, *ACTA2*, *MMP2*), adipocytes (*CFD*, *APOD*, *PLA2G2A*), endothelial cells (*PECAM1*, *MCAM*, *VWF*), red blood cells (*HBB*, *HBA2*, *HBA1*);
3. Immune – lymphoid lineage including T lymphocytes (*PTPRC*, *CD3E*, *CD3D*, *IL7R*, *CD2*) and B lymphocytes (*CD79A*, *MS4A1*, *C79B*, *BANK1*) and myeloid lineage (*CD14*, *C1QC*, *LYZ*, *C1QB*, *APOE*).

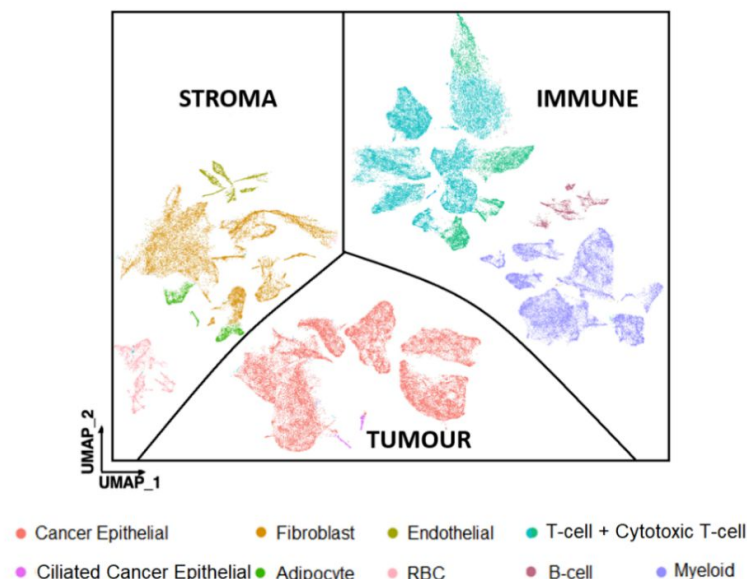


Figure 3.14 Distinct Cell Types across 4 HGSOC Patients with 8 Samples.

Table 3.23 Canonical Marker Genes Used for Cell Type Identification

No.	Cell type	Marker genes	References
1.	Ovarian cancer epithelial cell	<i>EPCAM, MUC16, WFDC2, CD24</i>	(Schelker et al., 2017; Andrew J. Shih et al., 2018; Xinxin Zhang et al., 2019)
2.	Ciliated cancer epithelial	<i>TPPP3, PIFO, FOXJ1</i>	(P. J. Thul et al., 2017)
3.	Fibroblast	<i>VIM, CD81, ENG, ACTA2, MMP2</i>	(Schelker et al., 2017; P. J. Thul et al., 2017; Xinxin Zhang et al., 2019)
4.	Adipocyte	<i>CFD, APOD, PLA2G2A</i>	(Xinxin Zhang et al., 2019)
5.	Endothelial cell	<i>PECAM1, MCAM, VWF</i>	(Schelker et al., 2017; Xinxin Zhang et al., 2019)
6.	Red blood cell	<i>HBB, HBA2, HBA1</i>	(Tsang et al., 2017; Xinxin Zhang et al., 2019)
7.	T cell	<i>PTPRC, CD3E, CD3D, IL7R, CD2</i>	(P. J. Thul et al., 2017; Xinxin Zhang et al., 2019)
8.	Myeloid cell	<i>CD14, C1QC, LYZ, C1QB, APOE</i>	(Schelker et al., 2017; Xinxin Zhang et al., 2019)
9.	B cell	<i>CD79A, MS4A1, C79B, BANK1</i>	(Schelker et al., 2017; Xinxin Zhang et al., 2019)

3.4.3.1 Confidence of cell type identification

The cell type identification method using known cellular gene markers in this study was highly reproducible throughout the 8 good quality libraries. These datasets originated from 4 different HGSOC patients (patients 5, 6, 7, and 9) with a mixture of pre- and post-chemotherapy samples from various sites representing heterogeneity within and between the patients. Despite this, using unsupervised hierarchical clustering of populations of cells from all patients resulted in 3 major groups of cells (cancer epithelial, stromal and immune) concordant with the above findings (see 3.5.3 Cell Identification). The Z-scores of shared highly variable genes (N = 411) across the average transcriptome of each cell type from every sample correlated highly with the same cell type (see Figure 3.29). Z-score represents a standardised expression of genes within each cluster, where $Z = (\text{Gene expression in tumour sample} - \text{Mean expression in reference sample}) / \text{Standard deviation of expression in reference sample}$. (Cheadle et al., 2003)

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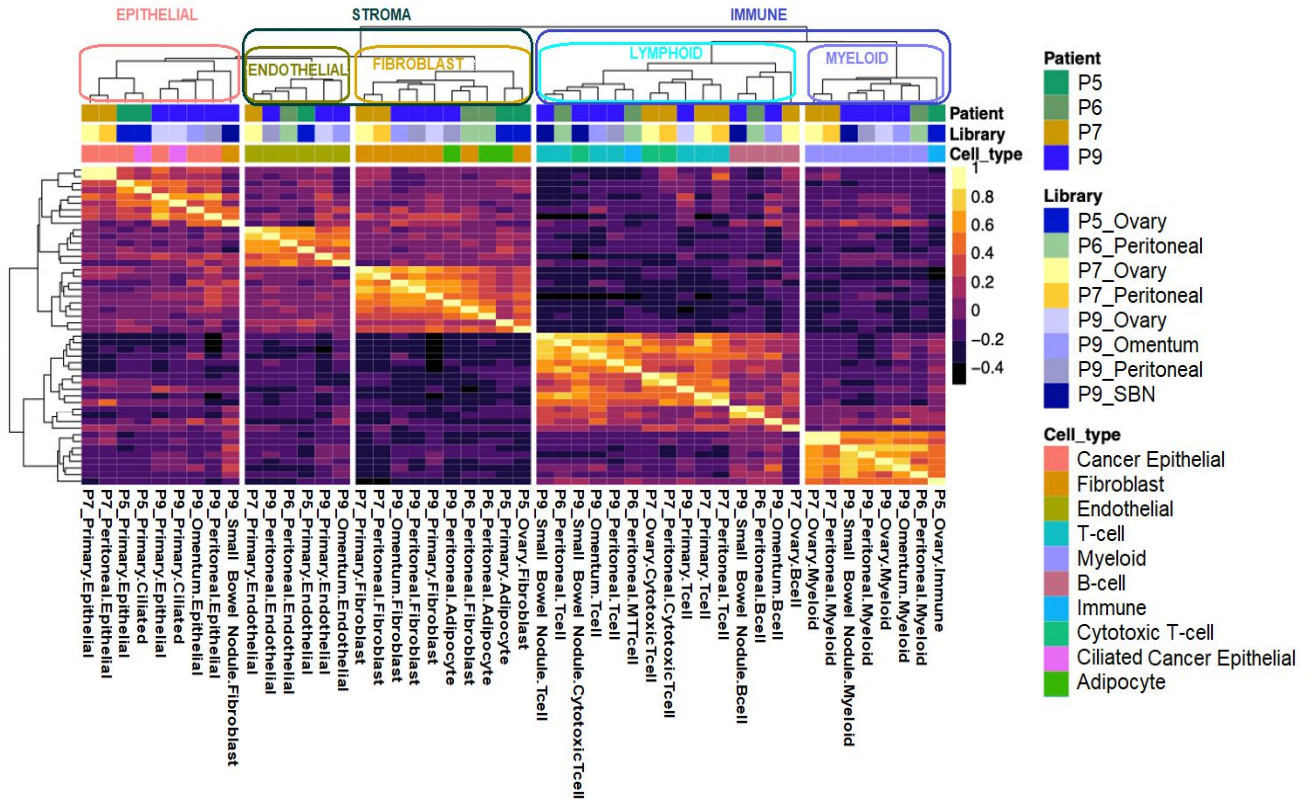


Figure 3.15 Z-score Pearson Correlation of Shared Highly Variable Genes (NHGVs = 411) across the Average Transcriptome of Each Cell Type from Every Sample. With the exception of P9_Small Bowel Nodule. Fibroblast cells, cancer epithelial and ciliated cancer epithelial clustered into the Tumour Epithelial group; endothelial, fibroblasts and adipocytes clustered into the Stromal group; and lymphoid (T cells, cytotoxic T cells and B cells) and myeloid cells clustered into the Immune group.

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3.4.3.2 Cancer epithelial and ciliated cancer epithelial cells belong to the same cell group (tumour epithelial cells)

Within the tumour compartment, the single cell datasets in this study illustrated two types of cells which expressed similar canonical markers for HGSOC epithelial genes but on their own entity have differentially expressed genes (DEGs). This implied that both these cells were epithelial in nature, yet were subtly distinct from each other. These cells were identified as cancer epithelial cells (non-ciliated) and ciliated cancer epithelial cells.

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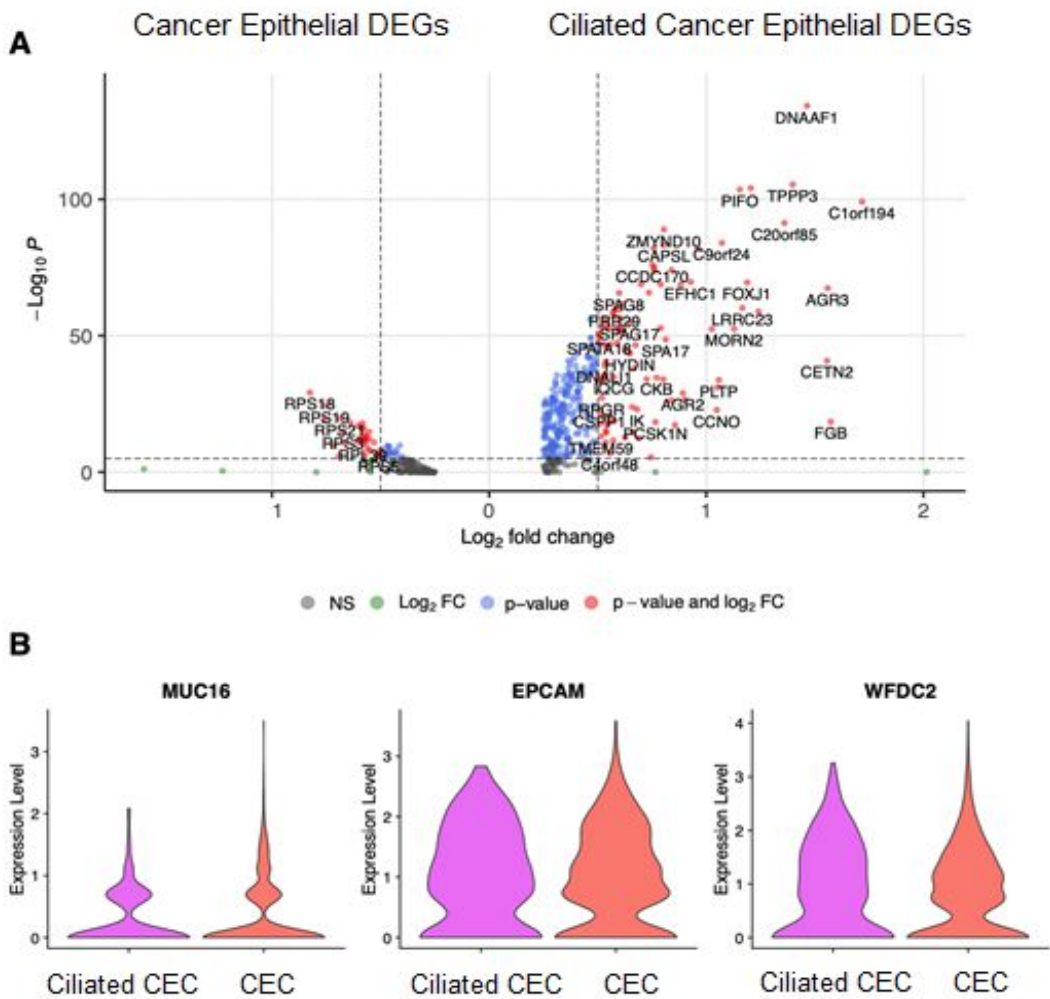


Figure 3.16 Comparison of Cancer Epithelial Cells and Ciliated Cancer Epithelial Cells. (A) Volcano plot of the differentially expressed genes between cancer epithelial (non-ciliated) and ciliated cancer epithelial cells from the datasets that feature both these cell types. Wilcoxon Rank Sum test detected statistical significant difference in the DEGs observed between these two cell types. (B) Violin plots of the expression of 3 canonical gene markers for HGSOC epithelial cells in the cancer epithelial and ciliated cancer epithelial cells. Wilcoxon Rank Sum test did not detect statistical differential expressed canonical genes between these two cells.

3.4.4 Cell population by patient and by site

Each patient sample had differing relative proportions of the identified cell types, see Figure 3.18 for proportions of cell types identified. The majority of the primary adnexal masses and metastatic samples contained cancer epithelial cells. Of note, the peritoneal nodule (metastatic site) of Patient 6 who had neoadjuvant chemotherapy was deficient in cancer epithelial cells. The primary adnexal mass sample of Patient 6 was dissociated but did not make it to library generation due to failing cell viability QC (final cell viability of 56-60%). At interval debulking surgery (IDS), this patient had good chemotherapy response with no gross residual disease at the end of surgery. There was also a unique population of T-cell within the peritoneal nodule of Patient 6 with metallothionein expression which was observed only in a post-chemotherapy sample and absent in all other pre-chemotherapy samples. Patient 5 and 7 both had >2cm residual disease at the end of primary debulking surgery (PDS). The primary adnexal sample of Patient 5 had more than 75% of cancer epithelial cells with minimal immune cells. Patient 7 had increasing cancer epithelial cells with decreasing immune cells from her primary to metastatic sample (> 50% cancer epithelial cells and < 50% immune cells in metastatic site). Patient 9 had a successful cytoreduction at PDS with no gross residual disease at the end of surgery. This patient had a good representation of the disease TME with primary adnexal and multiple metastatic sites sampled and sequenced. Moving from primary adnexal to metastatic sites, the proportion of cancer epithelial cells was decreased with complete absence in small bowel nodule site while immune cells (particularly T cells) increased.

Within the epithelial compartment, there was a small population of ciliated epithelial cells in the primary adnexal masses of patient 5 and 9. This population is specific to primary adnexal sites and absent from all metastatic sites.

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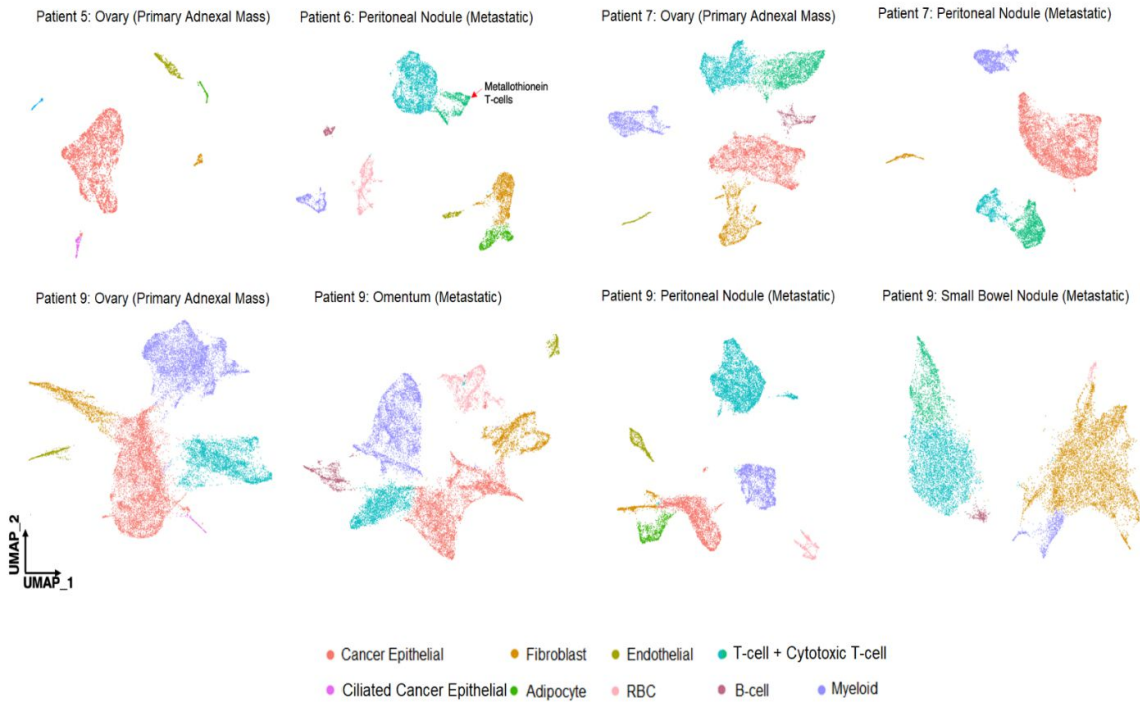


Figure 3.17 UMAPs of the individual samples of the 4 HGSOc Patients Used for Cell Identification.

Table 3.24 Proportion of Cell Types (%) Making up Each Sample

Samples	Patient 5	Patient 6	Patient 7	Patient 7	Patient 9	Patient 9	Patient 9	Patient 9
Cell Types	OV	PE	OV	PE	OV	OM	PE	SBN
Cancer Epithelial	80.3	0.0	30.8	57.3	37.7	31.3	22.8	0.0
Fibroblast	2.7	17.9	12.0	3.2	7.7	11.7	4.9	49.5
Adipocyte	3.5	9.5	0.0	0.0	0.0	0.0	6.6	0.0
Endothelial	8.2	3.2	1.9	0.0	2.3	2.5	5.3	0.0
Ciliated Cancer Epithelial	3.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
T cell	0.0	37.1	39.0	25.0	19.0	14.5	39.0	42.8
Myeloid	0.0	7.3	12.4	14.5	32.6	26.0	17.0	5.1
B-cell	0.0	2.9	3.9	0.0	0.0	5.3	0.0	1.8
RBC	0.0	12.3	0.0	0.0	0.0	8.7	4.4	0.8
Metallothionein T cell	0.0	9.8	0.0	0.0	0.0	0.0	0.0	0.0

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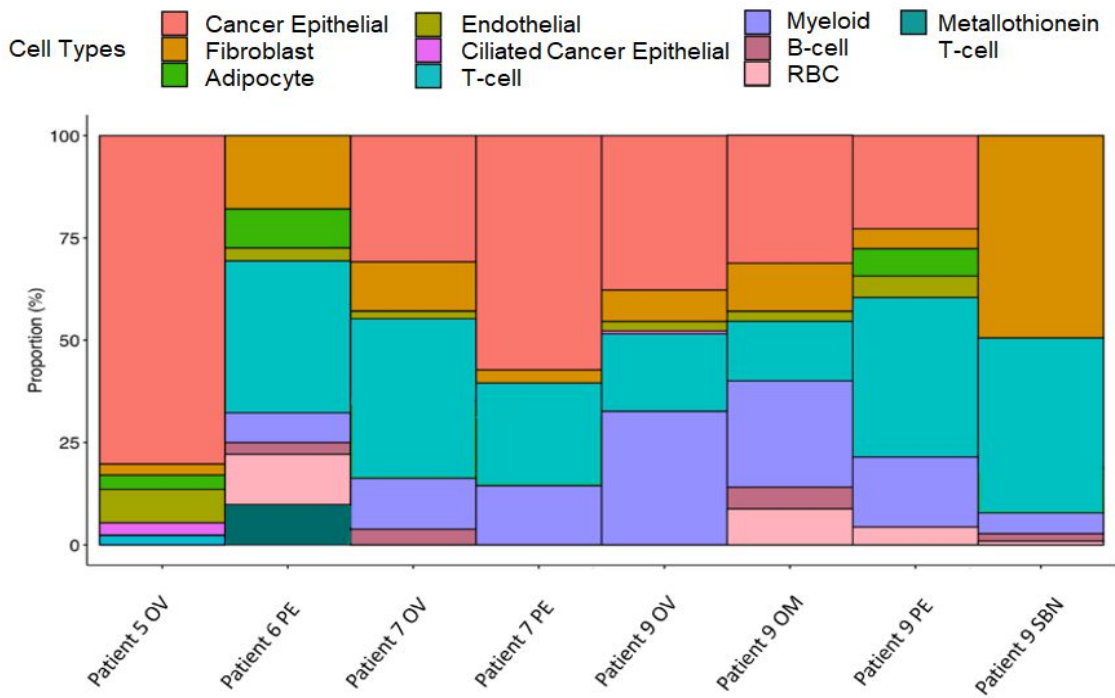


Figure 3.18 Proportions of Cells Making Up Each Sample.

Patients' sample sites: OV, ovary; PE, peritoneal nodule; OM, omentum; SBN, small bowel nodule. Cell type: RBC, red blood cell.

3.4.5 Inference of CNVs of HGSOC epithelial cells to determine cancer epithelial cells (aneuploid/ malignant) vs normal epithelial cells

Using the copy number karyotyping of aneuploidy tumours (CopyKAT) approach (R. Gao et al., 2021), the genomic copy number profiles in the HGSOC epithelial cells were estimated. A single epithelial cell is defined as a confident normal epithelial cell (i.e. diploid) when genes in neutral states account for at least 99% of the expressed genes with little or no copy number aberrations. Meanwhile, aneuploid epithelial cells (malignant) have genomic gain or losses resulting in aberrant chromosome or chromosome arm copy number (Taylor et al., 2018). CopyKAT assignment of aneuploidy to cancer epithelial cells distinguishing them from normal epithelial cell works well with HGSOC due to the fact that this tumour is highly aneuploidy. The patients' myeloid cells were used as the background normal cells. From the analysis of 21 249 HGSOC epithelial cells from 3 patient samples (Patient 5, 7 and 9), a total 18 738 cells were identified as cancer epithelial cells with copy number aberrations (88.18%) (Table 3.25).

The CNV heatmaps (see Figure 3.19) show that compared to normal myeloid cells, the cancer epithelial cells have greater CNV losses or gains as seen on the relative gene expression intensities across each chromosome. The inter-patient CNV signatures are broadly different from each other. However, the intra-patient differences are more subtle across different sites (as seen from Patient 7 and 9 with multiple samples) (Figure 3.19). It is interesting to note that within Patient 7 there are two subclones of CNV signatures with differing proportions across her primary ovarian and peritoneal tumour samples (see Figure 3.21). These CNV signatures suggest that

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there are intra- and inter-patient heterogeneity with specific clonal heterogeneity within the tumour cell populations.

Table 3.25 Proportions of HGSOC Epithelial Cells Classified as Cancer Epithelial or Normal Cells by CopyKAT

Experiment	Patient ID	Sample Site	Sample State	No. of Cancer Epithelial Cells (%)	No. of Normal Epithelial Cells (%)	Total Epithelial Cells
E21	5	Ovary	Cryo	1753 (73%)	640 (27%)	2393
E27	7	Ovary	Fresh	3039 (>99%)	11 (<1%)	3050
E27	7	Peritoneal nodule (Met) from left paracolic gutter	Fresh	5002 (99%)	39 (1%)	5041
E29	9	Ovary (Pri)	Fresh	5054 (95%)	248 (5%)	5302
E29	9	Hemi-diaphragm peritoneal nodule (Met)	Fresh	1107 (97%)	34 (3%)	1141
E29	9	Omentum (Met)	Fresh, PDS, A	2783 (64%)	1539 (36%)	4322

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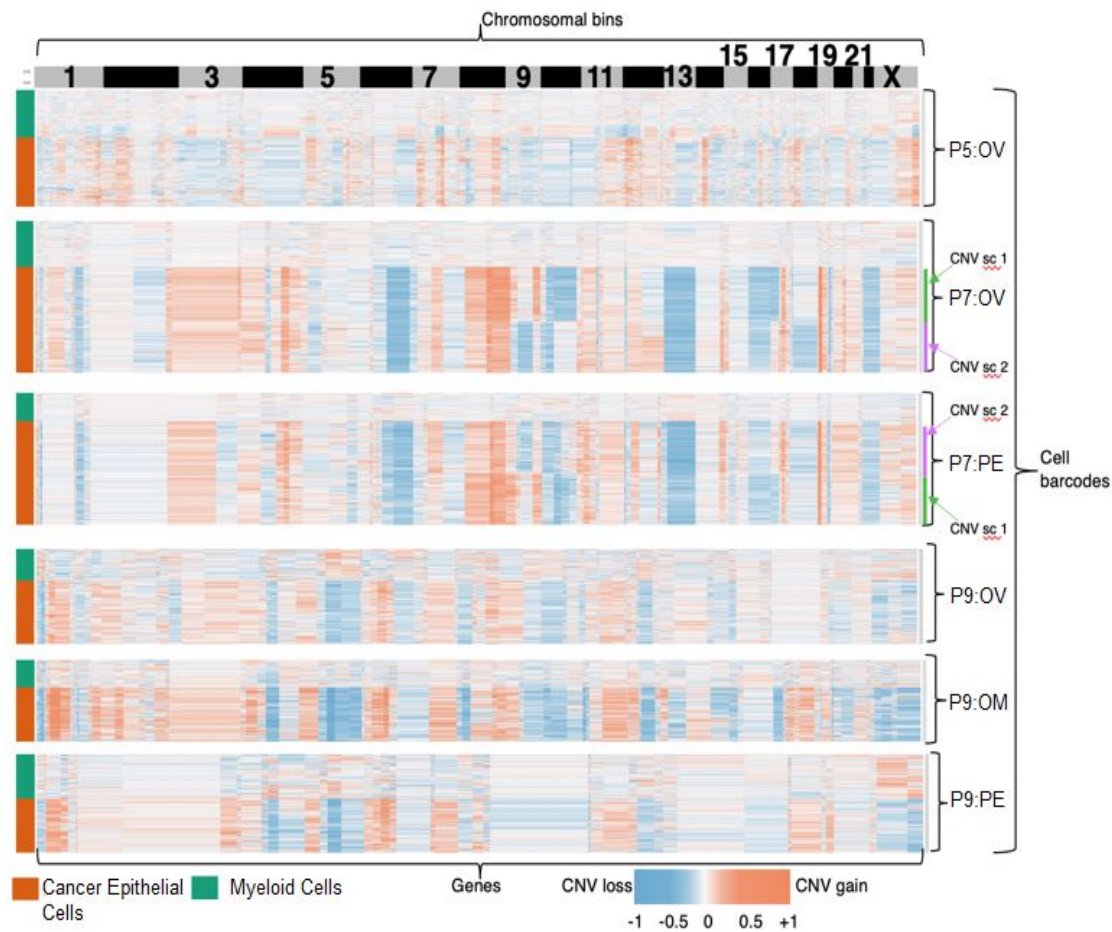


Figure 3.19 Copy Number Profile in Cancer Epithelial Cells vs Normal Background Myeloid Cells. CNVs heatmaps of 6 samples from patients 5,7, and 9 illustrate the CNV gains and losses in red and blue respectively. The orange bar on the far left represent cancer epithelial cells and green represent normal background cells generated from myeloid cells. Rows correspond to individual cells across the 6 samples from the 3 patients and columns correspond to genes ordered by chromosomal location (with black and grey bars at the top indicating different chromosomes).

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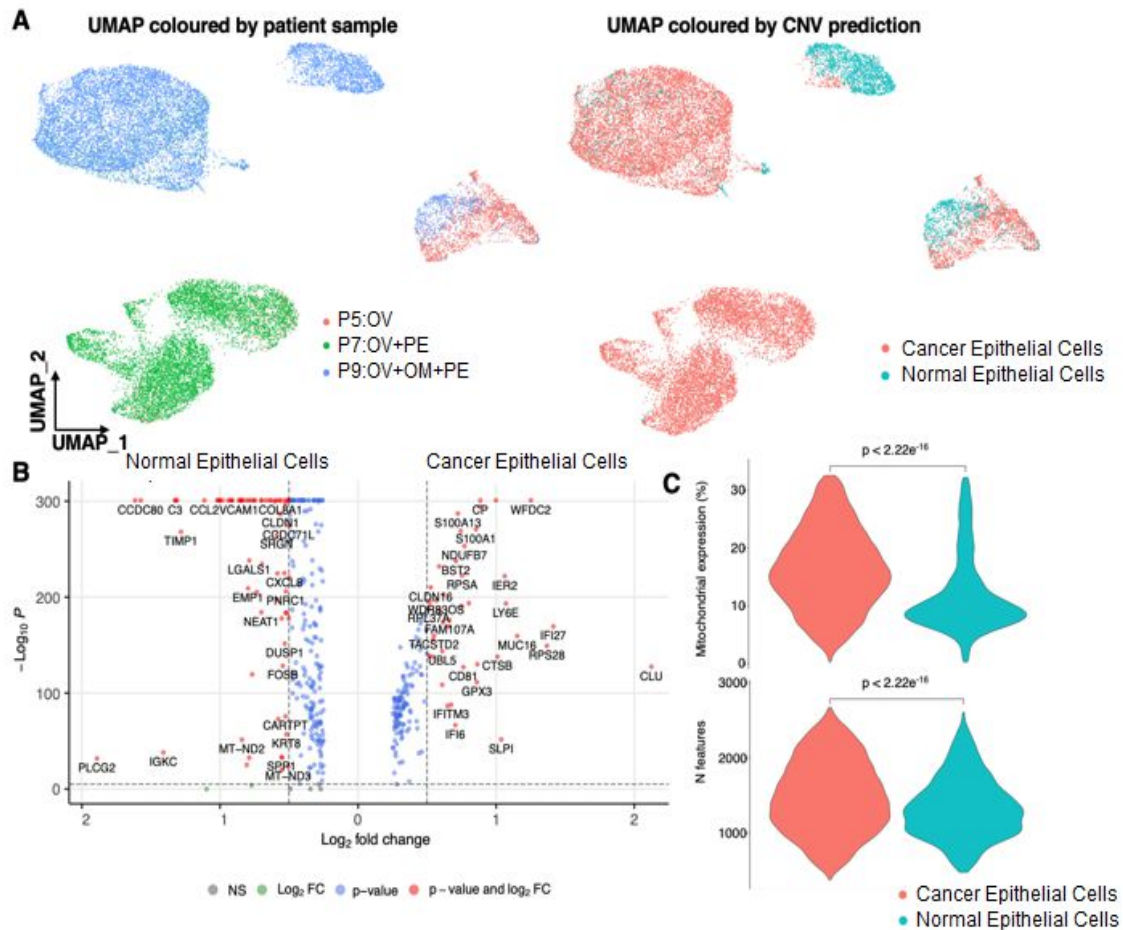


Figure 3.20 Differences between Cancer Epithelial Cells and Normal Epithelial Cells. (A) UMAP of HGSOC epithelial cells coloured by Patient ID/ Samples (left) and CNVs prediction (right). (B) Volcano plot of DEGs between cancer and normal epithelial cells further consolidating the CNV prediction of cancer cells where these cells have significant ovarian cancer canonical marker genes including WFDC2, MUC16 and claudin genes. (C) Violin plots illustrate that the predicted cancer epithelial cells express greater number of genes and mitochondrial expression (indicating higher stress response).

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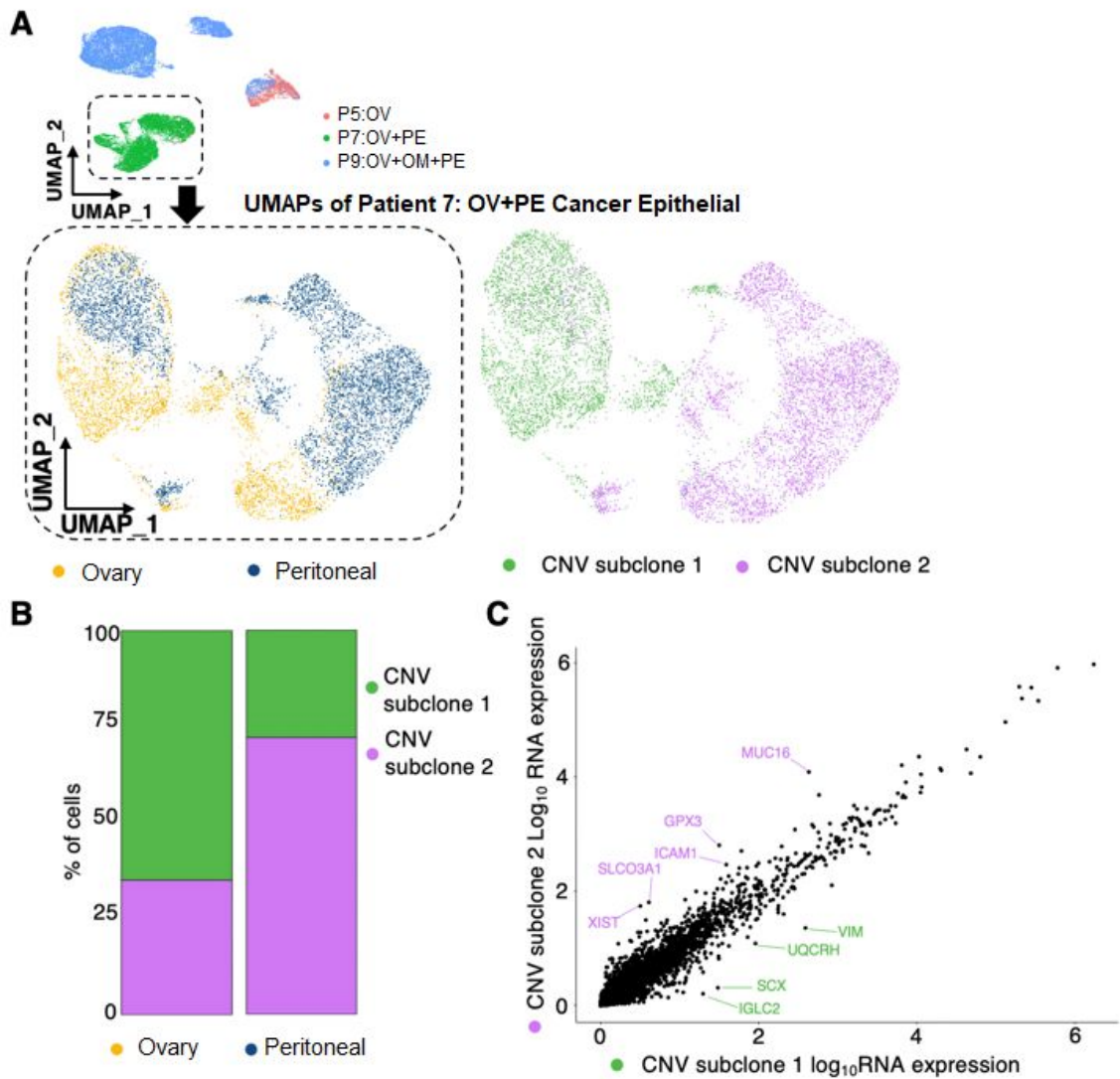


Figure 3.21 CNV Signature Sub-clones within Primary Ovarian and Peritoneal Samples of a Single Patient. (A) UMAPs of the cancer epithelial cells of Patient 7 separate based on CNV signature subclones rather than sample site. (B) Proportions of CNV subclone 1 and subclone 2 differ from primary and metastatic sites. (C) Scatter plot of the DEGs identified in each subclone shows the gene expression within these subclones are different further supporting the presence of separate clonality.

*Figure generated by Parry T.

3.4.6 Identification of subclusters of cells within the TME of HGSOc

3.4.6.1 Cancer epithelial cells subclustering and biological significance

In order to examine the tumour compartment of HGSOc in greater depth, the cancer epithelial cells (only aneuploidy cells) from all the datasets were integrated, batch corrected and re-clustered to uncover subclusters of cancer epithelial cells based on their transcriptomic signals. This strategy allows for the discovery of rare subtypes of cells within the tumour compartment which may explain the biological behaviour of the cancer. From this analysis, 5 subclusters were detected based on their DEGs (see Figure 3.23 for the top 10 DEGs for each sub-cluster):

1. Null – Only one DEG (COL3A1) which indicates that this cluster may be the ‘resident’ cancer epithelial cells with no major upregulation of genes or potentially different from other sub-clusters by virtue of downregulated genes;
2. Pro-tumourigenic – Increased expression of epithelial markers (KRT), claudin genes and other genes associated with OC proliferation and treatment resistance (SLPI, GPX3, TACSdT2, IER3, HES1 amongst others);
3. Stress-response – Upregulated genes responsive to heat shock, DNA-damage repair and mitochondrial genes which are all related to the stress response pathway (HSPA1A, HSPA6, DNAJB1, HSPH1, SERPINH1, HSPB1, HSP90AA1, FOS and JUN);
4. Ciliated – Increased expression of genes associated with cilium organisation, movement and microtubule functions (TPPP3, PIFO, TUBA1A, FOXJ1, FOXP1);

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5. Immuno-regulatory – Genes associated with the immune response and regulation (CXCL10, CXCL11, IFITs, HLAs).



Figure 3.22 Cancer Epithelial Cell Sub-clusters UMAP.

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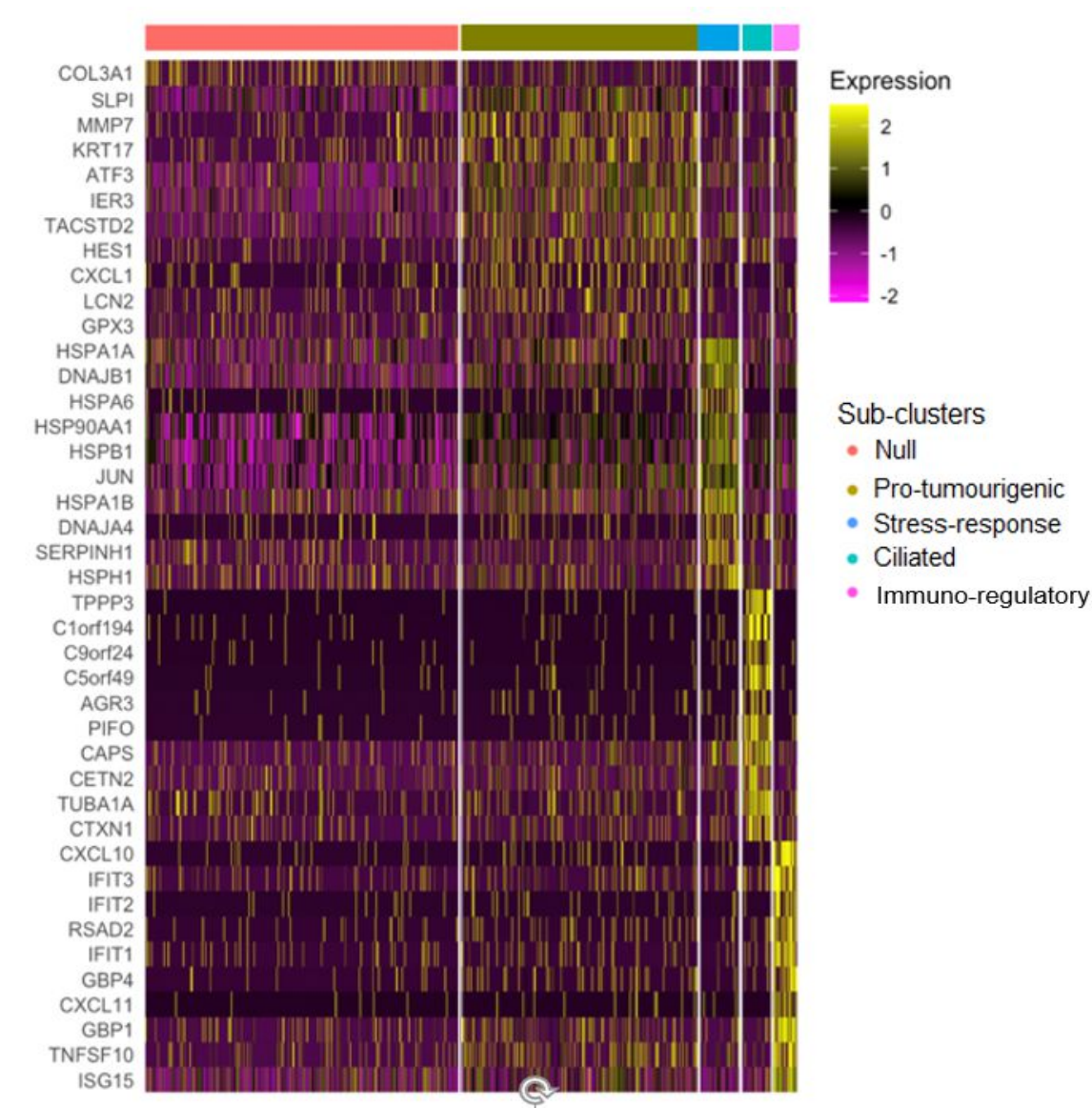


Figure 3.23 Gene Expression Heatmap of Top 10 Differentially Expressed Genes (DEGs) for Each Subcluster of Cancer Epithelial Cells.

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In order to assign biological significance to these subclusters, we used gene set enrichment analysis to identify significant signal pathways and functional processes of all the gene expression of these cells using MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer' and Gene Ontology 'Biological Processes' (Liberzon et al., 2015; Mi et al., 2018) (see Figure 3.24). From this analysis, the **'Pro-tumourigenic'** subcluster displayed cancer promoting properties including apoptosis resistance, immune-mediated destruction through inflammatory TNF- α signalling and epithelial-mesenchymal-transition. This subcluster also displayed proliferative functions including extra-cellular matrix (ECM) interaction and adopts immune-suppressive mechanisms to create a pro-inflammatory tumour microenvironment. The **'Stress response'** subcluster had genes associated with oxidative phosphorylation and metabolic ATP activities suggesting a high energy consumptive modulation within the cancer cells with recent evidence of ovarian cancer stem cells relying on oxidative phosphorylation for their metabolic needs.(Nayak et al., 2018) The **'Ciliated'** subcluster overlapped with the cluster detected in 3.5.3 with genes related to ciliary function. The **'Immuno-regulatory'** subcluster had increased gene expression associated with interferon signalling pathway and immune suppression suggesting the presence of immune-crosstalk between cancer cells and the immune microenvironment of HGSOC.

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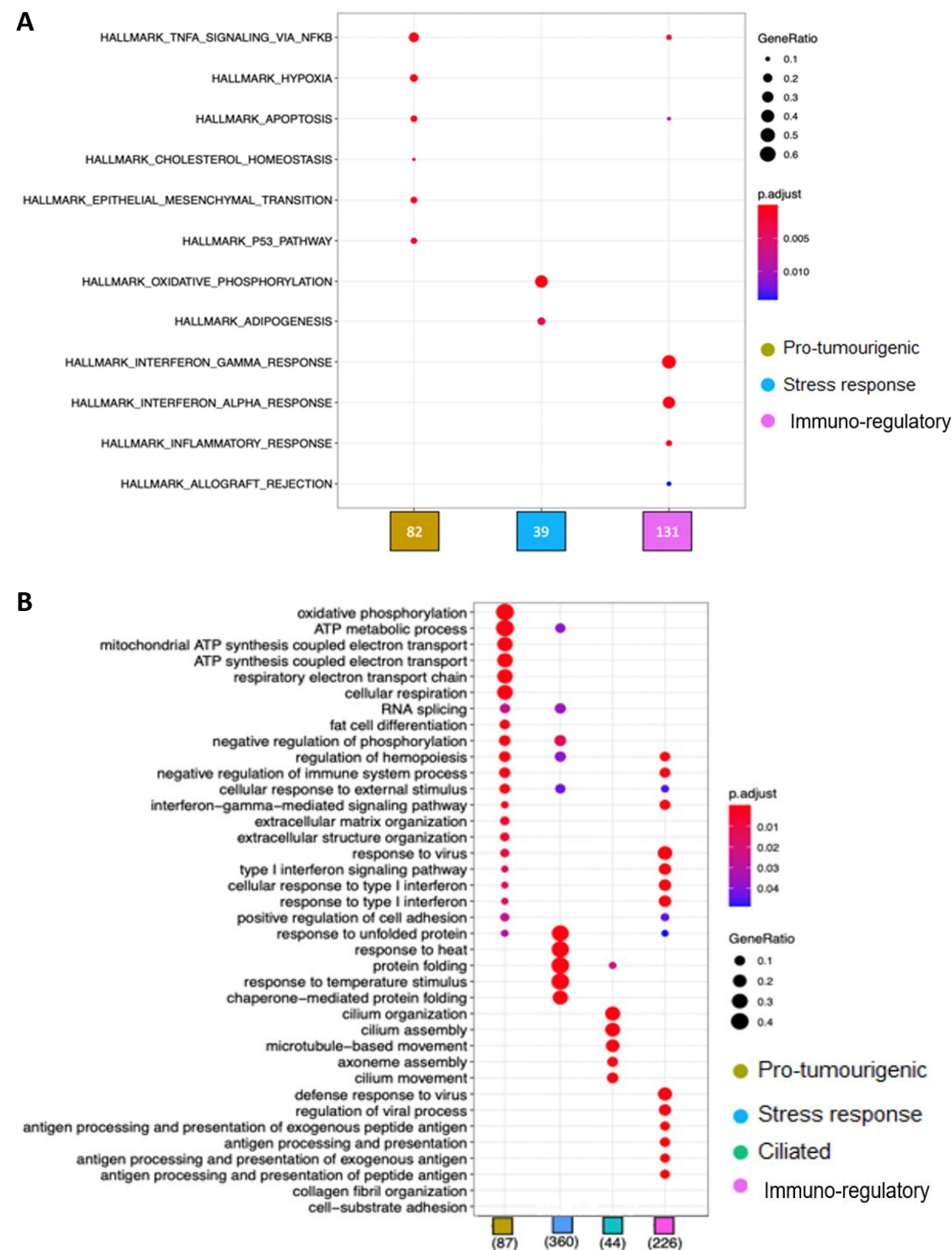


Figure 3.24 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of Cancer Epithelial Cells. (A) Enrichment dotplot illustrates significantly associated MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer'. (B) Enrichment dotplot illustrates significantly associated GO 'Biological processes' terms for each subcluster.

3.4.6.2 Fibroblast cells subclusters and their biological significance

Integration and reclustering of the fibroblasts from the datasets (of patients 5, 6, 7 and 9) revealed 5 subclusters of fibroblast cells within the stroma of HGSOC. The subclusters identified as fibroblasts by displaying the canonical markers (e.g. *VIM*, collagen genes, *CD81*, *ENG*) were segregated by their DEGs (see Figure 3.26):

1. Resident/Normal – Only 3 DEGs which were non-specific (*TFPI2*, *KRT*, *CLDN*);
2. Pro-tumourigenic CAFs – Striking upregulation of *ACTA2* (smooth muscle α -2 actin), collagen genes (*COL5A1/2*, *COL6A3*, *COL8A1*, *COL10A1*, *COL11A1*, *COL12A1*), MMP and MMP-related genes (*MMP2*, *MMP14*), *LUM*, *FAP*, TGF- β signalling-related genes (such as *POSTN*, *CTHRC1*, *VCAN*) compared to other subclusters;
3. Mesenchymal stromal stem cells from adipose tissue – Upregulated genes associated with stromal stem cells from adipose tissue including *CD34*, *CD105*, apolipoprotein, angiogenic factors such as *SERPINF1*, *IGFBP6* (Peter J. Thul et al., 2017);
4. Immuno-regulatory CAFs – 2 subclusters displayed CAF markers and had overexpression of immune-related genes (*CXCR4*, *CXCL8*, *CCL5*, *IL7R*).

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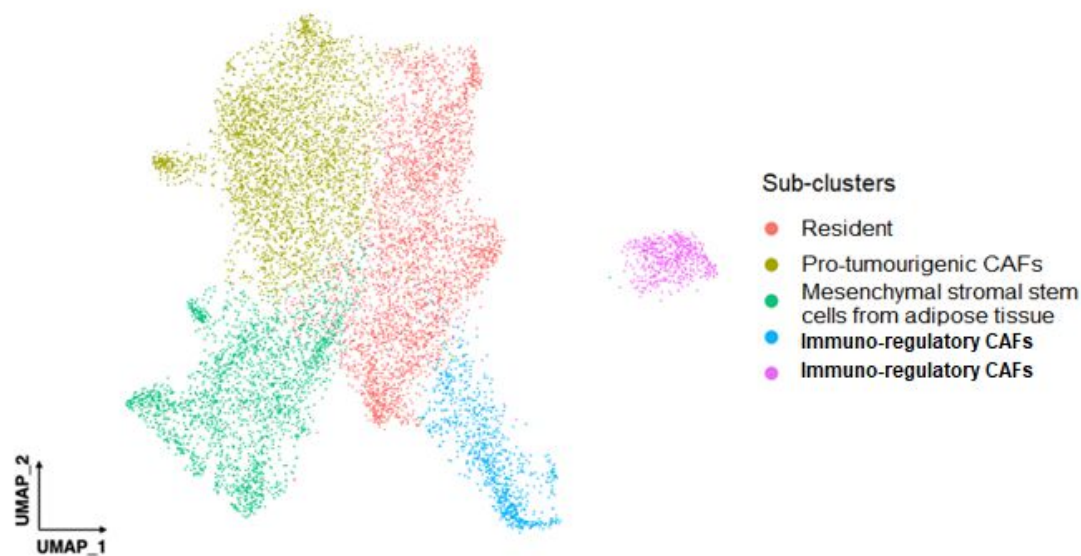


Figure 3.25 Fibroblast Cell Subclusters UMAP.

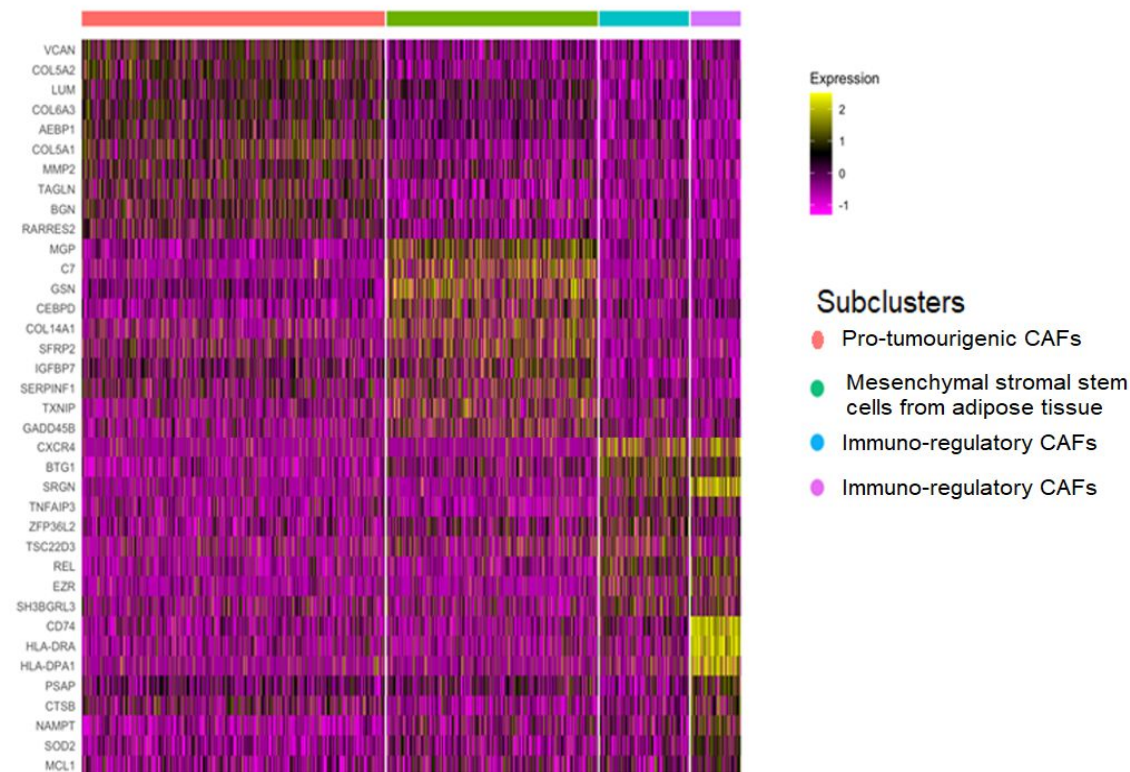


Figure 3.26 Gene Expression Heatmap of Top 10 Differentially Expressed Genes (DEGs) for Each Subcluster of Fibroblasts.

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To assign biological significance to these fibroblast subclusters, gene set enrichment analysis of the DEGs was performed using MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer' and Gene Ontology 'Biological Processes' (Liberzon et al., 2015; Mi et al., 2018) (see Figures 3.27 and 3.28). The '**Resident/normal**' subcluster did not return significant terms. The '**Pro-tumourigenic**' CAFs expressed markers that promote tumour growth through several mechanisms such as EMT (*via* TGF- β signalling and activation of STAT3), remodelling of ECM (*via* autocrine and paracrine signalling involving matrix metalloproteinases, MMPs, collagen genes, tissue inhibitors of metalloproteinases, TIMPs), and secretion of cytokines facilitating tumour proliferation and metastasis (e.g. mediated by *CTHRC1* which activates EGFR/ ERK/ AKT signalling pathway, *VCAN* activating NF- κ B pathway amongst others). The '**Mesenchymal stromal stem cells from adipose tissue**' had increased expression of genes related to myogenesis, EMT promotion, stem cell fate commitment and fibroblast migration. This subpopulation of cells promotes cancer progression by differentiating into CAFs or cancer-associated adipocytes (CAAs) and facilitate cancer stem cells self-renewal (Coffman et al., 2016). They are also capable of promoting OC chemoresistance by inhibiting the cleavage of caspase-3 and result in platinum accumulation in OC cells (Wen, Guo, et al., 2017). The other two subclusters of fibroblasts identified as '**Immuno-regulatory CAFs**' displayed genes relating to pro-inflammatory response and immune-related processes (such as IL2-STAT5 signalling).

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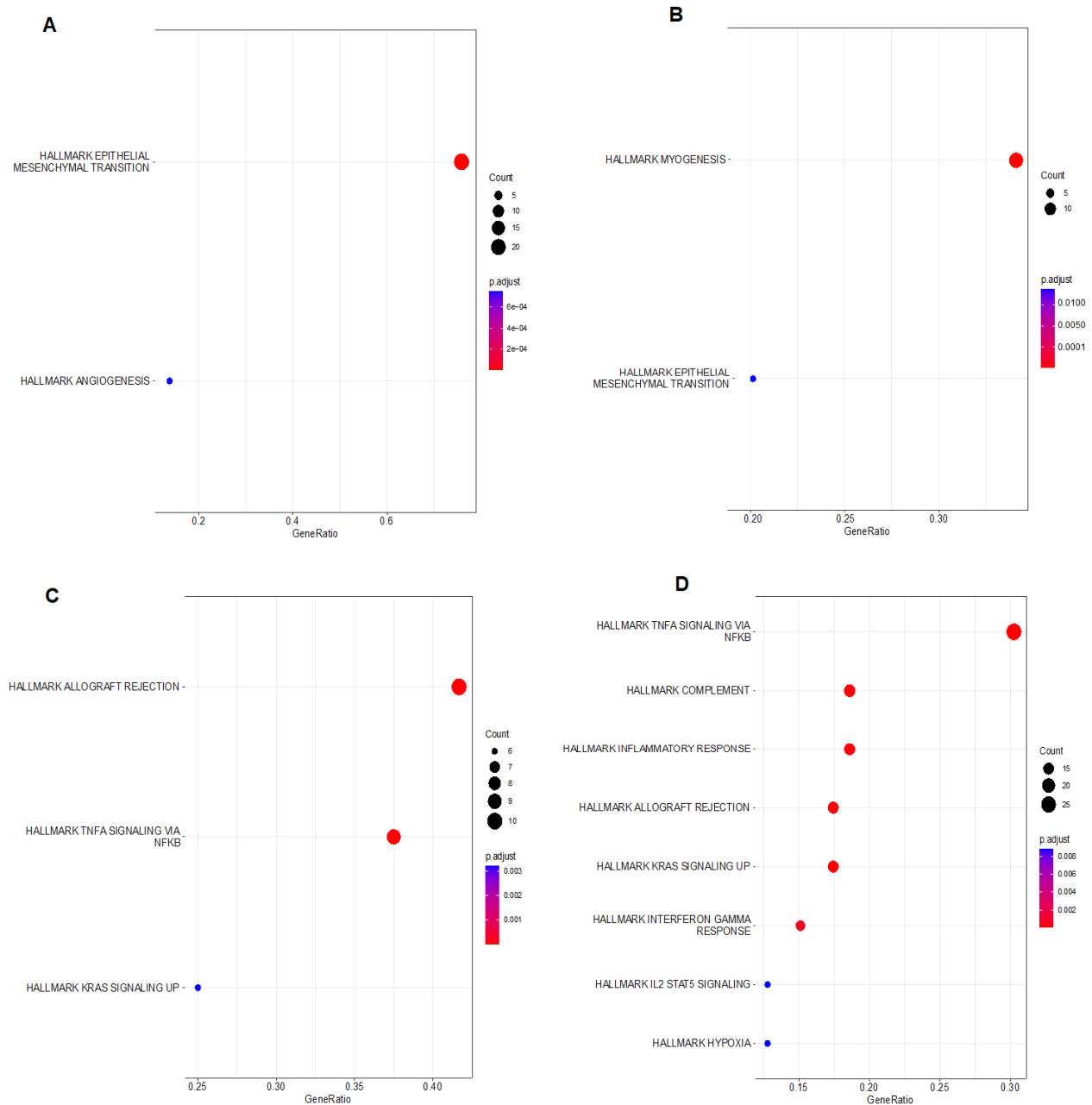


Figure 3.27 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of CAFs using MSigDB (Molecular Signature Database) curated geneset ‘Hallmark of Cancer. Enrichment dotplots for (A) ‘Pro-tumorigenic CAFs’, (B) ‘Mesenchymal stromal stem cells from adipose tissue’, (C) and (D) ‘Immuno-regulatory CAFs’.

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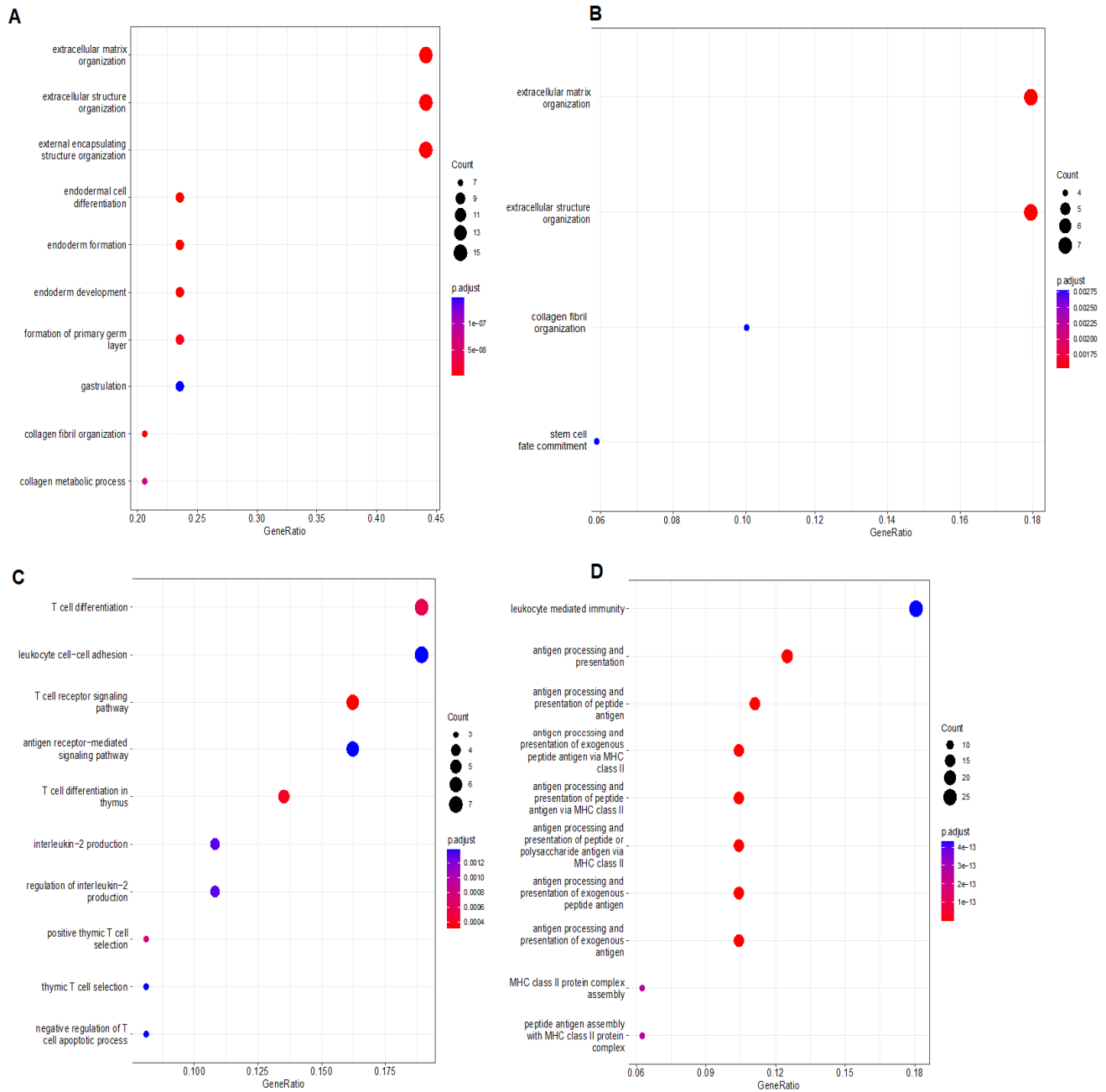


Figure 3.28 Geneset Enrichment Analysis of the Differentially Expressed Genes of the Subclusters of CAFs using Gene Ontology 'Biological Processes'. Enrichment dotplots for (A) 'Pro-tumorigenic CAFs', (B) 'Mesenchymal stromal stem cells from adipose tissue', (C) and (D) 'Immuno-regulatory CAFs'.

3.4.6.3 Immune cells subpopulations and their biological significance

Integrated analysis of the immune cells from the datasets (of patients 5, 6, 7 and 9) detected cells from 2 main immune lineage: the lymphoid (CD8⁺ T cells, CD4⁺ T cells, CD4⁺ Treg cells, B cells) and myeloid cells (monocytes, macrophages) (see Figures 3.28 and 3.29). CD4⁺ T regulatory cells were discriminated from CD4⁺ T cells by the expression of the transcription factor, FoxP3. Of these immune cells, macrophages were the most abundant (29.7%), followed by CD4⁺ T cells (27.9%), CD8⁺ T cells (25.9%), monocytes (12.5%), CD4⁺ Treg cells (3.9%) and very sparse B cells (0.1%). Percentage of each type of the immune cell were analysed from the total number of all the immune cells combined. CD4⁺ Treg cells and B cells were present in the samples of patients 6, 7 and 9 but absent in patient 5's sample which had the least immune cells (mainly comprised of CD4⁺ T cells).

Ratios of CD8⁺ T cells to CD4⁺ Treg cells were analysed in samples containing these cells. Patient 7 with > 2cm RD at debulking surgery had the lowest CD8⁺ T cells/CD4⁺ Treg cells ratio compared to patients 6 and 9 who had no gross RD at the end of surgery (Table 3.11).

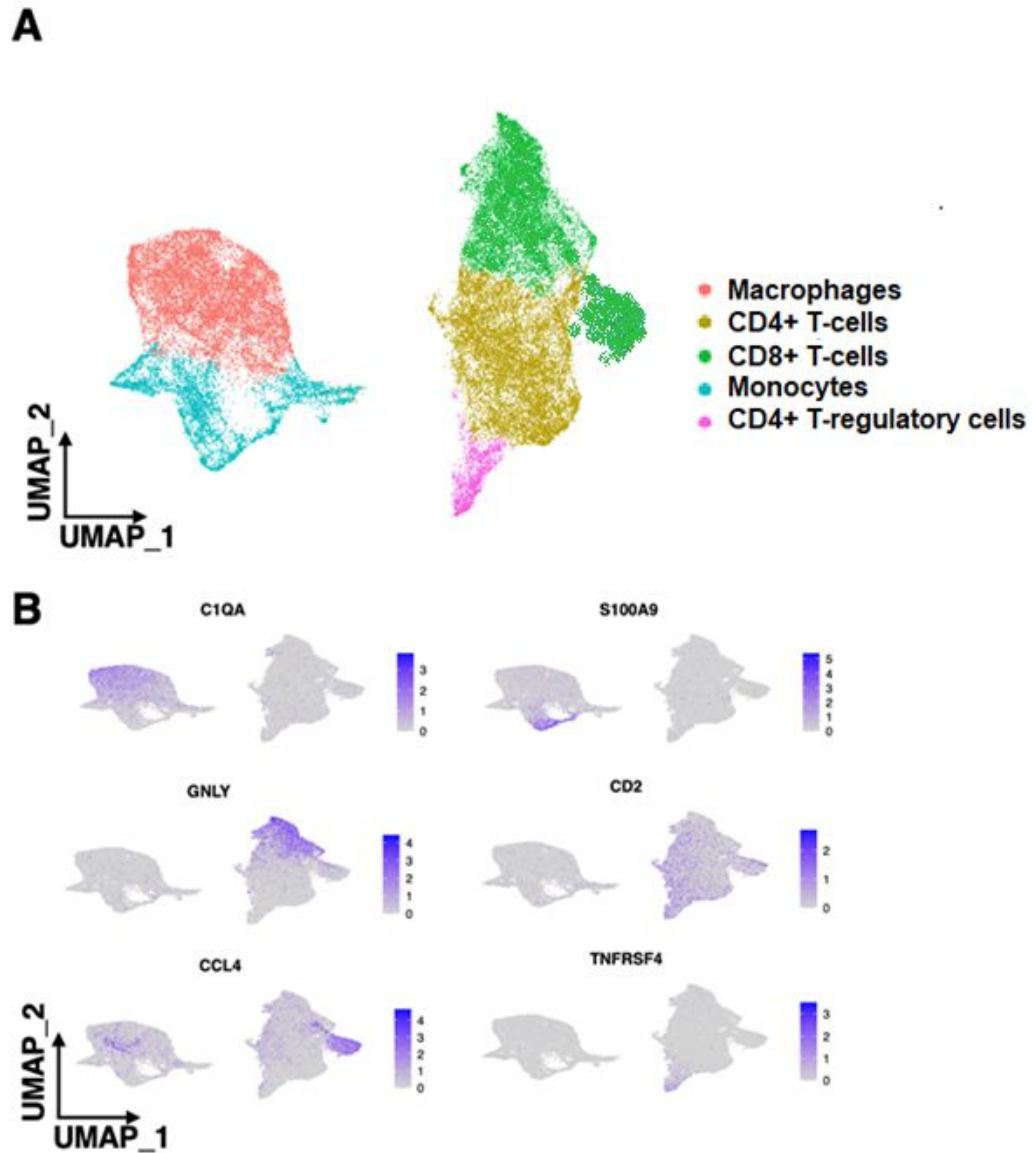


Figure 3.29 UMAP of Immune Cells. (A) Subpopulations of immune cells from myeloid (left) and lymphoid (right) lineages clustered into 2 major clusters. Note, B cells were present but too sparse to appear on the UMAP. (B) Marker genes for each immune cell subpopulation.

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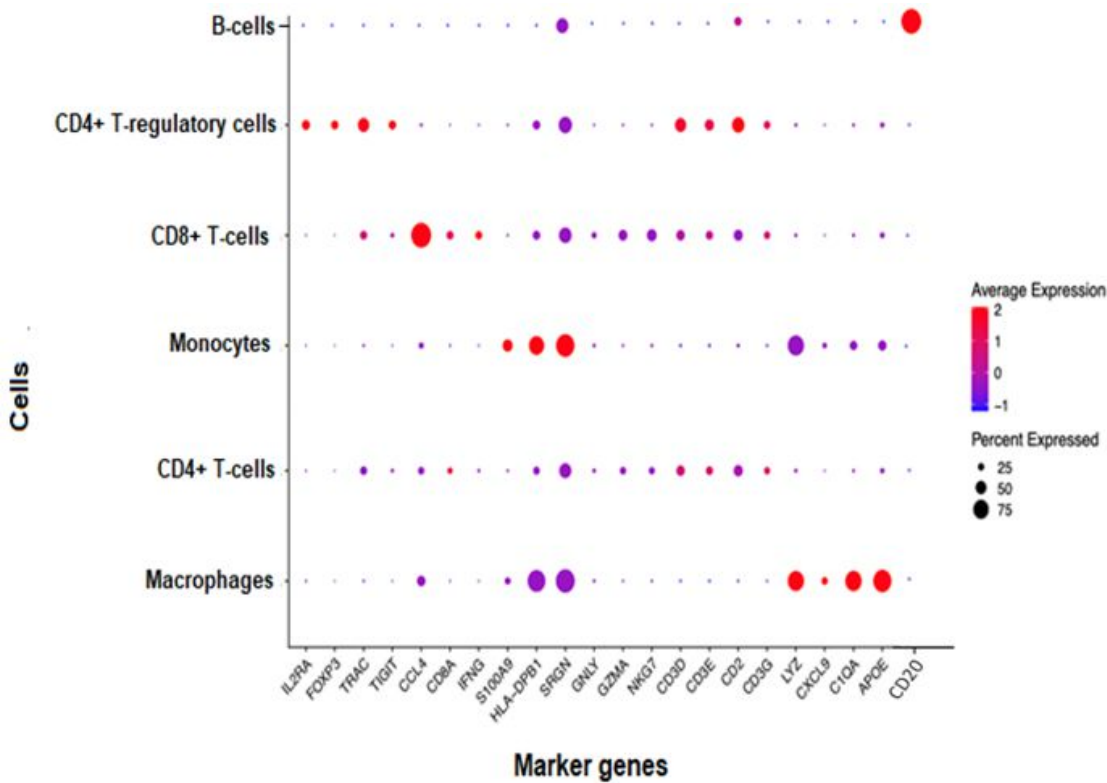


Figure 3.30 Marker Genes Identifying Subpopulations of Immune Cells.

Table 3.26 Ratio of CD8⁺ T cells to CD4⁺ Treg cells in Different Patient Samples

	Patient 6	Patient 7	Patient 9
CD8 ⁺ T cells/ CD4 ⁺ Treg cells	1.25	0.98	1.20
Residual Disease Status (cm)	0	> 2	0

*Patient 5's sample did not contain any CD4⁺ Treg cells. No ratio calculated for this sample.

3.4.7 Detecting cancer stem cells

The expression of potential cancer stem cell (CSC) markers were investigated in the integrated cancer epithelial cells. Within this group of cells, CSC markers that were detectable were *CD24*, *CD44*, *MYC*, *SOX2*, *ROR1* and *ABCB1* (see Figure 3.30). Refer to Table 3.27 for the putative roles of the CSC markers in OC.

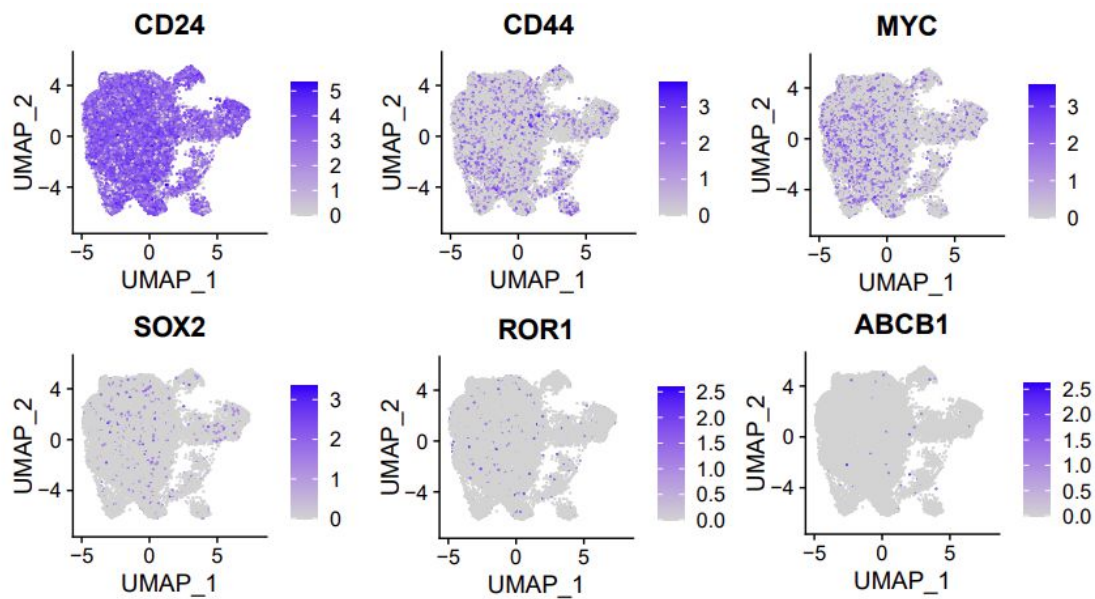


Figure 3.31 CSC Markers Detected in the Integrated Cancer Epithelial Cells Dataset. UMAPs illustrate the level of expression of the CSC markers with decreasing level of expression from blue to grey. Proportion of CECs with the following markers: *CD24*⁺ (74%), *CD44*⁺ (28%), *CD24*⁺/*CD44*⁻ (50%), *CD24*⁺/*CD44*⁺ (24%), *CD24*⁻/*CD44*⁺ (4%), *MYC* (38%), *SOX2* (14%), *ROR1* (6%) and *ABCB1* (3%).

Table 3.27 OC CSC Markers (Kenda Suster & Virant-Klun, 2019)

Marker	Type of protein	Putative role in OC
CD24	Cell surface transmembrane glycoprotein	Stem gene expression (Gao et al., 2010; Su et al., 2009), tumour formation (Burgos-Ojeda et al., 2012; Meirelles et al., 2012), metastasis (Jaggupilli & Elkord, 2012; Nakamura et al., 2017), chemoresistance (Nakamura et al., 2017), poor prognosis (Choi et al., 2005; Nakamura et al., 2017), recurrence of disease (Choi et al., 2005)
CD44	Cell surface transmembrane glycoprotein	Tumour formation (Zhang et al., 2008), progression (Casagrande et al., 2011), chemoresistance (Zhang et al., 2008), poor prognoses (Zhang et al., 2013), recurrence of disease (Meng et al., 2012)
MYC	Transcription factor	Tumour formation (Di et al., 2013), chemoresistance (Ning et al., 2017)
SOX2	Transcription factor	CSC maintenance and self-renewal (Ng & Lufkin, 2011; Z. Yu et al., 2012), poor prognosis and chemoresistance (Wen, Hou, et al., 2017)
ROR1	Tyrosine kinase receptor	Spheroid and tumour formation (S. Zhang et al., 2014), poor prognosis (H. Zhang et al., 2014)
ABCB1	ATP binding cassette transporter	Chemoresistance (Dou et al., 2011; Garson & Vanderhyden, 2015; Hu et al., 2010; Kobayashi et al., 2011)

3.4.8 Comparison of cell populations to TCGA HGSOC molecular subtypes

To classify the cell populations uncovered in this study by the 4 TCGA HGSOC subtypes, the Verhaak's 100 genes (Appendix 1) were utilised here as this gene set was concise and well-accepted by other major studies. Using the Verhaak's gene sets defining the 4 TCGA HGSOC subtypes, the average expression of all the cells (within each patient sample and also within each cell type) were used to classify the samples. The threshold for classification was defined as 2SDs above the mean of the combined group comparisons. For example, classification by patient's samples, the mean gene expression was the combined mean RNA expression of all the patients' samples (P5-9) for the different TCGA HGSOC subtypes upregulated genes. Meanwhile, classifying by the cell type, the mean gene expression was calculated using the combined average RNA expression of cells in all the cell types for the different TCGA HGSOC subtypes upregulated genes. This method for classification was adapted from Shih's study (A. J. Shih et al., 2018).

Using patient sample IDs, only one patient's sample (Patient 7 peritoneum) identified as "Differentiated" HGSOC subtype (mean gene expression value of 4.120 compared to a threshold value of 3.885) (Table 3.28). However using identified cell populations, the Cancer epithelial cells classified as "Differentiated" subtype (mean gene expression value of 2.079 compared to threshold value of 1.937), fibroblasts as "Mesenchymal" subtype (mean gene expression value of 3.625 against threshold of 3.471), endothelial cells as "Proliferative" (mean gene expression value of 0.492 vs threshold of 0.491), myeloid and cytotoxic T cells approached close to the threshold for "Immunoreactive" subtype (mean gene expression value of 1.476-1.742 against

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threshold of 1.863) (Table 3.30). Here, it was suggested that the bulk TCGA HGSOC subtypes correlated with the specific cell types rather than necessarily with the patients' samples.

Table 3.28 Comparison to TCGA HGSOC Subtypes by Patient Sample ID

TCGA HGSOC Subtypes Patient Sample ID	Differentiated	Immuno-reactive	Mesenchymal	Proliferative
Patient 5 OV	[^] 0.878	0.128	0.469	0.515
Patient 6 PE	0.839	0.783	1.290	0.205
Patient 7 OV	2.370	1.023	1.130	0.313
Patient 7 PE	*4.120	0.953	0.619	0.309
Patient 9 OV	0.540	0.760	1.009	0.247
Patient 9 OM	0.600	0.673	1.987	0.277
Patient 9 PE	0.580	0.740	1.547	0.307
Patient 9 SBN	0.650	0.948	1.185	0.261
+Mean Gene Expression (All samples)	1.322	0.751	1.155	0.304
Standard Deviation	1.281	0.280	0.485	0.093
Threshold	3.884	1.311	2.125	0.490

*Patient 7 PE = Mean gene expression of all the cells in this sample for “Differentiated” subtype genes exceeded the threshold used for classification. Hence, this sample identified as “Differentiated” subtype.

Each individual value denote the average gene expression of all the cells within that particular sample for the different TCGA HGSOC subtypes upregulated genes.

[^]Example of how the value was obtained (refer to Table 3.29).

+Mean Gene Expression = mean of the combined patients’ samples (P5-9) for the different TCGA HGSOC subtype.

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Table 3.29 Example of Calculation for the Average of RNA Expression of All Cells in Patient 5 OV for All the Genes of the TCGA 'Differentiated' Subtype from Verhaak's List

TCGA HGSOC 'Differentiated' Subtype Genes	Average RNA expression of All cells in Patient 5 OV
<i>STEAP3</i>	0.012
<i>LCN2</i>	6.418
<i>AGFG2</i>	0.416
<i>MYO5C</i>	0.115
<i>ALS2CL</i>	0.072
<i>ANXA1</i>	3.718
<i>PTGER2</i>	0.086
<i>MVP</i>	0.311
<i>SSH3</i>	0.185
<i>DTX4</i>	0.165
<i>MLPH</i>	0.128
<i>TNFRSF14</i>	0.090
<i>MGLL</i>	0.142
<i>CLU</i>	2.762
<i>C2CD2L</i>	0.175
<i>SLC37A1</i>	0.121
<i>RIN1</i>	0.019
Average of all the genes within 'Differentiated' subtype gene set	0.878 [^]

[^]Mean of the average RNA expression of all cells in patient 5 OV for all the genes within the 'Differentiated' subtype gene set.

Table 3.30 Comparison to TCGA HGSOC Subtypes by Cell Types

TCGA HGSOC Subtypes Cell Types	Differentiated	Immuno- reactive	Mesenchymal	Proliferative
Cancer Epithelial	*2.079	0.255	0.419	0.334
Ciliated Cancer Epithelial	0.710	0.208	0.404	0.273
Fibroblast	0.914	0.263	*3.625	0.405
Adipocyte	0.961	0.135	2.456	0.257
Endothelial	0.970	0.239	1.277	*0.492
Myeloid	0.510	*1.742	1.301	0.217
T cell	0.698	0.979	0.571	0.224
Cytotoxic T cell	0.704	*1.476	0.689	0.226
B cell	1.350	0.602	0.441	0.199
+Mean Gene Expression (All cell types)	0.988	0.655	1.243	0.291
Standard Deviation	0.474	0.604	1.114	0.100
Threshold	1.936	1.863	3.471	0.491

*Mean gene expression values for each cell type highlighted in **green** exceeded the threshold for classification, hence the cell type/ population identified as the TCGA HGSOC subtype. Mean gene expression values for each cell type highlighted in **blue** approached the threshold for classification and was accepted as the TCGA HGSOC subtype.

Each individual value denote the average gene expression of all the cells within that particular cell type for the different TCGA HGSOC subtypes upregulated genes.

+Mean Gene Expression = mean of the combined average gene expression of cells in all the cell types for the different TCGA HGSOC subtype.

3.4.9 Cells uncovered in lower quality datasets

The first batch of sequencing (Patients 1-4, Experiments E05-E14) returned samples with lower quality data which made analysis and cell identification in this batch difficult. This batch had lowest % cell viability, total cells recovered and mean transcriptome size compared to the later 2 batches of sequencing. Of note, all the samples in this batch were cryopreserved tissues.

Patient 1 returned mainly immune and neuronal cells. Apart from low library complexity, the chemotherapy effects on this patient who had neo-adjuvant treatment may explain the absent of cancer epithelial cells with an immune shift in this patient's TME. The two samples (E09A and E09B) from patient 2 underwent different dissociative processes with E09A manually dissociated and E09B dissociated by automated protocol with the additional tumour cell isolation performed. In both samples, ribosomal and 'stressed' cells and cancer epithelial signatures were present but there were immune cells present in E09A (non-tumour isolated sample). This illustrated that the tumour isolation step removed other cell types and retained tumour epithelial cells. Samples from Patients 3 and 4 did not yield meaningful results with mainly non-significant genes including ribosomal (RPL) and 'stressed' cells signatures such as heat-shock related genes (*HSP90AA1*, *HSP90AB1*, *DNAJB1*), apoptosis regulator (*MCL1*), and mitochondrial MT-genes among others.

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Table 3.31 Summary of the Lower Quality Datasets

Experiment	Patient ID	Sample Site	Sample State, Surgery, Dissociation	Mean % Cell Viability	Total No. of Cells	Mean Transcriptome Size (Avg No. of Genes)
E05+	1	Small bowel nodule (Met)	Cryo,IDS, M	72.80	7113	16117
E09A+	2	Ovary(Pri)	Cryo, PDS, M			
E09B*	2	Ovary (Pri)	Cryo, PDS, A			
E11*	3	Ovary (Pri)	Cryo, PDS, A			
E14*	4	Ovary (Pri)	Cryo, PDS, A			

+Dissociated using manual protocol without DCR as processed earlier before we started using DCR kits.

*Dissociated using automated protocol with tumour cell isolation and DCR performed.

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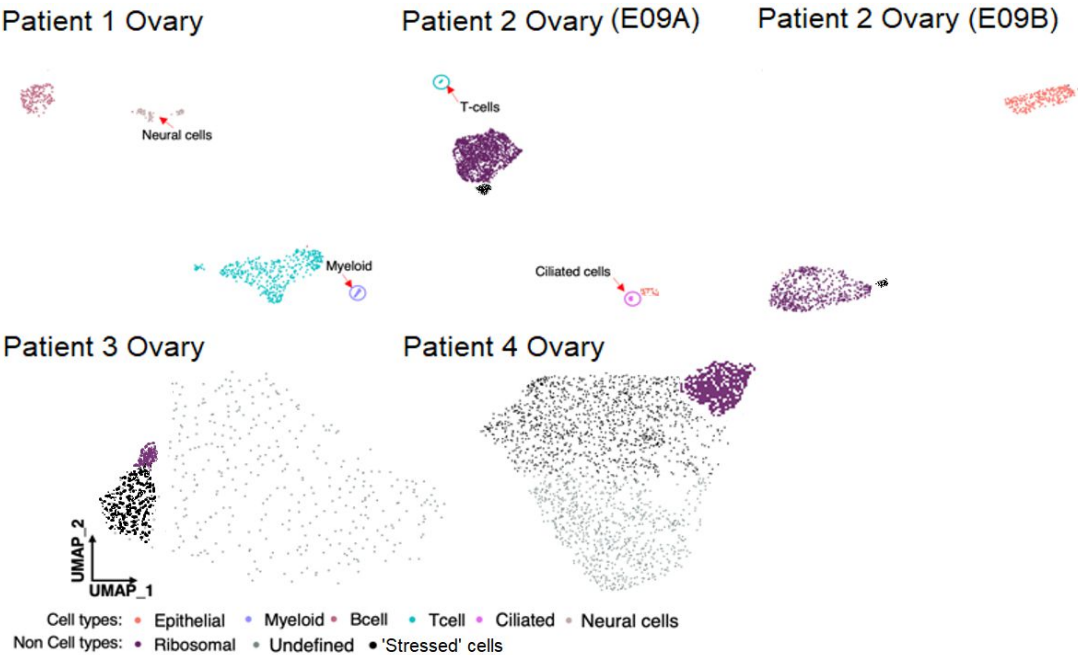


Figure 3.32 UMAPs of Lower Quality Libraries.

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3.4.10 Broad cell composition of a low grade serous ovarian cancer (LGSOC) case

A LGSOC case was included in our study as it was not known upfront whether the primary debulking surgery was a HGSOC or LGSOC (in which for this case the samples had been processed prior to confirmation on histology that required time). Duplicate samples were processed from the same single cell suspension from a primary adnexal mass site. This experiment returned relatively similar proportions of cell types between the duplicate samples based on 17438 and 19427 genes in each sample. This support the notion that random sampling of cells from a single cell suspension post dissociation that were sent for sequencing does not have significant impact on the downstream determination of the cellular composition or make up within the sample.

Cell Types in 2 LGSOC (Duplicate Sample)

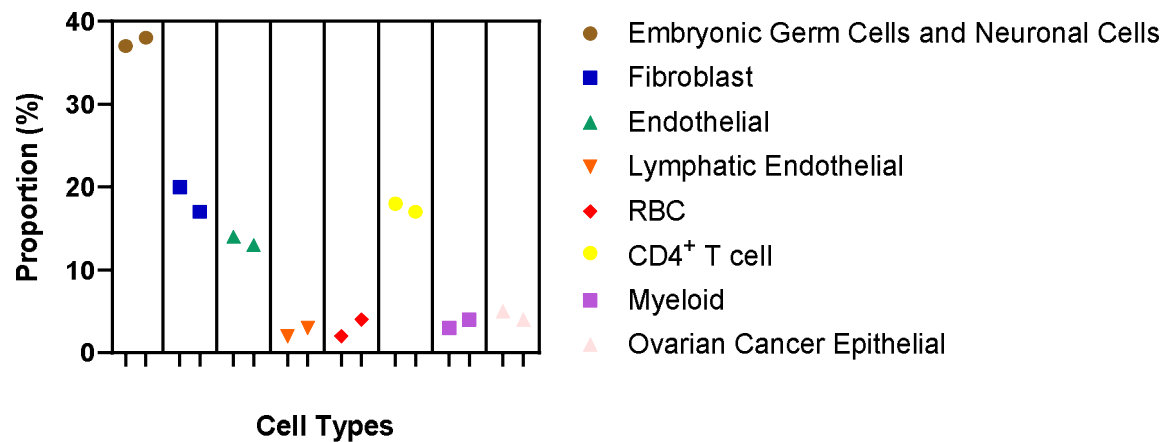


Figure 3.33 Cellular Make-up of the Duplicate LGSOC Samples from the Same Single Cell Suspension Post-dissociation. Both samples have almost similar proportions and types of cells uncovered.

3.5 Discussion

This scRNA-seq study allows an in depth characterisation of the transcriptomic landscape of the dynamic TME of OC, helping to unravel the tumour as well as intra- and inter-patient heterogeneity. This study has sequenced data from a cell number in excess of that in previous single cell OC studies revealing a rich transcriptomic repertoire of the disease with analysis of the tumour-stromal-immune interfaces.

Cryopreservation is a common method of conserving cell viability and plays a key role for tissue biobanking, particularly when studying rare clinical samples (Guillaumet-Adkins et al., 2017; Hu et al., 2020). This study has examined the effects of cryopreservation by comparing the transcriptomes between fresh and cryopreserved HGSOC cells. A significant finding was the increased transcriptional artefact detected in cryopreserved cells which most likely was attributed to the effects of cryopreservation and thawing with the upregulation of cellular stress response pathways (GO:0033554) and regulation of cell death (GO:0010941) (Ashburner et al., 2000; "The Gene Ontology resource: enriching a GOld mine," 2021). Despite this, cryopreserved tissues were capable of revealing gene expression of cellular phenotypes correlating to that of fresh tissues. This was concordant with observations from single cell studies of breast cancer, prostate cancer and melanoma where the authors noted a high correlation of gene expression between freshly analysed and cryopreserved tissues (S. Z. Wu et al., 2021). However, in this study the analysis of fresh tissues were preferred due to the reduced stress artefacts. A study of single cell profiling of fallopian tube epithelial (FTE) cells by Hu et al. noted that the cryopreserved cells which were recovered in culture medium overnight after thawing displayed striking effects induced by the process on the transcriptomes including the expression of rarely expressed genes in FTE cells (e.g. CD44), reduced expression of key

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markers of secretory cells (e.g. ESR1) and alteration of key pathways (e.g. Wnt signalling) (Hu et al., 2020).

This study has consistently uncovered major cell types which can be categorised into 3 broad groups including tumour cells (cancer epithelial cells, ciliated cancer epithelial cells), stromal cells (fibroblasts, adipocytes, endothelial cells, RBCs) and immune cells (of lymphoid and myeloid lineages). As part of validation of the existence of the diverse cell types within the OC TME, this study employed the following techniques: 1) by processing the tissue sample through tumour isolation kit and the presence of malignant cells was confirmed on FACS analysis (using EPCAM⁺/CD45⁻) and upon cell clustering by bioinformatics analysis on cancer epithelial cells were present vs non-tumour isolated sample where FACS analysis and cell clustering confirmed the presence of tumour and non-tumour cells (see section 2.4.4.3.3); and 2) using bioinformatics analysis where Z-scores of each cell type from each patient sample correlated highly with the same cell type (see section 3.4.3.1). Additionally, the cell type labelling within this study was performed based on known canonical markers described by major cell marker gene resources such as CellMarker database and Human Protein Atlas (Peter J. Thul et al., 2017; X. Zhang et al., 2019), and the presence of these cells were also described by other single cell ovarian cancer studies (Izar et al., 2020; Schelker et al., 2017; A. J. Shih et al., 2018).

Within the epithelial compartment, ciliated cancer epithelial cells were detected expressing functional genes related to their origin which was concordant to findings by Shih et al (A. J. Shih et al., 2018). This small population of ciliated cancer epithelial cells was specific to primary adnexal sites (detected in patient 5 and 9) and absent from all metastatic sites (see section 3.5.4). The fallopian tube epithelium has been increasingly acknowledged as a precursor for HGSOC with serous tubal intraepithelial

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carcinoma (STIC) lesion commonly known as a non-invasive tumour origin of HGSOC, often arising from the distal fallopian tube epithelium (Kim et al., 2018). Genomic analysis identifying alterations in *BRCA1*, *BRCA2*, *TP53*, and *PTEN* as critical early events in the initiation of STICs suggested the possibility of invasive tumours (high grade serous) developing in the fallopian tube; and then subsequently spreading to the ovary and peritoneal cavity (Bijron et al., 2013; Kurman & Shih le, 2010; Labidi-Galy et al., 2017; Xiang et al., 2018). The expression of genes characteristic of fallopian tube cells in cancer epithelial cells within primary adnexal masses further supports this theory regarding the cellular origin of HGSOC. The biomarkers expressed by this subset of cancer epithelial cells include *TPPP3* (tubulin polymerisation-promoting protein which is expressed by ciliated cells and is involved in the progression of colorectal and lung cancer) (Li et al., 2016; Ye et al., 2017); *FOXJ1* (a transcription factor involved in ciliogenesis which originates from the reproductive tract and is associated with adverse outcomes in ovarian cancers) (Hackett et al., 1995; Siu et al., 2013); *RSPH1*, *DNAAF1*, *CCDC170*, *LRRC46* and *STK33* (are expressed by ciliated cells in the fallopian tube and found to have predisposition to HGSOC) (Coan et al., 2018); and *ZMYND10* (responsible for ciliary function and involved in the apoptosis cascade which is important in ovarian cancer cells survival) (Cheng et al., 2015).

Through the integrated analysis of the tumour compartment, further intra-tumour heterogeneity could be uncovered allowing for the discovery of rare subtypes of cancer epithelial cells which may explain the biological behaviour of the cancer. Specifically, 5 subclusters were detected based on their DEGs ('Null/ Resident', 'Pro-tumorigenic', 'Stress-response', 'Ciliated' and 'Immuno-regulatory'). An interesting subcluster was the 'Pro-tumourigenic' which displayed common malignant features of HGSOC with

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most cancer promoting properties including apoptosis resistance, immune-mediated destruction through inflammatory TNF- α signalling, EMT and perturbation of p53 pathway (an almost ubiquitous occurrence in HGSOC) (Vang et al., 2016). This subcluster also expressed proliferative functions including ECM interaction and adopted immune-suppressive mechanisms to create a pro-inflammatory TME. The top upregulated markers in this subcluster (had been described by Shih et al.) included the *KRT*, claudin genes (involved in regulation of EMT), *WFDC2* (an important biomarker for OC and overexpression promotes OC tumour growth) (A. J. Shih et al., 2018). Other pro-tumourigenic marker genes expressed by this subcluster included *SLPI* (anti-protease associated with paclitaxel chemo-resistance in OC), *GPX3* (conferring protection against platinum and taxane-based chemotherapy), *TACSDT2* (described as a potential immune-therapeutic target associated with aggressive malignancy in OC) (Bignotti et al., 2010; Lee et al., 2011; Rasool et al., 2010).

Within the cancer epithelial cells, another notable subcluster was the 'Immuno-regulatory' displaying immuno-suppressive functions with upregulation of genes such as the *IFITs* (interferon induced proteins family involved in malignancy through EMT, AKT activation and acquired platinum-based chemo-resistance) and *CXCL10/11* chemokines (acting along an autocrine axis within the TME to promote cancer cell proliferation, immune evasion and metastasis) (Niess et al., 2015; Pidugu et al., 2019; Tokunaga et al., 2018). The discovery of these cancer epithelial subclusters shows that a dynamic process drives the HGSOC disease through synergistic pro-tumourigenic activities (promotion of cancer cell proliferation and migration via overexpression of epithelial cell proliferative markers), increased resilience to stress and cell death, and immuno-suppressive mechanisms (through interferon release creating a pro-inflammatory TME).

Integrative analysis of the fibroblasts in the stromal compartment revealed novel subclusters of CAFs. The 'Pro-tumourigenic' CAFs and adipose-derived stromal stem cells identified in this study may both react with the tumour cells to promote OC growth through the stimulation of EMT (*via* TGF- β signalling) (Liu et al., 2021), angiogenesis, and ECM remodelling (by MMP degradation of the ECM to increase tumour invasion) (A. J. Shih et al., 2018). The 'Immuno-regulatory' CAFs detected here displayed immune-related genes which closely resembled the immunomodulatory CAFs in the single cell study of malignant ascites in HGSOC by Izar and colleagues (Izar et al., 2020). Some of the markers expressed by the 'Immuno-regulatory' CAFs included complement factors (*C1QA/B/C*), chemokines (*CXCL8/10/16*) and cytokines (*IL1/2/7/32*) which are involved in pro-inflammatory response such as the activation of IL-2-STAT5 pathway (where secretion of IL-2 by CAFs contributes to *FOXP3* induction through the activation of STAT5 leading to the differentiation of naïve CD4⁺ T cells to Tregs) (Worzfeld et al., 2017). Unravelling of the subpopulations of CAFs in this study potentially sheds light onto important biomarkers/ signalling molecules that could be further evaluated to generate new stromal targeting drugs which could aid the efficacy of conventional chemotherapy and anti-VEGF therapies.

Tumour-immune crosstalk plays an important role in HGSOC. The presence of TILs have been associated with improved survival outcomes (L. Zhang et al., 2003). Sato and colleagues in their studies of 117 OCs demonstrated a significant prolonged median survival of 55 months in patients with high levels of CD8⁺ T cells compared to 26 months in those with little or no CD8⁺ T cells (E. Sato et al., 2005). However, immuno-suppressive cells and factors interact dynamically with the anti-tumour immunity within the rich TME. CD4⁺ Treg cells have been recognised as a major

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immune-suppressor which acts by blocking the function of activated T cells (Preston et al., 2011). Several studies have shown that low CD8⁺ T cells and CD8⁺/CD4⁺ Treg cells ratio were associated with poorer survival outcomes in OC (Preston et al., 2013; E. Sato et al., 2005). When the CD8⁺/CD4⁺ Tregs ratio was analysed, this scRNA-seq study found that a higher ratio was associated with one of the positive prognostic factors of HGSOc (i.e. no gross RD at the end of surgery) (Table 3.11). Future study should seek to examine the levels/ratio of various TILs (including CD8⁺, CD3⁺, CD4⁺, CD4⁺ Treg cells) at single cellular level (with IHC and quantitative microscopy validation) and correlate these to clinical outcomes.

It was recognised that the small number of patients included within this scRNA-seq study remained an issue which limited the analysis of inter-patient and inter-site heterogeneity. Additionally, the RNA-sequencing of a small amount of tumour (1 g) using the 10x Genomics platform which targeted only the 3' ends of poly(A) transcripts (in comparison to other deeper sequencing tools such as smartSeq-2 using full-length total RNA approach with high transcriptome coverage per cell and greater sensitivity) somehow limited the ability of this study to fully analyse the disease (Baran-Gale et al., 2018). However, this study still enabled the detection of rare cell subtypes through profiling of thousands of cells with lower associated costs, hence covering breadth rather than depth.

Nevertheless, this study managed to detect subtle differences between patients and multi-site heterogeneity. Notably, the chemotherapy effects reflected in the metastatic sample from a patient who had NACT (refer to patient 6 in section 3.5.4) with deficient cancer epithelial cells (concordant with findings by Shih et al. where neoadjuvant treatment 'killed off' tumour epithelial cells) (A. J. Shih et al., 2018) and the presence of a metallothionein (MT) expressing T cell population absent in all other pre-

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chemotherapy cases. MT overexpression has been associated with chemoresistance and poor prognosis in several cancers, particularly ovarian, breast, prostate, lung, melanoma, neuroblastoma and soft tissue sarcoma (H. H. W. Chen et al., 2015; Hayden et al., 2014; Krizkova et al., 2012; Lai et al., 2018; J.-H. Lee et al., 2015; Raudenska et al., 2013; Si & Lang, 2018; Tariba et al., 2015; Weinlich et al., 2003; Wong & Stillman, 2018) Platinum acts by causing DNA damage in cancer cells, interrupting cell division and induces apoptosis (Dasari & Bernard Tchounwou, 2014). In this case, the surviving cells with overexpression of MT within the T cells may indicate that MT plays an important role in modulating the cellular responsiveness to the DNA-targeted platinum agent. It has been demonstrated that MT mediates chemoresistance through cellular inactivation and subsequent sequestration of platinum *via* chelation and eventually preventing platinum interaction with tumour cell DNA (Cheng et al., 2006; Hou et al., 2017; Surowiak et al., 2005). Therefore, further interrogation of these 'surviving' cells with overexpression of MTs in post-chemotherapy samples may reveal crucial biomarkers and mechanisms associated with chemoresistance; and potentially aid in patient stratification as it had been reported in few studies whereby MT expression was increased in cisplatin-resistant OC cell lines and also linked with poor outcomes in cisplatin treated OC patients (Kawahara et al., 2019; Surowiak et al., 2005).

This study also noted a shift in the proportion of cancer epithelial cells vs immune cells between primary and metastatic sites in association with RD status (see section 3.4.4). Patient 7 with gross macroscopic RD at PDS had increasing cancer epithelial cells and decreasing immune cells moving from primary to metastatic site; the opposite was seen in Patient 9 with no gross RD at the end of surgery. Shih et al. in their scRNA-seq study of ovarian tumours also noted a shift in cell populations between primary

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and metastatic samples but did not correlate to RD status. (A. J. Shih et al., 2018) This shift towards immune cells moving from primary to metastatic sites at single cellular level coinciding with zero RD status at PDS may present as a potential favourable biomarker and complement the widely accepted positive survival prognostic factor where presence of TILs (particularly CD8⁺ T cells and Th-1 cytokines in tumours) correlates with improved OS and PFS in many cancers including HGSOC. (Fridman et al., 2012) Nonetheless, this finding will require further validation with more samples and comparison of the cell populations should be performed between matched pre- and post-chemotherapy samples.

The CSC population is rare (Burgos-Ojeda et al., 2012; Shah & Landen, 2014) and this may be an underestimate due to the technical difficulty in detecting these cells (Quintana et al., 2008). Based on the aggressive nature of OC with a remitting and relapsing course of disease, it is believed that a rare but highly potent subpopulation of CSCs may evade treatment, evolve to be chemoresistant and cause refractory disease (Clevers, 2011). Thus far, the molecular characterisation of ovarian CSCs is still not well-defined (Bast et al., 2009; Shah & Landen, 2014). However, this study was able to identify several ovarian CSCs based on common markers within the cancer epithelial cell population. Of note, the detected common CSC surface markers here were CD24 and CD44 (associated with tumour formation, metastasis, poor prognosis, chemoresistance and disease recurrence) (Burgos-Ojeda et al., 2012; Choi et al., 2005; Meirelles et al., 2012; Nakamura et al., 2015). The proportions of CD24 and CD44 vary substantially within OC evident from studies on different OC cell lines. Khayrani et al. detected a high amount of CD24⁺/CD44⁻ in their OVCAR3 cell lines, meanwhile their SKOV3 cells exhibited CD44⁺, consisting of both CD24⁻/CD44⁺ and CD24⁺/CD44⁺ population (Khayrani et al., 2019). Several other studies found a high

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proportion of CD44 in SKOV3 but little or no expression in OVCAR3 (Gao et al., 2010; Strauss et al., 2011; Ween et al., 2011). Jaggupilli A et al. detected high levels of CD24⁺/CD44⁻ in their OVCAR3 and CAOV3 cells (Jaggupilli & Elkord, 2012). This study found higher levels of CD24⁺/CD44⁻ compared to CD24⁺/CD44⁺ possibly reflecting the predominance of CD24 within HGSOC stem cells as demonstrated by published data on HGSOC cell lines (OVCAR3, OVCAR8, CAOV3) (Jaggupilli & Elkord, 2012; Zheng et al., 2024). Compared to bulk studies, the detection of these common ovarian CSCs within the tumour population paved an important platform whereby future studies should seek to quantify the frequency of these cells in HGSOC tumours at single cell level, followed by functional analyses to determine their stemness which will allow for direct therapeutic targeting and eradication of these cells (Kenda Suster & Virant-Klun, 2019; Winterhoff et al., 2017).

Classifying the cell populations uncovered in this scRNA-seq study by the TCGA HGSOC transcriptomic subtypes suggest that the bulk signatures are likely to have arisen from the abundance of the various cell types within the TME rather than being driven by intrinsic subtypes within the malignant tumour cells themselves. This finding is concordant with other scRNA-seq studies of HGSOC, thence raising the avenue for replacing the bulk HGSOC transcriptomic subtypes with a multi-omics model comprised of analysis of subclones, somatic aberrations, immune and stromal interface with correlations to tumour staging and tissue origin (Geistlinger et al., 2020; Izar et al., 2020; A. J. Shih et al., 2018; Winterhoff et al., 2019).

HGSOC is a highly aneuploid (unbalanced whole chromosome or chromosome arm) C class tumour, driven by recurrent copy number changes with two-thirds of the genes altered by copy number aberrations (Bell et al., 2011; Ciriello et al., 2013b). A recent bulk whole genome sequencing study revealed advanced staged HGSOC have high

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ploidy and whole genome duplication compared to early stages (Cheng et al., 2021). In addition to characterising the HGSOC TME transcriptomic landscape, this single cell datasets enabled genomic profiling of the tumour cells through inference of CNVs from these single cell RNA-seq data which allows the determination of normal vs malignant epithelial cells, and also revealing the intra-and inter-patient heterogeneity. CNVs are mainly genomic events where the number of copies of a particular gene varies from cell to cell (Boen et al., 2021). Sequencing both the genome and transcriptome from the same single cell presents technical challenges. As scDNA-sequencing is less ubiquitous, there has been increasing interest in inferring CNVs from scRNA-seq data (Boen et al., 2021) and the assessment of copy number alterations (CNAs) may be utilised to inform treatment regimens. For example, taxanes used in HGSOC exploit the effectiveness of this chemotherapy in targeting aneuploid tumour cells (Kawakami et al., 2019). Single cell CNAs study has unravelled that on-average 2-4% of diploid cells in HGSOC tumours may undergo spontaneous ploidy change (randomly gain or lose an entire genome). This small subpopulation of cells contributes to tumour heterogeneity and may account for long-term drug resistance (Kumar et al., 2020). Through CNV inference, this study found differing CNV signatures between patients and also distinct intra-patient subclonal CNV signatures across different sites (see Figure 3.33).

In summary, this scRNA-seq study contributes towards the enrichment of the transcriptomic landscape of HGSOC as well as informing CNVs prediction with the analysis of samples from multi- patients and –sites. One of the major advantages afforded by this scRNA-seq study over bulk transcriptomic profiling is the ability to identify rare important subpopulations (including metallothionein-expressing T cells and ovarian CSCs) that could lead to potential targeted treatments specifically at these

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treatment resistant cells. Selected treatment resistant cells should be interrogated further using multi-omics studies in order to understand their expression, signalling pathways and their inhibition correlated clinically with disease and treatment outcomes through in vitro and in vivo experiments. This study also allows for deeper analysis of the heterogeneous subpopulations within the tumour epithelial, stromal and immune compartments with functional enrichment for their biological processes highlighting the existence of a rich crosstalk between these cells in the TME and revealing important biomarkers. Further extrapolation of these differential gene expression at single cellular level along with bulk RNA data onto larger dataset could potentially help in determining the prognostic or translational clinical significance of these cell subpopulations.

**CHAPTER 4: ANALYSIS OF HGSOC TUMOUR
MICROENVIRONMENT USING QUANTITATIVE
HISTOPATHOLOGICAL EXAMINATION**

4 CHAPTER 4: ANALYSIS OF HGSOE TUMOUR MICROENVIRONMENT USING QUANTITATIVE HISTOPATHOLOGICAL EXAMINATION

4.1 Introduction

4.1.1 Quantitative histopathological analysis of HGSOE TME

Molecular profiling of HGSOE has advanced significantly and revealed the diverse molecular nature of the cancer. (Bell et al., 2011; Tothill et al., 2008) However, these molecular studies have yet to be widely implemented in clinical practice mostly due to the expense and complex technical issues associated with the molecular tests. Only sequencing of *BRCA* and homologous recombination deficiency (HRD) tests have been used to prognosticate HGSOE cases. (Ngoi & Tan, 2021) Moreover, recent single cell transcriptomic studies indicate that the bulk molecular subtypes of HGSOE may be driven by the widespread heterogeneity in the tumour cell type composition rather than arising from the tumour epithelial cell subtypes. (Geistlinger et al., 2020; A. J. Shih et al., 2018; Winterhoff et al., 2017) Therefore, some authors have suggested the dismissal of HGSOE bulk transcriptomic subtypes, instead replacing with model of mixtures of sub-clones, somatic aberrations, tumour, immune and stromal cell proportions correlating with tumour stage and tissue origin. (Geistlinger et al., 2020)

In line with this, there has been increasing interest in quantitative histopathological analysis which offers one of the cost effective alternatives in helping to stratify and identify the prognosis of HGSOE due to widely available histological resources such as large archives of slides or digitized images.

4.1.2 Role of tumour and stromal component in modulating TME of HGSOC

4.1.2.1 Tumour component

The full understanding of the biology and composition of the TME of HGSOC may afford the opportunity for directed targeting of both the cancer cells and stromal components. The percentage of cancer cells within HGSOC tumour samples is variable as evidenced by histopathological analysis both at pre-chemotherapy and post-chemotherapy stages. (Böhm et al., 2015; Lan et al., 2015b) These tumour sites that are thought to be comprised of cancer cells are often replaced by necrotic and fibrotic areas. At pre-chemotherapy stage, necrotic cells within tumours may contribute to more aggressive disease as reported in breast cancer. (Le et al., 2006) However, little is known about the prognostic significance of tumour necrosis in ovarian cancer at pre-chemotherapy stage where limited data from small cohort studies have found positive association of necrosis with improved survival outcomes. (Green et al., 2005; Penault-Llorca et al., 2000) Meanwhile, at post-chemotherapy stage, the increased necrosis reflects better response to chemotherapy and consequently reduced recurrence and better prognosis. (Le et al., 2006) Interestingly, Soslow et al. described HGSOC with specific SET (Solid, pseudoEndometrioid and Transitional) histological features characterised by higher mitotic activity, tumour-infiltrating lymphocytes, and either geographic or comedo necrosis to be more associated with germline and/ or somatic *BRCA1/2* mutations. (Soslow et al., 2012) This raised the potential for correlating morphological subtypes with different sensitivity to chemotherapy and PARP inhibitors. (De Leo et al., 2021) Hence, demonstrating the potential for utilising histopathological features of HGSOC to guide risk and treatment stratification.

4.1.2.2 Stromal component

The tumour stroma is comprised of a complex mixture of fibroblasts, immune cells and endothelial cells embedded in the proteins of the extracellular matrix (ECM). (van Pelt, Sandberg, et al., 2018) The fibroblast cells surrounding or in close proximity to HGSOC tumours are termed cancer associated fibroblasts (CAFs) and may be in a state of quiescence or activated. The activated CAFs contribute to a reactive or 'desmoplastic' stroma which promotes tumour development through secretion of growth factors, mediates tumour-promoting inflammation, ECM remodelling, angiogenesis, tumour epithelial-mesenchymal transition (EMT), invasion, metastasis, recurrence and leads to poor overall survival. (Desbois & Wang, 2021; Lan et al., 2015a) The reactive stromal changes are caused by the interactions during tumour progression between cancer cells, ECM and the CAFs and involve also inflammatory cells and blood vessels. This may explain that tumours with high stromal content represent a pro-metastatic phenotype group linked to worse prognosis. (van Pelt, Sandberg, et al., 2018)

4.1.2.2.1 Desmoplasia and role of cancer associated fibroblasts (CAFs) in HGSOC

Desmoplasia (or also synonymously termed as fibrosis) is the formation of excess connective tissue causing stromal stiffening and scar formation as a result of tissue injury such as cancer or infection. (Chandler et al., 2019) It originates from the Greek words '*desmos*' meaning 'band' and '*plassein*' which stands for 'form' or 'mold'; and it can manifest clinically through 1) the overproduction of extra-cellular matrix proteins

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and 2) extensive proliferation of myofibroblast-like cells (Jemal et al., 2010; Whatcott et al., 2012; Yen et al., 2002).

There are dual roles of desmoplasia in cancer. During early stages of tumour growth, fibrosis acts to restrain cancer initiation, proliferation, and metastasis. However, at later stages of the disease, desmoplasia promotes cancer growth through a variety of mechanisms including stiffened ECM, extensive angiogenesis, immune modulation, increased resistance to chemoradiotherapy, anti-angiogenics, and immunotherapy. (Chandler et al., 2019; De Leo et al., 2021; Provenzano et al., 2012)

Desmoplasia occurs due to inter- and intra-cellular biological signalling where molecules such as transforming growth factor- β (TGF- β), basic fibroblast growth factor (FGF), connective tissue growth factor (CTGF), interleukin-1 β (IL-1 β), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) have been shown to stimulate ECM production, angiogenesis, inflammation, EMT, and proliferation of myofibroblast-like cells. (Shimizu, 2008)

The Tothill and TCGA studies which made a revolutionary impact in the discovery of molecular subgroups of HGSOC have found that the mesenchymal subtype (C1) with distinct reactivated and desmoplastic stroma presented the worst prognosis. (The Cancer Genome Atlas Research, 2011; Tothill et al., 2008) Activated CAFs play a key role in the desmoplastic reaction resulting in fibrosis around the tumour cells. Different markers have been used to characterise the activated CAFs and indirectly measure the desmoplastic response in which increased expression of these markers correlate with greater extent of desmoplasia, higher incidence of metastases and poorer survival outcomes. (Dolznic et al., 2005; Park et al., 1999) Some of the important markers include FAP (a type II membrane-bound glycoprotein of the serine protease family with

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dipeptidylpeptidase and collagenolytic activity) (Park et al., 1999), α -smooth muscle actin (α -SMA) or also known as ACTA2, low caveolin1 (CAV1), CD10, G protein-coupled receptor 77 (GPR77), fibroblast specific protein 1 (FSP-1), periostin (POSTN), platelet-derived growth factor receptor- α or - β (PDGFR- α /PDGFR- β), podoplanin (PDPN), integrin β 1 (CD29) and matrix metalloproteinase (MMP). (Abbas & Mahalingam, 2011; Kanzaki & Pietras, 2020; Mhaidly & Mechta-Grigoriou, 2020)

4.1.3 Tumour stroma ratio (TSR) or tumour stroma proportion (TSP)

There has been increasing interest in the evaluation of the tumour stroma ratio (TSR), also known as the tumour stroma percentage as a reflection of the TME and survival outcome in several solid tumours such as colon cancer (Huijbers et al., 2013b; van Pelt, Sandberg, et al., 2018), breast cancer (de Kruijf et al., 2011; Qian et al., 2021), oesophageal cancer (Wang et al., 2012), cervical cancer (Liu et al., 2014) and epithelial ovarian cancer (Ying Chen et al., 2015). The TSR has been found to be a strong independent prognostic factor particularly in colon cancer and other epithelial cancers. (de Kruijf et al., 2011; Huijbers et al., 2013b; Liu et al., 2014; Lv et al., 2015; Mesker et al., 2007) TSR is normally assessed on haematoxylin and eosin (H&E) stained paraffin sections at the invasive front of the tumour which was decided through the findings of a colon cancer study whereby the highest stroma percentages were seen in the tumour areas with the deepest invasion. (Mesker et al., 2007) Correspondingly, the most invasive tumour area is generally used to determine the T-status in routine pathology for colon cancer. (van Pelt, Sandberg, et al., 2018) Tumour cells have to be present at the four borders of the selected image and the stroma percentages are scored manually to the nearest 10% on image fields of x10 objective.

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Most studies used a statistically determined cut-off value of 50% to differentiate between stroma-rich and stroma-poor patients. (Ying Chen et al., 2015; de Kruijf et al., 2011; Mesker et al., 2007) These set criteria helped to reduce the inter-observer variation in various published validation studies. (Huijbers et al., 2013b; Hynes et al., 2017; Mesker et al., 2007; van Pelt, Sandberg, et al., 2018) TSR is normally scored on patients prior to adjuvant therapies.

The TSR assessment is based on the amount of stroma present surrounding the tumour. On deeper reflection, this study has noted that tumour stroma proportion (TSP) would be a better terminology in place of TSR to represent the stromal proportion or percentage out of the total tumour and stroma areas. Therefore, the term TSP is adopted here.

4.2 Study aims

This part of my PhD utilises retrospective cohorts of Edinburgh patients diagnosed with HGSOE to investigate the tumour and stromal components of the cancer using automated and semi-automated quantitative histopathological analysis. Of note, the third cohort originates from a large dataset which is well annotated clinically and molecularly at bulk level.

The specific objectives include:

1. Using pilot cohorts (A and B), identify and quantify the tumour and stromal regions on histological sections and develop an automated pipeline for the detection of these tumour compartments.
2. Determine the best histological stain for identifying necrosis and desmoplasia. Identify and quantify these features on the histological sections and develop an automated workflow of this process.
3. Analyse and quantify the tumour stroma proportion (TSP) from these histological sections.
4. Correlate the tumour stroma ratio, necrosis and desmoplasia to clinical characteristics available from the Edinburgh Ovarian Cancer Database
5. Determine if TSP could be utilised as an independent prognostic factor using the large validation cohort
6. Compare the molecular landscape of the stroma-rich and stroma-poor groups, including immune infiltration, genomic sequencing data and transcriptomic subtype
7. Generate a prediction model based on TSP to help in risk stratification for HGSOE patients

4.3 Materials and methods

4.3.1 Patient's samples and cohorts

Three cohorts (A, B and C) of archival HGSOC tumour specimens were used for histopathological analysis. Ethical approval was obtained from the Lothian NRS Human Annotated Bioresource (Table 4.2). All the diagnostic tumour tissues from multiple sites of the patients have been requested from the Edinburgh Bioresource in the form of formalin-fixed paraffin-embedded (FFPE) tissue sections. Five μ m sections were cut from these FFPE tissue, then stained with Haematoxylin-Eosin (H&E) and scanned using Nanozoomer at a 40x yielding digital images. The details of each cohort are summarised in Table 4.2.

Table 4.1 Cohorts. Characteristics of Cohort A, B and C.

Cohort	Number of patients (n)	Date of Diagnosis	Surgery	Stages	Ethics Reference
A (Pilot cohort)	9	2012-2013	Primary debulking surgery	III & IV	15/ES/0094-SR1145
B (Pilot cohort)	14	2000-2006	Primary debulking surgery	III & IV	13/ES/0126-SR489
C	374	1984-2006	Primary debulking surgery	I – IV & Unknown	15/ES/0094-SR705, SR752

4.3.2 Pilot cohorts (A and B)

For the first cohort (A), 9 patients diagnosed with advanced HGSOC between 2012 and 2013 were identified from the Edinburgh Ovarian Cancer database. The criteria for selection of these patients included HGSOC diagnosis, Stages III/ IV, primary debulking surgery, FFPE tissue available from at least three sites (primary, omentum and other metastatic sites such as peritoneum, colon, abdominal wall, etc) and a minimum of five years of follow-up clinical records. Three 5µm sections from all the FFPE sites available for the 9 patients were cut and mounted onto glass slides. These were prepared by the Lothian NRS Human Annotated Bioresource according to the ethics for the use of the human tissue specimens in research.

Initially, in order to identify the best staining for the detection of desmoplasia and necrosis, 39 slides from multiple sites of a single patient were stained with H&E, Masson's trichome and Haematoxylin and Van Gieson's (HVG) by the Division of Pathology Laboratories, Western General Hospital, University of Edinburgh, UK. The three different staining methods were compared. Areas of desmoplasia were defined as the presence of a newly formed, immature collagenous stroma surrounding the clusters of tumour cells. These desmoplastic collagen are more sparse, less organised and have a storiform pattern where the fibroblasts appear as loosely-arranged whorls of elongated, spindled-like cells. Necrosis was identified as areas of ghosting of the tumour cells with nuclear pyknosis and/or karyorrhexis, cytoplasmic eosinophilia and cellular swelling or shrinkage. (Evans, 1992) The slides were evaluated by Professor C Simon Herrington (CSH), an expert Gynaecological Pathologist. Masson's trichome and H&E were comparable in performance for the detection of desmoplasia (Figure 4.1). H&E staining provided the best discrimination for detection of necrosis (Figure 4.2). Therefore, further staining was performed using H&E alone. Once stained with

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H&E, slides were scanned using Nanozoomer at 40x magnification yielding digital images and analysed using QuPath open source software for quantitative pathology and bioimage analysis (QuPath-0.2.0-m8). (Bankhead et al., 2017) Slides with tumour present and the following histological features assessable at 40x magnification (where features of viable and necrotic tumour, and desmoplastic/ non-desmoplastic stroma could be assessed) on QuPath were included in this pilot study. The following steps were used when analysing the images on QuPath: a) set image type as H&E (Figure 4.3A), b) perform simple tissue detection to define boundary of the image (where this is set to areas surrounding the tumour ensuring that all four borders of the image field contain tumour cells), c) run difference of Gaussians (DoG) superpixel creator to divide the image into small segments of 8 sigmaMicrons (Figure 4.3B), d) add shape measurements to allow calculation of the size of the areas of interest, e) select object by class to define tumour and stromal cells, f) run the automated object classification based on cells defined in step e), g) define the necrosis and desmoplasia areas manually within the image by setting different colours respectively (Figure 4.3C), and h) calculate the viable tumour, necrotic tumour, desmoplastic and non-desmoplastic stromal areas from the measurements detected by QuPath.

To assess for interobserver variation in defining the histological features of interest (including tumour, stroma, necrosis and desmoplasia), approximately 30 images out of 137 were randomly selected and evaluated by CSH. This represented a validation set of > 20% of the total images available from this cohort. The interobserver agreement evaluation between observer 1 (JHCL) and 2 (CSH) was reported as % of agreement and kappa values calculated from observer agreements using Cohen's kappa statistics. (Cohen, 1968; Cross, 1996; Germolec et al., 2004; Viera & Garrett, 2005) Generally, kappa values of < 0.4 reflect relatively poor agreement, values

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between 0.4-0.6 moderate agreement, 0.6-0.8 good agreement and values > 0.8 are representative of excellent agreement. (Cohen, 1968) The viable tumour, necrotic tumour, desmoplastic and non-desmoplastic stromal areas were independently assessed using whole section slides and a high level of concordance was achieved when defining these areas (Table 4.2). Specifically, 100% agreement ($k = 1.0$) was achieved when defining tumour and stroma (without the distinction of absence or presence of necrosis or desmoplasia). However, $\geq 90\%$ concordance was achieved when defining necrosis ($k = 0.9$) and desmoplasia ($k = 0.8$) (Table 4.2).

When scoring the TSP, total stromal areas from all the images were divided by the total tumour and stroma areas (summed from all the images available for each patient). In cases where the whole image field surrounding the tumour could not be analysed due to technical image focussing issues, at least 25% of the image was included ensuring that tumour cells were present at the four borders. This was to enable adequate representation of the tumour and stroma from the image analysed.

To calculate the desmoplasia and necrosis percentages for each patient, the size of desmoplastic stroma or necrotic tumour for each image were added up and divided by the total tumour and stroma areas (from all the images available for each patient) respectively. This gave rise to total desmoplasia or necrosis percentages for each patient.

The same process above was repeated for the second cohort (B) where 14 patients diagnosed with advanced HGSOc between 2000 to 2006 were identified from the Edinburgh Ovarian Cancer Database and selected for this cohort's analysis. The same criteria used for selection cohort A's patients were used including HGSOc diagnosis,

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stages III/IV, primary debulking surgery, FFPE tissue available from at least three site and a minimum of five years of follow-up clinical records.

Once TSP, necrosis and desmoplasia were quantified, they were correlated to clinical data including age of patients, residual disease at primary debulking surgery, overall survival (OS) and progression free survival (PFS).

In this study, the interobserver variation was eliminated during the scoring of TSP by training QuPath to recognise tumour and stromal cells through artificial intelligence and measuring these compartments automatically. Additionally, there had been 100% concordance observed in the validation set for the tumour and stromal cells classification.

Table 4.2 Interobserver Agreement for Classification of Histologic Features in Validation Set

Histologic Feature	Observer (n=no. of slides)		2		Interobserver agreement ^a	
			Yes	No	% agreement	kappa value
Tumour	1	Yes	30	NA	100.0	1.0
		No	NA	NA		
Stroma	1	Yes	30	NA	100.0	1.0
		No	NA	NA		
Necrosis	1	Yes	23	1	96.7	0.9
		No	0	6		
Desmoplasia	1	Yes	7	3	90.0	0.8
		No	0	20		

^a Between observer 1 (JHCL) and 2 (CSH)

NA, not applicable as all 30 slides contained tumour and stroma

Formula for TSP, % Desmoplasia and % Necrosis Calculations

$$\text{*Tumour stroma proportion (TSP)} = \frac{\text{Total stroma areas} + \text{(desmoplastic + non-desmoplastic)}}{\text{Total tumour + stroma areas}}$$

$$\% \text{ Desmoplasia} = \frac{\text{Total desmoplastic stroma areas}}{\text{Total tumour + stroma areas}} \times 100$$

$$\% \text{ Necrosis} = \frac{\text{Total necrotic tumour areas}}{\text{Total tumour + stroma areas}} \times 100$$

* Adapted from (de Kruijf et al., 2011; Huijbers et al., 2013a; Mesker et al., 2007; van Pelt, Sandberg, et al., 2018; Yang et al., 2017).

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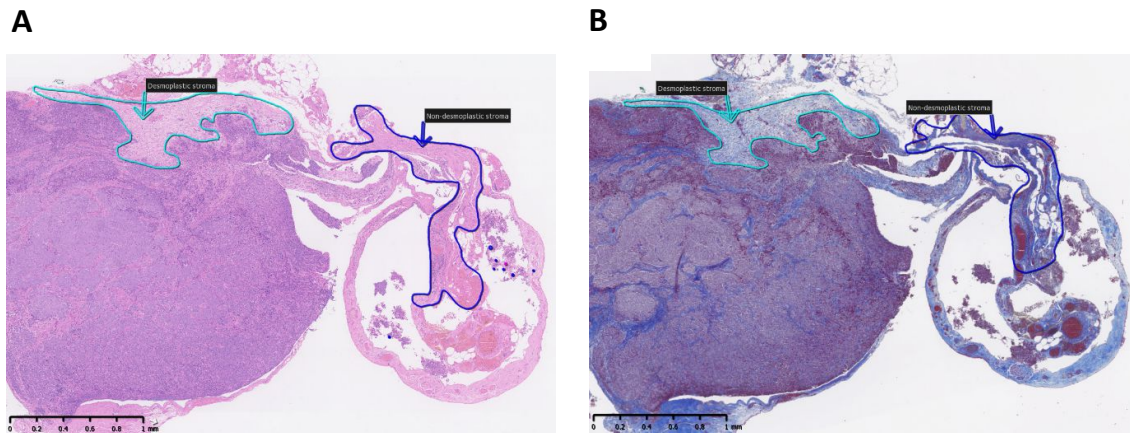


Figure 4.1 Metastatic nodule. H&E stained (A) and Masson's trichrome stained (B) sections of a HGSOc metastatic nodule. Desmoplastic stroma (encircled cyan) can be distinguished from non-desmoplastic stroma (encircled blue) on both staining methods.

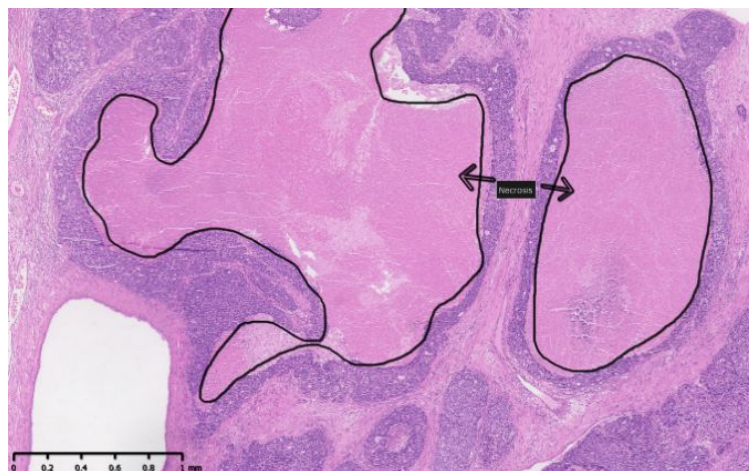


Figure 4.2 H&E stained section of a HGSOc ovary. Necrosis (encircled black) appears as 'ghosted' cells within the section.

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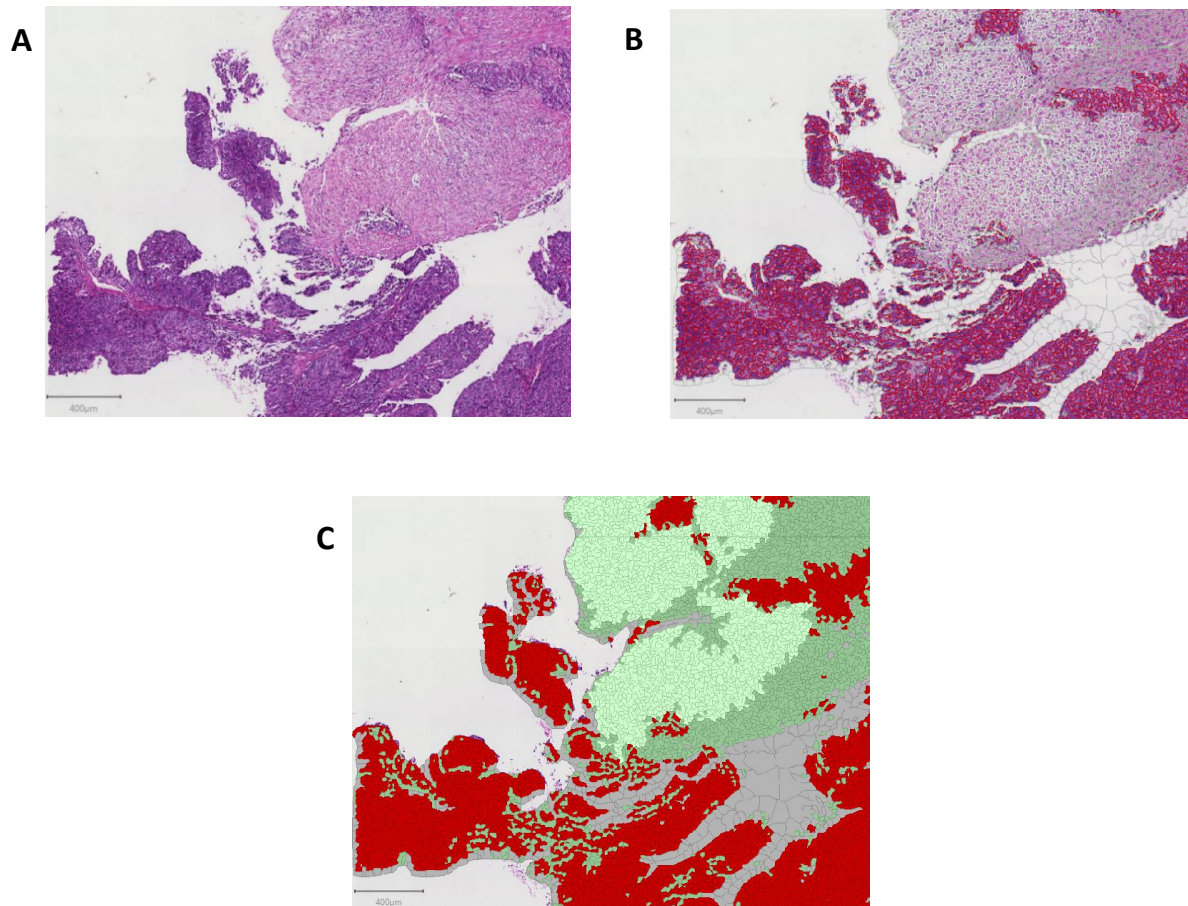


Figure 4.3 Section from an Ovary: Workflow of QuPath Image Analysis. Image set to brightfield (H&E) (A). DoG superpixel segmentation of image (B). Final annotated image on QuPath (C) with tumour cells (red), non-desmoplastic stroma (dark green), desmoplastic stroma (light green) and excluded areas (grey).

4.3.3 Cohort C

Cohort C was generated from a cohort available to our research group which had extensive molecular profiling performed by Dr Robert Hollis (RLH), a Research Fellow at the CRUK Scotland Centre, University of Edinburgh. This cohort met the following criteria including HGSOC diagnosis, Stages III/ IV disease and primary debulking surgery. Each patient from this cohort had FFPE tissue available from a single site (either primary ovarian, metastatic omental or colonic site).

H&E slides were scanned at 40x magnification using Nanozoomer and analysed using QuPath following the aforementioned steps. For Cohort B and C, the viable tumour, necrosis, non-desmoplastic stromal components and desmoplastic areas were analysed using same techniques and steps as the pilot study above (which helped to optimise the methodology adopted throughout this study).

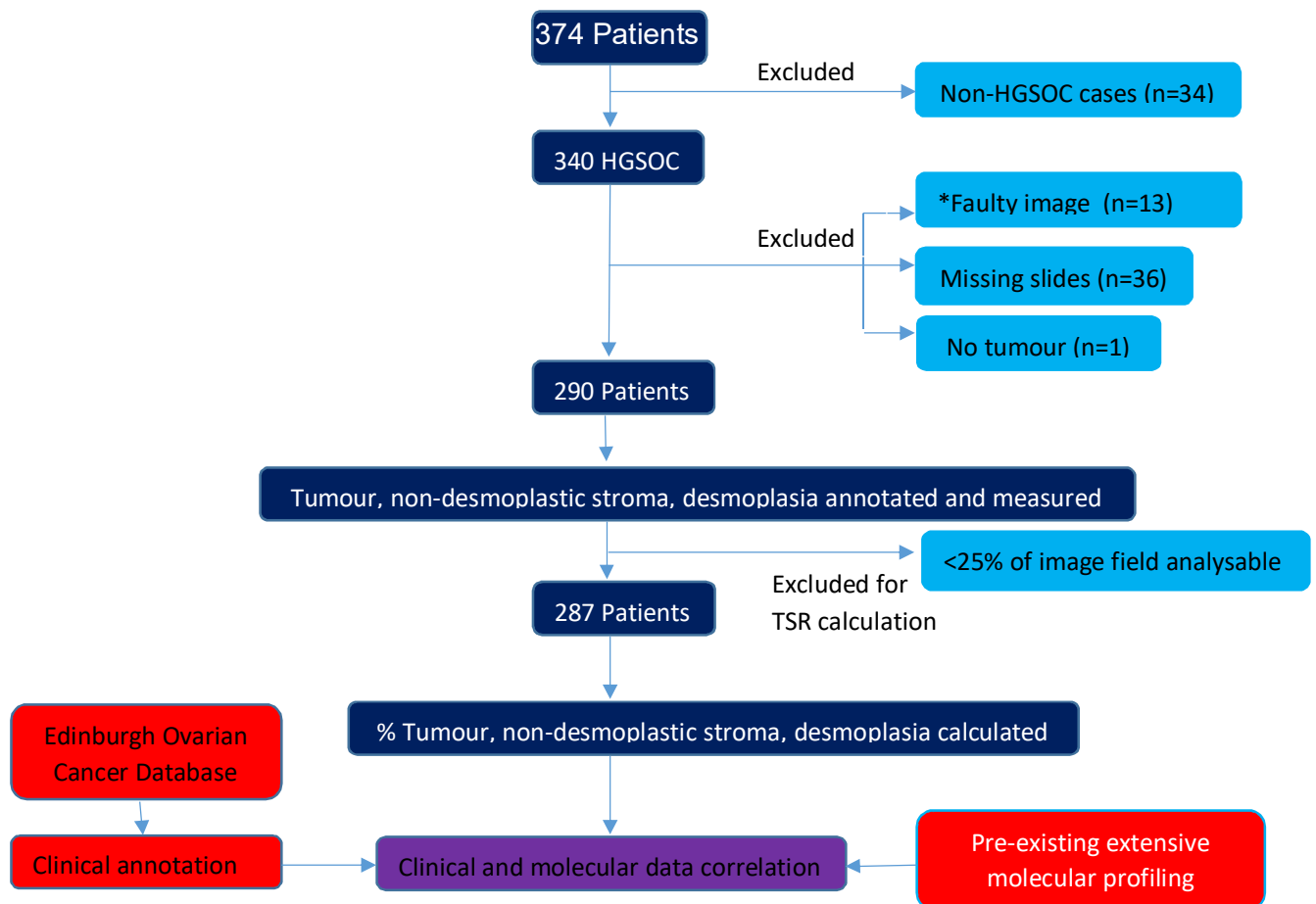


Figure 4.4 Cohort C Identification. Consort diagram of HGSOC Cohort C generated for quantitative histopathological analysis with clinical and molecular data correlation. *Faulty images on QuPath where there was lack of focus at 40x magnification or presence of artefacts within the digital images precluding analysis of the tumour or stromal features were excluded.

4.3.4 Clinical data collection

Clinical data were gathered from the Edinburgh Ovarian Cancer Database and patients' paper and electronic health records. The access, ethics approval for clinical data collection and correlation to histopathological and molecular analyses were obtained from NHS Lothian Research and Development (reference ID 2007/W/ON/29). The clinical responses including response detected radiologically after platinum-containing chemotherapy were reported based on World Health Organisation (WHO) or Response Evaluation Criteria In Solid Tumors (RECIST) criteria, and response detected through CA-125 tumour marker were defined by the Gynecological Cancer Intergroup (GCIG) guidelines (Rustin et al., 2011).

4.3.5 Molecular subtyping, phosphatase and tensin homolog (PTEN) and retinoblastoma protein (RB) status, and immune cell infiltration data procurement for cohort C

Data for Cohort C including the molecular subtyping, PTEN and RB status, and immune cell infiltration were obtained from previous work by the Gourley laboratory (Gourley et al., 2014b; Hollis et al., 2019; R. L. Hollis et al., 2022). As a background, these previous works were related to bevacizumab sensitivity and multi-omic characterisation of HGSOC where the samples were comprised of a group of OC patients who underwent PDS and then received adjuvant platinum-based chemotherapy, and were divided into a training (2/3) and validation (1/3) cohort. Cohort C in this study was a subset of this larger data selected via aforementioned inclusion criteria.

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For the transcriptomic subtyping, the following steps were performed: 1) macrodissection of FFPE, 2) extraction of mRNA, 3) cDNA formation and amplification, 4) cDNA fragmentation and labelling, 5) hybridisation to the Ovarian DSA cDNA microarray platform and sequenced, and 6) normalisation of raw transcriptomic data using the Robust Multi-Array Average (RMA). Tothill (C1, C2, C4, C5) and TCGA (Mesenchymal, Immuno-reactive, Differentiated, Proliferative) transcriptomic subtyping were performed using consensusOv R package (Gregory M. Chen et al., 2018; R. L. Hollis et al., 2022). The HGSOC patients were also classified using an HR-centric taxonomy with homologous recombination repair-aberrant (HRR-aberrant) (including *BRCA1/2* mutation, *EMSY* overexpression, non-*BRCA* HRR gene mutation), homologous recombination proficient (HRP) and Cyclin E1 gain (CCNE1g) (R. L. Hollis et al., 2022).

PTEN and RB status were determined by immunohistochemistry (IHC) using HGSOC tumour microarray (TMA) sections where the complete absence of positive staining within tumour cells represented a loss of the protein (with appropriate positive internal control of stromal staining) (R. L. Hollis et al., 2022).

Infiltration by tumour-infiltrating lymphocytes including CD3⁺ and CD8⁺ T cells were quantified using IHC of TMAs. The quantification of these cells were performed as a % of marker-positive cells within the tumour areas using QuPath version 0.1.2 (R. L. Hollis et al., 2022).

4.3.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.3.1. A P value of <0.05 was considered statistically significant. Kaplan-Meier survival analysis was used for the analysis of OS and PFS and log-rank statistics were used to determine

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the differences in survival curves. Cox proportional hazard model was used to perform the uni- and multi-variate survival analyses. Fisher's exact and Chi-square tests were utilised to determine if there were significant association between the histological subgrouping and clinicopathological/ molecular factors.

For the prediction models, statistical analyses were carried out using R (R version 3.6.2) with the following packages: *survival*, *survivalROC*, *survAUC*, *timeROC* and *Hmisc* (Therneau, 2020). The Harrell's C-statistics (C-index) was used to assess the discrimination performance of the prediction models.

4.4 Results

4.4.1 Pilot cohorts (A and B) results as proof of principle

4.4.1.1 Patient demographics, clinical characteristics and histopathological findings

For the pilot cohorts A and B, 9 and 14 patients met the criteria set out as detailed in section 4.4.2 for each cohort respectively. Tables 4.3 detailed the patient demographics and clinical characteristics for both cohorts. Tables 4.4 and 4.5 summarised the histopathological findings for both cohorts. QuPath was able to detect viable tumour and stromal areas (without the distinction of desmoplastic or non-desmoplastic stroma) which enabled automatic quantification of these regions once the software was trained to recognise the tumour (viable) and stromal cells (Figure 4.5). However, training QuPath to detect necrosis and differentiate desmoplastic from non-desmoplastic stromal cells was less successful as the software did not have adequate sensitivity or specificity for the detection of these features even after consultation with Dr Peter Bankhead (creator of QuPath). The software could not accurately define the necrosis/ desmoplasia within the tumour/stroma respectively. An attempt using another digital pathology software Ilastik returned the same results (Figure 4.6). Therefore, the tumour (viable) and stromal (desmoplastic and non-desmoplastic) region annotations were performed using QuPath automation while more discrete features (necrosis and desmoplasia) were annotated manually on each slide.

Table 4.3 Table Summary of Patients Demographics and Clinical Characteristics

Cohort (n = No. of patients)	Mean Age	FIGO Staging (at diagnosis)	Overall Survival (Days)	Progression Free Survival (Days)	Residual Disease (n = No. of patients)
A (n = 9)	61	III (n = 6) IV (n = 3)	278-2369 (Median OS 1842)	278-2160 (Median PFS 832)	0 cm (n = 2) < 2 cm (n = 6) > 2cm (n = 1)
B (n = 14)	62	III (n = 9) IV (n = 5)	122-3620 (Median OS 829)	78-2005 (Median PFS 444)	NA (n = 2) < 2 cm (n = 4) > 2 cm (n = 8)

Table 4.4 Table Histopathological Findings of Cohort A

Patient ID	Tumour size (mm ²)		Stromal size (mm ²)		TSP	% Desmo-plasia	% Necro-sis	No. of sites
	Necrotic	Viable	Non-desmoplastic	Desmo plastic				
A1	120.27	2996.70	1017.42	142.59	0.28	3.33	2.81	10P, 16M
A2	78.92	939.84	1224.27	57.94	0.58	2.52	3.43	3P, 14M
A3	4.14	210.37	83.73	7.57	0.30	2.47	1.44	5P, 2M
A4	8.85	52.66	29.89	2.01	0.38	2.15	9.47	5P, 2M
A5	55.20	591.37	484.87	20.27	0.46	1.76	4.79	5P, 10M
A6	87.92	975.16	598.28	13.02	0.39	0.77	5.25	5P, 9M
A7	47.08	509.95	446.72	27.71	0.48	2.69	4.56	6P, 4M
A8	234.23	2023.4	1150.27	60.67	0.37	1.75	6.75	12P, 15M
A9	1432.61	1716.9	800.64	16.10	0.32	0.41	36.12	7P, 7M

P, primary; M, metastatic

TSP, tumour stroma proportion

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Table 4.5 Table Histopathological Findings of Cohort B

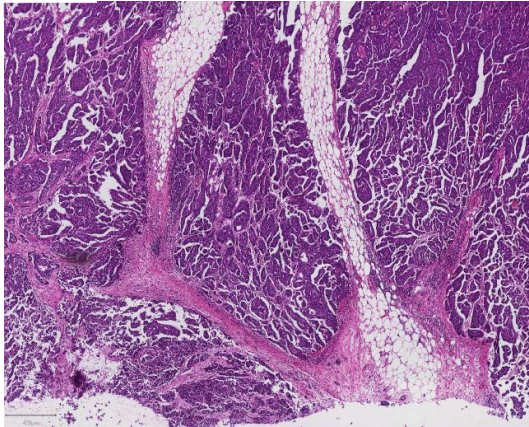
Patient ID	Tumour size (mm ²)		Stromal size (mm ²)		TSP	% Desmo-plasia	% Necro-sis	No. of sites
	Necrotic	Viable	Non-desmoplastic	Desmo-plastic				
B1	15.14	157.63	148.20	72.64	0.58	18.45	3.85	2P, 2M
B2	33.44	948.71	547.59	231.15	0.45	13.13	1.90	3P, 8M
B3	120.09	471.85	309.64	111.19	0.47	10.98	11.86	4P, 3M
B4	63.30	239.34	373.51	81.86	0.66	10.80	8.35	0P, 3M
B5	2.70	218.14	339.15	60.05	0.65	9.68	0.44	3P, 2M
B6	60.63	327.44	420.19	61.33	0.60	7.05	6.97	3P, 2M
B7	0.00	38.09	37.35	5.02	0.53	6.24	0.00	1P, 1M
B8	0.75	271.47	126.52	25.20	0.36	5.94	0.18	2P, 3M
B9	9.16	293.85	175.2	12.29	0.39	2.51	1.87	2P, 2M
B10	3.12	193.76	68.55	206.11	0.59	14.54	0.66	1P, 1M
B11	0.00	25.99	99.69	16.21	0.82	11.42	0.00	5P, 2M
B12	90.34	259.35	139.72	22.15	0.38	4.33	17.66	2P, 3M
B13	2.33	100.56	150.48	13.33	0.62	5.00	0.87	1P, 2M
B14	0.00	310.81	385.08	23.76	0.57	3.30	0.00	3P, 3M

P, primary; M, metastatic

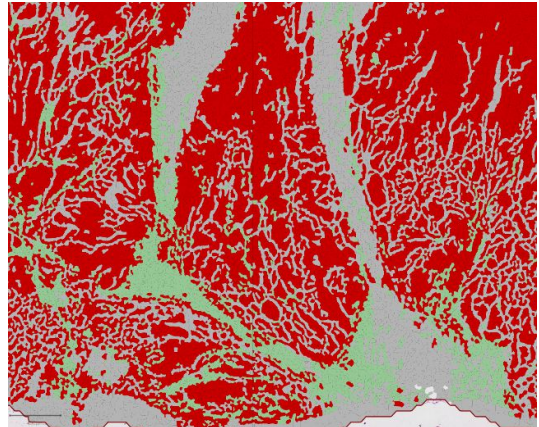
TSP, tumour stroma proportion

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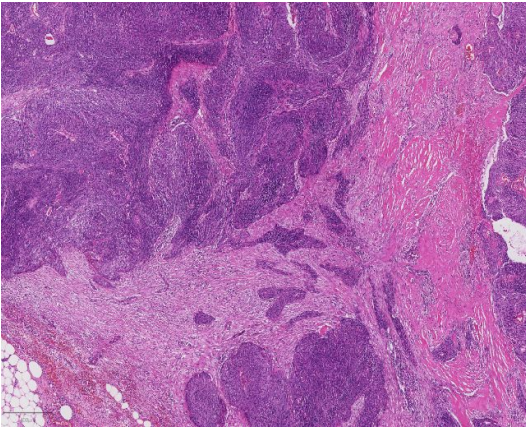
A1



A2



B1



B2

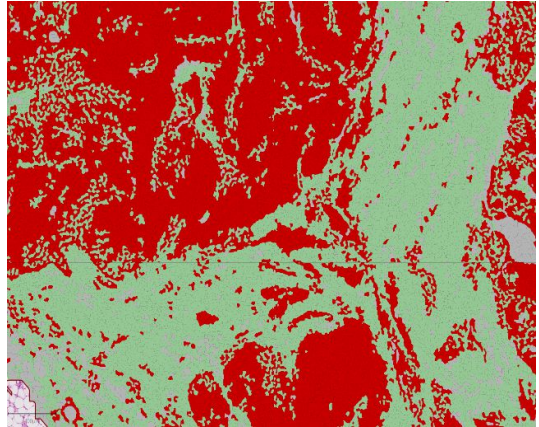


Figure 4.5 Images Pre- and Post-QuPath Classification. (A1) and (B1) were the original histological images at 40x magnification. (A2) and (B2) were examples of images with QuPath classification of tumour (red) and stromal (green) areas following training of the software.

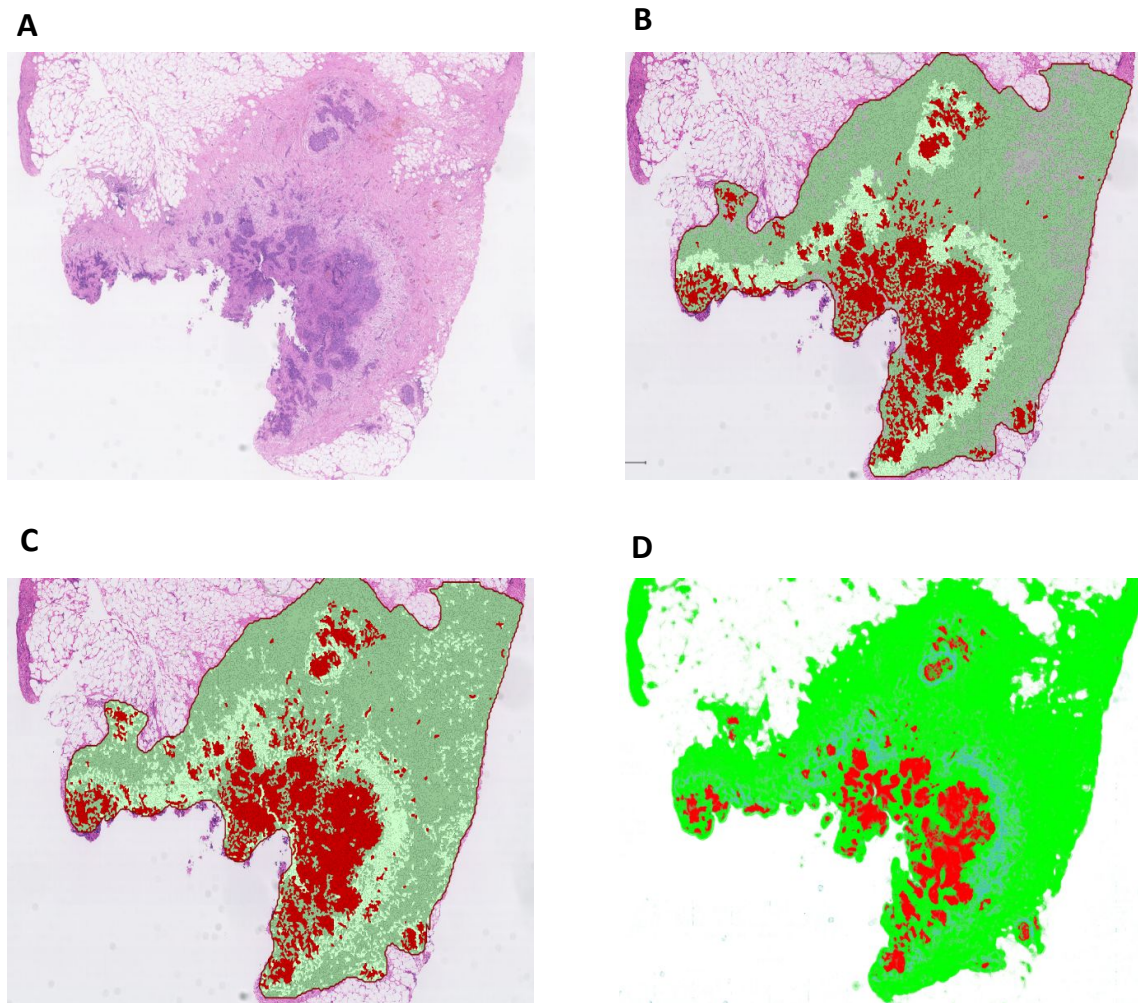


Figure 4.6 Desmoplasia Detection Using Manual and Automated Methods. (A) Original image at 40x magnification. (B) Same image with manual annotation of desmoplastic stroma (light green) with QuPath. Tumour labelled as red, non-desmoplastic stroma as dark green and excluded areas (grey). (C) Automated annotation of desmoplastic stroma attempted with QuPath which the software over-classified desmoplasia by highlighting non-desmoplastic stroma as light green areas. (D) Automated annotation of desmoplastic stroma with Ilastik which the software under-classified desmoplastic stroma despite optimising the pixel classification. Tumour labelled as bright red, desmoplastic stroma labelled as dark green and non-desmoplastic stroma labelled as bright green.

4.4.1.2 Evaluation of the prognostic value of TSP and correlation with clinicopathological features in pilot cohorts

The TSP evaluation was performed by dividing the stromal area by total viable tumour (excluding necrotic tumour) and stromal areas. This calculation was based on published methodology from TSP measurements in colorectal cancer where TSP is more widely adopted (Sirinukunwattana et al., 2018; van Pelt, Kjær-Frifeldt, et al., 2018). To evaluate if there was any clinical significance of the TSP analysis, the OS and PFS for both cohorts were correlated to the TSP values (excluding necrosis) without any threshold cut-off for TSP. The initial findings showed that higher TSP (i.e. greater stromal composition) was associated with shorter OS (Spearman's rho -0.40, P value 0.075) and PFS (Spearman's rho -0.51, P value 0.0126) (Figure 4.5). As the preliminary analysis showed that greater TSP was associated with poorer survival outcomes, further evaluation of TSP using the most commonly utilised cut-off of 0.5 from the literature (or 50% stromal proportion) against the survival data was performed. (de Kruijf et al., 2011; J. Gao et al., 2021; Xi et al., 2017) The Kaplan-Meier analysis for both cohorts demonstrated inferior PFS in the stroma-rich group (TSP $\geq$ 0.5) compared to stroma-poor group (TSP < 0.5) (log-rank P value 0.0349; median PFS for stroma-rich 445 days compared to stroma-poor 832 days; HR 2.93, 95% CI 1.08-7.95); the corresponding difference in OS approached statistical significance (log-rank P value 0.0658; median OS for stroma-rich 905 days compared to stroma-poor 2160 days; HR 2.53 95% CI 0.94-6.79) .

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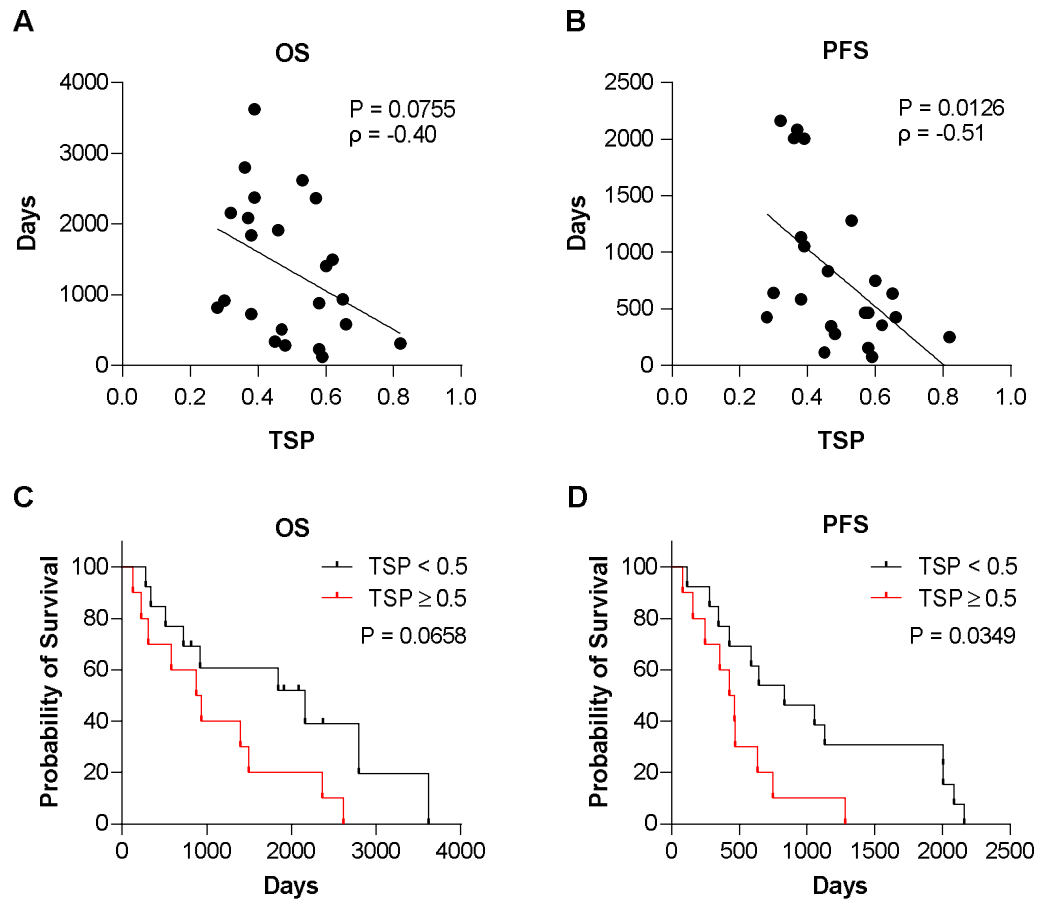


Figure 4.7 Relationship between TSP and Survival Outcomes for Cohorts A and B. (A) and (B) TSP is inversely correlated with OS and PFS. (C) and (D) Kaplan-Meier curves for the survival of pilot cohorts patients. Stroma-rich patients (TSP ≥ 0.5) have poorer OS and PFS compared to stroma-poor group. P, P value; ρ , Spearman's rho value

4.4.1.3 Evaluation of the relationship between TSP and desmoplasia and necrosis using pilot cohorts

Using these pilot cohorts, the TSP values were correlated to desmoplasia and necrosis to evaluate if there were any relationship between the tumour stroma proportion and these histopathological features. From these small number of cases, we demonstrated a moderate correlation between TSP and % desmoplasia (Spearman's rho 0.57, P value 0.0049) (Figure 4.8A). But, the relationship between TSP and % necrosis was not clear (Spearman's rho -0.30, P value 0.1673) (Figure 4.8B). When the TSPs are divided into stroma-poor (< 0.5) and stroma-rich (≥ 0.5) groups, a clearer relationship was observed between TSP and desmoplasia and necrosis (Figure 4.9). Stroma-rich patients have a greater extent of desmoplasia with a median % desmoplasia of 7.84% vs 2.51% for stroma-poor patients (Mann-Whitney U test P value 0.008). In terms of necrosis, stroma-rich patients have a lower extent of necrosis with a median % necrosis of 0.77% vs 4.79% for stroma-poor patients (Mann-Whitney U test P value 0.0357).

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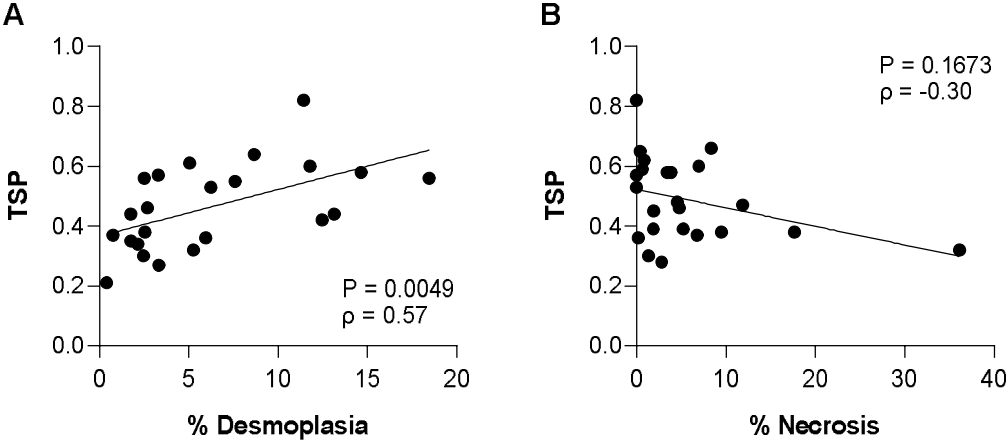


Figure 4.8 Correlation of TSP with Desmoplasia and Necrosis. (A) There is a moderate correlation between TSP and %desmoplasia in these pilot cohorts. (B) The correlation between TSP and %necrosis is not clear from these pilot cohorts. P, P value; ρ , Spearman's rho value

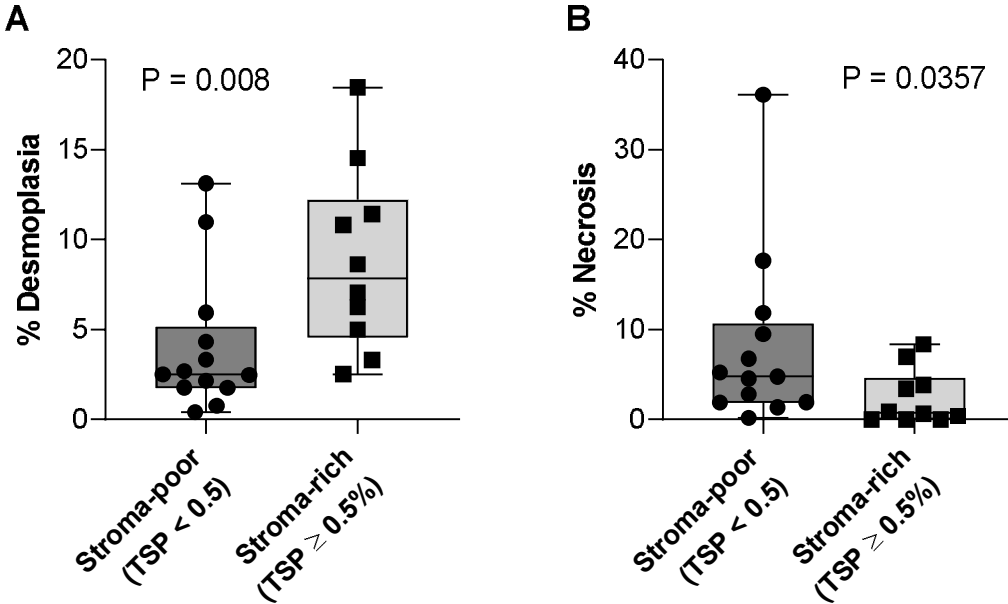


Figure 4.9 % Desmoplasia and % Necrosis in Stroma-poor and Stroma-rich Groups. (A) Stroma-rich patients have higher % desmoplasia. (B) Stroma-rich patients have lower % necrosis.

4.4.1.4 Evaluation of prognostic value of desmoplasia by sites in pilot cohorts

In both pilot cohorts, the desmoplasia proportions were analysed from a series of primary and metastatic sites available for each patient. To evaluate if there were any differences in the contribution of desmoplasia by disease sites, the proportion of desmoplasia were worked out by adding up the % of desmoplasia for all the primary sites and metastatic sites separately. Six out of 9 patients (66.7%) in Cohort A and 8 out of 14 patients (57.1%) in Cohort B had greater %desmoplasia in metastatic sites compared to primary sites. In addition, desmoplasia in primary and metastatic sites moderately correlate to overall survival when both cohorts were analysed together. There was a slight increased correlation of % desmoplasia in primary sites to OS (Spearman's rho -0.55, P value 0.0070) compared to metastatic sites (Spearman's rho -0.44, P value 0.0353) (Figure 4.10). This pilot study showed that although there were more patients with a greater proportion of desmoplasia in metastatic than primary sites, the presence of desmoplasia in both sites had potential impact on survival. It was recognised that the sample size in the pilot cohorts were somehow limited, therefore the prognostic significance of desmoplasia in both primary and metastatic sites should be further investigated in the larger validation cohort (C).

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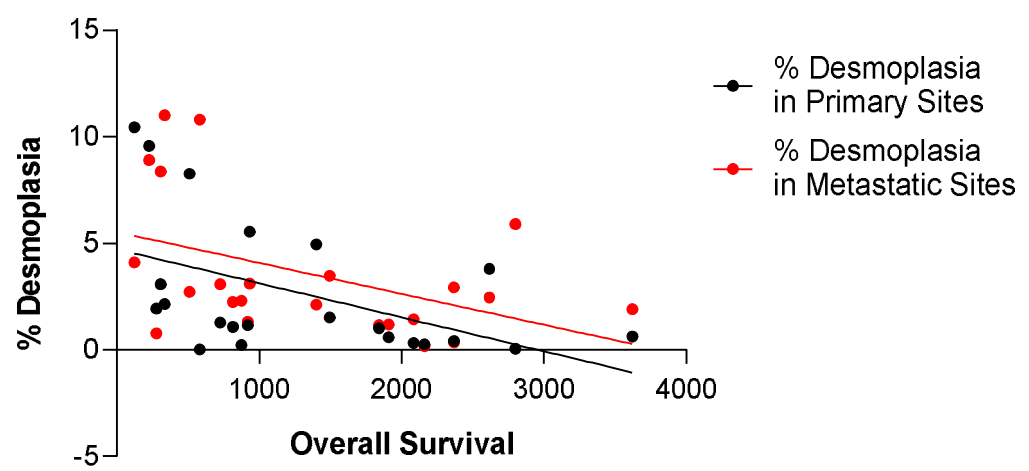


Figure 4.10 Correlation of %Desmoplasia and OS by Sites for Cohorts A and B.

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4.4.2 Cohort C results (Large dataset of 290 patients)

4.4.2.1 Patient characteristics

Cohort C was comprised of a total of 290 patients where TSP was evaluable in 287 patients. The histopathological and molecular landscape is depicted in Figure 4.11. The age range of this cohort was between 33-86 years old with a median age of 62. The maximum follow-up period for this cohort was 8301 days (22.7 years) with a median follow up of 912.5 days (2.5 years).

Representative images of H&E stain for histopathological characterisation are shown in Figure 4.12.

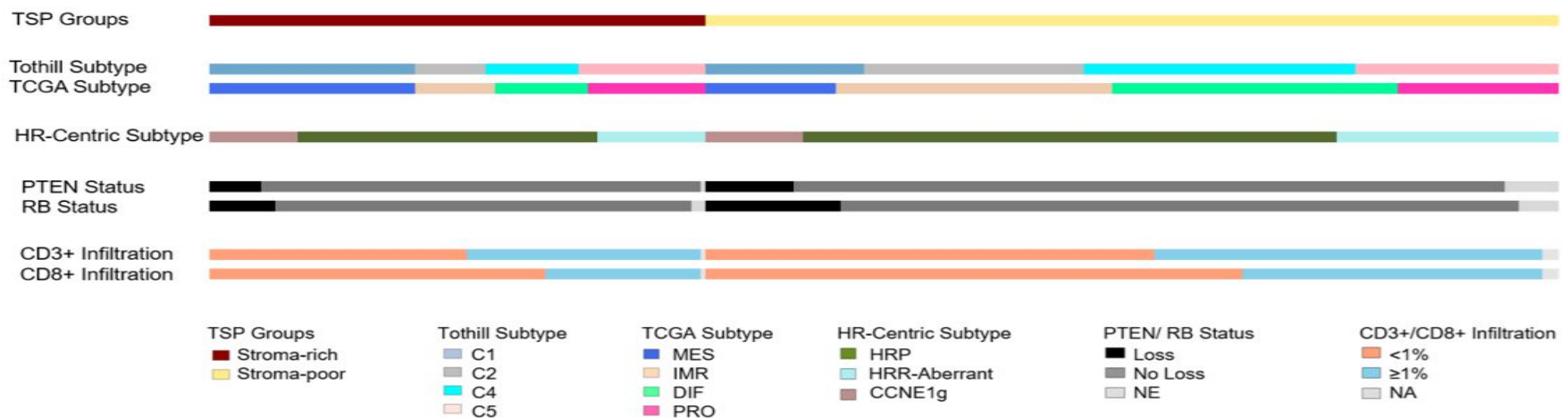


Figure 4.11 Histopathological and Molecular Landscape of Cohort C. The distribution of 287 patients by histopathological classifications (TSP groups and immune cells infiltration) and by molecular classifications (Tothill/ TCGA subtypes, HR-centric subtype, PTEN / RB status). HR-centric subtype were classified as homologous recombinant proficient (HRP), HRR-aberrant (*BRCA* mutant, *EMSY*-overexpression, non-*BRCA* HRR gene mutant) and gain of *CCNE1*. Within the stroma-rich group, the proportion of C1/ MES by Tothill/ TCGA subtypes were greater compared to the stroma-poor group.

4.4.2.2 Determination of TSP cut-off to categorise patients into stroma-poor and stroma-rich groups

With regards to ovarian cancer, the TSP threshold had not been optimised. Therefore, an exploration of a cut-off value was warranted. In this study, to work out an optimal TSP threshold to categorise the patients into stroma-poor and stroma-rich groups, comparisons of the overall survival using different TSP values were performed. TSP of 0.5 (50% stromal content) gave rise to the most statistically significant different overall survival when the patients were divided into stroma-poor < 0.5 and stroma-rich ≥ 0.5 groups (adjusted for multiple testing using Bonferroni's correction) (Table 4.6).

Table 4.6 Table Determining Cut-off for TSP (Stroma-poor and stroma-rich groups)

TSP (%stroma)	No. of patients	Median OS	χ^2	P
TSP<0.2 (<20%) TSP≥0.2 (≥20%)	49 238	868 972	0.9199	0.3375
TSP<0.3 (<30%) TSP≥0.3 (≥30%)	84 203	1109 907	1.209	0.2715
TSP<0.4 (<40%) TSP≥0.4 (≥40%)	134 153	1215 865	8.926	0.0028
TSP<0.5 (<50%) TSP≥0.5 (≥50%)	180 107	1136 733	14.22	*0.0005
TSP<0.6 (<60%) TSP≥0.6 (≥60%)	219 68	1075 720	6.239	0.0125
TSP<0.7 (<70%) TSP≥0.7 (≥70%)	256 31	1004 702	1.849	0.1739
TSP<0.8 (<80%) TSP≥0.8 (≥80%)	282 5	953 707	0.0216	0.8831
TSP<0.9 (<90%) TSP≥0.9 (≥90%)	287 0	953 NA	NA	NA
Bonferroni- corrected threshold	^a Bonferroni = ^a Family/ K = 0.05/ 16 = 0.0031 *TSP with a cut-off of 0.0005 has the lowest P value and this P value is significant as it is lower than the Bonferroni-corrected threshold.			

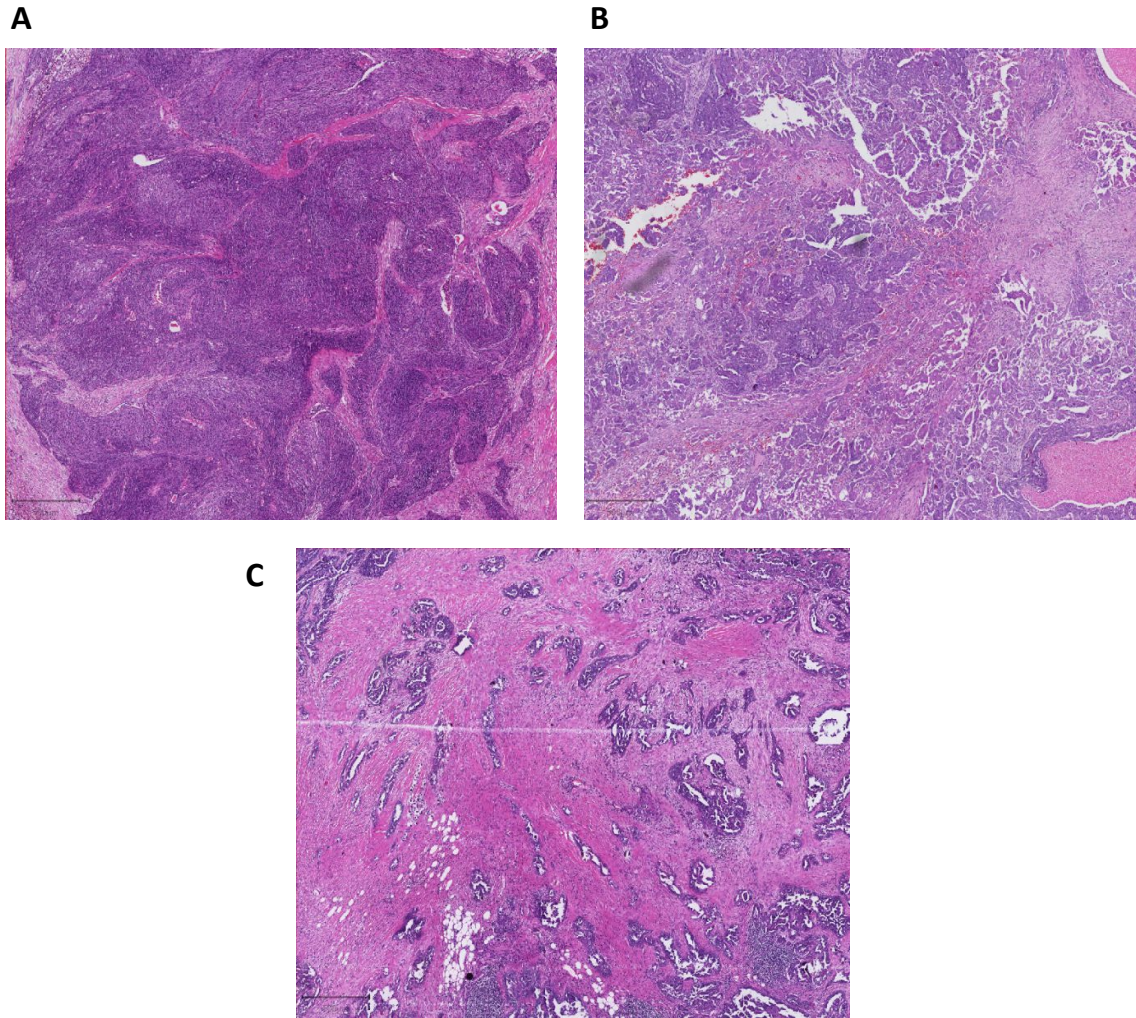


Figure 4.12 Representative images of H&E stained 5 µm sections of HGSOc patients from Cohort C (original magnification 40X). (A) HGSOc of stroma-poor type (TSP 0.11). (B) HGSOc of stroma-rich type (TSP 0.51). (C) HGSOc of stroma-rich type (TSP 0.70).

4.4.2.3 Evaluation of the TSP by sites

The TSP values of Cohort C patients were examined from a series of histopathological sections of both primary (77.7%, n=223) and metastatic sites (22.3%, n=64) with one site available for each patient. When the TSP values were categorised by sites, the median TSP was higher for metastatic sites (0.59) compared to primary sites (0.39), Mann-Whitney test P value <0.0001 (Figure 4.13).

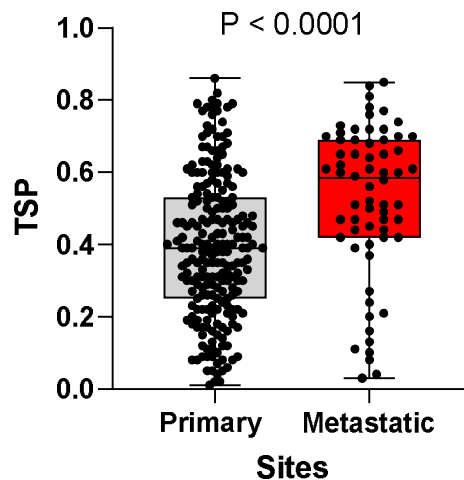


Figure 4.13 TSP by Sites. More than 2/3 of patients in Cohort C had histological sections from primary sites. The median TSP for primary sites (0.39) was lower than metastatic sites (0.59).

4.4.2.4 Relationship between TSP and clinicopathological features and molecular characteristics

Table 4.7 illustrates the clinicopathological features and molecular characteristics of the patients in Cohort C (a retrospective cohort). In this cohort (n = 287), 182 (63.4%) patients were categorised into the stroma-poor group and 105 (36.6%) patients into the stroma-rich group. Clinicopathological and molecular characteristics that were significantly different between stromal categories included residual disease at primary debulking surgery (PDS), presence/ absence of desmoplasia and molecular subtyping of HGSOc by Tothill and TCGA classification (Figure 4.14). In comparison to stroma-poor group, the stroma-rich group had greater proportions of patients with macroscopic residual disease at PDS (88.7% vs 78.3%, P value 0.0346), desmoplastic stroma (86% vs 25.6%, P value < 0.0001) and C1 (Mesenchymal) subtype (by Tothill classification 42.1%/by TCGA classification 42.6%, P value < 0.0001 vs by Tothill classification 18.3%/by TCGA classification 15.2%, P value < 0.0001).

The clinical responses detected radiologically and by CA-125 levels after platinum-based chemotherapies were reported according to WHO/ RECIST and GCIG guidelines (Rustin et al., 2011) respectively where patients with complete or partial response (CR/PR) were regarded as responders, meanwhile progressive disease or no change (stable disease) detected were defined as non-responders. In terms of radiological response after 1st line treatment, the stroma-rich group in comparison to stroma-poor group had greater non-responders (32.6% vs 14.5%, P value 0.0311). In terms of CA-125 response after 2nd line treatment, the stroma-rich group when compared to stroma-poor group had greater non-responders (48.7% vs 29.2%, P value 0.0334).

Table 4.7 Table Patient Clinicopathological Features and Molecular Characteristics (Grouped by tumour stroma proportion)

Characteristics	Total N (%)	Stroma-poor (TSP<0.5) N (%)	Stroma- rich (TSP≥0.5) N (%)	Stroma-rich proportion N (%)	P	Test
Age (in years)					0.3717	Chi- square
≤55	80 (27.9)	54 (29.7)	26 (24.8)	26 (32.5)		
>55	207 (72.1)	128 (70.3)	79 (75.2)	79 (38.2)		
FIGO stage					0.0954	Chi- square
I-II	34 (12.1)	26 (14.6)	8 (7.8)	8 (23.5)		
III-IV	246 (87.9)	152 (85.4)	94 (92.2)	94 (38.2)		
Residual Disease					*0.0346	Chi- square
Zero	47 (17.9)	36 (21.7)	11 (11.6)	11 (23.4)		
Macroscopic	216 (82.1)	130 (78.3)	86 (88.7)	86 (39.8)		
Radiological Response						
After 1 st Line Treatment					*0.0311	Chi- square
CR/PR	78 (77.2)	47 (85.5)	31 (67.4)	31 (39.7)		
PD/NC	23 (22.8)	8 (14.5)	15 (32.6)	6 (65.2)		
After 2 nd Line Treatment					0.6171	Chi- square
CR/PR	37 (39.8)	25 (41.7)	12 (36.4)	12 (32.4)		
PD/NC	56 (60.2)	35 (58.3)	21 (63.6)	21 (37.5)		
CA-125 Response						
After 1 st Line Treatment					0.3597	Chi- square
CR/PR	174 (57.6)	103 (89.6)	71(93.4)	71 (40.8)		
PD/NC	17 (2.1)	12 (10.4)	5 (6.6)	5 (29.4)		
After 2 nd Line Treatment					*0.0334	Chi- square
CR/PR	83 (64.8)	63 (70.8)	20 (51.3)	20 (24.1)		
PD/NC	45 (35.2)	26 (29.2)	19 (48.7)	19 (42.2)		
Desmoplasia					*<0.0001	Chi- square
Present	138 (48.1)	46 (25.6)	92 (86.0)	92 (66.7)		
Absent	149 (51.9)	134 (74.4)	15 (14.0)	15 (10.1)		
Necrosis					0.8697	Chi- square
Present	190 (66.2)	120 (66.7)	69 (65.4)	69 (36.8)		
Absent	97 (33.8)	60 (33.3)	36 (34.6)	36 (38.1)		
Tothill Transcriptomic Subtype					*<0.0001	Chi- square
C1 (MES)	78 (27.2)	33 (18.3)	45 (42.1)	45 (57.7)		
C2 (IMR)	62 (29.7)	47 (26.1)	15 (14.0)	15 (24.2)		
C4 (DIF)	78 (53.1)	58 (32.2)	20 (18.7)	20 (25.6)		
C5 (PRO)	69 (24.0)	42 (41.6)	27 (25.2)	27 (39.1)		
TCGA Transcriptomics Subtype					*<0.0001	Chi- square
MES	72 (25.1)	28 (15.4)	44 (41.9)	44 (61.1)		

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IMR	76 (26.5)	59 (32.4)	17 (16.2)	17 (22.4)		
DIF	81 (28.2)	62 (34.1)	19 (18.1)	19 (23.5)		
PRO	58 (20.2)	33 (18.1)	25 (23.8)	25 (43.1)		
HR-Centric Subtype					0.0957	Chi-square
HRR-aberrant	69 (24.0)	46 (25.6)	23 (21.5)	23 (33.3)		
HRP	178 (62.0)	115 (63.9)	63 (58.9)	63 (35.4)		
CCNE1g	40 (14.0)	19 (10.6)	21 (19.6)	21 (52.5)		
PTEN					0.9033	Chi-square
Loss	30 (10.9)	19 (11.0)	11 (10.6)	11 (36.7)		
No Loss	246 (89.1)	153 (89.0)	93 (89.4)	93 (37.8)		
RB					0.5281	Chi-square
Loss	43 (15.5)	29 (16.6)	14 (13.7)	14 (32.6)		
No Loss	234 (84.5)	146 (83.4)	88 (86.3)	88 (37.6)		
CD3+ Infiltration					0.7491	Chi-square
CD3+<1%	151 (53.2)	97 (53.9)	54 (51.9)	54 (35.8)		
CD3+≥1%	133 (46.8)	83 (46.1)	50 (48.1)	50 (37.6)		
CD8+ Infiltration					0.5126	Chi-square
CD8+<1%	187 (65.9)	116 (64.4)	71 (68.3)	71 (38.0)		
CD8+≥1%	97 (34.1)	64 (35.6)	33 (31.7)	33 (34.0)		

*Significant P value

Chi-square (when number of patients ≥5) and Fisher's exact (when number of patients <5) tests were performed to compare the differences between the stromal groups for each variable.

FIGO, International Federation of Gynaecology and Obstetrics; CR, complete response; PR, partial response; PD, progressive disease; NC, no change; TCGA, the Cancer Genome Atlas; MES, Mesenchymal subtype; IMR, Immuno-reactive subtype; DIF, Differentiated subtype; PRO, Proliferative subtype; HRR-aberrant (homologous recombination repair-aberrant) group = *BRC*Am, non-*BRCA*-HRm, High-EMSY patients; HRP group, Homologous recombination repair proficient patients; CCNE1g, Cyclin E1 gain patients; PTEN, Phosphatase and tensin homolog; RB, Retinoblastoma protein.

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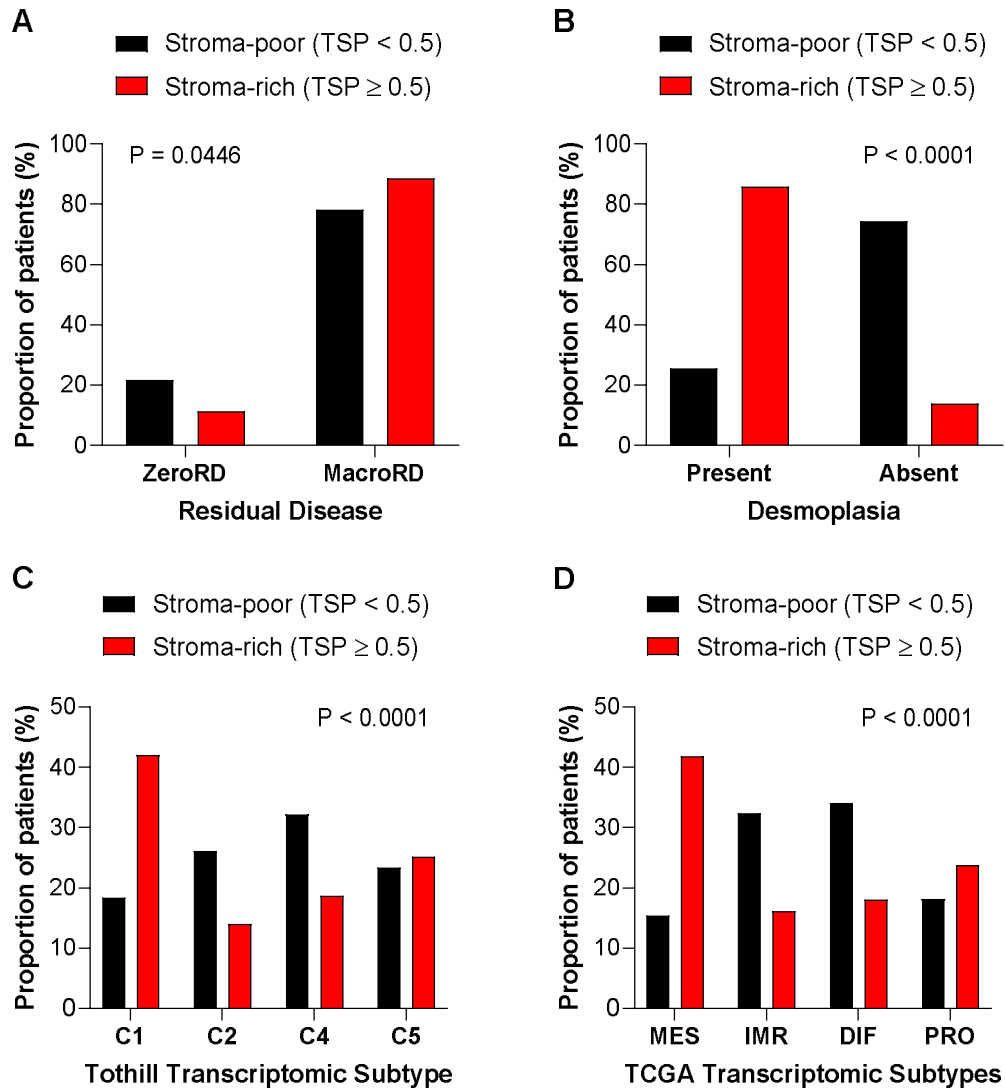


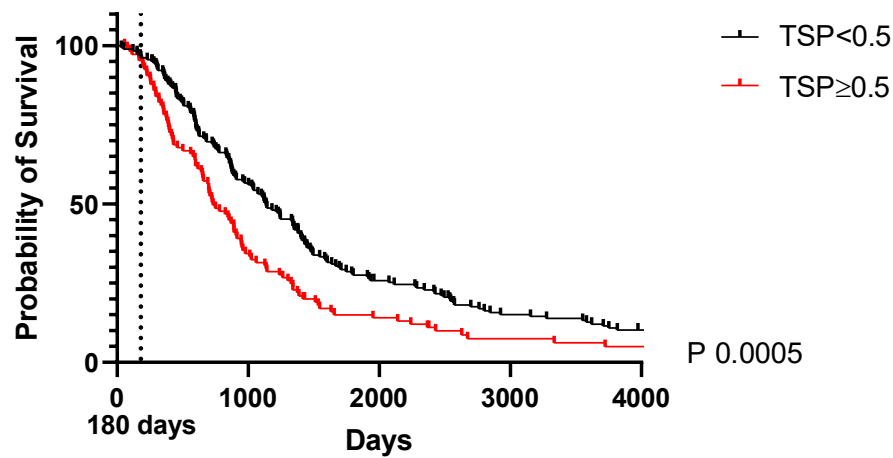
Figure 4.14 Clinicopathological and Molecular Characteristics of Stroma-poor and Stroma-rich Groups. P values for graphs (A-D) denotes that there were significant differences between the stromal groups for residual disease, extent of desmoplasia and by Tothill/TCGA transcriptomic subtypes.

4.4.2.5 The prognostic value of TSP and other clinicopathological variables

In all patients examined in Cohort C, the median OS was 953 days (2.6 years). The Kaplan-Meier analysis demonstrated statistically significant different survival outcomes between the two stromal groups in favour of stroma-poor patients (median OS 1136 days vs 733 days, log-rank P value 0.0005; median PFS 435 days vs 401 days, log-rank P value 0.0095) (Figure 4.15).

Cox univariate and multivariate models were applied to evaluate the association between clinicopathological variables and survival outcomes (Table 4.8). The results of Cox univariate analysis revealed the age, FIGO staging, residual disease status and TSP to be important factors of OS and PFS. When TSP was compared, the HR was 1.52 (95% CI 1.18-1.95, P value 0.0010) for OS and 1.40 (95% CI 1.07-1.83, P value 0.0135) for PFS. On Cox multivariate analysis, the FIGO staging, residual disease status and TSP remained vital factors for OS and PFS. The HR of the TSP on multivariate analysis was 1.30 (95% CI 1.00-1.67, P value 0.0482) and 1.22 (95% CI 0.93-1.61, P value 0.1528) for PFS. On both Cox univariate and multivariate analysis, the TSP had a significant prognostic value for OS.

OS in Stroma-rich (TSP $\geq$ 0.5) and Stroma-poor (TSP<0.5) Groups



PFS in Stroma-rich (TSP $\geq$ 0.5) and Stroma-poor (TSP<0.5) Groups

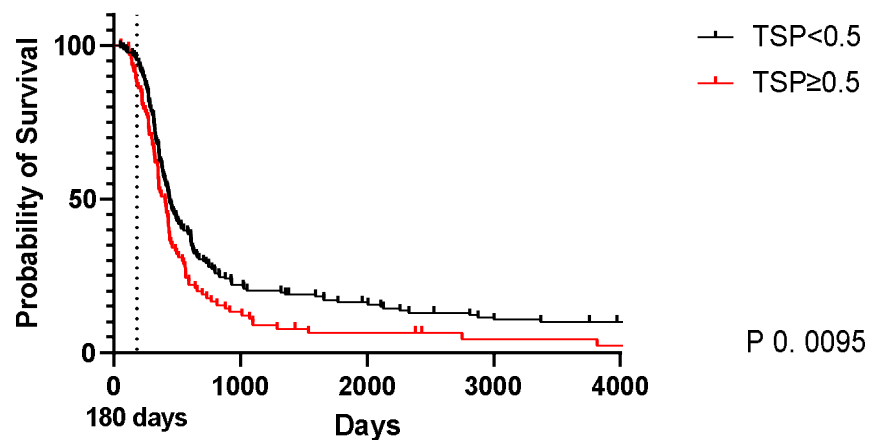


Figure 4.15 Kaplan-Meier Curves for the Survival of 287 Patients in Cohort C Stratified by TSP. Stroma-poor patients showed significantly increased OS and PFS compared to stroma-rich patients.

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Table 4.8 Cox Univariate and Multivariate Analysis for OS and PFS for Cohort C patients.

Clinicopathological Variables		OS				PFS			
		Univariate		Multivariate		Univariate		Multivariate	
	N	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Age	290	1.01 (1.00- 1.03)	0.0230	1.02 (1.00- 1.03)	0.0200	1.01 (1.00- 1.02)	0.1111	1.01 (1.00- 1.03)	0.0467
FIGO Stages									
I-II	34	Ref.	<0.0001	Ref.	0.0075	Ref.	<0.0001	Ref.	0.0186
III-IV	248	2.67 (1.78- 4.19)		2.03 (1.23- 3.46)		2.84 (1.85- 4.61)		2.07 (1.14- 3.84)	
Residual Disease									
Zero	47	Ref.	<0.0001	Ref.	0.0905	Ref.	<0.0001	Ref.	0.0788
Macroscopic	219	2.24 (1.59- 3.26)		1.47 (0.96- 2.35)		2.64 (1.83- 3.94)		1.58 (0.97- 2.70)	
TSP									
Stroma-poor	182	Ref.	0.0010	Ref.	0.0482	Ref.	0.0135	Ref.	0.1528
Stroma-rich	105	1.52 (1.18- 1.95)		1.30 (1.00- 1.67)		1.40 (1.07- 1.83)		1.22 (0.93- 1.61)	

4.4.2.6 Evaluation of the prognostic value of desmoplasia and correlation with clinicopathological features in cohort C

51.9% of cases had low levels of desmoplasia ($< 0.5\%$), 16% had $< 5\%$ desmoplasia and 32.1% had $> 5\%$ of desmoplasia (Figure 4.16). There was one histological site per patient for this cohort ($N = 223$ primary sites and $N = 64$ metastatic sites) unlike the pilot cohorts where there were a combination of primary and metastatic sites for each patient. From the pilot studies above, although there were greater desmoplasia in metastatic sites compared to primary sites, there were significant associations between the extent of desmoplasia in both primary and metastatic sites with OS (Spearman's rho correlation -0.55 and -0.44 respectively). In Cohort C, % desmoplasia was also noted to be greater in metastatic sites compared to primary sites. Kaplan-Meier analysis of the OS and PFS for all the patients in Cohort C (with a combination of 223 primary and 64 metastatic site cases) demonstrated that patients with severe desmoplasia ($\geq 5\%$) had the worst survival outcomes (median OS 505 days and median PFS 316 days) compared to moderate ($< 5\%$) (median OS 1182 and median PFS 455 days) or low desmoplasia ($< 0.5\%$) (median OS 1304 days and median PFS 491 days). Similar survival outcomes were seen when the Kaplan-Meier analyses were performed separately on primary and metastatic cases alone in favour of patients with low or moderate desmoplasia over severe desmoplasia (Figure 4.17).

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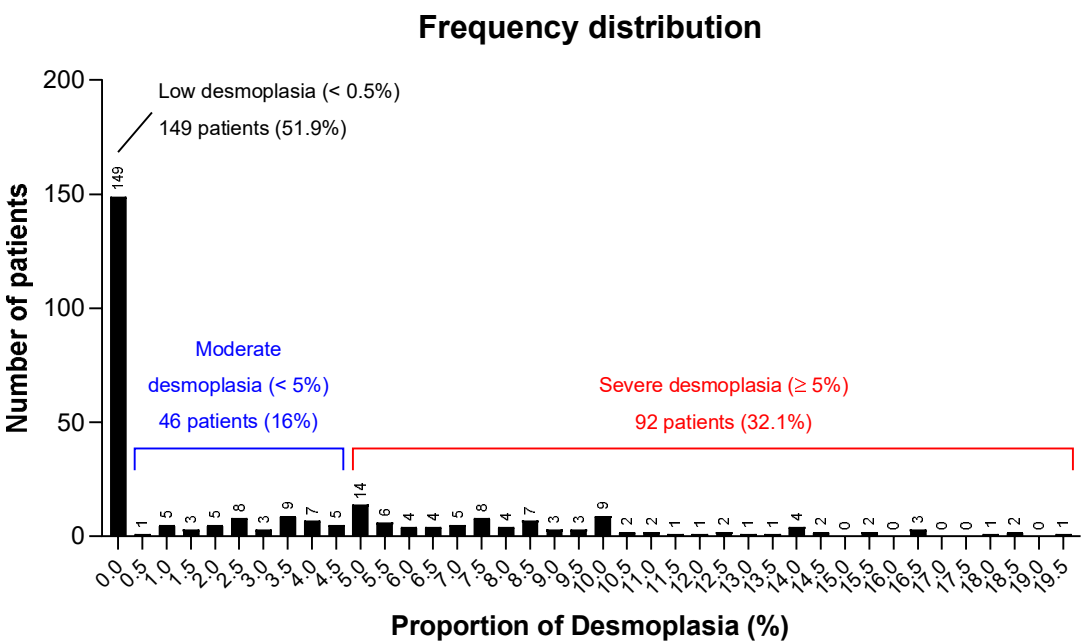


Figure 4.16 Frequency Distribution of Desmoplasia for Cohort C.

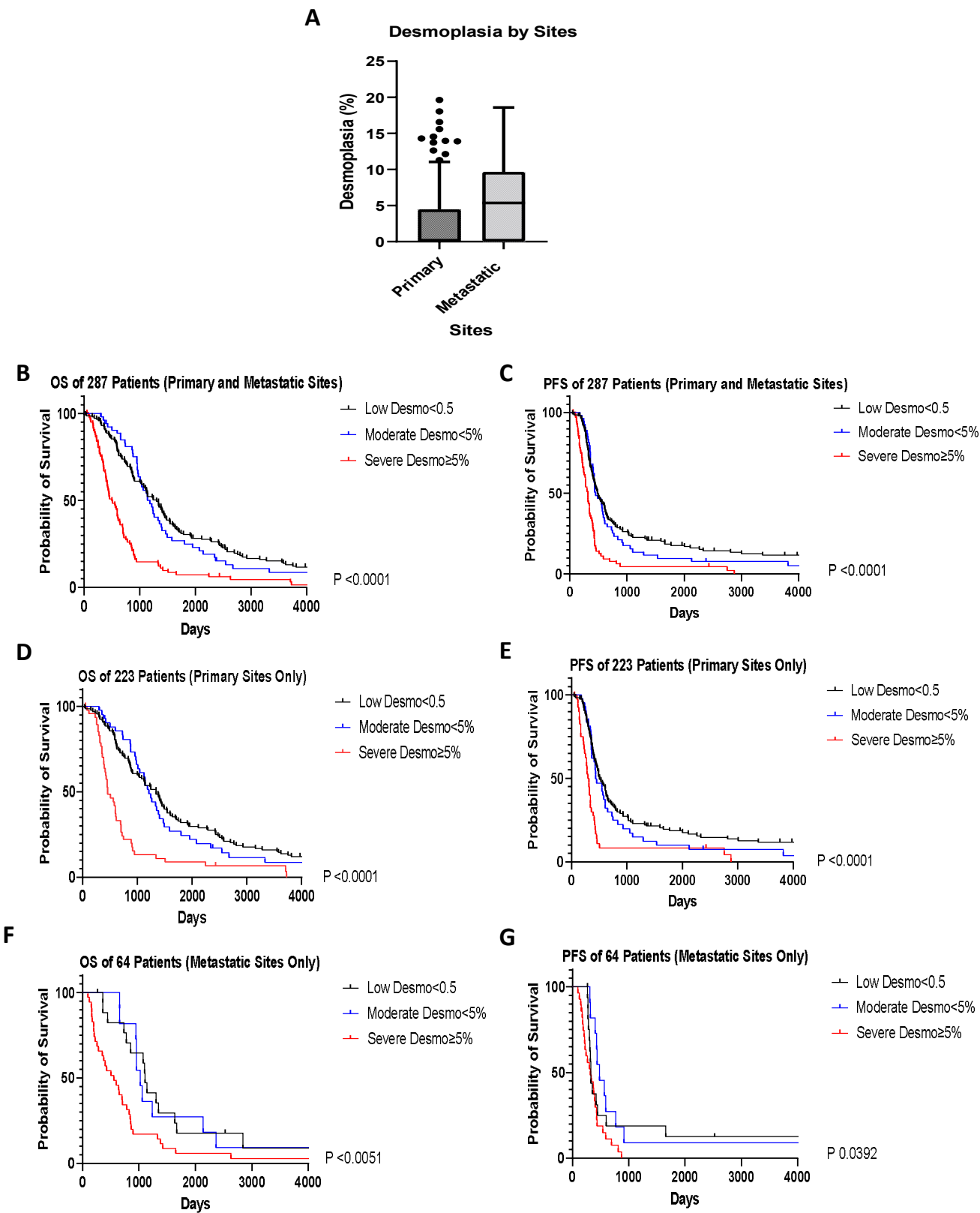


Figure 4.17 Figure Desmoplasia by Sites for Cohort C. (A) % Desmoplasia was greater in metastatic sites compared to primary sites. Kaplan-Meier OS (B) and PFS (C) curves for all 287 HGSOC patients with severe desmoplasia cases ($> 5\%$) having the worst survival outcomes compared to low ($< 0.5\%$) or moderate ($< 5\%$) desmoplasia cases. Kaplan-Meier OS (D) and PFS (E) curves for 223 HGSOC patients with primary sites. Kaplan-Meier OS (F) and PFS (G) curves of 64 HGSOC patient with metastatic sites.

As the low ($< 0.5\%$) and moderate ($< 5\%$) desmoplastic groups had better survival outcomes (both superior to severe ($\geq 5\%$) desmoplastic cases), further clinicopathological and molecular correlation analyses were performed by stratifying the patients into low desmoplasia ($< 5\%$) and high desmoplasia ($\geq 5\%$). There were no significant differences between the desmoplastic groups in terms of patient age, radiological and CA-125 responses after 2nd line treatments, presence/ absence of necrosis, HR-centric subtypes, PTEN or RB status and immune cells infiltration (Table 4.9). However, the proportions of desmoplasia were significantly associated with FIGO stage, residual disease at PDS, radiological and CA-125 responses after 1st line treatments, stromal proportions and HGSOC molecular subtypes by Tothill and TCGA classifications (Table 4.9). Higher desmoplasia proportions were found in HGSOC patients with advanced stage disease (32.1% vs 5.9%), macroscopic residual disease at PDS (31.5% vs 12.8%), non-responsive to 1st line treatments ($> 50\%$ vs $< 50\%$), and stroma-rich feature (62.9% vs 9.3%) and mesenchymal subtype (C1). When correlated to TSP, the extent of desmoplasia was greater in stroma-rich patients (Figures 4.18 and 4.19).

Table 4.9 Patient Clinicopathological Features and Molecular Characteristics
(Grouped by proportions of desmoplasia).

Characteristics	Total N (%)	Low Desmoplasia (5%) N (%)	High Desmoplasia (≥ 5%) N (%)	High Desmoplasia Proportion N (%)	P	Test
Age (in years)					0.1359	Chi-square
≤ 55	80 (27.9)	62 (30.4)	18 (21.7)	18 (22.5)		
> 55	207 (72.1)	142 (69.6)	65 (78.3)	65 (31.4)		
FIGO stage					*0.0009	Fisher's exact
I-II	34 (12.1)	32 (16.1)	2 (2.5)	2 (5.9)		
III-IV	246 (87.9)	167 (83.9)	79 (97.5)	79 (32.1)		
Residual Disease					*0.0097	Chi-square
Zero	47 (17.9)	41 (21.7)	6 (8.1)	6(12.8)		
Macroscopic	216 (82.1)	148 (78.3)	68 (91.9)	68(31.5)		
Radiological Response						
After 1 st Line Treatments					*<0.0001	Chi-square
CR/PR	78 (77.2)	61 (91.0)	17 (50.0)	17 (21.8)		
PD/NC	23 (22.8)	6 (0.9)	17 (50.0)	17 (73.9)		
After 2 nd Line Treatments					0.7437	Chi-square
CR/PR	37 (39.8)	28 (38.9)	9 (42.9)	9 (24.3)		
PD/NC	56 (60.2)	44 (61.1)	12 (57.1)	12 (21.4)		
CA-125 Response						
After 1 st Line Treatments					*0.0412	Chi-square
CR/PR	174 (91.1)	115 (94.3)	59 (85.5)	31 (33.9)		
PD/NC	17 (8.9)	7 (5.7)	10 (14.5)	10 (58.8)		
After 2 nd Line Treatments					0.1115	Chi-square
CR/PR	83 (64.8)	69 (68.3)	14 (51.9)	14 (16.9)		

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PD/NC	45 (35.2)	32 (31.7)	13 (48.1)	13 (28.9)		
TSP					*<0.0001	Chi-square
TSP < 0.5	182 (63.4)	165 (80.9)	17 (20.5)	12 (9.3)		
TSP ≥ 0.5	105 (36.6)	39 (19.1)	66 (79.5)	66 (62.9)		
Necrosis					0.4172	Chi-square
Present	190 (66.2)	138 (67.7)	52 (62.7)	52 (27.4)		
Absent	97 (33.8)	66 (32.4)	31 (37.3)	31 (32.0)		
Tothill Subtype					0.0549	Chi-square
C1 (MES)	78 (27.2)	48 (23.5)	30 (36.1)	30 (38.5)		
C2 (IMR)	62 (29.7)	46 (22.6)	16 (19.3)	16 (25.8)		
C4 (DIF)	78 (53.1)	63 (30.9)	15 (18.1)	15 (19.2)		
C5 (PRO)	69 (24.0)	47 (23.0)	22 (26.5)	22 (31.9)		
TCGA Transcriptomics Subtype					*0.0036	Chi-square
MES	72 (25.1)	42 (20.6)	30 (36.2)	30 (41.7)		
IMR	76 (26.5)	63 (30.9)	13 (15.7)	13 (17.1)		
DIF	81 (28.2)	62 (30.4)	19 (22.9)	19 (23.5)		
PRO	58 (20.2)	37 (18.1)	21 (25.2)	21 (36.2)		
HR-Centric Subtype					0.6663	Chi-square
HRR-aberrant	69 (24.0)	52 (25.5)	17 (20.5)	17 (24.6)		
HRP	178 (62.0)	124 (60.8)	54 (65.0)	54 (30.3)		
CCNE1g	40 (14.0)	28 (13.7)	12 (14.5)	12 (30.0)		
PTEN					0.4182	Chi-square
Loss	30 (10.9)	23 (11.9)	7 (8.5)	7 (23.3)		
No Loss	246 (89.1)	171 (88.1)	75 (91.5)	75 (30.5)		
RB					0.1753	Chi-square

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Loss	43 (15.5)	34 (17.4)	9 (11.0)	9 (20.9)		
No Loss	234 (84.5)	161 (82.6)	73 (89.0)	73 (31.2)		
CD3+ Infiltration					0.6749	Chi-square
CD3+<1%	151 (53.2)	109 (54.0)	42 (51.2)	55 (27.8)		
CD3+≥1%	133 (46.8)	93 (46.0)	40 (48.8)	51 (30.1)		
CD8+ Infiltration					0.7810	Chi-square
CD8+<1%	187 (66.1)	132 (65.4)	55 (67.1)	72 (29.4)		
CD8+≥1%	97 (33.9)	70 (34.6)	27 (32.9)	32 (27.8)		

*Significant P value

Chi-square (when number of patients ≥5) and Fisher's exact (when number of patients <5) tests were performed to compare the differences between the stromal groups for each variable.

FIGO, International Federation of Gynaecology and Obstetrics; CR, complete response; PR, partial response; PD, progressive disease; NC, no change; TCGA, the Cancer Genome Atlas; MES, Mesenchymal subtype; IMR, Immuno-reactive subtype; DIF, Differentiated subtype; PRO, Proliferative subtype; HRR-aberrant (homologous recombination repair-aberrant) group = *BRC*Am, non-*BRCA*-HRm, High-EMSY patients; HRP group, Homologous recombination repair proficient patients; CCNE1g, Cyclin E1 gain patients; PTEN, Phosphatase and tensin homolog; RB, Retinoblastoma protein.

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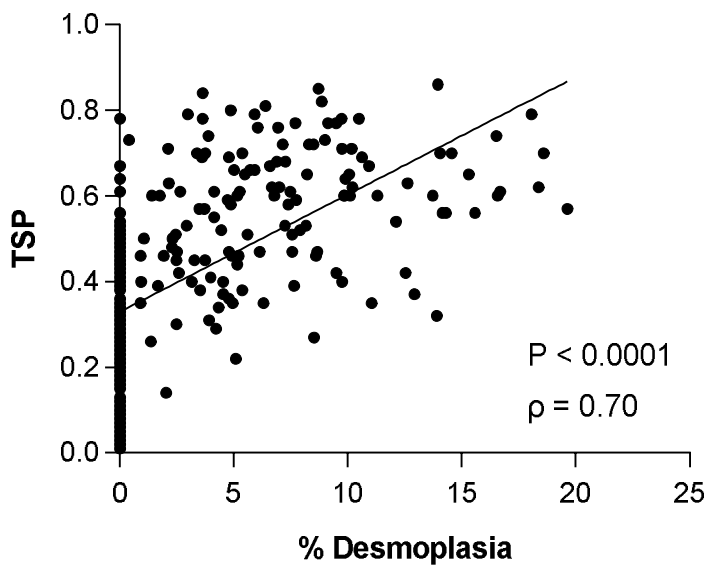


Figure 4.18 Correlation of % Desmoplasia and TSP.

% Desmoplasia vs Stroma-poor (TSP < 0.5) and Stroma-rich (TSP ≥ 0.5) Groups

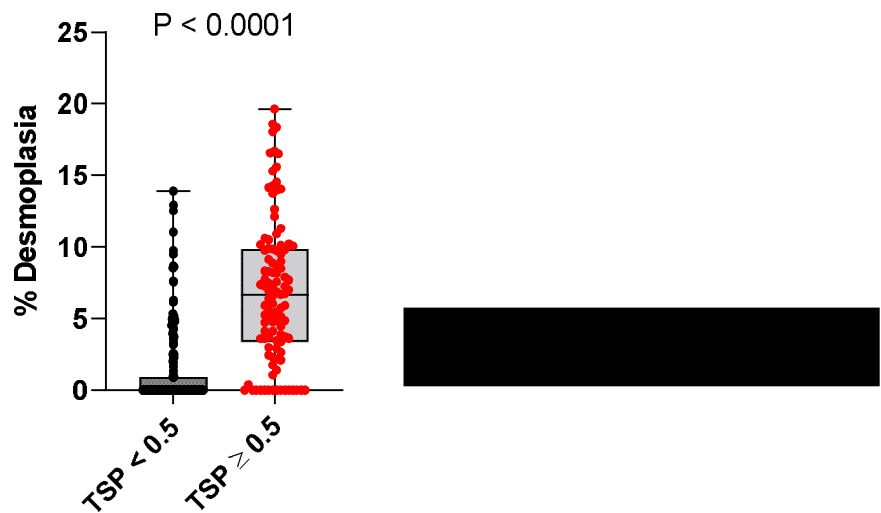


Figure 4.19 % Desmoplasia in Stroma-poor and Stroma-rich Groups. There was higher desmoplastic reaction within the stroma-rich patients compared to stroma-poor group.

4.4.2.7 Evaluation of the prognostic value of necrosis and correlation with clinicopathological features in cohort C

The distribution of % necrosis for 287 evaluable cases found that more than a third of the patients had no necrosis (35.9%) (Figure 4.20). To evaluate the prognostic value of necrosis, the patients were stratified into 2 groups according to the presence or absence of necrosis. Kaplan-Meier analysis of the OS and PFS demonstrated that patients with lack of necrosis had inferior survival outcomes (median OS 856 days and median PFS 401 days) compared to patients with necrosis present (median OS 1040 days and median PFS 434 days) (Figure 4.21).

Further correlation of necrosis with clinicopathological and molecular features were performed by stratifying the patients into necrosis and lack of necrosis groups. The group with necrosis present had lower macroscopic residual disease compared to patients with lack of necrosis (31.5% vs 68.5%, Chi-square P value 0.0097). Apart from this, there were no significant differences in other clinicopathological/molecular features between the groups (Table 4.10).

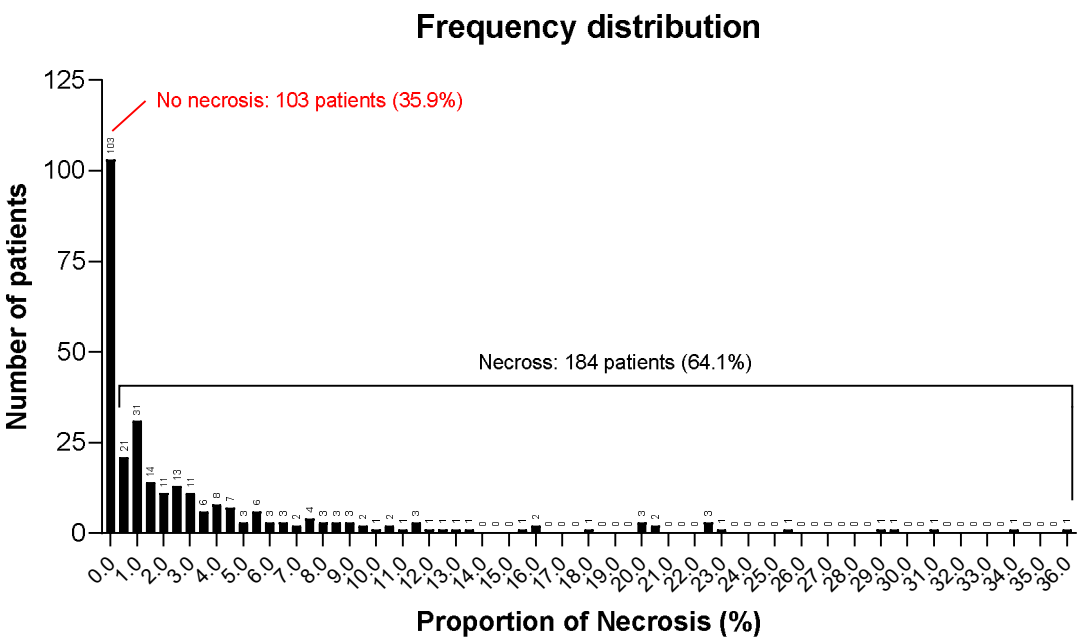
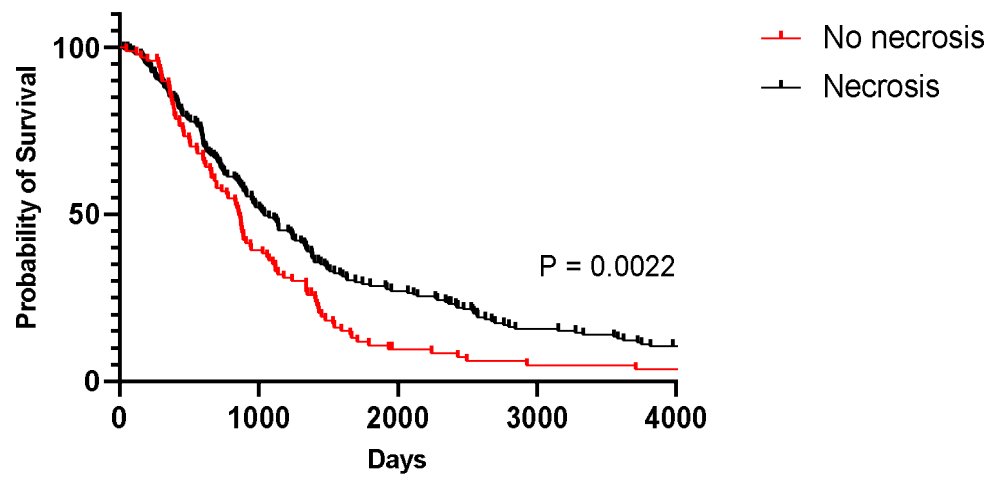


Figure 4.20 Distribution of % Necrosis in Cohort C patients.

OS in Necrosis and No Necrosis Groups (Cohort C)



PFS in Necrosis and No Necrosis Groups (Cohort C)

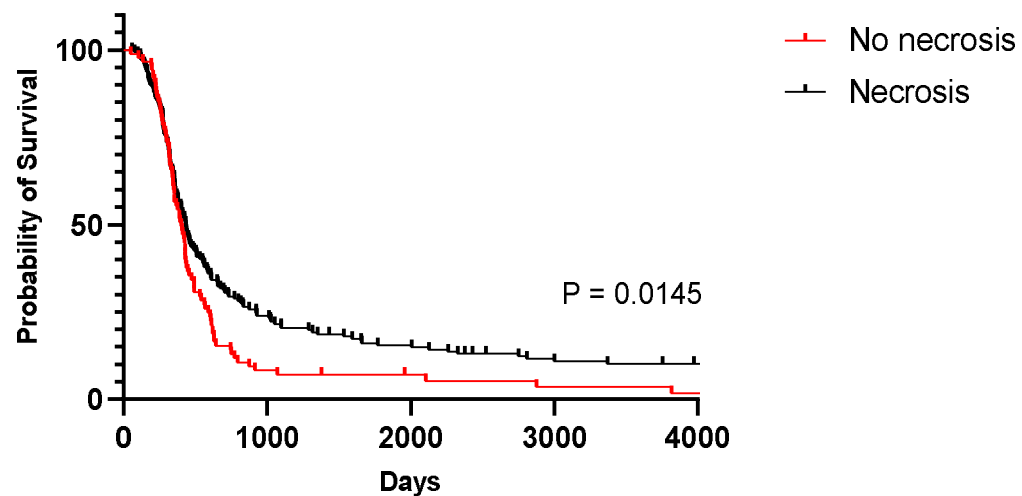


Figure 4.21 Kaplan-Meier Curves for the Survival of 287 Patients in Cohort C Stratified by Necrosis. Patients with lack of necrosis had inferior OS and PFS compared to the necrosis group.

Table 4.10 Patient Clinicopathological Features and Molecular Characteristics (Grouped by presence and absence of necrosis).

Characteristics	Total N (%)	No Necrosis N (%)	Necrosis N (%)	Necrosis proportion N (%)	P	Test
Age (in years)					0.2611	Chi-square
≤55	80 (27.9)	23 (23.7)	57 (30.0)	57 (71.3)		
>55	207 (72.1)	74 (76.3)	133 (70.0)	133 (64.3)		
FIGO stage					0.3496	Chi-square
I-II	34 (12.1)	9 (9.6)	25 (13.4)	25 (73.5)		
III-IV	246 (87.9)	85 (90.4)	161 (86.6)	161 (65.5)		
Residual Disease					0.0097	Chi-square
Zero	47 (17.9)	41 (21.7)	6 (8.1)	6(12.8)		
Macroscopic	216 (82.1)	148 (78.3)	68 (91.9)	68(31.5)		
Radiological Response						
After 1 st Line Treatments					0.2570	Chi-square
CR/PR	78 (77.2)	24 (70.6)	54 (80.6)	55 (69.2)		
PD/NC	23 (22.8)	10 (29.4)	13 (19.4)	13 (56.5)		
After 2 nd Line Treatments					0.1659	Chi-square
CR/PR	37 (39.8)	10 (30.3)	27 (45.0)	27 (73.0)		
PD/NC	56 (60.2)	23 (69.7)	33 (55.0)	33 (58.9)		
CA-125 Response						
After 1 st Line Treatments					0.6497	Chi-square
CR/PR	174 (91.1)	62 (89.9)	112 (91.8)	112 (64.4)		
PD/NC	17 (8.9)	7 (10.1)	10 (8.2)	10 (58.8)		
After 2 nd Line Treatments					0.2457	Chi-square
CR/PR	83 (21.9)	30 58.8)	53 (68.8)	53 (63.9)		
PD/NC	45 (3.1)	21 (41.2)	24 (31.2)	24 (53.3)		
TSP					0.8944	Chi-square
TSP<0.5	182 (63.4)	61 (62.9)	121 (63.7)	121 (66.5)		
TSP≥0.5	105 (36.6)	36 (37.1)	69 (36.3)	69 (65.7)		
Desmoplasia					0.4172	Chi-square
Low (<5%)	204 (71.1)	66 (68.0)	138 (72.6)	138 (67.7)		
High (≥5%)	83 (28.9)	31 (32.0)	52 (27.4)	52 (62.7)		
Tothill Subtype					0.1882	Chi-square
C1 (MES)	78 (27.2)	29 (29.9)	49 (25.8)	49 (62.8)		

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C2 (IMR)	62 (29.7)	14 (14.5)	48 (25.2)	48 (77.4)		
C4 (DIF)	78 (53.1)	27 (27.8)	51 (26.8)	51 (65.4)		
C5 (PRO)	69 (24.0)	27 (27.8)	42 (22.2)	42 (60.9)		
TCGA Transcriptomics Subtype					0.5875	Chi-square
MES	72 (25.1)	26 (26.8)	46 (24.2)	46 (63.9)		
IMR	76 (26.5)	21 (21.7)	55 (29.0)	55 (72.4)		
DIF	81 (28.2)	28 (28.9)	53 (27.9)	53 (65.4)		
PRO	58 (20.2)	22 (22.6)	36 (18.9)	36 (62.1)		
HR-Centric Subtype					0.1846	Chi-square
HRR-aberrant	69 (24.0)	25 (25.8)	44 (23.2)	44 (63.8)		
HRP	178 (62.0)	54 (55.7)	124 (65.3)	124 (69.7)		
CCNE1g	40 (14.0)	18 (18.5)	22 (11.5)	22 (55.0)		
PTEN					0.0783	Chi-square
Loss	30 (10.9)	6 (6.3)	24 (13.3)	24 (80.0)		
No Loss	246 (89.1)	89 (93.7)	157 (86.7)	157 (63.8)		
RB					0.9209	Chi-square
Loss	43 (15.4)	14 (15.1)	29 (15.5)	29 (67.4)		
No Loss	237 (84.6)	79 (84.9)	158 (84.5)	84 (66.7)		
CD3+ Infiltration					0.3542	Chi-square
CD3+<1%	152 (53.0)	54 (56.8)	98 (51.0)	98 (64.5)		
CD3+≥1%	135 (47.0)	41 (43.2)	94 (49.0)	94 (69.6)		
CD8+ Infiltration					0.6404	Chi-square
CD8+<1%	188 (65.5)	64 (67.4)	124 (64.6)	124 (66.0)		
CD8+≥1%	99 (34.5)	31 (32.6)	68 (35.4)	68 (68.7)		

*Significant P value

Chi-square (when number of patients ≥5) and Fisher's exact (when number of patients <5) tests were performed to compare the differences between the stromal groups for each variable.

FIGO, International Federation of Gynaecology and Obstetrics; CR, complete response; PR, partial response; PD, progressive disease; NC, no change; TCGA, the Cancer Genome Atlas; MES, Mesenchymal subtype; IMR, Immuno-reactive subtype; DIF, Differentiated subtype; PRO, Proliferative subtype; HRR-aberrant (homologous recombination repair-aberrant) group = *BRC*Am, non-*BRCA*-HRm, High-EMSY patients; HRP group, Homologous recombination repair proficient patients; CCNE1g, Cyclin E1 gain patients; PTEN, Phosphatase and tensin homolog; RB, Retinoblastoma protein.

4.4.3 Construction and evaluation of prediction models based on TSP

From the above studies, TSP had prognostic significance on OS outcomes. A survival prediction model was developed using Cohort C which was divided into a discovery cohort (n = 188) and a validation cohort (n = 70). On univariate and multivariate analysis of Cohort C, age, FIGO staging (I-II vs III-IV), and TSP were identified as independent predictors of OS. Therefore, prediction TSP models incorporating the above independent predictors (TSP – age – stage) and reference models (age – stage) were developed for the discovery and validation cohorts of Cohort C.

On both discovery and validation cohorts, the prediction TSP model demonstrated better discrimination performance than the reference model (see C-index values from Table 4.11). The resulting time-dependent AUC plotted over time are shown in Figure 4.22. The TSP model showed higher AUC across the time-points compared to the reference model.

Table 4.11 Table Discrimination Performance of the Reference and TSP Models in the Discovery and Validation Cohorts

	Discovery Cohort (n=188)			Validation Cohort (n=70)		
	Reference model	TSP model	P	Reference model	TSP model	P
C-index (95%CI)	0.583 (0.530-0.637)	0.607 (0.555-0.661)	0.0019	0.626 (0.543-0.709)	0.634 (0.551-0.716)	0.0011
AUC at 5 years	0.663	0.690		0.714	0.737	

C-index, Harrell's C-statistics

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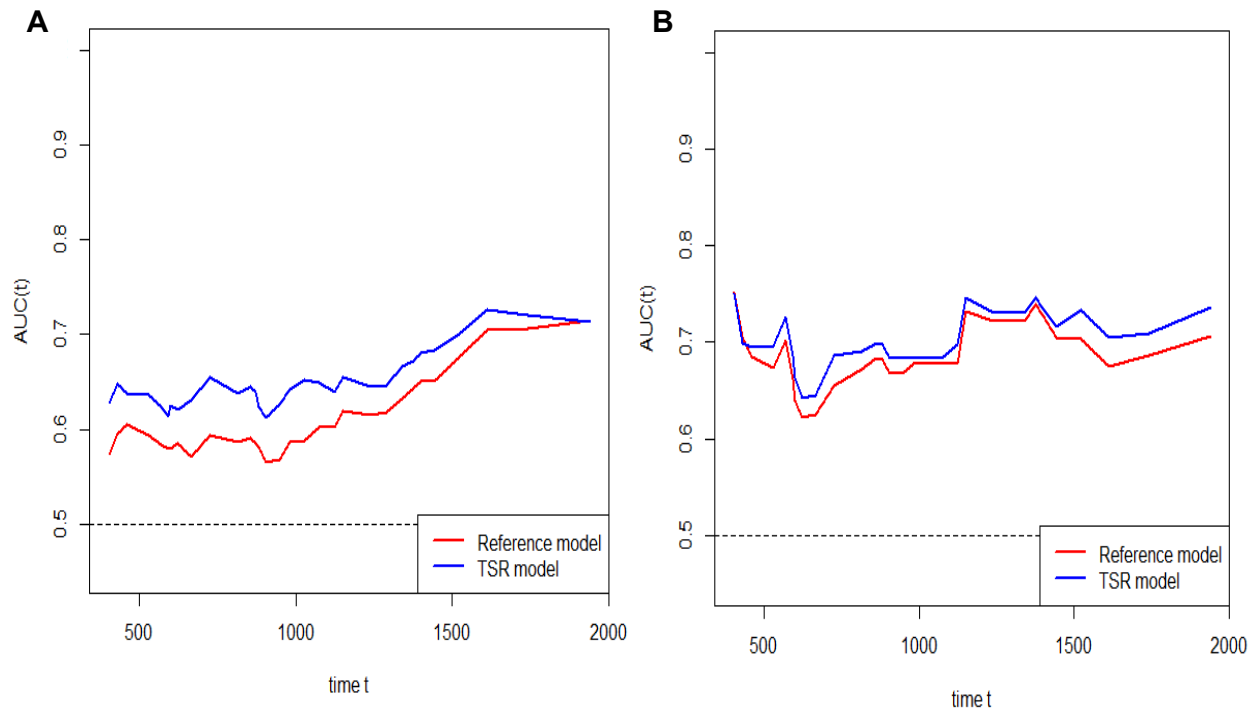


Figure 4.22 Performance of the TSP Model. Time-dependent AUC was measured for at least 5 years at daily intervals, demonstrating the prediction performance at different time-points for the reference vs TSP model. (A) Discovery cohort. (B) Validation cohort. TSP, tumour-stroma ratio; AUC, area under curve.

4.5 Discussion

The analysis of prognostic histological features in the tumour and stromal compartments has received increasing interest over the last decade. The TSP (also commonly known as TSR) is defined as the proportion of surrounding stroma relative to tumour tissue. TSP first gained traction in the field of colorectal cancer and has recently been introduced as a promising prognostic feature for many other solid tumours (Huijbers et al., 2013a; Niranjana & Sarathy, 2018; Vangangelt et al., 2020; Zong et al., 2020). For colorectal cancer (CRC) specifically, TSP may impact on improved decision-making for adjuvant treatment such as stroma-poor (low-risk of recurrence) stage III CRC patients (normally treated with adjuvant chemotherapy, ACT) can be spared from ACT, and stroma-rich (high-risk of recurrence) stage II CRC patients (not normally treated with ACT) could be considered for ACT (Argilés et al., 2020). In ovarian cancer, TSP evaluation and its prognostic implication is still not well recognised. A study of 838 EOC patients using manual scoring of TSP found that stroma-rich tumours in comparison to stroma-poor tumours were associated with more advanced stage disease, had worse survival outcomes (OS of 50 months vs 58 months), higher recurrence risk (34.3% vs 6.8%) and more lymph node metastasis (51.9% vs 27.3%) (Ying Chen et al., 2015). In this study, the prognostic value of TSP was examined by evaluating its impact on survival outcomes, correlation with clinicopathological and molecular features.

The pilot cohorts served as a proof of principle in the evaluation of the clinical and prognostic significance of TSP, necrosis and desmoplasia in HGSOc through quantitative histopathological analysis which set the grounds for further validation using the larger dataset, Cohort C. From the preliminary analysis of the pilot study, TSP was significantly associated with survival, despite the small numbers of cases

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within these cohorts ($n = 23$). There were positive correlation between TSP and desmoplasia (where stroma-rich patients had higher desmoplasia). As this study specifically focused on the stroma surrounding the tumour (whereby sections without tumours were excluded from analysis), the demonstration of desmoplasia occurring within stroma-rich tumours through histological examination further supports that the stroma surrounding tumour is normally activated and contributes to tumourigenesis.

This study had explored different cut-offs for TSP in ovarian cancer where the 0.5 threshold was demonstrated to be optimal in categorising stroma-rich vs stroma-poor patients. The 0.5 threshold had the highest predictive value in this study as well as other cancers particularly for digestive system malignancies evident from a meta-analysis of 8 studies (1959 patients) which demonstrated a greater stromal content was associated with worse clinical outcomes (Gao, Shen, Deng, & Mei, 2021). Similar cut-off level had been reported for hepatocellular carcinoma (Lv et al., 2015), oesophageal squamous cell carcinoma (Wang et al., 2012), breast carcinoma (de Kruijf et al., 2011) and lung cancer (Xi et al., 2017) with prognostic significance for survival.

In the larger dataset Cohort C study, the Kaplan-Meier survival curves showed that the survival in the first 6 months (180 days) between the stroma-rich and stroma-poor patient groups was almost equal. From this observation, factors other than tumour progression could be contributing towards event occurrence such as attenuating co-morbidities, post-operative or post-treatment complications. However, beyond the first 6 months, survival outcomes differ significantly between the TSP groups favouring stroma-poor patients. This study had evaluated the prognostic value of TSP in independent cohorts of patients (pilot cohorts and Cohort C). Despite the presence of differences in patient characteristics between the cohorts, particularly with regards to

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FIGO staging, the results showed that TSP achieved comparable prognostic performance in these cohorts, reflecting the independent prognostic value of TSP. This was further confirmed by multivariate analysis that TSP remained a stage-independent prognostic factor of decreased OS, consistent with studies from other tumours (Bell et al., 2011; Huijbers et al., 2013a; Park et al., 2015). Notably, this was concordant with the findings of an important landmark study in colorectal cancer (published in eBioMedicine as part of the Lancet Discovery Science Journal) where TSP was shown to be a strong stage-independent prognostic factor for OS (Zhao et al., 2020).

Taking the TSP further for individualised prognostic prediction in HGSOC, this study proposed a prediction model incorporating TSP with other risk factors found to be independently prognostic through multivariate analysis. In both the discovery and validation cohorts, the TSP model demonstrated better prognostic capability compared to the reference models (with just age or age and stage). Therefore, TSP could be considered as a potential addition to the FIGO staging system in helping to stratify patients' disease risk. This had been explored more widely and was considered to be integrated into the TNM staging system for colorectal cancer by the TNM Evaluation Committee (UICC) and the College of American Pathologists (van Pelt, Sandberg, et al., 2018).

The strength of the TSP evaluation here was that it could be performed fully automated through artificial intelligence (AI) by training the QuPath software to recognise tumour and stroma, thereby reducing the workload for pathologists. However, challenges in training AI to score TSP include cases with high amounts of tumour necrosis (these regions are commonly excluded from TSP scoring due to potential confounding effects) and especially in post-chemotherapy cases whereby there are good treatment

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response resulting in little or no viable tumour cells remaining resulting in non-evaluable TSP.

Additionally, by using a well clinically and molecularly annotated cohort of patients to validate the hypotheses set out proved to be another strength of this study. However, the limitations of this study remained that the cohorts were retrospective (where the pilot cohorts had multiple sites available for each patient, meanwhile the larger Cohort C only had one site per patient but remained a clinically and molecularly well annotated resource). Therefore, this warrants more prospective studies to include multiple sites per patient with good clinical and molecular annotations in order to further validate the automatic scoring of TSP for potential use in routine clinical practice. Nevertheless, the key finding of this study whereby greater stromal proportions was associated with poor prognosis in HGSOC is concordant with gene expression analyses by Tothill et al. and TCGA and other important studies including by Lan et al. (using automated scoring of tumour, stromal and immune cells at single-cell resolution which found higher stromal infiltration correlate with less favourable prognosis) and by Chen et al. (where stroma-rich tumours was associated with worse prognosis and higher relapse risk in OC) (Bell et al., 2011; Y. Chen et al., 2015; Lan et al., 2015b; The Cancer Genome Atlas Research, 2011; Tothill et al., 2008). It is important to note that the work here is unique from the aforementioned studies as the approach utilised here involved automated quantitative histopathological analysis with clinical and molecular correlations. In the following chapter, the bulk transcriptomic landscape of these high stromal tumours will be investigated to further understand the biological mechanisms responsible for their pro-tumourigenic effects conferring poorer survival outcomes.

As part of a more specific assessment of the stromal compartment, the tissue sections were scored for desmoplasia as a measure of stromal activation. The extent of

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desmoplasia was positively correlated with stromal proportions (as demonstrated in both pilot and validation cohorts of this study, Spearman's rho 0.57 and 0.70 respectively). This finding was consistent with gene expression studies by Tothill et al. and TCGA (Bell et al., 2011; Tothill et al., 2008) but the work here added value by using quantitative analysis of the desmoplasia extent rather than just qualitative scoring. It has also been demonstrated here that higher desmoplastic reaction were associated with patients of advanced stage HGSOc, greater residual disease at PDS, non-response to 1st line treatments and mesenchymal subtype (C1). Another concordant finding of this study with Tothill/ TCGA was that the desmoplasia proportions were greater in metastatic sites but presence of desmoplasia in both primary ovarian and metastatic sections demonstrated the significance of the prognostic value of desmoplasia rather than a sampling artefact due to inadvertent inclusion of stroma in a given sample. One of the limitations of the histopathological evaluation of desmoplasia was the presence of inter-observer variation. Future work should focus on the validation of the desmoplasia annotation of the large dataset here using specific CAF markers such as FAP, α -SMA, SNAI2 amongst others. Yang et al. demonstrated that SNAI2 was specifically involved in stromal fibroblasts activation contributing to OC progression and worse patient outcomes (Yang et al., 2017).

CAFs mediate desmoplasia by remodelling the ECM through increased expression of cytokines and growth factors responsible for collagen deposition with resultant increased tissue stiffness (Junttila & de Sauvage, 2013). In Chapter 3, it has been shown that there were distinct populations of CAFs existing within the stromal compartment of HGSOc. The CAFs subtype signatures discovered from the single cell study in Chapter 3 will be utilised along with bulk transcriptomic TCGA mesenchymal signatures to reflect the transcriptomes within the stromal-rich vs

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stromal-poor patients in Cohort C. The integration of single cell and bulk transcriptomes of key players of the stromal cells will shed further light into the biological processes contributing towards cancer progression and unravel the potential impact on clinical outcomes (Zhang et al., 2021).

The prognostic significance of tumour necrosis in OC has only been rigorously analysed in patients who had neoadjuvant chemotherapy (NACT) and interval debulking surgeries (IDS). T. Le et al. in their study of 101 retrospective patients who had NACT followed by IDS (with data on tumour necrosis evaluable in 72% of their patients) found that the lack of or minimal tumour necrosis after NACT was an independent significant risk factor for disease recurrence after adjusting for other prognostic factors including age, FIGO staging, CA-125 response and residual disease status (Le et al., 2006). The three-tiered chemotherapy response score (CRS) proposed by Bohm et al. which measured the tumour response in post-treatment tissue was another histopathological evaluation looking at the degree of viable tumour left after NACT whereby CRS 3 with minimal viable tumour denoting complete or near-complete response was associated with better OS (Böhm et al., 2015). Therefore, the analysis of necrosis at pre-treatment stage serves as a useful addition to the current available research. Interestingly, it has been demonstrated here that the presence of necrosis in treatment naïve patients (pre-chemotherapy) conferred survival advantage with improved OS and PFS in the large validation cohort C. This study together with the available limited data on tumour necrosis at pre-treatment stage suggest that tumour necrosis reflecting an increased biological marker for fast-growing ovarian cancers may be a good prognostic factor possibly attributed to improved response to cytotoxics (Green et al., 2005; Penault-Llorca et al., 2000). This is in contrast to reports of other solid tumours such as breast cancer, hepatocellular carcinoma,

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osteosarcoma, renal cancer, pancreatic cancer amongst others where tumour necrosis was found to be associated with poorer prognosis and more aggressive disease (Björnsson et al., 1993; Gilchrist et al., 1993; Karsch-Bluman et al., 2019; Ling et al., 2020). Further research evaluating the degree of necrosis should be directed at matched samples pre- and post-chemotherapy in order to fully analyse the effects of tumour necrosis in OC. This may be performed by examining the degree of necrosis from laparoscopic biopsy samples prior to NACT and matching the samples post-NACT at IDS. Alternately, radiological scans may be utilised to study tumour necrosis. For example, thallium-201 and Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) scan pre- and post-operatively had been found to be effective at correlating the degree of necrosis and chemotherapy response in sarcoma (Menendez et al., 1993; Schulte et al., 1999). When correlated to clinicopathological factors, this study found that the patient group with necrosis present had lower macroscopic disease at PDS which correlated towards positive survival outcomes. In order to further unravel the prognostic significance of tumour necrosis in OC, it is important to research further into the biological process of necroptosis, a form of programmed necrotic cell death which has both anti-tumourigenic and pro-tumourigenic roles. In OC, a pan-ALDH1A inhibitor has been detected to have synergistic effects with chemotherapy in triggering necroptosis in OC stem-like cells thereby reducing tumour growth and increasing tumour eradication (Chefetz et al., 2019). Necroptosis has been found to effect anti-tumour immunity through the activation of CD8⁺ T cells (Chefetz et al., 2019). In the next chapter of our study, the bulk transcriptomic landscape of the necrosis group which had superior survival outcomes will be compared to the lack of necrosis group in order to detect any important anti-tumourigenic biological effects of necroptosis.

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It was recognised that the pilot cohorts sample sizes were small (total N = 23). Therefore, the larger cohort C was utilised as a validation cohort to further evaluate the prognostic significance of the 3 histopathological features being examined here. One of the limitations of this study was the multiple statistical tests being performed in various sections to determine if TSP, desmoplasia extent and presence of necrosis had any clinical impact on survival outcomes. An interesting point to note where the application of Bonferroni correction when the 3 histopathological features were analysed together, the adjusted log rank P value remained statistically significant for the OS. This demonstrated that these histopathological features had important survival prognostic contributions and should be further evaluated for any significant biological processes.

In summary, this quantitative histopathological evaluation of HGSOC collectively shown that the histological features of both the malignant and non-malignant compartments of the OC contribute independently to clinical outcomes and are associated with specific molecular features. The analysis of TSP in a total of 310 patients in this study demonstrated that stroma-rich patients have inferior survival outcomes (shorter OS and PFS), higher association with poor prognostic factors including greater macroscopic disease at primary surgery and more extensive desmoplasia with mesenchymal signature. TSP could potentially serve as an adjunct biomarker to stratify patients into high risk groups which may benefit from targeted therapies. This study also presented automated histopathological analysis using deep learning as an emerging promising tool in aiding clinical prognostication and decision-making.

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CHAPTER 5: INTEGRATION OF BULK AND SINGLE CELL TRANSCRIPTOMIC DATA IN HGSOC

5 CHAPTER 5: INTEGRATION OF BULK AND SINGLE CELL TRANSCRIPTOMIC DATA IN HGSOE

5.1 Introduction

5.1.1 Tumour stroma proportion (TSP) in HGSOE

Previous chapter has demonstrated that the TSP can be utilised as an independent prognostic factor for HGSOE. The assessment for TSP can be easily performed using conventional H&E stained paraffin sections and this process can be automated to reduce inter-observer bias. Stroma-rich tumours were associated with poorer survival outcomes, greater desmoplasia, advanced stage disease and comprised of tumours with poor-prognosis molecular features such as Mesenchymal TCGA subtypes and *CCNE1*g. This is consistent with findings of major studies including the Cancer Genome Atlas which has shown that 'Mesenchymal' subtypes have increased stromal components by virtue of higher HOX genes, myofibroblast markers (FAP) and microvascular pericytes (ANGPT2, ANGPT1) along with greater activation of an EMT transcriptional programme and exhibiting poorer survival (Bell et al., 2011; Sohn et al., 2021).

5.1.2 Transcriptomic profiles of stroma-rich and stroma-poor HGSOE tumours stratified by TSP

While two major studies (the TCGA and AOCs) (Bell et al., 2011; Tothill et al., 2008) have explored the gene expression of the bulk molecular HGSOE subtypes amongst many other studies (Gourley et al., 2014a; Talhouk et al., 2020; Verhaak et al., 2013; Waldron et al., 2014), it will be interesting to uncover the transcriptomic profiles of our stroma-rich vs stroma-poor tumours stratified by TSP. This will allow for a deeper

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understanding of the molecular characteristics of the stroma-rich tumours using bulk and single cell markers which will strengthen the value for using TSP as a risk stratification tool in HGSOC. Sohn et al. identified 2 molecular subtypes of HGSOC: 1) HRR-activation subtype and 2) Mesenchymal subtype which was characterised by increased mesenchymal cell markers and transcriptional activation of EMT-transcription factors such as upregulation of β -catenin, TCF4 and ZEB1 (Sohn et al., 2021). In their study, they invented the EMT index comprised of 38 genes and demonstrated significantly worse OS in high EMT index groups, both in their samples and TCGA HGSOC data. They also suggested that high EMT index patients (with expected poorer prognosis) may benefit from additional targeted agents (e.g. bevacizumab) and dose-dense chemotherapy or extended chemotherapy cycles (Sohn et al., 2021). Directly targeting the EMT pathway, TGF- β is one of the most researched therapeutic targets in cancer. In OC specifically, studies have shown that blocking the TGF- β signalling pathway regulates EMT plasticity and reversed the process in epithelial OC ascites-derived spheroids (Rafehi et al., 2016) and enhanced platinum sensitivity in metastatic OC models (Newsted et al., 2019).

This study hypothesised that 1) TSP stratification is a useful stratification tool in HGSOC; 2) stroma-rich tumours stratified by TSP have enriched Mesenchymal subtype gene signatures and biological pathways associated with EMT, one of the cancer hallmarks involved in tumour proliferation, metastasis and chemoresistance; and 3) these molecular characteristics are likely driven by the various CAF subpopulations.

5.1.3 Transcriptomic profiles of necrotic HGSOC tumours

The histopathological analysis of a large cohort (N = 287) in Chapter 4 demonstrated that the pre-treatment HGSOC patients with necrotic tumours had better survival outcomes (greater median OS/PFS) and lower macroscopic residual disease compared to patients with non-necrotic tumours. Necroptosis, a form of programmed non-apoptotic inflammatory cell death is detected in the necrotic regions of solid tumours and has both anti- and pro-tumourigenic roles (Jiao et al., 2018). Multiple mechanisms have been involved in triggering tumour necroptosis including RIPK1-RIPK3-MLKL (a canonical death receptor-mediated pathway), TNF signalling, Toll-like receptor-3/4, glucose deprivation, amongst others (Baik et al., 2021; Pennica et al., 1984; Zhao et al., 2012). Tumour cell necroptosis has been known to induce anti-tumour immunity through the activation of CD8⁺ T cells via antigen cross-priming (Chefetz et al., 2019; Snyder et al., 2019; Yatim et al., 2015). RIPK1 expression and NF- κ B activation during necroptosis are essential for effective cross-priming and anti-tumour immunity (Yatim et al., 2015). In an experimental mouse model, vaccination with necroptotic cancer cells has been demonstrated to induce efficient anti-tumour immunity by promoting the maturation of dendritic cells, the cross-priming of CD8⁺ T cells and the production of IFN- γ (Snyder et al., 2019). In the presence of treatment, the acute and massive induction of necroptosis by chemotherapy or radiotherapy is believed to facilitate tumour antigen recognition through cross priming between antigen-presenting dendritic cells and T cells leading to activated CD8⁺ T cell infiltration which promotes the recession of tumour (Yan et al., 2022; Yatim et al., 2015).

Conversely, necroptosis can also induce the blockade of anti-tumour immunity in certain types of cancer by creating an immuno-suppressive TME. For example, in

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pancreatic ductal adenocarcinoma, activation of necroptosis triggers immunotherapeutic resistance through switching of tumour-associated macrophages towards an M2-like phenotype (Wang et al., 2018).

Tumour necrosis remains a complex process which has anti- and pro-tumourigenic functions in different types of cancers. Beyond necroptosis, other forms of cell death such as pyroptosis (secondary lytic cell death following apoptosis) and ferroptosis (iron-dependent non-apoptotic cell death) may also contribute to tumour necrosis. (Dixon et al., 2012; Rogers et al., 2017) Nevertheless, evaluation of the transcriptomic profiles of necrotic tumours in HGSOC provides a better understanding of the underlying mechanisms of necroptosis which may help to delineate the exact role of necrosis in OC.

5.2 Study aims

This study relies on gene expression data of Cohort C patients in Chapter 4 which was previously generated by the Gourley laboratory investigating the transcriptomic features of HGSOC (Gourley et al., 2014a; Hollis et al., 2019; R. L. Hollis et al., 2022), and also single cell transcriptomic data available from Chapter 3.

The following objectives will be addressed:

1. Investigate the differentially expressed gene (DEG) profiles of tumours stratified by TSP in Cohort C
2. Investigate the biological significance of DEGs in the stroma-rich group
3. Compare the gene expression of stroma-rich and stroma-poor tumours to TCGA HGSOC subtype signatures
4. Integrate the fibroblast subcluster markers (from single cell study in Chapter 3) and TCGA mesenchymal markers (from publicly available TCGA bulk transcriptomic data), and examine the level of expression of these markers within the stroma-rich vs stromal-poor groups
5. Investigate the DEG profiles of tumours stratified by presence and absence of necrosis in Cohort C
6. Identify potential biological mechanisms of necroptosis leading to improved survival outcomes in HGSOC patients detected from Chapter 4

5.3 Materials and methods

5.3.1 Patient's samples

A total of 287 HGSOC patients in Chapter 4 (cohort C) were stratified into groups based on TSP and necrosis status. Transcriptomic data was available for 279 patients out of a total of 287. Here, the transcriptomic profiles of these patients were further analysed and comparisons made between the stroma-rich vs stroma-poor and necrosis present vs absent groups. HGSOC patients from TCGA (N = 488) database were included and their transcriptomic profiles examined.

5.3.2 Data processing

This study examined the gene expression array which was available for 279 patients evaluated as part of Cohort C in Chapter 4. Samples were divided into a larger training cohort C1 (N = 213 HGSOC patients) and another smaller validation cohort C2 (N = 66 HGSOC patients). The mRNA expression array was previously processed by the Gourley laboratory through the extraction of RNA from macrodissected FFPE tumours using the Roche High Pure FFPE RNA Isolation kit. Next, cDNA amplification was performed using the NuGEN FFPE WT-Ovation FFPE System kit, then cDNA fragmented and labelled using the NuGEN Encore Biotin Module. The resultant products were hybridised to the Ovarian DSA™ cDNA microarray platform. Finally, before passing through quality control steps, the raw data were processed and normalised using the Robust Multi-Array Average (RMA) method.

The bulk transcriptome data of 488 HGSOCs were available from TCGA published database (Bell et al., 2011).

5.3.3 Differential gene expression analysis of Cohort C patients

Normalised gene expression data available for Cohort C patients were further processed using R. Genes with low expression values were filtered out using mean values where genes below the threshold of lower 10% were considered lowly expressed and removed (Draghici, 2012). SAM (Significance Analysis of Microarray) R package was used to perform the differential gene expression analysis with a false discovery rate threshold of < 0.10 (Tusher et al., 2001). The differential gene expression analysis was first performed on the training dataset (C1) and validated on cohort C2. Volcano plots were generated to illustrate the top upregulated and downregulated DEGs between the stromal-poor/-rich and necrosis/non-necrosis groups.

5.3.4 Correlation of stroma-rich gene signature to bulk RNA-seq in TCGA data

A stroma-rich gene signature was defined using the top 45 upregulated genes of stroma-rich tumours identified through differential gene expression analysis of the 2 stromal groups in cohort C1. The expression level of these genes were examined in 488 HGSOCs from TCGA bulk RNA-seq data. The expression levels of the stroma-rich gene signature were compared between the original TCGA HGSOC subtypes ('DIF', 'IMR', 'MES' and 'PRO').

5.3.5 Functional geneset enrichment analysis (GSEA)

Functional GSEA was performed to explore the potential biological significance of the DEGs detected between the stromal groups and necrosis/non-necrosis groups. The R

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package clusterProfiler (G. Yu et al., 2012) was used to perform the GSEA and mapping of the significant DEGs to MSigDB (Molecular Signature Database) curated geneset 'Hallmark of Cancer' and Gene Ontology 'Biological Processes'. (Liberzon et al., 2015; Mi et al., 2018)

5.3.6 Integration of single cell fibroblast subclusters markers and bulk TCGA 'Mesenchymal' markers

Gene markers from fibroblast subclusters identified through single cell RNA-sequencing in Chapter 3 were integrated along with bulk TCGA 'Mesenchymal' markers. The expression levels of these marker genes were then compared between the stromal-rich and stroma-poor tumours from Cohort C. Average expression of each of these marker genes for stroma-rich vs stroma-poor groups were illustrated using a scatter plot. Wilcoxon signed rank test was performed to identify if there were statistically significant differences between each of the marker gene expression levels of the 2 stromal groups.

5.3.7 Predictive prognostic novel gene signature in HGSOC

A multi-gene signature was developed by intersecting the upregulated DEGs of the stroma-rich patients in Cohort C1, bulk TCGA 'Mesenchymal' and CAFs subclusters markers. To explore the prognostic values of these genes, the levels of expression were then compared between the stroma-rich vs stroma-poor groups in Cohort C with Wilcoxon signed rank test performed to establish any statistically significant differences.

5.4 Results

5.4.1 Transcriptomic profiles of stroma-rich vs stroma-poor tumours from Cohort C

A total of 279 HGSOC patients from Cohort C (with available gene expression data) was divided into a training dataset group, C1 (N = 213) and validation dataset group, C2 (N = 66). This case assignment was based on the 2 separate batches which the tumours were processed. Within cohort C1, 133 patients had stroma-poor tumours (62%) and 80 patients had stroma-rich tumours (38%). Meanwhile, cohort C2 had 43 patients with stroma-poor tumours (65%) and 23 patients with stroma-rich tumours (35%).

Firstly, DEG analysis of cohort C1 showed that the stroma-rich tumours had distinct transcriptomic profiles from stroma-poor tumours. The top 20 upregulated (N = 429) and top 10 downregulated (N = 11) genes of the stroma-rich tumours of this cohort are listed in Tables 5.1 and 5.2. Volcano plot in Figure 5.1 illustrated the upregulated and downregulated genes in stroma-rich compared to stroma-poor tumours of cohort C1. GSEA of the upregulated genes in stroma-rich tumours of this cohort returned biological processes related to EMT and angiogenesis (using Hallmark gene sets) (see Figure 5.2). The downregulated genes of stroma-rich tumours in cohort C1 did not return significant biological processes on Hallmark gene sets (with adjusted P value threshold of 0.10).

Table 5.1 Top 20 Upregulated Genes by Stroma-rich Tumours of Cohort C1

Gene	Description	Log ₂ Fold Change	q value (%)
<i>COL10A1</i>	collagen type X alpha 1 chain	2.0212	0.0000
<i>THBS2</i>	thrombospondin 2	1.9916	0.0000
<i>VCAN</i>	versican	1.9415	0.0000
<i>MIR1245</i>	microRNA 1245	1.9148	0.0000
<i>GJB2</i>	gap junction protein beta 2	1.9010	0.0000
<i>NKD2</i>	NKD inhibitor of WNT signaling pathway 2	1.8303	0.0000
<i>FAP</i>	fibroblast activation protein alpha	1.8080	0.0000
<i>LUM</i>	lumican	1.7880	0.0000
<i>IGF2</i>	insulin like growth factor 2	1.7780	7.9168
<i>COL11A1</i>	collagen type XI alpha 1 chain	1.7091	0.0000
<i>SFRP2</i>	secreted frizzled related protein 2	1.7087	0.0000
<i>SPARC</i>	secreted protein acidic and cysteine rich	1.7033	0.0000
<i>CTSK</i>	cathepsin K	1.6891	0.0000
<i>DLX5</i>	distal-less homeobox 5	1.6842	3.9771
<i>CTGF</i>	connective tissue growth factor	1.6709	0.0000
<i>MMP13</i>	matrix metalloproteinase 13	1.6685	5.0385
<i>SRPX2</i>	sushi repeat containing protein X-linked 2	1.6516	0.0000
<i>TMEM158</i>	transmembrane protein 158	1.6430	2.8999
<i>ERRFI1</i>	ERBB receptor feedback inhibitor 1	1.6384	0.0000
<i>ISLR</i>	immunoglobulin superfamily containing leucine rich repeat	1.6366	0.0000

Table 5.2 Top 10 Downregulated Genes by Stroma-rich Tumours of Cohort C1

Gene	Description	Log ₂ Fold Change	q value (%)
<i>TMPRSS3</i>	transmembrane serine protease 3	0.6959	2.0844
<i>NDUFB5</i>	NADH:ubiquinone oxidoreductase subunit B5	0.7770	2.0844
<i>C6orf203</i>	chromosome 6 open reading frame 203	0.7846	6.0998
<i>PTGS1</i>	prostaglandin -endoperoxide synthase	0.7369	6.0998
<i>INTS6</i>	integrator complex subunit 6	0.8044	6.0998
<i>L3MBTL4</i>	L3MBTL histone methyl-lysine binding protein 4	0.7723	7.9168
<i>GDPD2</i>	glycerophosphodiester phosphodiesterase domain containing 2	0.6018	9.6256
<i>C7orf47</i>	chromosome 7 open reading frame 47	0.8223	9.6256
<i>MTM1</i>	myotubularin 1	0.8055	9.6256
<i>AFAP1L1</i>	actin filament associated protein 1 like 1	0.7890	9.6256

Volcano Plot of Gene Expression

This volcano plot displays the differential expression of genes. The x-axis represents the log fold change (logFC), and the y-axis represents the negative log base 10 of the p-value (-log10(pvalue)).

Differential Expression Status

- Normally expressed (black dots)
- Significant, downregulated (blue dots)
- Significant, upregulated (red dots)

Key genes identified in the plot include:

- Significant, downregulated (blue dots):** OR2L2, GDPD2, TMPRSS3, OVGP1, NXNL2, CYP4Z1, SCGB1D1, SCX17, CKMT1B, SUSD3.
- Significant, upregulated (red dots):** CMTM3, COL10A1, PTRF, CDH11, BGN, PLS3, ERRF1, ISLR, NKD2, VCAN, THBS2, MIR1245, LUM, CTGF, TAGLN, TMEM200A, FBLN2, COL1A2, ITGA5, CREB3L1, MMP2, NUA1, SRPX, SRP2, INHBA, SRP2, SPARC, GJB2, FAP, SFRP2, TMEM45A, GREM1, CILP, TMEM158, DLX5, SNORD114-1, POSTN, TNFAIP6, VGLL3, RCAN2, MIR614, ZNF469, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7, CXCR8, CXCR9, CXCR10, CXCR11, CXCR12, CXCR13, CXCR14, CXCR15, CXCR16, CXCR17, CXCR18, CXCR19, CXCR20, CXCR21, CXCR22, CXCR23, CXCR24, CXCR25, CXCR26, CXCR27, CXCR28, CXCR29, CXCR30, CXCR31, CXCR32, CXCR33, CXCR34, CXCR35, CXCR36, CXCR37, CXCR38, CXCR39, CXCR40, CXCR41, CXCR42, CXCR43, CXCR44, CXCR45, CXCR46, CXCR47, CXCR48, CXCR49, CXCR50, CXCR51, CXCR52, CXCR53, CXCR54, CXCR55, CXCR56, CXCR57, CXCR58, CXCR59, CXCR60, CXCR61, CXCR62, CXCR63, CXCR64, CXCR65, CXCR66, CXCR67, CXCR68, CXCR69, CXCR70, CXCR71, CXCR72, CXCR73, CXCR74, CXCR75, CXCR76, CXCR77, CXCR78, CXCR79, CXCR80, CXCR81, CXCR82, CXCR83, CXCR84, CXCR85, CXCR86, CXCR87, CXCR88, CXCR89, CXCR90, CXCR91, CXCR92, CXCR93, CXCR94, CXCR95, CXCR96, CXCR97, CXCR98, CXCR99, CXCR100.

299

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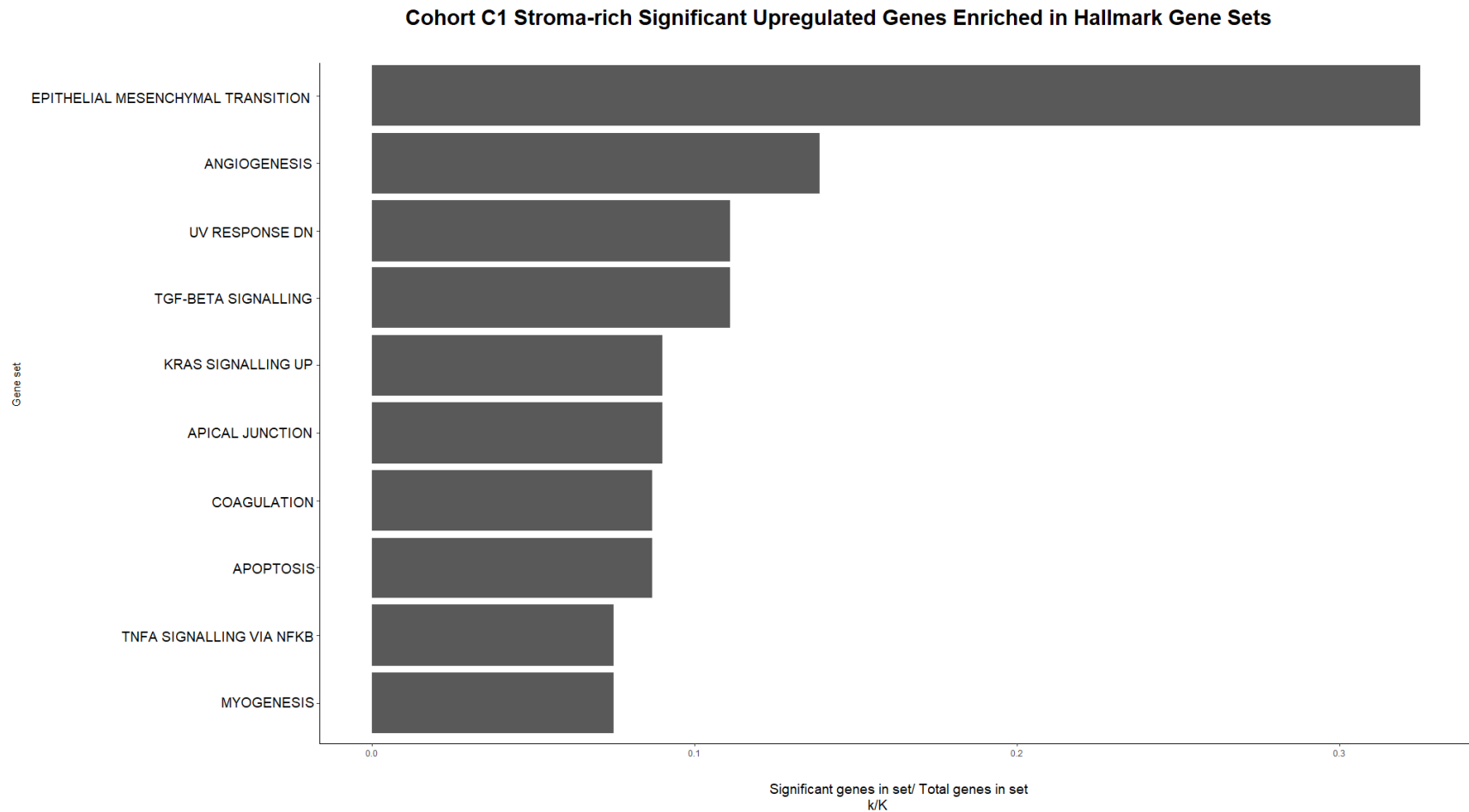


Figure 5.2 Cohort C1 Stroma-rich Significantly Upregulated Gene Expression Enrichment Analysis. Barplot showing the main enriched processes within the Hallmark Gene Sets curated by MSigDB for the stroma-rich gene signatures. The top overlapping term/ biological processes (denoted by k/K value on x-axis) returned as 'EMT' and 'Angiogenesis' for the stroma-rich tumours.

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In order to validate the DEGs detected in cohort C1 between the stromal groups, the top upregulated (N = 20) and top downregulated (N = 10) genes of the stroma-rich tumours of cohort C1 were directly interrogated in cohort C2 tumours. The expression levels of these gene were examined in cohort C2 and comparison made with cohort C1. In both the training (C1) and validation (C2) cohorts, the levels of the genes expression were not statistically different (see Figures 5.3 and 5.4). For the upregulated DEGs, Mann-Whitney U test P values between the cohorts were 0.7381 for stroma-poor tumours and 0.9893 for stroma-rich tumours. For the downregulated DEGs, Mann-Whitney U test P values between the cohorts were 0.7394 for stroma-poor tumours and 0.8534 for stroma-rich tumours.

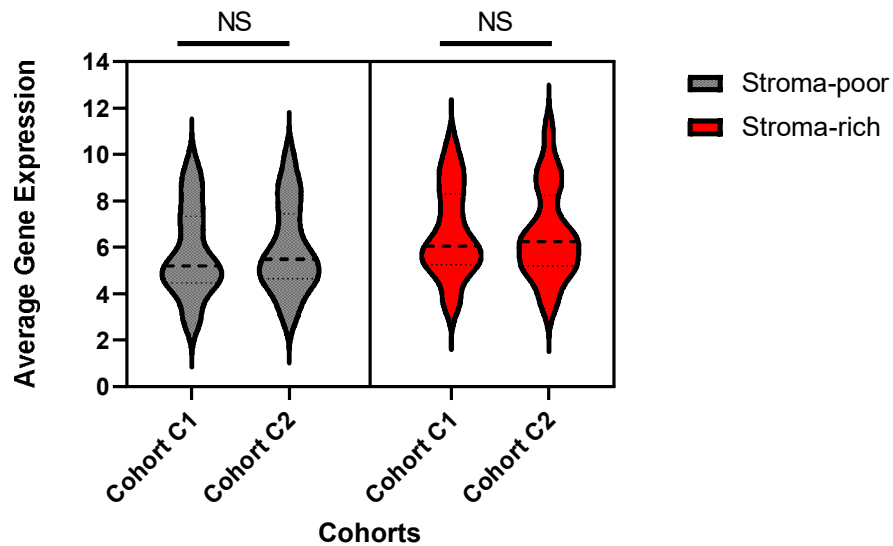


Figure 5.3 Levels of Expression of the Upregulated Genes (Top 20) of C1 Stroma-rich Tumours in both C1 and C2 Cohorts. Violin plots showing the average expression levels of the C1 stroma-rich upregulated genes are higher in the stroma-rich tumours of both cohorts compared to stroma-poor tumours (median expression 6.130 vs 5.329, Wilcoxon signed rank test P value <0.0001). Mann-Whitney U test P values non-significant (NS) when testing for difference between the cohorts C1 and C2.

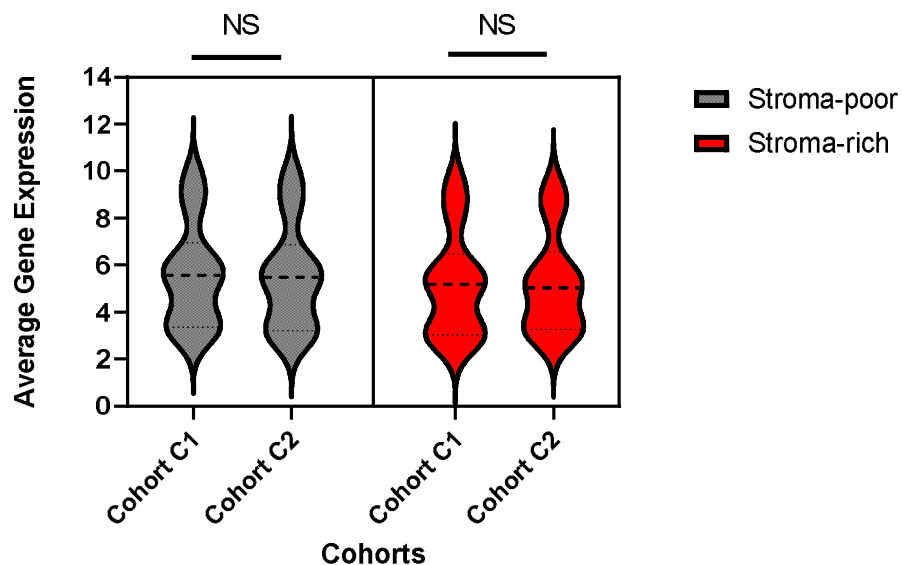


Figure 5.4 Levels of Expression of the Downregulated Genes (Top 10) of C1 Stroma-rich Tumours in both C1 and C2 Cohorts. Violin plots showing the average expression levels of the C1 stroma-rich downregulated genes are lower in the stroma-rich tumours of both cohorts compared to stroma-poor tumours (median expression 5.103 vs 5.529, Wilcoxon signed rank test P value 0.0020). Mann-Whitney U test P values non-significant (NS) when testing for difference between the cohorts C1 and C2.

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5.4.2 Stroma-rich gene signature displayed by TCGA HGSOC 'Mesenchymal' subtype

The top 45 upregulated gene markers derived from the stroma-rich tumours of cohort C1 were selected and termed as stroma-rich gene signature. The expression levels of these gene markers were examined in 488 HGSOC tumours from the TCGA database. Within these TCGA HGSOC tumours, the 'Mesenchymal' subtype demonstrated the highest expression levels of the stroma-rich gene signature (see Figure 5.5).

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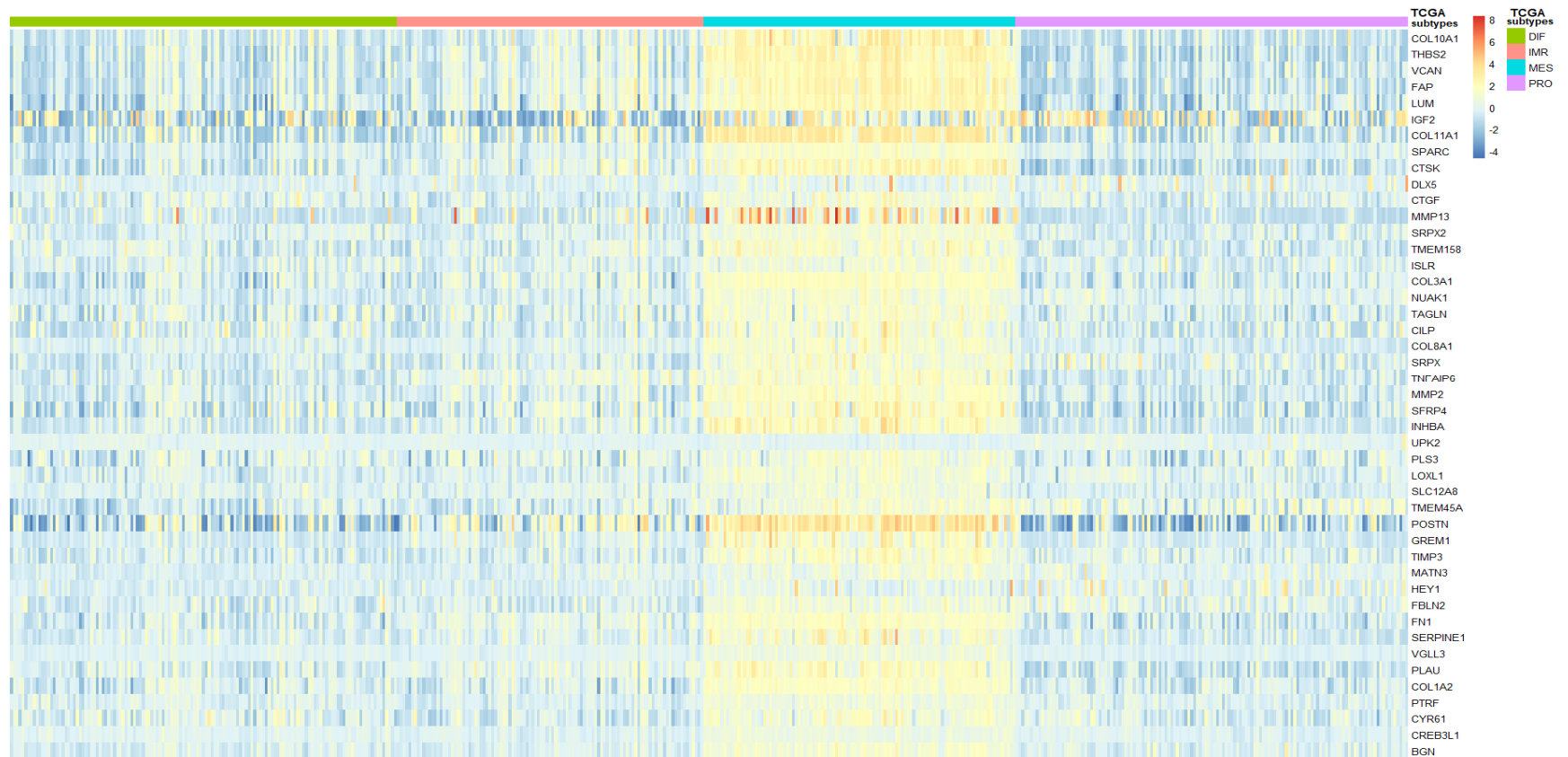


Figure 5.5 Expression of Stroma-rich Gene Signature (Top 45 Upregulated Genes) in TCGA tumours. Heatmap of the level of expression of the stroma-rich gene signature in clustered by the 4 TCGA molecular subtypes. Each column represents the tumour sample (N = 488) and each bar represents the level of gene expression with increasing levels from blue to red. The 'Mesenchymal' subtype expressed the highest levels of the stroma-rich gene signature.

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5.4.3 CAF subclusters and TCGA 'Mesenchymal' markers were associated with stroma-rich tumours

Marker genes from the single cell fibroblast subclusters (available from Chapter 3) were integrated with bulk TCGA HGSOC 'Mesenchymal' subtype genes (available from TCGA published database) (Bell et al., 2011). The marker genes for each fibroblast subcluster and TCGA HSGOC 'Mesenchymal' subtype are listed in Table 5.4.

Average expression levels of each marker gene were examined and comparison made between the stroma-rich vs stroma-poor tumours in Cohort C (Figures 5.6 and 5.7). All of the CAF subclusters and TCGA 'Mesenchymal' markers showed statistically significant higher expression in stroma-rich tumours compared to stroma-poor tumours in both cohorts C1 and C2 (P values < 0.0001 on Wilcoxon signed rank test) (Table 5.3). Only normal fibroblasts markers did not show statistically significant difference between the stromal groups (P value > 0.05 on Wilcoxon signed rank test) (Table 5.3).

Table 5.3 Statistical Comparison of the Expression Levels of Marker Genes for the Different CAF Subclusters and TCGA 'Mesenchymal' Subtype between Stroma-rich and Stroma-poor Tumours

P values (Wilcoxon Signed Rank Test) Subcluster/ Subtype	Cohort C1	Cohort C2
Pro-tumourigenic CAFs	<0.0001	<0.0001
Mesenchymal Stromal Stem Cells from Adipose Tissue	<0.0001	<0.0001
Immuno-regulatory CAFs	0.0073	<0.0001
TCGA Mesenchymal	<0.0001	<0.0001
Pro-tumourigenic CAFs & TCGA Mesenchymal	<0.0001	<0.0001
Normal Fibroblasts	0.0781	0.2969

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Table 5.4 List of Marker Genes for the Fibroblast Subclusters and TCGA HGSOC ‘Mesenchymal’ Subtype

Marker Genes							
Pro-tumourigenic CAFs	Mesenchymal Stromal Stem Cells from Adipose Tissue	Immuno-regulatory CAFs				Normal Fibroblasts	TCGA HGSOC ‘Mesenchymal’ Subtype
<i>ACTA2</i>	<i>ADAMTS1</i>	<i>ACTR2</i>	<i>COTL1</i>	<i>HMOX1</i>	<i>RBM47</i>	<i>TFPI2</i>	<i>AEBP1</i>
<i>AEBP1</i>	<i>ADH1B</i>	<i>AIF1</i>	<i>CREM</i>	<i>ID2</i>	<i>REL</i>	<i>KRT18</i>	<i>ANTXR1</i>
<i>ANTXR1</i>	<i>AKAP12</i>	<i>ALOX5AP</i>	<i>CSTB</i>	<i>IL1RN</i>	<i>RGS1</i>	<i>CLDN1</i>	<i>BGN</i>
<i>BGN</i>	<i>ANK2</i>	<i>AP1S2</i>	<i>CTSB</i>	<i>IL32</i>	<i>RGS2</i>	<i>VIM</i>	<i>CTGF</i>
<i>CDH11</i>	<i>APOD</i>	<i>APOC1</i>	<i>CTSD</i>	<i>IL7R</i>	<i>RNASE1</i>	<i>PDGFRA</i>	<i>CACNA1C</i>
<i>COL10A1</i>	<i>C7</i>	<i>APOE</i>	<i>CTSS</i>	<i>INSIG1</i>	<i>RNF19A</i>	<i>DLK1</i>	<i>CALD1</i>
<i>COL11A1</i>	<i>CD248</i>	<i>AREG</i>	<i>CTSZ</i>	<i>ITGAX</i>	<i>RUNX3</i>	<i>S100A4</i>	<i>CDH11</i>
<i>COL12A1</i>	<i>CD34</i>	<i>ARHGDIB</i>	<i>CXCL16</i>	<i>ITGB2</i>	<i>SAMSN1</i>		<i>COL5A1</i>
<i>COL5A1</i>	<i>CEBPD</i>	<i>ARL4C</i>	<i>CXCR4</i>	<i>LAPTM5</i>	<i>SDCBP</i>		<i>COL5A2</i>
<i>COL5A2</i>	<i>CFD</i>	<i>ASAH1</i>	<i>CYBB</i>	<i>LCP1</i>	<i>SDS</i>		<i>COL6A3</i>
<i>COL6A3</i>	<i>COL14A1</i>	<i>ATP13A3</i>	<i>DSE</i>	<i>LGMN</i>	<i>SERPINA1</i>		<i>COL8A1</i>
<i>COL8A1</i>	<i>CXCL14</i>	<i>ATP1B3</i>	<i>DUSP4</i>	<i>LIMS1</i>	<i>SERPINB9</i>		<i>COL11A1</i>
<i>CTGF</i>	<i>CYGB</i>	<i>BIRC3</i>	<i>EREG</i>	<i>LITAF</i>	<i>SGK1</i>		<i>CTGF</i>
<i>CTHRC1</i>	<i>DPT</i>	<i>BTG1</i>	<i>ETS1</i>	<i>LRRFIP1</i>	<i>SH3BGRL3</i>		<i>CTSK</i>
<i>CTSK</i>	<i>EBF1</i>	<i>C15orf48</i>	<i>EZR</i>	<i>LST1</i>	<i>SLA</i>		<i>DDR2</i>
<i>FAP</i>	<i>FBLN2</i>	<i>C1QA</i>	<i>FCER1G</i>	<i>LYZ</i>	<i>SLC11A1</i>		<i>DLC1</i>
<i>IGFBP2</i>	<i>FBLN5</i>	<i>C1QB</i>	<i>FCGR2A</i>	<i>MS4A6A</i>	<i>SLC16A10</i>		<i>EVI2A</i>
<i>INHBA</i>	<i>FXYP1</i>	<i>C1QC</i>	<i>FCGR3A</i>	<i>MS4A7</i>	<i>SLC16A3</i>		<i>FAP</i>
<i>ISLR</i>	<i>GADD45B</i>	<i>C5AR1</i>	<i>FGL2</i>	<i>MSR1</i>	<i>SLC2A3</i>		<i>F2R</i>
<i>LUM</i>	<i>GPX3</i>	<i>CAPG</i>	<i>FPR3</i>	<i>MXD1</i>	<i>SLC8A1</i>		<i>FN1</i>
<i>MDK</i>	<i>GSN</i>	<i>CCL3</i>	<i>G0S2</i>	<i>NAMPT</i>	<i>SMCHD1</i>		<i>FSCN1</i>
<i>MMP11</i>	<i>IGFBP6</i>	<i>CCL4</i>	<i>GK</i>	<i>NFKB1</i>	<i>SOD2</i>		<i>GAS7</i>
							<i>HSPA13</i>
							<i>INHBA</i>

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MMP14	IGFBP7	CCL5	GLUL	NPC2	SPP1		ISLR
MMP2	ITIH5	CD14	GNA13	NR4A2	SRGN		KDELC1
MXRA5	LTBP4	CD2	GPR183	NR4A3	STK4		LAMB1
MYL9	MGP	CD3D	GRB2	OLR1	SYNE2		LGALS1
NBL1	NTRK2	CD3E	GRN	PCBP1	SYNGR2		LUM
PALLD	PI16	CD4	GZMA	PFKFB3	TET2		MAPRE2
PDLIM3	PLAC9	CD44	HBEGF	PFN1	TFRC		MARCKS
POSTN	PODN	CD52	HCST	PGK1	THBD		MCAM
RARRES2	SCARA5	CD53	HIF1A	PLAUR	TNFAIP3		MMP2
SFRP4	SERPINF1	CD68	HLA-	PLEK	TNFRSF1B		MMP14
TAGLN	SFRP1	CD69	DMA	PLIN2	TNFSF13B		MXRA8
THBS2	SFRP2	CD74	HLA-	PPP2R5C	TPP1		NBL1
VCAN	SOD3	CD83	DMB	PPT1	TRBC2		NNMT
	SRPX	CFLAR	HLA-	PRDM1	TSC22D3		NUAK1
	TFPI	CLEC7A	DPA1	PSAP	TYMP		PALLD
	TNXB	CMTM6	HLA-	PTPRC	TYROBP		PCOLCE
	TXNIP	CNOT6L	DPB1	RANBP2	VAMP8		PDLIM3
	WISP2		HLA-	RASGEF1B	VEGFA		PDPN
			DQA1		VEGFA		PLEKHO1
			HLA-		ZFP36L2		PMEPA1
			DQB1		ZFYVE16		POSTN
			HLA-		ZNF331		RHOBTB3
			DRA				SRPX2
			HLA-				SFRP4
			DRB1				TAGLN
			HLA-				THBS2
			DRB5				TPST1
							VCAN
							WIPF1

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Expression of Fibroblasts and TCGA Mesenchymal Markers in Stroma-Poor and Stroma-Rich Patients in Cohort C1

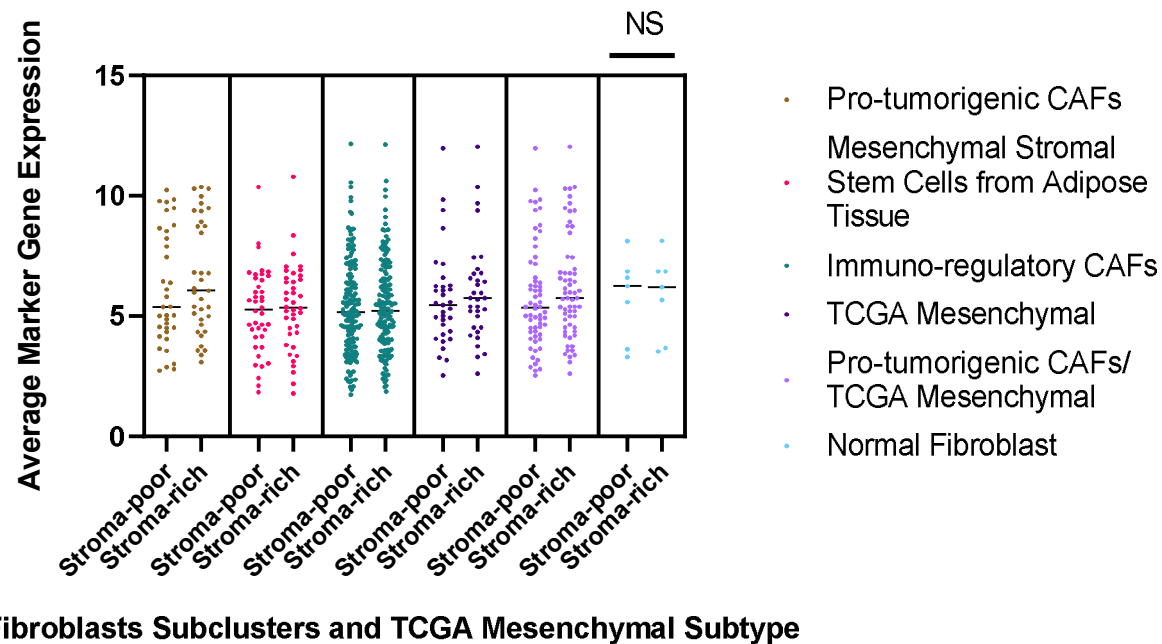


Figure 5.6 Expression of Fibroblast Subclusters and TCGA 'Mesenchymal' Gene Markers in Stroma-poor vs Stroma-rich Tumours of Cohort C1. Barplot illustrating the average gene expression level for each marker gene derived from the single cell fibroblast subclusters and TCGA 'Mesenchymal' Subtype. Stroma-rich tumours displayed higher median gene expression levels for all markers except for normal fibroblast markers. Each point represents the average expression of a marker gene within the subcluster or subtype for stroma-poor vs stroma-rich patients. Line represents median expression of all the genes within the subcluster or subtype for stroma-poor vs stroma-rich patients. NS, P value non-significant (Wilcoxon signed rank test).

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Expression of Fibroblasts and TCGA Mesenchymal Markers in Stroma-Poor and Stroma-Rich Patients in Cohort C2

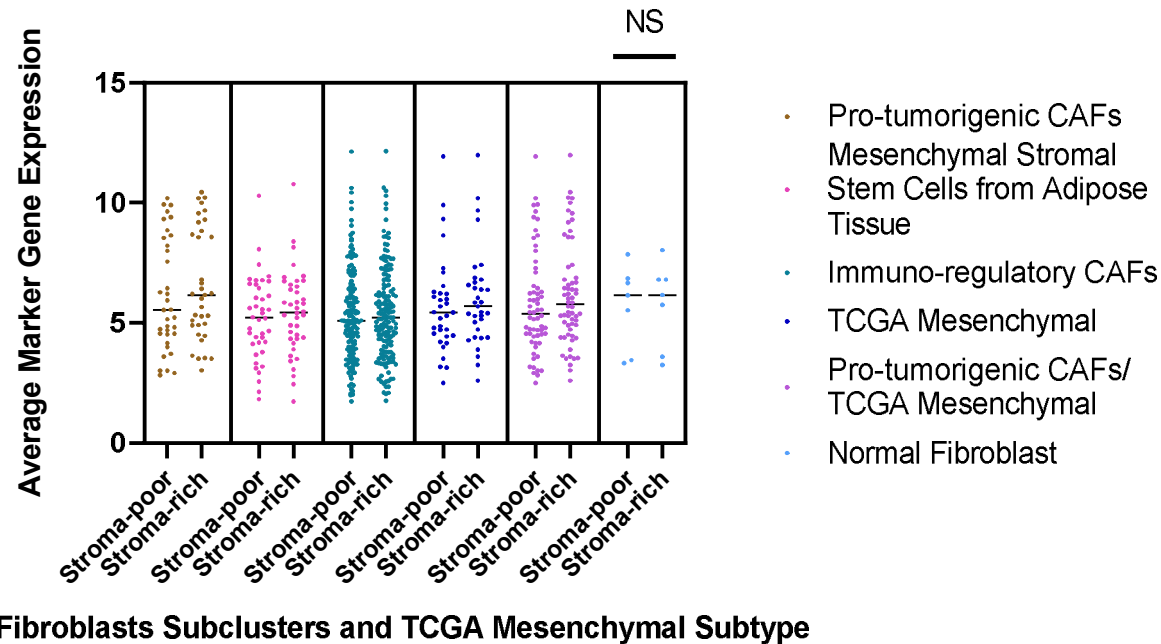


Figure 5.7 Expression of Fibroblast Subclusters and TCGA ‘Mesenchymal’ Gene Markers in Stroma-poor vs Stroma-rich Tumours of Cohort C2. Barplot illustrating the average gene expression level for each marker gene derived from the single cell fibroblast subclusters and TCGA ‘Mesenchymal’ Subtype. Stroma-rich tumours displayed higher median gene expression levels for all markers except for normal fibroblast markers. Each point represents the average expression of a marker gene within the subcluster or subtype for stroma-poor vs stroma-rich patients. Line represents median expression of all the genes within the subcluster or subtype for stroma-poor vs stroma-rich patients. NS, P value non-significant (Wilcoxon signed rank test).

5.4.4 Predictive prognostic novel gene signature in HGSOC

A novel 26-gene signature was obtained by intersecting the upregulated genes from the stroma-rich group from Cohort C1, fibroblast subclusters (pro-tumorigenic CAFs, mesenchymal stromal stem cells from adipose tissue and immuno-regulatory CAFs), and TCGA ‘Mesenchymal’ subtype marker genes. Figure 5.8 showed the heatmap of the 26-gene signature expression in Cohort C patients. In comparison to stroma-poor group, the stroma-rich patients of Cohort C had poorer survival outcomes compared and they displayed higher levels of the 26 genes (see Figure 5.9).

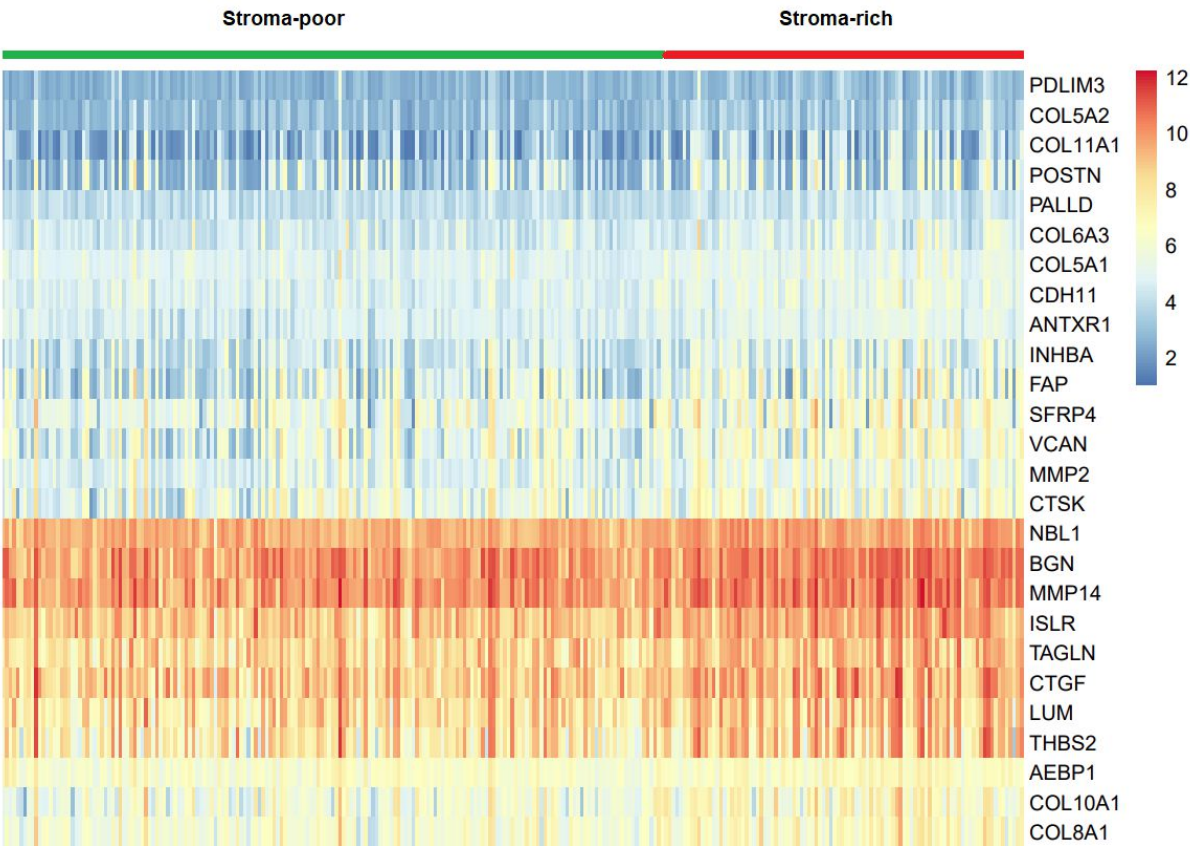


Figure 5.8 Novel Gene Signature Expression in Cohort C. Heatmap of the 26 genes expression in Cohort C with increasing levels of expression from blue to red bars.

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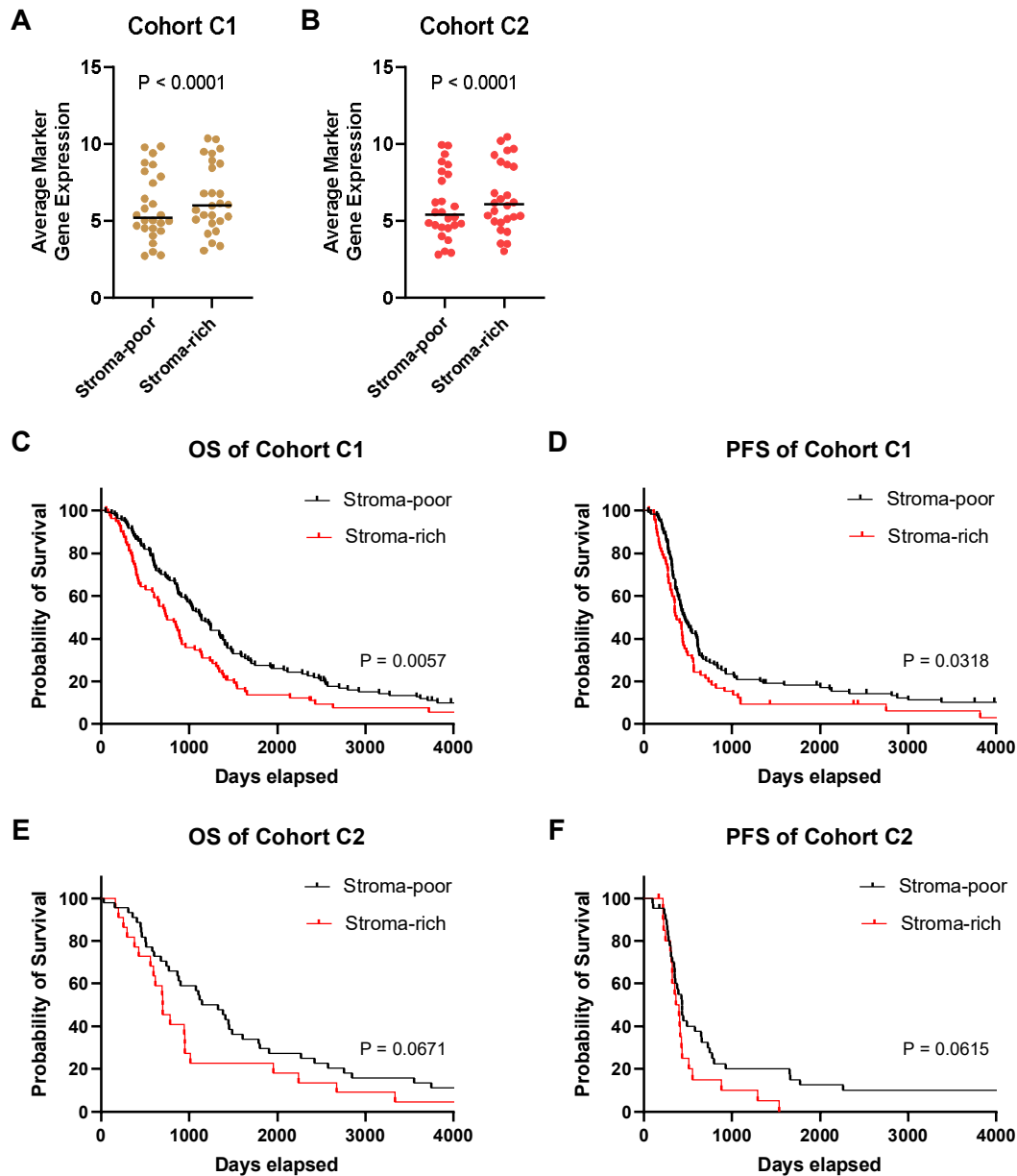


Figure 5.9 The Correlation between Signature Gene Expression and Survival Outcomes of Patients in Cohort C. (A) and (B) showed scattered plots of the average gene expression levels of the novel 26-gene signature in stroma-poor vs stroma-rich patients in both cohorts C1 and C2. The median level of the 26 genes expression for stroma-rich patients were higher in both cohorts (Wilcoxon signed rank test P values < 0.0001). (C-F) KM survival curves for OS and PFS of stroma-rich vs stroma-poor patients for both cohorts C1 and C2. Stroma-rich patients (with higher expression of the 26 genes) had poorer survival outcomes compared to stroma-poor patients. Survival outcomes of cohort C2 patients were almost approaching statistical significance. This cohort had a smaller number of patients ($N = 66$) compare to C1 ($N = 213$).

5.4.5 Transcriptomic profiles of necrotic vs non-necrotic tumours from cohort C

Within cohort C1 (N = 213), necrosis was absent in tumours of 64 patients (30%) and present in tumours of 149 patients (70%). Meanwhile, in cohort C2 (N = 66), necrosis was absent in tumours of 26 patients (39%) and present in tumours of 40 patients (61%). Differential gene expression analysis of these tumours returned a list of 91 significantly downregulated genes in tumours with necrosis in cohort C1 compared to non-necrotic tumours. The top 20 downregulated genes of the necrotic tumours of this cohort are listed in Table 5.5.

GSEA of the downregulated genes in necrosis group using Hallmark terms returned biological processes related to MYC targeting and MTORC1 signalling pathway (Figure 5.10). Using the GO terms, enriched processes were related to protein sorting and transport, nonsense-mediated RNA decay (involved in targeting abnormal mRNAs for degradation) (Maquat, 2004), and upregulation of activated T cell proliferation (Figure 5.11).

Table 5.5 Top 20 Downregulated Genes by Necrotic Tumours of Cohort C1

Gene	Description	Log ₂ Fold Change	q value (%)
<i>TRA2A</i>	transformer 2 alpha homolog	0.8888	6.7864
<i>MRP63</i>	mitochondrial ribosomal protein 63	0.8677	7.4133
<i>C16orf53</i>	chromosome 16 open reading frame 53 human recombinant	0.8627	6.7864
<i>TSFM</i>	ts translation elongation factor, mitochondrial	0.8574	6.7864
<i>N4BP2L1</i>	NEDD4 binding protein 2 like 1	0.8556	5.6973
<i>TPTE2P2</i>	TPTE2 pseudogene 2	0.8531	7.4133
<i>ADCYAP1R1</i>	ADCYAP receptor type I	0.8477	0.0000
<i>PTGES3</i>	prostaglandin E synthase 3	0.8471	6.7864
<i>WDR36</i>	WD repeat domain 36	0.8467	5.6973
<i>HNRPDL</i>	heterogeneous nuclear ribonucleoprotein D like	0.8425	6.7864
<i>FAM160A2</i>	family with sequence similarity 160 member A2	0.8414	6.7864
<i>C15orf54</i>	chromosome 15 open reading frame 54 human recombinant	0.8387	5.6973
<i>USP21</i>	ubiquitin specific peptidase 21	0.8365	6.7864
<i>MT-CYB</i>	mitochondrially encoded cytochrome b	0.8359	7.4133
<i>RPL15</i>	ribosomal protein L15	0.8354	6.7864
<i>RPL27A</i>	ribosomal protein L27A	0.8354	6.7864
<i>UBXN8</i>	UBX domain protein 8	0.8340	5.6973
<i>ADPRHL1</i>	ADP-ribosylhydrolase like 1	0.8326	4.0480
<i>DNAJC24</i>	dnaJ heat shock protein family (Hsp40) member C24	0.8296	9.2972
<i>HMGB1</i>	high mobility group box 1	0.8285	4.0480

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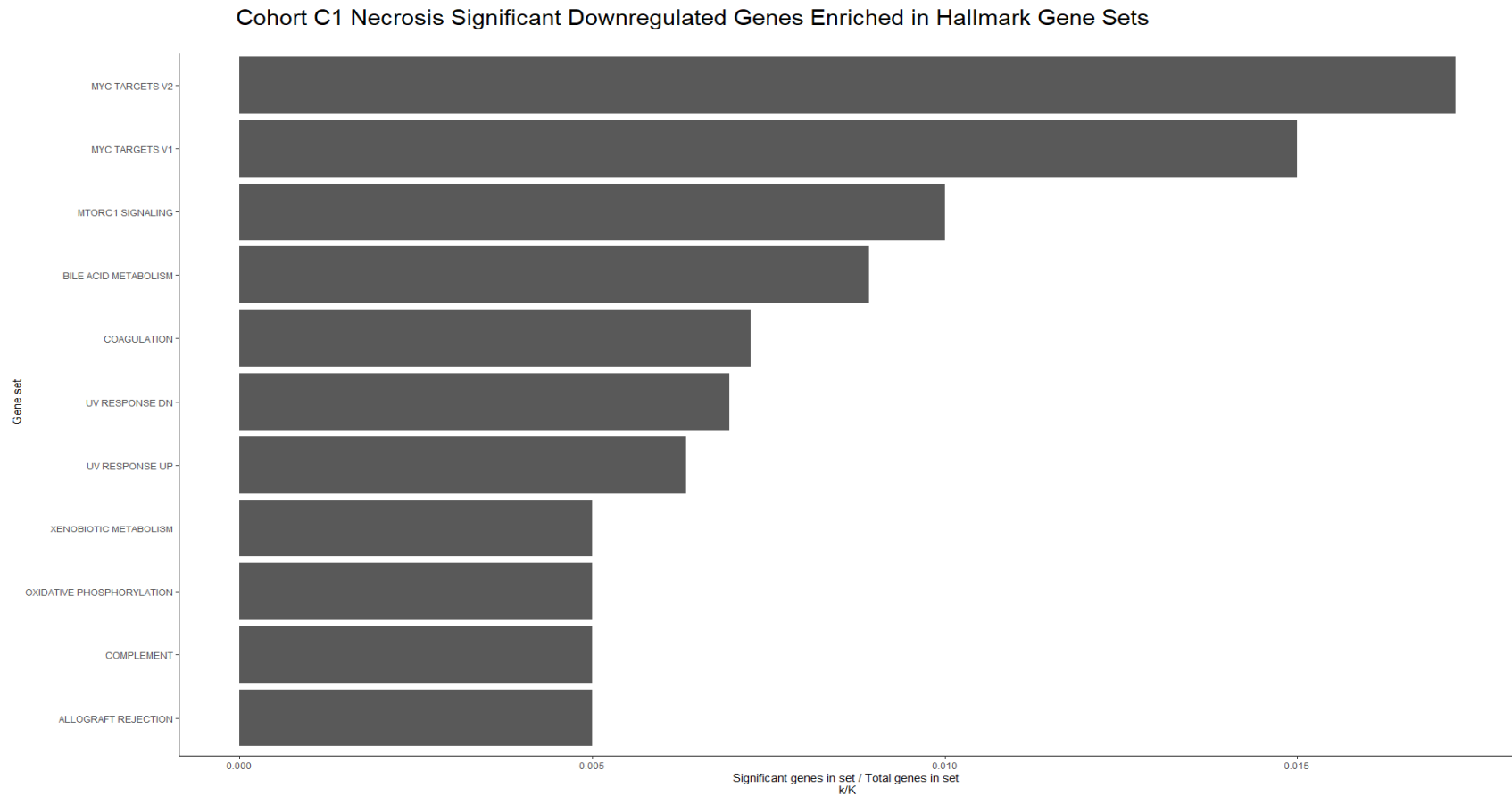


Figure 5.10 Cohort C1 Necrosis Significant Downregulated Gene Expression Enrichment Analysis. Barplot showing the main enriched processes within the Hallmark Gene Sets curated by MSigDB for the downregulated genes within the necrosis group of Cohort C1.

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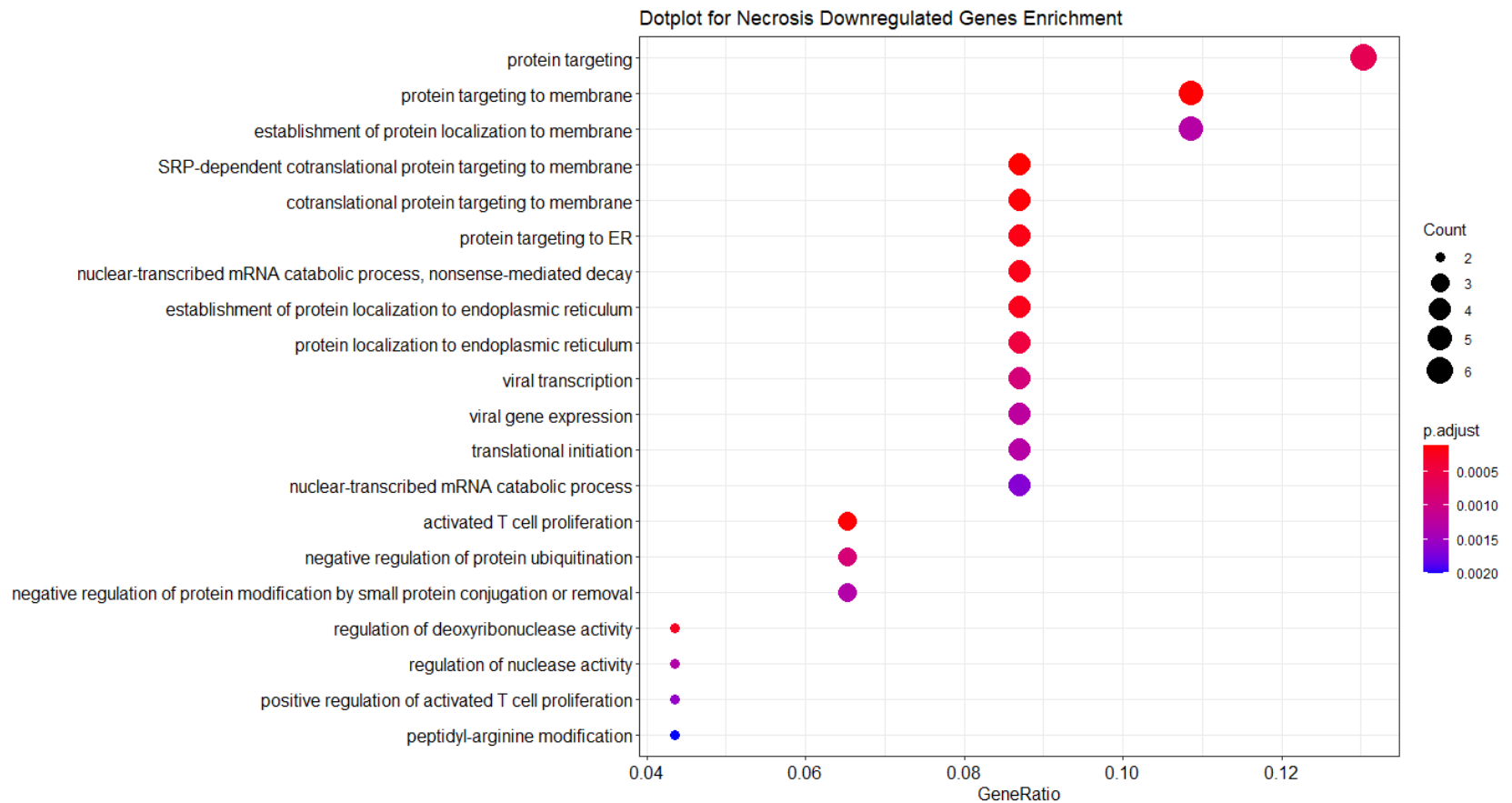


Figure 5.11 Cohort C1 Necrosis Significant Downregulated Gene Expression Enrichment Analysis. Dotplot showing significantly associated Gene Ontology 'Biological Processes' terms for the downregulated genes within the necrosis group of Cohort C1.

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In order to validate the DEGs detected in cohort C1 between the necrosis and non-necrosis groups, the top downregulated (N = 20) genes of the necrosis tumours of cohort C1 were directly interrogated in cohort C2 tumours. The expression levels of these gene were examined in cohort C2 and comparison made with cohort C1. In both the training (C1) and validation (C2) cohorts, the levels of the genes expression were not statistically different (see Figure 5.12). For the downregulated DEGs, Mann-Whitney U test P values between the cohorts were 0.7381 for lack of necrosis tumours and 0.9254 for necrotic tumours.

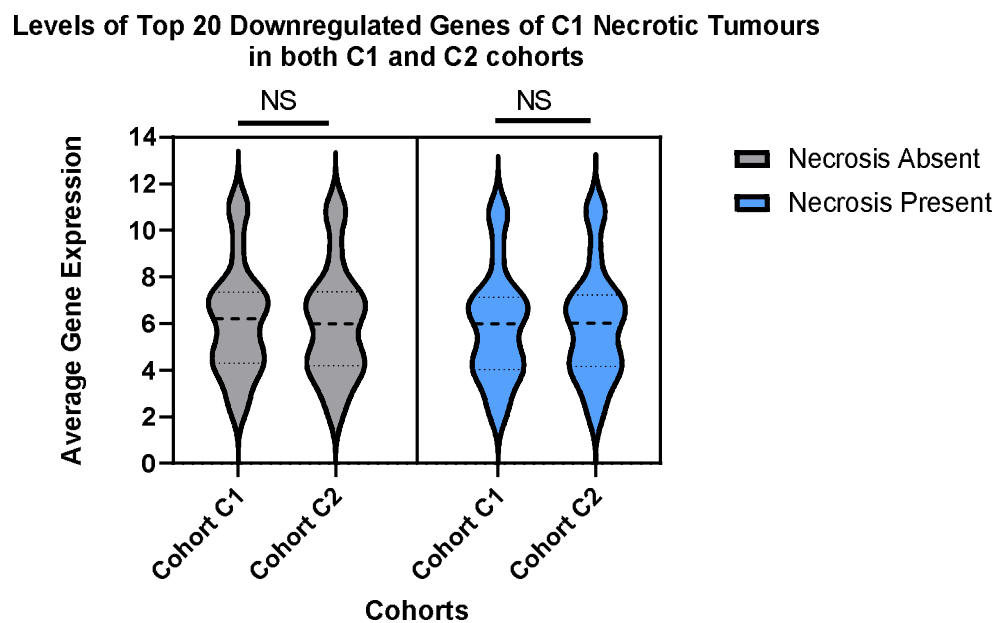


Figure 5.12 Levels of Expression of the Downregulated Genes (Top 20) of C1 Necrotic Tumours in both C1 and C2 Cohorts. Violin plots showing the average expression levels of the C1 necrosis downregulated genes are lower in the necrotic tumours of both cohorts compared to non-necrotic tumours (median expression 6.105 vs 4.018, Wilcoxon signed rank test P value < 0.0001). Mann-Whitney U test P values non-significant (NS) when testing for difference between the cohorts C1 and C2.

5.5 Discussion

The transcriptomic profiling of the TME of HGSOC tumours using bulk and single cell-RNA sequencing presents a useful method of characterising the tumour which may help to unravel the intra- and inter-tumour heterogeneity of the disease. One of the main findings of Chapter 3 is the distinct subclusters of fibroblasts differentiated by their DEGs and enriched biological processes/ pathways. Chapter 4 through histopathological analyses of a large well-annotated clinical cohort (C) has enabled stratification of HGSOC patients using TSP and necrosis. Chapter 5 explored the transcriptomic profiles of 279 patients from cohort C whereby 63% had < 50% of stroma (> 50% tumour, high tumour content), while 37% had $\geq$ 50% of stroma ($\leq$ 50% tumour, low tumour content). The gene expression profile of bulk tumour is the mean signal from a mixture of tumour, stromal and other cells which may impact on the molecular subtyping of the cancer (Wang et al., 2010). The tumour purity is highly variable within and between studies, such as in the Australian Ovarian Cancer Study (AOCS) (40% C1 subtype tumours had $\leq$ 50% tumour content), and in the TCGA study (median proportion of stromal cells was 10%) (Bell et al., 2011; Tothill et al., 2008). Nevertheless, the tumour purity in cohort C was relatively high with more than 2/3 of the samples having > 50% tumour.

When stratifying the patients by TSP, in comparison with the stroma-poor patients, the stroma-rich group has been demonstrated to have poorer prognosis with more inferior survival outcomes. The bulk transcriptomic profiling of this group of patients found a stroma-rich signature (derived from top 45 upregulated DEGs expressed by the TSP-rich tumours). This signature was enriched for EMT, angiogenesis and coagulation. This signature mainly comprised of collagen genes (*COL1A2*, *COL3A1*, *COL8A1*, *COL10A1*, *COL11A1*), extracellular matrix (*VCAN*, *LUM*), and myofibroblast markers

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(*FAP*, *POSTN*) which have been found to be highly expressed in stroma in other studies including breast cancer (Allinen et al., 2004; Ma et al., 2009). Mapping onto the bulk TCGA HGSOC data, this stroma-rich signature was expressed highly in the 'Mesenchymal' subtype which is known to have the worst prognosis compared to other subtypes. This stroma-rich signature provides a useful addition to the current knowledge which could be used for future studies as an adjunct to other clinicopathological features for tumour stratification of HGSOC.

Additionally, the stroma-rich patients in this study displayed statistically significant higher expression of the integrated CAFs subclusters and TCGA 'Mesenchymal' subtype markers. This discovery supports that fibroblasts (specifically CAFs) are likely to account for the stroma-rich signature as well as the bulk TCGA HGSOC 'Mesenchymal' subtype signature (see Chapter section 3.4.8). This finding also allowed for the exploration of the clinical roles of the different single cell CAFs subclusters in HGSOC which indicated potential pro-tumour functions of the 'Pro-tumourigenic' CAFs, 'Mesenchymal stromal stem cells' and 'Immuno-regulatory' CAFs. The main markers shared by the 'Pro-tumourigenic' CAFs, stroma-rich signature and TCGA 'Mesenchymal' subtype were collagen related genes, matrix metalloproteinase genes, TGF- β signalling genes such as *POSTN*, *VCAN*, *THBS1* (associated with tumour growth and metastasis) (Lin et al., 2012) and *BGN* (which has been shown to form complexes with TGF- β receptors to intensify the phosphorylation of Smad2/3 cultured endothelial cells) (Hara et al., 2017). TGF- β signalling is central to cancer-stromal crosstalk and drives fibroblast activation (Cheon et al., 2014).

A multi-gene signature was constructed from the intersection of the bulk (stroma-rich upregulated DEGs and TCGA 'Mesenchymal' markers) and single cell (CAFs subclusters) biomarkers. This 26-gene signatures were consistently expressed at

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higher levels in the stroma-rich patients with poorer survival outcomes compared to stroma-poor patients in both the training (C1) and validation (C2) datasets. This novel gene signature adds value for prognosis prediction in HGSOC and further work should seek to validate this multi-gene signature in internal and external datasets.

From the previous chapter, when patients were stratified by presence or absence of necrosis, the group with necrotic tumour had better survival outcomes in comparison to the group with lack of necrosis. Bulk transcriptomic profiling of these patients detected interesting downregulated DEGs related to necrosis with top 10 genes including *NUFIP1*, *MIR320D1*, *THSD1P1*, *OTUD6B*, *SPATA13*, *ADCYAP1R1*, *CCDC17*, *FANCF*, *IKBKB* and *SNORA45*. These 'necrosis signature' genes were enriched for processes related to the targeting of c-MYC, MTORC1 signalling pathway, protein sorting and transport, nonsense-mediated RNA decay and upregulation of activated T-cell proliferation. The proto-oncogene *c-myc* regulates the expression of genes involved in cellular processes, including cell replication, growth, metabolism, differentiation and apoptosis (Bui & Mendell, 2010; Eilers & Eisenman, 2008; Grandori et al., 2000). The TCGA study found that 30-60% of human ovarian tumours have amplified *c-myc* gene and OC patients with high c-MYC mRNA levels had decreased disease-free survival (DFS) and OS (Bell et al., 2011). Reyes-González et al. in their study using murine xenograft models observed that c-MYC silencing in cisplatin-resistant OC cells activates apoptosis, hence suggesting that c-MYC could be used as a potential therapeutic target for OCs expressing high levels of this oncoprotein (Reyes-González et al., 2015). Necrosis genes were detected to target c-MYC which may explain the induction and acceleration of tumour cell death, hence the better prognostic outcomes of the cohort C patients with necrotic tumours compared to non-necrotic tumours. However, it is noted that c-MYC is extremely difficult to target.

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MTORC1/2 signalling has been shown to cause carboplatin resistance in OC via selective translation of mRNAs involved in DNA damage and repair, cell cycle and anti-apoptosis (survival) pathways (David-West et al., 2018). Inhibition or targeting of MTORC1 signalling pathway may explain the anti-tumourigenic effect of necrosis in HGSOC. Tumour necrosis factor (TNF) plays a central role in immune homeostasis, inflammation and host defence via the induction of a wide range of cellular processes such as apoptosis, necrosis, angiogenesis, immune cell activation, differentiation and cell migration (Wajant, 2009). TNF is produced by activated immune cells (particularly macrophages and T cells), fibroblasts and tumour cells (Wajant et al., 2003). Necrosis can exert anti-tumourigenic effects via TNF signalling which in turn activates tumour antigen-specific T cells (Müller-Hermelink et al., 2008; Wajant et al., 2003).

In summary, the work here strengthens the argument for the use of TSP as a histological stratification tool in HGSOC. Additionally, necrosis emerges as a potential prognostic histological feature, particularly at pre-chemotherapy stage. This will be a useful addition in view of the inclusion of tumour necrosis as part of the chemotherapy response score by the International Collaboration on Cancer Reporting (ICCR) and the College of American Pathologists (CAP) guidelines for histopathologic reporting of OC (McCluggage et al., 2015), which has been proven useful in guiding clinical decision-making and identifying platinum resistant patients (Ditzel et al., 2019; Lee et al., 2017; Singh et al., 2018). Future studies should seek to examine and validate the 'stroma-rich' and 'necrosis' biomarkers/gene signatures using well-annotated clinical database (such as the TCGA and AOCS) and prospective clinical cohorts. Moving forward, patient risk stratification and treatment response may benefit from a multi-model characterisation of the disease using automated histopathological evaluation of

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the tumour (including TSP and necrosis) combined with deep molecular profiling using potentially prognostic biomarkers detected through single cell sequencing.

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CHAPTER 6: DISCUSSION AND CONCLUSION

6 CHAPTER 6: DISCUSSION AND CONCLUSION

OC is one of the leading lethal gynaecologic malignancies with HGSOC by far the most dominant subtype accounting for 70-80% of all OC deaths (Bowtell et al., 2015; Cortez et al., 2018; Kurman & Shih, 2016). This disease frequently relapses leading to therapy resistance in 80-90% of patients with widespread disease, eventual treatment failure and ultimate poor prognosis (Bowtell et al., 2015). Cellular and molecular diversity accounts for the highly heterogenous nature of the disease. Current advances in the management of HGSOC arose from extensive work in molecular stratification of the disease with one of the major successes being the use of PARP inhibitor and BRCAm characterisation of the tumour (Ledermann et al., 2012; Mirza et al., 2016).

It has been recognised that there is the need for a more refined approach to research and clinical trials in HGSOC, with multi-omic technology being adopted to unravel the molecular landscape in order to facilitate future therapeutic strategies. The complex TME plays a crucial role in the tumourigenesis of the cancer and presents a major potential target for the development of effective therapies through genome-, transcriptome- and proteome-wide studies (Yang et al., 2020). Conventional studies relied heavily on the examination of the TME as a bulk entity. However, the presence of intra- and inter-tumour as well as inter-patient heterogeneity necessitates the in depth exploration of the TME at single cell resolution. The work described here explores the complex TME of HGSOC at both single cellular and bulk transcriptomic level combined with histopathological examination using clinically well-annotated Edinburgh cohorts.

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Single cell sequencing technology has become increasingly popular providing a powerful tool in studying the heterogeneity of many diseases in particular cancer. To explore the dynamic TME of cancer, many studies have employed scRNA-seq to characterise the phenotypic cell composition and states of the cancer cells (Q. Hao et al., 2021; Hu et al., 2020; Izar et al., 2020; Kan et al., 2020; A. J. Shih et al., 2018; Winterhoff et al., 2019; Zhao et al., 2021). One of the limitations in obtaining high quality scRNA-seq data relies on the cell viability after tumour dissociation and single cell isolation. The work in Chapter 2 described the extensive optimisation experiments using human ovarian cancer tumours (mainly HGSOC and LGSOC) for dissociation and live single cell isolation. The main findings from this study provide an effective protocol for obtaining high quality single cells (with > 70% viability and low ambient mRNA contaminants) from multiple sites of OC tumours (pre-and post-chemotherapy) starting from tumour harvesting through to single cell suspensions for downstream RNA-sequencing. Various scRNA-seq studies of OC have utilised fresh and cryopreserved samples. The analyses here suggest that although a good viability (> 70%) single cell suspensions could be obtained from both fresh and cryopreserved samples, the effects of cryopreservation may impact on the final quality of the single cell libraries with the generation of less cells and lower number of genes recovered. This study also builds upon the commercialised automated dissociation method using GentleMACs – which have been widely adopted by other research for tumour dissociation and single cell isolation – identifying that the chronology of tumour dissociation, DCR and RBC lysis steps are important and should be specific to the tissue and site of origin. The work presented here also explored the importance of techniques used for tissue harvesting during surgery to minimise ischaemia and tissue handling/ selection during the dissociation step which should be tailored to the type,

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nature and consistency of the tissues (such as ovarian, omental and metastatic nodules requiring specific approaches).

Future work should investigate matched samples pre- and post-chemotherapy using fresh and cryopreserved tissues from the same patients in order to evaluate the effects of treatment on the quality of single cell suspensions other than the impact of the dissociation methodologies employed. Ideally, even samples with cell viability of < 70% should be included for scRNA-sequencing in order to detect if the treatment itself has detrimental effects on the single cells and potential true signals may be further uncovered and not filtered out by premature stringent QC checks. The optimised adapted automated dissociation and single cell isolation protocol here may also be tested in xenograft murine models or experimental models including cell lines and spheroids to test the efficacy of the single cell isolation in tumours other than live human OCs. This may prove beneficial as the study of live human tissues are often hindered by multiple factors including complex time-consuming surgeries and the lack of readily available tissues for repeated validation studies unlike experimental models. Moreover, further studies using different platforms of scRNA-seq may be employed in future on the same single cell suspensions to allow for comparison of the efficacy of the sequencing platform itself such as microfluidic-, plate-based and droplet-based technologies (e.g. Fluidigm C1, WaferGen iCell8, 10x Genomics Chromium Controller, and Illumina/BioRad ddSEQ) which have potential impact on the total cells and depth of genes recovery (Ashton et al., 2021).

Emerging interest in understanding the diverse TME of HGSOC through bulk and single cell transcriptomic profiling has revealed complex cellular and molecular network involved in OC tumourigenesis, progression and treatment resistance. The

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HGSOC TME comprises of cancer cells, immune cells and stromal cells actively interacting with each other through autocrine and paracrine signalling of secreted molecules including cytokines, chemokines, integrins, ECM proteins and others, forming an evolving microenvironment (Hanahan & Weinberg, 2011). The work in Chapter 3 here represents the bioinformatics analysis of the good quality single cell libraries of OC samples generated from Chapter 2. Specifically, Chapter 3 describes the consistent discovery of the cellular composition of HGSOC tumours from multiple sites of different patients pre- and post-chemotherapy, which was concordant with findings of other single cell OC studies (Izar et al., 2020; A. J. Shih et al., 2018; Winterhoff et al., 2017). These cells were categorised into 3 broad groups including tumour cells (cancer epithelial cells, ciliated cancer epithelial cells), stromal cells (fibroblasts, adipocytes, endothelial cells, RBCs) and immune cells (of lymphoid and myeloid lineages). This study adds further knowledge to available current scRNA-seq research on HGSOC through the identification of subpopulations of cells within the tumour, stromal and immune compartments with distinct functional enriched genes. Within the tumour compartment, the detection of 5 subclusters of cancer epithelial cells based on their DEGs - 'Null/ resident', 'Pro-tumorigenic', 'Stress-response', 'Ciliated' and 'Immuno-regulatory' – sheds light into the potential mechanisms of OC tumourigenesis primarily through apoptosis resistance, immune-mediated destruction via TNF- α signalling, EMT, perturbation of p53 pathway and immuno-suppressive functions. In the stromal compartment, 3 novel subclusters of CAFs were identified – 'Pro-tumourigenic', 'Adipose-derived stromal stem cells' and 'Immuno-regulatory' with major enriched biological processes for EMT, angiogenesis and pro-inflammatory responses. Within the immune compartment, this study described cells from 2 main immune lineage - the lymphoid (CD8⁺ T cells, CD4⁺ T cells, CD4⁺ Treg cells, natural

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killer T cells, B cells) and myeloid cells (monocytes, macrophages). This study did note an interesting observation whereby CD8⁺/CD4⁺ Tregs ratio potentially may be associated with one of the positive prognostic factors of HGSOC (zero RD). It has been noted in other studies where low CD8⁺ T cells and CD8⁺/CD4⁺ T-regulatory cells ratio were associated with poorer survival outcomes in OC (Preston et al., 2013; E. Sato et al., 2005). However, this finding should be validated in future studies by including a larger sample study. Further exploration of the immune compartment using scRNA-seq should focus on how best to score immune infiltrates particularly TILs which have been confirmed to have prognostic significance. The development of an 'immunoscore' using single cell technology would facilitate further progress of immunotherapeutic options in HGSOC including the potential stratification of tumours according to their immune infiltration and determining those which may benefit from immune checkpoint blockade or adoptive T cell therapies (Galon et al., 2013); agents that stimulate CD8⁺ T cell infiltration; and vaccines that prime new T cell responses or with engineered T cells (such as chimeric antigen receptor, CAR-T cells) (Bowtell et al., 2015).

While the work in Chapter 3 is limited due to the number of patient samples, this is to some extent compensated by the large number of cells (134,306) and total genes studied which allows for the detection of more subtle differences between patients and multi-site heterogeneity. Other interesting findings which bolstered the confidence that these single cell transcriptomes were true signatures of the tumours included the fact that within the metastatic NACT sample, there was a lack of tumour cells which had been stripped off by the treatment (a finding concordant with Shih et al.) (A. J. Shih et al., 2018) and metallothionein expressing T cells absent in other pre-chemotherapy samples.

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Additionally, one of the main advantages of this scRNA-seq study over bulk transcriptomic profiling is the identification of rare important subpopulations of cells (ovarian CSCs and the chemo-resistant metallothionein T cells) which may be utilised for personalised therapies specifically targeted at these treatment resistant cells. With still limited scRNA-seq studies specific to HGSOC, this work adds further value by demonstrating that the bulk TCGA HGSOC transcriptomic subtype classify according to the different single cell populations (i.e. cancer epithelial cells accounting for 'DIF', immune cells for 'IMR', fibroblasts for 'MES' and endothelial cells for 'PRO' subtype). In essence, this supports the suggestion that the cellular components of the TME are responsible for the bulk signatures of HGSOC and future studies may be more accurately represented by a multi-omics molecular model combined with tumour staging and other clinicopathological correlations (Geistlinger et al., 2020; Izar et al., 2020; Winterhoff et al., 2019).

Building onto this multi-model characterisation of HGSOC and its TME, quantitative histopathological analysis using artificial intelligence (AI) offers a cost-effective adjunct to help stratify and prognosticate the disease given the widely available histological resources. The work in Chapter 4 outlines the rationale for adopting TSP analysis using AI in HGSOC. Using prediction models this histological classification method had demonstrated good predictive prognostic capability, thence could be considered as a potential addition to the FIGO staging system in HGSOC patient risk stratification. When classified by TSP, a clear stratification was demonstrated in the rich clinical annotation Edinburgh cohort (C) whereby the stroma-rich group had poorer prognosis compared to the stroma-poor group with superior survival outcomes. Apart from inferior survival outcomes, the stroma-rich group had more patients who were associated with advanced stage HGSOC, greater RD at PDS, greater desmoplastic

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reaction and TCGA 'Mesenchymal' subtype. The analysis of HGSOC using necrosis as a histopathological feature in Chapter 4 detected an interesting finding where the presence of necrosis within HGSOC tumours at pre-chemotherapy could confer positive prognostic effects. Patients with necrotic tumours had better OS and lower macroscopic disease at PDS compared to those without necrosis. Along with limited studies of tumour necrosis in OC at pre-chemotherapy stage, this work raised the potential for further correlation of morphological subtypes of HGSOC (utilising necrosis as one of the features for subtyping) with patients' prognosis and response to treatment (Green et al., 2005; Penault-Llorca et al., 2000; Soslow et al., 2012).

The work in Chapter 5 analysed the bulk transcriptomic profiles of the tumours studied in Chapter 4 and stratified these tumours according to TSP and necrosis. This work strengthens the use of TSP and necrosis as histological stratification tools in HGSOC through the detection of significant biological pathways related to 'stroma-rich' and 'necrosis' signatures. Of note, the 'stroma-rich' signature was enriched for EMT, angiogenesis and coagulation. While the 'necrosis' signature was related to the targeting of c-MYC, MTORC1 signalling pathway, protein sorting and transport, nonsense-mediated RNA decay and upregulation of activated T-cell proliferation. Integrating the bulk transcriptomic profiles of the TCGA 'Mesenchymal' subtype with the single cell CAFs genes, and mapping of these markers onto cohort C had demonstrated that fibroblasts (particularly CAFs) are likely to account for the 'stroma-rich' signature. This also allows the exploration of the clinical roles of the different CAFs subclusters and their mechanism of actions in HGSOC tumourigenesis. Additionally, the generation of the prognostic multi-gene signature (comprised of 26 genes) from the intersection of the bulk and single cell stromal biomarkers adds

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novelty value to the work here which may serve as another risk stratification tool for HGSOC patients and facilitate the implementation of personalised therapies.

In terms of future clinical applications, this work potentially could facilitate individualised stratification of surgical candidates through identification of patients with positive or negative predictive factors detected by their TSP and necrosis score on histopathological examination combined with transcriptomic signatures. Advanced stage HGSOC patients with stroma-rich tumours combined with poor prognostic stromal markers identified through diagnostic biopsy may be stratified to neoadjuvant chemotherapy (NACT) rather than extensive upfront cytoreductive surgery. These patients generally have poorer prognosis, less successful maximal cytoreduction and greater platinum resistance (Bell et al., 2011; Tothill et al., 2008). Additionally, it has been demonstrated that necrosis at pre-chemotherapy stage contributes to good prognostic outcomes within the Edinburgh cohort. Further studies using larger international cohorts may seek to evaluate the presence of necrosis as a biomarker to stratify patients with high necrosis score to upfront primary debulking surgery rather than NACT. Importantly, the work here provides a novel platform for combining histopathological and transcriptomic scoring in HGSOC through a comprehensive disease score comprised of TSP, necrosis, stromal and other molecular signatures which may aid the well-recognised HRD clinical assay in order to help predict disease operability, platinum-sensitivity and survival.

Interestingly, the single cell transcriptomic landscape of HGSOC in this study demonstrated variation between different sites of a single patient (intra-patient heterogeneity) and between multiple patients (inter-patient heterogeneity). There was cellular shift from epithelial to immune cells and vice versa when comparing primary

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to metastatic sites of pre- and post-chemotherapy patients. Moreover, the immune cells composition (particularly CD8⁺/CD4⁺ T-regulatory cells obtained from combined proportions of multiple sites in a single patient) showed variation between different patients which may correspond to their surgical cytoreductive outcomes. Therefore, this study has highlighted the importance of multi- versus single-site biopsies for prediction of individualised treatment response.

In conclusion, the work carried out here forms an integrative analysis of single cell and bulk RNA-sequencing and digital histopathology in evaluating the TME of HGSOc and correlation of their clinical significance using well-annotated Edinburgh cohorts. The direct clinical translation of the single cell work in this study is still premature and requires further exploration and validation of the biomarkers using RNAscope, ovarian cancer cell lines or 2D/3D models in order to translate these RNA biomarkers into clinical use. Future studies may seek to validate this using matched FFPE samples from adjacent sites available for all the samples included in the scRNA-seq study here.

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Appendix 1 100 Gene Symbols Defining TCGA HGSOC Subtype Signatures
(Verhaak et al., 2013)

Gene Symbol	Class	Description
<i>FUT2</i>	DIF	fucosyltransferase 2 (secretor status included) [Source:HGNC Symbol;Acc:4013]
<i>STEAP3</i>	DIF	STEAP family member 3 [Source:HGNC Symbol;Acc:24592]
<i>LCN2</i>	DIF	lipocalin 2 [Source:HGNC Symbol;Acc:6526]
<i>HRBL</i>	DIF	---
<i>MYO5C</i>	DIF	myosin VC [Source:HGNC Symbol;Acc:7604]
<i>ALS2CL</i>	DIF	ALS2 C-terminal like [Source:HGNC Symbol;Acc:20605]
<i>ANXA1</i>	DIF	annexin A1 [Source:HGNC Symbol;Acc:533]
<i>PTGER2</i>	DIF	prostaglandin E receptor 2 (subtype EP2), 53kDa [Source:HGNC Symbol;Acc:9594]
<i>MVP</i>	DIF	major vault protein [Source:HGNC Symbol;Acc:7531]
<i>SSH3</i>	DIF	slingshot homolog 3 (Drosophila) [Source:HGNC Symbol;Acc:30581]
<i>C1ORF116</i>	DIF	chromosome 1 open reading frame 116 [Source:HGNC Symbol;Acc:28667]
<i>DTX4</i>	DIF	deltex homolog 4 (Drosophila) [Source:HGNC Symbol;Acc:29151]
<i>MLPH</i>	DIF	melanophilin [Source:HGNC Symbol;Acc:29643]
<i>TNFRSF14</i>	DIF	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator) [Source:HGNC Symbol;Acc:11912]
<i>MGLL</i>	DIF	monoglyceride lipase [Source:HGNC Symbol;Acc:17038]
<i>CLU</i>	DIF	clusterin [Source:HGNC Symbol;Acc:2095]
<i>TMEM24</i>	DIF	---
<i>SLC37A1</i>	DIF	solute carrier family 37 (glycerol-3-phosphate transporter), member 1 [Source:HGNC Symbol;Acc:11024]
<i>RIN1</i>	DIF	Ras and Rab interactor 1 [Source:HGNC Symbol;Acc:18749]
<i>SCGB2A2</i>	DIF	secretoglobin, family 2A, member 2 [Source:HGNC Symbol;Acc:7050]

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<i>FBXO5</i>	IMR	F-box protein 5 [Source:HGNC Symbol;Acc:13584]
<i>LAG3</i>	IMR	lymphocyte-activation gene 3 [Source:HGNC Symbol;Acc:6476]
<i>AIM2</i>	IMR	absent in melanoma 2 [Source:HGNC Symbol;Acc:357]
<i>CXCL10</i>	IMR	chemokine (C-X-C motif) ligand 10 [Source:HGNC Symbol;Acc:10637]
<i>IFI30</i>	IMR	interferon, gamma-inducible protein 30 [Source:HGNC Symbol;Acc:5398]
<i>CASP1</i>	IMR	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) [Source:HGNC Symbol;Acc:1499]
<i>ECGF1</i>	IMR	---
<i>LAIR1</i>	IMR	leukocyte-associated immunoglobulin-like receptor 1 [Source:HGNC Symbol;Acc:6477]
<i>IL21R</i>	IMR	interleukin 21 receptor [Source:HGNC Symbol;Acc:6006]
<i>SLC31A2</i>	IMR	solute carrier family 31 (copper transporters), member 2 [Source:HGNC Symbol;Acc:11017]
<i>PDZK1IP1</i>	IMR	PDZK1 interacting protein 1 [Source:HGNC Symbol;Acc:16887]
<i>SAMSN1</i>	IMR	SAM domain, SH3 domain and nuclear localization signals 1 [Source:HGNC Symbol;Acc:10528]
<i>FCER1G</i>	IMR	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide [Source:HGNC Symbol;Acc:3611]
<i>RARRES3</i>	IMR	retinoic acid receptor responder (tazarotene induced) 3 [Source:HGNC Symbol;Acc:9869]
<i>ADAMDEC1</i>	IMR	ADAM-like, decysin 1 [Source:HGNC Symbol;Acc:16299]
<i>SLA</i>	IMR	Src-like-adaptor [Source:HGNC Symbol;Acc:10902]
<i>LAPTM5</i>	IMR	lysosomal protein transmembrane 5 [Source:HGNC Symbol;Acc:29612]
<i>UBE2L6</i>	IMR	ubiquitin-conjugating enzyme E2L 6 [Source:HGNC Symbol;Acc:12490]
<i>KMO</i>	IMR	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase) [Source:HGNC Symbol;Acc:6381]
<i>CIITA</i>	IMR	class II, major histocompatibility complex, transactivator [Source:HGNC Symbol;Acc:7067]
<i>GIMAP5</i>	IMR	GTPase, IMAP family member 5 [Source:HGNC Symbol;Acc:18005]
<i>APOBEC3G</i>	IMR	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G [Source:HGNC Symbol;Acc:17357]

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<i>PCOLCE</i>	MES	procollagen C-endopeptidase enhancer [Source:HGNC Symbol;Acc:8738]
<i>MXRA8</i>	MES	matrix-remodelling associated 8 [Source:HGNC Symbol;Acc:7542]
<i>MAPRE2</i>	MES	microtubule-associated protein, RP/EB family, member 2 [Source:HGNC Symbol;Acc:6891]
<i>CXCR7</i>	MES	chemokine (C-X-C motif) receptor 7 [Source:HGNC Symbol;Acc:23692]
<i>MMP14</i>	MES	matrix metalloproteinase 14 (membrane-inserted) [Source:HGNC Symbol;Acc:7160]
<i>PDPN</i>	MES	podoplanin [Source:HGNC Symbol;Acc:29602]
<i>PLEKHO1</i>	MES	pleckstrin homology domain containing, family O member 1 [Source:HGNC Symbol;Acc:24310]
<i>TPST1</i>	MES	tyrosylprotein sulfotransferase 1 [Source:HGNC Symbol;Acc:12020]
<i>MARCKS</i>	MES	myristoylated alanine-rich protein kinase C substrate [Source:HGNC Symbol;Acc:6759]
<i>CALD1</i>	MES	caldesmon 1 [Source:HGNC Symbol;Acc:1441]
<i>TMEPAI</i>	MES	---
<i>NNMT</i>	MES	nicotinamide N-methyltransferase [Source:HGNC Symbol;Acc:7861]
<i>GAS7</i>	MES	growth arrest-specific 7 [Source:HGNC Symbol;Acc:4169]
<i>WIPF1</i>	MES	WAS/WASL interacting protein family, member 1 [Source:HGNC Symbol;Acc:12736]
<i>FSCN1</i>	MES	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus) [Source:HGNC Symbol;Acc:11148]
<i>NBL1</i>	MES	neuroblastoma, suppression of tumorigenicity 1 [Source:HGNC Symbol;Acc:7650]
<i>LAMB1</i>	MES	laminin, beta 1 [Source:HGNC Symbol;Acc:6486]
<i>CACNA1C</i>	MES	calcium channel, voltage-dependent, L type, alpha 1C subunit [Source:HGNC Symbol;Acc:1390]
<i>DLC1</i>	MES	deleted in liver cancer 1 [Source:HGNC Symbol;Acc:2897]
<i>RUNX1</i>	MES	runt-related transcription factor 1 [Source:HGNC Symbol;Acc:10471]
<i>KIAA0247</i>	MES	KIAA0247 [Source:HGNC Symbol;Acc:19956]
<i>PALLD</i>	MES	palladin, cytoskeletal associated protein [Source:HGNC Symbol;Acc:17068]

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<i>RHOBTB3</i>	MES	Rho-related BTB domain containing 3 [Source:HGNC Symbol;Acc:18757]
<i>NUAK1</i>	MES	NUAK family, SNF1-like kinase, 1 [Source:HGNC Symbol;Acc:14311]
<i>MYO1B</i>	MES	myosin IB [Source:HGNC Symbol;Acc:7596]
<i>COL5A1</i>	MES	collagen, type V, alpha 1 [Source:HGNC Symbol;Acc:2209]
<i>COL8A1</i>	MES	collagen, type VIII, alpha 1 [Source:HGNC Symbol;Acc:2215]
<i>SRPX2</i>	MES	sushi-repeat containing protein, X-linked 2 [Source:HGNC Symbol;Acc:30668]
<i>LGALS1</i>	MES	lectin, galactoside-binding, soluble, 1 [Source:HGNC Symbol;Acc:6561]
<i>CTGF</i>	MES	connective tissue growth factor [Source:HGNC Symbol;Acc:2500]
<i>DDR2</i>	MES	discoidin domain receptor tyrosine kinase 2 [Source:HGNC Symbol;Acc:2731]
<i>EVI2A</i>	MES	ecotropic viral integration site 2A [Source:HGNC Symbol;Acc:3499]
<i>MCAM</i>	MES	melanoma cell adhesion molecule [Source:HGNC Symbol;Acc:6934]
<i>FN1</i>	MES	fibronectin 1 [Source:HGNC Symbol;Acc:3778]
<i>KDELC1</i>	MES	KDEL (Lys-Asp-Glu-Leu) containing 1 [Source:HGNC Symbol;Acc:19350]
<i>STCH</i>	MES	---
<i>F2R</i>	MES	coagulation factor II (thrombin) receptor [Source:HGNC Symbol;Acc:3537]
<i>COPS3</i>	PRO	COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) [Source:HGNC Symbol;Acc:2239]
<i>EFS</i>	PRO	embryonal Fyn-associated substrate [Source:HGNC Symbol;Acc:16898]
<i>MARCKSL1</i>	PRO	MARCKS-like 1 [Source:HGNC Symbol;Acc:7142]
<i>RAD51AP1</i>	PRO	RAD51 associated protein 1 [Source:HGNC Symbol;Acc:16956]
<i>PKIA</i>	PRO	protein kinase (cAMP-dependent, catalytic) inhibitor alpha [Source:HGNC Symbol;Acc:9017]
<i>SALL2</i>	PRO	sal-like 2 (Drosophila) [Source:HGNC Symbol;Acc:10526]
<i>NETO2</i>	PRO	neuropilin (NRP) and tolloid (TLL)-like 2 [Source:HGNC Symbol;Acc:14644]

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<i>SPATS2</i>	PRO	spermatogenesis associated, serine-rich 2 [Source:HGNC Symbol;Acc:18650]
<i>MAPRE1</i>	PRO	microtubule-associated protein, RP/EB family, member 1 [Source:HGNC Symbol;Acc:6890]
<i>UCHL1</i>	PRO	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) [Source:HGNC Symbol;Acc:12513]
<i>MFAP2</i>	PRO	microfibrillar-associated protein 2 [Source:HGNC Symbol;Acc:7033]
<i>TCF7L1</i>	PRO	transcription factor 7-like 1 (T-cell specific, HMG-box) [Source:HGNC Symbol;Acc:11640]
<i>LRP4</i>	PRO	low density lipoprotein receptor-related protein 4 [Source:HGNC Symbol;Acc:6696]
<i>BEX1</i>	PRO	brain expressed, X-linked 1 [Source:HGNC Symbol;Acc:1036]
<i>KIF1A</i>	PRO	kinesin family member 1A [Source:HGNC Symbol;Acc:888]
<i>COL4A6</i>	PRO	collagen, type IV, alpha 6 [Source:HGNC Symbol;Acc:2208]
<i>TRO</i>	PRO	trophinin [Source:HGNC Symbol;Acc:12326]
<i>BLMH</i>	PRO	bleomycin hydrolase [Source:HGNC Symbol;Acc:1059]
<i>STMN1</i>	PRO	stathmin 1 [Source:HGNC Symbol;Acc:6510]
<i>SMARCD1</i>	PRO	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1 [Source:HGNC Symbol;Acc:11106]
<i>C5ORF13</i>	PRO	chromosome 5 open reading frame 13 [Source:HGNC Symbol;Acc:16834]

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Appendix 2 Work for Publication in Progress

Lim, JHC., Parry, T., Hollis, R., Thomson, J., Churchman, M., Herrington, SC., Semple, C., Gourley, C. Single cell RNA sequencing of the tumour microenvironment characterises malignant epithelial and cancer-associated fibroblasts in HGSOC.

Lim, JHC., Hollis, R., Herrington, SC., Gourley, C. Analysis of HGSOC tumour microenvironment using quantitative digital histopathological examination.

Currently in progress and planned for submission to ***Scientific Reports***.