
***Characterisation of appendiceal tumours:
a step towards rational therapeutics***

Madeleine Cornelia Churchill (née Strach)

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work.
This thesis has not been submitted for any degree or other purposes.

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Madeleine Cornelia Churchill, (née Strach)

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Abstract

The **overall aim** of this project is to characterise the biology of appendiceal tumour peritoneal disease (ATPD) to identify new treatment strategies and ultimately improve clinical outcomes.

The optimal approach for the combination and sequencing of systemic chemotherapy, cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) is not known. The **first aim** is to evaluate the role of perioperative chemotherapy and establish predictors of clinical outcomes in patients with ATPD who undergo CRS with or without HIPEC.

The biological understanding of appendiceal tumours lags behind that of other tumours. Exploring the tumour microenvironment (TME) provides greater understanding of both tumour-host interplay and tumour specific characteristics. Single cell transcriptomic analysis has the power to accurately characterise all cells in the TME and to better understand their individual roles in the malignant process. Spatial transcriptomic profiling can provide insights into the influence of the architecture of the microenvironment, particularly relevant for the mucinous disease that is characteristic of ATPD. The **second aim** is to perform a comprehensive biological analysis of ATPD to better understand the biology of the disease.

Robust preclinical models that recapitulate the TME are essential for understanding patient-specific tumour biology and *ex vivo* testing of therapies. Patient-derived explants (PDEs) are a novel method to characterise the TME. PDEs maintain native tissue architecture, microenvironment and tumour heterogeneity and can be used to inform biomarker discovery. The **third aim** is to establish a PDE model of ATPD.

This study will address the urgent need to define relevant clinical predictors of outcome and the role of perioperative systemic chemotherapy in ATPD. Comprehensive biological profiling of the TME may identify active therapeutic agents and lay the foundation for rationally designed clinical studies. This study will provide a step towards optimising treatment options, identifying prognostic and predictive biomarkers, and ultimately selecting patients for a rational therapeutic approach to improve clinical outcomes.

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Abbreviations

AA	Appendiceal adenocarcinoma
ACD	Australian Cancer Database
AGITG	Australian Gastro-intestinal Trials Group
AI	Apoptotic index
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
AMN	Appendiceal mucinous neoplasm
ANOS	Adenocarcinoma not otherwise specified
ANOVA	Analysis of variance
APC	Allophycocyanin
ASCO	American Society for Clinical Oncology
ATPD	Appendiceal tumour peritoneal disease
AT	Appendiceal tumour
ATR	Ataxia telangiectasia and Rad3-related protein kinase
AWD	Alive with Disease
BEV	Bevacizumab
Bro	Bromelain
BSC	Best supportive care
BV	Brilliant violet
CA	California
CAF	Cancer-associated fibroblast
CAP	Capecitabine
CAPOX	Capecitabine + Oxaliplatin
CAR	Chimeric antigen receptor
CC	Completeness of cytoreduction score
CDK	Cyclin dependent kinase
CE	Carboplatin + etoposide
CEA	Carcinoembryonic antigen
CI	Confidence Interval
CMP	Common myeloid progenitor
COX	Cyclo-oxygenase
CR	Complete response
CRC	Colorectal cancer
CRPM	Colorectal cancer peritoneal metastases
CRS	Cytoreductive surgery
CSF	Colony-stimulating factor
CT	Computed tomography
CTX	Cetuximab
CYC	Cyclophosphamide
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DOD	Died of disease
DSP	Digital spatial profiler

DSS	Disease-specific survival
DTT	Dithiothreitol
DWI	Diffusion-weighted magnetic resonance imaging
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Epitope retrieval
ESMO	European Society of Medical Oncology
ETS	E-twenty-six
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FC	Fold change
FDA	Food and drug administration
FDG	¹⁸ F-fluorodeoxyglucose
FFPE	Formalin-fixed paraffin-embedded
FITC	Fluorescein isothiocyanate
FOLFIRI	5-Fluorouracil/folinic acid + irinotecan
FOLFOXIRI	5-Fluorouracil/folinic acid + oxaliplatin + irinotecan
GALNT	N-acetylgalactosaminyltransferase
GCA	Goblet cell adenocarcinoma
GI	Gastrointestinal
GIMR	Garvan Institute of Medical Research
GMP	Granulocyte-monocyte progenitor
GNAS	G-protein α -subunit
GWCCG	Garvan-Weizmann Centre for Cellular Genomics
HAMN	High grade appendiceal mucinous neoplasm
HG	High grade
HIER	Heat-induced epitope retrieval
HIPEC	Heated intraperitoneal chemotherapy
HR	Hazard ratio
HREC	Human Research Ethics Committee
HSC	Haematopoietic stem cells
ICAM	Intercellular adhesion molecule
ID	Identification
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IMUP	Immortalisation up-regulated protein
IQR	Interquartile range
IRI	Irinotecan
JSS	Joo-Shik Shin
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRAS	Kirsten rat sarcoma

LAMN	Low grade appendiceal mucinous neoplasm
LG	Low grade
MAC	Mucinous adenocarcinoma
MAIT	Mucosal-associated invariant T-cell
MAPK	Mitogen-activate protein kinase
MC	Madeleine Churchill
MCAP	Mitomycin + Capecitabine
MCP	Mucinous carcinoma peritonei
MD	Moderately differentiated
MDSC	Myeloid derived suppressor cell
MEK	Mitogen-activated extracellular signal-regulated kinase
MMC	Mitomycin C
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
MSI	Microsatellite instability
NA	Not applicable
NAC	N-acetylcysteine
NED	No evidence of disease
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NK	Natural Killer
NMD	National Mortality Database
NOD	nucleotide-binding oligomerisation domain
NOS	Not otherwise specified
NR	Not reached
NSAID	Non-steroidal anti-inflammatory drug
NSW	New South Wales
NTC	No template control
NZ	New Zealand
ORR	Objective response rate
OS	Overall survival
OX	Oxaliplatin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCI	Peritoneal cancer index
PD	Progressive disease
PDE	Patient-derived explant
PDO	Patient-derived organoid
PDX	Patient-derived xenograft
PE	Phycoerythrin
PET	Positron emission tomography
PFA	Paraformaldehyde
PFS	Progression-free survival
PI	Proliferative index
PM	Peritoneal metastasis
PMN	Polymorphonuclear

PMP	Pseudomyxoma peritonei
PNS	Peripheral nervous system
PR	Partial response
PSOGI	Peritoneal Surface Oncology Group International
QS	Quality score
RAGE	Receptor for advanced glycosylation endpoint
RAS	Rat Sarcoma
RECIST	Response Evaluation criteria in Solid Tumours
RFS	Recurrence-free survival
RNA	Ribonucleic acid
ROI	Region of interest
RPMI	Roswell Park Memorial Institute
RTM	Resident tissue macrophage
Rx	Treatment
SD	Stable disease
SDF	stromal cell-derived factor
SEER	Surveillance, Epidemiology and End-Results
SRC	Signet-ring cell adenocarcinoma
srcs	Signet-ring cells
T _{CM}	Central memory T cell
TCR	T cell receptor
T _{EM}	Effector memory T cell
T _{EMRA}	Terminally differentiated effector memory cells
TFF	Trefoil factor
T _{FH}	Follicular helper T cell
TGF	Transforming growth factor
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TME	Tumour microenvironment
TMP	Tumour-stromal matrix proteins
TNF	Tumour necrosis factor
TNM	Tumour, node, metastasis
UK	United Kingdom
UMAP	Uniform manifold approximation projection
UMI	Unique molecular identifier
US	United States
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIC	Victoria
WD	Well differentiated
WHO	World Health Organisation
WTA	Whole Transcriptome Assay
XDE	Xenograft-derived explant

Publications from this thesis

Please note I publish under my maiden name, Strach, which remains my professional name and have provided the University of Sydney with my marriage certificate as proof of identity.

Publications from this thesis in peer-review journals

1. **Strach, M. C.**, Sutherland, S., Horvath, L. G. and Mahon, K. The role of chemotherapy in the treatment of advanced appendiceal cancers: summary of the literature and future directions. *Ther Adv Med Oncol.* 2022;14. Doi:[10.1177/17588359221112478](https://doi.org/10.1177/17588359221112478)

[Appendix 1.1](#) of this thesis is published as this citation. I performed the literature review, performed the analysis and wrote drafts of the manuscript.

2. **Strach, M. C.**, Chakrabarty, B., Nagaraju, R. T., Mullamitha, S., Braun, M., O'Dwyer, S. T., Aziz, O. and Barriuso, J. Defining a role for systemic chemotherapy in local and advanced appendix adenocarcinoma. *ESMO Open.* 2023;8(5):101619. Doi:[10.1016/j.esmoop.2023.101619](https://doi.org/10.1016/j.esmoop.2023.101619)

[Appendix 1.2](#) of this thesis is published as this citation. I performed the data collection, analysis and wrote drafts of the manuscript.

Other publications completed during PhD candidature

3. Yousef, A., Yousef, M., Zeineddine, M. A., More, A., Fanaeian, M., Chowdhury, S., Knafl, M., Edelkamp, P., Ito, I., Gu, Y., Pattalachinti, V., Naini, Z. A., Zeineddine, F. A., Peterson, J., Alfaro, K., Foo, W. C., Jin, J., Bhutiani, N., Higbie, V., ... **Strach, M.**, ...Shen, J. P. 1. Serum Tumor Markers and Outcomes in Patients With Appendiceal Adenocarcinoma. *JAMA Netw Open.* 2024;7(2):e240260. Doi: [10.1001/jamanetworkopen.2024.0260](https://doi.org/10.1001/jamanetworkopen.2024.0260)

[Appendix 1.3](#) of this thesis is published as this citation. I performed the data collection, analysis and draft for the UK validation cohort.

4. **Strach, M. C.**, Mahon, K. and Barriuso, J. Genomic Subtypes of Appendiceal Adenocarcinoma: Enough to Guide Clinical Decision Making? *J Clin Oncol.* 2023;41(19):3559–3559. Doi: [10.1200/JCO.22.02895](https://doi.org/10.1200/JCO.22.02895)

[Appendix 1.4](#) of this thesis is published as this citation. I critically appraised the manuscript that this correspondence article describes and wrote drafts of the article.

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In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Madeleine Cornelia Churchill (née Strach)

DATE: October 2024

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Kate Lynette Mahon

DATE: October 2024

Chapter 1. Introduction

1.1. Appendiceal tumours

Appendiceal tumours (AT) are rare and understudied.^{1,2} They encompass a diverse group with wide-ranging clinical behaviour and prognosis ranging from indolent disease with prolonged overall survival (OS) to aggressive ATs with short OS.^{3,4} ATs have a propensity to spread directly into the peritoneal cavity causing peritoneal implants and large volume mucinous ascites. The burden and distribution of AT peritoneal disease (ATPD) determines the clinical impact, which results in considerable morbidity, as well as life-threatening complications for patients.^{1,3} Treatment for patients with ATPD is unique, compared to for other advanced cancers, as curative intent cytoreductive surgery (CRS) followed by heated intraperitoneal chemotherapy (HIPEC) can be performed.⁵⁻⁷

1.1.1. Terminology and disease classification

Nomenclature in this field has changed frequently in attempts to improve understanding of the relationship between the pathology, clinical behaviour and outcomes.^{3,8-12} The existing literature can be challenging to interpret due to this continuous effort to refine complex descriptive and prognostic capabilities.^{3,13} Primary AT are classified by the WHO 2019 grading system as appendiceal mucinous neoplasms (AMN) or appendiceal adenocarcinomas (AA) (WHO 2019).¹⁴ AT peritoneal disease (ATPD) is classified separately to the primary tumour as acellular mucin, mucinous carcinoma peritonei (MCP) or non-mucinous peritoneal metastases (PM).^{14,15}

The word ‘tumour’ has been carefully selected over similar words (neoplasm, malignancy and cancer) to be used to describe AT, due to the spectrum of disease incorporated within this term. Furthermore, for many experts in the field the term ‘appendiceal cancer’ refers only to AAs with invasive potential (such as loss of the cell-to-cell adhesion molecule E-cadherin, increase in cell-migration molecule N-cadherin, resistance to cell death ‘apoptosis’ and lymphovascular spread) as defined by the canonical hallmarks of cancer.¹⁶ AMNs are not thought to have these capabilities so it is not appropriate to refer to them as cancers.¹ Other illustrative examples of entities that are most accurately described as ‘tumours’ rather than ‘cancers’ include desmoid tumours,¹⁷ giant cell tumour of bone¹⁸ and gastrointestinal stromal tumours.¹⁹ These diagnoses represent abnormal cell masses that have not acquired the hallmarks of cancer which impart the malignant phenotype.¹⁶ However, the term ‘tumour’ can still be used for carcinomas with the qualifying term ‘malignant’. Therefore, there is justification in referring to appendiceal neoplasms as ‘tumours’.

To assist with conveying the results from this research project, the nomenclature has been harmonised and the applied terminology summarised in Table 1.1. These terms are a simplified form but they correspond with the current World Health Organisation (WHO) 2019 grading.¹⁴ The terms low-grade (LG) and high-grade (HG) are used to describe disease originating from AMNs and AAs respectively (Table 1.1). These terms that include the word ‘grade’ but are misnomers as they do not describe, and should not be confused with, histological grading.¹⁴ They were pragmatically chosen as they reflect the terms used in clinical practice.

Table 1.1 | Harmonisation of terminology used for this research[Back to paragraph](#)

Chapter Short title	Primary appendiceal tumour (AT)		Appendiceal tumour peritoneal disease (ATPD)	
1 Introduction ^a	LG AT / AMN	HG AT / AA	LG ATPD / AMN peritoneal disease	HG ATPD / AA peritoneal metastases
2 Outcomes (Aus)	Appendiceal mucinous neoplasm (AMN)	Appendiceal adenocarcinoma (AA)	AMN peritoneal disease	AA peritoneal metastases
3 Tumour microenvironment	LG AT	HG AT	LG ATPD	HG ATPD
4 Immune				
5 Explants				
6 Discussion ^a	LG AT / AMN	HG AT / AA	LG ATPD / AMN peritoneal disease	HG ATPD / AA peritoneal metastases
Appendices of published papers				
1.1 Chemotherapy review ^b	Primary AMN	Primary AA	Advanced appendiceal cancers	
	LG disease	HG disease	LG disease	HG disease
1.2 Outcomes (UK)	AMN	AA	AMN	Peritoneal metastases

^aBoth terms are used in the relevant context and reference to the current or historical nomenclature is provided if this helps with interpretation

^bThe term appendiceal ‘cancer’ was chosen at the time of writing the narrative review

Abbrev. AT, appendiceal tumour; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; AMN, appendiceal mucinous neoplasm; AA, appendiceal adenocarcinoma; Aus, Australia; UK, United Kingdom.

The term pseudomyxoma peritonei (PMP) is commonly used to describe LG ATPD, but can also be applied more loosely to include HG ATPD. However, this is an outdated term as it can arise from non-appendiceal primary tumours and other benign conditions.²⁰ PMP should only be used to describe the clinical syndrome of abdominal mucinous disease, as it fails to capture the heterogeneous biology of the underlying histopathological diagnoses and associated tumour biology.^{3, 9}

1.1.2. Epidemiology

1.1.2.1. Global burden

ATs are rare and listed internationally by the National Organisation for Rare Disorders.²¹ It should be noted that historically, ATs were misclassified as colon cancers, with some registry coding deriving from misclassification on death certificates.²² This issue may still exist, and statistical analyses are likely still underestimating the impact and burden of ATs.

A study of the nationwide pathology database of the Netherlands over 10 years (1995 to 2005) found that an AT was in 0.22% of all appendectomy specimens and that the incidence of ATPD was 1.7 per million people per year (Smeenk 2008).¹ A study in the United States (US) of the National Cancer Institute’s Surveillance, Epidemiology and End-Results (SEER) database (1973 to 1998) described an age-adjusted incidence of primary HG ATPD of 0.12 per 1,000,000 people per year.²

An updated analysis of the SEER database (2000 to 2017) found an overall incidence of ATs of 0.93 per 100,000 people per year, with an increase of the age-adjusted incidence from 0.47 to 1.72 per 100,000 people per year. This was attributed to a rise in incidence of appendiceal neuroendocrine tumours (sometimes grouped with LG and HG ATs) over the study period.²³ Other reports indicated that there was also an increasing incidence for LG and mucinous HG ATs.²⁴⁻²⁶ In fact, ATs were recently reported to have the fastest-growing incidence rate (15% increase each year,

2010-2019) in people with early-onset cancers (younger than 50 years).²⁷ An incidence rate of 3.2 people per million per year was reported in both Norway (2009 to 2018) and in England (2012 to 2017).²⁸ However, this was based on data from two peritoneal malignancy centres. Real-world incidence is likely thought to be higher than what is reported in these studies due to misdiagnosis, under-reporting and lack of centralised referral.²⁸

Specific data for global mortality regarding ATs is lacking in the literature, with most studies reporting survival statistics. One study reorganised published data from a previous publication of the Peritoneal Surface Oncology Group International (PSOGI) registry study⁵ to calculate an annual death rate (range 8% to 37% over the 10-year study period) reflecting data from multiple specialised peritoneal malignancy centres (n=2298).²⁸

1.1.2.2. Australian burden

In Australia, ATs were classified as ‘cancer of the appendix’ under the colorectal cancer disease code (C18.1) until the most recent update to the data. Currently ATs are classified separately (C18.0), but there is have no differentiation between LG and HG AT.²⁹ While Australian data is limited due to the historically inaccurate classification of ATs, current data available from the Australian Institute of Health and Welfare suggests increasing incidence from 0.4 per 100,000 people per year in 1982, 2.06 per 100,000 people per year in 2013 and 2.5 per 100,000 people per year in 2019 (Figure 1.1).^{22, 29} Potential reasons for this increasing incidence include a general rise in lower gastrointestinal cancers with an ageing population, genetic factors and environmental exposures.³⁰ More accurate AT diagnoses from increasing use of computed tomography (CT) and changing patterns in pathological assessment of appendicectomy specimens may also contribute to this apparent rise in incidence.^{22, 31}

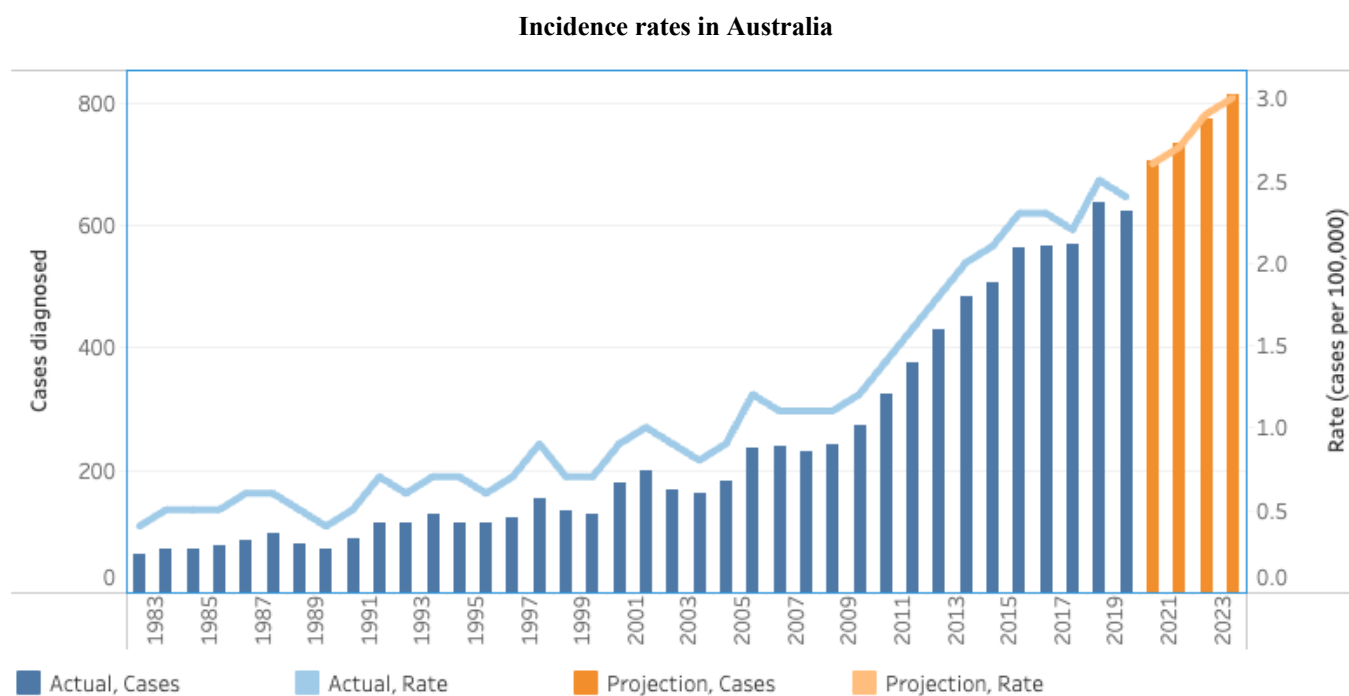


Figure 1.1 | Trends in age-specific incidence rates for Australian patients with ‘appendiceal cancer’ between 1982 to 2023

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Adapted from aihw.gov.au, accessed 09-Sep-2022²⁹

<https://www.aihw.gov.au/reports/cancer/cancer-data-in-australia/contents/cancer-incidence-by-age-visualisation>

Crude mortality increased from 12 deaths in 1979 to 68 deaths in 2021 with a corresponding increase in the age-standardised mortality rate 0.1 to 0.3 per 100,000 persons (for the 2023 Australian population; Figure 1.2).²⁹ One explanation for this may be improved documentation of the accurate cause of death as it is likely, as previously described, that some older death certificates will misclassify the death as due to ‘colon cancer’.²²

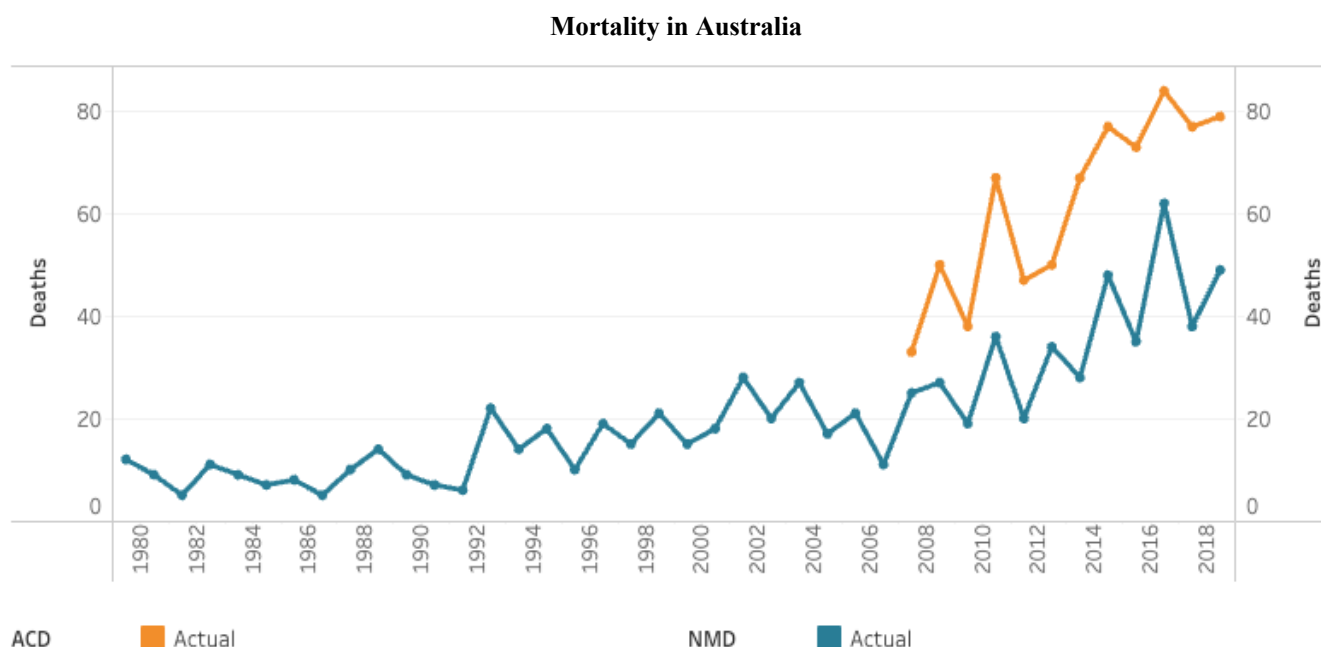


Figure 1.2 | Trends in overall mortality of Australian patients with ‘appendiceal cancer’ between 1971 to 2023

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Abbrev. ACD, Australian Cancer Database; NMD, National Mortality Database

Adapted from [aihw.gov.au](https://www.aihw.gov.au), accessed 09-Sep-2022²⁹

<https://www.aihw.gov.au/reports/cancer/cancer-data-in-australia/contents/cancer-mortality-by-age-visualisation>

1.1.3. Clinical presentation

Appendicitis is the most common presentation (32%) for patients with AT.^{32, 33} It either prompts an incidental diagnosis of an otherwise asymptomatic LG AT (23%), results from mucinous distension of the appendix or is the result of HG AT that has invaded and/or perforated the appendix due to obstruction of the orifice.^{32, 33} HG AT may also present with altered bowel habit or right iliac fossa pain or lump.^{34, 35}

A recent systematic review and meta-analysis evaluated the association of ATs with uncomplicated and complicated appendicitis (defined as abscess, phlegmon and/or perforation at the time of surgery).³⁶ They evaluated seven cohort studies (six retrospective, one prospective) published between 2017 and 2022. The risk of having an AT was similar between complicated and uncomplicated appendicitis (odds ratio 0.44, 95% confidence interval, CI, 0.16 to 1.23). One study reported a complicated appendicitis had a higher rate of adenocarcinoma (50%) compared to uncomplicated appendicitis (13%).³⁷

While most ATPD is a synchronous diagnosis, in those with metachronous disease (development of ATPD more than six months after primary AT diagnosis) the time to ATPD is highly variable with a median of 21 months, but can be more than 10 years.¹ ATPD can present symptomatically with abdominal distension and/or bowel obstruction or be an incidental diagnosis at the time of an abdominal procedure such as laparoscopy or repair of hernia.³⁴

1.1.4. Aetiology

The cause and specific pathogenesis of ATs is unknown. There has been understandable interest in the relationship between acute appendicitis and development of ATs. Factors that predict for ATs in the context of acute appendicitis include older than 50 years and appendix diameter more than 13 mm on ultrasonography.³⁸ There also exists a theory that conservative management of acute appendicitis with antibiotics instead of surgery alters the microbiome and triggers chronic inflammation leading to the development of ATs.³⁹

Given the association of ATs with appendicitis there has been interest in the role that microbial infection may have in triggering and/or propagating oncogenic signals.^{40, 41} One research group has studied this extensively based on their hypothesis that spillage of microbial contents into the abdominal cavity during acute appendicitis may lead to progression or recurrence of disease.⁴⁰ One specific relevant observation was that *MUC2* (MUC2, secreted mucin 2) expression correlated with bacterial density and that bacterial presence and MUC2 expression was higher in HG compared to LG ATs.⁴¹ *H. pylori* presence has been proposed as a potential aetiological agent. Patients with HG ATPD who were treated with oral antibiotics for two weeks prior to surgical resection had lower bacterial densities as well as reduced β -catenin levels.⁴² There is additional data suggesting the role of the microbiome in the development of colorectal cancer metastasis.⁴³ It has been postulated that bacterial contamination of the peritoneal cavity may induce a pro-metastatic niche and favourable microenvironment.⁴⁴

1.1.5. Natural History

Accurate representation of the natural history of ATs and ATPD has been challenging due to heterogeneity of the inclusion criteria of study cohorts (historical cohorts often included benign appendiceal mucoeles).⁴⁵ The change in nomenclature for classification of tumours,³ and evolving curative intent treatments have impacted prognosis, in particular CRS/HIPEC.⁴⁶ Older literature is less relevant for disease outcomes in a curative setting, however, some of these studies shed light on the natural history of disease.

1.1.5.1. Distribution phenomenon

The spread of peritoneal tumour cells around the abdominal cavity has been described by the pioneering surgeon Professor Sugarbaker, who developed the modern-day CRS/HIPEC technique.⁴⁷ This theory is based on his extensive surgical experience as well as clinical and preclinical studies using labelled cells and dyes to track peritoneal fluid movement for evaluation of lymphatic drainage of the peritoneal cavity and the effect of perturbations induced by ovarian cancer.⁴⁸ Typical sites of disease result from intraperitoneal “puddles” where free-floating tumour cells gravitate to and accumulate within the pelvis, the right subhepatic space, the left abdominal gutter and the ligament of Treitz (Figure 1.3).⁴⁷

1.1.1.1. Extraperitoneal disease

Cases of extraperitoneal disease from LG ATPD such as pleural or pericardial disease are thought to be due to transdiaphragmatic seeding.⁴⁹ Haematogenous spread in HG ATPD occurs sporadically, but distant extraperitoneal metastases are uncommon.^{32, 50}

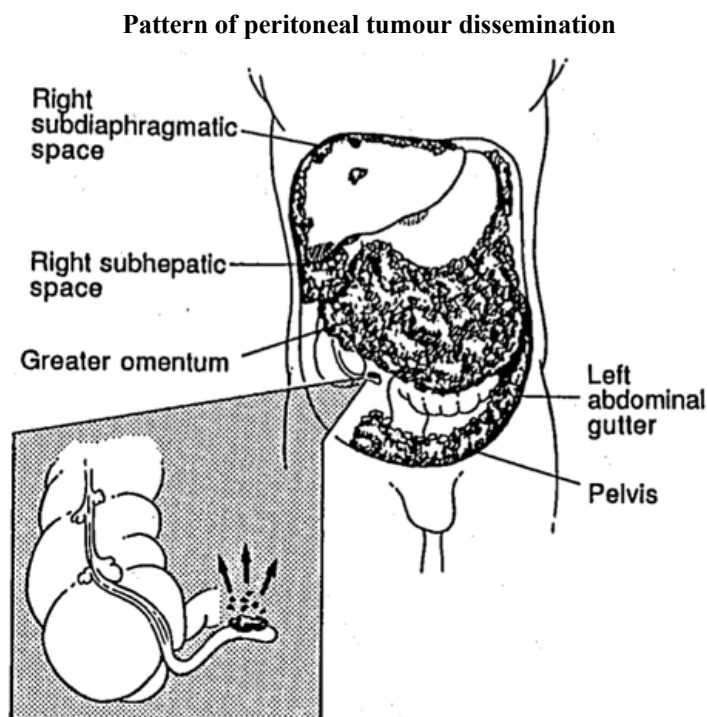


Figure 1.3 | Typical distribution following dissemination of intraperitoneal tumour cells

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Reproduced from Sugarbaker, *Ann Surg* 1994;219(2):109–111⁴⁷

1.1.6. Diagnosis and staging

1.1.6.1. Imaging

As part of the initial evaluation, determining disease burden and presence of extraperitoneal sites is performed.⁵¹ Ultrasound will often be the modality to suggest the presence of mucinous ascites and to facilitate initial sampling of peritoneal disease.³⁴

Cross-sectional imaging is strongly recommended after incidental or suspected finding of an AT. CT is the most accessible modality and mucinous ascites is suggested by high density fluid in the peritoneal cavity (5 to 20 Hounsfield units).³⁴ However, sensitivity for detection of solid tumour nodules varies depending on the size and location of tumour, as extrapolated from findings in studies of patients with colorectal cancer peritoneal metastases.^{52, 53} In peritoneal metastases from colorectal cancer, the sensitivity for detecting tumour nodules drops from 59% to 94% for more than 5 cm nodules to 11% to 28% for less than 0.5 cm nodules and detection of small bowel or mesenteric involvement, important for surgical decision making ranges from 8% to 55%.^{52, 53} Diffusion-weighted magnetic resonance imaging (DWI-MRI), while more technically challenging, demonstrates increasing utility for ATPD with particular use in imaging the hepatic hilar and small bowel regions.^{51, 54, 55} Furthermore, CT and MRI characteristics

have been used to define scoring systems to predict resectability of LG ATPD.^{54, 56, 57} CT chest is also recommended for all HG AT and advanced or recurrent LG AT.⁵¹

¹⁸F-fluorodeoxyglucose positron emission tomography/CT (FDG PET/CT) is not a standard modality for diagnosis of ATPD, due to generally low FDG avidity of mucinous disease, however, some studies indicate evidence for its utility for non-mucinous HG AT.⁵⁸⁻⁶¹

1.1.6.2. Diagnostic interventions

Colonoscopy is not useful as a diagnostic procedure for ATs. There is evidence to support colonoscopy in patients suitable for curative intent treatment to exclude synchronous colorectal malignancies due to reports of an increased risk in the setting of ATs.⁶²⁻⁶⁴

The use of routine diagnostic laparoscopy is debated as a diagnostic and staging tool. The advantages include improved assessment of limiting factors for curative intent treatment such as low volume ‘miliary’ small bowel, mesenteric or serosal involvement. It also provides more accurate assessment of disease burden and prediction of completeness of cytoreduction, access to biopsy and macroscopic evaluation of response to neoadjuvant treatment.^{65, 66} Limitations include inability to assess retroperitoneal structures.

1.1.6.3. Staging

Appendiceal tumour staging according to the Union for International Cancer Control staging system is shown in Table 1.2.⁶⁷ Staging is performed by evaluation of the patient’s clinical, radiological and laparoscopic findings.

Table 1.2 | Tumour, nodal and metastasis stage of appendiceal tumours

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TNM Stage	Definition			
T – Primary tumour				
TX	Not assessed			
T0	No tumour			
Tis	Carcinoma in situ: intraepithelial (tumour cells within glandular basement membrane) or invasion of lamina propria (intramucosal) with no extension through muscularis mucosae into submucosa			
Tis (LAMN)	Low-grade appendiceal mucinous neoplasm confined to the appendix (defined as involvement by acellular mucin or mucinous epithelium that may extend into muscularis propria)			
T1	Tumour invades submucosa			
T2	Tumour invades muscularis propria			
T3	Tumour invades subserosa			
T4	Tumour perforates visceral peritoneum, including mucinous peritoneal tumour or acellular mucin on the serosa of the appendix or mesoappendix and/or directly invades other organs or structures			
T4a	Tumour perforates visceral peritoneum, including mucinous peritoneal tumour or acellular mucin on the serosa of the appendix or mesoappendix			
T4b	Tumour directly invades other organs or structures			
N – Regional lymph nodes ^a				
NX	Not assessed			
N0	No regional lymph node metastasis			
N1	Metastasis in 1-3 regional lymph nodes			
N1a	Metastasis in 1 regional lymph node			
N1b	Metastasis in 2-3 regional lymph nodes			
N1c	Tumour deposit(s), i.e. satellites, ^b in the subserosa, or in non-peritonealised pericolic or perirectal soft tissue without regional lymph node metastasis			
N2	Metastasis in 4 or more regional lymph nodes			
M – Distant metastasis				
M0	No distant metastasis			
M1	Distant metastasis			
M1a	Intraperitoneal acellular mucin only			
M1b	Intraperitoneal metastasis only, including mucinous epithelium			
M1c	Non-peritoneal metastasis			
Overall stage				
Stage	T	N	M	Grade
0	Tis	N0	M0	
0	Tis (LAMN)	N0	M0	
I	T1, T2	N0	M0	
IIA	T3	N0	M0	
IIB	T4a	N0	M0	
IIC	T4b	N0	M0	
IIIA	T1, T2	N1	M0	
IIIB	T3, T4	N1	M0	
IIIC	Any T	N2	M0	
IVA	Any T	Any N	M1a	Any G
	Any T	Any N	M1b	G1
IVB	Any T	Any N	M1b	G2, G3, GX
IVC	Any T	Any N	M1c	Any G

^aRegional lymph nodes are the ileocolic nodes

^bDiscrete macroscopic or microscopic nodules of cancer in the peri-colorectal adipose tissue's lymph drainage area of a primary carcinoma that are discontinuous from the primary and without histological evidence of residual lymph node or identifiable vascular or neural structures

Adapted from Brierley et. al. TNM classification of malignant tumours; 2017: 70-72⁶⁷

1.1.6.4. Peritoneal disease burden

The peritoneal cancer index (PCI) describes the burden of peritoneal disease and is calculated by scoring different regions of the peritoneum as depicted in Figure 1.4. PCI is calculated at the time of diagnostic laparoscopy and at the time of CRS/HIPEC. It describes both the quantity (0 to > 5 cm tumour) and the distribution of disease (score 0 to 39). PCI performed at the time of diagnostic laparoscopy assists optimal selection of patients for CRS/HIPEC.^{68, 69} It identifies patients who have a prohibitive tumour burden and are not suitable CRS candidates which in turn reduces the risk of non-therapeutic laparotomy.^{68, 69} The PCI determined at the time of CRS/HIPEC is prognostic for OS in LG and HG ATPD.^{70, 71} Radiological PCI does not predict extent of CRS.⁷²

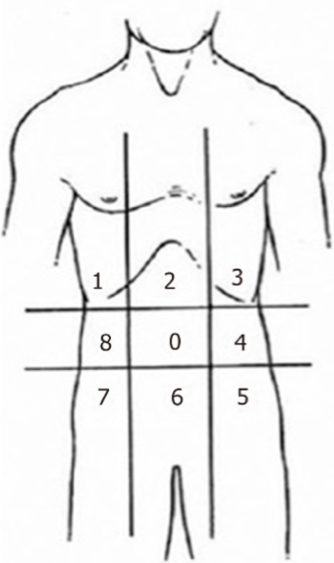
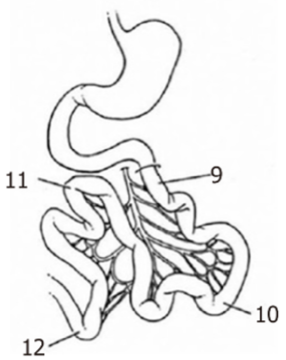
Peritoneal Cancer Index (PCI)			
	Regions	Lesion size	Lesion size score
	0 Central	_____	LS0 No tumor seen
	1 Right upper	_____	LS1 Tumor up to 0.5 cm
	2 Epigastrium	_____	LS2 Tumor up to 5.0 cm
	3 Left upper	_____	LS3 Tumor > 5.0 cm or confluence
	4 Left flank	_____	
	5 Left lower	_____	
	6 Pelvis	_____	
	7 Right lower	_____	
	8 Right flank	_____	
	9 Upper jejunum	_____	
	10 Lower jejunum	_____	
	11 Upper ileum	_____	
12 Lower ileum	_____		
PCI			

Figure 1.4 | Peritoneal cancer index (PCI)

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Reproduced from Sánchez-Hidalgo et. al. *World J Gastroenterol*, 2019;25(27):3484–502⁷³; originally from Jacquet and Sugarbaker, *J Exp Clin Canc Res*, 1996; 15(49):359-360⁷⁴

1.1.7. Histological grading

The current histological grading system is the WHO 2019 fifth edition (Table 1.3).¹⁴ For mucinous AT (more than 50% mucin observed) this includes three-tiered grading as per the American Joint Committee on Cancer (AJCC) of grade 1, well differentiated; grade 2, moderately differentiated and grade 3, poorly differentiated.^{12, 14, 75} Acellular mucin refers to ATPD where no epithelial tumour cells are observed. The presence of signet-ring cells in mucinous AT contributes to the grade: (1) grade three is less than 50% signet ring cells, (2) signet ring cell adenocarcinoma has more than 50% signet ring cells. For non-mucinous HG AT (less than 50% mucin observed), two-tiered grading is the same as for colorectal cancer: (1) low grade (well and moderately differentiated) and (2) high grade (poorly differentiated). The three-tiered grading system for goblet cell adenocarcinoma is based on the proportion of low- (classic goblet cells) and high-grade patterns (Table 1.3). Primary AT and ATPD are graded separately due to the potential for discordant grading, i.e. a grade 2 primary tumour could be associated with a grade 3 peritoneal tumour.⁷⁶⁻

Table 1.3 | World Health Organisation 2019 histological grading for appendiceal tumours*

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Histological Type	Definition	Primary grade (description) ^c	Peritoneal Grade (Description) ^c
Appendiceal mucinous neoplasms			
Low-grade AMN (LAMN)	Mucinous neoplasms are characterised by mucinous epithelial proliferation with extracellular mucin and pushing tumour margins.	Low-grade (mucinous epithelium with tall cytoplasmic mucin vacuoles and compressed bland nuclei, only mild atypia)	Grade 1 MCP ^d (Hypocellular mucinous deposits, neoplastic epithelial elements have low-grade cytology, no infiltrative-type invasion)
High-grade AMN (HAMN)		High-grade (micropapillary features, cribriforming, piling up of epithelial cells with high-grade features including enlarged, hyperchromatic, pleomorphic nuclei, numerous mitotic figures, single cell necrosis, sloughed necrotic epithelial cells)	Grade 2 MCP (Hypercellular mucinous deposits, high-grade cytological features, infiltrative type invasion) Grade 3 MCP (1-50% signet-ring cells)
Appendiceal adenocarcinoma	Malignant glandular neoplasms characterised by invasion.		
Mucinous adenocarcinoma	>50% mucin content	Grade 1 (equivalent to AMN as above)	Grade 1 MCP ^d (hypocellular mucinous deposits, neoplastic epithelial elements have low-grade cytology, no infiltrative-type invasion)
		Grade 2 (Invasive mucinous adenocarcinoma without a signet-ring cell component)	Grade 2 MCP (hypercellular mucinous deposits, high-grade cytological features, infiltrative type invasion)
		Grade 3 (Signet-ring cell adenocarcinoma, with numerous signet-ring cells in mucin pools or infiltrating tissue)	Grade 3 MCP (1-50% signet-ring cells)
Adenocarcinoma not otherwise specified	<50% mucin content	Low grade (well and moderately differentiated)	Low-grade metastases (well and moderately differentiated)
		High grade (poorly differentiated)	High-grade metastases (poorly differentiated)
Goblet cell adenocarcinoma	These are an amphicrine tumour composed of gobletlike mucinous cells, as well as variable numbers of endocrine cells and paneth-like cells, typically arranged as tubules resembling intestinal crypts.	Primary and peritoneal tumour Definition	
		Grade (High-grade pattern)	
		Grade 1 (<25%)	Classic low-grade tumour composed of goblet-like mucinous cells that grow as tubules and can include endocrine cells and paneth-like cells with granular eosinophilic cytoplasm. Mild architectural disarray or tubular fusion can be present, nuclear atypia is mild and mitoses infrequent. Extracellular mucin is often present and can be abundant.
		Grade 2 (25-50%)	Mix of low-grade and high-grade pattern components.
		Grade 3 (>50%)	High-grade pattern component is characterised by loss of tubular or clustered growth pattern. Features can include infiltrating single mucinous or non-mucinous cells, complex anastomosing tubules, cribriform masses, sheets of tumour cells, large aggregates of goblet cells or signet ring cells. Desmoplastic stromal response, high grade cytology, numerous and atypical mitoses and necrosis can be present. Areas of conventional adenocarcinoma can be seen with irregular glands lined by columnar cells with malignant nuclei.
Signet-ring cell adenocarcinoma	>50% signet-ring cells	Grade 3 (100%) or mucinous adenocarcinoma with >50% signet-ring cells	Same pattern as for grade 3 goblet cell adenocarcinoma above but with no recognisable low-grade pattern.

*Excludes appendiceal neuroendocrine tumours

^aMucinous adenocarcinoma/MCP are defined as >50% mucin in WHO 2019¹⁴

^bNon-mucinous adenocarcinoma ($\leq 50\%$ mucin) resembling traditional colorectal type and peritoneal metastasis classification is the same as for mucinous primaries, which has been noted to conflict with colorectal metastasis nomenclature. Peritoneal grading is based upon primary tumour grade.

^cThere can be discordance between primary and peritoneal grading; grading both is recommended by WHO 2019. ¹⁴

^dThe WHO 2019 classification includes acellular mucin in G1 MCP.

Abbrev. MCP, mucinous carcinoma peritonei.

Adapted from Washington and Nagtegaal, WHO Classification of Tumours [online](#), 2019; accessed 22-Sep-2024¹⁴

<https://tumourclassification.iarc.who.int/chaptercontent/31/42>

1.1.8. Treatment

1.1.8.1. Surgery

The only treatment to confer a survival benefit is an aggressive cytoreductive surgical procedure involving resection of organs and peritoneum followed by heated intraperitoneal chemotherapy (CRS/HIPEC) which was first described by Professor Sugarbaker in 1995.⁷⁹ Survival benefits gained since then are due to improved surgical techniques, better patient selection and the surgical “learning curve”.⁸⁰

a) Appendicectomy

As ATs can be diagnosed incidentally after appendicectomy, there is literature focusing on management of incidentally diagnosed primary and peritoneal lesions. There are studies supporting expectant management for low-risk LG AT (LAMN) i.e. those with normal serum tumour markers, PCI ≤ 3 and disease confined to right lower quadrant or pelvis.^{81, 82} These tumours have been classified as ‘LAMN I’ by one high volume centre.⁸¹ However, there are a number of studies supporting CRS/HIPEC for patients with perforated LG AT due to higher recurrence rate in the absence of surgery.⁸³⁻⁸⁵ A study of patients with perforated LG AT (LAMN, n=1000) who had CRS between 1994 and 2014, reported a 10-year OS of 70.3% for CRS/HIPEC compared to a 8.1% for maximal tumour debulking.⁸⁶ There is also data supporting risk-reducing CRS/HIPEC for patients with ‘LAMN II’ (not a category in the WHO 2019 grading). The authors of this study defined this to be as mucin or neoplastic epithelium in the appendiceal submucosa, wall or peri-appendiceal tissue; with or without perforation.⁸¹

b) Right hemicolectomy

Completion right hemicolectomy is routinely offered for HG AT at high-volume centres, although at some it is an individualised approach based on suspicion of margin and lymph node positivity.^{87, 88} However, prospective data from a large cohort from Washington Cancer Centre (n=501)⁸⁹ and registry data from the SEER-database (n=2101)⁹⁰ indicates that this may not confer a survival benefit for mucinous AT.^{89, 90}

c) Cytoreductive surgery

CRS is a radical surgical procedure that entails resection of peritoneal surface and major organ resection. Initial reports were compared to historical controls with improved outcomes (five-year OS was 86% for CRS versus 32% for aggressive debulking) such that the technique has been adopted in peritoneal centres worldwide.⁹¹ The curative intent CRS/HIPEC is recommended for LG ATPD and often recommended at high volume peritonectomy centres following multidisciplinary team discussion for selected patients with HG ATPD, or those high risk of developing peritoneal disease.^{35, 92-98}

(i) Completeness of cytoreduction score (CC)

The completeness of cytoreduction (CC) score is calculated by the surgeon at the conclusion of the CRS procedure. It ranges from CC0 (no residual disease) through to CC3 (residual disease) and is scored according to the thresholds shown in Figure 1.5. Complete CRS has been defined for LG ATPD as CC0-1.⁹⁹ However, data suggests for HG ATPD complete CRS should be defined as CC0 CRS (i.e. no residual disease) given worse outcomes for patients with residual disease (CC1-3).^{88, 100}

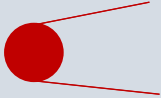
Completeness of cytoreduction (CC) score		
Score	Residual disease	
CC0	No disease	
CC1		<0.25 cm
CC2		0.25-2.5 cm
CC3		>2.5 cm

Figure 1.5 | Completeness of cytoreduction (CC) score according to the quantity of macroscopic residual disease

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Adapted from Gilly *et. al.* *Eur J Surg Oncol*, 2006; 32(6):597-601¹⁰¹; originally from Sugarbaker *Sem Surg Oncol*, 1998; 14(3): 254-261¹⁰²

(ii) Predictive factors for complete CRS

The CC score is the most important determinant of OS and risk of recurrence.¹⁰⁰ Factors that predict the ability to achieve no or minimal residual disease at the time of CRS (CC0-1) help select suitable patients for CRS/HIPEC. Known factors that predict for incomplete CRS include elevated PCI (over 20), small bowel involvement and high-grade histological groups (including presence of signet ring cells).^{5, 100} The predictive value of elevated serum tumour markers have also been assessed. Tumour markers elevated in ATPD include carcinoembryonic antigen (CEA), cancer antigen 125 and carbohydrate antigen 19-9.^{70, 103} One study (n=519) found that 97% of patients who had normal tumour markers had a complete CRS compared to 50% in those who had elevated tumour markers.¹⁰³ While elevated tumour markers likely reflect a higher peritoneal disease burden, the study did not evaluate PCI score as a confounding variable.¹⁰³ Similar findings were reported in another study except the cohort favoured high burden disease (mean PCI 22).¹⁰⁴

d) Maximal tumour debulking

Maximal tumour debulking can be performed when it is not possible to aim for complete tumour clearance. Maximal tumour debulking and incomplete CRS (CC3) are similar in the quantity of disease remaining. The difference is in the

planned intent of the procedure. For example, patients who have a planned curative intent CRS procedure that reveals higher burden disease at the time of surgery may still have some degree of tumour clearance, but HIPEC is abandoned, and the disease is scored by the surgeon as CC3 (> 2.5 cm residual disease). Whereas a planned maximal tumour debulking procedure is a non-curative procedure where HIPEC is not planned to be delivered. This type of procedure is performed for patients who require symptomatic relief of peritoneal tumour burden, such as bowel obstruction. Outcomes of maximal tumour debulking are akin to those after CC3 CRS with shorter OS compared to patients who have complete CRS (CC0-1).^{5, 86} Regardless, maximal tumour debulking can be warranted to improve patients' quality of life and to increase 'obstruction-free survival'.^{105, 106} For patients with surgically challenging locations of peritoneal disease, such as small bowel involvement, it has been used as an initial treatment strategy to defer curative CRS/HIPEC. However, a study evaluating this strategy showed upfront CRS/HIPEC was associated with reduced morbidity and recurrence.¹⁰⁷ Although, one study showed longer median OS of 36.8 months for patients having more than one maximal tumour debulking procedure compared to 18 months for those who only had one procedure.¹⁰⁸

1.1.8.2. Mucin directed therapies

a) Wide-bore percutaneous mucin aspiration

Some patients with advanced ATPD who develop recurrent large volume accumulation of peritoneal mucin can benefit from drainage of mucin. This can help relieve the compressive symptoms of abdominal discomfort, anorexia, dyspnoea and altered bowel habit. An audit at a UK national peritoneal tumour centre of ultrasound-guided percutaneous wide-bore mucin drain aspirations found the procedure was well tolerated.¹⁰⁹ Patients had improvement in abdominal discomfort, dyspnoea and appetite. Further study is needed to determine impact on clinical outcomes and quality of life.

b) Mucolytics

Strategies that assist the dissolution of mucin are a rational approach in ATPD. Mucolytic agents that can break the disulfide bonds between mucin strands can be repurposed from respiratory diseases like cystic fibrosis, such as N-acetylcysteine.¹¹⁰ A phase 1 clinical trial tested the combination of bromelain (pineapple stem enzymes) and N-acetylcysteine given intraperitoneally to patients with ATPD (n=20) with large volume mucin accumulation and 13 of 15 (86%) patients had a clinical and radiological response.¹¹¹ However, there was a high rate of adverse events (85%), the majority serious or leading to discontinuation, but no deaths.¹¹¹ The impact of clinical outcomes has not been described, and the high rate of adverse events are concerning for patients with advanced disease requiring effective palliative treatment strategies.

1.1.8.3. Chemotherapy

The following sections are selected excerpts describing the most relevant studies from a published literature review which is presented in full in [Appendix 1.1](#).*

* Minor amendments made in excerpts from the published literature review to ensure consistency of style include: (1) altering the term 'appendiceal cancer' (AC) to 'appendiceal tumour' (AT), (2) terminology has been amended to use the term AT or ATPD and to reflect the LG and HG descriptors, and (3) changing from first to third person voice.

“The role of chemotherapy in the multidisciplinary setting is a dilemma. Chemotherapy regimens are extrapolated from the colorectal cancer literature. While some data is within colorectal cancer directed studies there has been increasingly homogenous data published specifically for AT populations over recent years.^{112, 113} Furthermore, distinctions have emerged between ATs and colorectal cancer with regard the biological understanding of peritoneal disease and subsequent outcomes.¹¹⁴⁻¹¹⁹ Despite significant limitations and challenges that exist in interpreting this literature, an evidence-based and rational therapeutic approach can still be employed. Treatment decisions should be made by a multidisciplinary team at centralised, high-volume treatment centres.”

a) Heated intraperitoneal chemotherapy (HIPEC)

(i) Mechanism of action and rationale

“The aim of intraperitoneal chemotherapy after cytoreduction is to sterilise the peritoneal cavity of occult tumour cells. Intraperitoneal delivery of the chemotherapy intends to improve drug exposure to the peritoneal surface which is limited with systemic administration.¹²⁰ Active drug concentrates in tissue to a few millimetres, therefore, resection to a minimal volume is required for definitive treatment.¹²¹⁻¹²⁴ Further studies confirm immediate chemotherapy is needed so that tumour cells do not seed in surgical adhesions.^{124, 125} Factors associated with altered HIPEC clearance include the extent of resection, contracted peritoneal space and completeness of cytoreduction.^{126, 127}”

(ii) Hyperthermia

“Hyperthermic infusion of chemotherapy was first clinically demonstrated after CRS in 1980.¹²⁸ Hyperthermia alone may confer a therapeutic benefit by inducing heat stress in tumour cells, denaturing proteins, and impairing deoxyribonucleic acid (DNA) repair mechanisms especially important after a cytotoxic insult.^{129, 130} Heat improves delivery of the chemotherapy into tissues and has a synergistic effect with particular cytotoxic agents.¹³¹⁻¹³⁶ The optimal temperature was established initially by *in vitro* studies showing 42.5°C was more effective than 39°C.¹³⁷ Elias *et. al.* evaluated HIPEC at 43°C, the maximal tolerable temperature in animals, but found higher morbidity and mortality compared to other published literature.¹²⁴”

(iii) Evidence for benefit of HIPEC in addition to CRS

“CRS/HIPEC evolved together, with neither alone demonstrating success.⁴⁶ The specific question of whether HIPEC contributes to improved outcomes compared to CRS alone has not been directly addressed. However, there is reasonable evidence in the literature of case series to suggest independent benefit in both LG and HG ATPD.^{6, 107, 138-143}”

“A recent retrospective study evaluating outcomes of CRS/HIPEC compared to CRS-alone is the best evidence to date of the efficacy of HIPEC. This study evaluated 1924 patients with ATPD from more than 20 centres over 24 years.⁶ The addition of HIPEC to CRS in LG ATPD was associated with a 35% reduction in the risk of death (HR 0.65, 95% CI 0.50-0.83). This was a statistically robust cohort study with propensity matched analysis and inverse probability treatment weighting. Subgroup analysis further confirmed the benefit of HIPEC in AT and those with complete and incomplete cytoreduction. However, there were several limitations of this study. The reasons the CRS-alone group did not receive HIPEC were not available.¹⁴⁴ Additionally, the registry itself was set up for prospective data collection in

2010, yet the period captured was from 1993 requiring a long period of retrospective data collection with incomplete data in 45% of the patients in the registry.”

“While all studies investigating the addition of HIPEC to CRS in appendiceal cancer are flawed and results are mixed, no data suggests worse survival outcomes with HIPEC. On the whole, the data available suggests a probable benefit for HIPEC supporting its continued use.”

(iv) Evidence for HIPEC after maximal tumour debulking

“Historically, most patients who underwent CRS or debulking received concomitant intraperitoneal chemotherapy. However, the poor outcomes of patients who undergo debulking despite also receiving HIPEC questions its utility. The addition of HIPEC to debulking surgery appears not to improve OS in one Swedish study comparing 110 patients who had CRS/HIPEC compared to 40 patients who had debulking and HIPEC.¹⁴⁵ However, recent literature contradicts this suggesting the even those patients with incomplete cytoreduction benefit.^{6, 138} The challenge in this setting is that a proportion of patients who have a surgical intention of debulking (anticipated macroscopic residual disease) can still achieve surgical outcomes of no residual macroscopic disease. This occurred in 25% of the debulking group in the study by Andreasson *et. al.*¹⁴⁵ It is this subgroup of patients that may still benefit from provision of HIPEC. Therefore, the literature currently supports current practice of abandoning HIPEC in the setting of gross macroscopic residual disease and continuing its use in the setting of achieving complete cytoreduction (regardless of the initial surgical intention).”

(v) Mitomycin C HIPEC

“Mitomycin C is the most ubiquitous of agents used for HIPEC with most of the evidence for the combination of CRS/HIPEC historically using this agent.^{6, 7, 107, 114, 138-141, 146-156} In the randomised control trial that compared mitomycin C- to oxaliplatin-HIPEC, no significant difference was found in survival rates consistent with prior data.^{146, 154} In a follow-up study, patients reported improved physical and functional well-being from oxaliplatin-HIPEC.^{157, 158} The recent PSOGI registry analysis did not show survival benefit in the group receiving mitomycin C-HIPEC compared to no HIPEC (HR 0.93, 95% CI 0.65-1.34) noting that this was not the most common agent in this cohort and has flaws of retrospective analysis.⁶ Other clinical considerations in the choice of HIPEC agent include prior receipt and intended sequencing of perioperative chemotherapy. This has not been clearly evaluated in the literature, and current clinical practice at most institutions favour initial mitomycin C-HIPEC to facilitate initial oxaliplatin-based systemic chemotherapy to reduce the dose-limiting risk of neurotoxicity.^{157, 159}

(vi) Oxaliplatin HIPEC

“The use of oxaliplatin-HIPEC has been controversial due to conflicting data regarding its benefits in patients with peritoneal metastases from colorectal cancer.¹⁶⁰ In ATPD, some studies show similar outcomes compared to mitomycin C-HIPEC.^{146, 161, 162} In the recent PSOGI registry study, oxaliplatin-HIPEC was shown to reduce the risk of death compared to no HIPEC (HR 0.42, 95% CI 0.19-0.93).⁶ However, only two patients in the HIPEC group are evaluable at five-years compared to 47 in the no HIPEC group, due to significant attrition in this group or short

follow-up.¹⁴⁴ Oxaliplatin-HIPEC has also been shown to have survival benefit when compared to irinotecan-HIPEC.¹⁶³”

b) Perioperative systemic chemotherapy

“The literature evaluating the role of perioperative chemotherapy is conflicting with some studies showing benefit and others suggesting worse outcomes in patients with ATPD.”

“There have been two prospective trials in the perioperative setting. The COMBATAC trial was a prospective phase 2 single arm study, which reported on 25 patients of whom 10 had ATPD^{164, 165} Patients received three months of pre- and post-operative chemotherapy using 5-fluorouracil/folinic acid and oxaliplatin (FOLFOX), 5-fluorouracil/folinic acid and irinotecan (FOLFIRI), both regimens with cetuximab. The median PFS was 14.9 months, which met the target threshold and is comparable to the literature.^{112, 166, 167} While this study supported the role of systemic chemotherapy, there was no control arm and no subgroup analysis of the ATPD cohort so further conclusions about the role of perioperative systemic chemotherapy are limited.”

“Asare *et. al.* interrogated the US National Cancer Database and reported on 5049 patients with stage IV appendiceal adenocarcinoma.¹⁶⁸ In the non-mucinous group, there was an improvement in survival with chemotherapy compared to no chemotherapy (HR 0.73, 95% CI 0.65-0.83). In the mucinous group there appeared to be no benefit from chemotherapy (HR 0.95, 95% CI 0.86-1.04). This effect is driven by the lack of benefit in the well differentiated group (median OS 6.4 versus 6.5 years, $p>0.05$), but with survival improvement in the moderate and poorly differentiated groups (3.0 versus 1.6 years, $p=0.0005$; 1.6 versus 1.0 years, $p=0.0007$).¹⁶⁸ A flaw with this study was that HIPEC may have been included as systemic chemotherapy, especially as the 5-fluorouracil/folinic acid bolus given with oxaliplatin-HIPEC is administered intravenously. Furthermore, there was no specific code for CRS/HIPEC which amplifies the potential inaccuracies of this analysis.”

“The subsequent study of the National Cancer Database by Lu *et. al.* focused on the role of chemotherapy in 639 patients with stage IV well differentiated mucinous HG ATPD.¹⁶⁹ The majority (90%) of this patient population underwent a surgical resection. Patients who had chemotherapy had improved five-year OS (61% versus 53%), but after multivariate analysis including surgical resection there was no association of benefit with chemotherapy (HR 1.1, 95% CI 0.82 -1.4). This study further supports the concept that well differentiated[†] HG ATPD remain relatively chemoresistant (similarly to LG ATPD) despite the increasing use of combination chemotherapy regimens.^{168, 170}”

“Some studies, albeit with significant limitations, suggested that perioperative chemotherapy could in fact cause harm.^{6, 171, 172} Thus, assessing which patient groups may be at increased risk of harm from a proposed treatment is essential. HG ATPD is expected to derive potential benefit from perioperative chemotherapy due to biological aggressiveness, and is recommended before and/or after at multiple institutions, regardless of data suggesting

[†] Well differentiated HG ATPD refers to histological low-grade tumour

otherwise.^{51, 172} However, the literature does not necessarily support this. For example, Cummins *et al.* evaluated 165 patients, 110 with HG ATPD which compared outcomes to 55 patients with colorectal cancer peritoneal metastases.¹⁷¹ Most of the cohort received perioperative chemotherapy. Pre-operative chemotherapy was associated with poorer OS (14 versus 20 months, $p=0.01$) and post-operative chemotherapy with improved OS outcomes (5 versus 35 months, $p<0.0001$). Furthermore, Levinsky *et al.* evaluated prognostic factors in a subgroup of 125 patients with AA with signet ring cells from the same study population described by Chen *et al.*^{120, 173} They have not described the proportion of signet ring cells within the tumour specimen so it is unclear if these meet the current classification for signet-ring cell adenocarcinoma (more than 50% signet ring cells). Multivariate analysis of the entire cohort suggested worse OS in those receiving systemic chemotherapy (HR 1.98, 95% CI 1.23-3.19, $p<0.01$).

This propensity for HG ATPD to have worse OS after perioperative chemotherapy could be explained by selection bias given both these studies are retrospective. However, in the second study described by Levinsky, the majority of the non-signet ring cell subgroup included well differentiated HG ATPD¹⁷³ These well differentiated adenocarcinomas are conversely, less chemosensitive which could also account for worse outcomes with systemic chemotherapy. This argument is strengthened by the study by Shaib *et al.* who in their multicentre study demonstrated worse survival outcomes in patients with LG ATPD who received perioperative chemotherapy compared to those who did not (HR 1.92, 95% CI 1.14-3.23, $p=0.013$).¹³⁸ This demonstrates the selection bias that although lower grade and well differentiated tumours may have more indolent biology, at the time clinicians select patients for systemic chemotherapy, the burden of the disease is such that it is more imminently life-threatening. Thereby, if systemic agents do not achieve response these patients will have worse overall outcomes.”

c) Systemic anticancer agents for unresectable disease

“There is limited literature demonstrating the activity of systemic chemotherapy in the unresectable setting, with few prospective trials.^{119, 174-180}”

(i) 5-Fluorouracil or Capecitabine

“The chemotherapy backbone in ATPD is fluoropyrimidine as in colorectal cancer. In a single institutional retrospective analysis of chemotherapy agents, 30% of the participants were treated with single agent 5-fluorouracil/folinic acid or capecitabine.¹⁷⁷ It is likely that single agent treatment was given in the advanced setting to those less fit for combination chemotherapy. The benefit of capecitabine in combination with cyclophosphamide and mitomycin has been demonstrated by two studies.^{176, 180} These were both small cohorts, but demonstrated activity of these agents with disease control rate of 27% in the first by Raimondi *et al.* and a clinical benefit rate of 38% in the second by Farquharson *et al.* The Raimondi study of low-grade ATPD included those with progressing disease on consecutive scans, whereas the Farquharson study of both low and high-grade ATPD did not require this. The Farquharson study demonstrated one-year OS of 84% and two-year OS 61%.¹⁷⁶ Interestingly, two patients originally deemed unresectable achieved CRS following treatment with this regimen. This study attempted to overcome the challenges of measuring radiological responses by systematic application of disease volume assessment, discrete deposit measurement and compressive effects on intraperitoneal organs assessed by experienced radiologists.”

(ii) Oxaliplatin-based combinations

“Oxaliplatin and fluoropyrimidine chemotherapy regimens have been the mainstay of colorectal cancer chemotherapy for many decades.¹⁸¹⁻¹⁸⁵ Its use has been extrapolated to the treatment of settings of ATPD and is often the preferential first-line regimen.^{170, 177, 186} The best evidence for this doublet treatment is from a single arm prospective study by Pietrantonio *et. al.*¹⁷⁴ This study evaluated survival outcomes in 20 consecutive patients with unresectable or recurrent LG and HG ATPD treated with FOLFOX. This was a high burden disease group and 45% patients achieved stable disease. This compares to a 24% partial response rate in a retrospective study by Shapiro *et. al.* which did not use Response Evaluation criteria in Solid Tumours (RECIST) and defined partial response as any degree of response.¹⁷⁷ Median PFS was 8 months and median OS 26 months in the Pietrantonio study.¹⁷⁴ This contrasts to longer median OS of 56 months likely driven by a mostly well differentiated tumour population in the retrospective analysis by Shapiro.¹⁷⁷ The poorer survival in this prospective study, is not inconsistent with a high disease burden population, some of whom have HG disease. Two of the six initially unresectable patients underwent laparotomy with one achieving complete CRS.”

“The impact of systemic chemotherapy on the survival outcomes in both these studies is unclear, but would appear to not overly influence the natural trajectory of high burden unresectable disease. The modest response rate suggests some degree of treatment activity and confirms oxaliplatin-based treatments as having the best evidence in this setting.”

(iii) Irinotecan-based combinations

“There are small numbers of patients with ATPD treated with irinotecan-based combinations.^{177, 178} No further details are provided in these studies on the outcomes of this subgroup due to low patient numbers. In the retrospective analysis by Lieu *et. al.*, there was a trend towards improved PFS in patients who had first-line irinotecan (1.0 versus 0.5 years, $p=0.07$).¹⁸⁶ In the control arm of the Verwaal *et. al.* randomised control trial, a small number of patients were given single agent irinotecan as the second line chemotherapy agent.^{187, 188} No further detail is available about these patients to draw any conclusions.”

(iv) Biologic therapy: anti-angiogenic agents

“Vascular endothelial growth factor (VEGF) expression has been shown to be associated with poor OS in ATPD.¹⁸⁹⁻¹⁹¹ Studies that have evaluated the role of anti-VEGF therapy with bevacizumab are conflicting, confounded by the chemotherapy regimen.^{170, 174, 178, 179, 186} The most recent was a prospective phase 2 study that evaluated 15 patients with mostly LG ATPD who relapsed after prior CRS/HIPEC and received capecitabine and bevacizumab.¹⁷⁴ Median PFS was 8.2 months (95% CI 5.3-not assessable) which met the prespecified non-inferiority threshold of five months and median OS was not reached, with one-year OS 91%. Three of the 15 patients had a partial response (ORR 20%).

Choe *et. al.* evaluated the role of biological therapy through analysis of 130 of 353 patients with ATPD.¹⁷⁸ Most patients (91%) received bevacizumab in addition to combination chemotherapy, so that the comparison is to those who received single agent chemotherapy. Median PFS was improved with bevacizumab compared to no bevacizumab (nine versus four months, HR 0.69, 95% CI 0.470-0.995). Median OS was improved by 34 months for patients receiving additional bevacizumab, but this finding could be confounded by the use of combination chemotherapy. Jimenez *et.*

al. evaluated differential *VEGFR2* gene expression in a cohort of 59 of 89 patients with HG ATPD.¹⁷⁹ Twelve of the 47 high expressors received adjuvant bevacizumab. There was no statistical difference between these groups, yet there was a trend to better outcomes in *VEGFR2* low expressors, noting that this comparison is underpowered. Conversely, a subgroup analysis of 112 patients with HG ATPD (51% received bevacizumab) suggested worse PFS in those who received bevacizumab (HR 1.91, 95% CI 1.17-3.14, p=0.01) and no advantage to OS.¹⁷⁰ Most patients who received bevacizumab had combination chemotherapy, likely reflecting selection of more aggressive disease biology, however, this study does not support the use of bevacizumab in treatment of advanced HG ATPD.

There are no studies that provide strong evidence for the benefit of the addition of anti-angiogenic agents to systemic chemotherapy agents in the unresectable setting.”

(v) Epidermal growth factor receptor inhibitors

“In the study by Shapiro *et. al.*, 11 (20%) of the patients studied received biologic therapy alone or in combination with chemotherapy.¹⁷⁷ Five patients were treated with gefitinib alone, which is interesting as this is not an extrapolated colorectal cancer regimen. Another study by Choe *et. al.* also included a small subgroup of patients treated with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies in addition to systemic chemotherapy.¹⁷⁸ In this group, OS outcomes were worse, (18 vs 20 months; HR 3.83 95%CI 1.04-14.14). The authors have not described *Rat sarcoma (RAS)* testing and the study period included the time prior to knowledge of the lack of benefit from EGFR-antibodies in *RAS*-mutant colorectal cancer.¹⁹² In the COMBATAC trial, perioperative systemic chemotherapy (48% FOLFOX; 48% FOLFIRI) and cetuximab was given to Kirsten rat sarcoma (*KRAS*) wild type patients with ATPD in a cohort of patients with CRC.^{164, 165} No additional conclusions for the role of cetuximab could be made from this study as the AT subgroup was not evaluated independently.”

This concludes the excerpts from the narrative review ([Appendix 1.1](#)). A summary of selected literature that supports existing clinical practice is shown in Table 1.4.

Table 1.4 | Selected literature supporting existing clinical practice for systemic chemotherapy in ATPD^a

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ATPD type	Treatment setting	Author	Study Design	Population (n)	Agents	Overall survival (OS)	Progression-free survival (PFS)	comments
Perioperative								
HG	Pre-operative	Bijelic <i>et. al.</i> , 2012 ¹⁶⁶	Retrospective review of prospective database 2005-2009 Prospective study 2005-2009	HG (58) (34 preop, 24 no preop) 34 preop	5FU+OX CAP+OX +BEV FOLFOX	Median preop: 37m No preop: 51m (p=0.56)	Median not reached	Response rate: 65% SD on CT 50% progressed intraop 7 PR and 3 CR on path response preop chemo showed improved OS in those with complete response (p=0.032)
		Sugarbaker <i>et. al.</i> , 2010 ¹⁹³						
	Post-operative	Votanopoulos <i>et. al.</i> , 2015 ¹⁷²	Retrospective review of prospective database 1991-2013	LG + HG (430) LG 77% HG 23%	Not reported	HG preop: 17m No preop 30m (p=0.02) Adj chemo: 32m No postop chemo: 6m (p=0.001) LG preop: p=0.003 Adj chemo: no effect (p=0.88)	Median not reached	Multivariate preop was predictor of poor OS HG: HR 2.5, p=0.006 LG: HR 2.2, =0.05 preop worse Benefit of postop chemo in HG
Pre-and/or Post-operative	Lieu <i>et. al.</i> , 2012 ¹⁸⁶	Retrospective review, 1992-2010	HG (442) (106 stage IV, 78 1L chemo) 26 complete CRS (5 preop, 12 postop chemo)	5FU OX IRI BEV CTX	1L Chemo: 20.4m Periop chemo: HR 0.12 [95% CI 0.01-1.59] (p=0.11)	1L Chemo: 6.9m RFS Periop chemo: HR 0.22 [95% CI 0.04-1.25] (p=0.09)	ORR 1L chemo 44% (correlates with improved PFS on multivariate analysis p=0.02)	
	Kabbani <i>et. al.</i> , 2002 ¹⁹⁴	Retrospective review, 1995-2000	HG (30) (25 chemotherapy) (23 mucinous adenoca, 7 nonmucinous adenoca)	Carboplatin Cisplatin Paclitaxel doxorubicin	Mean OS Mucinous 26m Non-mucinous 13m (p=0.0002)	Mean DFS Mucinous 18m Non-mucinous 7m (p=0.04)		
Palliative intent								
LG and HG	First line	Pietrantonio <i>et. al.</i> , 2014 ¹⁷⁵	Single arm prospective observational study, 2011-2013	LG (20) unresectable/recurrent	5FU+OX	Median 26 m	Median 8m	Response rate 20% 5 prior CAP Chemo activity
		Pietrantonio <i>et. al.</i> , 2016 ¹⁷⁴	Prospective single arm, single institution, 2014-2015	LG + HG (15) (33% HG, 67% LG)	CAP+BEV	Median not reached 1yr OS 91%	Median 8.2 m (95% CI 5.3-NA)	Noninferior to historical control ORR 20% GNAS 60% assoc with reduced PFS
LG	Later line	Raimondi <i>et. al.</i> , 2019 ¹⁸⁰	Prospective, 2015-2017	LG (23) unresectable, relapsed	Metronomic: CAP+CYC	1y 74%	Median 9.5 m	Disease control 27% >12m
		Farquharson <i>et. al.</i> , 2008 ¹⁷⁶	Prospective phase 2 single arm study, 2003-2006	LG (40)	MMC+CAP	1y: 84% 2y: 61%	Not reported	6 tumour reduction 17 stable disease Chemo activity
	Any line	Shapiro <i>et. al.</i> , 2010 ¹⁷⁷	Retrospective review, 2000-2005	LG (54) suboptimal for surgery (24 WD, 11 MD, 15 PD)	5FU CAP Platinum IRI Gefitinib BEV CTX	Median 55.6 m	Median 7.6 m	Response rate 24% Activity of chemo

^aThis table is adapted from Table 5 from published literature review (Appendix 1.1). It has been abbreviated to focus on the key studies that inform clinical practice in the ATPD only literature

Abbrev. 5FU, 5-Fluoruracil/folinic acid; OX, Oxaliplatin; CAP, capecitabine; BEV, bevacizumab; IRI, Irinotecan; CTX, cetuximab; MMC, mitomycin C; CYC, cyclophosphamide; LG, low-grade; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; preop, preoperative; Lymph node; HR, Hazard ratio; 95%CI, 95% confidence interval; RFS, recurrence-free survival; DFS, disease free survival.

1.1.9. Prognosis

The prognosis for ATs is broad, varying considerably depending on tumour stage and subtype from five-year OS 14% for HG ATPD to 96% for LG localised disease.^{32, 76, 116, 147, 149, 195-201} Ten-year survival for LG and HG AT subtypes has been reported to be 63%.⁵ Unlike other advanced malignancies, a cure rate for ATPD has been defined as 10-year PFS.²⁸ PFS ranges from 32-86% and mostly reflects the outcomes for LG ATPD which comprised ~70% of patients in these studies.^{5, 76, 198, 202, 203} Acellular mucin is associated with long-term OS.¹⁹⁶ For patients with LG ATPD the largest cohorts with long term outcomes reveal 10-year OS 63-70%.^{70, 86} HG ATPD has shorter OS but ranges 52% to 55% for those who have complete CRS.^{86, 88, 147} Signet ring cell carcinoma has the worst prognosis of 14% to 25% five-year OS,¹⁹⁵ but despite discussion by experts about potential futility of CRS/HIPEC, this approach remains recommended.²⁰⁴

1.1.10. Surveillance recommendations

After curative intent treatment (either localised resection for primary tumour or CRS/HIPEC for peritoneal disease) surveillance for recurrent disease in conjunction with a peritoneal centre is imperative. Surveillance is important to detected early recurrence of peritoneal disease when disease burden is lowest. This allows consideration of repeat ('redo') curative intent CRS/HIPEC in selected patients with favourable survival outcomes.²⁰⁵ Surveillance should continue for a decade as most recurrences (98.7%) occur within 10 years of treatment.^{32, 76, 116, 149, 197-201}

1.1.10.1. Tumour markers

Studies evaluating preoperative serum tumour markers show an association with prognosis supporting their inclusion in surveillance protocols. One study demonstrated correlation of CEA and CA19-9 levels with tumour load, but only patients with persistently elevated CA19-9 were associated with reduced two-year DFS (55%) compared to normal levels (94%).²⁰⁶ Other studies have found normal preoperative levels are associated with a reduced time to recurrence.^{5, 103, 104, 207-210} A pre-operative CA19-9/PCI ratio also has prognostic value.²¹¹ In LG ATPD, patients with an elevated CA19-9/PCI ratio had shorter median OS (83 months) compared to those with a normal ratio (median OS not reached, $p < 0.001$).²¹¹

1.1.10.2. Imaging

Following CRS/HIPEC, chest/abdominal/pelvic CT imaging (with oral and intravenous contrast) is performed six monthly until five years, although institutional practice varies. Some places do annual surveillance indefinitely and others image a certain intervals as determined by histology.^{212, 213} Recommendations for surveillance based on long-term data from a UK national peritoneal centre are indicated in Figure 1.6.

A	Surveillance recommendations											
	LG ATPD											
	1 year	2 years	3 years	4 years	5 years	6 years	8 years	10 years	11 years	15 years	20 years	
CT Abdomen & Pelvis	X	X	X	X	X	X	X		X	X	X	
Tumour marker (CEA, CA 19.9 & CA 125)	X	X	X	X	X	X	X		X	X	X	
Colonoscopy					X			X		X	X	

B	HG ATPD													
	6 months	1 year	18 months	2 years	3 years	4 years	5 years	6 years	8 years	10 years	11 years	15 years	20 years	
CT Abdomen & Pelvis	X	X	X	X	X	X	X	X	X		X	X	X	
Tumour marker (CEA, CA 19.9 & CA 125)	X	X	X	X	X	X	X	X	X		X	X	X	
Colonoscopy							X			X		X	X	

Figure 1.6 | Surveillance scheme recommended for patients with LG ATPD (A) and HG ATPD (B)

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Abbrev. ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; CT, computed tomography; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA125, cancer antigen 125.

Reproduced from Govaerts et. al. *Eur J Sur Oncol*, 2018; 44(9): 1371-1377²¹³

1.2. Peritoneal tumour microenvironment and genomics of appendiceal tumours

The biological understanding of peritoneal cancers lags behind that of other malignancies. For example, the molecular biology of ATPD is predominantly limited to genomic analysis of formalin-fixed paraffin-embedded tumour samples (FFPE), often without reference to clinical outcomes.^{118, 214} In addition, the tumour microenvironment (TME) has not been characterised in ATPD. Exploring the tumour microenvironment provides greater understanding of both tumour/host interplay and tumour specific characteristics.²¹⁵⁻²¹⁷ With the emerging role of the immune system in host anticancer responses, further understanding of this biological microenvironment is essential for rational therapeutic design.^{218, 219}

There is growing interest in the biology of ATPD to better understand the role of CRS and HIPEC as well as systemic chemotherapy and why response to conventional cytotoxic agents has limited efficacy. The cornerstones to elucidating mechanisms of response and resistance and identifying disease and treatment biomarkers involves understanding the tumour microenvironment (TME), genomic alterations of the tumour and expression of molecular pathways.^{220, 221}

Firstly, the TME is defined as the cells, structures and signalling proteins that are found in the vicinity of the tumour, but which also acts at distant sites to modulate the tumour and its surrounds.²¹⁵⁻²¹⁷ Cancer cells have complicated cross-talk between each other as well as interaction with surrounding cells and extracellular matrix, which influences drug sensitivity and resistance.^{219, 222} There is limited literature on the peritoneal TME of ATPD but analysis of the peritoneal TME related to other solid tumours including ovarian carcinoma, colorectal cancer and peritoneal mesothelioma provides some insights.²²³⁻²²⁵ A description of the composition of the peritoneal TME described in the literature is detailed below along with hypotheses and observations that are unique to the peritoneum and may play a role in this metastatic niche.

Secondly, understanding the molecular alterations that contribute to carcinogenesis and modulate treatment effects is essential for all solid tumours, especially those that are rare and conventional treatments are limited. While there have

been a handful of studies evaluating the genomic alterations of appendiceal tumours, they have limited clinical data to correlate outcomes and more studies are needed to evaluate tumour evolution.^{118, 214, 226}

Thirdly, transcriptomic analyses are informative for providing observations regarding the upregulation of oncogenic pathways and activation or suppression of cellular programs that may influence disease progression.²²⁷ There has been little investigation on the expression of genes linked with oncogenic molecular pathways for this tumour type. Multi-omic analyses in other more common solid tumours are identifying novel targets for therapeutics.²²⁷

1.2.1. Genomics

KRAS, *G-protein α -subunit (GNAS)* and *tumour protein 53 (TP53)* mutations have been consistently identified in ATs.^{119, 228} The most relevant is *KRAS* mutations as *KRAS*^{G12C} inhibitors have been recently approved for treating *KRAS*^{G12C} non-small cell lung cancer and pan-*RAS* inhibitors are in development.^{229, 230} A recent study classified ATPD into molecular subtypes defined as *RAS* mutation predominant with an indolent phenotype, *GNAS* mutation predominant associated with chemoresistance and an aggressive *TP53* mutation subtype.²²⁶ *GNAS* mutations have been suggested as a potential prognostic biomarker, and have been associated with reduced PFS in patients with ATPD who have relapsed disease.¹⁷⁴ Furthermore, *GNAS* mutations correlated with MUC5AC expression in LG ATPD and a role in mucin production.²³¹ *SMAD* mutations along with *TGFBR1/2* mutations were identified in some studies suggesting a role for impaired transforming growth factor (TGF)- β signalling in ATPD.^{214, 232} Similarly, identification of *WNT7A/10A* mutations and *CTNBB1* mutations implicated the Wnt signalling pathway in ATPD.²³²

1.2.2. Transcriptomics

The genetic profile of ATPD has been shown to be distinct from that of peritoneal metastasis from colorectal cancers.²³³ Individual genes found to be associated with worse prognosis in high-risk ATPD included *MUC5AC*, *MUC2*, *Trefoil factor (TFF)1* and *TFF3*.²³³ Pathways associated with high-risk ATPD included oncogenic pathways *MYC*, *E2F* and *SRC*; immune pathways *TNFA* and *TGFB*; and chemosensitive and resistance pathways.²³³ Building on this work, a 139-gene classifier was developed which predicted poor survival outcomes.¹¹⁷ Poor prognostic tumours had enriched genes for pathways including epithelial-mesenchymal transition, E2F targets, p53 mutation, AKT (protein kinase B) activation, HER2 overexpression and cancer stem cells. Patients with tumours that had genes enriched for antigen processing and presentation had better outcomes.¹¹⁷

Another study of a larger cohort (n=194) described a 17-gene signature that classified LG and HG ATPD into immune-enriched, oncogene-enriched and mixed subgroups that corresponded to survival outcomes. Immune-enriched had the longest OS and was followed by the oncogene-enriched then the mixed group.²³⁴ On multivariable analysis including established prognostic factors, two genes (*PRSS*, a protease and *EFNA1*, involved in cell adhesion) were identified in LG ATPD that were associated with worse OS. In HG ATPD, poor survival was associated with cell cycle, proliferation, cell adhesion, mucin production and immune pathways (p<0.05).²³⁵ Conversely, five genes associated with immune pathways (*IGHA1*, *KLRG1*, *TPSAB1*, *TRA*, *TSC22D3*; p<0.05) were associated with better survival after multivariable analysis.²³⁵

To date, gene profiling of ATPD has focused on bulk ribonucleic acid (RNA) sequencing which evaluates the pooled gene expression profile of multiple cell types and tumour clones.^{227, 236} Single cell RNA sequencing (scRNAseq) delineates the gene (messenger RNA) profile from individual cells, providing more dynamic information regarding the role of individual cell types in the TME.²³⁷ There is sparse literature reporting scRNAseq in peritoneal disease from ATPD. A non-peer reviewed pre-print manuscript of scRNAseq in ATPD (n=31) observed gene profiles indicating major cell lineages in the peritoneal TME including immune, endothelial and stromal cells. These are depicted in the uniform manifold approximation projection (UMAP) visualisation by the different coloured groups of individual cells (clusters) that group together by similarities and differences in cellular gene profiles (Figure 1.7A). The main observations that the authors suggested were: (1) tumour cells consisted of progenitor epithelial cells that differentiate into goblet cells (mucin producing cells), (2) peritoneal tumour cells had different gene expression patterns to primary tumours, (3) peritoneal tumour cells were associated with mTOR and RAS signalling pathways (Figure 1.7B), and (4) the presence of T cell infiltration in the TME suggested the potential for anti-tumoural immunity (Figure 1.7A).²³⁸ Additional observations were a moderate macrophage population relevant for conveying an immunosuppressive TME, and, the association of peritoneal tumour cells with cell cycle (*MYC*, *G2M*, *E2F*), *TGFB*, *TP53* and Wnt/ β -catenin signalling pathways (Figure 1.7B).²³⁸ Additionally ATPD appears to have active oncogenic processes that are amenable for further functional study as well as for therapeutic opportunities.

Studies evaluating the biology of ATPD are limited. However, the role of abnormal *RAS*, *GNAS*, TGF- β , Wnt/ β -catenin and cell cycle has been consistently observed. Validating these findings in other cohorts is essential to prioritise research efforts in the setting of rare tumours such as ATPD.

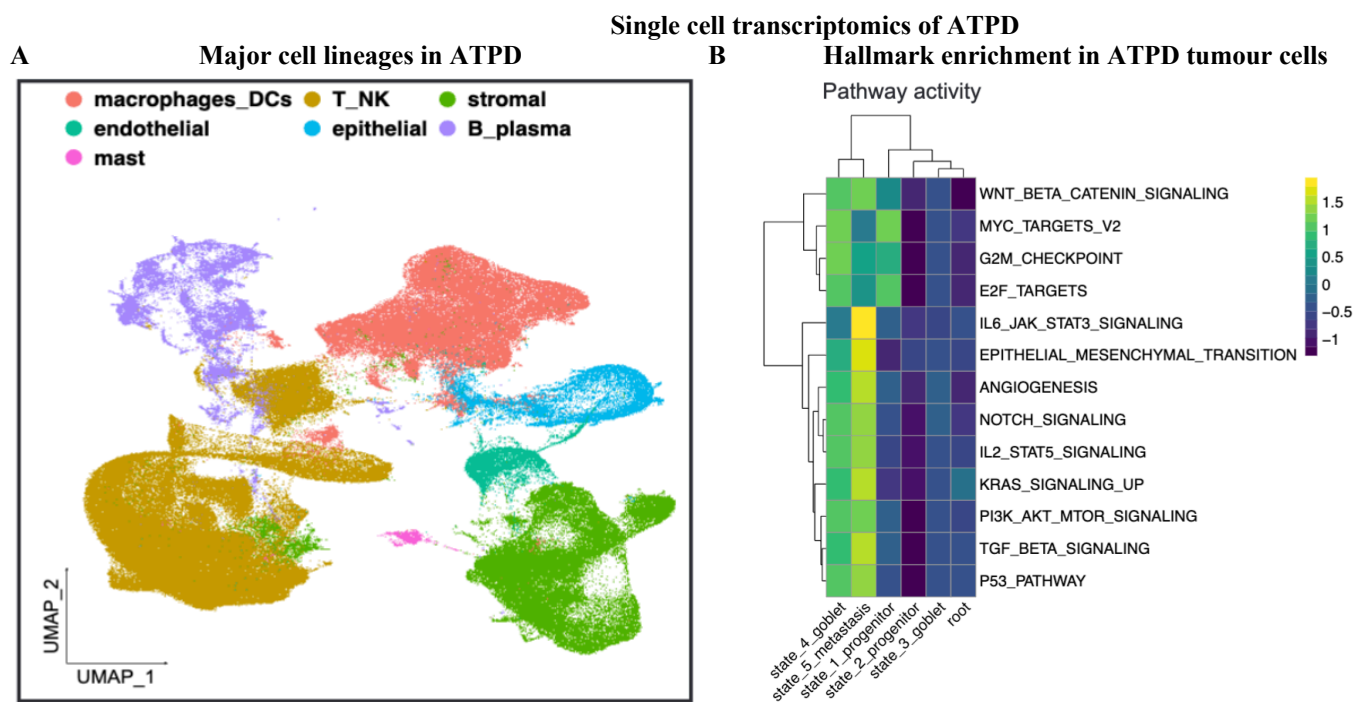


Figure 1.7 | Single cell RNA sequencing of ATPD and functional gene signature enrichment

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UMAP visualisation shows major cell lineages (A) and heatmap indicates average gene set variation analysis enrichment score of selected Hallmark gene signatures comparing tumour cell states of ATPD, yellow represents high and dark blue low enrichment score (B).

Abbrev. ATPD, appendiceal tumour peritoneal disease; DCs, dendritic cells, UMAP, uniform manifold approximation projection
 Reproduced from Ayala et. al. *BioRxiv Preprint*, 2022 (2022.0526.493618)²³⁸

1.2.3. The peritoneal tumour microenvironment

The typical cell compartments described in the tumour microenvironment (TME) include stromal fibroblasts, immune cells, endothelial cells and extracellular matrix.²³⁹ The peritoneal tumour microenvironment is also shaped by mesothelial cells and mucin.²⁴⁰

1.2.3.1. Mesothelial cells of the peritoneum

The peritoneal lining is composed of mesothelial cells that provide a small amount of lubricating fluid to ensure minimal friction between viscera.²⁴¹ Tumour cell adherence to the mesothelium is a key step in peritoneal dissemination of ATPD.²⁴⁰ There are a number of mechanisms for this. One of the most important interactions is that between MUC16 (CA125) on tumour cells and mesothelin on mesothelial cells, as described in ovarian cancer.²⁴² A small proportion (6.5%) of ATPD has been reported to express CA125.²⁴³ Other interactions that have been demonstrated *in vitro* to mediate mesothelial cell adhesion include: (1) integrins and integrin ligands, (2) proteoglycans such as CD44, and their ligands, such as hyaluronan, and (3) the immunoglobulin superfamily including intercellular adhesion molecule 1 (ICAM 1) and vascular cell adhesion molecule 1 (VCAM 1).²⁴⁴ Interruption of this binding with therapeutic agents has been explored *in vitro* using heparin (downregulated ICAM-1 on mesothelial cells).²⁴⁵ and simvastatin (downregulated VCAM-1 on mesothelial cells and its ligand integrin $\alpha_4\beta_1$ on ovarian tumour cells).²⁴⁶ Further characterisation of the role of mesothelial cells in ATPD is warranted.

1.2.3.2. Mucins

Mucinous ascites is a hallmark clinical manifestation of ATPD. Mucins are large glycosylated extracellular proteins that exist in health at various epithelial sites including the digestive system with a role in the maintenance of homeostasis.²⁴⁷ Mucins in the gut play an important role in maintaining the microbiome.²⁴⁸⁻²⁵⁰ Biochemically, mucin structures are characterised by a tandem repeat domain²⁵¹ (except MUC16²⁵²) and the presence of a mucin domain which is a 'bottle brush-like' structure created from O-glycosylation of serine and threonine residues.²⁵³⁻²⁵⁶ These are found in the 21 canonical mucins (MUC1-21), mucin-domain glycoproteins (e.g. CD45 mucin domain suppresses T cell activation)²⁵⁷ and multipass transmembrane mucins.^{247, 253} Recent studies of the mucinome demonstrated high mucin overlap between ovarian cancer ascitic fluid and identification of new mucin-domain glycoproteins.²⁵³

Other cancers have mucinous subtypes, including colorectal cancer.²⁵⁸ It is known that mucinous compared to non-mucinous colorectal cancers display chemoresistance.²⁵⁹⁻²⁶¹ Mucin may contribute to chemoresistance by creating a physical layer of protection around tumour cells preventing the cytotoxic effects of anticancer drugs.^{259, 262} Reduced drug penetration to tumour cells through mucin is due to: (1) the presence of small pores between large mucin strands, (2) its hydrophilic and charged nature, (3) the high interstitial pressure, and (4) microvascular compression.²⁶³⁻²⁶⁵ These factors may contribute to the reduced efficacy of conventional chemotherapy in ATPD compared to colorectal cancer.^{175, 266}

Secreted and transmembrane mucins are associated with different body tissues, such as MUC2 which is a gastrointestinal secreted mucin and MUC5AC, a respiratory secreted mucin (Table 1.5).^{267 268} Intraperitoneal mucin in ATPD is associated with multiple types of secreted and transmembrane mucins. MUC2, is the main component and is

present at higher levels than MUC5B, MUC5AC and MUC6.^{233, 262} MUC2 is secreted by the goblet cells of normal colonic epithelium and it has been suggested that ATPD is a goblet cell neoplastic process.²⁶⁹ Secreted mucin subtypes have differing physical and chemical characteristics and can be classified according to their ability to form a gel (Table 1.5).²⁷⁰ Mucin can be described as soft, semi-hard and hard, according to the ratio of mucin type (MUC2:MUC5B:MUC5AC), as well as hydration status, glucose, lipid and thiol content.²⁷⁰ This classification of mucin has been regarded clinically relevant in the setting of ATPD.²⁷¹ The components that contribute to the differing physical characteristics are important to understand to identify effective mucolytics and mucin-directed strategies.

Table 1.5 | Summary of types of mucins

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Mucins	Tissue
Secreted	
Gel forming	
MUC2	Jejunum, ileum, colon, endometrium
MUC5AC	Lung, stomach, conjunctiva, endocervix, endometrium
MUC5B	Lung, submandibular glands, endocervix
MUC6	Stomach, ileum, gallbladder, endocervix, endometrium
MUC19	Unknown
Non-gel forming	
MUC7	Sublingual and submandibular glands
MUC8	Lung, uterus, endocervix, endometrium
MUC9	Fallopian tubes
Transmembrane	
MUC1	Breast, pancreas, duodenum, ileum, colon, trachea, lung, cornea, conjunctiva, fallopian tubes, uterus, endometrium, cervix, vagina
MUC3A/B	Small intestine, colon, gallbladder
MUC4	Breast, lung, small intestine, colon, conjunctiva, cornea, cervix, vagina, endometrium
MUC11	unknown
MUC12	Colon, pancreas, prostate, uterus
MUC13	Colon, trachea, kidney, small intestine
MUC15	Colon, lung, small intestine, prostate
MUC16	Ovary, cornea, conjunctiva, lung, endometrium
MUC17	Stomach, duodenum, colon
MUC20	Placenta, colon, lung, prostate, liver
MUC21	Lung, thymus, colon
Atypical²⁶⁸ (Jonckheere 2021)	
MUC10	
MUC14	
MUC18	

Adapted from Corfield et. al. Biochim Biophys Acta - Gen Subj, 2015;1850(1):236-252 2015²⁶⁷ and Jonckheere et. al. Biochim Biophys Acta (BBA) - Rev Cancer, 2021;1876(1):188-538 2021²⁶⁸

1.2.4. Tumour immune microenvironment

The field of immunotherapy has progressed rapidly for treatment of other cancer types. Immune cell subsets have been characterised in detail in solid tumour malignancies.^{272, 273} Studies of peritoneal dissemination of primary ATs are limited. Existing biological understanding derives from studies of the peritoneum in the following contexts: (1) peritoneal metastases from solid tumours especially ovarian and gastrointestinal tumours,^{223, 274} (2) peritoneal dialysis,²⁷⁵⁻²⁷⁹ (3) peritoneal changes during intra-abdominal surgery,²⁸⁰⁻²⁸² and (4) endometriosis literature.²⁸³

The normal human peritoneal immune environment comprises a high proportion of T lymphocytes including memory subsets and macrophages.²⁸⁴ The lineages of immune cells described in the peritoneal microenvironment are mostly from studies of gastric cancer peritoneal disease²⁷⁴ and the presence of pro- and anti-tumoural subsets in the peritoneal

TME has not been well elucidated for ATPD. An understanding of the immune cells present would greatly assist efforts for developing rational therapeutic strategies. A summary of major immune cell groups and lineage-derived subsets found (and expected to be found) in the peritoneal immune microenvironment is outlined in Table 1.6

Table 1.6 | Summary of tumour microenvironment immune cell subsets by identifying genes

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Immune cell subset	Genes (proteins*)	References
T cells	<i>CD3D, CD3E, TRBC2, CD2</i>	Zheng <i>et. al.</i> 2021 ²⁸⁵
CD4	<i>CD3D, CD4</i>	Zheng <i>et. al.</i> 2021 ²⁸⁵
CD4 classical	<i>CD3D, CD3E, CXCR4, IL7R, LTB, TRBC2</i>	Zheng <i>et. al.</i> 2021, Hua <i>et. al.</i> 2023 ^{285, 286}
CD4 naïve	<i>PTPRC (CD45RA), CCR7, SELL (CD62L), PECAM-1 (CD31), CD27, CD28</i>	Van den Broek <i>et. al.</i> 2018 ²⁸⁷
CD4 Th1	<i>T-bet, STAT1</i>	
CD4 Th2	<i>IL2RA, IL2RB, IL2RG, IFNGR1, IL12RB1</i>	
CD4 Th9	<i>GATA3, STAT6, IL4R, IL10RB</i>	Tay <i>et. al.</i> 2021, Mitchell <i>et. al.</i> 2015 ^{288, 289}
CD4 Th17	<i>PU.1, IRF4</i>	
	<i>RORC (RORγT), STAT3</i>	
	<i>IL17RA, IL17RC, IL21R</i>	
T_{FH}	<i>BCL6, STAT3</i>	Tay <i>et. al.</i> 2021 ²⁸⁸
Treg	<i>FOXP3, STAT5</i>	
	<i>IL2RA (CD25), low CD127</i>	Van den Broek <i>et. al.</i> 2018 ²⁸⁷
CD8	<i>CD3D (CD3), CD8A (CD8)</i>	Zheng <i>et. al.</i> 2021 ²⁸⁵
T_{CM}	<i>PTPRC (CD45R0), CCR7, SELL (CD62L)</i>	
T_{EM}	<i>PTPRC (CD45RA)⁻, CCR7⁻, SELL (CD62L)^{+/-}</i>	Sallusto <i>et. al.</i> 2004 ²⁹⁰
T_{EMRA}	<i>PTPRC (CD45RA)⁺, CCR7⁻</i>	
MAIT T cell	<i>CD3D, KLRB1 (CD161)^{hi}, IL18R1, Va7.2-Ja33 (TCR Va7.2)⁺, γδ⁻</i>	Dusseaux <i>et. al.</i> 2011, Treiner <i>et. al.</i> 2003 ^{291, 292}
NK	<i>EOMES, GZMA, IRF8, JAK1, NKG7, PLEK, PRF1, ZEB2</i>	Lopes <i>et. al.</i> 2022 ²⁹³
CD56 ^{bright}	<i>NCAM1 (CD56)⁺ FCGR3A (CD16)⁻, GZMK, XCL1, XCL2, CD44, SELL, KLRC1</i>	Vivier <i>et. al.</i> 2024, Kurioka <i>et. al.</i> 2018 ^{294, 295}
CD56 ^{dim}	<i>FCGR3A (CD16)⁺, GZMA/B, PRF1</i>	
B cells	<i>MS4A1 (CD20), CD79A, CD19, IGKC</i>	Gentles <i>et. al.</i> 2015 ²⁷³
Plasma cells		
Myeloid	<i>CD68, CD163</i>	Engblom <i>et. al.</i> 2016 ²⁹⁶
Classical Monocyte	<i>CD14⁺, FCGR3A (CD16)⁻</i>	
Non-classical monocyte	<i>CD14⁺, FCGR3A (CD16)⁺</i>	Fendl <i>et. al.</i> 2023 ²⁹⁷
Macrophage (MΦ)		
M1 MΦ	<i>CD40, FCGR1A (CD64), CXCL11, CCR7</i>	
M2 MΦ	<i>NR3C2 (MR), STAB2 (stabilin-1), CD180, CD163, TREM2</i>	Vogel <i>et. al.</i> 2014 ²⁹⁸
M3 MΦ	<i>FOLR1</i>	Rodriguez-Garcia <i>et. al.</i> 2021 ²⁹⁹
Gastrointestinal MΦ	<i>TIMD4, CD4</i>	Shaw <i>et. al.</i> 2018 ³⁰⁰
Proangiogenic MΦ	<i>SPPI⁺</i>	Zhang <i>et. al.</i> 2020 ³⁰¹
RTM	<i>ITGAM (CD11b), ADGRE1 (F4/80), MERTK, FCGR1A (CD64), LYVE1</i>	Cao <i>et. al.</i> 2024, Chakarov <i>et. al.</i> 2019 ^{302, 303}
Dendritic cells (DCs)		
Plasmacytoid DCs	<i>LILRA4</i>	
Conventional DCs	<i>HLA-DQA1, FCER1A</i>	Cheng <i>et. al.</i> 2021 ³⁰⁴
Myeloid-derived suppressor cells (MDSCs)	<i>ITGAM (CD11b)⁺, PECAM1 (CD31)</i>	
PMN-MDSCs	<i>ITGAM (CD11b)⁺, CD14⁺, FUT4 (CD15)⁺, CEACAM8 (CD66b)⁺</i>	Bronte <i>et. al.</i> 2000, Bronte <i>et. al.</i> 2016 ^{305, 306}
M-MDSCs	<i>ITGAM (CD11b)⁺, HLA-DR^{-/low}, CD14⁺, FUT4 (CD15)⁻</i>	
Neutrophils		
Immature PMN	<i>FCGR3A (CD16)⁺, CEACAM8 (CD66b)⁺</i>	Trellakis <i>et. al.</i> 2011 ³⁰⁷
Mature neutrophil	<i>S100A8, S100A9</i>	Vogl <i>et. al.</i> 1999 ³⁰⁸
Mast	<i>KIT, TPSAB1, CPA3, MS4A2</i>	Liao <i>et. al.</i> 2021 ³⁰⁹

*Selected proteins where not obvious from the gene name are displayed

Abbrev. Th1, T helper 1; Th2, T helper 2; MAIT, mucosal-associated invariant T-cells; TCR, T cell receptor; NK, natural killer; MΦ, macrophage; pDCs, plasmacytoid dendritic cells, cDCs, conventional dendritic cells; TAMs, tumour associated. Macrophages; GI, gastrointestinal; RTM, resident tissue macrophage; MDSCs, myeloid-derived suppressor cells; PMN, polymorphonuclear; M-MDSCs, monocytic-MDSCs

1.2.4.1. Lymphoid aggregates and tertiary lymphoid structures

Tumour infiltrating lymphocytes have been documented in several studies to form aggregates of T and B cells that structurally resemble the arrangement of these immune cells in lymph nodes. These ectopic lymphoid structures near tumour cells have been termed tertiary lymphoid structures (TLS) and in other solid tumours, they appear to provide a favourable prognosis and can independently predict the response to checkpoint inhibitors.³¹⁰⁻³¹³ It has been observed that TLS evolve through distinct maturation phases which culminate in the formation of a germinal centre defined by the presence of follicular dendritic cells (CD21⁺) and germinal centre B cells (CD23⁺).³¹⁴ The arrangement and composition of TLS has not been described in ATPD. One study in peritoneal mesothelioma, found 38% of cases exhibited TLS.³¹⁵ There was no association with the presence of TLS and survival outcomes, however, not all TLS in this study contained germinal centres. The germinal centre is thought to be essential for the *in-situ* generation of the anti-tumoural immune response.³¹⁶ Characterisation of the composition and structural arrangement of immune cells in ATPD could reveal insights into the disease biology and provide further understanding as to a potential role for immunotherapies.

1.3. Preclinical models and drug testing in appendiceal tumours

Drug development in oncology has a high attrition rate from phase 0/1 to phase 3 randomised control trials.³¹⁷⁻³¹⁹ While there exist excellent safety reasons for the prolonged drug development process, studies failing due to lack of efficacy need to be minimised.³¹⁹ The inefficiencies in this process limit availability of investigational agents and particularly impact patients with rare cancers and limited treatment options.³²⁰ Reliable and reproducible preclinical models are needed to accelerate the assessment of therapeutic agents and provide a platform to examine the biology of the TME and molecular pathways underpinning sensitivity and resistance to existing drugs.^{321, 322} Different experimental approaches have been used to investigate biological questions and there are advantages and disadvantages to these methods (Table 1.7). Preclinical models specific to ATPD are detailed in Table 1.8, but more work is required to establish robust, accessible preclinical models in ATPD.

Table 1.7 | Comparison of the advantages and disadvantages of different preclinical models used to study tumour biology

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Method	Advantages	Disadvantages
<i>In vitro</i>		
Cell lines	Widely available for common solid tumours. ³²³ Indefinite source of biological material. ³²⁴	Less available for rare cancers. ³²⁵ Artefactual genetic changes of cells compared to primary tissue. ³²⁶ Does not account for intra-tumour heterogeneity. ³²⁷
Organoids	Cells more accurately reflect original tissue, possibility of cryopreservation. ^{328, 329}	Do not recapitulate microenvironment. Uncertainty in early stages of generation of the cell type of interest. Lack of microenvironmental cues for mature tissue architecture. ³³⁰
Spheroids	Cell-cell contact effects, evaluation of cell growth and drug response, demonstration of drug and nutrient diffusion limitations, possibility for cryopreservation. ³²⁸	Development of central hypoxia and necrosis, cell aggregation, fragmentation, variability in size, lack of microenvironment. ³²⁸
<i>Ex vivo</i>		
Patient-derived explant	Lower cost than <i>in vivo</i> models, replicates TME cells and architecture, accounts for intra-tumour heterogeneity, maximises use of tissue, multiple conditions can be tested in parallel for the same patient, patient-specific. ³²⁷ No toxicity threshold, short-term culture permits timely clinical decision making. ³³¹	Less suitable for studying drugs that rely on recruitment of blood vessels or peripheral immune cells. ³³¹ Does not assess pharmacokinetic aspects of sensitivity. ³²⁷ Finite resource compared to immortal cell lines and sustainable xenograft models. ³³²
<i>In vivo</i>		
Patient-derived xenograft	Gold standard for drug sensitivity testing. ^{333, 334} Maintain histological appearance. ^{334, 335} Stability over generations allows for indefinite resource, allows direct measurement of tumour growth enrichment for tumour cells over stromal cells to facilitate detection of low frequency genetic mutations. ³³⁵	Resource intensive and high cost, use of sacrificial animals, not suitable for all tumours. ^{333, 334} Toxicity threshold. ³³¹ Altered physiology and gene expression after repeated passaging. ^{336, 337} Loss of representative TME by enrichment of tumour cells, Use of nude mice eliminates assessment of host-tumour immune interplay. ³³⁵ Long time for establishment that hinders information used for patient-specific decision making. ^{331, 338}
Genetically engineered mouse models	Allows study of early disease initiation, demonstration of predetermined genetic events, alterations associated with progression and metastasis. ³³⁹ More accurate recapitulation of the genetic features of specific tumours, improved preclinical testing for targeted drug therapies. ³⁴⁰	Expensive. Lack of genetic diversity found in human tumours. ³³⁹ Long time for models to develop tumours, unpredictability, low penetrance, tumorigenic variance.

Abbrev. TME, tumour microenvironment

Table 1.8 | Comparison of different methods of preclinical models studied in peritoneal disease from appendiceal tumours

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Method	Reference	ATPD type (n)	Key findings
Two-dimensional methods			
Cell lines	Roberts, <i>et. al.</i> 2015 ²⁶⁹	LG (2)	• MMC IC₅₀ >Ox IC₅₀ • IC₅₀ phloretin similar to oxaliplatin • Different gene profile to CRC
	Noguchi, <i>et. al.</i> 2022 ³⁴¹	HG (1)	• Similar genetic profile to parent tissue • High throughput drug screen found anti-proliferative effects for daunorubicin, homoharringtonine, MMC and ponatinib
Three-dimensional methods			
Organoids (PDO)			
Immune-enhanced	Votanopoulous, <i>et. al.</i> 2019 ³⁴²	LG (6) HG (3)	• LG PDOs: no drug responses • HG PDOs: variable response to drugs: 5FU (2/3), Ox (1/3), FOLFOX (3/3), FOLFIRI (3/3), Reg (3/3)
		LG (6)	• Immune-enhanced LG PDOs increased mitochondrial metabolism (=T cell activation) after nivolumab at 24h and 72h, reduced metabolism at 96h (tumour cell killing)
	Forsythe, <i>et. al.</i> ³⁴³	LG (16) HG (10)	• Abundant CK20⁺ epithelial cells and CD8⁺ T cells observed D1 • 6/19 (31%) at least 50% tumour cell death (50% in HG, 23% LG)
Patient-derived explants (PDE)			
No matrix ^a	Dilly, <i>et. al.</i> 2021 ²⁶²	LG (8) Mucinous (5) Non-mucinous (3)	• Apoptosis: mucinous<non-mucinous after Ox • Bro+Nac treatment of mucinous: mucin weight reduced, no change in viscosity, no MUC2
Matrix	Weitz, <i>et. al.</i> 2022 ³⁴⁴	LG (10) HG (9)	• Maintained cell compositions over 7d: epithelial, immune, fibroblast • Immune cells functional x 7d • 5/6 reduced viability and Tregs after treatment with TAK981, SUMOylation inhibitor
In vivo models			
Patient-derived xenograft (PDX)	Flatmark, <i>et. al.</i> 2007 ³⁴⁵	HG (2)	• Subcutaneous (5 passages to engraft) • Intraperitoneal (14 passages to engraft)
	Mavanur, <i>et. al.</i> 2010 ³³⁵	HG (1)	• 2/17 attempted successfully implanted; 1 successfully engrafted
	Kuracha, <i>et. al.</i> 2016 ³⁴⁶	LG ATPD (5) HG ATPD (1)	• 13 samples grafted in nude mice • 6 passaged for at least 3 generations • Similar histologically to original tumour • reduction in mucin parameters after Bro+Nac
Xenograft-derived explants (XDE)	Dilly, <i>et. al.</i> 2021 ²⁶² (Mavanur ³³⁵ method)	LG (8) Mucinous (5) Non-mucinous (3)	• Nude rate: cultured tissue from Bro+NAC treated PDX and control in Ox • Higher DNA adducts in tissues previously exposed to Bro+NAC than control

^aTissue is cultured directly in media and no gelatin sponge or other matrix

Abbrev. ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; 5FU, 5 fluorouracil/folinic acid, Ox, oxaliplatin, FOLFOX, 5FU + Ox; FOLFIRI, 5FU + irinotecan; Bro+Nac, bromelain + N-acetylcysteine; Reg, regorafenib; PDO, patient-derived organoids; PDE, patient-derived explant; PDX, patient-derived xenograft; XDE, xenograft derived explant.

1.3.1. Cell lines

There are only three cell lines from ATPD reported in the literature.^{269, 341} There are no reports of cell lines developed from primary HG ATs. Some of the literature evaluating mechanisms of mucin production and effect of mucolytic agents has been performed in cell lines from human mucinous colorectal cancer (e.g. LS174T),²⁶² which may have similarities to ATPD, but also has distinct differences. The first reported immortalised cell lines derived from two cases of ATPD (N14A, N15A; Table 1.8).²⁶⁹ Only two sustainable lines out of 50 primary cell lines were characterised.²⁶⁹ Interestingly, the AT cells in primary culture produced a layer of macroscopic mucin visible in the flask and exhibited a doubling time of 48 hours.²⁶⁹ Importantly, genetic analysis demonstrated matched profiles to that taken from control tissue samples from the same patients and did not match to any known profile of cell lines in the American Type Culture Collection.²⁶⁹ The authors used the established cell lines to assist validation of candidate genes identified from differential gene expression analysis by exon-array analysis comparing ATPD tissue with normal appendiceal and colonic tissue.²⁶⁹ They identified *ARHGAP24* (encodes Rho GTPase-activating protein 24, involved in p53 regulation³⁴⁷ as consistently upregulated in the ATPD cell lines compared to colonic cell lines (HCT116, CaCo2, HT-29, Lovo and C32).²⁶⁹

The third cell line, NCC-PMP1-C1, was established from disseminated HG ATPD that had metastasised to the thigh (Table 1.8).³⁴¹ Extraperitoneal metastases are unusual,^{32, 50} so this cell line seems less relevant for studying ATPD. Yet, on review of the case imaging provided in the published manuscript, the ‘thigh’ metastasis anatomically appeared to result from metastasis through the obturator foramen (i.e. direct local extension rather than haematogenous spread), which supports the biological relevance of this cell line. Regardless, the investigators documented these cells to harbour a *KRAS*^{G12V} mutation. Furthermore, transplantation of the tumour cells in BALB/c nude mice resulted in abdominal mucinous tumours similar in morphology and protein expression to the original tissue. A high throughput drug screen of 210 agents was conducted and inhibitory effects of mitomycin C, daunorubicin, homoharringtonine and ponatinib were suggested (the latter three agents are not reported in use for this tumour type).

These three cell lines are important for preclinical research. However, they lack diversity for the clinical spectrum of the disease, limiting their utility.

1.3.2. Patient-derived organoids

Patient-derived organoids have been reported in the literature for investigating gastrointestinal tumours.^{342, 343} Votanopoulos *et. al.* successfully generated organoids from nine patients (out of twelve total attempts) with AT and ATPD (Table 1.8).³⁴² There was no response observed (viability > 95%) in the organoids from LG ATPD.³⁴² Exposure to chemotherapy demonstrated variable cell viability with LIVE/DEAD³ staining of organoids from HG ATPD (viability 18% for oxaliplatin and 5-fluorouracil/folinic acid to 100% for oxaliplatin alone), perhaps due to previous exposure to systemic chemotherapy (Table 1.8).³⁴²

1.3.3. Patient-derived explants

Ex vivo cultures or patient-derived explants (PDE) of peritoneal malignancies are a novel way to evaluate biological processes affecting both the tumour cells and the microenvironment. PDEs have been shown in breast and prostate tumours to provide a high throughput, cost effective model that retains tissue architecture and microenvironment.^{348, 349} Other tumour types studied by this method include ovarian, endometrial, pancreatic and mesenchymal tumours.³⁵⁰⁻³⁵³ PDEs provide a robust preclinical tool to evaluate drug efficacy, resistance mechanisms and biomarker evaluation which are essential for development of new therapeutic strategies.³⁴⁹

Preclinical models that recapitulate the TME, including heterogeneity of cells and architecture, provide an important platform for understanding patient-specific tumour biology, potential mechanisms of drug sensitivity and testing of novel therapeutics.^{331, 349} PDE models are made from fresh tissue pieces that are obtained at the time of a procedure and then placed on or in matrix surrounded by culture medium and can be maintained for many days.³²⁷

While advantages to the PDE method are outlined in Table 1.7, a key advantage is the preservation of an intact TME, which helps evaluate short-term biological responses.³²⁷ There have been no previous reports of this method in ATPD. A similar *ex vivo* organotypic culture method was described for ATPD (n=19).³⁴⁴ This was similar to that for PDEs, but involved solidifying fresh tissue fragments (5-10 ml) into agarose gel which were then sectioned using a vibroslicer.³⁴⁴ These tissue ‘slices’ were cultured in medium within polyester membranes in wells or formalin-fixed and paraffin-embedded.³⁴⁴ They were reported to maintain viability, consistent cellular proportions and functional immune cells for seven days.³⁴⁴

Other researchers have cultured *ex vivo* cultures of ATPD in a similar manner to the PDE method, but with no matrix support (i.e. the tissue pieces were cultured directly in the medium with no gelatine sponge or other matrix).²⁶² The characteristics of mucin and role for mucolytics were investigated in non-matrix PDE models from mucinous and non-mucinous ATPD.²⁶² They tested dilutions of patient-derived mucin laid over a colorectal cell line (LS174T) that was treated with oxaliplatin. Increased cell death corresponding to increased mucin dilutions was observed. This was confirmed by increasing intracellular platinum levels in serial mucin dilutions compared to undiluted mucin.²⁶²

³LIVE/DEAD Viability/Cytotoxicity Kit, ThermoFisher, Waltham MA

Furthermore, exposure of patient-derived mucin from ATPD to the mucolytic combination of bromelain and N-acetylcysteine, resulted in reduced weight of the mucin gel, reduced viscosity as measured by a syringe pump viscometer assay and no detection of MUC2 protein on western blot.²⁶² Finally, they demonstrated increased tumour cell death in ATPD tissue treated with a combination of mucolytics and mitomycin C compared to mitomycin C alone.

1.3.4. Patient-derived xenografts

There has been limited study of *in vivo* animal models of ATPD. Three groups have developed ATPD patient-derived (PDX) models.^{335, 345, 346} Similar to the cell lines described, these models derive from only a small number of patients and do not fully represent the full spectrum of disease. They are also challenging to generate and sustain. Six of 13 engrafted tumours passaged for at least three generations in one study³⁴⁶ and two out of 17 in another.³³⁵ Histological, features and *KRAS* mutation status were similar in mouse compared to human tumours.^{335, 345, 346} Although in one model there were differences in morphology compared to the human tumour which included adherence to mouse intestinal serosa, mouse stromal and vascular infiltration, and lack of fibrosis in the mouse tumour, the latter thought to relate to a lack of host immune response.³³⁵

Interestingly, only one of the cases implanted subcutaneously engrafted and achieved less passages compared to those implanted intraperitoneally (5 versus 14 passages).³⁴⁵ This suggests that there may be a role in the host environment (exclusive of the host immune response) with the peritoneal niche more conducive to tumour growth. Another notable difference was a higher Antigen Kiel 67 (Ki67) proliferation rate mouse tumour (over 50%) compared to the human tumour (primary tumour less than 10% and peritoneal tumour 10-50%).³⁴⁵ The reason for this discrepancy is unclear and interpretation of this difficult. Ki67 has not been widely validated for ATPD.³⁴⁵ There has been only one published study evaluating Ki67 which demonstrated an average Ki67 of 15% with focal hotspots up to 50%.³⁵⁴ Thus, a conceivable explanation for this is that the selection pressure of highly proliferative tumour cells, which are more likely to survive the engraftment process, results in enrichment of high Ki67 proliferating tumour cells in the PDX model.

While PDX models are the gold standard for preclinical research, they are resource intensive with a prolonged turnaround time. This limits capacity for use in personalised precision oncology. They have been demonstrated to be challenging to generate in ATPD due to specific difficulties in this tissue type related to low tumour cellularity and high mucinous components. However, the biological insights from the established PDX models are informative and valuable for *in vivo* preclinical drug testing.

1.3.5. *Ex vivo* drug testing

1.3.5.1. Conventional chemotherapy

Intraperitoneal administration of chemotherapy agents resulted in growth inhibition in two PDX models of ATPD. Capecitabine was found to be the least beneficial with regorafenib suggested to have moderate effect.³⁵⁵ Table 1.9 summarises *ex vivo* drug testing studies in PDE and organoid models for the agents relevant to ATPD. Overall, results are heterogeneous, but responses are higher in HG than LG ATPD, consistent with the clinical literature.

1.3.5.2. *Mucolytics*

A PDX model designed to mimic low and high peritoneal disease burden (early growth days 8-14; late growth days 14-28) was exposed to *ex vivo* drug testing of combination mucolytics bromelain and N-acetylcysteine.²⁶² Treatment of low peritoneal disease burden compared to control mouse PDX models had reduced mean mouse body weight (310 g versus 270 g), mean abdominal girth (32 mm vs 27 mm), and reduced mean quantity of tumour (15 g versus 7.5 g). High burden mouse PDX models did not tolerate the treatment. Instead, high burden rat PDX models were investigated and the mucolytic treated animals compared to controls had less imageable ascites on pre- and post-drainage MRIs and more ascites could be drained (~100 ml vs 10 ml).²⁶²

1.3.5.3. *Immunotherapy*

Evaluation of *ex vivo* responses to immune modulating agents has specific challenges that relies on presence of an intact local immune microenvironment and an ability to mimic the peripheral immune system.^{327, 331, 356} Preclinical models without a functional immune system (including tumour-derived cell lines, organoids and nude PDXs) cannot be used for these experiments. Some investigators have devised models where stromal and immune compartments from autologous peripheral blood are added to the culture or stromal components are preserved.^{342, 343, 357}

1.3.5.4. *Ex vivo HIPEC simulation*

The administration of intraperitoneal chemotherapy in ATPD provides a unique opportunity for *ex vivo* topical treatment of anticancer agents. One study evaluated organoids from colorectal cancer peritoneal metastases to mimic *ex vivo* HIPEC conditions.³⁵⁸ The investigators found increased efficacy of mitomycin C by the addition of ATR (ataxia telangiectasia and Rad3-related protein kinase) inhibitors, which was proposed to prevent the DNA repair during cell cycle arrest that is activated by ATR after mitomycin C induced DNA damage.³⁵⁸

Table 1.9 | Summary of *ex vivo* studies testing anti-cancer agents

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Agent	Study	Tissue tested (model)	Doses tested (duration)
Monotherapy			
Oxaliplatin	Votanopoulos, <i>et. al.</i> 2019 ³⁴²	Appendiceal tumour (PDO)	1 µM (4d Rx, 7d culture)
	Dilly, <i>et. al.</i> 2021 ²⁶²	Appendiceal tumour (PDE no matrix)	100 µM (24h)
	Martin, <i>et. al.</i> 2019 ³⁵⁹	Colorectal cancer (PDE no matrix)	5 µM (72h)
	Tan, <i>et. al.</i> 2023 ³⁶⁰	Colorectal cancer (PDO)	20 µM (72h) °75 µM (7d)
5-fluorouracil/folinic acid	Votanopoulos, <i>et. al.</i> 2019 ³⁴²	Appendiceal tumour (PDO)	10 µM (4d Rx, 7d culture)
	Forsythe, <i>et. al.</i> 2020 ³⁶¹	Colorectal cancer (3D cell line/PDO/microtumour constructs)	^a LD50 78.7 µM 25 µM} (72h Rx, 96h, 50 µM} 7d and 14d)
	Tan, <i>et. al.</i> 2023 ³⁶⁰	Colorectal cancer (PDO)	°50 µM (7d)
Irinotecan (SN38)	Votanopoulos, <i>et. al.</i> 2019 ³⁴²	Appendiceal tumour (PDO)	1 µM (4d Rx, 7d culture)
	Forsythe, <i>et. al.</i> 2020 ³⁶¹	Colorectal cancer (3D cell line)	^a LD50 30.3 µM
	Tan, <i>et. al.</i> 2023 ³⁶⁰	Colorectal cancer (PDO)	°0.5 µM (7d)
Mitomycin C	Dilly, <i>et. al.</i> 2021	Appendiceal tumour (PDE no matrix)	5 µM (24h)
	Bolenz, <i>et. al.</i> 2009 ³⁶²	Urothelial cancer (PDE)	1 mg/ml (2h)
	Ali, <i>et. al.</i> 2013 ³⁶³	Nasal fibroblasts (cell culture)	0.1 – 0.5 mg/ml (x 3, 5, 10m)
Cetuximab	Martin, <i>et. al.</i> 2019 ³⁵⁹	Colorectal cancer (PDE no matrix)	20 nM (72h)
			200 nM (72h)
	Majumder, <i>et. al.</i> 2014 ³⁶⁴	Colorectal cancer / Head and neck squamous cell carcinoma (PDE TMP ^b matrix)	NR (72h)
Combination therapies			
FOLFOX	Votanopoulos, <i>et. al.</i> 2019 ³⁴²	Appendiceal tumour (PDO)	1:10:1 µM (4d Rx, 7d culture)
	Tan, <i>et. al.</i> 2023 ³⁶⁰	Colorectal cancer (PDO)	°50:75 µM (7d)
FOLFIRI (SN38)	Votanopoulos, <i>et. al.</i> 2019 ³⁴²	Appendiceal tumour (PDO)	1:10:1 µM (4d Rx, 7d culture)
	Tan, <i>et. al.</i> 2023 ³⁶⁰	Colorectal cancer (PDO)	°50: 0.5 µM (7d)
FOLFIRI + cetuximab			
	Majumder, <i>et. al.</i> 2014 ³⁶⁴	Colorectal cancer / head and neck squamous cell carcinoma (PDE TMP ^b matrix)	NR (72h)
Mucolytics			
Bromelain + N-acetylcysteine	Pillai, <i>et. al.</i> 2014 ²⁷¹	Appendiceal tumour (PDE no matrix)	300 µg/ml + 250nM (24h)
	Dilly, <i>et. al.</i> 2021 ²⁶²		

^a3D cell line composite LD50 comparing Caco-2, SW480, HT-29 and HC-116³⁶¹

^bThis study evaluated 3D matrix support using human TMPs³⁶⁴

^cStarting dose followed by 4-fold dilution series³⁶⁰

Abbrev. PDE, patient-derived explant; SN38, 7-ethyl-10-hydroxycamptothecin (an irinotecan metabolite); µTCs, microtumour constructs; LD50, lethal dose to achieve 50% cell death; TMP, tumour-stromal matrix proteins; Rx, treatment.

1.3.6. Summary of preclinical models

Multiple different methods are described for studying tumour biology, but no single model is able to comprehensively assess the composition of the TME, molecular pathways, genetic alterations and drug sensitivity.³³¹ These models all have a role in identifying potential biomarkers for validation in clinical studies which in turn should lead to improved success rate of clinical trials.³³¹ PDEs have the most utility for application as a ‘companion assay’ for patient-specific drug sensitivity testing³³¹ and have been chosen as a model to optimise for further study of ATPD.

1.4. Aims of this thesis

The overall **aim** of this project is to characterise the biology of ATPD to identify new treatment strategies and ultimately improve clinical outcomes.

The specific aims of the work described in this thesis are:

1. To evaluate the role of perioperative chemotherapy and establish predictors of clinical outcomes in patients with ATPD who undergo CRS/HIPEC.
2. To describe a comprehensive biological profile of ATPD.
3. To establish patient-derived explant (PDE) models of ATPD.

Chapter 2. Outcomes of patients with appendiceal tumours: a contemporary prospective cohort treated at a state peritonectomy service

Chapter 2 presents an Australian cohort study of a prospective database of patients with appendiceal tumours. A parallel UK cohort, which was completed as part of my ESMO Translational Research Fellowship during my PhD candidature, is presented as [Appendix 1.2](#). Components of this study also contributed to the analysis by collaborators presented in [Appendix 1.3](#).

An abbreviated preliminary analysis was presented as a poster at the American Society of Clinical Oncology (ASCO) 2022 Annual Meeting (Abstract 3629): **Strach, M.C.**, Ansari, N., Koh, C., Solomon, M., Horvath, L. and Mahon, K. Outcomes of appendiceal cancer treated at a state peritonectomy service. *J Clin Oncol.* 2022;40(16_suppl):3629–3629. Doi: [10.1200/JCO.2022.40.16_suppl.3629](https://doi.org/10.1200/JCO.2022.40.16_suppl.3629)

Terminology note: Classification of AT and ATPD is described in Table 1.1 (Chapter 1). This chapter uses clinical classification where LG AT is AMN and HG ATPD is AA; LG ATPD is acellular mucin or mucinous carcinoma peritonei and HG ATPD is peritoneal metastases.

2.1 Introduction

Appendiceal tumours are rare with an estimated incidence of 1 to 2 per million people per year.^{2, 365, 366} These tumours encompass a spectrum of clinical behaviours from more indolent appendiceal mucinous neoplasms (AMN) to more aggressive appendiceal adenocarcinomas (AA).³ Prognosis varies according to these subtypes with 5-year overall survival (OS) 46 to 96% for AMNs^{5, 6, 367} and 14 to 63% for AA.^{116, 147, 173, 368-371}

The aim of this study was to evaluate predictors of survival outcomes and the role of systemic chemotherapy in patients with appendiceal tumours treated on this campus since the establishment of a peritoneal malignancy centre in 2017. This peritoneal malignancy programme used a previously described external mentor model with the goal of efficiently establishing a highly informed and evidence-based subspeciality service to deliver safe and optimised oncological outcomes.⁸⁰ This prospective cohort is a contemporaneous series based on modern evidence-based treatment protocols.

The primary endpoint was progression-free survival (PFS) for the AMN cohort and OS for the AA cohort. Secondary endpoints were PFS (AA cohort) and OS (AMN cohort), the proportion of patients treated with systemic chemotherapy and the response to treatment.

2.2 Methods

Patients with histological confirmed appendiceal tumours who were referred for discussion by the multidisciplinary team were identified from a prospective database between inception April 2017 and October 2022 at a peritoneal malignancy centre in Sydney, Australia (Royal Prince Alfred Hospital and Chris O'Brien Lifehouse). Of 253 patients identified, 45 patients were excluded as demonstrated in the consort diagram (Figure 2.1). Data obtained included demographics, clinicopathological variables, treatment characteristics and survival status. Missing data was referenced from the medical record. The study was approved by the institutional human research ethics committee with approval for waiver of informed consent (HREC reference X20-0083, 2020/ETHO00514; [Appendix 2](#)).

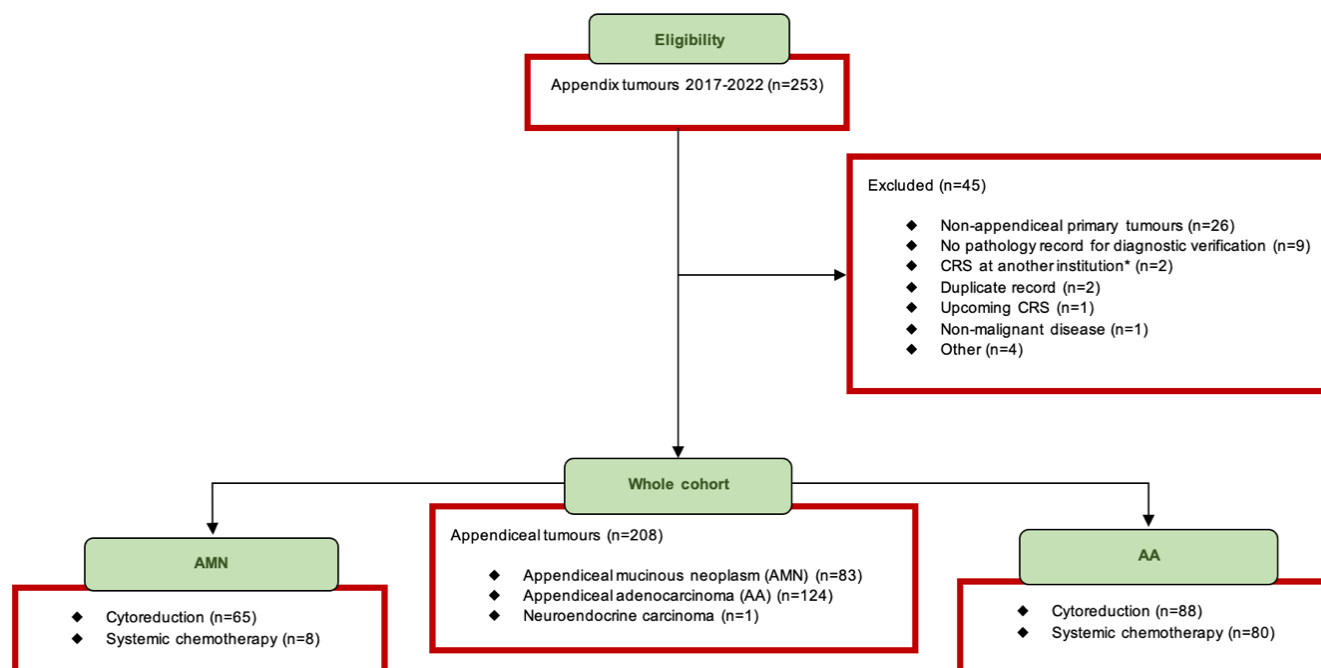


Figure 2.1 | Consort diagram

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*Included patients that had multiple CRS as long as at least one procedure was performed at this campus.

Abbrev. CRS, cytoreductive surgery; AMN, appendiceal mucinous neoplasm; AA, appendiceal adenocarcinoma.

All cases had pathology records reviewed and documented according to the current WHO 2019 grading (Chapter 1, Table 1.3),¹⁴ but the classification of peritoneal acellular mucin was retained from the PSOGI 2016 classification.³⁷² All cases on this campus that have been externally referred have pathology re-reviewed by expert gastrointestinal pathologists. Tumour cohorts were classified according to primary appendiceal tumour of origin (AMN or AA) and where primary tumour record was missing or unavailable for review, classified according to peritoneal pathology. For the AA cohort, primary and peritoneal tumour grade (defined by WHO 2019 grading)¹⁴ was classified into low and high grade to facilitate analysis (Table 2.1). Additionally, for the purpose of analysis, patients with unknown nodal status (NX) were grouped with those with node negative (N0) disease. This was based on the higher likelihood of truly having negative lymph nodes in the AMN cohort⁶ and due to a selective surgical strategy for including right hemicolectomy and lymph node sampling during CRS in the AA cohort (i.e. these procedures are only performed during CRS for high-grade tumours if local resection margin is positive, presence of lymphovascular invasion and/or evidence of nodal involvement).^{89, 373, 374}

The operative approach including short-term operative outcomes has been described in detail previously.⁸⁰ The PCI was calculated by the operating surgeon at the time of CRS and scored 0-39.³⁷⁵ The CC score was calculated after maximal CRS and defined by amount of residual disease: none (CC0), < 0.25 cm (CC1), 0.25-2.5 cm (CC2) and > 2.5 cm (CC3).³⁷⁵ Mitomycin C (15 mg/m² over 60 minutes) or oxaliplatin (350 mg/m² over 30 mins and intravenous 5-fluorouracil).

Table 2.1 | Classification of primary and peritoneal pathology for patients with appendiceal adenocarcinoma^a

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Classification	Primary tumour	Peritoneal tumour
Low grade	Grade 2 ^b mucinous adenocarcinoma	Grade 1 and 2 mucinous carcinoma peritonei
	Low grade ^c adenocarcinoma NOS	Low grade ^c adenocarcinoma NOS metastases
	Grade 1 and 2 goblet cell adenocarcinoma	Grade 1 and 2 goblet cell adenocarcinoma metastases
High grade	Grade 3 mucinous adenocarcinoma (1-50% srcs)	Grade 3 mucinous carcinoma peritonei (1-50% srcs)
	High grade ^c adenocarcinoma NOS	High grade ^c adenocarcinoma NOS metastases
	Grade 3 goblet cell adenocarcinoma	Grade 3 goblet cell adenocarcinoma metastases
	Signet-ring cell adenocarcinoma (>50% srcs)	Signet-ring cell adenocarcinoma metastases (>50% srcs)

^aHistological grading is per the WHO 2019 grading¹⁴

^bThere is no grade 1 mucinous adenocarcinoma

^cIn the WHO 2019 grading system, adenocarcinoma NOS is graded into two tiers (low grade and high grade) in the same manner as colorectal cancer¹⁴

Abbrev. NOS, not otherwise specified; srcs, signet-ring cells; WHO, World Health Organisation.

Systemic chemotherapy was given as per standardised protocols. Systemic chemotherapy was defined by treatment context as: (1) ‘neoadjuvant’, within six months before CRS/HIPEC, (2) ‘adjuvant’, within six months after CC0-1 CRS/HIPEC or primary tumour resection if no CRS/HIPEC (such as right hemicolectomy), (3) ‘prior’, more than six months before CRS/HIPEC, and (4) ‘palliative’, following CC2-3 CRS/HIPEC (noting that HIPEC was omitted for CC3 disease) or no CRS/HIPEC. Patients with peritoneal metastasis who did not receive active treatment received best supportive care (BSC). For the purposes of analysis, patients who had prior, neoadjuvant and adjuvant systemic chemotherapy were combined into one group termed ‘neo/adjuvant’.

Tumour markers were defined by laboratory limits with CEA, 3 µg/L or less, CA125, 35 kU/L or less, and CA19.9, 37 kU/L or less, considered normal. PFS was defined as the time of diagnosis to the first of disease recurrence, progression or death, and censored for last follow-up. OS was defined as the time from diagnosis to death of any cause and censored for last follow-up. Response rate to chemotherapy was recorded based on the modified Response Evaluation Criteria in Solid Tumours (RECIST 1.1)³⁷⁶ or clinician report (including response assessed at diagnostic laparoscopy). Clinical benefit rate was defined as proportion of patients who had response as well as stable disease.

2.2.1 Statistical analyses

The cohort was described with descriptive statistics. Survival analysis was performed using the Kaplan-Meier method with log-rank p-value <0.05 to establish statistical significance. Univariate and multivariable analysis was performed using Cox regression. Analyses were performed in R version 4.3.2 (2023-10-31).

2.3 Results

2.3.1 Patient cohort

A total of 208 patients with appendiceal tumours (40% AMN, 60% AA) with a median age at diagnosis of 56 years were identified for further analysis. There were baseline differences as expected comparing the AMN cohort to the AA cohort (Table 2.2). The median follow-up of the whole cohort was 33 months (1 to 306 months); 30 months (1 to 306 months) and 35 months (2 to 148 months) for the AMN and AA cohorts respectively. Most of the patients in the AMN cohort were node negative (N0, 73%) and had peritoneal disease (M1 stage 83%, noting 17% was acellular mucin). In the AA cohort, 40% had node positive disease and 66% had peritoneal metastases. The most common primary tumour type was AMN (38%) and in the AA cohort, mucinous adenocarcinoma (24%). There were slightly more patients with AMN who had CRS/HIPEC (78%) compared to those with AA (71%). Mitomycin C was the most used HIPEC agent (89% in AMN and 73% in AA cohort). Peritoneal disease burden at the time of CRS/HIPEC was higher in those with AMN compared with AA (median PCI 27 compared to 19) and this is reflected in higher pre-CRS tumour markers (AMN versus AA: CEA 10 versus 3 and CA125 42 versus 25). Complete or minimal residual disease (CC0-1 cytoreduction) was achieved in 80% of the cohort, similar in AMN and AA.

Systemic chemotherapy was given to more patients with AA than with AMN (65% compared to 10%) reflective of clinical treatment patterns. Of AA patients who received chemotherapy (n=80), 12 (15%) received it in the neoadjuvant setting and 22 (28%) in the adjuvant setting. The most common neo/adjuvant regimen was fluoropyrimidine and oxaliplatin (82%); including one patient who received neoadjuvant fluoropyrimidine, oxaliplatin and irinotecan (FOLFOXIRI regimen). There were 52 patients who had tumour molecular profiling for whom 12 revealed *KRAS* mutations (11 were AA). Microsatellite stability status or mismatch repair (MMR) testing was performed in 65 patients and only one patient (with AA) was found to be deficient in MMR.

Overall, these findings were consistent with the expected characteristics for patients with AMN and AA. The higher proportion of AA compared to AMN cases may relate to underlying disease epidemiology and referral patterns.

Table 2.2 | Patient characteristics at baseline by tumour type cohort
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Characteristic	Whole Cohort N = 208 (%)	AMN Cohort n = 83 (%)	AA Cohort n = 124 (%)
Age at diagnosis, median (range), years	56 (18-81)	57 (25-78)	56 (18-81)
Sex			
Female	110 (53)	43 (52)	66 (53)
Male	98 (47)	40 (48)	58 (47)
Node stage at diagnosis			
NX	53 (25)	21 (25)	32 (26)
N0	104 (50)	61 (73)	43 (35)
N1	32 (15)	1 (1)	31 (25)
N2	19 (9)	0 (0)	18 (15)
Metastasis stage at diagnosis			
MX	2 (1)	0 (0)	2 (2)
M0	54 (26)	14 (17)	42 (34)
M1	152 (73)	69 (83)	82 (66)
M1a (acellular mucin)	13 (6)	14 (17)	0 (0)
M1b/c	139 (67)	55 (66)	82 (66)
Primary AMN and grade^a			
Low grade (LAMN)	79 (38)	79 (95)	-
High grade (HAMN)	72 (35)	72 (87)	-
	7 (3)	7 (8)	-
Primary AA and grade	112 (54)		112 (90)
MAC	49 (24)	-	49 (40)
Grade 2	23 (11)	-	23 (19)
Grade 3	21 (10)	-	21 (17)
Missing	5 (2)	-	5 (4)
ANOS	26 (13)	-	26 (21)
Low grade	19 (9)	-	19 (15)
High grade	7 (3)	-	7 (6)
Missing	1 (0.5)	-	1 (1)
GCA	32 (15)	-	32 (26)
Grade 1	4 (2)	-	4 (3)
Grade 2	6 (3)	-	6 (5)
Grade 3	13 (6)	-	13 (10)
Missing	9 (4)	-	9 (7)
SRC	5 (2)	-	5 (1)
Neuroendocrine carcinoma	1 (0.5)	-	-
Missing ^b	16 (8)	4 (5)	12 (10)
Peritoneal tumour pathology and grade^a			
Mucinous (AMN/MAC)			
Acellular mucin	14 (7)	14 (17)	0 (0)
Grade 1 MCP ^c	56 (27)	54 (65)	2 (2)
Grade 2 MCP ^c	17 (8)	1 (1)	16 (13)
Grade 3 MCP ^c	20 (14)	0 (0)	20 (16)
Non-mucinous metastases			
ANOS			
Low grade	9 (4)	-	9 (7)
High grade	9 (4)	-	9 (7)
Missing	2 (1)	-	2 (2)
GCA			
Grade 1	1 (0.5)	-	1 (1)
Grade 2	3 (1)	-	3 (2)
Grade 3	16 (8)	-	16 (13)
SRC ^d	4 (2)	-	4 (3)
Molecular profiling	52 (25)	5 (6)	46 (37)
Mutation identified	17 (8)	1 (1)	16 (13)
KRAS mutations	12 (6)	1 (1)	11 (9)
G12V	6 (3)	-	5 (4)
G12D	5 (2)	-	5 (4)
G13D/N	1 (0.5)	-	1 (1)
Missing	2 (1)	-	-
Non-KRAS mutations			
BRAF V600E	2 (1)	-	2 (2)
NRAS Q61R	2 (1)	-	2 (2)
GNAS R201H	1 (0.5)	-	1 (1)
IDH1 R132C	1 (0.5)	-	1 (1)
PI3KCA	1 (0.5)	-	1 (1)
Other ^k	1 (0.5)	1 (1)	1 (1)
Mismatch repair (MMR)/microsatellite instability (MSI)	65 (31)	16 (19)	49 (40)
Deficient MMR/MSI-high	1 (0.5)	0 (0)	1 (1)
Proficient MMR/MSI-low/stable	64 (31)	16 (19)	48 (39)
CRS	154 (74)	65 (78)	88 (71)
No surgery	11 (5)	1 (1)	10 (8)
Non-CRS surgery ^c	44 (21)	18 (22)	26 (21)
Appendicectomy	24 (12)	13 (16)	11 (9)
Right hemicolectomy	11 (5)	3 (4)	8 (6)
Total abdominal hysterectomy and bilateral salpingoophorectomy	6 (3)	4 (5)	2 (2)
Other ^f	6 (3)	0 (0)	6 (5)
Unknown	1 (0.5)	1 (1)	0 (0)

Characteristic	Whole Cohort N = 208 (%)	AMN Cohort n = 83 (%)	AA Cohort n = 124 (%)
CRS Type			
CRS	132 (63)	56 (67)	75 (60)
Debulking	22 (11)	9 (11)	13 (10)
Patients who had CRS (n=154)			
HIPEC	135 (65)	58 (70)	77 (62)
Mitomycin C	122 (59)	58 (70)	64 (52)
Oxaliplatin	11 (5)	0 (0)	11 (9)
No HIPEC [§]	15 (7)	5 (6)	9 (7)
Missing	2 (1)	2 (2)	0 (0)
PCI, median (range)	23 (0-39)	27 (2-39)	19 (0-39)
Missing	9 (4)	3 (4)	6 (5)
CC score			
0	100 (48)	45 (54)	54 (44)
1	23 (11)	8 (10)	15 (12)
2	1 (0.5)	0 (0)	1 (1)
3	21 (10)	9 (11)	13 (10)
Missing	8 (4)	3 (4)	5 (4)
Tumour markers (pre-CRS), median (range)			
CEA (µg/L)	5 (1-455)	10 (0.9-455)	3 (0.8-150)
CA125 (kU/L)	31 (2-417)	42 (2-307)	25 (2-2502)
CA19.9 (kU/L)	24 (1-2502)	25 (<1-570)	27 (4-417)
Missing	6 (3)	2 (2)	4 (2)
Radiotherapy	3 (1.4)	0	3
Patients who had systemic chemotherapy (n=89)			
Systemic chemotherapy (definition)	89 ^b (43)	8 (10)	80 (65)
Prior (>6m CRS/HIPEC)	7 (3)	1 (1)	6 (5)
Neoadjuvant (<6m pre-CRS/HIPEC)	14 (7)	2 (2)	12 (10)
Adjuvant (<6m post-CC0/1 CRS/HIPEC)	38 ^b (18)	2 (2)	35 (28)
Both neoadjuvant and adjuvant	6 (3)	0 (0)	6 (5)
Palliative (post-CC2/3 CRS/HIPEC or no CRS)	24 (12)	3 (4)	21 (17)
First line systemic chemotherapy regimen			
Fluoropyrimidine	12 (6)	5 (6) ⁱ	7 (6)
+ Oxaliplatin	65 (31)	1 (1)	64 (52)
+ Irinotecan	3 (1)	0 (0)	3 (2)
+ Oxaliplatin + irinotecan	4 (2)	0 (0)	4 (3)
+ Biologic	18 (9)	0 (0)	
Bevacizumab	16 (7)	0 (0)	16 (13)
Cetuximab	2 (1)	0 (0)	2 (2)
Other ^j	4 (2)	2 (2)	2 (2)
Missing	1 (0.5)	0 (0)	1 (1)

^aPathology classification is based on the WHO 2019 grading,³⁷² but also includes PSOGI 2016 grading¹⁵ of acellular mucin as a discrete entity.

^bFor the n=16 cases where primary tumour pathology was missing, morphology was based on the peritoneal pathology by the reporting pathologist: AMN (n=4), ANOS (n=3), MAC (n=5), GCA (n=3), SRC (n=1).

^cMCP results from AMN or MAC primary tumours, and if arising from MAC primary where minimum grade is G2 then the peritoneal metastases are classified as discordant.

^dSRC on peritoneal pathology arising from SRC primary (n=1), MAC primary (n=1), GCA primary (n=1), primary tumour pathology missing (n=1).

^eNon-CRS surgical interventions were for the 1st procedure done, some done at external institutions prior to referral. Patients could have more than one of the procedures listed, thus numbers do not add to total.

^fOther non-CRS procedures included: ileocaecectomy (n=1), excision of primary tumour (n=1), small bowel bypass (n=1), Open low Hartmann's (n=1), loop ileostomy (n=1), ileocolic resection + ileostomy (n=1)

^gCC3 n=13 (HIPEC is not routinely administered for CC3 residual disease due to evidence of poor outcomes,¹⁴⁵ CC0 n=1 (patient had metabolic acidosis at CRS and HIPEC was withheld for concern regarding excess toxicity)

^hNeuroendocrine carcinoma (n=1)

ⁱOne of the patients who had neoadjuvant chemotherapy had radiologically stable disease after 12 cycles of capecitabine but had no evidence of any pathological response at the time of CRS.

^jOther chemotherapy regimens: carboplatin + etoposide (n=2), mitomycin C + capecitabine (n=2).

^kSMAD4 loss, P53 Loss and VHL loss (n=1)

Abbrev. AMN, appendiceal mucinous neoplasm; AA, appendiceal adenocarcinoma; MAC, mucinous adenocarcinoma; ANOS, adenocarcinoma not otherwise specified; GCA, goblet cell adenocarcinoma; SRC, signet ring cell adenocarcinoma; LAMN, low grade appendiceal mucinous neoplasm; HAMN, high grade appendiceal mucinous neoplasm; MCP, mucinous carcinoma peritonei; CRS, cytoreductive surgery; HIPEC, heated intraperitoneal chemotherapy; PCI, peritoneal cancer index; CC, completeness of cytoreduction; MMR, mismatch repair; MSI, microsatellite instability.

2.3.2 Survival outcomes

There were 95 PFS events and 53 deaths in the whole cohort. Median PFS was 42 months (range 1 to 185 months) and 5-year PFS was 39% (Figure 2.2A). Median OS for the whole cohort was 84 months (range 1 to 306 months) and 5-year OS was 59% (Figure 2.2B). Patients with AMN had improved PFS and OS compared to patients with AA (Figure 2.2C,D).

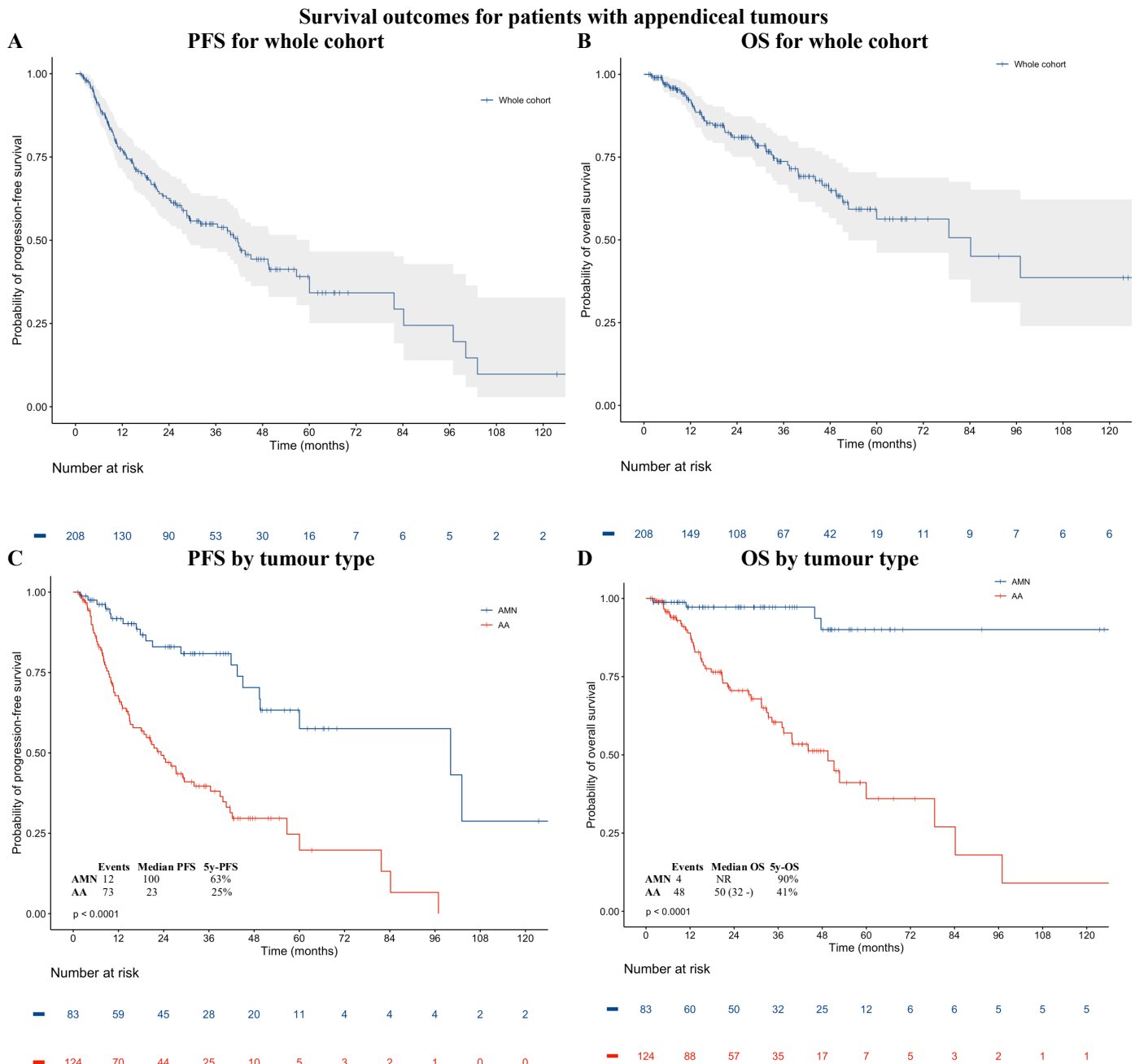


Figure 2.2 | Survival outcomes of patients with appendiceal tumours

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Kaplan-Meier curves show the OS (A) and PFS (B) for the whole cohort (A) and OS (C) and PFS (D) by tumour subtype comparing AMN to AA.

Abbrev. OS, overall survival; PFS, progression-free survival; AMN, appendiceal mucinous neoplasm; AA, appendiceal adenocarcinoma.

2.3.2.1 Outcomes for AMN cohort

Analysis of prognostic variables suggested shorter PFS in this cohort on univariate analysis for M stage, CRS, PCI, CC score, elevated pre-CRS CA125 and receipt of systemic chemotherapy ($p < 0.05$; Table 2.3). Multivariable analysis indicated that systemic chemotherapy ($p = 0.018$) and high burden peritoneal disease (PCI 30-39, $p = 0.015$) retained prognostic significance (Table 2.3 and Figure 2.3). Elevated pre-CRS tumour markers CA125 ($p = 0.035$) and CA19.9 ($p = 0.0061$) were associated with shorter OS on univariate analysis (multivariable analysis was not performed due to the low event rate).

Table 2.3 | Univariate and multivariable analysis of prognostic factors for PFS for patients with AMN

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Variable	n	Events	Median PFS, months	5-year PFS	Univariate analysis (n = 83)		Multivariable analysis ^b (n = 62, Events = 18)	
					HR (95% CI)	p-value	HR (95% CI)	p-value
AMN	83	21	100	63%	-	-	-	-
Sex								
Male	40	9	NR	61%	ref		-	-
Female	43	12	100	66%	0.95 (0.39-2.30)	0.90	-	-
Age, years								
≤ 55	40	11	185	55%	ref		-	-
> 55	43	10	100	71%	0.76 (0.31-1.83)	0.53	-	-
N Stage								
N0/X	82	21	100	63%	-	-	-	-
N1/2	1	0						
M Stage								
M0	14	2	NR	61%	} ref		-	-
M1a	13	0	NR	4y 100%	} ref		-	-
M1b/c	56	19	60	55%	4.20 (0.96-18.40)	0.038*	-	-
Primary grade								
Low grade	72	18	100	58%	-	0.23	-	-
High grade	7	0	NR	4y 100%			-	-
Peritoneal grade								
No disease	13	1	NR	67%	ref		-	-
Acellular mucin	14	0	NR	4y 100%	-		-	-
Grade 1 MCP	54	20	60	53%	4.02 (0.53-30.3)	0.074	-	-
Grade 2 MCP	1	0	NR		-		-	-
CRS								
Yes	65	20	100	57%	-		-	-
No	18	1	185	100%		0.024*	-	-
HIPEC								
Yes ^a	58	16	103	61%	ref		-	-
No	5	2	29	2y 67%	3.23 (0.70-14.80)	0.11	-	-
PCI								
<10	11	1	NR	83%	ref		ref	
10-19	7	2	103	86%	2.74 (0.24-32.0)		3.85 (0.25-33.2)	0.40
20-29	16	4	60	68%	3.92 (0.43-36.0)	0.0031*	3.53 (0.38-32.6)	0.27
30-39	28	11	29	4y 24%	19.40 (2.18-173.00)		15.6 (1.71-142)	0.015*
CC score								
0	45	11	103	67%	ref		-	-
1	8	2	45	4y 38%	1.51 (0.33-6.97)	0.033*	-	-
2/3	0/9	6	42	18%	3.57 (1.29-9.87)		-	-
CEA (µg/L)								
≤ 3	11	1	NR	90%	ref		-	-
> 3	52	18	60	53%	3.74 (0.50-28.20)	0.17	-	-
CA125 (kU/L)								
≤ 35	30	5	103	84%	ref		-	-
> 35	34	14	44	34%	3.55 (1.26-10.0)	0.011*	-	-
CA19.9 (kU/L)								
≤ 37	41	9	103	68%	ref		-	-
> 37	23	10	42	43%	2.31 (0.93-5.78)	0.066	-	-
Systemic chemotherapy								
No	75	15	103	74%	ref		ref	
Yes	8	6	21	3y 43%	7.24 (2.49-21.0)	<0.0001*	3.73 (1.26-11.0)	0.018*

^aAll had mitomycin C HIPEC.

^bMultivariate analysis: Included two most significant univariate variables due to low event rate

*Denotes significant $p < 0.05$

Abbrev. PFS, progression-free survival; CI, confidence interval; HR, hazard ratio; NR, not reached/calculable; AMN, appendiceal mucinous neoplasm; MCP, mucinous carcinoma peritonei; CRS, cytoreductive surgery; HIPEC, heated intraperitoneal chemotherapy; PCI, peritoneal cancer index; CC, completeness of cytoreduction.

Effect of significant prognostic variables on PFS in AMN cohort

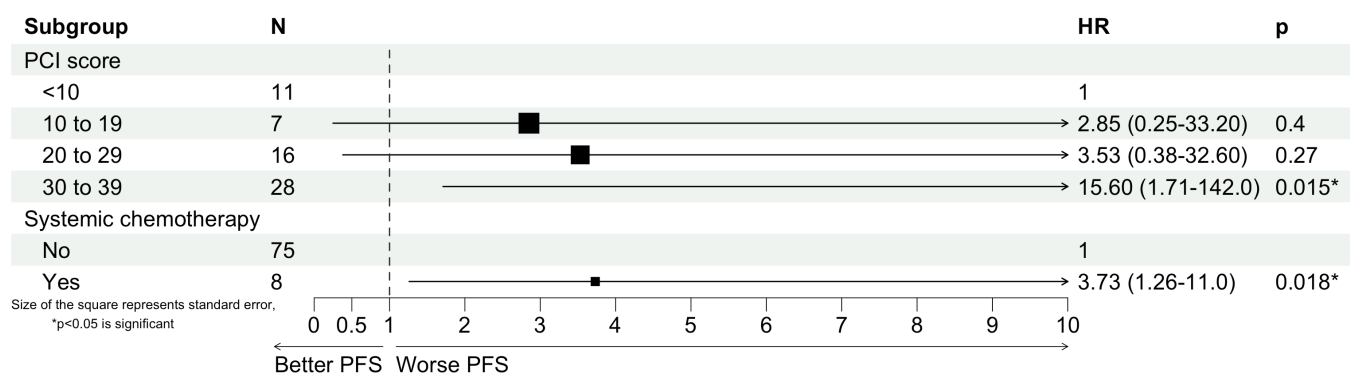


Figure 2.3 | Forest plot after Cox regression analysis in AMN cohort

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Abbrev. PFS, progression-free survival; AMN, appendiceal mucinous neoplasm; PCI, peritoneal cancer index.

2.3.2.2 Outcomes for AA cohort

a) CRS cohort

For patients with AA who had CRS, factors associated with shorter PFS on univariate analysis included M1 stage, primary tumour type of mucinous and signet-ring cell adenocarcinoma, high-grade primary or peritoneal grade, higher PCI, residual disease post CRS, no HIPEC, elevated pre-CRS CEA and CA125, receipt of systemic chemotherapy and palliative intent chemotherapy ($p < 0.05$; Table 2.4). Following multivariable analysis, the only factor significantly associated with shorter PFS was CC1 cytoreduction ($p = 0.020$; Table 2.4).

In this cohort, factors associated with shorter OS on univariate analysis were similar to those described previously for PFS, except for the addition of positive lymph node status ($p = 0.0053$; Table 2.5). On multivariable analysis CC1 status ($p = 0.016$) and high-grade primary tumour grade ($p = 0.047$) remained significantly associated with shorter OS (Table 2.5 and Figure 2.4).

Table 2.4 | Univariate and multivariable analysis of prognostic factors for PFS for patients with AA who had CRS

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Variable	n	Events	Median PFS, months	5-year PFS	Univariate analysis (n = 88)		Multivariable analysis ^a (n = 73, events = 39)	
					HR (95% CI)	p-value	HR (95% CI)	p-value
AA and CRS	88	48	29	30%				
Sex								
Male	41	22	29	33%	ref			
Female	47	26	27	24%	0.92 (0.52-1.65)	0.79	-	
Age, years								
≤ 55	47	25	24	34%	ref			
> 55	41	23	36	27%	0.86 (0.48-1.53)	0.60	-	
N Stage								
N0/X	49	23	36	31%	ref			
N1/2	39	25	19	27%	1.54 (0.86-2.74)	0.14		
M Stage								
M0	24	4	57	74% 4y	ref		ref	
M1b/c	64	44	21	23%	2.28 (1.36-3.81)	0.00049*	1.67 (0.73-3.84)	0.23
Primary tumour type								
ANOS	18	11	22	17%	ref			
MAC	40	23	24	33%	1.08 (0.50-2.30)			
GCA	25	9	41	48%	0.54 (0.22-1.34)			
SRC	3	3	8	33% 12m	4.63 (1.22-17.70)	0.0078*		
Missing	2							
Primary grade^b								
Low grade	39	15	57	40%	ref		ref	
High grade	41	29	16	19% 4y	3.13 (1.60-6.15)	0.00047*	1.86 (0.84-4.08)	0.12
Missing	8							
Peritoneal grade^b								
Low grade	25	14	30	28%	ref			
High grade	43	33	16	17%	2.01 (1.06-3.83)	0.03*		
No disease	20							
HIPEC								
Yes	77	40	29	36%	ref			
No	9	6	6	14% 2y	2.88 (1.21-6.87)	0.013*		
Missing	2							
PCI								
<10	26	5	NR	64%	ref		ref	
10-19	17	14	21	22%	4.04 (1.41-11.5)		1.40 (0.34-5.81)	0.64
20-29	13	6	36	36% 4y	2.75 (0.84-9.04)		1.08 (0.22-5.31)	0.93
30-39	26	18	12	23%	6.06 (2.24-16.4)	0.0012*	0.79 (0.15-4.21)	0.78
Missing	6							
CC								
0	54	21	41	48%	ref		ref	
1	15	13	15	20% 2y	4.90 (2.35-10.2)	<0.0001*	3.31 (1.20-9.08)	0.020*
2/3	14	11	11	19%	3.50 (1.65-7.42)		2.26 (0.60-8.48)	0.23
Missing	5							
CEA, µg/L								
≤ 3	40	15	41	49%	ref			
> 3	44	30	19	15%	2.40 (1.29-4.50)	0.0046*		
Missing	4							
CA125, kU/L								
≤ 35	50	19	41	45%	ref			
> 35	32	25	13	15%	2.37 (1.28-4.38)	0.0046*		
Missing	6							
CA19.9, kU/L								
≤ 37	52	23	36	32%	ref			
> 37	31	22	19	27%	1.43 (0.77-2.64)	0.25	-	
Missing	5							
Systemic chemotherapy								
No	37	12	82	56%	ref		ref	
Yes	51	36	21	11%	2.68 (1.35-5.31)	0.0033*	1.56 (0.63-3.87)	0.33
Setting								
None	37	12	82	56%	ref			
Neo/adjuvant	46	31	21	12%	2.44 (1.22-4.90)	0.00043*	-	-
Palliative	5	5	6	20% 3y	6.95 (2.37-20.40)			

^aMultivariable model: included p<0.10 on univariate analysis, and N status as known to be prognostic. Systemic chemotherapy by treatment setting not included in the model due to definitions according to extent of cytoreduction which is already measured in the model in the CC variable.

^bSimplified grading classification based on WHO 2019 grading¹⁴ used for this analysis as defined in Table 2.1.

*Denotes significant p<0.05

Abbrev. OS, overall survival; HR, hazard ratio; NR, not reached; CI, confidence interval; CRS, cytoreductive surgery; AMN, appendiceal mucinous neoplasm; AA, appendiceal adenocarcinoma; LAMN, low grade AMN; HAMN, high grade AMN; MAC, mucinous adenocarcinoma; ANOS, adenocarcinoma not otherwise specified; GCA, goblet cell adenocarcinoma; SRC, signet ring carcinoma; MMC, mitomycin C; Ox, oxaliplatin.

Table 2.5 | Univariate and multivariable analysis of prognostic factors for OS for patients with AA who had CRS

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Variable	n	Events	Median OS, months	5-year OS	Univariate analysis (N = 88)		Multivariable analysis ^a (n = 75, events = 27)	
					HR (95% CI)	p-value	HR (95% CI)	p-value
AA and CRS	88	29	60	52%				
Sex								
Male	41	13	NR	52%	ref			
Female	47	16	60	55%	0.95 (0.45-1.98)	0.88		
Age, years								
≤ 55	47	13	84	58%	ref			
> 55	41	16	50	46%	1.18 (0.56-2.49)	0.67		
N Stage								
N0/X	49	10	97	65%	ref		ref	
N1/2	39	19	34	41%	2.95 (1.33-6.56)	0.0053*	1.70 (0.64-5.51)	0.29
M Stage^a								
M0	24	1	60	100%	ref			
M1b/c	64	28	40	41%	3.07 (1.13-8.33)	0.0072*		
Primary tumour type								
ANOS	18	6	97	63%	ref			
MAC	40	14	NR	51%	1.02 (0.39-2.67)			
GCA	25	6	50	50%	0.66 (0.21-2.08)			
SRC	3	3	21	2y 33%	5.19 (1.24 -21.7)	0.0013*		
Missing	2							
Primary grade^b								
Low grade	39	6	97	81%	ref		ref	
High grade	41	22	32	21%	5.11 (1.92 -13.6)	0.00028*	3.05 (1.01-9.20)	0.047*
Missing	8							
Peritoneal grade^b								
Low grade	25	8	97	63%	ref			
High grade	43	21	38	37%	1.93 (0.85-4.37)	0.11		
No disease	20							
HIPEC								
Yes	77	26	60	51%	ref			
No	9	2	NR	3y 67%	1.72 (0.40-7.37)	0.46	-	
Missing	2							
PCI								
<10	26	3	60	69%	ref			
10-19	17	11	38	37%	4.52 (1.25-16.4)			
20-29	13	2	NR	4y 79%	1.39 (0.23-8.37)			
30-39	26	11	37	36%	4.71 (1.30-17.0)	0.025*		
Missing	6							
CC score								
0	54	13	84	63%	ref		ref	
1	15	9	28	4y 20%	4.10 (1.71-9.87)	0.0023*	3.18 (1.24-8.15)	0.016*
2/3	14	6	40	38%	2.52 (0.93-6.88)		2.09 (0.75-5.80)	0.16
Missing	5							
CEA, µg/L								
≤ 3	40	9	60	69%	ref			
> 3	44	20	37	37%	2.15 (0.97-4.77)	0.054		
Missing	4							
CA125, kU/L								
≤ 35	50	12	50	49%	ref			
> 35	32	17	37	40%	2.29 (1.09-4.82)	0.025*		
Missing	6							
CA19.9, kU/L								
≤ 37	52	15	50	44%	ref			
> 37	31	14	44	48%	1.12 (0.52-2.42)	0.77	-	-
Missing	5							
Systemic chemotherapy								
No	37	6	97	84%	Ref		ref	
Yes	51	23	40	40%	2.79 (1.05-7.41)	0.031*	1.01 (0.35-2.98)	0.98
Setting								
None	37	6	97	84%	ref			
Neo/adjuvant	46	19	50	44%	2.48 (0.92-7.70)	0.0058*	-	-
Palliative	5	4	23	2y 30%	7.58 (1.98-29.0)			

^aMultivariable model: included p<0.10 on univariate analysis. Systemic chemotherapy by treatment setting not included in the model due to definitions according to extent of cytoreduction which is already measured in the model in the CC variable.

^bSimplified grading classification based on WHO 2019 grading¹⁴ used for this analysis as defined in Table 2.1.

*Denotes significant p<0.05

Abbrev. OS, overall survival; AA, appendiceal adenocarcinoma; CRS, cytoreductive surgery; CI, confidence interval; HR, hazard ratio; NR, not reached/calculable; CRS, cytoreductive surgery; ANOS, adenocarcinoma not otherwise specified; MAC, mucinous adenocarcinoma; GCA, goblet cell adenocarcinoma; SRC, signet ring carcinoma; HIPEC, heated intraperitoneal chemotherapy; PCI, peritoneal cancer index; CC, completeness of cytoreduction.

Effect of significant prognostic variables on OS in patients with AA who had CRS

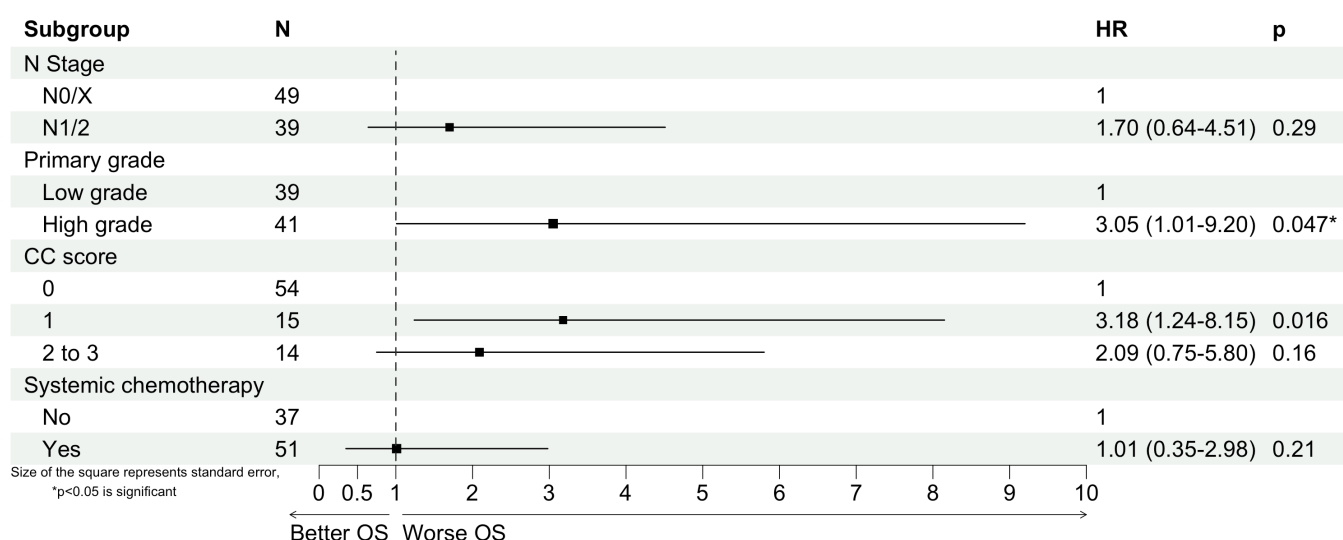


Figure 2.4 | Forest plot after Cox regression of prognostic variables on OS in AA cohort who had CRS

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Abbrev. OS, overall survival; AA, appendiceal adenocarcinoma; CRS, cytoreductive surgery; N, nodal; CC, completeness of cytoreduction; PCI, peritoneal cancer index.

b) Localised disease (M0)

Patients with localised AA who had risk-reducing CRS (n=24) had prolonged survival outcomes, with low event rates, compared to those who did not have CRS. In those who had CRS, 5-year OS was 100% and 4-year PFS was 74% compared to 5-year OS 28% (p=0.087) and 4-year PFS 36% (p=0.098) respectively in those who did not have CRS.

c) Metastatic disease (M1)

The variables associated with shorter PFS in patients with AA peritoneal metastases (n=82) were signet-ring cell adenocarcinoma tumour subtype, and high-grade primary and peritoneal tumour, no CRS and residual disease after CRS (p<0.05; Table 2.6). These were not significant on multivariable analysis (Table 2.6). Univariate analysis for OS showed similar associations, except high-grade peritoneal grade was not significant (p=0.25; Table 2.7). Following multivariable analysis, mucinous adenocarcinoma remained associated with longer OS while high-grade primary tumour and no CRS remained associated with shorter OS (p<0.05; Table 2.7).

Table 2.6 | Univariate and multivariable analysis of prognostic factors for PFS for patients with AA peritoneal metastasis

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Variable	n	Events	Median PFS, months	5-year PFS	Univariate analysis (n = 82)		Multivariable analysis ^a (n = 64, events 45)	
					HR (95% CI)	p-value	HR (95% CI)	p-value
AA M1	82	60	15	18%				
Sex								
Male	37	29	14	10%	ref	0.22	-	-
Female	45	31	19	25%	0.72 (0.43-1.22)			
Age, years								
≤ 55	40	30	19	13%	ref	0.51	-	-
> 55	42	30	14	24%	0.84 (0.50-1.41)			
N Stage								
N0/X	47	33	14	22%	ref	0.64	-	-
N1/2	35	27	15	13%	1.13 (0.67-1.90)			
Primary tumour type								
ANOS	13	24	23	28%	ref		Ref	
MAC	39	11	14	10%	0.62 (0.29-1.34)		0.53 (0.23-1.20)	0.13
GCA	13	9	26	22%	0.63 (0.25-1.57)	0.02*	0.42 (0.14-1.29)	0.13
SRC	5	5	8	1y 20%	2.66 (0.88-8.06)		0.91 (0.24-3.48)	0.90
Missing	12							
Primary grade								
Low grade	26	15	23	38%	ref	0.0034*	ref	
High grade	40	31	9	4y 10%	2.57 (1.34-4.994)		2.54 (0.96-6.72)	0.060
Missing	16							
Peritoneal grade								
Low grade	31	20	21	28%	ref		ref	
High grade	49	39	12	13%	1.74 (1.00-3.02)	0.046*	1.23 (0.48-3.13)	0.67
No disease	2							
CRS								
Yes	64	44	20	23%	ref	<0.0001*	ref	
No	18	16	9	1y 12	4.25 (2.18-8.32)		2.60 (0.76-8.88)	0.13
PCI								
<10	6	3	30	27%	ref			
10-19	15	13	20	21%	1.24 (0.34-4.46)			
20-29	13	6	36	4y 36%	0.85 (0.21-3.42)	0.28	-	-
30-39	26	18	12	23%	1.92 (0.56-6.55)			
Missing	22							
CC								
0	32	18	30	35%	ref			
1	15	13	15	2y 13%	3.29 (1.54-7.01)	0.0027*	-	-
2/3	14	11	11	4y 19%	2.46 (1.14-5.35)			
Missing	21							
CEA, µg/L								
≤ 3	21	12	29	36%	ref	0.13	-	-
> 3	40	29	18	18%	1.67 (0.85-3.29)			
Missing	21							
CA125, kU/L								
≤ 35	30	16	21	33%	ref	0.094	-	-
> 35	30	24	12	20%	1.73 (0.90-3.31)			
Missing	22							
CA19.9, kU/L								
≤ 37	32	20	21	18%	ref	0.85	-	-
> 37	29	21	19	31%	1.06 (0.56-2.01)			
Missing	21							
Systemic chemotherapy								
Neo/adjuvant	42	30	19	14%	ref		ref	
Palliative	40	30	9	19%	1.30 (0.77-2.18)	0.33	1.04 (0.50-2.18)	0.92

^aMultivariable model: included *p<0.05 on univariate analysis except for CC score as exclusive of no CRS group, included systemic chemotherapy as variable of interest.

^bSimplified grading classification based on WHO 2019 grading¹⁴ used for this analysis as defined in Table 2.1.

*Denotes significant p<0.05

Abbrev. PFS, progression-free survival; HR, hazard ratio; NR, not reached; CI, confidence interval; CRS, cytoreductive surgery; PCI, peritoneal cancer index; CC, completeness of cytoreduction; AA, appendiceal adenocarcinoma; MAC, mucinous adenocarcinoma; ANOS, adenocarcinoma not otherwise specified; GCA, goblet cell adenocarcinoma; SRC, signet ring carcinoma.

Table 2.7 | Univariate and multivariable analysis of prognostic factors for OS for patients with AA peritoneal metastasis
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Variable	n	Events	Median OS, months	5-year OS	Univariate analysis (n = 82)		Multivariable analysis ^a (n = 66, events = 33)	
					HR (95% CI)	p-value	HR (95% CI)	p-value
AA M1	82	41	33	33%				
Sex								
Male	37	19	32	26%	ref	0.61	-	-
Female	45	22	38	39%	0.85 (0.46-1.58)			
Age, years								
≤ 55	40	18	38	40%	ref	0.61	-	-
> 55	42	23	29	27%	1.17 (0.63-2.19)			
N Stage								
N0/X	47	21	44	40%	ref	0.15	-	-
N1/2	35	20	32	23%	1.58 (0.85-2.95)			
Primary tumour type								
ANOS	13	8	29	34%	ref		ref	
MAC	39	15	44	47%	0.58 (0.24-1.38)		0.37 (0.14-0.99)	0.048*
GCA	13	6	50	26%	0.61 (0.21-1.80)	0.0051*	0.39 (0.11-1.42)	0.15
SRC	5	5	12	2y 20%	3.09 (0.97-9.82)		1.02 (0.27-3.79)	0.98
Missing	12							
Primary grade^b								
Low grade	26	8	97	66%	ref		ref	
High grade	40	25	33	10%	3.64 (1.56-8.49)	0.0015*	6.43 (2.26-18.30)	0.00050*
Missing	16							
Peritoneal grade^b								
Low grade	31	14	50	45%	ref	0.25	-	-
High grade	49	26	33	28%	1.47 (0.76-2.82)			
Missing	2							
CRS								
Yes	64	28	40	41%	ref	<0.0001*	ref	
No	18	13	13	2y 18%	5.10 (2.41-10.80)		4.02 (1.29-12.50)	0.016*
PCI								
<10	6	2	50	50%	ref			
10-19	15	11	35	36%	2.16 (0.47-9.89)			
20-29	13	2	-	4y 79%	0.59 (0.02-4.25)	0.25	-	-
30-39	26	11	37	36%	2.06 (0.45-9.41)			
Missing	22							
CC								
0	32	12	84	53%	ref			
1	15	9	28	4y 20%	2.81 (1.15-6.86)	0.055	-	-
2/3	14	6	40	38%	1.93 (0.71-5.29)			
Missing	21							
CEA, µg/L								
≤ 3	21	8	84	55%	ref	0.33	-	-
> 3	40	20	33	33%	1.51 (0.66-3.47)			
Missing	21							
CA125, kU/L								
≤ 35	30	11	44	37%	ref	0.12	-	-
> 35	30	17	32	37%	1.81 (0.84-3.87)			
Missing	22							
CA19.9, kU/L								
≤ 37	32	14	40	29%	ref	0.76	-	-
> 37	29	14	44	45%	0.89 (0.41-1.92)			
Missing	21							
Systemic chemotherapy								
Neo/adjuvant	42	19	37	33%	ref	0.16	ref	
Palliative	40	22	21	33%	1.56 (0.84-2.92)		2.00 (0.82-4.89)	0.13

^aMultivariate model: included p<0.05 on univariate analysis with chemotherapy as the variable of interest.

^bSimplified grading classification based on WHO 2019 grading¹⁴ used for this analysis as defined in Table 2.1.

*Denotes significant p<0.05

Abbrev. OS, overall survival; HR, hazard ratio; NR, not reached; CI, confidence interval; CRS, cytoreductive surgery; PCI, peritoneal cancer index; CC, completeness of cytoreduction; MAC, mucinous adenocarcinoma; ANOS, adenocarcinoma not otherwise specified; GCA, goblet cell adenocarcinoma; SRC, signet ring carcinoma.

2.3.3 Systemic chemotherapy

2.3.3.1 AMN cohort

Only eight patients (10%) with AMN received systemic chemotherapy due to disease with higher risk features not meeting pathological criteria for high-grade tumours (Table 2.1)

2.3.3.2 AA cohort

Treatment setting had an impact on both PFS and OS in the AA cohort on univariate analysis ($p < 0.0001$, Figure 2.5). The potential benefit was most notable for neoadjuvant, adjuvant and prior chemotherapy (Figure 2.5). In AA patients with peritoneal metastasis (M1), systemic chemotherapy was analysed comparing neo/adjuvant to palliative chemotherapy and BSC. There was longer median OS for patients treated with neo/adjuvant chemotherapy (37 months) compared to those having BSC (21 months), but this was not statistically significant ($p = 0.16$). Exploratory analysis of chemotherapy groups by different variables indicated no significant difference in OS by adenocarcinoma subtype (mucinous adenocarcinoma, $p = 0.14$; adenocarcinoma not otherwise specified, $p = 0.11$; and goblet cell adenocarcinoma, $p = 0.19$). On analysis of outcomes by PCI, there were no significant differences in OS for intent of systemic chemotherapy.

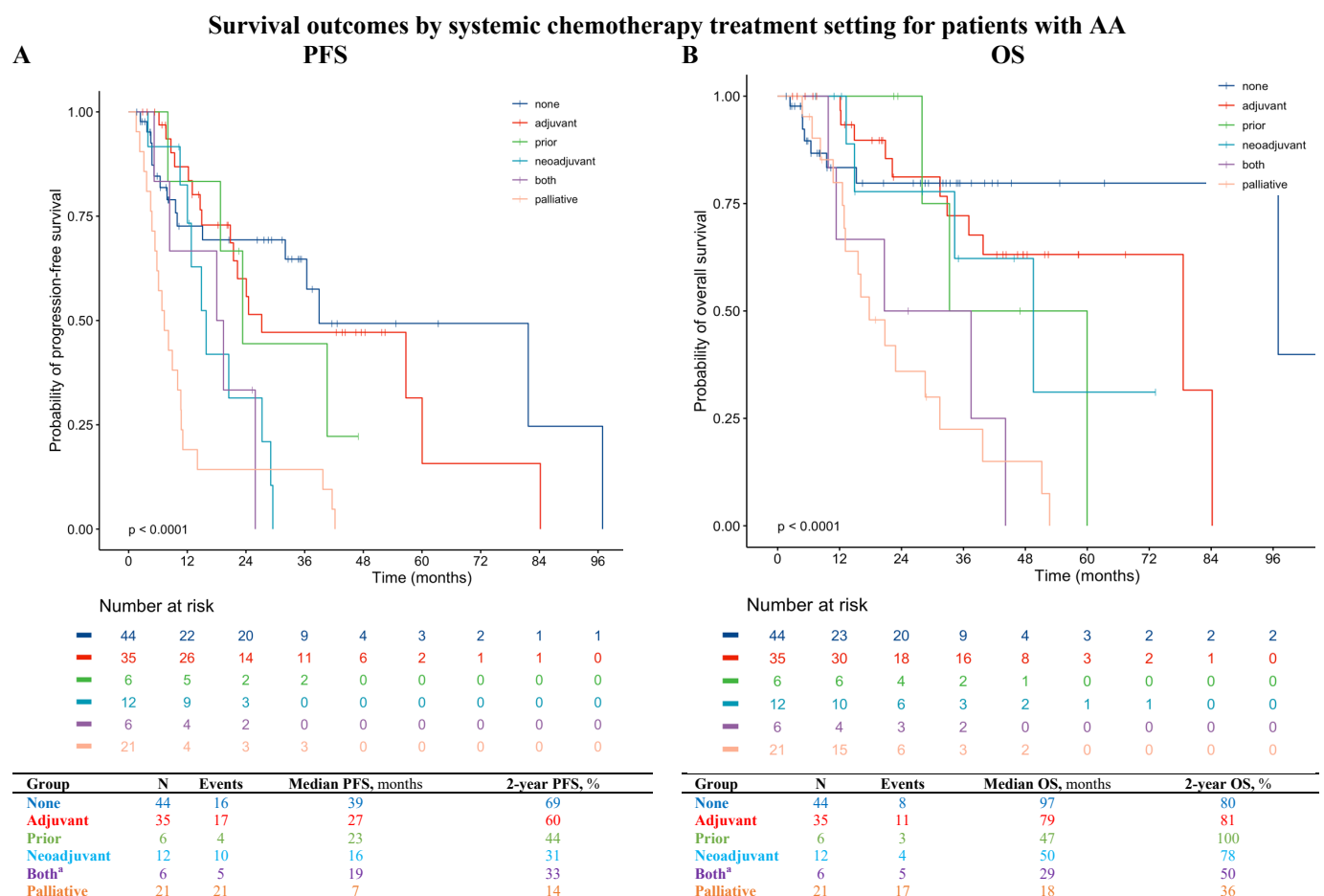


Figure 2.5 | Survival outcomes by systemic chemotherapy treatment setting

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Kaplan-Meier curves show the PFS (**A**) and OS (**B**) by systemic chemotherapy treatment setting for all patients with AA. Tables of outcomes below each plot display the median and 2-year PFS (**A**) and OS (**B**) for each group.

Abbrev. OS, overall survival; PFS, progression-free survival; CI, confidence interval; AA, appendiceal adenocarcinoma.

a) Positive lymph nodes

In patients with AA who had positive lymph nodes, patients received systemic chemotherapy appeared to have longer median OS (median not reached; 2-year OS 72%) compared to no chemotherapy (38 months; 2-year OS 54%), however, this was not statistically significant ($p=0.33$). Findings were similar for PFS. This was especially evident in patients with peritoneal metastases who also had positive lymph nodes where receipt of systemic chemotherapy was associated with longer OS and PFS ($p<0.0001$; Figure 2.6).

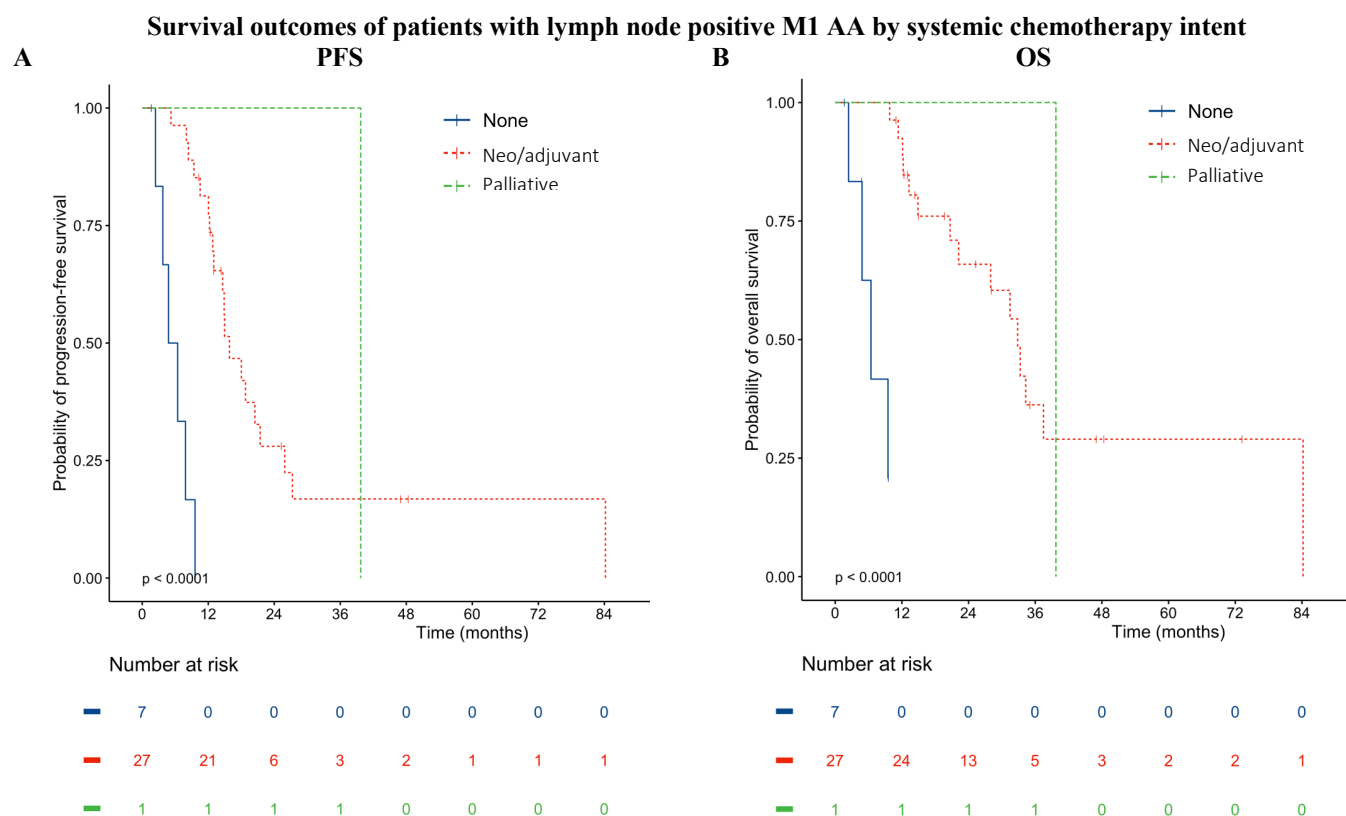


Figure 2.6 | Survival outcomes of patients with lymph node positive M1 AA

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Kaplan-Meier curves show the PFS (A) and OS (B) by systemic chemotherapy intent.

Abbrev. OS, overall survival; PFS, progression-free survival; AA, appendiceal adenocarcinoma; CRS, cytoreductive surgery.

b) Residual disease after CRS

For patients with AA who had CRS, those who had no residual disease (CC0) after CRS had shorter PFS and OS with neo/adjuvant systemic chemotherapy than those who did not have chemotherapy ($p=0.0004$; Figure 2.7). However, in the small subgroup of patients who had CC1 residual disease ($n=15$), there was improved median OS with neo/adjuvant chemotherapy (41 months) compared to palliative (15 months) or no chemotherapy (11 months), which was not statistically significant ($p=0.09$). There were similar outcomes regardless of chemotherapy intent for patients with CC2-3 macroscopic residual disease ($p=0.57$).

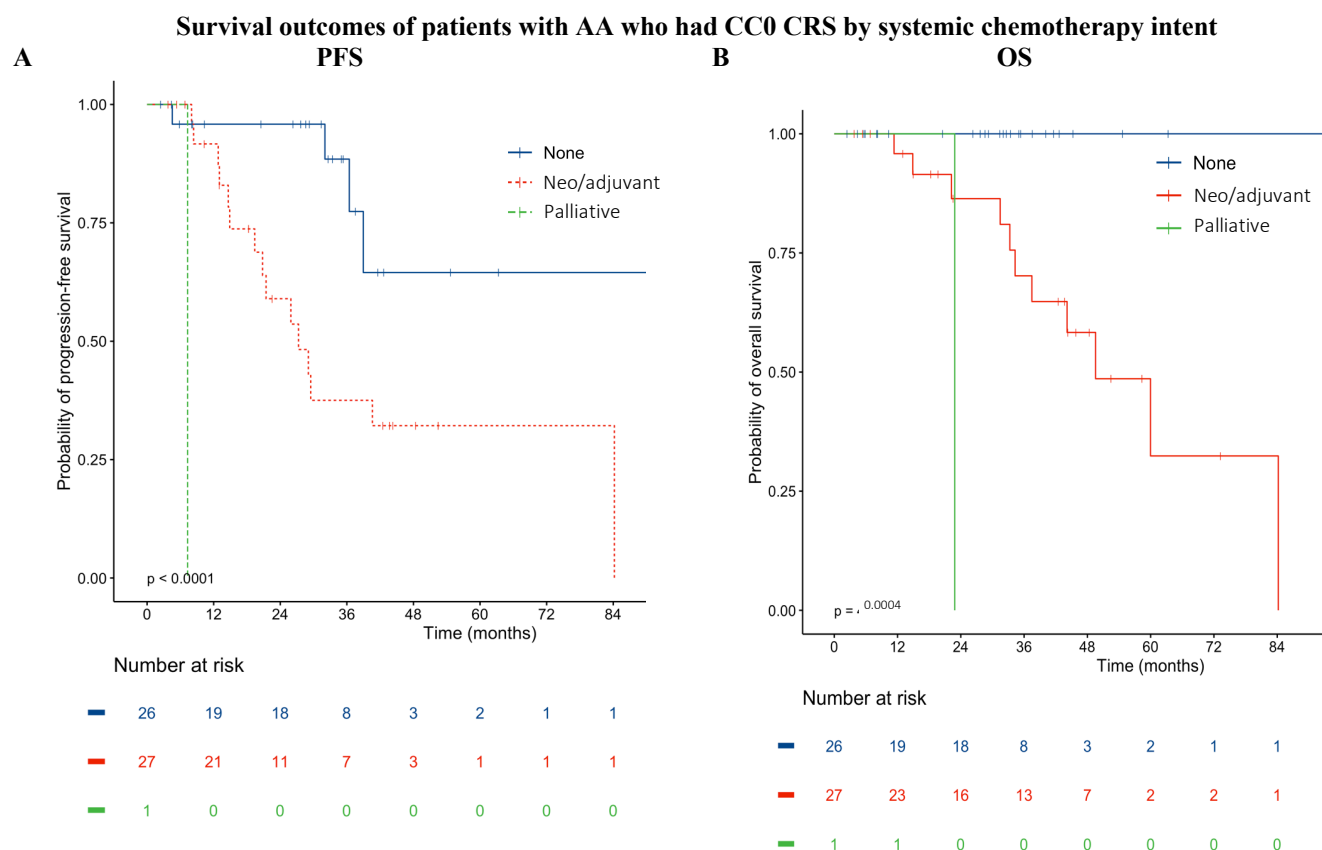


Figure 2.7 | Survival outcomes of patients with AA who have CC0 CRS by systemic chemotherapy intent

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Kaplan-Meier curves show the PFS (A) and OS (B) by systemic chemotherapy intent.

Abbrev. OS, overall survival; PFS, progression-free survival; AA, appendiceal adenocarcinoma; CRS, cytoreductive surgery.

c) Tumour grade

In an individual patient, discordance in tumour grade between the primary appendiceal tumour and the peritoneal metastases can be seen. Peritoneal grade rather than primary tumour grade is a better determinant of prognosis⁷⁸ so outcomes according to peritoneal grade are described. In patients with AA who had with low-grade peritoneal metastases, there was a significantly longer PFS and OS in those who had no chemotherapy comparing to neo/adjuvant and palliative chemotherapy ($p < 0.05$; Figure 2.8A,B). This suggests that selected patients with low-grade peritoneal metastases from AAs, may not benefit from neo/adjuvant systemic chemotherapy. In contrast, patients with AA who had high-grade peritoneal metastases had significantly longer PFS in those who had neo/adjuvant chemotherapy compared to no and palliative chemotherapy ($p = 0.011$; Figure 2.8C). OS was also improved but did not reach statistical significance for OS ($p = 0.056$; Figure 2.8D). This suggests that the presence of high-grade peritoneal metastases may help select patients with AA who benefit more from neo/adjuvant systemic chemotherapy.

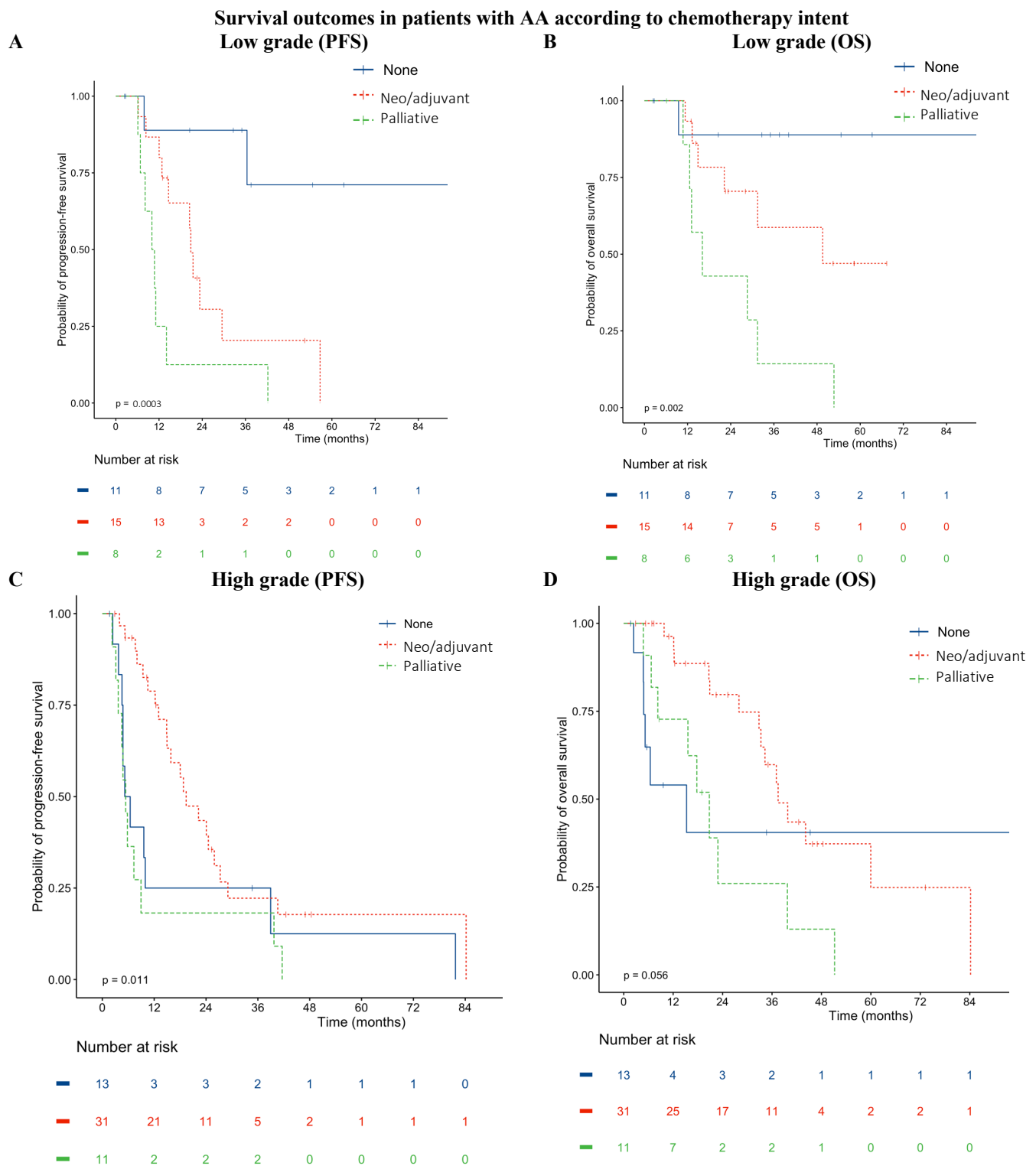


Figure 2.8 | Survival outcomes of AA patients with low and high-grade peritoneal tumour according to chemotherapy intent

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Kaplan-Meier curves show the PFS (A,C) and OS (B,D) for patients with low-grade (A,B) and high-grade (C,D) peritoneal tumour by systemic chemotherapy intent.

Abbrev. OS, overall survival; PFS, progression-free survival; AA, appendiceal adenocarcinoma; CRS, cytoreductive surgery.

2.3.3.3 Response rate

There were 29/89 patients (33%) who received first line chemotherapy with response to treatment evaluable. The remainder were either not evaluable due to curative intent treatment (i.e. no measurable disease, n=45) or had missing data (n=15). Of the evaluable patients, the overall response rate was 41% and clinical benefit rate was 72% (Table 2.8). These results are much higher than expected from clinical experience, and so a sensitivity calculation was performed with the assumption that those with missing data also had progressive disease, resulting in a total denominator of 44 patients. This resulted in an overall response rate of 27% and clinical benefit rate of 48%. These rates are consistent with the published literature (response range 20-44%).^{175, 186}

Table 2.8 | Response rate to first line systemic chemotherapy in patients with appendiceal tumours

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		First line chemotherapy ^a n=29 (%)
Complete response (CR)		2 (7)
Partial response (PR)		10 (34)
	Overall response rate (CR+PR) ^b	12 (41)
Stable disease (SD)		9 (31)
	Clinical benefit rate (CR+PR+SD) ^b	21 (72)
Progression		8 (28)

^aExcluded patients without evaluable disease (n=45) or missing data (n=15).

^bSensitivity analysis assuming patients with missing data all had progression is described in the text.

Abbrev. CI, confidence interval; CR, complete response, PR, partial response; SD, stable disease.

2.4 Discussion

This study evaluates predictors of survival outcomes in a modern prospective cohort of 208 patients with appendiceal tumours, of whom 83 (40%) had AMN and 124 (60%) had AA. Most patients had peritoneal disease (73%) and underwent CRS with mitomycin C HIPEC (63%). Previously established prognostic factors including stage, primary tumour pathology, tumour grade, surgical status, peritoneal and residual disease burden, elevated tumour markers and receipt of systemic chemotherapy were confirmed to be associated with survival outcomes in the AMN and AA cohorts.^{5, 71, 103, 147, 207} The data suggested there was no added benefit for systemic chemotherapy for all patients with AA who have no residual disease after CRS/HIPEC (CC0) or for those with low-grade AA. However, a potential benefit was seen for neo/adjuvant systemic chemotherapy in select subgroups of patients with AA such as those with involved lymph nodes and high-grade peritoneal metastases. This is supported by the findings from the analysis of the parallel UK cohort presented in [Appendix 1.2](#).

Compared to published data the survival outcomes reported in the AMN subgroup were better than expected.^{5, 6} In the AA cohort, survival outcomes were similar to that reported in literature.^{172, 186, 377} This discrepancy may relate to a more contemporary histopathological classification in this cohort. The largest studies of AMN have been reported by the PSOGI international registry, however, results are challenging to compare due to their long inclusion period from 1993, disease classifications defined by the term ‘PMP’ and including patients with high-grade peritoneal disease which would now be classified as AA.^{5, 6} Similarly, the AA cohort described in the literature have mixed populations with poorer outcomes driven by cohorts focusing on signet-ring cell adenocarcinoma and better outcomes in those groups that have higher proportions of well-differentiated adenocarcinomas.^{370, 371}

Patients with localised (M0) AA who had risk-reducing CRS/HIPEC had exceptional outcomes compared to those who did not (5-year OS 100% versus 28%, $p=0.087$). This is important preliminary data as there is sparse literature to guide surgical decision making in this group. An retrospective observational study evaluated outcomes of ‘prophylactic’ CRS/HIPEC in 62 patients with AA, but it did not have a comparison group.⁸⁷ While 37% had unexpected peritoneal metastases found at the time of procedure, 5-year OS was 83%, supportive for this approach.⁸⁷

The literature regarding the prognostic impact of pre-operative tumour markers is mixed with most studies finding correlation of poor outcomes with elevation of least one of CEA, CA125 or CA19.9.^{70, 103, 207} Most of this literature has been evaluated in AMNs. Studies in AA are less convincing with a cohort from the UK demonstrating an elevated CEA level was associated with poorer OS but there was no impact from elevation of CA125 or CA19.9.⁸⁸ In the UK parallel cohort, CEA was associated with poorer outcomes on univariate analysis, but the effect was not retained after multivariable analysis ([Appendix 1.2](#)). A recent large cohort study in an American peritoneal malignancy centre, contributed to by findings from the UK parallel cohort study, found an 11-fold risk of death for elevation at any time-point of all three tumour markers compared to none elevated ([Appendix 1.3](#)). Tumour marker levels have not been evaluated during systemic chemotherapy or in the non-CRS setting.

After CRS, 65% of patients had no residual disease (CC0). These patients had the longest survival outcomes in both AA and AMN subgroups. In contrast, patients with AA who had residual tumour nodules less than 0.25 cm (CC1) after CRS had similar outcomes to those with larger volumes of residual disease (CC2-3). This was the only variable significant for shorter PFS on multivariable analysis. Complete cytoreduction is defined in the literature for the AMN cohort as CC0-1,²¹³ however, this definition may be less relevant in AA cohorts where CC1 after CRS has been associated with worse prognoses.^{147, 371} One study evaluated outcomes of CC0 and CC1 after CRS by histopathology and suggested that for high-grade mucinous carcinoma peritonei with signet ring cells and goblet cell adenocarcinomas, only no residual disease (CC0) after CRS should be considered as complete cytoreduction.¹⁰⁰ These data lends support to this definition.

Data from this cohort and the parallel UK cohort ([Appendix 1.2](#)) support using lymph node status to help select patients who may benefit from neo/adjuvant chemotherapy. This raises the issue of lymph node assessment. One-quarter of the whole cohort had unknown lymph node status (NX). In AMN, most patients will have uninvolved lymph nodes.⁶ In AA, a selective surgical strategy is used, evaluating risk factors for positive lymph nodes which include tumour grade, radiological findings and patient factors.⁸⁹ The 40% node positive rate in the AA cohort is higher than expected but is influenced by the higher rates of involved lymph nodes reported in appendiceal goblet cell adenocarcinomas which are included in this AA cohort.³⁷⁸ Furthermore, in one study of AA, 50 to 61% of sampled lymph nodes were negative.³⁶⁸ Therefore, if after thorough patient assessment, lymph node sampling is not performed, it is likely that these are truly negative. However, there should be a low threshold for consideration of lymph node sampling and biopsy in order to identify patients who may be selected to benefit from neo/adjuvant systemic chemotherapy.

Patients who received palliative intent chemotherapy had the poorest outcomes as anticipated and these patients should be the focus of evolving treatment strategies and novel clinical trials. Tumour mutations were observed in one-third of patients who underwent molecular tumour profiling of which the majority were *KRAS* mutations. This is a potential promising avenue for future targeted treatments, although this cohort did not yield any actionable *KRAS*^{G12C} mutations.^{379, 380} Appendiceal tumours with their high proportion *KRAS* mutations^{214, 226} can be considered an ideal population to evaluate novel all-RAS inhibitors, which are under development.³⁸¹

The assessment of response to chemotherapy was limited by missing data. The raw response rate was much higher than what would be expected for AA (20%),¹⁷⁵ although not inconsistent with the highest response rate reported in a high-risk AA cohort (44%).¹⁸⁶ Recalculation of the response, assuming patients with missing data had progressive disease, resulted in a modest response and clinical benefit rate to first-line chemotherapy. The revised rate was similar to what the literature suggests for the AA group and was consistent with clinical experience.^{166, 175, 177, 382} This highlights the difficulties in obtaining accurate information even from prospectively collected and well maintained databases. Furthermore, response assessment in peritoneal disease is challenging with modified RECIST criteria poorly reflecting the burden of peritoneal disease. Other groups have described CT scoring systems for predicting the ability for successful cytoreduction in AMNs but this has not been validated for AA.^{56, 57} Furthermore, criteria for response assessment during systemic chemotherapy has not been formalised. One prospective chemotherapy study used a semiquantitative method to describe disease as stable, reduced or progressed, but again this has not been

validated.¹⁷⁶ There is scope to develop a formalised response assessment based on clinical, radiological, operative and pathological parameters, with specific relevance to patients with appendiceal tumours.

The limitations of this study include a relatively short follow-up for the AMN cohort with associated low event rate precluding multivariable analysis for OS. In addition, small clinically relevant subgroups are underpowered to confirm comprehensively explore predictors of prognosis. This campus is a state referral centre, so while it is likely that all referred cases were captured in the prospective database used for this study, systemic chemotherapy is often delivered externally, leading to a loss of follow-up which particularly impacted the accurate assessment of response rate. However, manual record review of patients' external letters, centralised e-health record and external institution records were reviewed to optimise data curation. While most imaging studies, during surgical planning and for suspected recurrence, were centrally reviewed by expert peritoneal radiologists, scans performed at external institutions during patients' systemic chemotherapy were not routinely reviewed.

As this is a retrospective, observational study, multiple sources of bias may affect these findings. There may be referral bias, as this campus is a state referral service and patients who are deemed not fit enough for consideration of CRS may not be referred, leaning the cohort towards improved outcomes. However, referrals for second opinions and early phase clinical trials are captured, so patients judged not suitable for surgical intervention may still be referred for consideration of additional systemic treatments. These factors may tend towards worse outcomes in the palliative intent treatment group. There may also be selection bias in assessing systemic chemotherapy as a predictor of prognosis, as patients who are recommended systemic treatment in the neo/adjuvant setting are those with the highest-burden and/or rapidly progressing disease, portending worse outcomes in those patients.

Finally, as an emerging peritoneal malignancy centre within 10 years of commencement it is possible that aspects of the service are still on a learning curve. However, this centre was established using an external mentor model to accelerate the learning curve. Outcomes audited after the first year demonstrated comparable short-term surgical outcomes to established centres, and this updated data remains consistent with international outcomes.⁸⁰

2.5 Conclusions

This modern patient cohort reflective of contemporary practice demonstrates improved medium-term outcomes for patients with AMN and similar outcomes for patients with AA to the literature. Established prognostic factors related to pathological features, stage, disease burden and systemic chemotherapy are associated with survival outcomes in this cohort. Systemic chemotherapy does not provide benefit to all patients with AA after complete cytoreduction (CC0); however, those who have high-grade tumours and/or involved lymph nodes appear to benefit from neo/adjuvant systemic treatment. While this study adds to the published data from other cohorts, all available evidence is observational and heavily confounded. Individualised treatment recommendations by centralised multidisciplinary teams are still advocated. Prospective clinical trials of systemic therapy are now essential to improve clinical decision making but will require collaborative international effort in this rare disease.

Chapter 3. Transcriptomic profiling of the microenvironment of appendiceal tumour peritoneal disease

Chapter 3 presents a transcriptomic analysis focusing on the tumour and stromal compartments of the ATPD TME. The pilot analysis was presented as a poster at the ESMO 2022 Congress (Abstract 1713P):

Strach, M. C., Yeung, N., Apostolov, E., Lin, H.-M., Nagaraju, R. T., Ansari, N., Koh, C., Shin, J.-S., Kench, J., Aziz, O., Swarbrick, A., Horvath, L. G., Barriuso, J. and Mahon, K. 1713P Single-cell transcriptomic analysis of appendiceal cancer peritoneal disease. *Ann Oncol.* 2022;33:S1322. Doi: [10.1016/j.annonc.2022.07.1791](https://doi.org/10.1016/j.annonc.2022.07.1791)

Findings from the main analysis were presented as a poster at the AACR 2024 Annual Scientific Meeting (Abstract 5480):

Strach, M. C., Yeung, N., Apostolov, E., Wang, T., Lin, H.-M., Ansari, N., Koh, C., Shin, J.-S., Kench, J., Swarbrick, A., Horvath, L. and Mahon, K. L. Abstract 5480: Single cell and spatial transcriptomic profile of appendiceal tumour peritoneal disease. *Cancer Res.* 2024;84(6_Supplement):5480–5480. Doi: [10.1158/1538-7445.am2024-5480](https://doi.org/10.1158/1538-7445.am2024-5480)

Terminology note for Chapters 3-5: Classification of AT and ATPD is described in Table 1.1 (Chapter 1). This chapter uses LG and HG to distinguish the tumour groups.

3.1 Introduction

Single cell RNA sequencing (scRNAseq) is the analysis of the gene expression profile of individual cells and has been used across cancer types to build descriptive cell type atlases.^{237, 383} Spatial transcriptomic profiling combines high resolution imaging with gene profiling from specified intact tissue.³⁸⁴ It is especially suited for evaluating the molecular features of the TME as directed by cell and tissue architecture.³⁸⁵ Most studies to date have focused only on the tumour cell compartment.^{117, 233-235, 238, 269, 386} However, it is critical to go beyond this to a comprehensive biological characterisation of the TME of ATPD.

The primary objective of this study was to evaluate the biological profile of the TME of ATPD in order to understand: (1) the cell-types present, (2) the potential mechanisms for the overproduction of mucin, (3) the characteristics of the peritoneal mesothelium and (4) to compare tumour and non-tumour tissue architecture to identify potential novel targets and biomarkers. To achieve these objectives, a method of dissociating peritoneal tumour tissues for single cell analysis was developed. This study presents a comprehensive transcriptomic profile generated by scRNAseq and spatial transcriptomic profiling. A separate analysis of the immune microenvironment of ATPD is presented in the next chapter of this thesis (Chapter 4).

3.2 Methods

3.2.1 Patient cohort

Tissue was collected from 36 patients: 33 with ATPD (19 LG, 14 HG), two ‘normal’ controls and a patient with peritoneal metastasis from colorectal cancer (control for spatial analysis), during CRS prior to HIPEC. The normal peritoneal tissue was defined following histological evaluation where no tumour deposits were identified.

Transcriptomic analysis was performed on a total of 25 patients (22 with ATPD; Figure 3.1). Comprehensive scRNAseq was performed on fresh tissue specimens from nine patients (four HG, five LG). Spatial transcriptomic profiling was performed on formalin-fixed paraffin-embedded (FFPE) tissue from 19 patients with ATPD. FFPE tissues from 15 patients’ samples were combined as a tissue microarray section for the analysis, whereas the FFPE tissues of the other four patients (two LG, two HG) were analysed as whole sections (one section per patient sample, two sections per slide).

Patient demographic, clinicopathological variables and outcome measures were captured from a prospective institutional database and missing information referenced from the hospital record. Burden of disease measures included the PCI (defined Chapter 1 Figure 1.4) and the CC score (defined Chapter 1 Figure 1.5).^{74, 101, 387} All diagnostic pathology was reviewed by expert gastrointestinal pathologists at this campus. OS was defined from the date of initial diagnosis, until the date of death, censored for the date of last follow-up. Informed consent was obtained from all patients contributing to this project under research protocols approved by central human research ethics committees: St Vincent’s Hospital Ethics Committee (HREC/2020/ETH006897) and Sydney Local Health District Ethics Committee, Royal Prince Alfred Hospital zone (HREC/X13/0439) for all participating sites ([Appendix 2](#)).

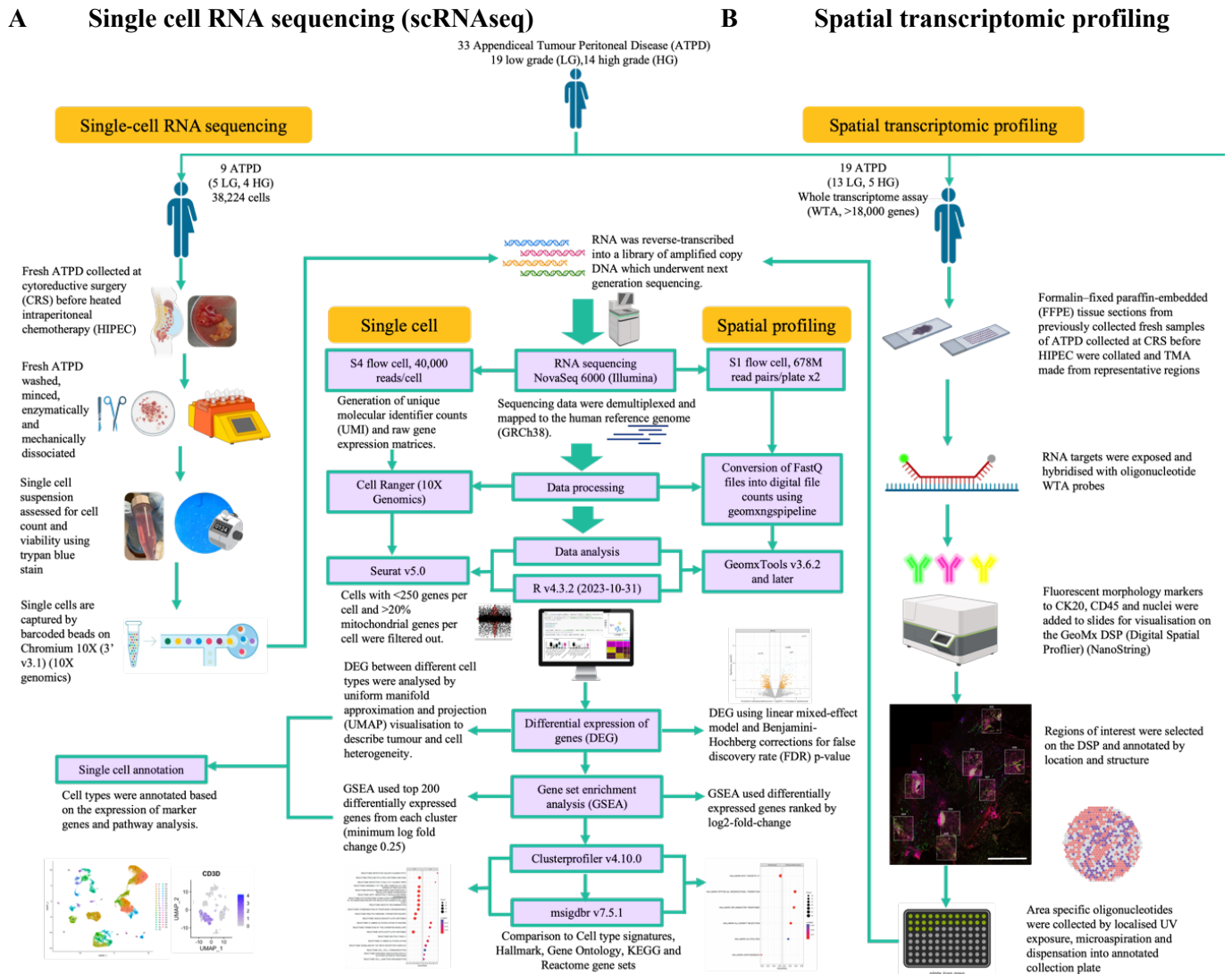


Figure 3.1 | Outline of transcriptomic analysis of ATPD

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Transcriptomic analysis was performed using scRNAseq (A) and spatial transcriptomic profiling (B) of LG and HG ATPD. Abbrev. scRNAseq, single cell RNA sequencing; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease. Image created by BioRender.com.

3.2.2 Tissue dissociation for scRNAseq

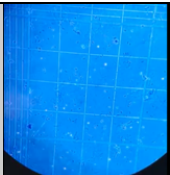
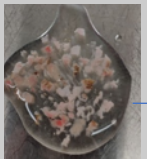
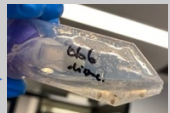
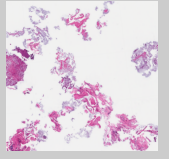
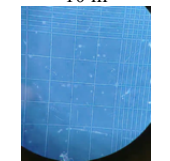
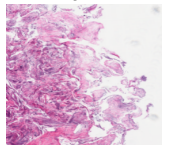
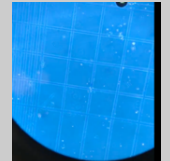
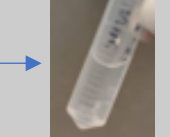
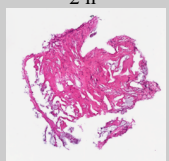
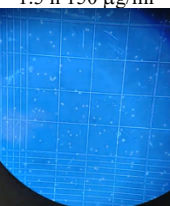
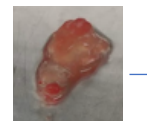
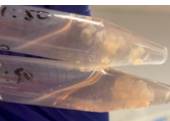
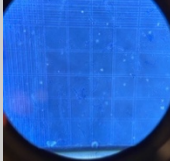
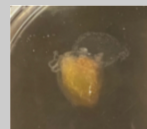
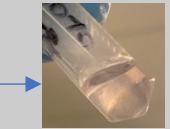
Fresh peritoneal samples that had been cold preserved overnight at 4°C were mechanically and enzymatically dissociated using Human Tumor Dissociation Kit (Miltenyi Biotec) following the manufacturer's protocol with modifications for mucinous samples based on a pre-digestion protocol (Miltenyi Biotec).^{388, 389} Throughout, tissue and reagents were kept on wet ice. Tissue was washed twice with Roswell Park Memorial Institute (RPMI)-1640 prior to enzymatic (Enzyme H, R and A; Miltenyi Biotec) and mechanical dissociation in a gentleMACS™ Dissociator (Miltenyi Biotec). The sample was then placed on a shaking incubator (Multitron Pro, Infors HT) at 37 °C for 30 to 60 min, checking the sample every 15 min for dissociation of the larger tissue pieces prior to further mechanical dissociation in the dissociator. After resuspension and filtration, a final digestion with Trypsin (TrypLE, ThermoFisher) was performed. Red blood cell lysis was performed with Lysing Buffer (Becton Dickinson). The final pellet was resuspended in phosphate buffered saline (PBS) with 10% fetal bovine serum (FBS, batch 2017-Apr 2021: HyClone NZ Sourced FBS Batch #DB0525, GE Healthcare Australia; current batch: Bovogen Cat Batch #2008A, Expiry Aug-2025, Scientifix Australia).

Optional pre-digestion steps with a mucolytic agent were incorporated for mucinous samples following optimisation experiments (Table 3.1). These experiments showed mucolytic effect of all agents, however, NAC and bromelain required a long duration of tissue digestion which resulted in suboptimal cell viability. Pre-digestion with dithiothreitol (DTT) preserved cell viability and was chosen as the preferred pre-digestion mucolytic. Pre-digestion was performed after the initial washing and mincing steps when the sample was added to 20 ml of pre-digestion solution containing 1 mM DTT (Sputolysin® Reagent; Cat #560000-10ML CAS 578517 Calbiochem®, Sigma-Aldrich). This was placed in a shaking incubator at 37 °C for 20 min followed by 10 sec vortex and filtration on a MACS® SmartStrainer (100 µM; Miltenyi Biotec). At this point the filtrate was used to continue the standard tissue dissociation protocol but if there were significant large tissue pieces in the filter a second pre-digestion step of these pieces was performed while the initial filtrate was kept at 4°C on wet ice. After mechanical and enzymatic digestion for especially mucinous samples, another digestion with DTT was performed instead of adding trypsin.

Cell viability of the single cell suspension was assessed by Trypan Blue staining. A 10 µl sample of cell suspension was mixed with an equal volume of Trypan Blue (ThermoFisher), loaded onto a haemocytometer and viewed with a light microscope. Live and dead cells were counted in five squares of the haemocytometer and multiplied by 2 (the dilution factor) to calculate the cell viability (number of live cells out of total cell number). If viability was less than 70% and more than 100,000 cells were present, viability enrichment was performed using the EasySep Dead Cell Removal (Annexin V) Kit (StemCell Technologies) as per the manufacturer's protocol.³⁹⁰ Following these steps, if the sample had >70% viability, minimal debris and a total cell concentration ~800-1,200 cells/µl, the dissociated cells were subjected to single cell capture. The patients' samples for the pilot experiment were pooled into one tissue sample for single cell capture. For the subsequent main experiments, single patient samples were processed individually and not pooled together i.e. one patient sample per reaction and one reaction per single cell capture session was processed.

Table 3.1 | Comparison of effect of mucolytics on cell viability for single cell suspensions from tissue samples from patients with ATPD

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Experiment	Method/Dose	Duration	Total live/dead cell count (x10 ⁴ cells/ml) ^a	Viability ^a	Haemocytometer image ^c	Macroscopic appearance after mucolysis	Microscopic appearance after mucolysis
Control (mechanical dissociation)	1. Centrifuged 450g x5min (no separation) 2. Physical dissociation using gentleMACS dissociate x2 'setting h_tumor_02.01' 3. Centrifuged 450g x 5 m	na	29.5	47%		 → 	
Trypsin	1. Minced sample submerged in 1 ml TrypLE 2. Shaking incubator at 37°C x5 m 3. Filtered through 70 µM strainer, washed with PBS 4. Centrifuge x450 g x5 m 5. Repeat on undissociated pieces at time intervals	5 m 10 m 15 m 30 m 45 m	65 18 12.5 70 10	53% 72% 48% 67% 75%	10 m 	 → 	45 m 
NAC^b	1. Minced sample submerged in 1 ml 4% NAC + 20% FBS 2. Shaking incubator at 37°C x30 m 3-5 m as above	30 m 1 h 2 h 2.5 h	(debris) (debris) 7 13.5	(debris) (debris) 57% 63%	2.5 h 	2.5 h  → 	2 h 
Bromelain^b	1. Minced sample submerged in 2 ml Bromelain 150 or 300 µg/ml 2. Shaking incubator at 37°C x30 m 3-5, as above (except 100 µM filter)	30 m 1 h 1.5 h 2 h	151 192 207 58.5 178.5 - 56.5 26	38% - 83% 76% 90% - 79% 23%	1.5 h 150 µg/ml 	2 h  → 	na
DTT[*]	6.5 mM concentration, equal volume added to mucin Added as pre-digestion step to full single cell dissociation protocol	15 m	F1 167 F2 112 Pre-digestion 39 Dissociation 20	43% 38% 33% 82%	 F2	 → 	na

^aCell viability of the single cell suspension was assessed by Trypan Blue staining. A 10 µl sample of cell suspension was mixed with an equal volume of Trypan Blue (ThermoFisher), loaded onto a haemocytometer and viewed with a light microscope. Live and dead cells were counted in five squares of the haemocytometer and multiplied by 2 (the dilution factor) to calculate the cell viability (number of live cells out of total cell number).

^bDosing based on Pillai *et. al.*²⁷¹

^cSingle cell suspensions visualised under light microscope, live cells are bright, dark cells are dead.

^{*}Preferred agent.

Abbrev. ATPD, appendiceal tumour peritoneal disease; NAC, N-Acetylcysteine, DTT, Dithiothreitol; m, minutes; h, hours; F1, filtrate 1; F2, filtrate 2.

3.2.3 Single cell capture and RNA sequencing

Single cell capture was performed at the Garvan-Weizmann Centre for Cellular Genomics (GWCCG), Garvan Institute of Medical Research (GIMR) using the Chromium Single-Cell v3 3' Chemistry Library, Gel Bead and Chip Kits (10x Genomics) per the manufacturer's protocol.³⁹¹ A total of 8,000 to 12,000 cells were targeted for each well. Single cells were captured by 10X barcoded gel beads (10X Genomics). RNA was reverse-transcribed into a libraries of amplified copy DNA. When all planned patient samples for the pilot study had been captured and libraries processed the first batch of sequencing was performed to facilitate the pilot analysis used to inform design of the main experiment. Similarly, when all planned patient samples for the main experiment had been captured and libraries processed a second batch of sequencing was performed. Libraries were sequenced in one batch using the S4 flow cell on the Novaseq 6000 platform (Illumina) with paired-end sequencing and dual indexing at 40,000 reads per cell. A total of 28 cycles were run for Read 1, 10 for i7 index and 90 cycles for Read 2 according to the recommendations for the Chromium platform.

3.2.4 Data processing and cell annotation for scRNAseq

Data processing and analysis that were performed with *R* version 4.3.2 (R Core Team 2023),³⁹² with *R* packages mentioned accordingly. Cell Ranger Single Cell Software v6.0.2 (pilot samples) to v7.1.0 (main samples) (10x Genomics) was used to perform read demultiplexing of raw Binary Base Call files and alignment to the human reference genome GRCh38-2020-A.³⁹³ Counts of unique molecular identifier (UMIs) and raw gene expression matrices were generated. Samples that were processed pooled together (in the pilot study) were assigned back to the sample of origin (demultiplexed) using genotype information using the Souporecell (singularity container image, built from commit 790b281dbe, <https://github.com/wheaton5/souporcell>)³⁹⁴ and VireoSNP v0.5.6³⁹⁵ *R* packages. Data was filtered, normalised, and subjected to dimensionality reduction and clustering using the *R* package Seurat v5.0.1.³⁹⁶ Clustering steps (*RunPCA*, *FindNeighbours*, *FindClusters*) was performed at resolution 1.2 on 100 principle components. Analyses were performed in the main experiments initially on each individual patient sample (n=6), on all combined ATPD samples and finally combined by tumour type as LG (n=3) and HG (n=6).

Cell clusters were grouped by the following terms: (1) Major cell lineages, described the cell compartments of tumour, immune, mesothelial, endothelial and stromal; (2) Major cell types, describes the broad groups of cells by subset (for example, T cells); and (3) Minor cell types or 'subclusters' are used to refer to smaller cell populations that contributed to the broader group (for example, CD4 T cells). These cell clusters were annotated for gene expression using the following four methods:

1. Manual annotation was performed firstly by evaluating canonical markers for known major and minor cell types based on a literature review (Chapter 1, Table 1.6).^{285, 304, 397} This included genes such as *KRT20* and *MUC2* for CK20 tumour cells³⁹⁸, *PTPRC* for CD45 immune cells³⁹⁹ and *CALB2* and *MSLN* for calretinin positive peritoneal mesothelial cells⁴⁰⁰ from LG and HG ATPD. Key marker genes are displayed in feature plots in [Appendix 3](#) Supplementary Figures SF3.1-3.6.
2. Minor cell type annotation and annotation of undefined subclusters was also based on reviewing the top 10 upregulated genes expressed per cluster, and for the most specific gene(s) visualised for that cluster reviewing cell attributions suggested by the Human Protein Atlas^{401, 402} and/or Gene Entrez⁴⁰³ and using gene set

enrichment analysis to identify genes overlapping with published signatures of specific cell types and associated functional pathways.⁴⁰⁴

3. Automated annotation with an annotated localised prostate cancer atlas as the reference (manuscript in preparation, courtesy of Ms Eva Apostolov and A/Prof Alexander Swarbrick), was performed using the R package singleCellNet v0.4.01.⁴⁰⁵
4. Automated annotation with the Human Primary Cell Atlas⁴⁰⁶ as the reference was performed using SingleR v2.4.1 and celldex v1.12.0 R packages.⁴⁰⁷

3.2.5 Flow cytometry

Flow cytometry experiments were also performed at the GWCCG, GIMR, on freshly dissociated tissue (two patients), viably cryopreserved single cell suspensions from previously dissociated fresh tissue (two patients) and dissociated tissue from previously viably cryopreserved tissue pieces (one patient). All cases were ATPD and three had matched samples processed for scRNAseq. The cell suspensions were fluorescently labelled for flow cytometry as follows.

Fresh tissue or thawed cryopreserved single cell suspensions were washed with PBS and incubated with 1 µl of LIVE/DEAD™ Fixable Violet Stain (1:1000, ThermoFisher Scientific #L34963). Cells were resuspended in Human TruStain FcX™ Fc Receptor Blocking Solution (5 µl per million cells in 100 µl; Biolegend #422301) to block nonspecific antibody binding. Cells were pelleted and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing salts, 2% FBS) containing the following antibodies for cell surface staining: anti-epithelial cell adhesion molecule (EpCAM) (1:100, Biolegend #324203), anti-CD31 (1:100, Biolegend #303115) and anti-CD45 (1:100, Biolegend #368523). Cells were incubated in the dark on ice for 30 mins prior to washing and resuspended in 4% paraformaldehyde (PFA in PBS) for fixation. Permeabilisation of cells for intracellular staining was done using 0.1% Triton™ X-100 (Sigma-Aldrich) prior to incubation with the following antibodies: anti-CK20 (1:500, clone EPR1622Y, Abcam #ab209923) and anti-calretinin (1:25, custom conjugate, BD Biosciences #624292). Cells were incubated for 30 mins on ice in the dark prior to washing and resuspension in 1% PFA for fixation.

Flow cytometry was performed on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) using BD FACSDIVA software. Unstained cells were used as negative compensation controls to adjust for background fluorescence. The fluorophores, emission and excitation parameters are summarised in Table 3.2. Results were analysed in FlowJo™ software v10.9.0 (BD Biosciences). Target cell populations were identified by marker^{HIGH(+)} expression (Table 3.2). The gating strategy selected live single cells from debris and multiplets (clumped cells) using forward and side scatter characteristics and live/dead viability stain.

3.2.6 Tissue microarray creation and slide preparation

A 50-core (5 x 10) tissue microarray was created with 1 mm tissue cores from 17 patient samples consisting of ten LG ATPD, three HG ATPD, two 'normal' tissue and two control tissues (one peritoneal metastasis from colorectal cancer and one tonsil tissue). Multiple representative cores from each sample were created as technical replicates.

Table 3.2 | Flow cytometry fluorescent antibodies and parameters[Back to paragraph](#)

Target cell	Marker (location)	Fluorophore	Wavelength (nm):		Laser name /parameter
			Excitation	Emission	
Dead cell	na	Fixable violet dead cell stain	416	451 (violet)	V450
Endothelial	CD31 (cell surface)	APC	650	660 (red)	R670
Immune cell	CD45 (cell surface)	BV605	405	605 (orange)	V610
Epithelial cells	EpCAM (cell surface)	FITC	490	525 (green)	B530
Appendiceal tumour cell	CK20 (intracellular)	PE	565	578 (yellow)	YG586
Mesothelial cell	Calretinin (intracellular)	BV786	407	786 (brown)	V780

Abbrev. EPCAM, epithelial cell adhesion molecule; CK20, cytokeratin 20; APC, allophycocyanin; BV, brilliant violet; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Serial unstained FFPE 4-6 µm thick sections were deparaffinised (CitriSolv™, Fisher Scientific), digested with proteinase K (ThermoFisher Scientific, AM2546) and then hybridised overnight with oligonucleotide probes (Human whole transcriptome assay (WTA) 18,269 gene targets, NanoString) according to the GeoMx-NGS RNA FFPE Manual Slide Preparation Protocol v2.3 (NanoString Technologies). The morphology markers used were: 1:1,600 anti-CK20 (recombinant Alexa Fluor 488, clone EPR1622Y, Abcam Australia, Cat. #ab75988), 1:20 anti-CD45 (Alexa Fluor 594, Novus, clone 2B11+PD7/26). Cat. #NBP2-34528AF594) and 1:10,000 SYTO83 (ThermoFisher Scientific, Cat. #S11364) in blocking buffer W (NanoString). These morphology markers were used to select cell segments: tumour (CK20), immune (CD45) and stroma (non-CK20/CD45). Consecutive sections were haematoxylin and eosin (H&E) and immunohistochemically stained.

3.2.7 Region of interest selection and spatial annotation

Regions of interest (ROIs) for spatial transcriptomic profiling were identified by light microscopy examination of H&E stained sections from FFPE specimens. Each ROI was segmented into marker specific areas when the tissue was processed by the spatial profiling instrument (Figure 3.2A). These segments corresponding to the main regions of the TME (immune, tumour, and stroma) were annotated by descriptions regarding their spatial location in the microenvironment and structural morphology (Figure 3.2B-D). Microenvironmental descriptors included peri-tumoural, intra-tumoural, peri-mucinous, stromal, peri-inflammatory or combinations of these (Figure 3.2B) and structural descriptors for tumour cells included single cells, epithelial structures or glandular formations (Figure 3.2C,D). Annotation of the microenvironment was based on visibility of the cell group near the region of interest within a field of view at 40X magnification.

3.2.8 Tissue processing for spatial profiling and RNA sequencing

A digital immunofluorescent image of the tissue section was generated on the Geomx digital spatial profiler instrument (NanoString) based on the tissue morphology determined by the fluorescent markers. This was performed according to the DSP Instrument User Manual v2.4 (NanoString Technologies). A total of 81 ROIs were selected on the spatial profiler with reference to preselected areas on adjacent tissue sections (Figure 3.2E-H). Each ROI was segmented into marker specific segments. Collection of area specific oligonucleotides from 192 segments was performed by localised ultraviolet exposure, microaspiration and dispensation into an annotated (barcoded) collection plate by the Geomx digital spatial profiler instrument. The collection plate contained RNA primers that target unique

nucleotides to identify specific genes. Library preparation was performed on the aspirates according to the manufacturer's instructions (Protocol v2.3) to perform polymerase-chain reaction amplification to add Illumina adapter sequences and unique dual sample indices added prior to pooling purification. Sequencing was performed on a NovaSeq 6000 S1 cell (Illumina). Cycling conditions were set for 100 cycles: 28, 8, 8, 28 for Read 1, i7, i5 and Read 2.

3.2.9 GeoMx spatial profiling data processing

FastQ files were converted into digital count files using the 'geomxngspipeline' (v2.0) tool.⁴⁰⁸ Analysis including quality control was done using GeomxTools (v3.6.2 and later)⁴⁰⁹ in R. Segment quality control was performed according to parameters in and displayed in (Table 3.3). Two 'no template controls' (NTC) were included in the experimental set up (one NTC well per 96-well plate). These wells do not contain an RNA template and measure potential contamination of segments. NTC values of more than 1000 could indicate contamination of segments. There were 95 segments found to have an NTC count of 15 indicating no significant contamination. There were a low number of segments with low gene signals (Figure 3.3A). Most segments had more than 5% of the genes detected (Figure 3.3B). Due to the explorative nature of this study and the possibility of relevant low frequency genes, filtering was set to 1%. Thus, no segments were excluded as all segments had more than 1% of genes detected. Data normalisation of the raw gene counts was performed using the upper quartile (Q3) normalisation method and background normalisation of negative control counts (Figure 3.3C-E)

Table 3.3 | Quality control parameters and results

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Parameter	Threshold	Number of segments:	Pass ^a	Flagged ^b
Minimum number of reads per segment	1000	Low reads	190	0
Minimum % of reads trimmed	80%	Low trimmed	190	0
Minimum % of reads stitched	80%	Low stitched	190	0
Minimum % of reads aligned	80%	Low aligned	190	0
Minimum sequencing saturation	50%	Low saturation	189	1
Minimum negative control counts	1	Low negative counts	190	0
Minimum segment area	100 μm^2	Low area	189	1
TOTAL			188	2

Segments flagged by this analysis did not meet quality control parameters and were removed. NTC results: 95 segments had a NTC count of 11 and 95 of 15. Values >1,000 could indicate contamination of segments associated with high NTC counts.

^aNumber of segments that passed quality control threshold parameters.

^bNumber of segments that did not pass quality control threshold parameters and were excluded from further analysis.

Abbrev. NTC, no template control.

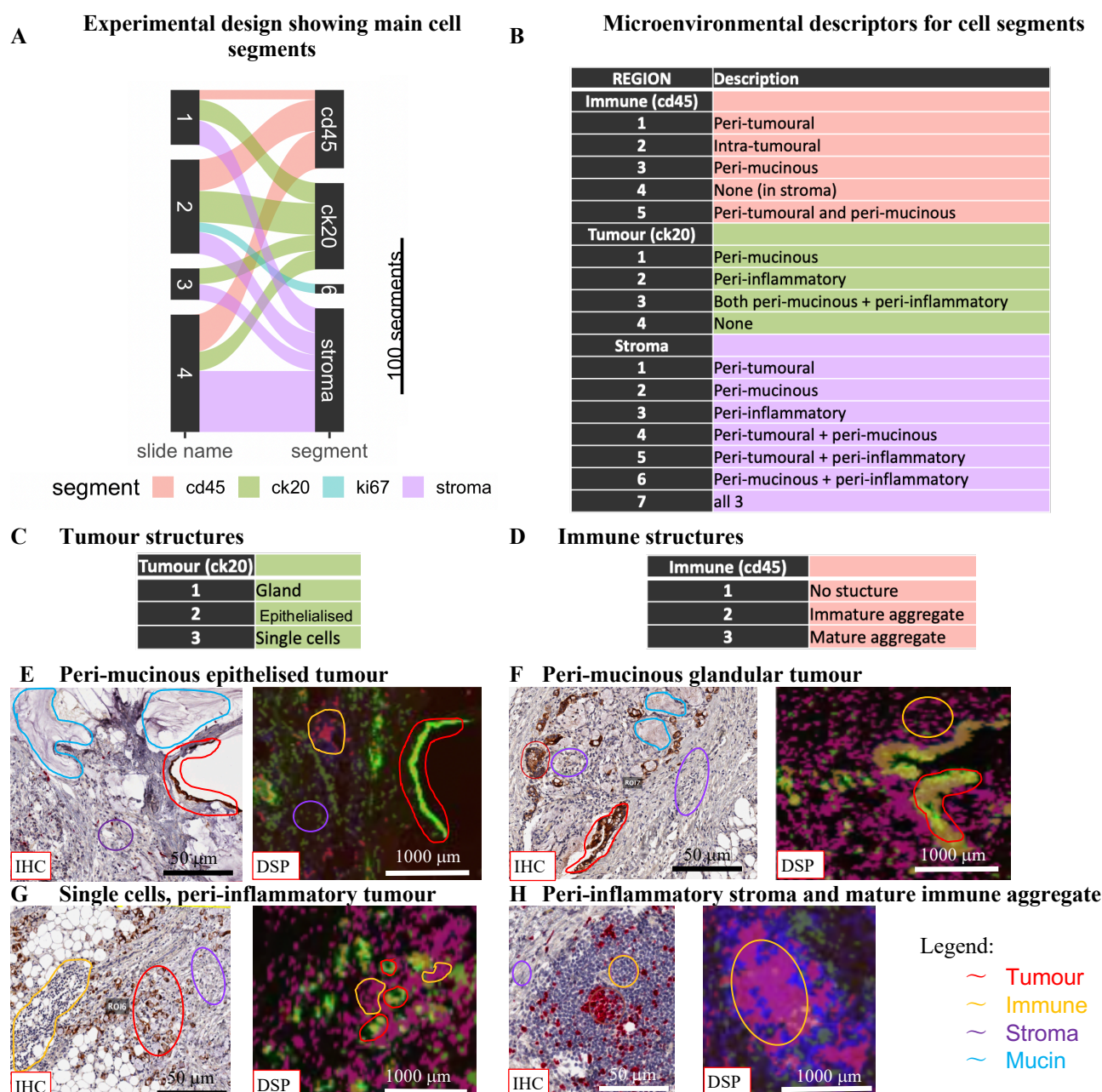


Figure 3.2 | Definitions of regions of interest (ROI) and cell type segments used for spatial transcriptomic profiling

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Sankey diagram of the analysis of four slides and associated cell segments generated from predefined ROIs. Cell segments were defined by three main compartments of the tumour microenvironment: (1) tumour cells (CK20⁺), (2) immune cells (CD45⁺) and (3) stroma cells (CK20⁻CD45⁻). A fourth group containing a small proportion of highly proliferative tumour cells (Ki67⁺) were manually masked based on viewing parallel sections stained with Ki67 (A). Cell type segments were annotated by geographic location in the microenvironment based on proximity to combinations of tumoural, inflammatory, mucinous or stromal regions (B). Tumour (C) and immune cells (D) were annotated by structural morphology. Examples of ATPD tissue sections showing tumour, immune and stromal ROIs selected for spatial transcriptomic profiling: low-grade (LG) peri-mucinous non-inflammatory epithelialised tumor cells and peri-mucinous peri-tumoural stroma (E), High-grade (HG) glandular tumour cells (F), HG peri-inflammatory single tumour cells and infiltrating immune cells (G) and LG immune cell aggregate (H). In each figure panel of E-H, the left image depicts immunohistochemistry staining with anti-CK20 (brown) for tumour cells, anti-Ki67 (red) for proliferating cells, and haematoxylin counterstained; the right panel depicts immunofluorescence staining of a consecutive section visualised on the digital spatial profiler with anti-CK20 (AF488, green) for tumour cells, anti-CD45 (AF594, pink) for immune cells, SYTO83 nuclei stain (yellow). Circle annotations are colored according to the legend in the bottom right: tumour (red), immune (yellow), stroma (purple) and mucin (blue).

Abbrev. ROIs, regions of interest; H&E, haematoxylin and eosin stain, DSP, digital spatial profiler; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease.

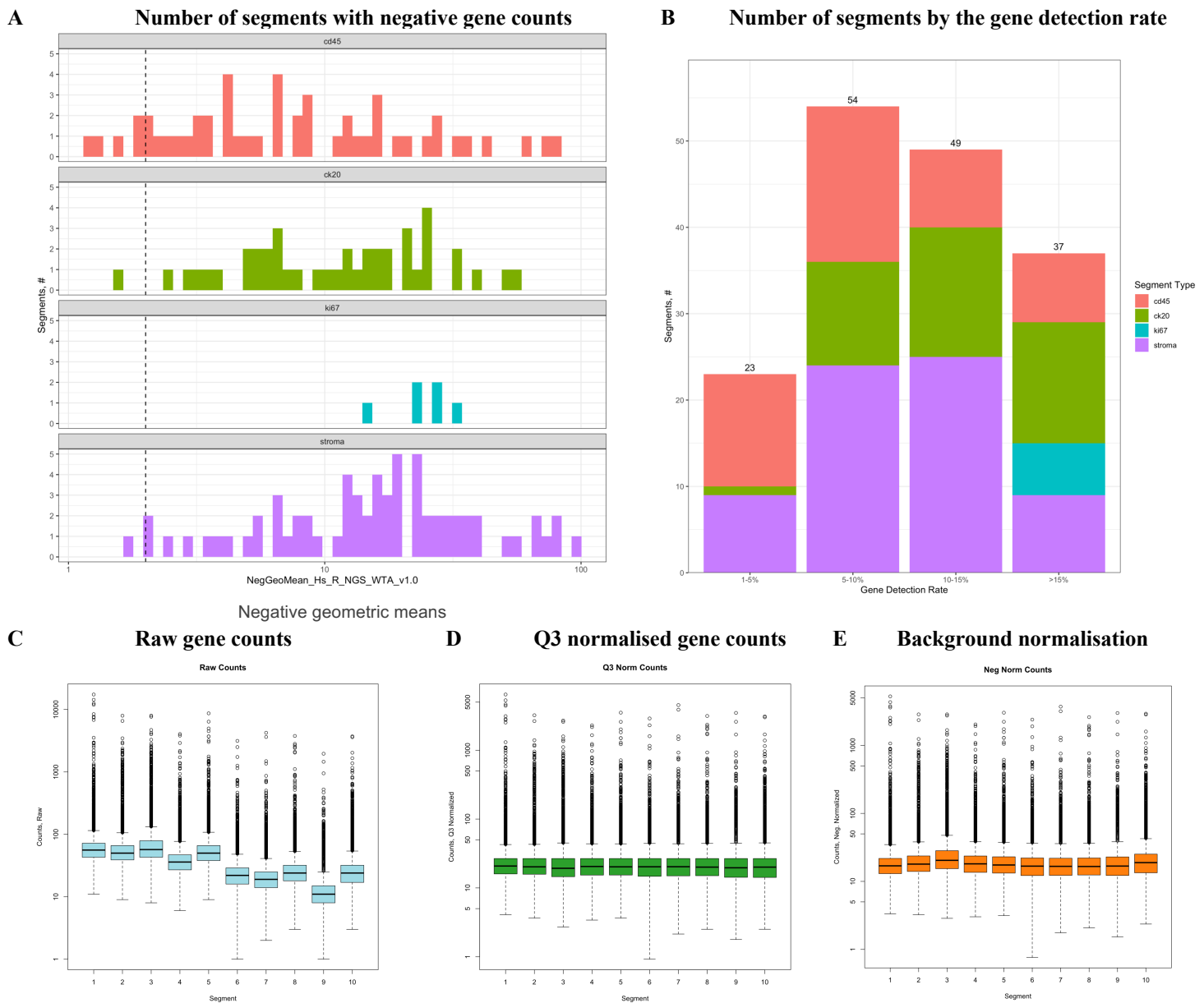


Figure 3.3 | Quality control parameters for spatial analysis of cell type segments^a from ATPD

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Bar plots show the number of segments with negative counts >1 (defined by the geometric mean of unique negative probes in GeoMx panel that do not target mRNA and establish the background count level per segment; segments with low counts ~1-10 may have low endogenous gene signals or insufficient tissue sampling (A), the number of segments by gene detection rate (B). Box plots show for the first 10 segments the raw RNA data (C) which is normalised using upper quartile (Q3) normalisation (D) and background normalisation of the negative control counts (E).

^aSegments refer to the main cell compartments: tumour, immune and stromal cells.

Abbrev. ATPD, appendiceal tumour peritoneal disease; Norm, normalised; Neg, negative
Figure text adapted from Reeves et. al.⁴¹⁰

3.2.10 Differential gene expression and gene set enrichment analysis

Differential gene expression analysis for scRNAseq was performed using the MAST (v1.25.1) method⁴¹¹ (*FindMarkers*) in Seurat using default parameters. The top 200 differentially expressed genes, ordered by log2 fold change from each cluster (minimum 0.25) were used for gene set enrichment analysis to evaluate statistically significant overlaps between the highest expressed genes and pre-existing gene signatures for known biological processes and functions⁴⁰⁴. For the spatial transcriptomic analysis, differentially expressed genes were identified using linear mixed-effect modelling (using case number to control for tissue subsampling) with the Benjamini-Hochberg multiple test correction to generate a false discovery rate (FDR) p-value using GeomxTools (v3.6.2 and later)⁴⁰⁹ in R. Genes were considered as significantly differentially expressed if the FDR was less than 0.05 and the log2 fold change was more than 0.5.

Gene set enrichment analysis utilised the ClusterProfiler package (v4.10.0)^{412, 413} and the msigdb package (v7.5.1) for comparison to Hallmark, Gene Ontology,^{229, 414} Kyoto Encyclopedia of Genes and Genomes (KEGG)⁴¹⁵ and Reactome gene sets.^{416, 417} Results were clustered, scaled and visualised using the packages enrichplot (v1.22.0) and pheatmap (v1.0.12).⁴¹⁸

3.3 Results

3.3.1 Patients and tissue samples

To identify the key cellular contributors to ATPD and the surrounding microenvironment, ATPD samples from nine patients were analysed by scRNAseq (Table 3.4). Single cell dissociation using the additional mucolytic pre-digestion with DTT was performed on tissue from LG ATPD (samples #13, #14, #17, #25, #30). The pilot study was included in the initial analysis (both LG and HG combined) but excluded from the dedicated tumour type analysis (LG and HG tumours analysis separately). This was due to assessment of a non-representative cell type biasing this sample. Individual analysis of this sample indicated a group of mostly neuroendocrine basal proliferating cells which was also verified on staining tissue sections for tumour and proliferation markers ([Appendix 3](#): Supplementary Figure SF3.7). Of the remaining six cases (three LG, three HG), a total of 55,762 single cells were captured and sequenced, with 49,313 single cells passing quality control (Figure 3.4). LG and HG cases each had 23,278 and 26,035 cells for analysis respectively.

Table 3.4 | Clinical and pathology details for appendiceal tumour patients analysed by transcriptomic profiling and immunohistochemical analysis of immune cell populations

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#	Analysis			Sex	Age (years)	Pathology classification ^a		ATPD group ^b	Stage		Clinical and treatment details				OS, months (status)
	scRNAseq (flow cytometry)	Spatial	IHC			Primary tumour	Peritoneal tumour		N	M	PCI	CC	Systemic chemotherapy ^c		
1		✓	✓	M	70	AMN	G2 MCP	LG	N0/X	M1	39	3	No	35.3 (NED)	
2		✓	✓	F	57	MAC	G3 MCP	HG	N1/2	M1	36	3	Adjuvant FOLFOX	47.0 (NED)	
3		✓	✓	F	76	AMN	G2 MCP	LG	N0/X	M1	27	0	No	33.5 (NED)	
4		✓	✓	F	57	AMN	G2 MCP	LG	N0/X	M1	20	6	No	32.3 (NED)	
5 ^c		✓	✓	F	51	AMN	No disease	LG	N0/X	M0	39	1	No	31.7 (NED)	
6		✓	✓	M	49	MAC	SRC ^a	HG	N0/X	M1	20	0	Neoadjuvant FOLFOX/Bevacizumab	45.8 (AWD)	
7 ^c		✓	✓	M	61	ANOS	No disease	HG	N1/2	M0	16	1	Neoadjuvant FOLFOX	13.3 (DOD)	
8		✓	✓	M	58	AMN	G2 MCP	LG	N0/X	M1	24	0	Neoadjuvant Capecitabine	50.7 (AWD)	
9		✓	✓	F	46	AMN	G2 MCP	LG	N0/X	M1	36	0	No	26.0 (NED)	
10 ^d		✓	✓	F	56	MAC	G2 MCP	HG	N0/X	M1	2	0	No	28.6 (NED)	
11		✓	✓	F	50	AMN	G2 MCP	LG	N0/X	M1	35	0	No	26.8 (NED)	
12		✓	✓	F	70	AMN	G1 MCP	LG	N0/X	M1	24	0	No	32.1 (NED)	
13	✓(✓)*		✓	F	43	AMN	G2 MCP	LG	N0/X	M1	33	0	No	25.2 (NED)	
14	✓(✓)*	✓	✓	F	31	AMN	G2 MCP	LG	N0/X	M1	34	0	No	25.1 (NED)	
15		✓	✓	M	71	AMN	G1 MCP	LG	N0/X	M1	31	3	No	25.6 (NED)	
16		✓	✓	F	47	GCA	G3 PM	HG	N1/2	M1	36	1	Neoadjuvant CE→FOLFOX ^f	28.1 (NED)	
17	✓	✓	✓	F	47	AMN	G2 MCP	LG	N0/X	M1	30	0	Palliative MCAP	21.8 (AWD)	
18		✓	✓	F	52	AMN	G2 MCP	LG	N0/X	M1	27	0	No	16.1 (NED)	
19	✓(✓)*	✓	✓	F	66	ANOS	HG PM	HG	N1/2	M1	12	0	Neoadjuvant FOLFOX	22.6 (NED)	
20		✓		F	29	AMN	G2 MCP	LG	N0/X	M1	33	0	Palliative MCAP	3.5 (NED)	
21			✓	F	66	MAC	LG PM	HG	N1/2	M1	35	1	Prior FOLFOX	25.3 (NED)	
22			✓	F	50	AMN	G2 MCP	LG	N0/X	M1	26	0	No	6.9 (NED)	
23			✓	M	74	AMN	G2 MCP	LG	N0/X	M1	34	3	No	10.0 (AWD)	
24			✓	M	55	GCA	G3 PM	HG	N0/X	M1	31	0	Adjuvant FOLFOX	5.3 (NED)	
25	✓		✓	M	52	AMN	G2 MCP	LG	N0/X	M1	27	0	No	3.3 (NED)	
26	✓(✓)		✓	F	76	MAC	G3 MCP	HG	N0/X	M1	20	0	No	2.4 (AWD)	
27			✓	F	73	AMN	G2 MCP	LG	N0/X	M1	30	0	No	14.6 (NED)	
28	✓		✓	F	53	GCA	G3 PM	HG	N0/X	M1	16	0	No	45.3 (AWD)	
29	✓		✓	M	51	MAC	G3 MCP	HG	N0/X	M1	39	1	Neoadjuvant FOLFOXIRI/Bevacizumab	7.4 (NED)	
30	✓(✓)		✓	M	47	AMN	G1 MCP	LG	N0/X	M1	18	0	No	13.4 (NED)	
31			✓	F	44	GCA	G3 PM	HG	N0/X	M1	16	0	Neoadjuvant FOLFOXIRI/Bevacizumab	17.0 (NED)	
32			✓	F	40	Not reported	G1 MCP	LG	N0/X	M1	39	1	No	306.4 (AWD)	
33			✓	M	55	GCA	LG PM	HG	N1/2	M1	29	0	Neoadjuvant FOLFOXIRI	20.8 (NED)	
34			✓	F	39	MAC	G3 GCA	HG	N0/X	M1	10	0	Adjuvant FOLFOX	10.0 (NED)	
35			✓	F	47	AMN	G1 MCP	LG	N0/X	M1	29	0	No	3.3 (NED)	

^aWorld Health Organisation 2019 classification.¹⁴

^bDichotomous categorisation for the purpose of this study.

^cThese two cases of ATPD had no tumour cells identified on research samples and were used as ‘normal’ controls for spatial and immune cell analysis (#5 had no tumour on diagnostic specimen, #7 had tumour present on diagnostic specimen).

^dThis case had no tumour present on research samples and was used to evaluate immune and stromal microenvironment.

^eSystemic chemotherapy was grouped by intent defined by relationship with CRS/HIPEC: adjuvant (<6m after CC0-1 CRS), neoadjuvant (<6m before CRS), prior (>6m before CRS); palliative (no or CC2-3 CRS).

^f#16 was initially treated with a small cell/neuroendocrine regimen when diagnosis was thought to be neuroendocrine carcinoma which was altered to appendiceal GCA after specialist review at this campus.

*Cases excluded from scRNAseq analysis of LG and HG tumour type.

Abbrev. ATPD, appendiceal tumour peritoneal disease; scRNAseq, single cell RNA sequencing; IHC, immunohistochemistry; M, male; F, female; AMN, appendiceal mucinous neoplasm; MAC, mucinous adenocarcinoma; ANOS, adenocarcinoma not otherwise specified; GCA, goblet cell adenocarcinoma; G1, grade 1, G2, grade 2; G3, grade 3; PM, peritoneal metastasis; MCP, mucinous carcinoma peritonei; SRC, signet ring cell adenocarcinoma (>50% signet ring cells); LG, low grade; HG, high grade; N, nodal stage; M0/I, metastasis stage; PCI, peritoneal cancer index; CC, completeness of cytoreduction score; FOLFOX, 5-fluorouracil + leucovorin + oxaliplatin; FOLFOXIRI, FOLFOX + irinotecan; MCAP, mitomycin C + capecitabine; CE, carboplatin + etoposide; OS, overall survival; NED, no evidence of disease; AWD, alive with disease; DOD, died of disease.

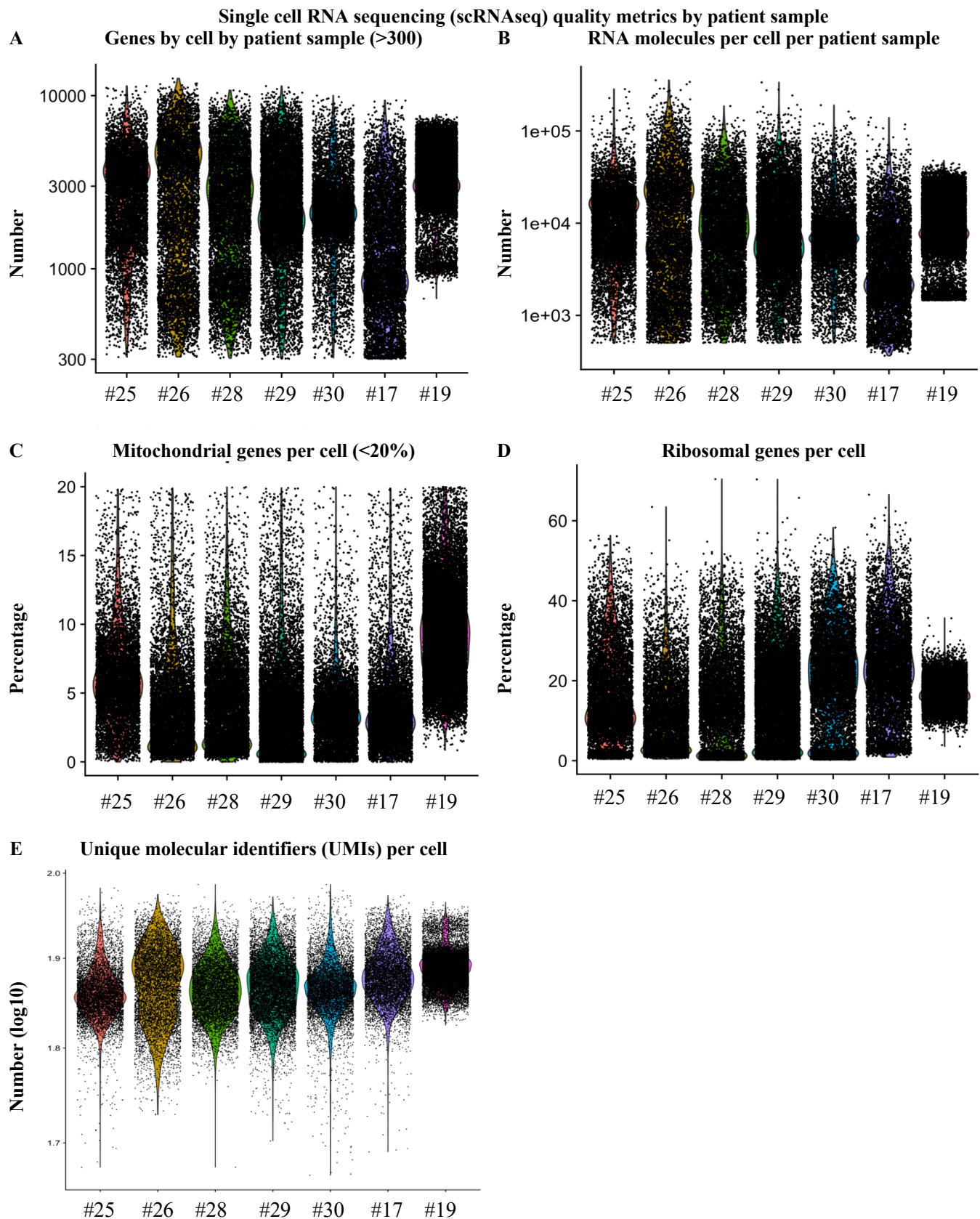


Figure 3.4 | scRNAseq quality metrics after post filtering out cells with more than 20% mitochondrial genes and cells with less than 300 genes per cell

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Number of genes per cell (A), number of RNA molecules per cell (B), percentage of mitochondrial genes per cell (C), percentage of ribosomal genes per cell (D) and number of UMIs per cell per tumour sample analysed by scRNAseq (E).

*Pilot study, excluded from main analysis.

Abbrev. UMI, unique molecular identifiers; scRNAseq, single cell RNA sequencing.

3.3.2 Single cell atlas of ATPD

Knowledge of the cell types present in ATPD and their roles in the TME is essential to understand the biological processes that may lead to new rational treatment strategies. It was hypothesised that tumour cells have altered genes related to mucin production and that cell types other than the tumour cells may contribute to the disease pathogenesis. To understand the potential different cell-types present in ATPD from scRNAseq, analysis of all samples combined was performed. This computational calculation is a data-driven, unbiased approach that groups ('clusters') the cell types by their transcriptomic similarity without any categorisation by the user.⁴¹⁹ Cell types that have different gene profiles from each other appear as clusters in a different location on a type of visualisation called the uniform manifold approximation and projection (UMAP; Figure 3.5A). The number of cell type clusters depends on the specified resolution chosen for analysis. Low resolution analysis of these data show there were 17 broad groupings of similar cell types (depicted by the dashed red lines; Figure 3.5A). High resolution analysis, as described in the methods (section 3.2.4), shows there were 38 smaller groupings of cells, with each subcluster assigned a different colour and number (Figure 3.5A). These groupings indicate the different major and minor cell subsets present in the ATPD microenvironment (Figure 3.5A).

Some of these subsets represent the same cell type, but have different gene profiles because they originate from different tumour types or patient samples. To inspect these possibilities on the UMAP, analysis was performed by classification of the cells by tumour type and patient sample (Figure 3.5B,C). This confirmed that the major cell populations had similar genetic profiles as they grouped together on the UMAP, but that some of the cell subclusters resulted from genetic differences in the cells. These differences were attributed mostly due to the LG (16/38 subclusters; 42%) or HG (12/38 subclusters; 32%) tumour type as only 3/38 (8%) clusters were associated with both LG and HG samples (given by the visual appearance of coloured groupings of cells on the UMAP; Figure 3.5B). The influence from patient heterogeneity was less (visually represented on the UMAP by mixing of the coloured cell groups; Figure 3.5C). Specific examples of this include: (1) subcluster 21 represented cells from LG case #17, which suggested this cell type was specific to the patient sample, (2) subclusters 12 and 14 represented cells from two HG cases (#26 and #29), which suggested a common cell type to HG disease, and (3) subclusters 5 and 6 represented multiple LG and HG case samples which suggested that the cell type was common across patient samples and tumour types (Figure 3.5A-C). This demonstrates that despite expected heterogeneity between patients' tissues and variation in technical aspects of the workflow due to individual sample processing, this analysis without batch correction preserved broad transcriptomic differences between cell types.

This informed the annotation process of the proposed cell types present in the analysis. The cell types were manually annotated for each of the major cell lineages expected in the TME using canonical gene markers (Figure 3.5D,E). This confirmed that this scRNAseq analysis of ATPD comprised all major cell lineages of the TME including tumour cells, mesothelial cells, immune cells and stromal cells.

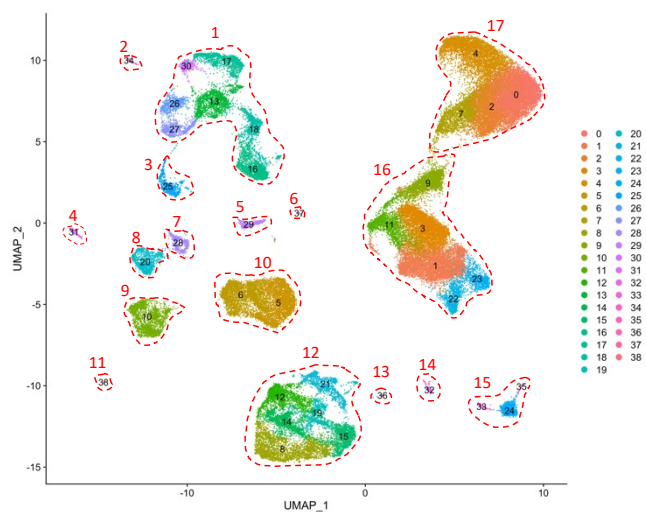
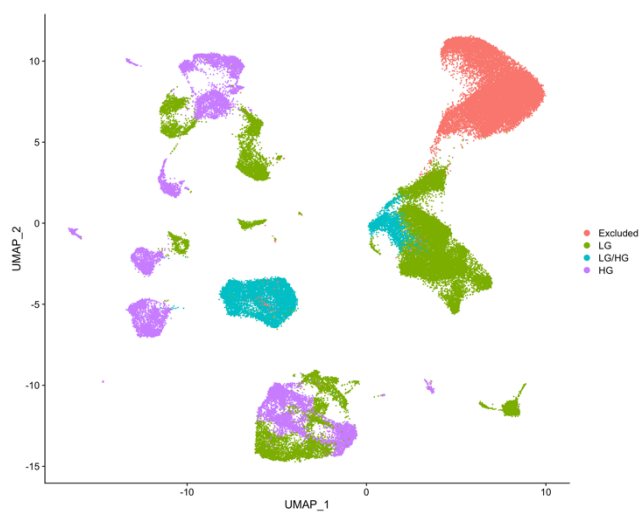
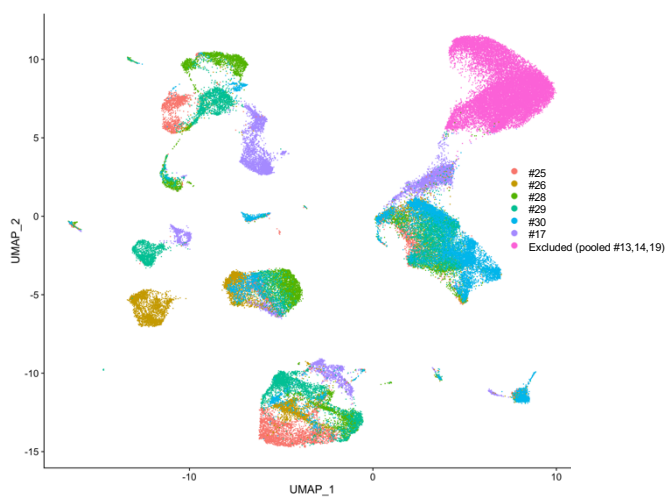
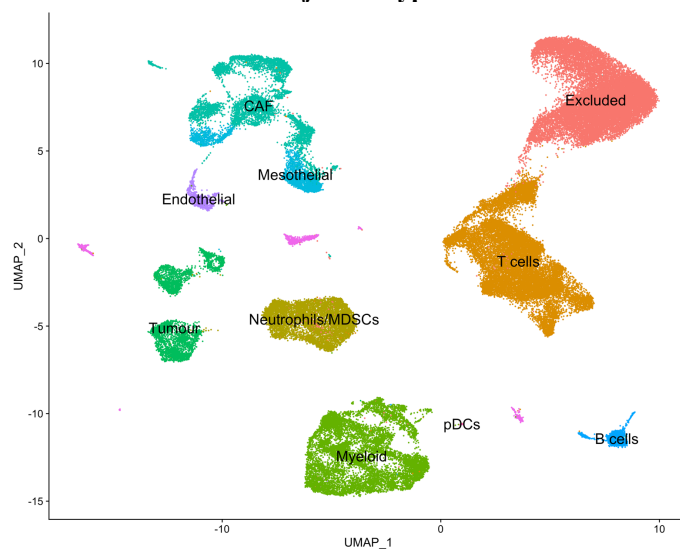
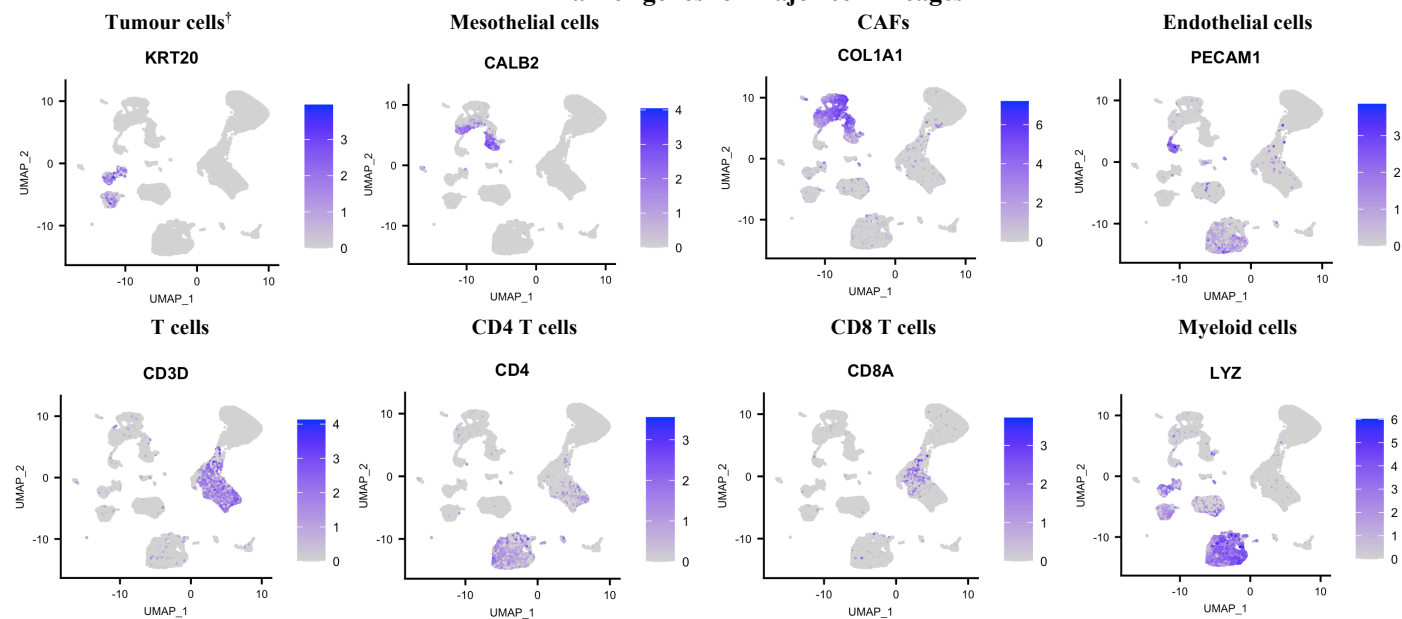
A Analysis of cell types in ATPD**B** Analysis by tumour type***C** Analysis by patient sample***D** Annotation of major cell types**E** Marker genes for major cell lineages

Figure 3.5 | UMAP and feature plot visualisation of cell compartments and canonical lineages for ATPD

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UMAP visualisation showing clustering of major (red dashed lines) and minor cell types (black numbers) for ATPD (A), identified by patient sample in (B) and tumour type in (C). UMAP visualisations showing annotation by cell compartment (C) and by major cell lineage in (D). Feature plots (UMAPs identifying cells expressing single genes of interest in blue; E) show log-normalised expression of canonical genes for major cell types: tumour cells (*KRT20*), peritoneal mesothelial cells (*CALB2*), T cell subsets (*CD3D*, *CD4*, *CD8A*), CAFs (*COL1A1*), endothelial cells (*PECAMI*) and monocyte/macrophages (*LYZ*) for ATPD. Additional canonical genes shown on the UMAP in (C) that are not displayed in (D) included: B cells (*CD79A*), neutrophils (*S100A9*) and plasmacytoid dendritic cells (*LILRA4*).

*One pooled sample from the pilot study (comprising tissues from patients #13, #14 and #19) was excluded from ongoing analysis due to assessment of a non-representative cell type biasing this sample. Individual analysis of this sample indicated a group of mostly neuroendocrine basal proliferating cells which was also verified on staining tissue sections for tumour and proliferation markers.

†Tumour cells were identified to be diploid on inferred copy number analysis on the pilot samples (pooled #13,14,19) and further analysis on subsequent samples was not performed.

Abbrev. UMAP, uniform manifold approximation and projection; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; CAFs, cancer associated fibroblasts, MDSCs, myeloid derived suppressor cells; pDCs, plasmacytoid dendritic cells.

Based on the previous analysis showing that tumour type influenced the cell type groupings and due to the varied clinical behaviour of LG compared to HG ATPD, analysis was performed for the scRNAseq profiles of each tumour type. The purpose of the analysis by LG and HG tumour type was to provide a description of the TME for each respective tumour type, rather than a direct comparison between LG and HG. For LG ATPD, this identified 11 major cell types (identified visually by the different shapes on the UMAP, outlined in red dashed lines and annotated with red numbers; Figure 3.6A) and 34 subclusters (identified visually by the different colours within each shape and annotated by smaller black numbers; Figure 3.6A) and for HG ATPD and 15 major cell types and 30 subclusters for HG ATPD (Figure 3.6B). Further analysis was performed within the LG and HG groups to assess the contribution from each patient sample. Most of the major cell populations were contributed to by each of the three patient samples for both LG and HG ATPD (Figure 3.6A-D). The most notable exception to this was HG major cell types 14 and 15 (red text annotated labels in Figure 3.6B) which formed discrete populations from patient samples #29 and #26 respectively, in keeping with intersubject heterogeneity (Figure 3.6D). Following cell annotation by canonical markers and the top 10 differential gene expression for each subcluster compared to all other subclusters for LG (Figure 3.7A,C) and HG ATPD (Figure 3.7B,D), these cell types represented were tumour cells (*KRT20*) with different gene profiles from each patient; despite the same histological diagnoses (grade 3 mucinous carcinoma peritonei). This is typical of tumour cell heterogeneity in tumour samples from different patients and has been reported in other cancer types.⁴²⁰

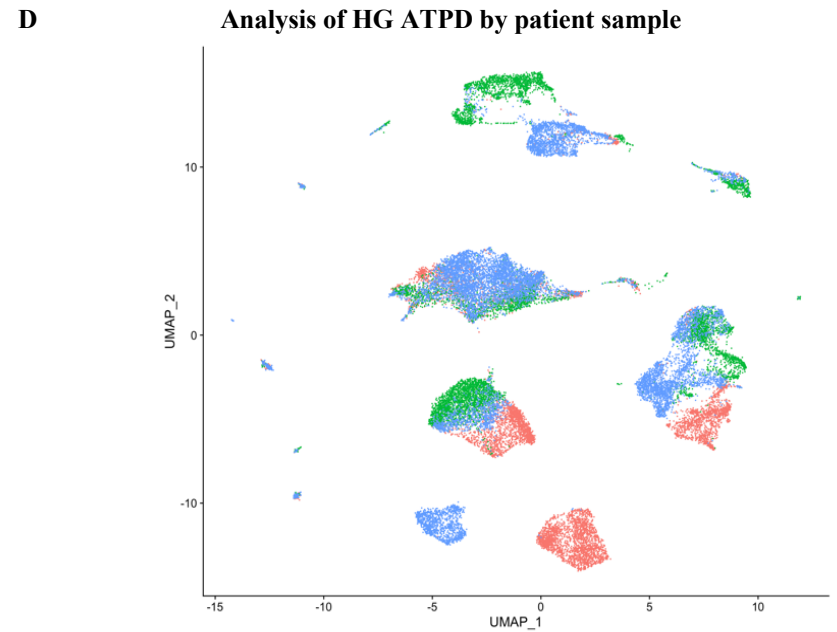
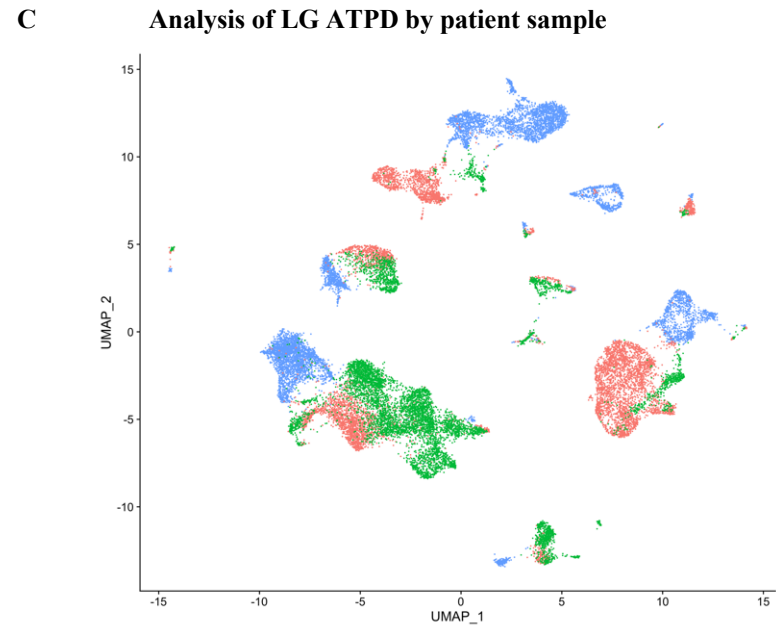
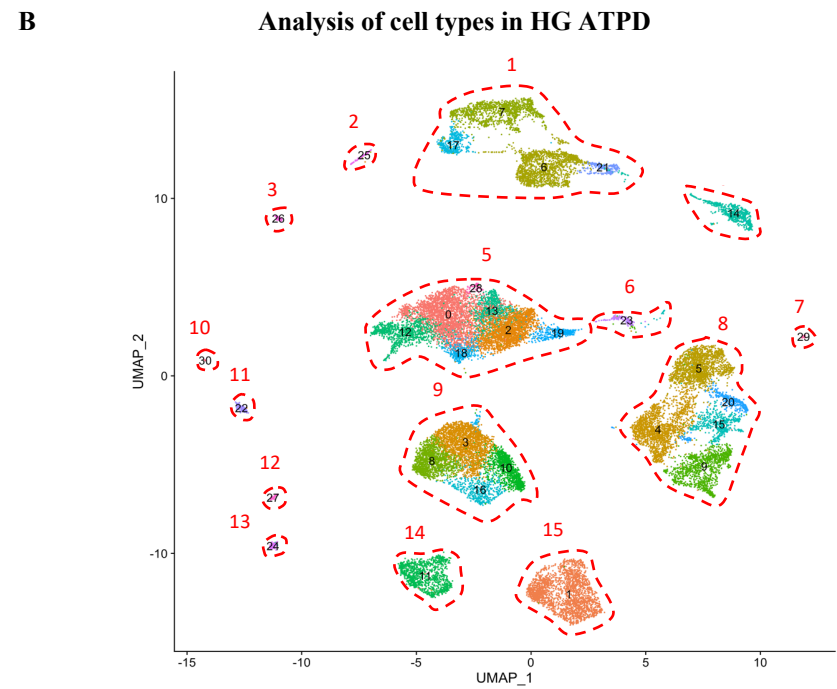
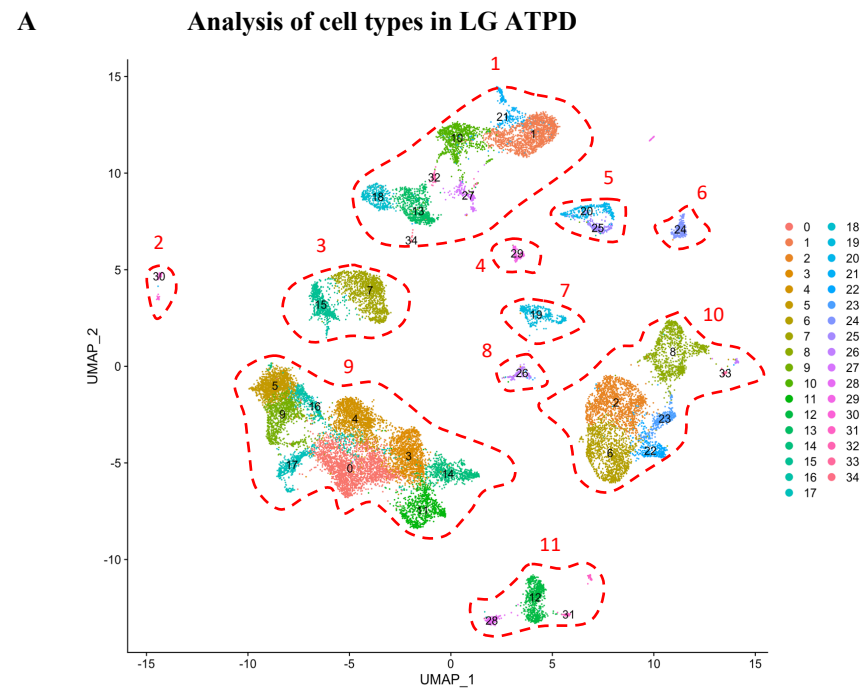


Figure 3.6 | UMAP visualisation of cell clustering for LG and HG ATPD by sample type

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UMAP visualisation showing clustering of major (red dashed line) and minor cell types for LG (**A**) and HG (**B**) ATPD, identified by patient sample in (**C**) and (**D**). Red text labels adjacent to the red dashed line annotations (**A**, **B**) correspond to the manual count of clusters after the analysis was performed whereas the black text labels overlying each coloured clusters corresponds to the automated subcluster count generated as part of the ‘nearest neighbour’ clustering analysis.

Abbrev. UMAP, uniform manifold approximation and projection; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade

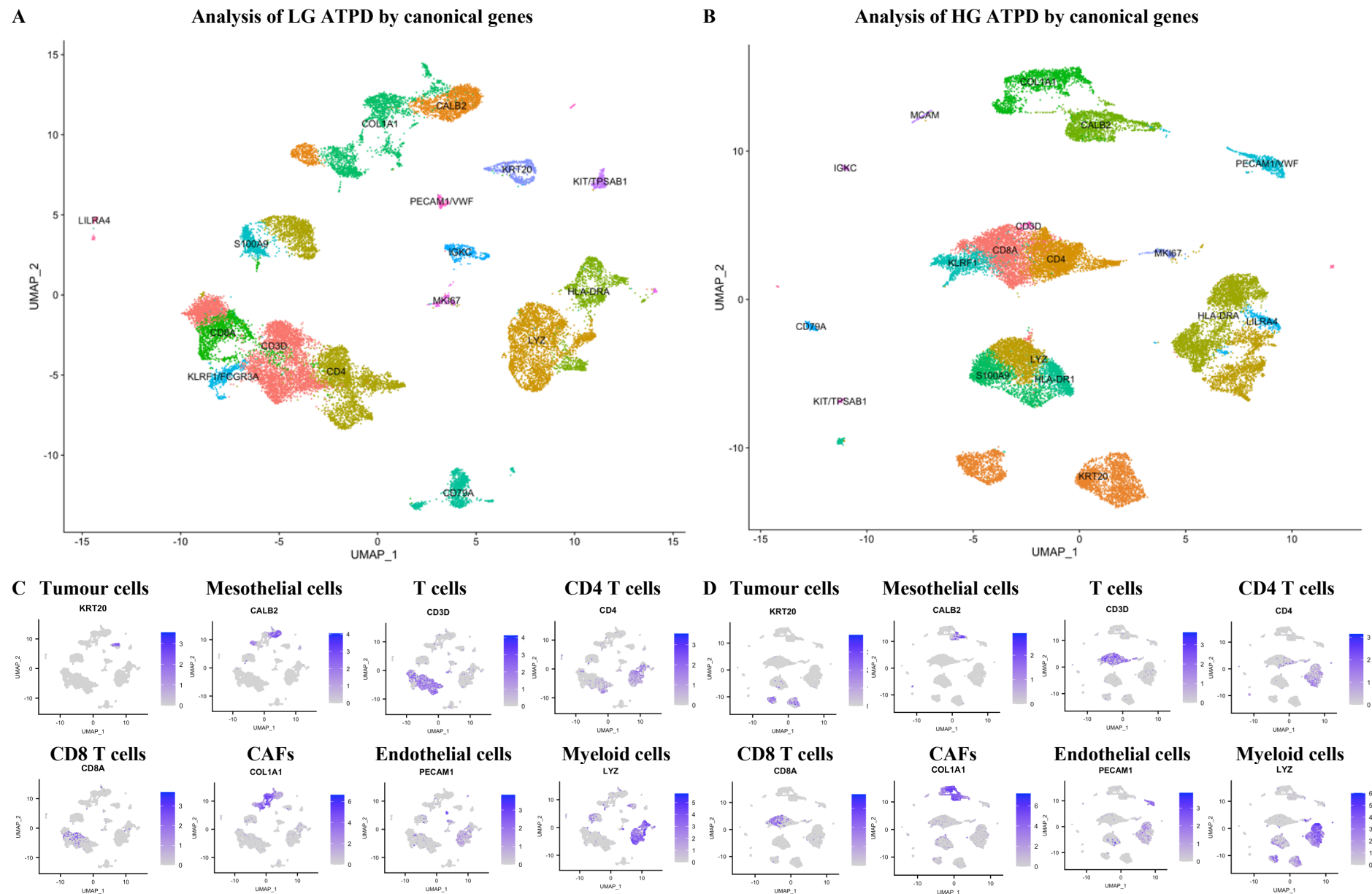


Figure 3.7 | UMAP and feature plot visualisation of cell compartments and canonical lineages for LG and HG ATPD

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UMAP visualisations showing annotation by canonical cell lineage for LG (**A**) and HG (**B**) ATPD. Feature plots (UMAPs identifying cells expressing single genes of interest; **C**, **D**) show colour scale represents the log-normalised expression of canonical genes for major cell types: tumour cells (*KRT20*), peritoneal mesothelial cells (*CALB2*), T cell subsets (*CD3D*, *CD4*, *CD8A*), CAFs (*COL1A1*), endothelial cells (*PECAM1*) and monocyte/macrophages (*LYZ*) for LG (**C**) and HG (**D**) ATPD. Additional canonical genes shown on the UMAPs in (**A**,**B**) that are not displayed in (**C**,**D**) included: B cells (*CD79A*), neutrophils (*S100A9*), plasmacytoid dendritic cells (*LILRA4*), natural killer cells (*KLRF1*, *FCGR3A*), macrophage/dendritic cells (*HLA-DRA*, *HLA-DRI*), Plasma cells (*IGKC*), cycling cells (*MKI67*), mast cells (*KIT*, *TPSAB1*), perivascular-like (*MCAM*).

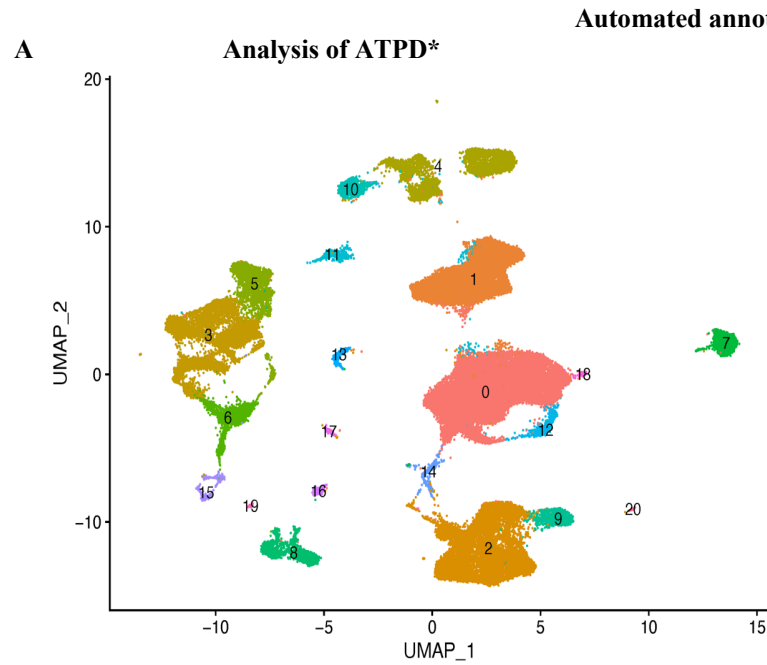
Abbrev. UMAP, uniform manifold approximation and projection; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; CAFs, cancer associated fibroblasts.

Given ATPD specific scRNAseq data was not available to verify the proposed cell type annotation, automated cell annotation was performed with reference to existing non-ATPD data. Reference data came from two sources: (1) localised prostate cancer (unpublished data courtesy of Ms Eva Apostolov who helped perform this analysis, with permission of A/Prof Swarbrick (Figure 3.8A,B); and (2) the Human Primary Cell Atlas (Figure 3.8C,D).⁴⁰⁶ These analyses were consistent with the manual annotations confirming accuracy of the summarised annotation, except for the following: (1) in the annotation using the prostate cancer reference (Figure 3.8B), there was no annotation for the mesothelial cell type as this is not a known cell type in prostate cancer; and (2) in the annotation using the Human Primary Cell Atlas there was erroneous identification of chondrocytes, hepatocytes and astrocytes in LG ATPD, and, chondrocytes and osteoblasts in HG ATPD. These cell types are not present in the peritoneum. Additionally, a HG tumour cell type was inaccurately predicted to be ‘embryonic stem cells’ using this automated annotation method (Figure 3.8D). While the tissue is not of embryonic origin it is possible that the tumour cells share stem cell properties. Furthermore, these inaccuracies of the predicted cell types using automated methods illustrate the challenges of annotating a novel cellular microenvironment.

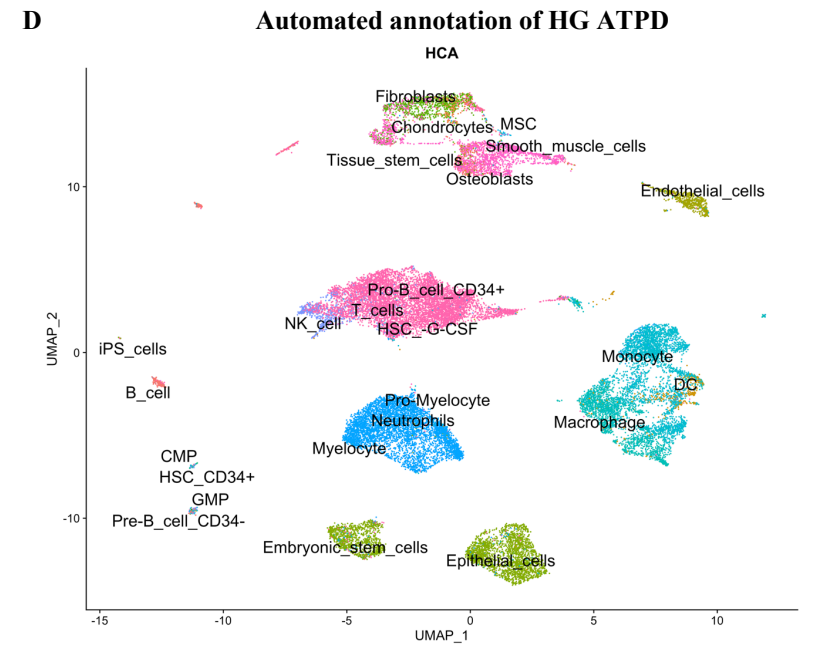
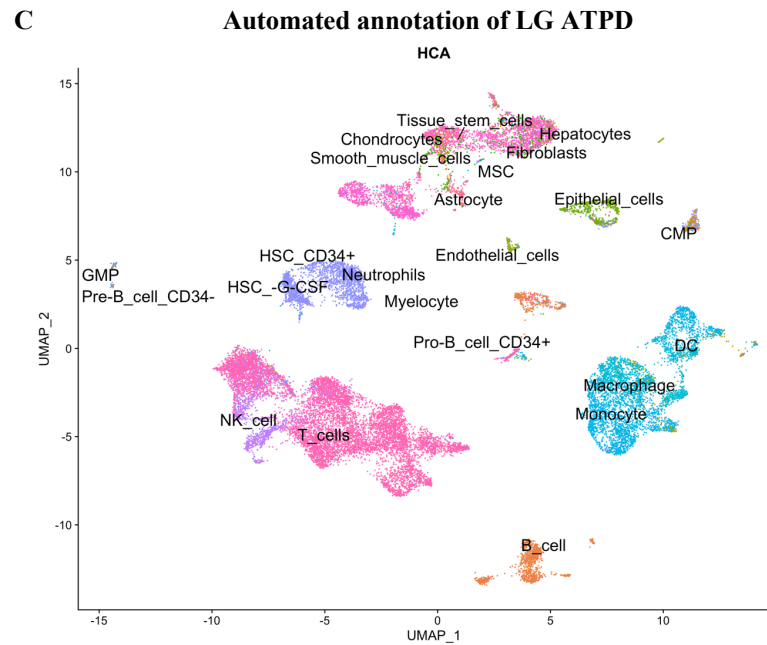
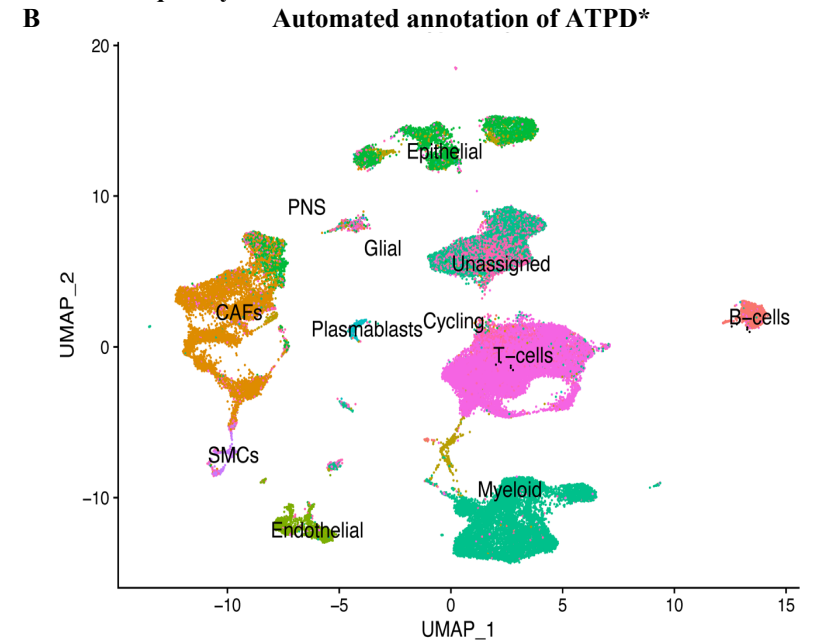
The summarised visualisation of the combined annotation methods for each tumour type is depicted in Figure 3.9. The top ten genes per cluster which supports this annotation are depicted in Appendix 3 for LG ATPD (Supplementary Table ST3.1 and Supplementary Figure SF3.8) and HG ATPD (Supplementary Table ST3.2 and Supplementary Figure SF3.9). The key finding from the summarised annotation was that ATPD contained all major cell lineages of the TME.⁴²¹ To validate the major cell lineages identified using scRNAseq, flow cytometry was performed on ATPD tissue samples to confirm the presence and proportion of each cell compartment (Figure 3.10). This was compared to the proportions of each cell group identified on scRNAseq analyses (Figure 3.11). Three patient samples had matched flow cytometry and scRNAseq analysis (#19, #26, #30). While the proportions of each cell type between patient samples varied, this analysis confirmed the larger immune and stromal cell compartment (including mesothelial cells) compared to endothelial and tumour cells. Therefore, while allowing for the differences between the cell types due to expected interpatient heterogeneity, this scRNAseq analysis accurately identified the major cell types of the ATPD TME. Minor cell type annotation identified similar cell subtypes particularly in the immune compartment (T cells and myeloid cells). LG appeared to have more prominent CD8 T cells, B cells and mast cells than HG ATPD, which appeared to have more prominent endothelial cells. The different tumour cell types in HG ATPD suggests more intersubject tumour heterogeneity than in LG ATPD.

Reference

Localised prostate cancer
TME*



Automated annotation of scRNAseq analysis



Human Primary Cell Atlas

Figure 3.8 | Automated cell annotation of ATPD with reference to localised prostate cancer and the Human Primary Cell Atlas

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UMAP visualisations show annotation of combined LG and HG ATPD (**A***), automated cell annotation with reference to a localised prostate cancer TME (Courtesy of Ms Apostolov and A/Prof Swarbrick) (**B***), automated annotation of LG (**C**) and HG ATPD (**D**) using the Human Primary Cell Atlas reference.⁴⁰⁶

*The UMAP visualisations appear different comparing A and B to C and D because the analysis for A and B was performed in a different analysis session by Ms Apostolov. This occurs due to the stochastic nature of the dimensionality reduction algorithm which means values are different each time the calculation is performed. This can be avoided by setting a common seed (e.g. `set.seed(42)`).

Abbrev. UMAP, uniform manifold approximation and projection; ATPD, appendiceal peritoneal disease; LG, low grade; HG, high grade; CAFs, cancer-associated fibroblasts; SMCs, smooth muscle cells; PNS, peripheral nervous system; CMP, common myeloid progenitor; DC, dendritic cell; GMP, granulocyte-monocyte progenitor; HSC, Haematopoietic stem cells; MSC, mesenchymal stem cells; NK, natural killer; iPS, induced pluripotent stem cells.

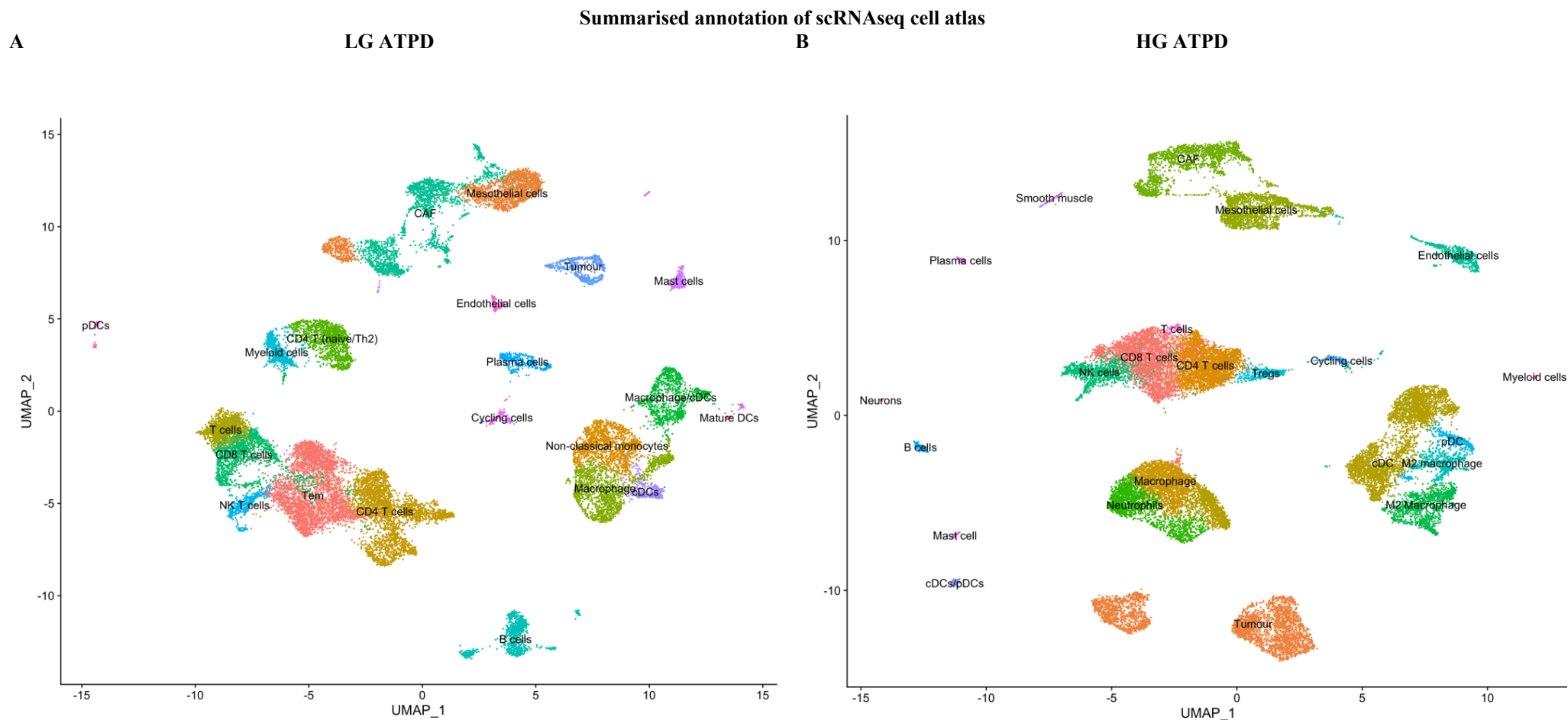


Figure 3.9 | Summarised annotation for a single cell atlas of LG and HG ATPD

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UMAP visualisations of LG (**A**) and HG (**B**) ATPD show the proposed annotation of clusters based on combined manual and automated annotation methods.

Abbrev. UMAP, uniform manifold approximation and projection; ATPD, appendiceal peritoneal disease; LG, low grade; HG, high grade; CAF, cancer-associated fibroblasts; Th2; T helper type 2; Tem, T effector memory cell; Treg, regulatory T cells; DC, dendritic cells; cDCs, conventional dendritic cells; pDCs, plasmacytoid dendritic cells; NK, natural killer

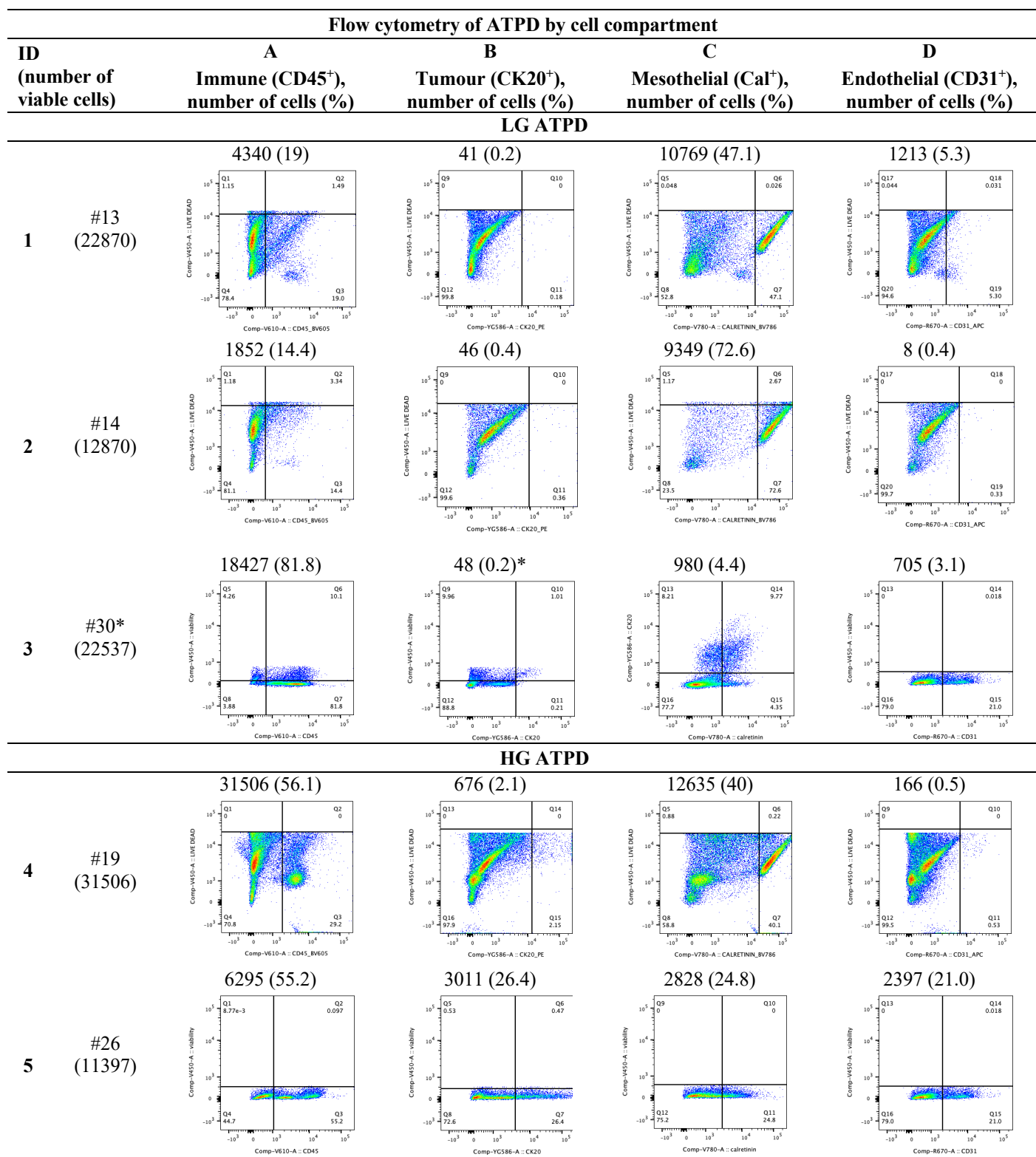


Figure 3.10 | Flow cytometry of ATPD patient samples assessing presence and proportions of major cell lineages

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Columns are cell compartment (**A-D**): Immune cells (CD45⁺) (**A**), tumour cells (CK20⁺) (**B**), mesothelial cells (Cal⁺) (**C**), endothelial cells (CD31⁺) (**D**). Rows are patient cases (1-5): LG ATPD (1-3) and HG ATPD (4-5). Sample IDs #19, 26 and 30 were matched to scRNAseq analysis.

*No tumour based on diagnostic report of acellular mucin.

Abbrev. Cal, calretinin.

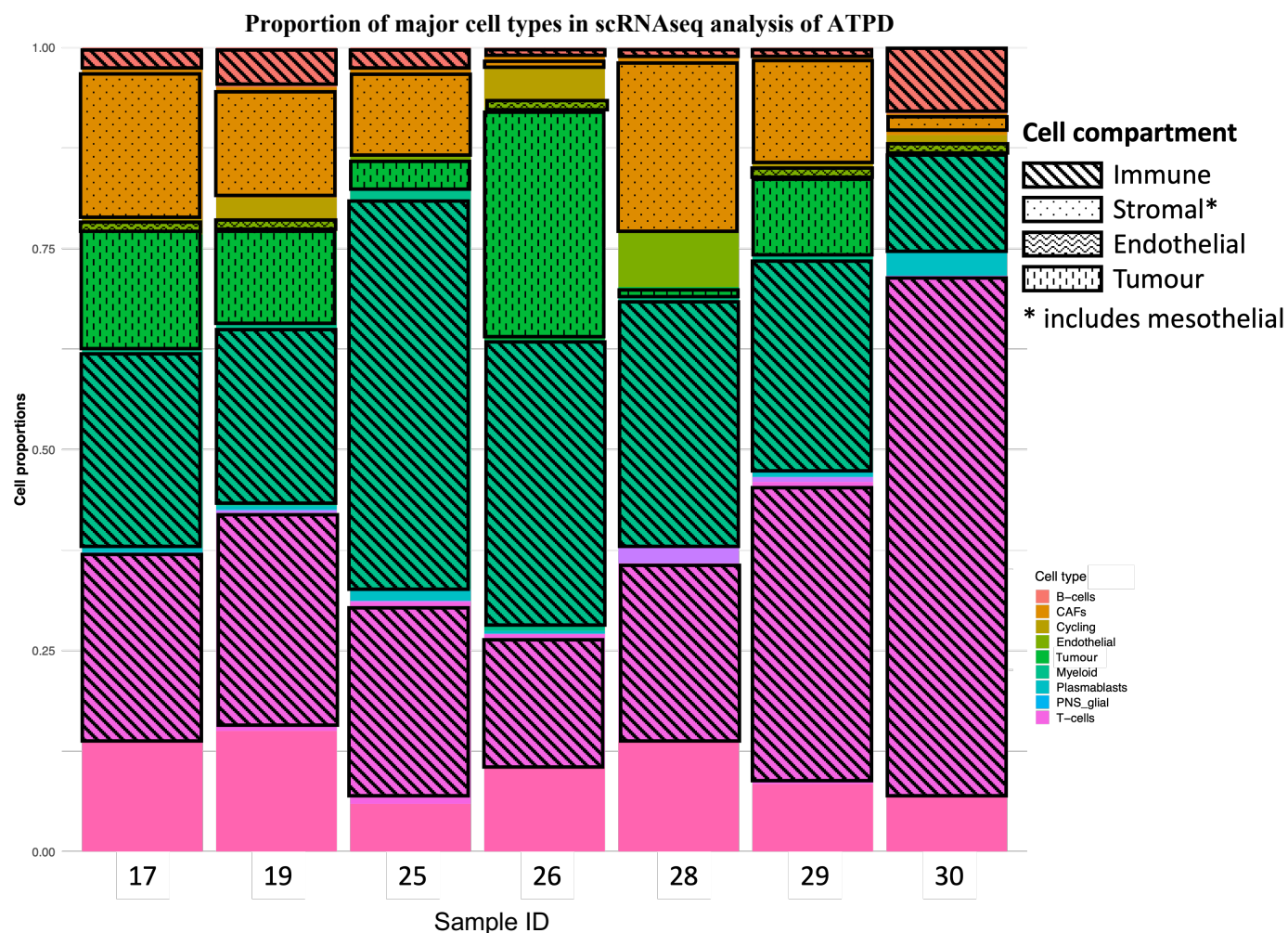


Figure 3.11 | Proportions of major cell lineages in scRNAseq analysis by patient sample

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Patterns show the main cell compartments and colours the cell type. Sample IDs #19, 26 and 30 were matched to flow cytometry analysis.

Abbrev. ATPD, appendiceal tumour peritoneal disease; CAFs, cancer associated fibroblasts; PNS, peripheral nervous system.

3.3.3 Spatial transcriptomic profiling of tumour, immune and stromal regions of ATPD

The single cell atlas details cell types present in ATPD but is limited by a lack of spatial annotation and reference to the architecture of the TME. This is highly relevant for ATPD, which is a disease predominantly characterised by excess mucin production. Mucin is an acellular structure and so the genetic profile cannot be characterised. Evaluation of mucin and its role in influencing the TME must be indirectly inferred by the transcriptomic characteristics of the surrounding cells. It was hypothesised that non-tumour supporting cells may modulate or directly contribute to the excess mucin production, which is a hallmark of this disease.

Spatial transcriptomic profiling was performed of the whole transcriptome (more than 18,000 genes) on FFPE slides from 22 patients with ATPD (13 LG, 5 HG), two 'normal' peritoneal samples, one peritoneal metastasis from a colorectal cancer control and one tonsil tissue control. The control tissues behaved as expected with the peritoneal metastasis from colorectal cancer sample displaying staining positive for anti-CK20 and tonsil tissue displaying staining negative for anti-CK20. Comparison of gene profiles of cell segments classified by tumour type and segment (tumour, immune or stromal) demonstrated differences in the profiles between tumour and immune/stromal segments (**Error! Reference source not found.**). LG and HG tumour cells clustered separately within the tumour segments on the UMAP, while immune cells from HG ATPD exhibited more transcriptomic variation interspersed within the LG immune segments (**Error! Reference source not found.**). The immune and stromal segments demonstrated some segregation but were closely located. This broad co-location may reflect the way immune cells infiltrate stromal areas making discrimination of fluorescent thresholds (using the CD45 fluorochrome) a technical challenge.

To confirm that the analysis captured accurate biological differences in the tumour tissues compared to non-tumour tissues, transcriptomic profiles of stromal and immune segments were compared to those from normal peritoneal tissues (noting the normal tissues were patient cases that had no identifiable tumour cells). This analysis showed differences between the differential gene expression comparing stromal segments from ATPD tissues to those from normal peritoneal tissues ($FDR < 0.05$; Figure 3.13A,B). There were not any statistically significant differentially expressed genes between immune cells within LG or HG ATPD compared to normal peritoneal tissue ($FDR > 0.05$; Figure 3.13C,D). Nevertheless, in both stromal and immune segments, both LG and HG ATPD showed significantly different profiles on gene set enrichment analysis ($P_{adj} < 0.05$; Figure 3.14). There were no major fundamental differences in the enriched pathways observed in LG and HG ATPD: Stromal segments were enriched with genes associated with 'cytoplasmic translation', 'ribosome' and 'focal adhesion' (Figure 3.14A,B) and immune segments were enriched with genes associated with 'ECM [extracellular matrix] proteoglycans' and 'extracellular matrix organisation' (Figure 3.14C,D). The one exception to this was the observation of enrichment for 'integrin cell surface interactions' ($P_{adj} < 0.05$; Figure 3.14C) in immune cells from LG ATPD compared to normal tissue. These findings suggest the transcriptomic profile of ATPD tissues reflects differences attributable to tumour presence. Furthermore, ATPD stromal segments reflect active protein synthesis pathways and immune segments involvement in extracellular matrix remodelling, including cell-adhesion interactions, as part of the evolving adaptation of the TME to the tumour.⁴²²

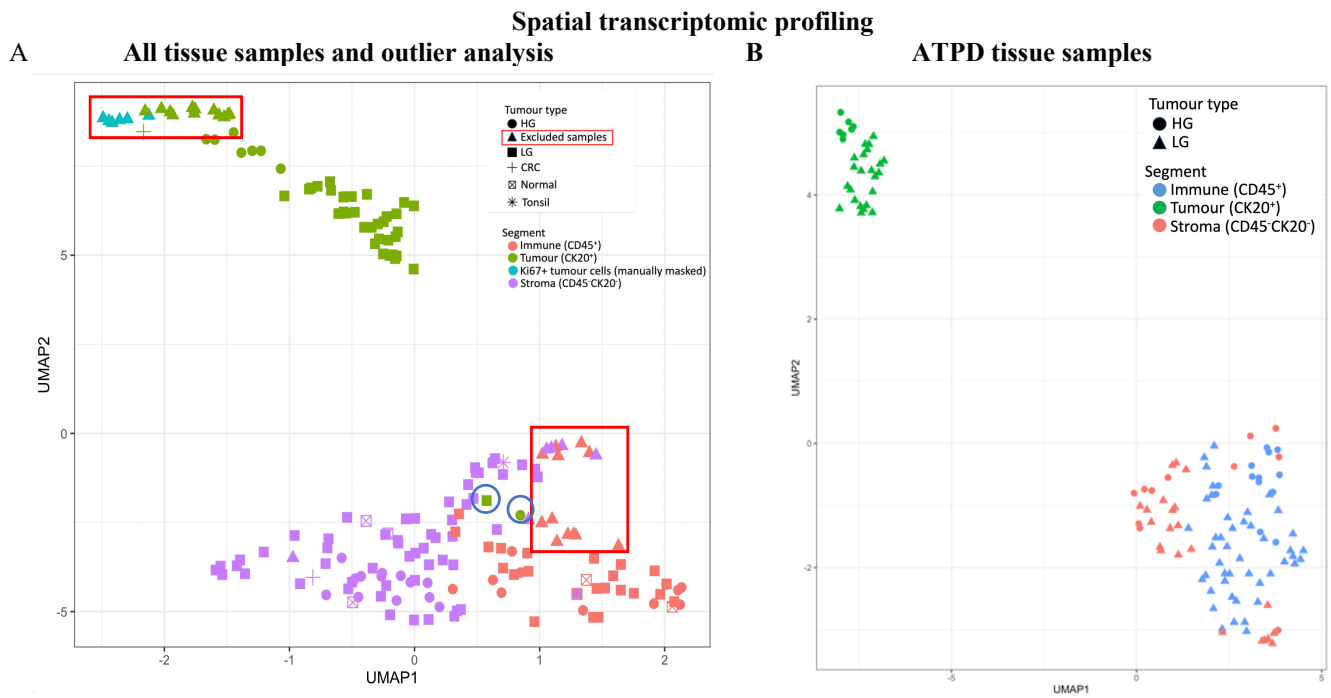


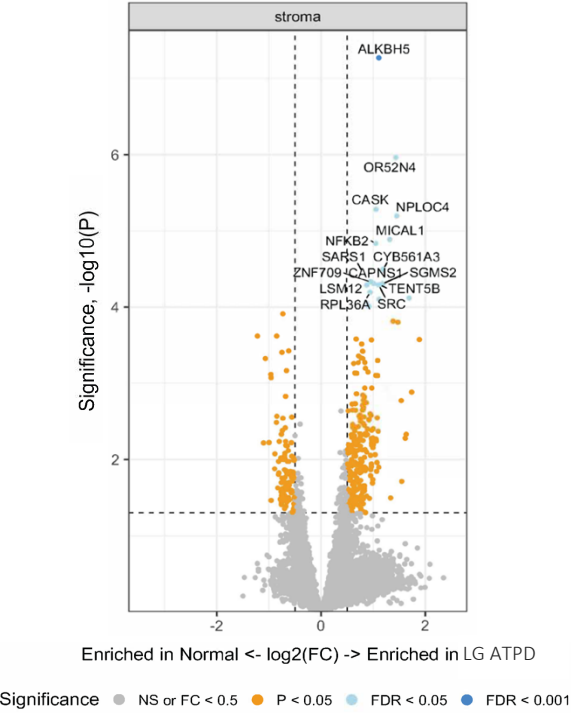
Figure 3.12 | Spatial transcriptomic profiling showing cell compartments

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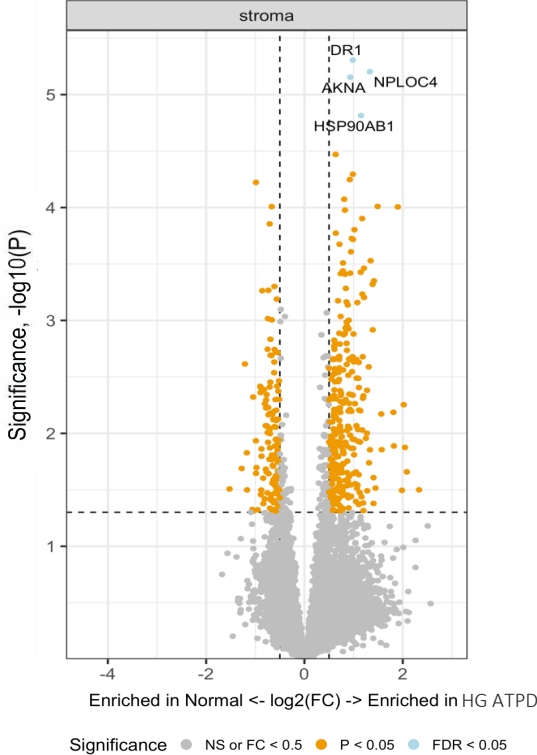
UMAP visualisations of immune, tumour (including proliferating ki67 positive cells) and stromal segments by tumour type. Comparison of ATPD tissues, including excluded samples in red box, to colorectal, tonsil and normal peritoneal control tissues is displayed. Two tumour outliers are shown in blue circles (A). ATPD comparing HG to LG with outliers and control tissues removed are shown in (B).

Abbrev. UMAP, uniform manifold approximation and projection; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; CRC, colorectal cancer.

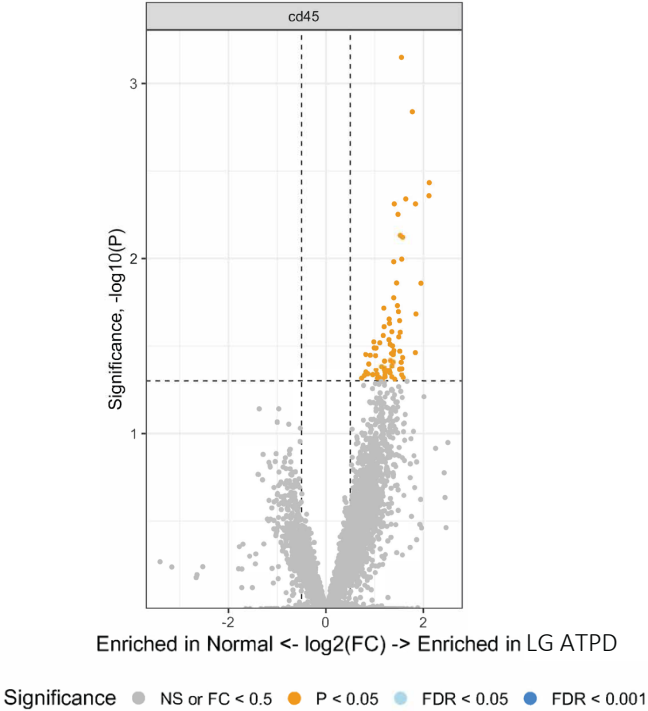
A Normal tissue versus LG ATPD stromal segments



B Normal tissue versus HG ATPD stromal segments



C Normal tissue versus LG ATPD immune segments



D Normal tissue versus HG ATPD immune segments

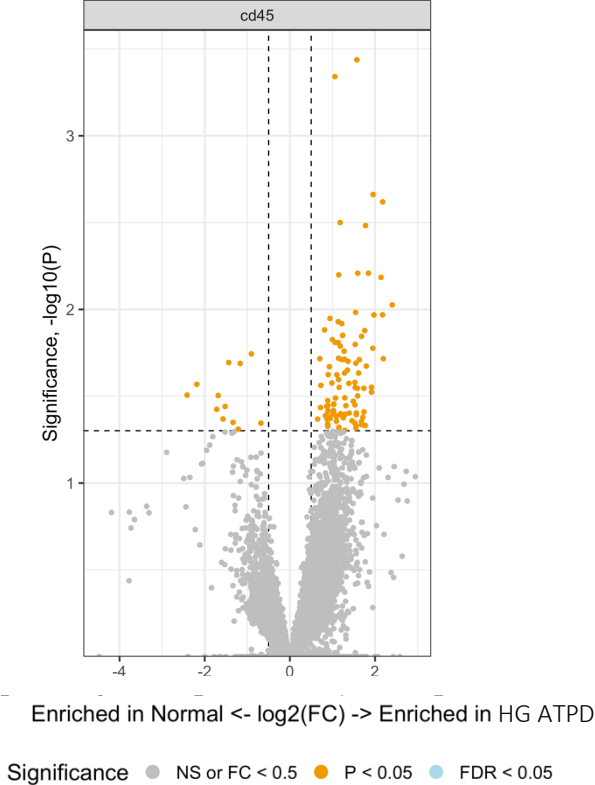


Figure 3.13 | Differentially expressed genes comparing normal tissue to ATPD

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The volcano plots show the differential gene expression by log2FC between stromal cell (A, B) and immune cell segments (cd45; C, D) in normal peritoneal tissue compared to LG (A, C) and HG ATPD (B, D). Significantly differentially expressed genes were defined as log2FC > 0.5 and FDR p-value < 0.05 (blue dots; A, B).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; log2FC, log2 fold change; FDR, false discovery rate

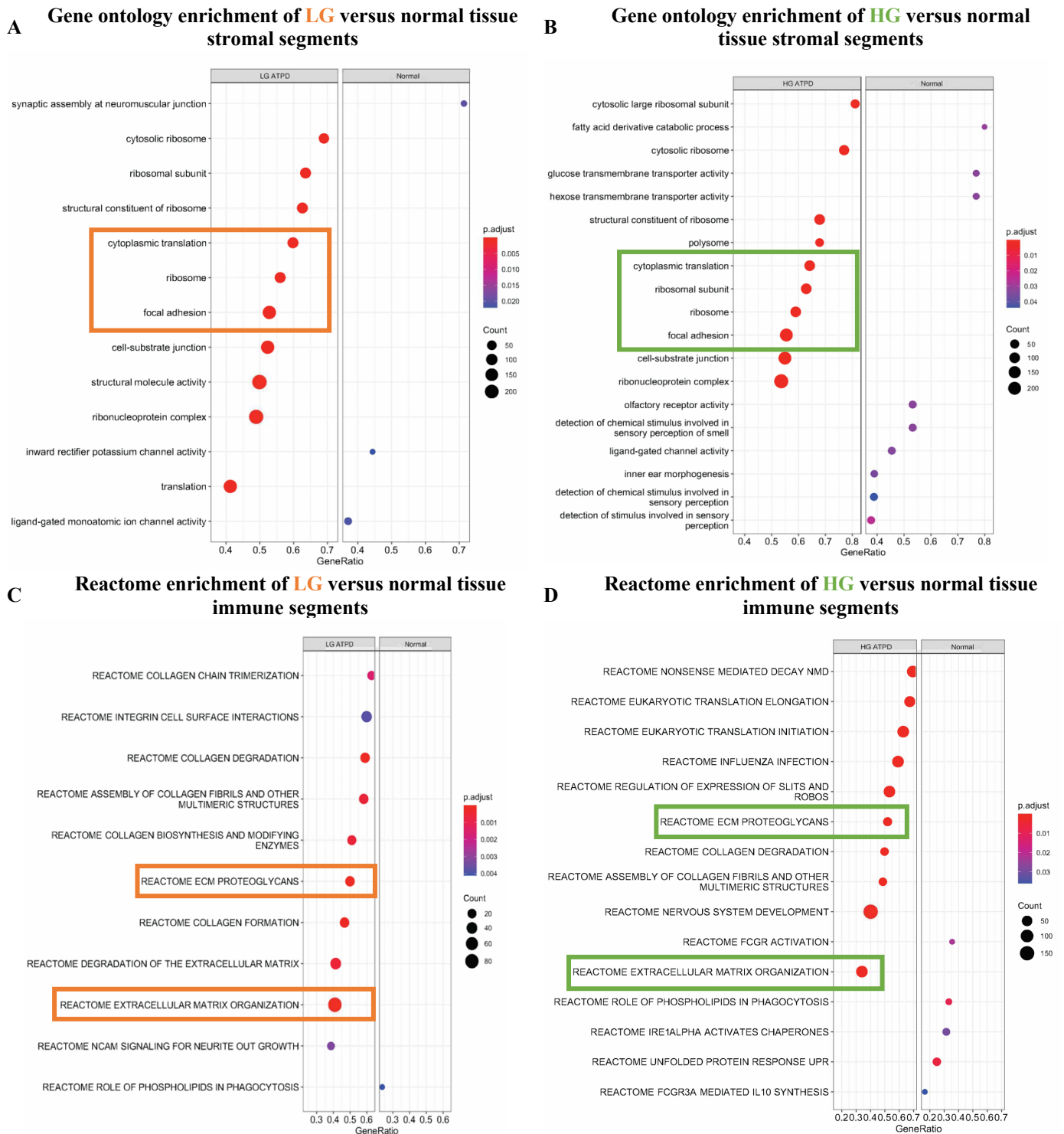


Figure 3.14 | Comparison of enriched gene sets in stromal and immune cells comparing ATPD to normal peritoneal tissue
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Dotplots show Gene Ontology enrichment of stromal cell segments (A,B) and Reactome enrichment of immune cell segments (C,D) comparing LG (A,C) and HG ATPD (B,D) to normal peritoneal tissue. The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted $p < 0.05$).

Abbrev. ATPD, appendiceal tumour peritoneal disease; HG, high grade; LG, low grade; ECM, extracellular matrix.

3.3.4 Transcriptomic profiles of tumour cells

Tumour-specific genes may identify potential biomarkers and therapeutic targets that are currently lacking in ATPD.⁴²³ In this study, transcriptomic analyses from both scRNAseq and spatial profiling confirmed gene expression differences between tumour and non-tumour cells and between LG and HG ATPD. These comparisons in genetic profiles, the corresponding biological themes and relevant signalling pathways are described below.

a) Tumour versus non-tumour cells

The annotated tumour cell clusters for LG and HG ATPD (Figure 3.9) were compared to the initial UMAP to examine the associated subclusters (Figure 3.6A,B). Two distinct tumour cell types were identified within each tumour type. When the gene expression of these tumour cell clusters were compared to other cell clusters (non-tumour cells) within the same tumour type, the top 10 differentially expressed genes upregulated in LG and HG tumour cells had an overlap of nine genes: *MUC2*, *TFF1*, *TFF3*, *CEACAM5*, *AGR2*, *CLDN4*, *ELF3*, *PHGR1* and *C19orf33* (Figure 3.15 and [Appendix 3](#) Supplementary Table ST3.1 and ST3.2).

MUC2 is the main mucin-producing gene attributed to intestinal goblet cells and is one of the dominant mucins in LG and HG mucinous ATPD.^{398, 424} Other relevant upregulated genes expressed in the top 10 genes in LG tumour cells included *CEACAM5* (encodes the tumour marker CEA) and *AGR2* (encodes anterior gradient protein 2). The presence of *CEACAM5* (CEA) is not surprising, as serum CEA has a prognostic role as a tumour marker for some patients with ATPD.²⁰⁷ *AGR2* encodes a goblet cell specific endoplasmic reticulum protein essential for normal intestinal mucin (*MUC2*) biosynthesis.^{425, 426}

Claudin genes (*CLDN4*) are involved in tight junction formation and may play a role in multiple stages of tumour development.⁴²⁷ E74-like factor 3 (*ELF3*) is a member of the E-twenty-six (ETS) transcription factor family and has been implicated in both oncogenic and tumour suppression pathways in multiple cancer types.⁴²⁸ The *PHGR1* (*proline-, histidine-, glycine-rich 1*) gene encodes the PHGR1 glycosylated protein found in mature gastrointestinal epithelial cells and has been proposed as a biomarker for lymph node metastases in colorectal cancer.⁴²⁹ *C19orf33* (chromosome 19 open reading frame 33) encodes immortalisation up-regulated protein 1 and 2 (IMUP-1 and IMUP-2) and may have a role in chemotherapy sensitivity as well as tumorigenesis.^{430, 431}

This group of upregulated genes relate to epithelial cell functions as well as mucin regulation and production. While some of these genes may be highly expressed in tumour cells as a bystander effect, some of these genes are associated with oncogenic effects in other tumour types. These tumour-specific genes shared between LG and HG tumour cells may warrant further evaluation as ATPD-biomarkers.

Top 10 upregulated differentially expressed genes in ATPD tumour cells

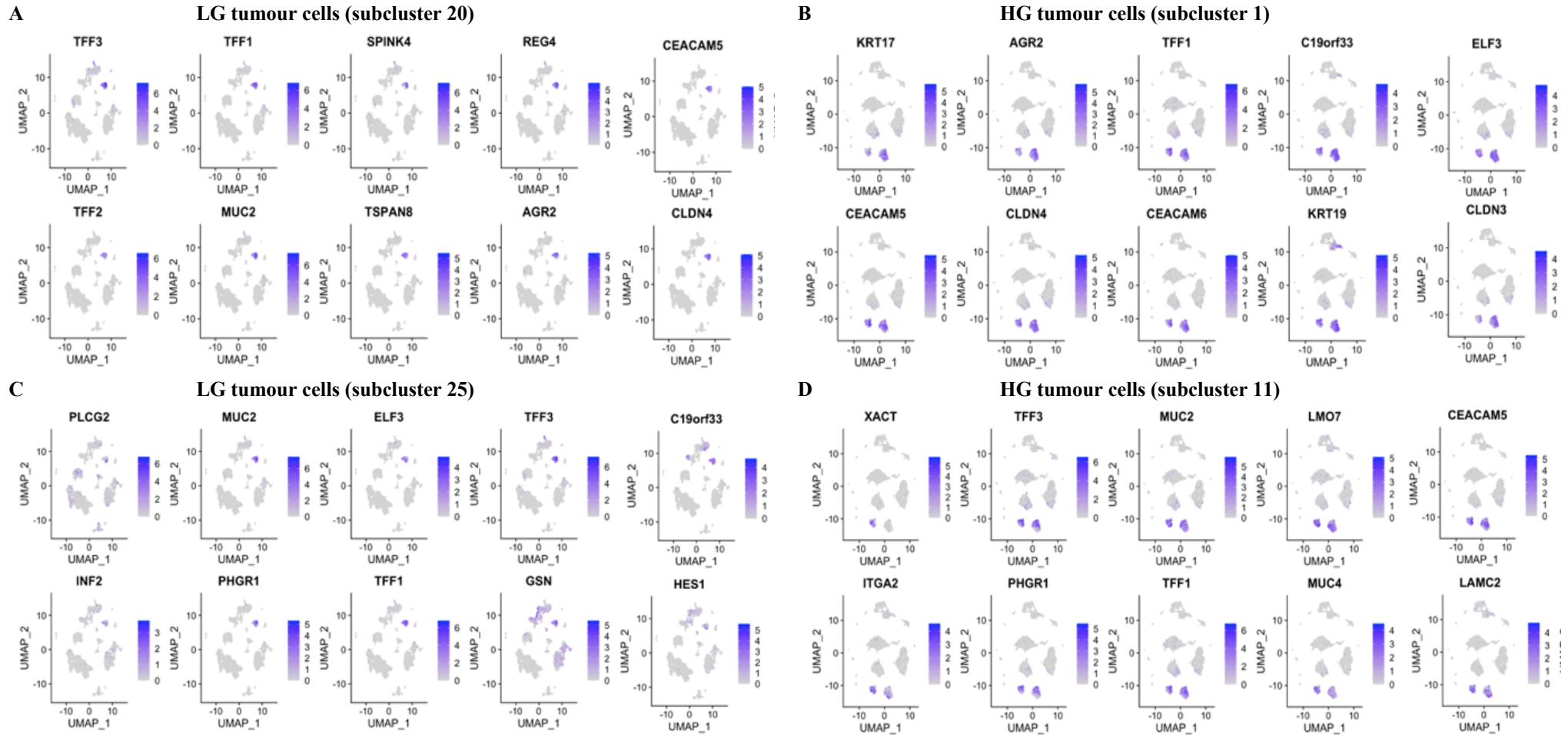


Figure 3.15 | Feature plots indicated the top 10 differentially expressed genes for tumour populations

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Feature plots (UMAPs identifying cells expressing single genes of interest in blue) show log-normalised expression of the top 10 differentially expressed genes for each tumour cell cluster compared to all other clusters. LG ATPD: subcluster 20 (A) and subcluster 25 (B). HG ATPD: subcluster 1 (C) and subcluster 11 (D). Subclusters refer to those annotated in Figure 3.6.

Abbrev. LG, low grade; HG, high grade, ATPD, appendiceal tumour peritoneal disease

b) *Expression of mucin genes in LG and HG tumour cells*

Abnormal accumulation of mucin in the peritoneal cavity causes significant morbidity and life-threatening complications for patients with ATPD. Copious mucin can be present despite scarce and at times no identifiable tumour cells (acellular mucin). Based on this, a hypothesis was developed that the microenvironmental signals from mucins may contribute to disease. Analysis of secreted and transmembrane mucin genes (Chapter 1, Table 1.5) revealed both LG and HG ATPD contained tumour cells expressing gel-forming *MUC2*, *MUC5AC* and *MUC5B* secreted mucin genes. Distribution of hallmark *MUC2* expression amongst LG and HG tumour and non-tumour cells are displayed in Figure 3.16A,C. Tumour cells also expressed the range of transmembrane mucin genes (*MUC1*, *MUC3A*, *MUC4*, *MUC12*, *MUC13*, *MUC16*, *MUC17* and *MUC20*). Notably, HG tumour cells had more cells expressing *MUC16* (tumour marker CA125) transmembrane mucin genes than LG tumour cells (Figure 3.16B,D). Transmembrane mucins are associated with intra- and inter-cellular signalling. In particular, *MUC1* (tumour marker CA15.3) has been associated with activation of β -catenin and canonical Wnt signalling, a known tumour-promoting pathway.⁴³²⁻⁴³⁴

Spatial transcriptomic profiling revealed one of the top upregulated genes in the HG compared to LG samples (FDR<0.001) was *GALNT2*, which encodes N-acetylgalactosaminyltransferase (GALNT)-2, a key glycosylation enzyme (Figure 3.17A and [Appendix 3](#) Supplementary Table ST3.3).²⁴⁷ Additionally, the most upregulated genes in LG tumour segments compared to HG tumour segments were enriched for genes associated with ‘O-linked glycosylation of mucins’ ($P_{\text{adj}}=0.00011$), suggesting that the glycosylation processes of mucin produced from LG tumours may be different from that from HG tumour cells (Figure 3.17B).

Overall, these observations indicate that while LG and HG tumour cells have transcriptomic similarities such as elevated secreted and transmembrane mucin gene expression, their differences in glycosylation processes may be more relevant to their pathogenesis. The expression of secreted mucin *MUC2* genes in LG and HG non-tumour cells was unexpected and may imply a role for these supporting cells in mucin regulation. Additionally, the abundance of *MUC16* expression in HG compared to in LG tumour cells suggests this transmembrane mucin (CA125) may have greater relevance for microenvironmental signalling in HG than LG tumours.

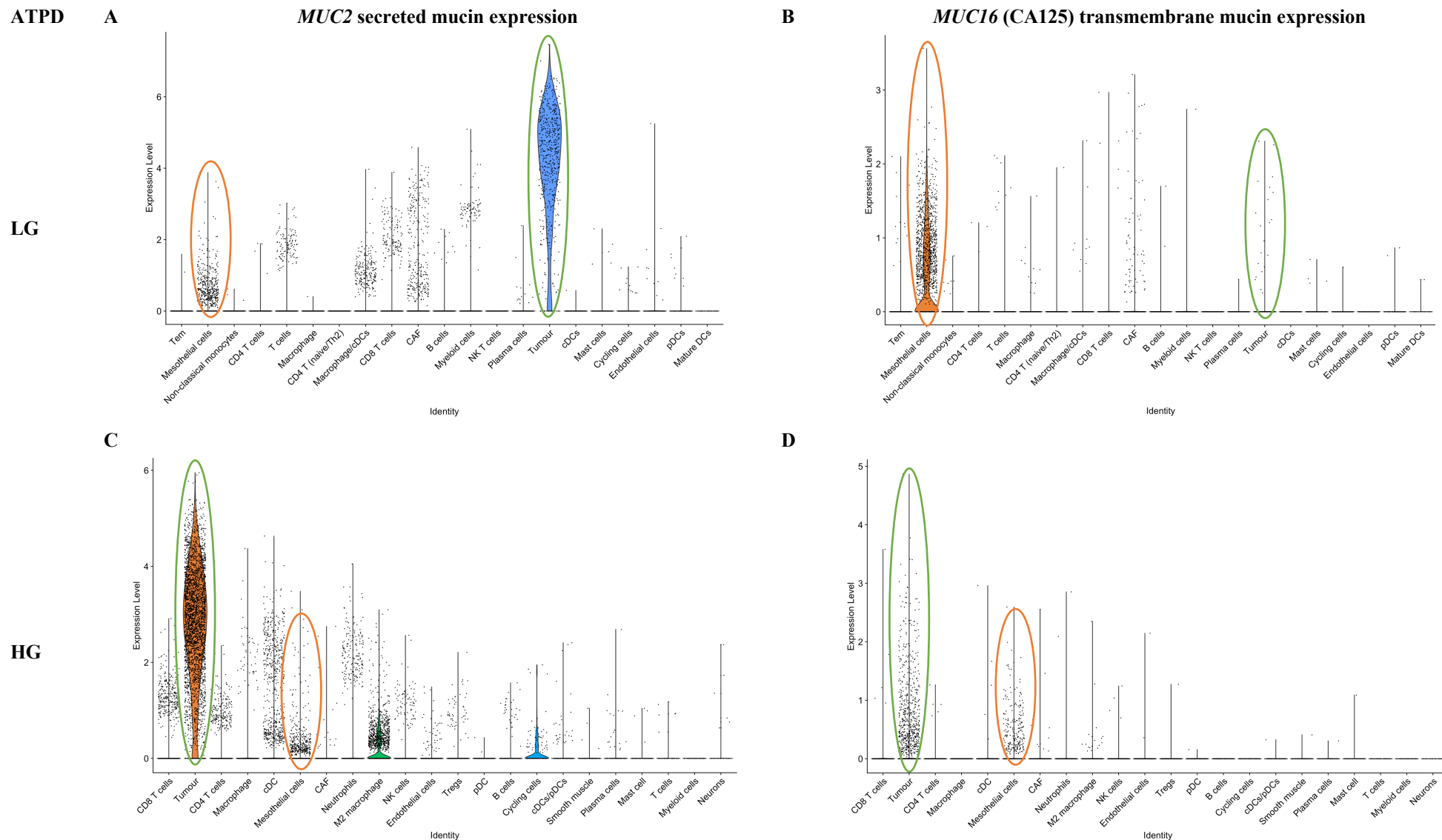


Figure 3.16 | Distribution of key secreted and transmembrane mucins in LG and HG ATPD

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Violin plots show the normalised expression level and distribution of the key mucin genes per annotated cell group for LG (**A,B**) and HG (**C,D**) ATPD: genes for secreted mucins *MUC2* (**A,C**) and genes for transmembrane mucin *MUC16* (tumour marker CA125; **B,D**).

c) *NF- κ B, p53 and TGF- β pathways are features of LG ATPD*

Patients with LG ATPD have a protracted clinical course with limited efficacy from treatments with systemic chemotherapy. A pro-tumoural and/or tolerant microenvironment that may contribute to this prolonged natural history and chemoresistance in LG ATPD. To explore this hypothesis further, the gene profiles of tumour segments from the spatial transcriptomic analysis were compared between LG and HG ATPD. The top differentially expressed genes upregulated in LG compared to HG tumours were *SRC* (FDR=0.001) encoding c-Src involved in mitogen-activated protein kinase (MAPK) signalling and *COL17A* (FDR=0.001) encoding collagen XVII (COL17) involved in cell matrix adhesion ([Appendix 3](#) Supplementary Table ST3.3).⁴³⁵ The expression of these genes were evaluated in the scRNAseq data which validated *COL17A* expression as specific to LG ATPD tumour cells, however, *SRC* demonstrated broader expression in other cell types suggesting influence of the surrounding tumour microenvironment on the gene expression profile of LG compared to HG tumour segments ([Appendix 3](#) Supplementary Figure 3.10A). Both these genes have been implicated in pathways leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pro-tumoural signalling via tumour necrosis factor (TNF)- α and interleukin (IL)-6.⁴³⁶ Furthermore, LG tumour segments had genes enriched for the Hallmark signature ‘TNFa signaling via NFKB’ ($P_{adj}<0.05$; Figure 3.17C). Other relevant pathways associated with the upregulated genes in LG compared to HG tumour segments included Hallmark ‘p53 pathway’ and Reactome ‘signaling by TGF beta complex’ ($P_{adj}<0.05$; Figure 3.17B,C). Alterations to both these pathways can result in perturbations in control of tumour growth.^{437, 438} This provides evidence for LG tumours existing under pro-tumoural immune conditions compared to HG tumours.

d) *Cell cycle progression, metabolic reprogramming and tumour invasion pathways are features of HG ATPD*

HG ATPD are more aggressive tumours with clinical behaviour more in keeping with the tempo of progression of colorectal cancers. Thus, transcriptomic profiles were anticipated to be consistent with high proliferation, altered metabolism and invasiveness. Consistent with this, HG compared to LG tumour segments were enriched with genes associated with Hallmark cell cycle progression gene signatures ‘MYC Targets’, ‘E2F targets’ and ‘G2M checkpoint’ ($P_{adj}<0.05$; Figure 3.17C). In addition, metabolic ‘oxidative phosphorylation’ and tumour invasion ‘epithelial mesenchymal transition’ signatures were identified ($P_{adj}<0.05$; Figure 3.17C). This provides evidence that HG tumour cells harbour abnormal cell cycle pathways that may promote cell cycle progression in the context of accumulating errors in replication rather than cell cycle exit.⁴³⁹ Furthermore, this suggests HG tumour cells have been metabolically reprogrammed to maintain their proliferative state in addition to development of a mesenchymal invasive phenotype compared to LG tumours.⁴⁴⁰ These mechanisms could explain the more aggressive phenotype of HG compared to LG tumours.

e) *Influence of the microenvironment on tumour cell profiles*

Spatial transcriptomic analysis was ideal to assess the influence of different microenvironments on the key cellular compartments in the ATPD TME, in particular the spatial relationship of cells to areas of mucin. It was postulated that excess mucin in the peritoneum (which is normally a sterile space) signals to tumour and non-tumour cells in the microenvironment, thereby stimulating a host defence response, which could inadvertently exacerbate the disease with further ‘protective’ mucin secretion. To interrogate this, the gene profile of tumour segments near mucin (‘peri-

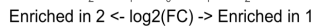
mucinous') were compared to that of tumour segments near immune cells ('peri-inflammatory', i.e. not near mucin; Figure 3.2B). This comparison did not identify statistically significant differential expression of genes ($FDR > 0.05$; Figure 3.17D). Nevertheless, gene set enrichment analysis identified statistically significant gene sets among the upregulated genes. Analysis of these regions indicated that tumour-promoting and abnormal mucin production pathways were upregulated in peri-mucinous compared to peri-inflammatory tumour regions. Peri-mucinous tumours, similarly to LG tumours, were enriched for genes associated with 'TNF α signalling via NF κ B', 'P53 pathway' and 'defective *GALNT3*' as well as the 'NOD-like receptor signaling pathway' ($P_{adj} < 0.05$; Figure 3.17E,F). These findings support the contribution of mucin to a pro-tumoural microenvironment.^{247, 441}

The pathways that were upregulated in peri-inflammatory compared to peri-mucinous tumour segments, similarly to HG tumours, were enriched for genes associated with cell cycle control 'E2F targets', 'MYC Targets V1/2' and 'G2M checkpoint' as well as metabolic reprogramming 'Oxidative phosphorylation' ($P_{adj} < 0.05$; Figure 3.17E,F). This connects peri-inflammatory tumour segments in LG and HG ATPD with a more aggressive phenotype to a potential role for inciting an immune response, or the reverse, that the presence of the local immune response influences the tumour behaviour via immune-tumour crosstalk. HG 'mucinous' tumours may be influenced by the surrounding mucin to upregulate genes involved in pro-tumoural pathways while peri-inflammatory LG tumour cells separated from areas of mucin may exhibit more aggressive features.

A

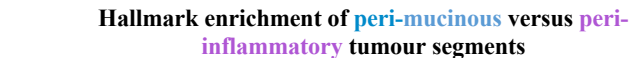


D



Significance ● NS or FC < 0.5 ● P < 0.05

B

**E**

C

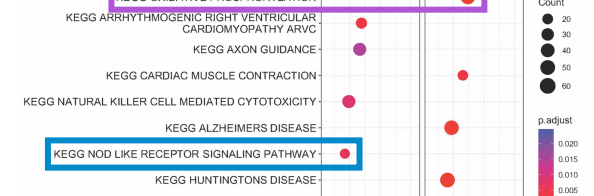
**F**

Figure 3.17 | Differential gene expression and gene set enrichment analysis of tumour segments by spatial transcriptomic profiling evaluating tumour type and microenvironment

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The volcano plots shows the differential gene expression between LG and HG ATPD (**A**) and peri-mucinous versus peri-inflammatory tumour cell microenvironments (by log2FC; **D**). Significantly differentially expressed genes are defined as log2FC >0.5 and FDR p-value <0.05 (blue dots) (**A,D**). Dotplots show the Reactome (**B**), Hallmark (**C, E**), and KEGG (**F**) themes associated with the upregulated genes between HG and LG ATPD (**B, C**) and between peri-mucinous and peri-inflammatory tumour cell microenvironments (**E, F**). The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted p<0.05).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; FC, fold change; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes

f) Differences between tumour cell morphological structures

Tumour cells were observed in the tissue sections in three distinct patterns: (1) 'epithelialised' cells, where single lines of tumour epithelium were observed often surrounding large pockets of mucin (Figure 3.2E); (2) glandular cells, where tumour cells formed cystic and spherical acinars (berry-like shapes; Figure 3.2F); and (3) single infiltrating cells, often interspersed within stromal and immune cells areas, but occasionally entrapped within mucin (Figure 3.2G). There were no statistically significant differentially expressed genes in the comparison between epithelialised tumour and infiltrating single tumour cells ($FDR > 0.05$). On comparison of tumour infiltrating cells to tumour forming glandular structures, there was one significantly upregulated gene (*THBS4*) in infiltrating single tumour cells and there were two significantly upregulated genes (*SPATA18* and *TEX10*) in glandular tumour cells ($FDR < 0.05$; Figure 3.18A). *THBS4* (thrombospondin-4) regulates progression through the epithelial mesenchymal transition and interacts with the oncogenic focal adhesion kinase (FAK)/phosphatidylinositol 3-kinase (PI3k)/Akt pathway.⁴⁴² *SPATA18* (encodes spermatogenesis-associated gene 18) and is thought to be induced by p53 activity playing a role in the cellular response to DNA damage.⁴⁴³ *TEX10* (Testis-specific transcript-10) is implicated in regulation of Wnt signalling as well as cancer stemness.⁴⁴⁴ Gene set enrichment analysis of the differentially expressed genes upregulated in tumour infiltrating single cells compared to glandular cells were enriched for genes associated with 'epithelial mesenchymal transition', whereas glandular-forming tumour cells compared to infiltrating single tumour cells were enriched for genes associated with protein synthesis ($P_{adj} < 0.05$; Figure 3.18C,D). These findings suggest that infiltrating single tumour cells may be responsible for intraperitoneal dissemination and that glandular tumour cells may be the source of abnormal mucin generation.

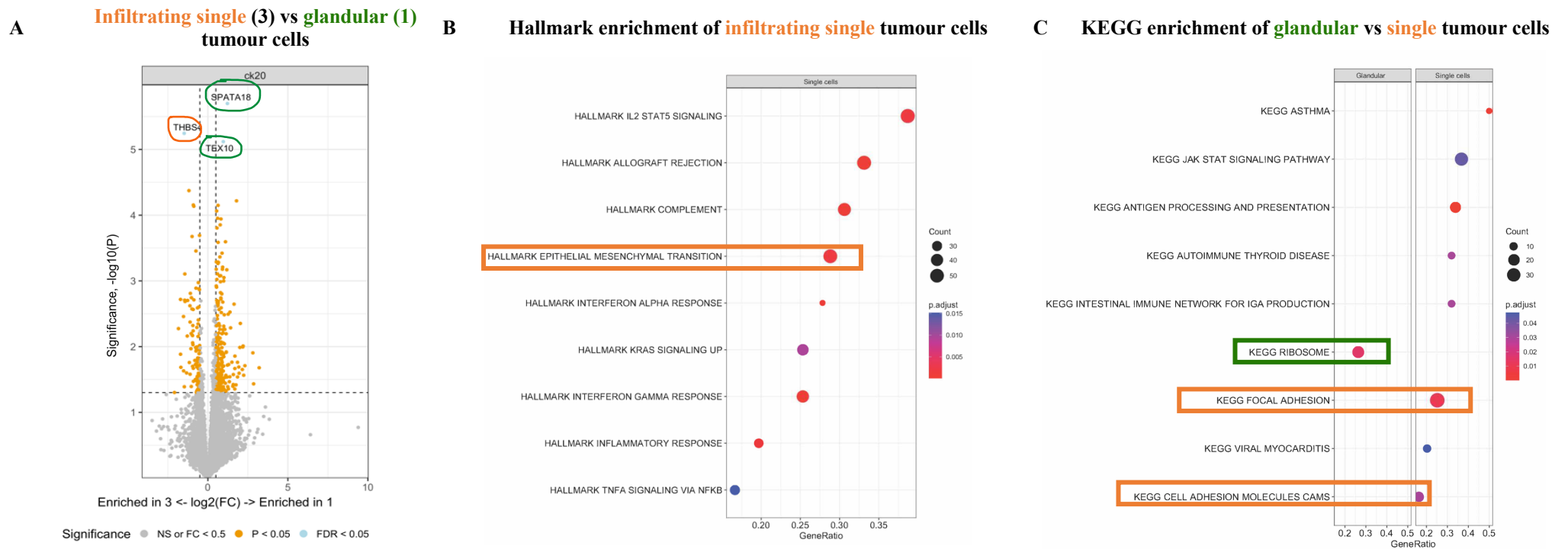


Figure 3.18 | Differential gene expression and gene set enrichment analysis of tumour segments by spatial transcriptomic profiling evaluating tumour cell structure

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The volcano plots shows the differential gene expression between infiltrating single and glandular tumour cells (by log2FC; **A**). Significantly differentially expressed genes are defined as log2FC > 0.5 and FDR p-value < 0.05 (blue dots). Dotplots show the Hallmark (**B**) themes associated with the upregulated genes between infiltrating single cells (**B**) and glandular tumour cells (no upregulated genes). The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted p < 0.05).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; FC, fold change; FDR, false discovery rate; AA, appendiceal adenocarcinoma; AMN, appendiceal mucinous neoplasm; KEGG, Kyoto Encyclopedia of Genes and Genomes

3.3.5 Transcriptomic profiles of stromal cells

There is a growing body of literature emphasising the key role that stromal cells play in the TME.⁴²² Peritoneal mesothelial cells are key players in the stromal cell compartment of ATPD are the peritoneal mesothelial cells. Based on the propensity of ATs to spread directly to and remain confined within the peritoneal cavity, the mesothelium not only provides a physical barrier to extraperitoneal spread but could be the responsible for orchestrating an aberrant immune defence as a response to abnormal mucin.

a) Peritoneal mesothelial cells and fibroblast stromal cells

In the single cell atlas of LG ATPD, there were two populations of peritoneal mesothelial cells (*CALB2*) each associated with a different patient sample (

A and Figure 3.6C). These mesothelial populations both clustered on the UMAP near to populations of CAFs (*COL1A1*) (

A and Figure 3.6C). On further examination, there were no overlapping genes in the top 10 differentially expressed genes between the two mesothelial cell populations.

Gene set enrichment analysis indicated that mesothelial cells from LG ATPD compared to all other cell clusters were enriched for genes associated with ‘complement’, ‘innate immune system’ and ‘cellular responses to stimuli’ ($P_{\text{adj}} > 0.05$; Figure 3.19A). This suggests that mesothelial cells play role in the innate immune response, consistent with published literature.⁴⁴⁵ Mesothelial cells from all HG ATPD samples clustered together and were also located on the UMAP nearby to the CAFs suggesting similar gene profiles and functions (

B and Figure 3.6D). Gene set enrichment analysis indicated the genes upregulated in mesothelial cells from HG ATPD compared to all other cell types were enriched for genes associated with ‘extracellular matrix proteoglycans’, ‘degradation of the extracellular matrix’ and ‘integrin cell surface interactions’ and ‘extracellular matrix organisation’ ($P_{\text{adj}} < 0.05$; Figure 3.19B). This suggests mesothelial cells in HG ATPD coordinate tumour invasion and dissemination. Analysis of the genes upregulated in CAFs demonstrated that they were enriched for the similar gene sets as to the mesothelial cells with upregulated pathways in LG ATPD for ‘complement’ ($P_{\text{adj}} < 0.05$; Figure 3.19C) and for HG ATPD for ‘extracellular matrix organisation’ ($P_{\text{adj}} < 0.05$; Figure 3.19D).

Therefore, these data show for the first-time transcriptomic similarities between mesothelial cells and CAFs in the ATPD TME. These cells appear to directly contribute to the disease by mesenchymal signalling that coordinates tumour cell invasion and the host innate immune response. The interactions between the CAFs and the mesothelium that are associated with extracellular matrix remodelling could further exacerbate mucin generation, which is a key component of the disease process.

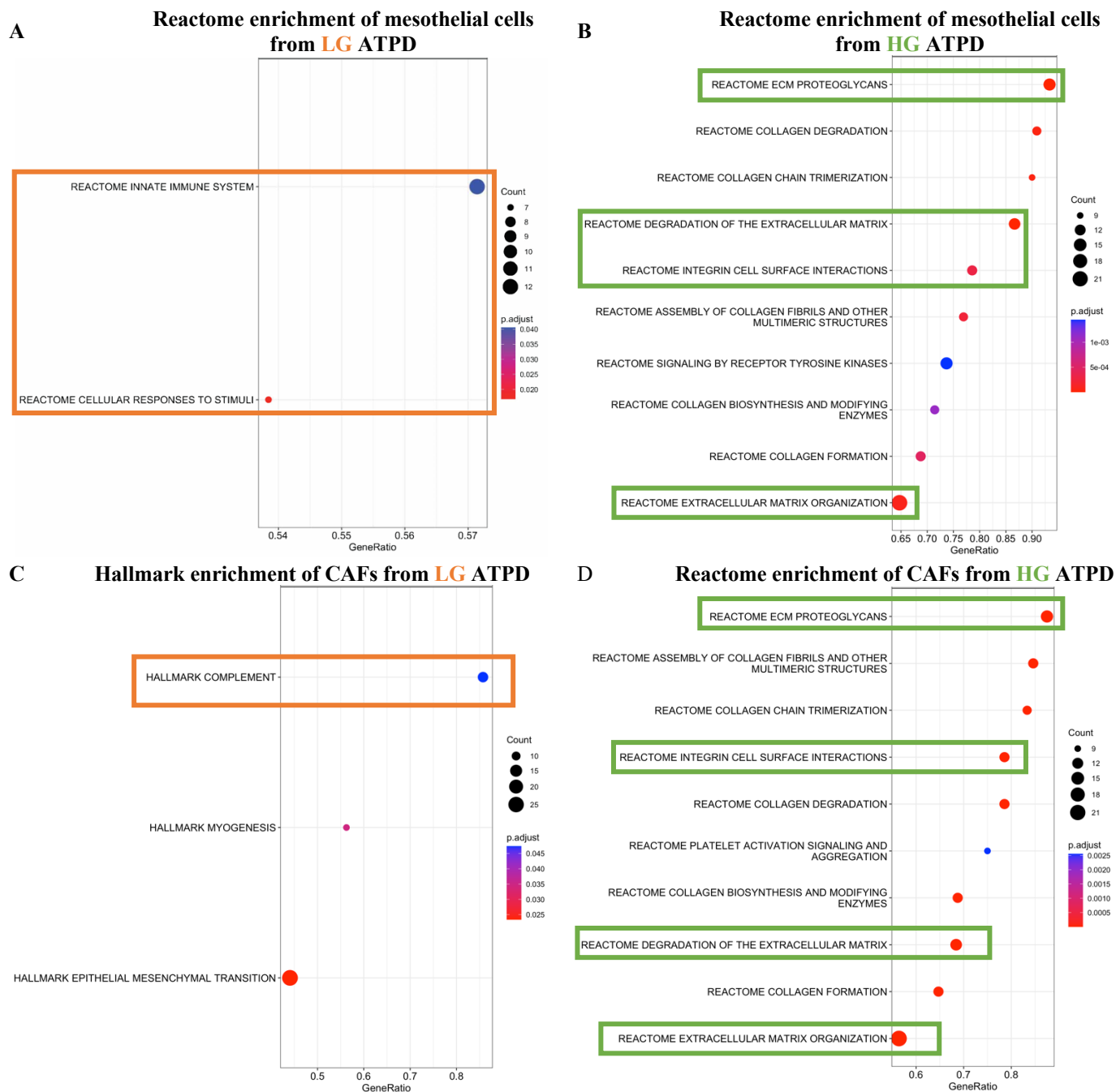


Figure 3.19 | Gene set enrichment analysis of peritoneal mesothelial cells and CAFs in ATPD

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Dotplots show the Reactome (A, B, D) and Hallmark (C) themes associated with the top upregulated genes of mesothelial cells (A,B) and CAFs (C,D) from LG (A,C) and HG (B,D) ATPD. The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted $p < 0.05$).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; CAFs, cancer-associated fibroblasts; ECM, extracellular matrix

b) Mesothelial cells and mucin gene expression

Consistent with the hypothesis that mesothelial cells play an important role in the TME of ATPD, it was observed that they had more cells expressing and higher normalised expression levels (compared to all other cells) of the transmembrane mucin gene *MUC16* (CA125), especially in LG ATPD (Figure 3.16B). *MUC16*/CA125 has been documented in mesothelial cells isolated from ovarian cancer peritoneal fluid (ascites).⁴⁴⁶ While protein studies are needed to validate these findings in ATPD, the presence of transmembrane mucin(s) on mesothelial cells could trigger pro-tumoural intracellular signalling pathways.⁴³²

c) Influence of the microenvironment on stromal cell profiles

Mesothelial and CAF populations were included as part of the stromal segments for the spatial profiling analysis. Stromal segments were annotated based on their location near tumour cells ('peri-tumoural'), near mucin ('peri-mucinous'), or near both tumour cells and mucin ('peri-tumoural/mucinous; Figure 3.2B). It was hypothesised that their location may influence their role in the TME, with the thought that peri-mucinous stromal segments may exhibit genetic profiles most in keeping with a role in extracellular matrix remodelling. Peri-tumoural compared to peri-tumoural/mucinous stromal cells had statistically significant expression of *CCL14* (chemokine ligand 14) (FDR<0.001), *IL6ST* (IL-6 signal transducer) (FDR=0.001) and *TGFBR2* (Transforming growth factor Beta 2) (FDR<0.001; Figure 3.20A). Peri-tumoural/mucinous stromal segments had increased expression of *ZNF793* (FDR=0.001; Figure 3.20A). These genes suggest that stromal cells in peri-tumoural microenvironment were involved with pro-tumoural signalling and that those in peri-tumoural and peri-mucinous microenvironments may be involved in tumour cell regulation.⁴⁴⁷

Gene set enrichment analysis indicated peri-mucinous compared to peri-tumoural stromal areas were enriched for genes associated with 'cell-cell adhesion' ($P_{\text{adj}} < 0.05$). Genes upregulated in peri-tumoural/mucinous compared to peri-tumoural stromal segments were associated with collagen remodelling including 'collagen formation' and 'extracellular matrix organisation' ($P_{\text{adj}} < 0.05$; Figure 3.20B). This is evidence of potential crosstalk between tumour and stromal cells present in the TME of LG and HG ATPD and the influence of mucin presence.

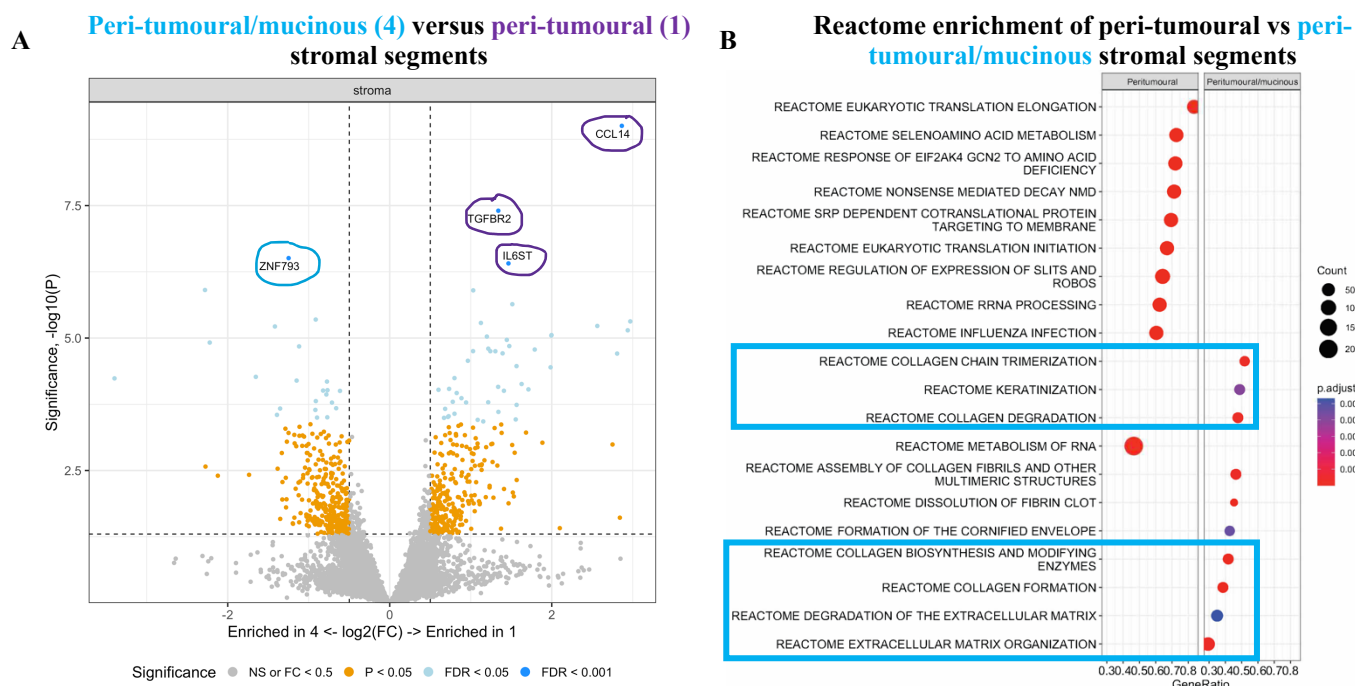


Figure 3.20 | Gene set enrichment analysis comparing peri-tumoural and peri-tumoural/mucinous stromal segments

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The volcano plot shows the differential gene expression between peri-tumoural and peri-tumoural/mucinous stromal segments (by log2FC). Significantly differentially expressed genes are defined as log2FC >0.5 and FDR p-value <0.05 (blue dots) (A). Dotplots show the Reactome enrichment of upregulated genes between peri-tumoural and peri-tumoural/mucinous stromal segments. The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted p<0.05).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; ECM, extracellular matrix

3.4 Discussion

3.4.1 Summary

This comprehensive transcriptomic profile of the TME of LG and HG ATPD provides the first proposed cell atlas for this disease type. A transcriptomic analysis of tumour and stromal cells is presented as classified by spatial location. This provides critical biological information to guide a rational therapeutic approach which is essential for improving outcomes for patients with this disease. The single cell atlas confirmed that all major cell lineages (tumour, mesothelial, immune and stromal cells) were represented in the TME of ATPD.

Tumour cells expressed genes associated with aberrant mucin production and glycosylation which may explain its abnormal accumulation in ATPD.^{247, 448} There was an association between tumour-promoting molecular pathways (β -catenin/Wnt, NF- κ B, p53 and TGF- β signalling) and mucin production in LG ATPD. Conversely, HG ATPD was associated with upregulated cell cycle progression (*MYC*, E2F and G2M pathways), altered metabolism (oxidative phosphorylation) and tumour invasion (epithelial mesenchymal transition). A mucinous microenvironment influenced the gene expression in nearby tumour segments, promoting tumour-permissive pathways (TNF α , NF- κ B, p53) and abnormal glycosylation (*GALNT3*), similar to LG ATPD.

Peritoneal mesothelial cells were similar in gene expression to populations of CAFs and the expression of transmembrane mucin genes, especially *MUC16*, indicates a potential role in microenvironment crosstalk and immunoediting which has been seen in other tumour types.^{223, 449} Peritoneal stromal segments (including mesothelial cells and CAFs) exhibited transcriptomic differences depending on their microenvironment. Peri-mucinous stromal segments had upregulated genes in pathways for cell adhesion and peri-tumoural stromal segments had genes upregulated in pathways for extracellular matrix organisation. This indicates the potential role of the peritoneal stromal cells in educating tumour and immune cells in the local microenvironment. This has been demonstrated in other tumour types that metastasise to the peritoneum.^{450, 451}

3.4.2 Proposed mechanisms of disease

Based on the transcriptomic evidence generated from this single cell atlas and spatial profile, explanations are proposed for the main clinical features of ATPD including: (1) the accumulation of mucin, (2) the peritoneal dissemination of tumour cells, (3) the indolent nature of LG ATPD and (4) for the limited efficacy for systemic chemotherapy.

3.4.2.1 Abnormal mucin production and Wnt/B-catenin signalling

This study provides evidence that the accumulation of mucin in ATPD may be driven by aberrant pathways signalling the overproduction of secreted mucins. This study confirmed previous findings that *TFFs* have a role in the abnormal mucin secreted in ATPD.²³³ These genes are associated with increasing viscosity and elasticity of mucin to make it more gel-like, which under physiological circumstances may impart mucosal defence and wound healing.⁴⁵² Mucin from ATPD has been documented to exhibit differing 'hardness' related to its viscosity, and while this may be due to different mucin subtypes (e.g. *MUC5B*)²⁷⁰, it may also be due to the contribution of TFFs.⁴⁵³ The physical consistency

of mucin has potential relevance to disease, as tumour mucin may aid tumour growth by providing a more favourable microenvironment, participating in defence against immunosurveillance and providing active immunosuppression.^{448, 454}

Mucin components may also contribute to tumourigenesis. TFFs have been implicated in epithelial cell migration,⁴⁵⁵ angiogenesis⁴⁵⁶ and chemotaxis.⁴⁵⁷ These mechanisms are essential for mucosal healing, but in an erroneous context may be tumour-promoting. Forced expression of TFF3 in a colorectal cancer cell line promoted proliferation, survival, migration and invasion of tumour cells.⁴⁵⁸ TFF1 may be a tumour suppressor gene, with downregulation associated with reduced apoptosis and increased proliferation of gastric cancer cells.⁴⁵⁹

Mucin synthesis is a demanding cellular process due to the extensive glycosylation steps required.⁴²⁶ The unfolded protein response is a biological process designed to manage endoplasmic reticulum stress when accumulation of un/misfolded proteins occurs.⁴⁶⁰ This response is physiological when MUC2 is produced by goblet cells⁸⁷ but it can also be exploited by tumour cells for pro-survival signals, promoting oncogenic transformation.⁴⁶¹ There is evidence that TFFs may be involved in protein folding processes in the endoplasmic reticulum and inhibition of TFFs may present a potential therapeutic opportunity to alter the abnormal mucin production that occurs in ATPD. An inhibitor of TFF3 showed promising preclinical activity in colorectal cancer along with enhanced chemotherapy sensitivity.⁴⁵⁸

These data highlight a role for the unfolded protein response via endoplasmic reticulum stress as a mechanism for overproduction of mucin (ATPD tumour cells highly expressed *AGR2*).⁵⁵ Overexpression of *AGR2* has also been found in multiple other solid tumours and in association with several oncogenic pathways.⁴⁶² It has been shown to be upregulated by mutant KRAS^{G12D} in pancreatic cancer cells, which is compelling for a similar role in ATPD which has high rates of KRAS mutations.²¹⁴ Given mucin synthesis is highly demanding,⁴²⁶ it would be plausible that tumour cells may develop mechanisms of metabolic reprogramming to cope with this process. While cancer cells are known to exhibit aerobic glycolysis due to impaired respiration,⁴⁶³ oxidative phosphorylation has emerged as an active process in cancer cells as part of metabolic reprogramming to maintain a state of proliferation.⁴⁴⁰ This process has been shown to be active in previous analyses of peritoneal metastases from other tumours.⁴⁶⁴ There was evidence for this occurring in HG tumour cells, which is plausible given the more aggressive nature of HG compared to LG tumours.

Mucin synthesis is demanding is due to the requirements for building the large polysaccharide ('bottle-brush-like') structure by the process of glycosylation. A key mechanism of glycosylation is called O'linked glycosylation, which is integral to mucin structure. It is a post-translational modification where sugars (oligosaccharides, the 'brush') are attached to a scaffold of serine and threonine residues (the 'handle').^{247, 448} LG and HG tumour cells were associated with glycosylation processes which may facilitate tumour progression and immune evasion.⁴⁶⁵ LG ATPD had increased enrichment of O'linked glycosylation processes compared to HG tumour, which is not unexpected given the dominant mucinous phenotype of LG disease. Truncated surface O'glycans have been reported to contribute to oncogenesis and their elongation confers resistance to immune-mediated killing.^{466, 467} Truncated surface O'glycans that form tumour-associated antigens have been found to be over expressed in colorectal cancer cells with enrichment

found in pathways involving MAPK signalling, cell migration, blood vessel development, antigen processing and presentation and cytotoxic T cell responses.⁴⁶⁸

There are additional steps in the glycosylation process that can be altered in the setting of malignancy. GALNTs (1-20) are involved in the initial steps of mucin O-glycosylation.²⁴⁷ Upregulation of GALNTs have a role in tumour cell proliferation, invasion and migration thought to be mediated by Wnt/ β -catenin induced epithelial mesenchymal transition.⁴⁶⁹ *GALNT2* has been shown to function as an oncogene in lung cancer.⁴⁷⁰ Its higher expression in HG ATPD suggests differences in mucin glycosylation processes between LG and HG tumours. The loss of *GALNT3* (identified in peri-mucinous LG ATPD) is associated with increased pancreatic cancer aggressiveness likely due to increased ErbB growth receptor (EGFR) phosphorylation mediated by altered glycosylation.⁴⁷¹

In addition to the proposed mechanism of abnormal mucin production due to aberrant glycosylation processes in ATPD, this study also revealed that mucin could influence tumour and non-tumour cells in the microenvironment. There was an association of peri-mucinous tumour cells with nucleotide-binding oligomerisation domain (NOD)-like receptor signalling. NOD-like receptors are pattern recognition receptors that function in the cytosol.⁴⁷² They have been shown to have a role in the pathogenesis of colorectal cancer in a number of studies, including promotion of tumour proliferation and the epithelial mesenchymal transition.⁴⁷² This is a potential pathway for aberrant mucin production in ATPD.

NF- κ B pathway activation is another key potential mechanism these data identified in association with abnormal mucin production in LG ATPD. NF- κ B activation promotes *MUC2* mucin transcription.⁴⁷³ One of the pathways that activates NF- κ B results from upstream MAPK signalling directed by Src, encoded by *SRC*, which was one of the top differentially expressed genes in LG ATPD. The protein Src also interacts with the transmembrane mucin MUC1 (CA15.3) which has been detected in tumour cell populations of both LG and HG ATPD.⁴⁷⁴ MUC1 binds to β -catenin which can activate canonical Wnt signalling.^{433, 434, 475} The oncogenic activity of Wnt signalling mediated by MUC1 has been shown in preclinical models.⁴⁷⁶ Enrichment of upregulated genes in LG ATPD associated with NF- κ B pathways is consistent with the downstream effects of *SRC* signalling and suggests a potential role for β -catenin/Wnt induced aberrant mucin production in LG ATPD.

3.4.2.2 Tumour promoting pathways, peritoneal dissemination and a permissive microenvironment

Cell cycle progression pathways (MYC, E2F and G2M) were associated with HG ATPD in this analysis, confirming previous findings that they have a role in ATPD.²³³ Abnormalities in cell cycle control are associated with oncogenesis, most likely because cancer cells are unable to exit the cell cycle rather than due to uncontrolled cell cycle progression.^{439, 477} E2F signalling governs G1-S phase transcription, committing the cell to replication and entry to S phase.^{439, 478} Deregulation of E2F transcription challenges the cell's ability to exit the cell cycle.⁴⁷⁷⁻⁴⁷⁹

One of the upregulated gene signature pathways associated with cell cycle progression identified in HG ATPD were MYC targets. Myc is expressed following activation of oncogenic pathways including MAPK, Wnt, SRC and activates E2F transcription.⁴⁸⁰⁻⁴⁸² The *c-Myc* (*MYC*) oncogene is a potent driver of cellular proliferation, one of the

hallmarks of cancer, and its deregulation interferes with the cell cycle, apoptosis, ribosome biogenesis and metabolism.^{16, 483-485} It is a fundamental regulator of the TME and responsible for tumour promoting processes such as immune evasion.⁴⁸⁵⁻⁴⁸⁷ Furthermore, *MYC* activation has been proposed to activate microRNA miR-105 which has been shown to enhance TNF α stimulated CRC cell migration and EMT, via the NF- κ B pathway described earlier.⁴⁸⁸

TP53 is a tumour suppressor gene known as the ‘guardian of the genome’ and its protein p53 has an integral role in cell cycle control. It regulates the G2M checkpoint which is essential for monitoring cells for errors in DNA replication and controls whether a cell enters mitosis, or needs growth arrest for DNA repair or induction of apoptosis.^{489, 490} Compromise of the G2M checkpoint, often by mutant p53, allows tumour cells to remain in the cell cycle but with accumulating genetic errors which promotes uncontrolled tumour proliferation.⁴⁹¹ This analysis also indicated G2M checkpoint pathway genes contribute to survival of HG ATPD cells. This checkpoint may also play a role in LG ATPD due to upregulated signalling of the p53 pathway.

Activation of cell cycle progression pathways in HG ATPD tumour cells suggests that they may develop genomic instability resulting from rapid cell cycling and deregulation.^{439, 492} Cell cycle inhibition would be a rational therapeutic approach using existing anticancer drugs that could be repurposed such as the oral CDK4/6 inhibitor palbociclib used for treatment of advanced breast cancer.⁴⁹³ There exists a case report of durable stable disease (57 months) of a patient with LG ATPD with a *CCND1* amplification who was treated on a precision oncology pathway with palbociclib, which works by preventing phosphorylation of Rb1, inducing G1 arrest and cell cycle exit in cycling cells.⁴⁹⁴ Furthermore, there is emerging preclinical and clinical data supporting potential activity for CDK4/6 inhibition in mucinous ATPD.⁴⁹⁵

3.4.2.3 Mesothelial cells function as cancer-associated fibroblasts and may impact therapeutics

One of the important findings from this study was the transcriptomic similarities between mesothelial cells and CAFs. Mesothelial cells are implicated in peritoneal fibrosis and metastasis through their ability to produce and regulate extracellular matrix.⁴⁹⁶ These processes are demonstrated in LG and HG ATPD in this analysis. Similarly to tumour cells, mesothelial cells have been documented to transform into myofibroblasts.²⁷⁹ This process has been termed the ‘mesothelial-mesenchymal transition’ and has been documented in peritoneal carcinomatosis in other cancers.^{497, 498} In this analysis the mesothelial cells in HG ATPD exhibit profiles suggesting this process (cells enriched for myogenesis and epithelial mesenchymal transition). Additionally, the erroneous automated annotation when using the Human Primary Cell Atlas reference of the mesothelial cell population as chondrocytes and osteoblasts (both mesenchymal origin cells) provides support for gene profiles consistent with mesenchymal differentiation. The myofibroblasts derived from mesothelial cells have been shown to function as CAFs during peritoneal metastasis for other tumour types, and have a role in rendering the peritoneum to be a receptive and permissive microenvironment for tumour formation.^{499, 500} Mesothelial-derived CAFs in disseminated ovarian cancer, have been shown to interact with other cells in the microenvironment and may contribute to chemotherapy resistance.⁵⁰¹

Mesothelial cells from LG and HG ATPD were unexpectedly found in this analysis to express genes for *MUC16*. This has not been observed previously in ATPD. However, this is consistent with published literature that documents

MUC16/CA125 protein expression on normal mesothelial cells in inflammation and fibrosis as well as on mesothelial cells in peritoneal disease from ATPD and ovarian cancers.^{243, 446, 502, 503} In ovarian cancer, MUC16/CA215 expression in ovarian cancer mesothelial cells was found to occur via the AKT pathway.⁴⁴⁶ Mesothelial cells also have been reported to have an antigen presenting role as part of the innate peritoneal defence. This may explain the unexpected observation that mucin genes are expressed in mesothelial cells.⁵⁰⁴

3.4.2.4 Potential biomarkers and therapeutic targets

This study identified tumour-specific genes among the top differentially expressed tumour genes, which could be evaluated further as potential ATPD biomarkers. Some of these have been briefly discussed in their relevant results section. The presence of CEACAM5 (CEA) in ATPD tumour cells has been documented in the published literature for this tumour type.⁵⁰⁵ Expression of *CEACAM5* in colorectal cancer has a tumour-promoting role and has been explored as a therapeutic target.⁵⁰⁶

Aberrant expression of *COL17A* is associated with invasion and metastasis in epithelial cancers, including in colorectal cancer.⁵⁰⁷ In a study of xenograft derived colorectal cancer organoids with a dormant population of cancer stem cells, *COL17A1* was found to be upregulated and persistent during chemotherapy.⁵⁰⁸ *COL17A* knock-out organoids that lost this dormant stem cell population became chemosensitive. Overexpression in pancreatic cancer has been associated with tumour promoting altered NF-κB signalling.⁵⁰⁹

XACT was noted in the top 10 upregulated genes for HG ATPD. It is expressed when another similarly functioning lncRNA called *XIST* is absent. While there has been no documented connection between *XACT* and tumourigenesis, *XIST* has been documented to function as an oncogene, including in colorectal cancer by modulating oncogenic pathways such as MAPK and Wnt signalling.⁵¹⁰ These tumour-specific genes have utility as candidates for disease markers that could be assessed diagnostically, prognostically and/or to assist with treatment selections.

3.4.3 Limitations

Limitations to this study include clinicopathological issues, challenges related to tissue collection and processing, platform dependent technical issues and potential sources of biases in the analysis. A significant challenge in translational research for rare tumours is low sample size and prolonged recruitment. The total number of patients evaluated in this study, while seemingly low compared to more common tumour types, is meaningful for this rare tumour type. Recruitment to the study was exceptional with negligible attrition and maximal use of the study period. Patients were recruited from a specialised peritoneal centre and the platforms chosen for transcriptomic analysis facilitated maximal use of patient specimens.

A significant challenge encountered performing this study was managing the large amounts of mucinous tissue that were collected in compensation for expected lower tumour yields, especially for LG ATPD. The difficulties related to this tumour type are well documented in the literature.^{269, 342} However, large amounts of tumour tissue was collected from patients with higher burden peritoneal disease to compensate for the low cellularity tissue. Lower tissue yields of sometimes sparse tumour cell populations also resulted in modest tumour cell populations for the single cell analysis.

To interrogate potential intra-patient transcriptomic differences in tumour cell populations (i.e. within a patient sample) higher cell numbers would be required.⁵¹¹ Fluorescent activated cell sorting was a method considered to ensure capture of cell types of interest such as the rare tumour cell population. However, this method required even higher starting cell numbers, which even with copious tissue was a challenge.

A further challenge to the scRNAseq workflow was the time of tissue collection on the day of surgery. This was usually in the afternoon which was too late to commence the tissue dissociation process and submission of the sample for single cell capture. Therefore, the fresh sample was placed in RPMI-1640 culture medium overnight at 4°C to preserve RNA and cell dissociation proceeded the following morning. The additional digestion process for mucinous tissues and the cold preservation of cells overnight could have led to higher loss of sensitive cells or changes in gene expression that convolute biological signals.⁵¹²

3.5 Conclusion

This study provides novel insights into the biological profile of ATPD by describing the first single cell atlas of the TME and a comprehensive analysis of the contribution of spatial architecture to disease mechanisms. It revealed pathways informing reasons for mucin overproduction, cell cycle regulation, epithelial mesenchymal transition and the role of oncogenic pathways such as Wnt/ β -catenin, NF- κ B, and TGF- β in tumoural growth and peritoneal dissemination. Mesothelial cells shared similar gene profiles to pro-tumoural CAFs with evidence of a role in extracellular matrix remodelling, facilitation of tumour dissemination by cell adhesion pathways and microenvironmental crosstalk. These findings may be used to identify clinically relevant biomarkers and new therapeutic targets. More research is needed to validate specific mechanisms and pathogenesis of disease inferred from this transcriptomic analysis. Application in preclinical models and window of opportunity/neoadjuvant early phase clinical studies is justified.