

Chapter 5. Patient-derived explant model of appendiceal tumour peritoneal disease.

Chapter 5 presents an optimised model of ATPD PDEs and the optimisation cohort was presented as a poster at ASCO Annual Meeting 2022 (Abstract 4160):

Strach, M. C., Yeung, N., Lin, H.-M., Ansari, N., Koh, C., Shin, J.-S., ... Mahon, K. Patient-derived explant model of appendiceal cancer. *J Clin Oncol.* 2022;Suppl 16 Abstr 4160(40). Doi: [10.1200/jco.2022.40.16_suppl.4160](https://doi.org/10.1200/jco.2022.40.16_suppl.4160)

5.1 Introduction

There are limited preclinical models of ATPD to enable study of the tumour and its microenvironment. The aim of this study was to develop and optimise a PDE matrix model, of ATPD which preserves the TME to facilitate further study of the biological profile of ATPD and testing of novel therapies. Five anti-cancer agents were chosen for *ex vivo* drug testing to provide a baseline comparison for future assessment of novel treatment strategies: (1) oxaliplatin, (2) folinic acid/5-fluorouracil, (3) irinotecan, (4) mitomycin C and (5) cetuximab. These agents are currently used via systemic or intraperitoneal (oxaliplatin and mitomycin C) administration in the routine clinical care of patients with appendiceal and colorectal tumours with peritoneal disease.^{6, 160, 165, 175-177, 183, 192, 575, 576} In addition, therapeutic strategies using mucolytic agents were evaluated on the PDE model based on previously demonstrated tumour-protective properties of mucin with activity demonstrated on patient-derived mucin and xenografts.^{262, 271} One of the key findings from Chapter 3 was that ATPD was associated with pathways involving cell cycle progression. Therefore, the effect of *ex vivo* treatment with palbociclib, a cyclin-dependent-kinase(CDK)-4/6 inhibitor, previously tested on PDEs in other solid tumours, was performed.^{577, 578} This study presents the development, optimisation and clinical implication of a PDE model of ATPD.

5.2 Methods

5.2.1 Tissue specimens

Fresh tissues were collected from patients managed at the Royal Prince Alfred Hospital/Chris O'Brien Lifehouse, Sydney with histopathology confirmed ATPD and colorectal cancer peritoneal metastases at the time of CRS prior to administration of HIPEC. Samples were collected from July 2020 until November 2022. Tissue was placed in ice-cold phosphate-buffered saline (PBS) or media (Gibco Roswell Park Memorial Institute (RPMI)-1640 Medium, ThermoFisher Scientific) and transported on wet ice. Informed written consent was obtained from all patients included in this study and all work was approved by St Vincent's Hospital, Sydney, Human Research Ethics Committee (2020/ETH00687). All clinical investigations were conducted according to the principles expressed in the declaration of Helsinki and good clinical practice guidelines.

5.2.2 Optimisation of PDE culture

Fresh ATPD tissue from ten consecutive patients was cultured under varying conditions relating to culture duration, supplementary media components, tissue size and matrix position (Table 5.1). Experimental conditions were designed to vary only one condition at a time while others remained constant.

Table 5.1 | Variables for PDE optimisation

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Variable	Conditions tested
Culture duration	24-72h; 4-7d
Tissue size , width x length (mm) ^b	3x3, 4x4, 5x5, 6x6, 10x10, 15x15
Supplementary media components ^a	EGF, 50 ng/ml (recombinant human protein, 100 µg, ThermoFisher Scientific) NAC, 1.25mM (5 g dissolved in PBS, Sigma Aldrich) Glutamine, 1x Gibco GlutaMAX™ Supplement (100x, ThermoFisher Scientific) B27, 1x Gibco B27™ Supplement (50x, ThermoFisher Scientific)
Matrix presence	Gelatin sponge No matrix (tissue fully submerged in media)
Matrix type	Spongostan Gelfoam

^aCombinations of media supplements were: EGF, EGF + NAC, EGF, NAC + Glutamine and EGF, NAC, Glutamine+ B27

^bThickness of all PDEs was ~2 mm.

Abbrev: PDE, patient-derived explant; EGF, epidermal growth factor; NAC, N-acetylcysteine; PBS, phosphate buffered saline; h, hours; d, days.

Fresh tissue from surgery was transported in ice-cold PBS or media to the laboratory for culture within one hour of excision. In the laboratory under sterile conditions in a tissue culture hood, the tissue was washed in cold PBS or media, and dissected with width and lengths varying according to pre-planned experimental sizes (Table 5.1). A representative uncultured specimen (Day 0) was fixed in 10% neutral-buffered formalin for paraffin embedding (formalin-fixed paraffin embedding; FFPE). The tissue pieces were placed on gelatine-sponges (Gelfoam, Pfizer Australia or Spongostan Dental, Ferrosan Medical Devices, Soeborg, Denmark), one sponge per well, submerged in culture media in a six-well (larger tissues; 2 ml media per well) or 24-well plate (smaller tissues; 500 µl media per well). The base culture media was composed of RPMI-1640 medium (500 ml, Gibco), 10% fetal bovine serum (FBS, batch used from 2017-Apr 2021: HyClone NZ Sourced FBS Batch #DB0525, GE Healthcare Australia; subsequent batch: Bovogen Cat Batch #2008A, Expiry Aug-2025, Scientifix Australia), 1x Antibiotic Antimycotic Solution[§] (100x, Sigma-Aldrich), 0.01 mg/ml insulin (Insulin Actrapid Penfill 100 IU/ml, Insulin Actrapid® Penfill® 100 IU/ml, Novo Nordisk, Baulkham Hills, Australia) and 10 mM HEPES (1M, Gibco). Base media was chosen based on this laboratory's pre-existing protocols.^{349, 579} Supplemental components for testing are summarised in Table 5.1. Technical replicates were placed on the same sponge and/or in additional wells with matched treatment conditions. All PDEs were incubated at 37°C in 5% CO₂.

[§]10, 000 units/ml Pencillin, 10 mg/ml Streptomycin and 25 µg/ml Amphotericin B in 0.9% sodium chloride

5.2.3 Drug treatment of PDEs

Tissue preparation was the same as described in the optimisation above, except that all tissues were dissected into 5 to 10 mm sizes and the sponge was half-submerged in the culture medium. Drug treatments or vehicle control were included in the culture media at the start of the PDE culture. The culture medium consisted of the base medium with all of the supplemental factors as listed in the optimisation. The exception was exclusion of epidermal growth factor for cetuximab (an epidermal growth factor receptor antibody) treated PDEs. One well per six- or 24-well plate was reserved for vehicle control treated PDE(s) and the remaining wells of PDEs contained single anti-cancer agents or combinations at various concentrations (Table 5.2). Dose titration was based on existing literature for preclinical drug evaluation (Chapter 1, Table 1.9).^{262, 271, 342, 359-364} Vehicle controls were matched where known to the master stock solvent of the anti-cancer agent (Table 5.2). PDEs were cultured for up to seven days and fixed in 10% neutral-buffered formalin prior to paraffin embedding at the end of the culture period. In some cultures, 10 μ M bromodeoxyuridine (BrdU, B5002, Sigma-Aldrich), a nucleoside analogue, was added to the culture 2 to 24 hours prior to ending the culture. This is incorporated into replicating DNA in the nuclei of proliferating cells and can be used to measure tumour cell proliferation.

Table 5.2 | Anti-cancer agents used for PDE *ex vivo* drug testing

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Anti-cancer agent		Stock concentration (manufacturer)	Doses	Durations (h)	Control vehicle ^d
Conventional agents					
Single agent					
Folinic acid/5-fluorouracil ^a	Folinic acid	50 mg/5 ml (Hospira Australia, Mulgrave, VIC, Australia)	0.1/1, 0.5/5, 1/10, 2/20, 2.5/25, 10/100, 25/250 µM	24, 48, 72	Sodium hydroxide
	5-fluorouracil	5000 mg/100 ml (Sandoz, Macquarie Park, NSW, Australia)			
Oxaliplatin		100 mg/20 ml (Pfizer, Sydney, NSW, Australia)	0.5, 1, 5, 10, 100, 200 µM	24, 48, 72	5% dextrose
Irinotecan		100 mg/5 ml (Accord Healthcare, Melbourne, Australia)	0.5, 1, 5, 10, 50, 100 µM	24, 48, 72	Baxter water (Baxter Healthcare, Old Toongabbie, NSW, Australia)
Mitomycin C		20 mg/20 ml (Omegapharm, Ormond, VIC, Australia)	0.05, 0.1, 0.2, 0.4 mg/ml	2, 24, 48, 72	media ^e
Cetuximab ^b		100 mg/ 20 ml (Merck Healthcare Pty Ltd, Macquarie Park, NSW, Australia)	10, 20, 50, 100, 150, 200, 250, 500, 1000 nM	24, 48, 72	media ^e
Combination					
Folinic acid/5-fluorouracil/oxaliplatin			0.1/1/0.1, 0.5/5/0.5, 1/10/1, 2/20/2, 2.5/25/2.5 µM	24	5% dextrose
Folinic acid/5-fluorouracil/irinotecan		As per single agents above	0.1/1/0.1, 1/10/1, 2/20/2 µM	24	Sodium hydroxide
Folinic acid/5-fluorouracil/irinotecan/cetuximab			0.1/1/0.1/0.05, 1/10/1/0.1, 2/20/2/0.2 µM	24	Sodium hydroxide
Novel agents^c					
N-acetylcysteine		5 g dissolved in PBS (Sigma-Aldrich)	1, 2, 4, 6%	48	PBS
Bromelain (from pineapple stem)		10 g (Sigma-Aldrich, #B4882-10G)	75, 150, 300, 450 µg/ml	72	Baxter water
Palbociclib		5 mg (SelleckChem, #PD-0332991)	0.02, 0.1, 0.5, 1 µM	24	Baxter water

^aConsidered a single agent despite being a combination of drugs, folinic acid enhances the cytotoxic effect of 5-fluorouracil and they are always used together in the clinical setting.⁵⁸⁰

^aPDE media excluded epidermal growth factor component, tissue from patients with known *RAS* mutations excluded and if unknown clinically correlated with later clinical testing.

^cThese agents were chosen for testing based on transcriptomic findings described in Chapter 3.

^dThe master solvent of the stock anti-cancer agent.

^eMaster solvent of the stock anti-cancer agent was not described, so media was used as the vehicle control.

^fPDEs were exposed to two hours mitomycin C incubation for a total 24-h culture (i.e. 22h without mitomycin C).

Abbrev. NAC, *N*-acetylcysteine; PDE, patient-derived explant; PBS, phosphate-buffered saline; h, hours.

5.2.4 Histological assessment

5.2.4.1 Haematoxylin and eosin stains

The FFPE Day 0 and PDE sections (4 µm thickness) were stained with haematoxylin and eosin (H&E) for assessment of PDE tumour cell presence and cell architecture. The tissue architecture, tumour cell and mucin proportions were assessed under light microscopy by two investigators** with blinding to the treatment conditions. The Day 0 sample served as the quality control sample for each individual case. Tissue architecture was rated on a four-point scale to describe low- to high-quality tissues (1-4; Figure 5.1).

5.2.4.2 Immunohistochemistry analysis

Day 0 and PDE samples containing adequate tumour cells for further analysis were stained immunohistochemically (IHC) with antibodies against the tumour marker cytokeratin-20 (CK20), apoptotic (programmed cell death) marker cleaved caspase-3 (CC3), proliferation markers Ki67 and BrdU. These were used to determine the endpoints of tumour cell death and proliferation. These endpoints informed the overall evaluation of the PDE recapitulation of ATPD as well as the tumour response to the anti-cancer agents. Staining for the retinoblastoma (Rb) protein was performed for cases treated with the CDK4/6 inhibitor. IHC staining of FFPE sections (4 µm thickness) was performed using a Leica Bond RX automated stainer (Leica Biosystems) according to the conditions listed in Table 5.3. These were done as single stains with the Bond polymer refine detection kit (Leica Biosystems) or as dual stains with the ChromoPlex 1 dual detection kit (Leica Biosystems). The dual staining used the DAB chromogen for primary mouse antibodies (brown staining) and Fast Red chromogen for primary rabbit antibodies (red staining).

Table 5.3 | IHC staining* conditions on the Leica Bond RX automated stainer for antibodies used to assess PDEs
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Antigen	Antihuman antibody (clone, source, cat.#)	Dilution	HIER (m)	Epitope retrieval solution	Staining protocol	Antibody incubation (m)
CK20	Mouse (Ks20.8, Agilent Dako, M701929-2)	1:400	30	ER2	IHC-K	60
CC3	Rabbit (polyclonal ASP175, Cell Signalling Technology, 9661)	1:200	30	ER2	IHC-K	60
Ki67	Rabbit (SP6, ThermoFisher Scientific, RM-9106-S1)	1:500	30	ER2	IHC-K	60
BrdU	Mouse (BU20A, ThermoFisher Scientific, 14-5071-80)	1:100	30	ER2	IHC-F	60
Rb	Mouse (4H1, Cell Signalling Technology, 9309)	1:500	30	ER2	IHC-K	60

*IHC staining performed according to manufacturer's instructions using the Leica Bond RX automated stainer.

Abbrev. IHC, immunohistochemistry; CK20, cytokeratin 20; CC3, cleaved caspase 3; BrdU, bromodeoxyuridine; Rb, retinoblastoma; HIER, heat-induced epitope retrieval; ER, epitope retrieval; Cat.#, catalogue number, m, minutes.

**Myself and Dr JSS (co-investigator and expert gastrointestinal pathologist)

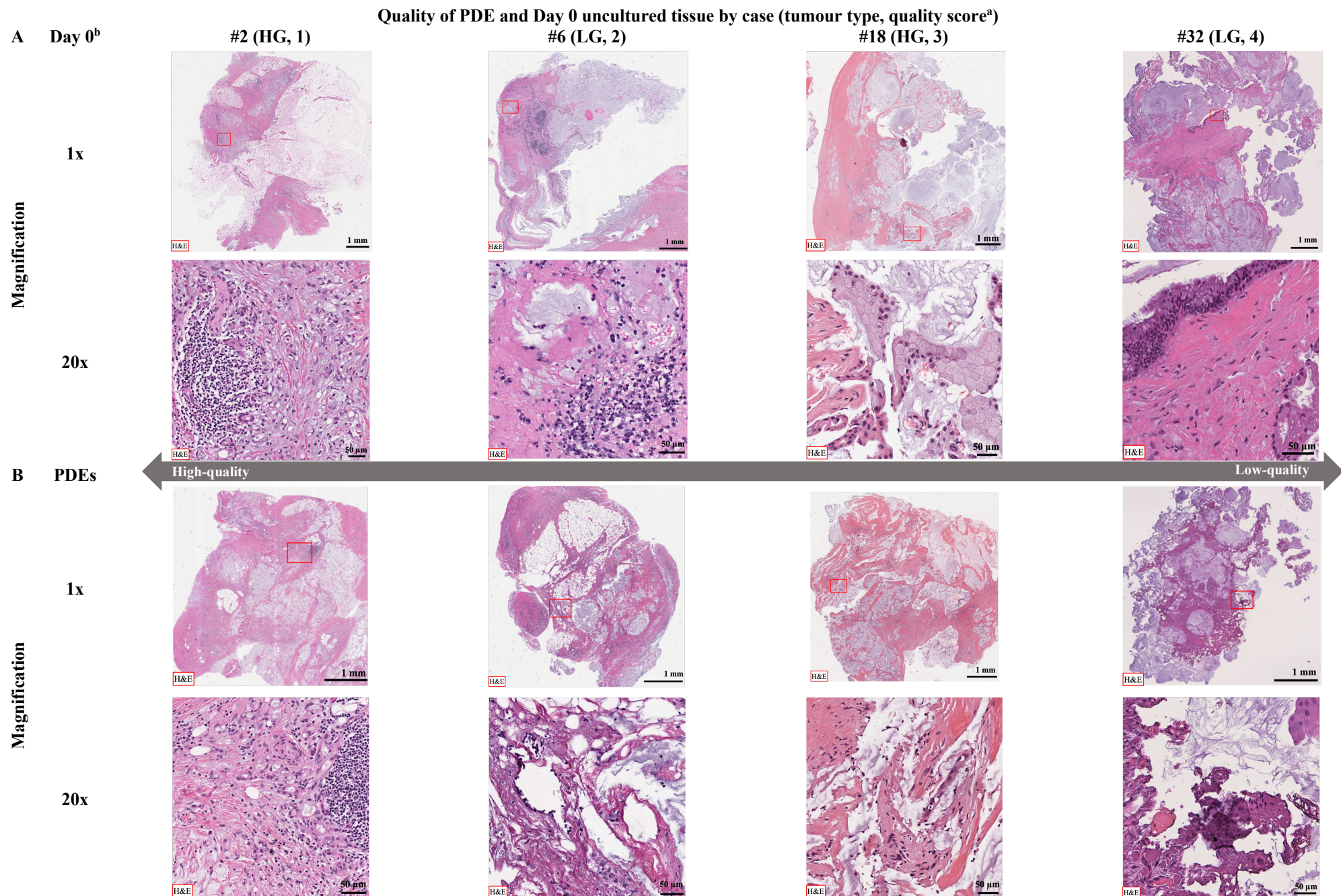


Figure 5.1 | Quality score of tissue sections

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Tissue section show H&E stains of ATPD tissue, at low (1x) and high power (20x), demonstrating examples of the four-point quality score comparing Day 0 uncultured tissues (A) to PDE tissues (B).

^aQuality scores: 1=high, 2=moderate-high, 3=moderate-low, 4=low

^bAll examples depicted in this figure have baseline Day 0 tissues rates with high-quality score (=1)

Abbrev. H&E, haematoxylin and eosin; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; QS, quality score

5.2.4.3 Assessment of tumour cell death and proliferation

IHC-stained tissue sections were digitised using an Aperio Slide Scanner (Leica Biosystems) or a NanoZoomer (S210, Hamamatsu Photonics). Cell counts were analysed using digital pathology software QuPath (version 0.4.3).⁵⁸¹ A representative section was used to train the software to recognise tumour cells compared to surrounding stroma. Analysis was performed by manual selection of clusters of tumour cells with automated cell counting performed by the software to generate the total tumour cell count per section. Quantification of tumour cell death (apoptosis, programmed cell death) was generated by reviewing each whole section PDE. Tumour cells (CK20) with co-staining for cell death (CC3) or cell proliferation markers (Ki67 or BrdU) were evaluated by both manual and automated cell counting (i.e. manual counting of individual cells for sparse clusters; automated counting within selected areas for more prolific tumour clusters; Figure 5.2). The apoptotic index was defined as the percentage of tumour cells with staining for CC3 (Figure 5.2A,B). The proliferative index was defined as the percentage of tumour cells with staining for Ki67 or BrdU (Ki67 shown in Figure 5.2C). An increased apoptotic index in treated PDE tissues compared to control PDEs meant increased tumour cell death whereas a reduced or unchanged apoptotic index meant there was no effect of the treatment on tumour cell death. Conversely, a reduced proliferative index in treated PDE tissues compared to control PDEs meant reduction in tumour cell proliferation, but increased or unchanged proliferative index meant no effect of the treatment on tumour proliferation. The hypotheses related to these endpoints were that effective anti-cancer agents would: (1) increase the apoptotic index and (2) reduce the proliferative index of treated PDEs compared to controls.

5.2.5 Statistical analysis

Statistical tests were performed using GraphPad Prism (v10.2.3 (347), La Jolla, CA) as follows. Chi-Square/Fisher's exact test was used for contingency table analysis of quality scores. Comparisons between groups with paired samples were analysed using paired t-test. For conditions that appeared to have a dose-response relationship for apoptotic index or proliferative index based on inspection of the bar graphs, a dose-response curve was fitted with a variable slope (four-parameter logistic curve) against the log concentration, where zero dose (vehicle control) was included as 0.1 or 0.01 (lower than the lowest starting drug dose) to facilitate the log-transformation. An agonist dose-response curve was fitted for the apoptotic index to derive the half maximal effective concentration (EC₅₀, the concentration of drug that achieved 50% tumour cell death). An inhibitory curve was fitted for the proliferative index to derive the half maximal inhibitory concentration (IC₅₀, the concentration of drug that reduced tumour cell proliferation by 50% proliferation). Outliers were not removed as it was deemed biologically plausible for some samples to exhibit extremes of cell death or proliferation.

Apoptotic index and proliferation index calculation of PDEs

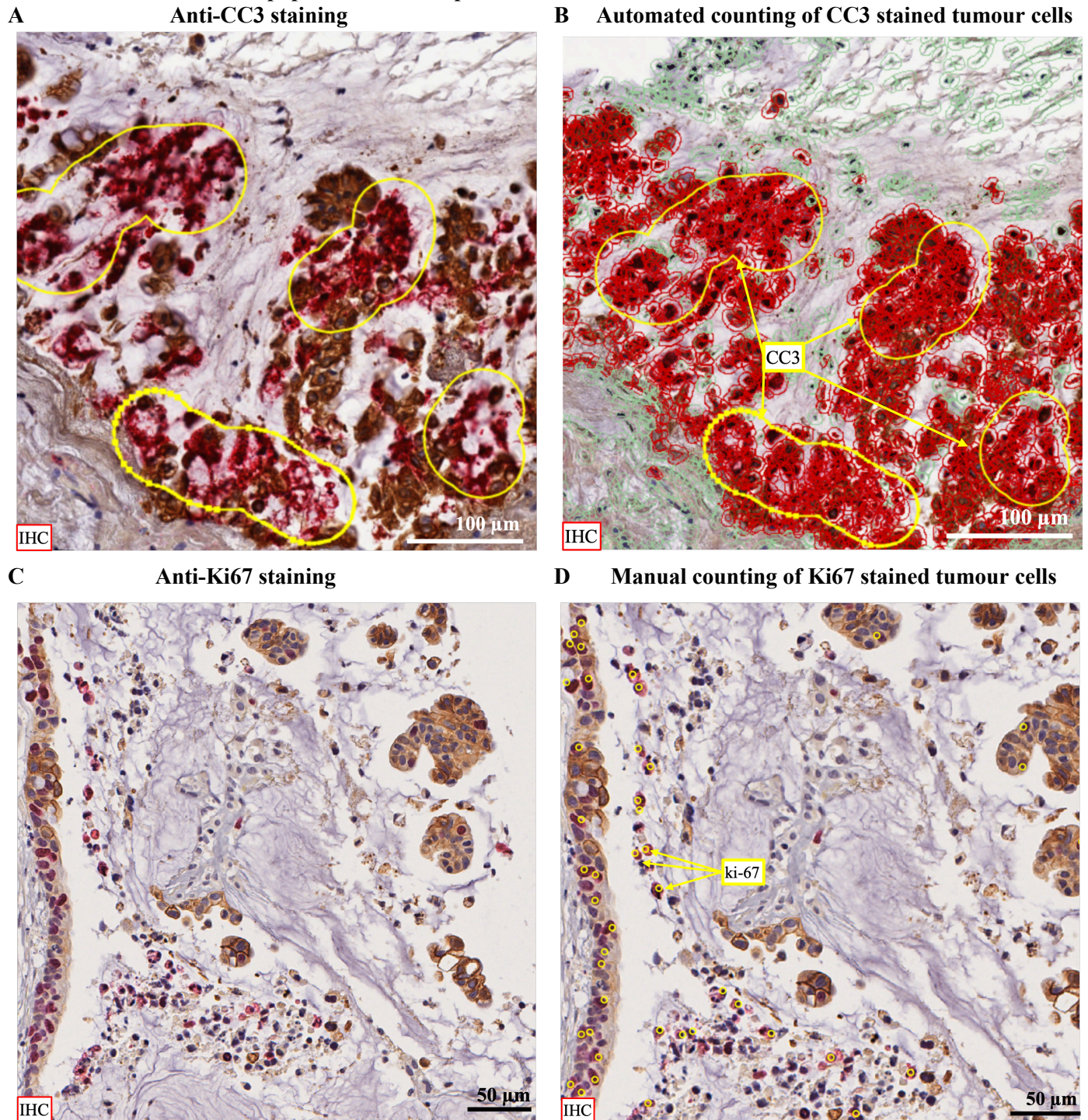


Figure 5.2 | Patient derived explant of ATPD tissue and calculations for cell death and cell proliferation endpoints

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FFPE tissue sections were co-immunostained with anti-CK20 (tumour marker, brown) and anti-CC3 (red) to calculate the proportion of apoptotic tumour cells (apoptotic index; **A,B**) or with anti-CK20 (brown) and Ki67 (red) to calculate the proportion of proliferating tumour cells (proliferative index; **C,D**). Sections show examples of the tumour cell quantification method performed using supervised automatic (**B**) and manual counting (**D**) of digitised images. Supervised automatic cell counting was performed by reviewing co-stained sections and manually selecting apoptotic tumour cells (tumour cells=brown, apoptotic cells=red, apoptotic tumour cells selected in yellow; **A**), which was followed by automated cell annotation and counting using a trained classifier (tumour=red, stroma=green; **B**). Manual cell counting of sparse tumour cell clusters was performed by reviewing co-stained sections (tumour cells=brown, proliferating cells=red; **C**), followed by manual cell marking of proliferating tumour cells (yellow markings; **D**).

Abbrev. ATPD, appendiceal tumour peritoneal disease; CC3, cleaved-caspase; CK20, cytokeratin 20.

5.3 Results

5.3.1 Patient cohort

PDEs were established from 34 patients: 28 ATPD, and six colorectal cancer peritoneal metastases. Baseline patient characteristics, the clinical course and outcomes for patients with PDEs from ATPD and colorectal cancer peritoneal metastases are summarised in Table 5.4. The ATPD optimisation cohort included 14 patients: 10 LG and four HG ATPD. Cases where tumour cells were not identified were not excluded as it was important in the optimisation stage to quantify the yield from the tissues and evaluation of the non-tumoural tissues remained informative for quality assessment of the PDEs. The *ex vivo* drug testing cohort included 24 patients: nine LG, nine HG ATPD and six with colorectal cancer peritoneal metastases. Three patients and their PDEs were excluded from this cohort due to no tumour cells (two HG ATPD and one colorectal cancer peritoneal metastases).

5.3.2 ATPD PDE optimisation cohort

Experimental conditions were tested for the first 10 consecutive patients. Four additional patients were used for analysis of extended cultures (5-7 days). Characteristics of the Day 0 tissue and PDEs are summarised in Table 5.5. Tumour cells were not identified in Day 0 tissues from four patients (29%) and PDEs from three patients (21%). Of Day 0 uncultured tissues, eight had tumour present (1-20%) and seven demonstrated mucinous areas (30-80%). PDEs, when compared to Day 0 uncultured tissue, had a similar proportion of tumour cells (2.0% versus 2.5%) and mucin content (50% versus 46%). Tumour cell proportion was lower in LG PDEs (2%, range 0.5-15.0%) than HG PDEs (10%, range 1-60%). Mucin content was higher in LG PDEs (60%, range 2-100%) compared to HG PDEs (7%, range 1-75%).

Table 5.4 | Clinical and pathology details for peritoneal patients who had cytoreductive surgery or debulking whose tissues formed patient-derived explants

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Case ID #	Sex	Age (years)	ATPD group	Stage		Clinical and treatment details			OS, months (status)
				N	M	PCI	CC	Systemic chemotherapy	
Optimisation cohort									
1	M	70	LG	N0/X	M1	39	3	No	35.3 (NED)
2	F	57	HG	N1/2	M1	36	3	Adjuvant FOLFOX	47.0 (NED)
3	F	76	LG	N0/X	M1	27	0	No	33.5 (NED)
4	F	50	LG	N0/X	M1	29	0	No	24.4 (NED)
5	F	57	LG	N0/X	M1	20	6	No	32.3 (NED)
6 ^a	F	51	LG	N0/X	M0	39	1	No	31.7 (NED)
7	M	49	HG	N0/X	M1	20	0	Neoadjuvant FOLFOX/bevacizumab	45.8 (AWD)
8 ^a	M	61	HG	N1/2	M0	16	1	Neoadjuvant FOLFOX	13.3 (DOD)
9	M	58	LG	N0/X	M1	24	0	Neoadjuvant capecitabine	50.7 (AWD)
10	F	52	LG	N0/X	M1	5	0	No	29.5 (NED)
Ex vivo culture cohort									
11	F	46	LG	N0/X	M1	36	0	No	26.0 (NED)
12 [*]	F	56	HG	N0/X	M1	2	0	No	28.6 (NED)
13	F	50	LG	N0/X	M1	35	0	No	26.8 (NED)
14	F	70	LG	N0/X	M1	24	0	No	32.1 (NED)
15 [*]	F	72	CRPM	N1/2	M1	5	3	Neoadjuvant FOLFOX	14.6 (DOD)
16	F	29	LG	N0/X	M1	33	0	Palliative mitomycin C/capecitabine	3.5(NED)
17	F	66	HG	N1/2	M1	35	1	Prior FOLFOX	25.3 (NED)
18	F	39	HG	N0/X	M1	35	3	Palliative FOLFOXIRI	19.0 (AWD)
19 [†]	F	50	LG	N0/X	M1	26	0	No	6.9 (NED)
20 [†]	M	74	LG	N0/X	M1	34	3	No	10.0 (AWD)
21 [*]	M	45	HG	N0/X	M0	6	0	No	8.2 (NED)
22	M	57	CRPM	N0/X	M1	8	0	Prior FOLFOX, pembrolizumab	80.9 (AWD)
23	M	55	HG	N0/X	M1	31	0	Adjuvant FOLFOX	5.3 (NED)
24	F	42	CRPM	N1/2	M1	6	0	Prior FOLFOXIRI, adjuvant CAPOX	41.3 (AWD)
25 [†]	M	47	HG	N1/2	M1	35	2	Neoadjuvant FOLFOX	12.4 (AWD)
26	F	45	CRPM	N1/2	M1	14	0	Prior FOLFOX, adjuvant capecitabine	41.6 (AWD)
27	F	35	CRPM	N1/2	M1	5	0	Prior FOLFOX	25.4 (NED)
28	F	42	CRPM	N1/2	M1	6	0	Neoadjuvant FOLFOX	52.7 (DOD)
29	F	76	HG	N0/X	M1	20	0	No	2.4 (AWD)
30	F	73	LG	N0/X	M1	30	0	No	14.6 (NED)
Novel drug testing cohort									
31	F	44	HG	N0/X	M1	16	0	Neoadjuvant FOLFOXIRI/bevacizumab	17.0 (NED)
32	F	40	LG	N0/X	M1	39	1	No	306.4 (AWD)
33	M	55	HG	N1/2	M1	29	0	Neoadjuvant FOLFOXIRI	20.8 (NED)
34	F	47	LG	N0/X	M1	29	0	No	3.3 (NED)

^aThese two cases of ATPD had no tumour cells identified on research samples and were used as 'normal' controls (#6 had no tumour on diagnostic specimen, #8 had tumour present on diagnostic specimen).

^{*}Excluded from analysis due to no tumour cells.

[†]PDEs for long duration used as part of optimisation set; Table 5.5.

Abbrev. ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; CRPM, colorectal cancer peritoneal metastases; OS, overall survival; IHC, immunohistochemistry; Stage: N, nodal stage; M, metastasis; PCI, peritoneal cancer index; CC, completeness of cytoreduction score; M, male; F, female; FOLFOX, 5-fluorouracil + folinic acid + oxaliplatin; FOLFOXIRI, FOLFOX + irinotecan; CAPOX, capecitabine + oxaliplatin; NED, no evidence of disease; AWD, alive with disease; DOD, died of disease.

Table 5.5 | Characteristics of ATPD PDE optimisation cohort

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Case ID # (extra cases)	Total (N)	Excluded (n)*	Analysed (n)	Tumour present, n (%)	% tissue containing tumour, median (range)	Mucin present, n (%)	% Mucin content, median (range) ^f	QS ^c =1, n (%)	Total tumour cell count, median (IQR) ^g	apoptotic index (%), mean, median (range)	proliferative index (%), mean, median (range) ^h
Day 0^a cases	14	0	14	10 (71)	2.5 (0.5-20)	10 (71)	46 (30-80)	12 (86)	3,803 (894-11,390)	7, 0 (0-40)	(0-0.02)
All PDEs	239	9	230	120 (50)	2 (0.5-60)	205 (86)	50 (1-100)	194 (81)	18,887 (11,158-39,986)	(0-92)	(0-7.2)
PDE optimisation cohort											
LG ATPD											
1	24	3	21	21 (100)	1 (0.5-15)	24 (100)	70 (20-95)	12 (50)	1,784 (408-3,708)	3, 0 (0-48)	-
3	24	1	23	20 (87)	2 (1-10)	23 (100)	40 (10-80)	21 (91)	1,620 (728-3,531)	54, 62 (0-92)	0 (0-0.6)
4^b	24	0	24	0		3 (13)	2 (2-20)	23 (96)	-	-	-
5	24	0	24	20 (83)	1 (1-15)	26 (100)	80 (50-90)	21 (81)	419 (203-842)	30, 27 (0-55)	-
6	16	0	16	15 (94)	6 (1-10)	16 (100)	40 (10-80)	14 (88)	497 (165-1,206)	12, 0 (0-67)	-
9	26	2	24	19 (73)	1 (1-5)	26 (100)	65 (2-90)	26 (100)	926 (457-2,391)	7, 0.1 (0-47)	-
10^b	24	0	24	0		19 (79)	80 (5-100)	20 (83)	-	-	-
HG ATPD											
2	30	3	27	26 (90)	10 (2-60)	29 (100)	30 (5-75)	17 (59)	3,923 (2,217-8,367)	5, 2 (0-30)	1.1, 0.7 (0-7.2)
7	6	0	6	2 (33)	5 (1-10)	1 (17)	1	4 (67)	112 (60-909)	17, 0 (0-50)	-
8^b	33	0	33	0		30 (91)	2.5 (1-7)	30 (91)	-	-	-
Extended culture duration extra PDEs^d											
LG ATPD											
12 (E1)	3	0	3	2	7 (0-15)	3 (100)	80 (60-90)	3	5,771 (3,040-6,358)	30, 13, 0	-
19 (E2)	2	0	2	2	5, 10	2 (100)	50	1	5,231, 16,948	8, 10	-
20 (E3)	2	0	2	2	0.5, 1	2 (100)	70, 90	2	7,772, 8,983	19, 11	-
HG ATPD											
25 (E4)	1	0	1	1	1	1 (100)	10	0 ⁱ	884	0	0

All histological assessment except for apoptotic index and proliferative index were performed blinded by (JSS) and (MC) on H&E sections.

*Missing data on digitised slide or missing digitised slide: PDE #1 (n=3), PDE #9 (n=2), PDE #2 (n=2); Missing tissue in histology cassette: PDE #3 (n=1), PDE #2 (n=1).

^aDay 0 were uncultured tissue sections from matched tissue created at the time of PDE creation.

^bCases with no tumour cells on H&E (n=3) PDE #4 was scar, PDE #8 and PDE #10.

^cQS=1, high; QS=2, moderate-high; QS=3, moderate-low; QS=4, low quality.

^d4 cases with extra PDEs were created for assessment of longer cultures: PDE #12 (E1) = 5, 6 & 7d culture; PDE #19 (E2) and #20 (E3) = 6- & 7-day culture.

^eCalculated for PDEs where tumour cells were present, i.e. PDEs with 0% were excluded; PDE #4 and #10 were excluded as no tumour in any PDE, PDE #8 (n=1) had 3% tumour cells.

^fCalculated for PDEs where mucin was present, i.e. PDEs with 0% mucin were excluded.

^gAutomated counting anti-CK20 stained PDE sections using digital Qupath software; statistics calculated excluding cases with no tumour.

^hProliferative index performed on HG cases except for PDE #7 (not enough PDEs with tumour present). Performed on 1 LG tumour (PDE #3), and minimal staining, therefore not performed on further cases.

ⁱQS=3.

Abbrev. ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; QS, quality score; PDE, patient-derived explant; apoptotic index, apoptotic index; proliferative index, proliferative index; IQR, interquartile range; H&E, haematoxylin and eosin

5.3.2.1 Assessment of apoptotic index

The PDEs were expected to have a baseline low to moderate apoptotic index, affected by the tumour type and reflected by findings in the Day 0 uncultured tissues. The apoptotic index was expected to vary according to the changes imposed by the varying experimental conditions. PDEs from LG ATPD were anticipated to have reduced tumour cell death compared to HG ATPD, as more aggressive tumour types are usually associated with metabolic stress, leading to tumour cell death.⁵⁸² To understand this further prior to performing *ex vivo* drug testing, the apoptotic index was evaluated comparing Day 0 uncultured tissues to PDEs from the ATPD optimisation cohort. As expected, cultured PDEs had a higher apoptotic index (17.3%) compared to uncultured tissue (Day 0) samples (6.1%), but the difference was not significant ($p=0.16$, Figure 5.3A). This confirms the PDE method (including the varying conditions) was not significantly detrimental to the tissue and that significant increases in apoptotic index following *ex vivo* treatments can be attributed to the anti-cancer agent. However, there was variation in the apoptotic index among tissue samples from individual patient PDEs (intra-patient variation) and between different PDE cases (inter-patient variation; Figure 5.3B). This was due to the significantly increased apoptotic indices of PDE #3 (56.5%) and PDE #5 (30.2%) compared to Day 0 controls (6.1%, $p<0.0001$ and $p=0.0028$ respectively; Figure 5.3B). Unexpectedly, LG PDEs had a significantly higher apoptotic index (20.7%) compared to HG PDEs (4.9%, $p=0.0020$; Figure 5.3C). However, there were a higher proportion of LG cases, which could influence the findings.

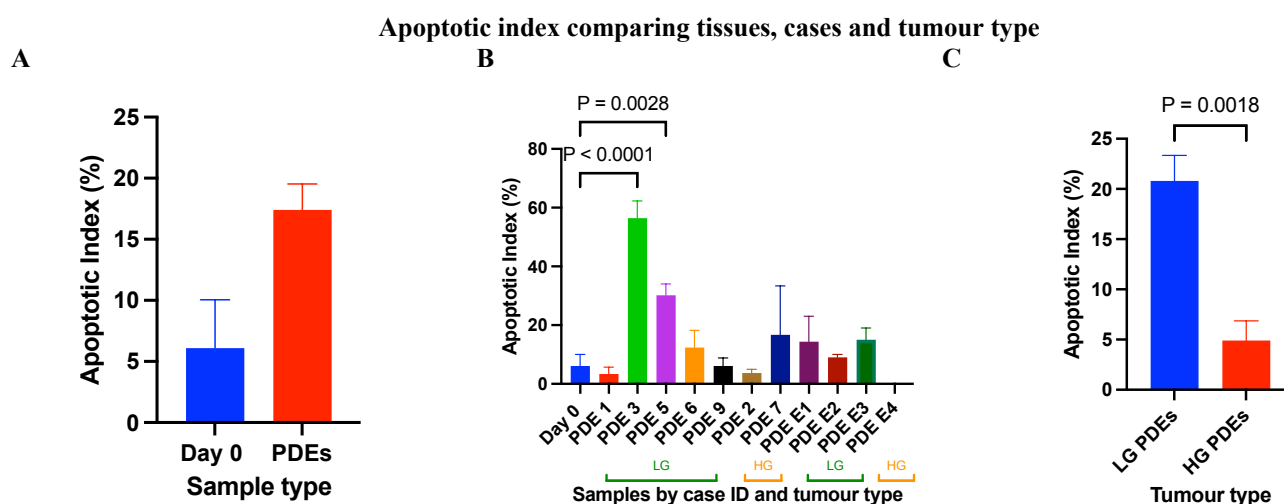


Figure 5.3 | Assessment of apoptotic index between uncultured Day 0 tissues and PDEs by tumour type

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Bar graphs show apoptotic index by sample type (A) individual patients (B) and tumour type. Statistical tests were ordinary one-way ANOVA using Dunnett's multiple comparisons post-test for (B) and unpaired T-tests for (A,C). Columns represent mean $\pm$ standard error of the mean.

Abbrev. PDE, patient-derived explant; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; ANOVA, analysis of variance.

5.3.2.2 Assessment of proliferative index

The degree of assessable tumour cell proliferation in ATPD was uncertain, although it was anticipated that there would be minimal measurable tumour cell proliferation in LG ATPD. Two methods of measuring tumour cell proliferation were investigated: Ki67 staining and BrdU incorporation. PDE from one LG ATPD case was evaluated for Ki67 proliferation (PDE #3) and only two tissue samples exhibited positive Ki67, giving a maximal proliferative index of 0.6% (Figure 5.4). Therefore, it was decided to not proceed with further Ki67 assessment of proliferation for LG ATPD. Only one (of three) HG cases evaluated for Ki67 proliferation (PDE #2) exhibited positive ki67, giving a maximal proliferative index of 7.2%. BrdU incorporation (n=7 cases) was evaluated by immunostaining tissue sections containing tumour cells: LG ATPD (n=2) and HG ATPD (n=2). Compared to the positive control, there was no BrdU positivity for any case, even with an incubation with BrdU of up to 24 hours (Figure 5.5). Thus, measuring tumour cell proliferation is a more challenging endpoint in ATPD tissues. Given a higher likelihood of HG cases exhibiting a higher baseline proliferative index, Ki67 remained as part of the assessment of the *ex vivo* treatment cohort.

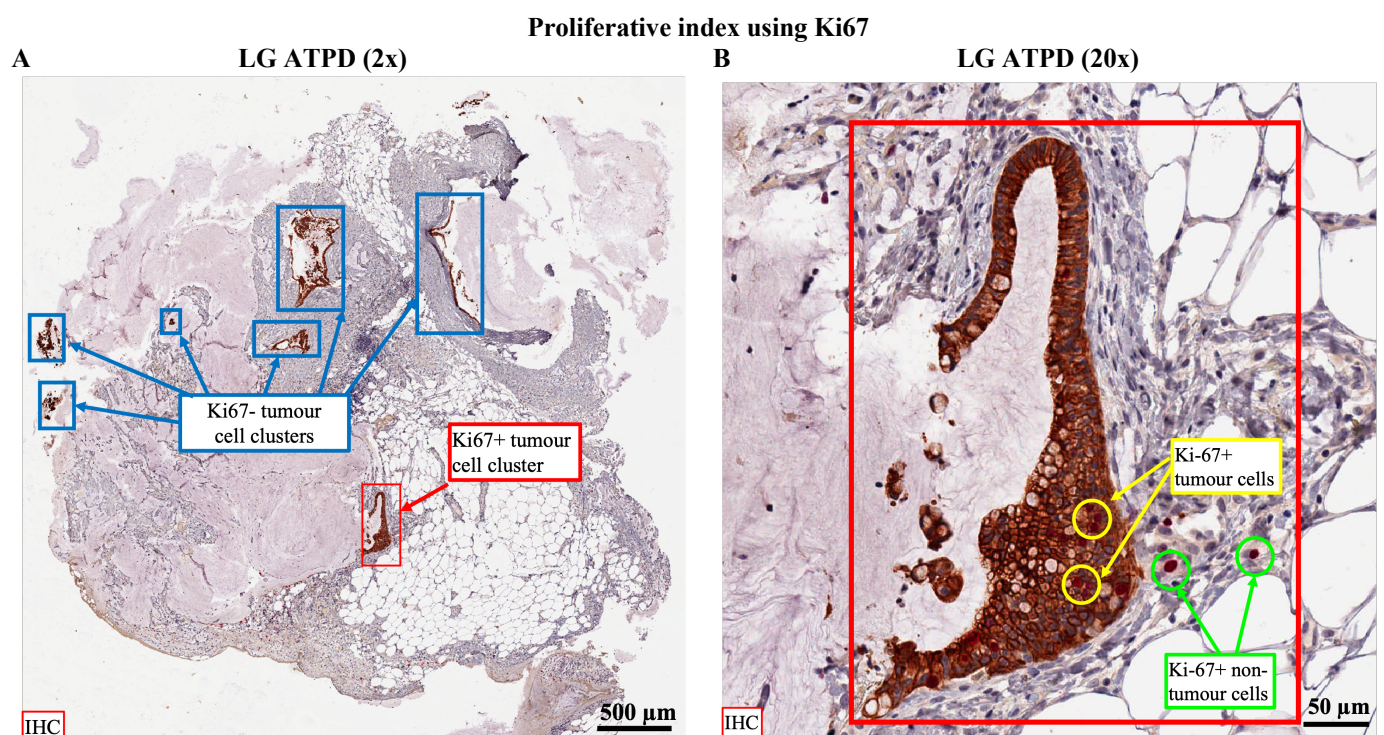


Figure 5.4 | Assessment of tumour cell proliferation by Ki67 immunostaining

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Tissue section of PDE #3 at low power (2x) shows the only tumour cluster (anti-CK20, brown) that positively stained for anti-Ki67 (red) (A). Higher power (20x) view (B) with positive Ki67 staining (red) of tumour cells (brown) marked with yellow circles. Green circles demonstrate non-tumour proliferating cells, used as an internal positive control (B).

Abbrev. PDE, patient-derived explant; LG, low grade; ATPD, appendiceal tumour peritoneal disease; CK20, cytokeratin 20.

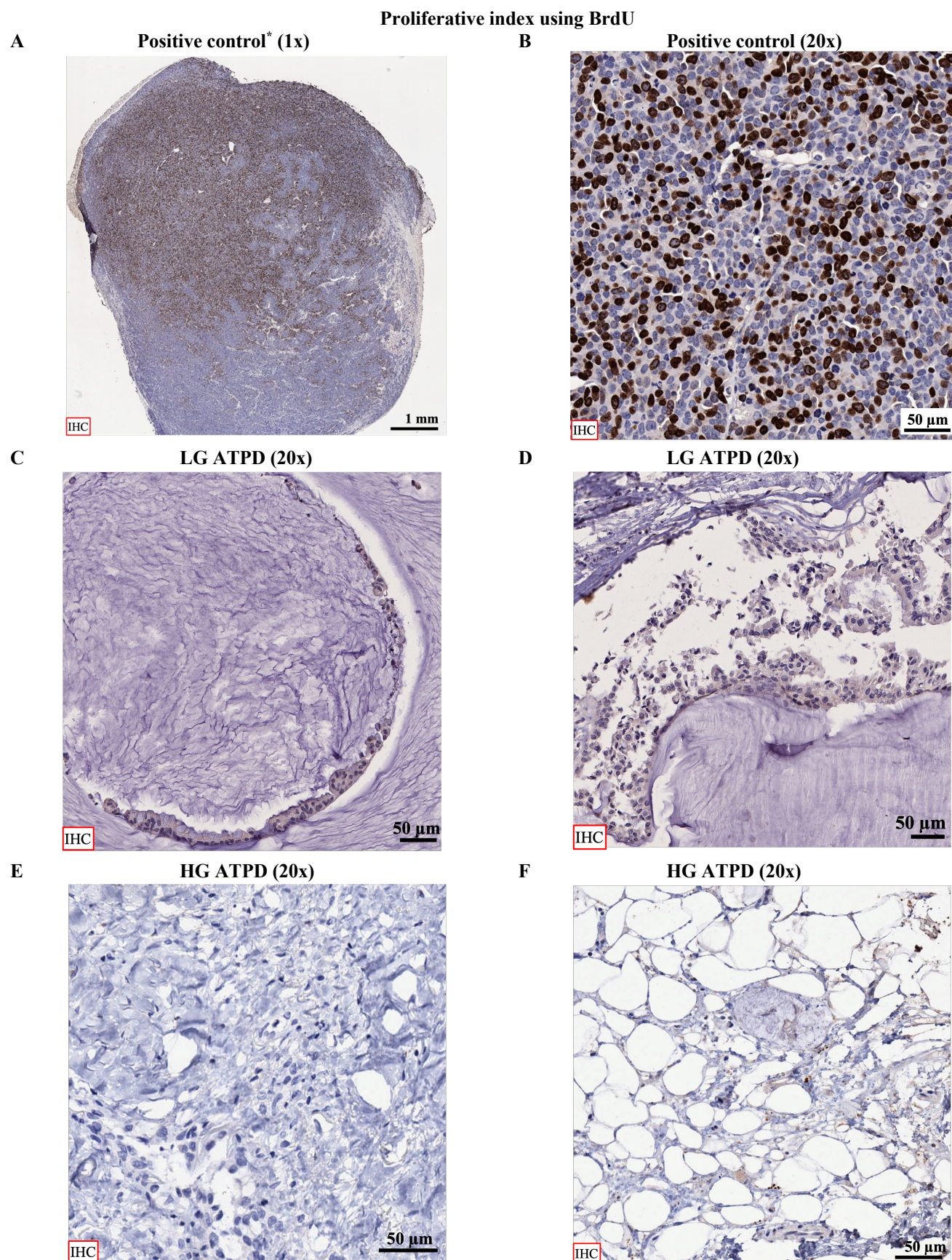


Figure 5.5 | Assessment of tumour cell proliferation by BrdU immunostaining

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Tissue sections show BrdU staining (brown) of tumour cells from the positive control at low (1x, **A**) and high power (20x, **B**). PDE tissue sections from LG (**C,D**) and HG (**E,F**) ATPD were BrdU negative despite incubation for up to 24 hours. PDEs were treated with vehicle control (**C,E,F**) or *ex vivo* anti-cancer agents e.g. irinotecan (**D**).

*The positive control was mouse neuroblastoma.

Abbrev. PDE, patient-derived explant; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; BrdU, bromodeoxyuridine.

5.3.2.3 Culture duration

In preparation for *ex vivo* testing of PDEs, it was important to understand the effect of varying culture durations on the PDEs. The exposure time for anti-cancer agents used for *ex vivo* testing can vary depending on their molecular size and mechanism of action.⁵⁸³ It was postulated that cytotoxic chemotherapies could have an effect on the PDEs between 24- and 72-hour cultures whereas targeted agents (often small molecules) may need longer exposure (such as one week) to modulate signalling in the PDEs. Therefore, the effect and feasibility of a variety of culture durations on the PDE model was evaluated. There was a higher proportion of high-quality PDEs (85%) for cultures between 24 and 72 hours compared to 96 hours or more (64%, $p=0.0027$; Figure 5.6A,B). Four PDEs were cultured for up to seven days and there were no low-quality tissues. The apoptotic index at 48 hours was significantly higher than Day 0 control ($p=0.001$; Figure 5.7A,B). However, the apoptotic index at 96 hours was similar to baseline. Extended cultures did not exhibit any significant difference in apoptotic index, although this analysis used a small number of PDEs. The conclusion was that culture durations between 24 and 72 hours were optimal for maintaining high-quality PDEs.

5.3.2.4 Tissue size

Optimising the size of the tissue samples was essential for ATPD PDEs due to lower tumour cell yields than typical solid tumours as well as accounting for large areas of mucin (often acellular). The need to create larger-sized PDEs than described in the literature ($\sim 1\text{mm}^3$) as anticipated to achieve adequate tumour cell yield to assess the preclinical endpoints. Small tissue pieces (3x3 mm and 4x4 mm) demonstrated the highest proportion of high-quality sections (92%), followed by medium size (5x5 mm and 6x6 mm; 74%) and large sizes (10x10 mm and 15x15 mm; 50%) and are displayed in Figure 5.6C,D. There was a higher proportion of high-quality sections for small-medium sized tissue samples compared to the large-sized tissue samples ($p=0.0019$, Figure 5.6C,D). Analysis of the apoptotic index for these comparisons indicated no significant difference between size groups (Figure 5.7C,D). The conclusion was that small-medium sized tissue samples were optimal for ATPD PDEs.

5.3.2.5 Media components

The nutrient supplementation was considered important to optimise the quality of the PDEs and various combinations of supplemental factors added to the base medium was studied. There was no difference in the proportion of high-quality PDE sections (83% versus 80%, $p=0.68$; Figure 5.6E,F) or of their apoptotic index (18% versus 17%, $p=0.85$; Figure 5.7E,F) comparing base media to base media containing some or all supplementary components. However, there appeared to be a relationship between reducing supplemental factors and increasing apoptotic index (Figure 5.7E). Therefore, media including all supplemental components was ideal for ATPD PDEs.

5.3.2.6 Matrix

While the gelatine-sponge matrix was a previously defined aspect of the PDE method,³⁴⁹ tissues placed in media without matrix support were also evaluated. There was no difference in the proportion of high-quality tissue pieces (82% versus 77% $p=0.49$; Figure 5.6) or of the apoptotic index (23% versus 17%, $p=0.32$; Figure 5.7) using the sponge method compared to no sponge. This was unexpected as the sponge assists with the nutrient uptake for the PDEs.³⁴⁹ However, there was a considerably lower proportion of tissue pieces that were evaluated with no sponge present (15%) which means a true difference cannot be excluded. There was no difference in the type of sponge used

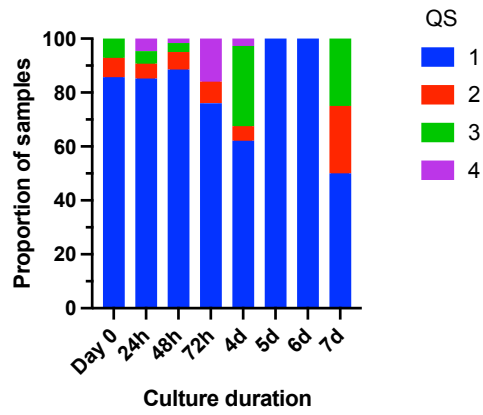
(spongostan versus gelfoam) in the proportion of high-quality PDEs (84% versus 71%, $p=0.085$; Figure 5.6) or of the apoptotic index (19% versus 11%, $p=0.57$; Figure 5.7). While there was not expected to be any difference in the type of sponge used, gelfoam sponges (which could be cut to size) were used mostly for larger tissue pieces (68%).

5.3.2.7 *Optimal method*

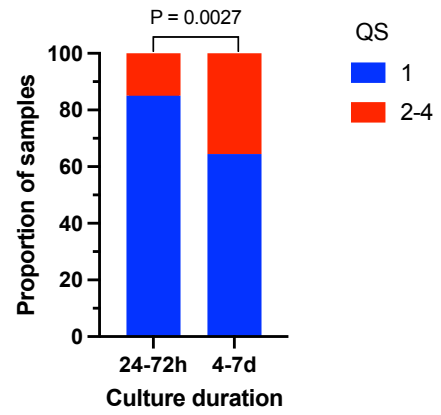
The optimal method for ATPD PDE involved the following parameters: (1) culture duration 24 to 72 hours, (2) small- to medium-sized tissue pieces (dimensions 3x3 to 10x10 mm) (3) base media and all supplemental components and (4) presence of a gelatine-sponge matrix.

Optimisation conditions of PDEs by quality score assessment

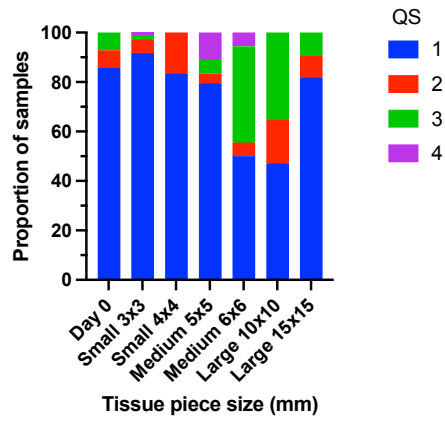
A



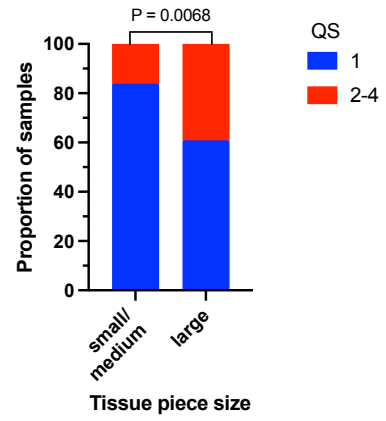
B



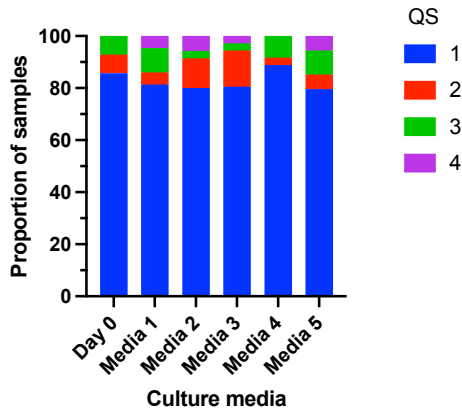
C



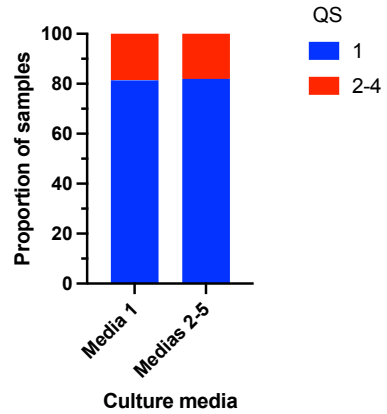
D



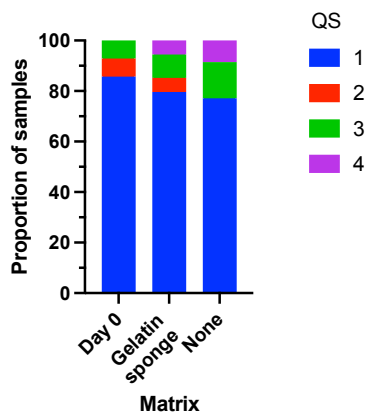
E*



F*



G



H

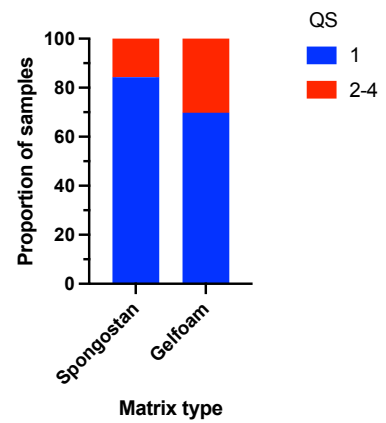


Figure 5.6 | Quality scores (QS) for different experimental conditions

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Bar graphs show the proportion of high-quality PDEs (QS=1) compared to moderate-high, moderate-low and low PDEs (QS=2-4) by culture duration (**A,B**), tissue piece size (**C,D**), culture media^a (**E,F**) matrix presence (**G**) and type of matrix (**H**). Comparison of each condition compared to the Day 0 uncultured controls (**A,C,E,F,G**) and between each grouped condition (**B,D,F,H**) were performed with contingency table analysis using Fisher's exact test. Significant comparisons are indicated where $p < 0.05$. Columns represent proportion of samples for QS=1 (blue) and QS=2-4 (red).

*Culture media comparisons where between base media (media condition 5) with varying combinations of support factors made of the supplemental components (media conditions 1-4). Each media condition is defined in the legend below. Comparisons excluded cultures without matrix.

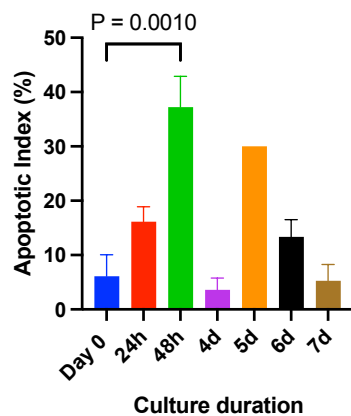
Legend:

Media condition	Description
1	Base media with EGF, NAC, Glutamine, B27
2	Base media with EGF, NAC, Glutamine
3	Base media with EGF, NAC
4	Base media with EGF
5	Base media only

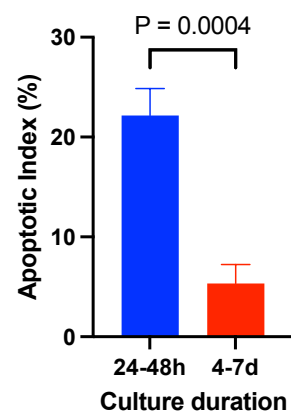
Abbrev. PDE, patient-derived explant; QS, quality score; EGF, epidermal growth factor; NAC, N-acetylcysteine; d, days; h, hours.

Optimisation conditions of PDEs by apoptotic index assessment

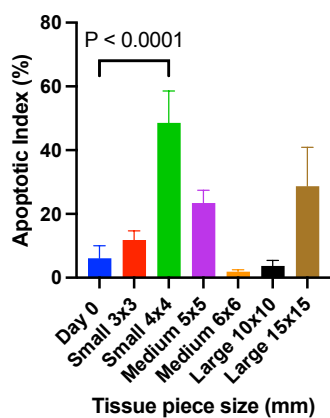
A



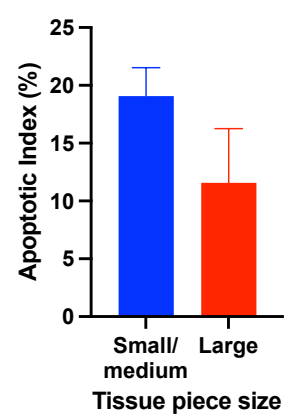
B



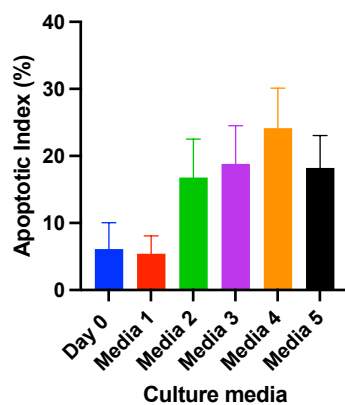
C



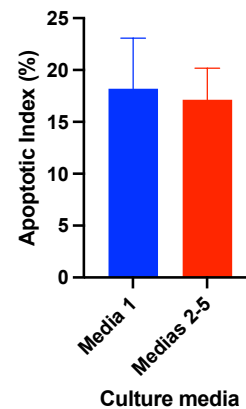
D



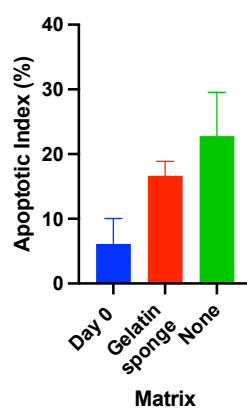
E*



F*



G



H

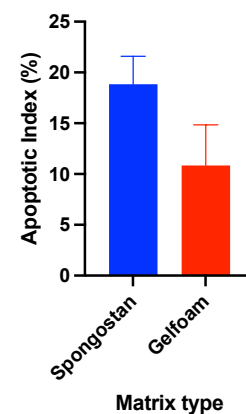


Figure 5.7 | Apoptotic index for different experimental conditions

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Bar graphs show the apoptotic index of PDEs by culture duration (**A,B**), tissue piece size (**C,D**), culture media^a (**E,F**) matrix presence (**G**) and type of matrix (**H**). Comparison of each condition compared to the Day 0 uncultured controls (**A,C,E,F,G**) and was performed using ordinary one-way ANOVA with Dunnett's post-test and comparisons between each grouped condition (**B,D,F,H**) were performed using unpaired t-tests. Significant comparisons are indicated where $p < 0.05$. Columns represent mean $\pm$ standard error of the mean).

*Culture media comparisons where between base media (media condition 5) with varying combinations of support factors made of the supplemental components (media conditions 1-4). Each media condition is defined in the legend below. Comparisons excluded cultures without matrix.

Legend:

Media condition	Description
1	Base media with EGF, NAC, Glutamine, B27
2	Base media with EGF, NAC, Glutamine
3	Base media with EGF, NAC
4	Base media with EGF
5	Base media only

Abbrev. PDE, patient-derived explant; ANOVA, analysis of variance; EGF, epidermal growth factor; NAC, N-acetylcysteine.

5.3.3 Ex vivo anti-cancer drug testing

5.3.3.1 Treatment of ATPD PDEs with conventional anti-cancer agents

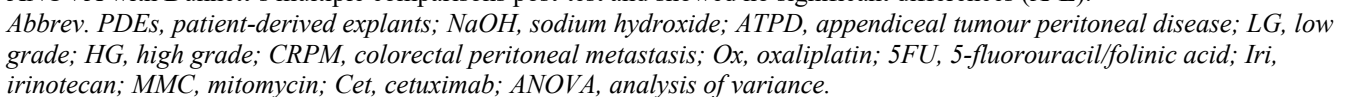
a) Effect of anti-cancer agents on ATPD PDEs

PDEs treated with vehicle controls had similar apoptotic index compared to matched uncultured Day 0 tissues (12% versus 11%, $p=0.81$). This was consistent across LG and HG. The vehicle control for folinic acid/5-fluorouracil treatments was sodium hydroxide ('caustic soda'), which may also have a cytotoxic effect due to its alkalinity.⁵⁸⁴ However, there was no significant difference in apoptotic ($p=0.83$) or proliferative index ($p=0.85$) between PDEs treated with vehicle controls composed of plain media, Baxter water and 5% dextrose compared to those treated with sodium hydroxide.

Pooled analysis of individual and combination treatments showed that there were no significant differences in apoptosis or proliferation compared to control ($p>0.05$; Figure 5.8). Evaluation of individual anti-cancer agents (all dose and durations combined) showed the following observations: (1) Cetuximab treated PDEs from HG ATPD had increased apoptotic index compared to control treatments (17% versus 8%, Figure 5.8B), (2) treatment of PDEs from HG ATPD with all agents, except oxaliplatin alone, had reduced proliferation compared to control PDEs (Figure 5.8C), and (3) mitomycin C treated PDEs from colorectal cancer peritoneal metastases had increased apoptotic index (11% versus 5%, Figure 5.8D).

A Assessment of tumour cell death Assessment of tumour cell proliferation

A Assessment of tumour cell death Assessment of tumour cell proliferation



b) Response of LG ATPD PDEs to chemotherapy dose

Evaluation of the optimal preclinical dose of anti-cancer agents was performed by titrating a wide range in dose. Proliferative index was not assessed in PDEs from LG ATPD due to low tumour cell proliferation observed previously in the optimisation cohort (section 5.3.2.2). The effect of dose titration on the apoptotic index from PDEs for each anti-cancer agent compared to vehicle control are shown for 24-hour and four-day (except mitomycin C) cultures in Figure 5.9. There were no significant differences between the response of each dose increment and control response for these conditions ($p>0.05$). The key findings were that there was a dose-response relationship between: (1) mitomycin C and increased apoptotic index for two-hour treatment after 24-hour culture ($EC_{50} = 0.06$ mg/ml; Figure 5.9A), (2) mitomycin C and increased apoptotic index after 24-hour treatment and culture ($EC_{50} = 0.19$ mg/ml; Figure 5.9B), (3) folinic acid/5-fluorouracil and increased apoptotic index after four-day culture ($EC_{50} = 0.53:5.3$ μ M; Figure 5.9F), (4) irinotecan and increased apoptotic index after four-day culture ($EC_{50} = 4.9$ μ M; Figure 5.9H), and (5) cetuximab and increased apoptotic index after four-day culture ($EC_{50} = 108$ nM; Figure 5.9J). PDEs from five patient cases treated with the combination of folinic acid/5-fluorouracil and oxaliplatin were not able to undergo further analysis due to no observed tumour cells in three cases and no apoptotic tumour cells in two cases (apoptotic index=0%).

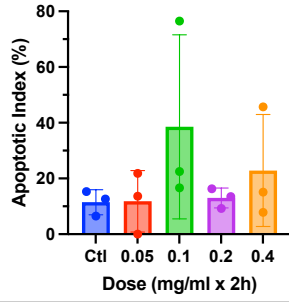
LG ATPD PDE response to anti-cancer agent dose titration*

24-hour culture

Mitomycin C

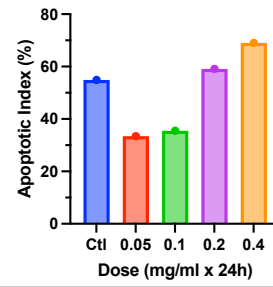
A

2h treatment



B

24h treatment

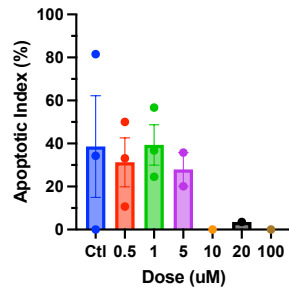


24-hour culture

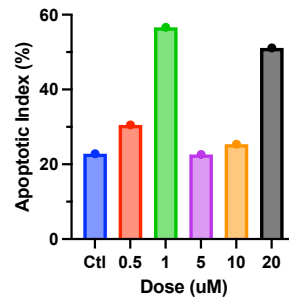
Four-day culture

Oxaliplatin

C

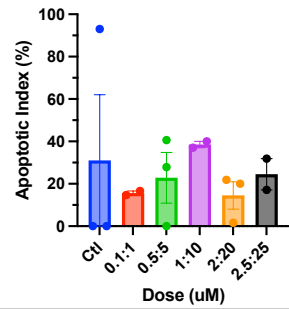


D

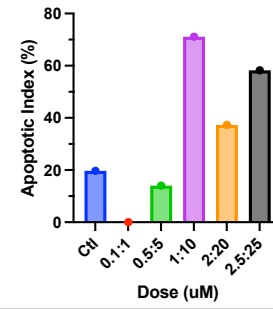


Folinic acid/5-fluorouracil

E

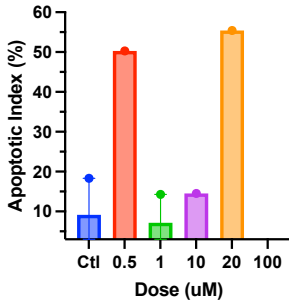


F

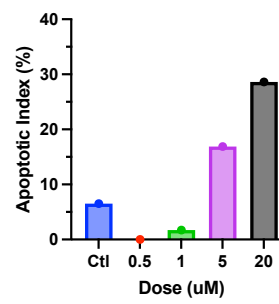


Irinotecan

G

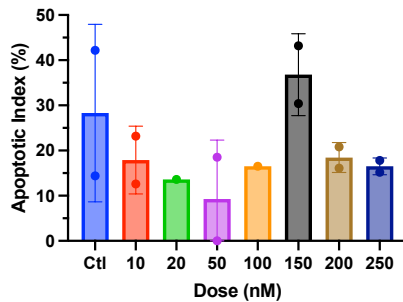


H



Cetuximab

I



J

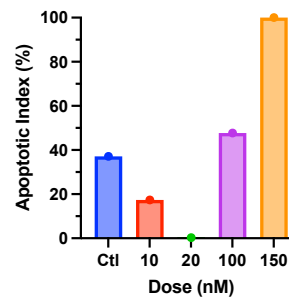


Figure 5.9 | Response of PDEs from LG ATPD to *ex vivo* titration of anti-cancer agent dose

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Plots show the apoptotic index of PDEs from LG ATPD cultured in various doses of anti-cancer agents or vehicle control (ctl). Mitomycin treated PDEs were cultured for 24 hours following either two-hour (**A**) or 24-hour incubation (**B**). The remaining *ex vivo* treated PDEs were cultured for 24 hours (**C,E,G,I**) and 4 days (**D,F,H,J**). Data points represent values of individual samples and columns represent mean $\pm$ standard error of the mean. There were no significant comparisons between each dose and control response using paired t tests.

*Only apoptotic index shown, as proliferative index not used for response assessment for LG ATPD due to low proliferation of tumour cells from this tumour type

Abbrev. PDEs, patient-derived explants; AI, apoptotic index; ATPD, appendiceal tumour peritoneal disease; LG, low grade; Ctl, control.

c) Response of HG ATPD PDEs to chemotherapy dose

Dose titration of anti-cancer agents was also performed for PDEs from HG ATPD. PDEs cultured for 24-hour duration had lower apoptotic index compared to the control (1% versus 14%). PDEs treated for 48- and 72-hour cultures had no tumour cell death (apoptotic index=0%), comparable with the matched control at 72 hours (the 48-hour matched control did not have any tumour cells identified). All PDEs treated with mitomycin C and matched vehicle control over 24-, 48- or 72-hour culture had no evidence of tumour proliferation (proliferative index=0%).

The effect of dose titration for the remaining anti-cancer agents on PDE tumour apoptotic index and proliferative index compared to vehicle control are shown for 24- and 48-hour cultures (Figure 5.10). There were no significant differences between each dose increment and control response for these conditions ($p > 0.05$). The key findings were that there was a dose-relationship between: (1) oxaliplatin and reduced proliferative index after 24-hour culture ($IC_{50} = 3.8 \mu M$; Figure 5.10B), (2) folinic acid/5-fluorouracil and reduced proliferative index after 24-hour ($IC_{50} = 4.2:42 \mu M$; Figure 5.10F), (3) folinic acid/5-fluorouracil and reduced proliferative index after 48-hour culture ($IC_{50} = 2.3:23 \mu M$; Figure 5.10H), and (4) irinotecan and increased apoptotic index after 24-hour culture ($EC_{50} = 9.9 \mu M$; Figure 5.10I).

There were few cetuximab treated PDEs, but there was an increased apoptotic index with higher doses after 24-hour culture, but this was not sustained for the 48-hour culture (Figure 5.10M,O). There was minimal tumour proliferation in cetuximab treated PDEs and their matched controls (Figure 5.10N,P). The PDEs treated with combinations of anti-cancer agents including folinic acid/5-fluorouracil, and irinotecan, folinic acid/5-fluorouracil, irinotecan and cetuximab, had no significant treatment effect (data not shown). PDEs from one patient treated for 72-hour cultures (data not shown) had minimal treatment effects when treated with anti-cancer agents except those treated with cetuximab had an increased apoptotic index compared to control PDEs (37% versus 19%, data not shown).

Overall, proliferative index appeared to be more consistently reduced in PDEs treated with anti-cancer agents compared to the matched controls. However, the baseline proliferative index in all, but one of the cases was under 5%, making this endpoint less reliable.⁵⁸⁵ The lack of clear dose-response for most agents with the apoptotic index may be due to the culture durations of 48 hours or less.

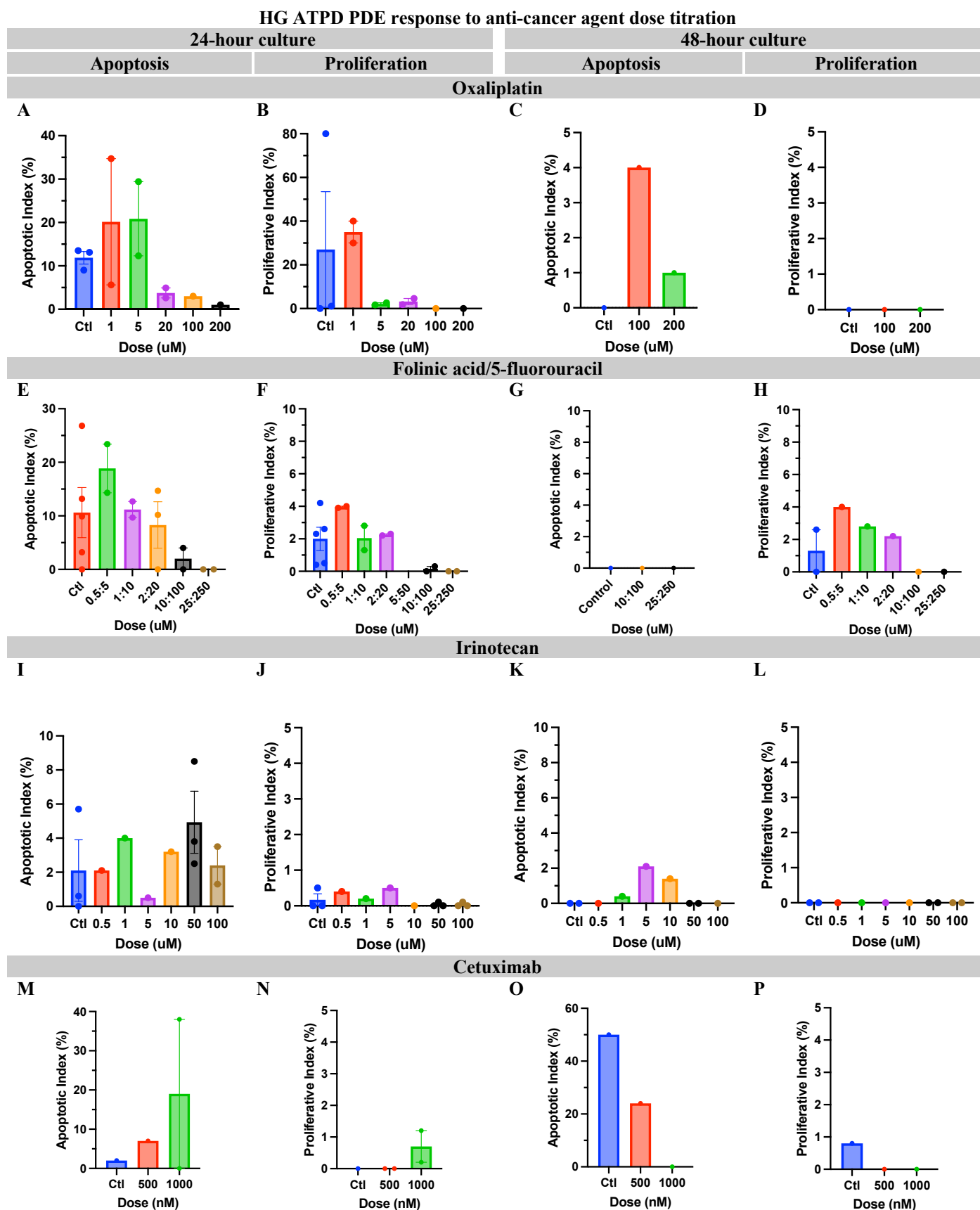


Figure 5.10 | Response of PDEs from HG ATPD to *ex vivo* titration of anti-cancer agent dose

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Plots show the apoptotic index (A,E,I,M) and proliferative index (B,F,J,N) for 24-hour culture and apoptotic index (C,G,K,O) and proliferative index (D,H,L,P) for 48-hour culture of various doses of anti-cancer agents compared to vehicle control on PDEs from HG ATPD. Data points represent values of individual samples and columns represent mean $\pm$ standard error of the mean. There were no significant differences in apoptotic index or proliferative index between each dose and control response according to paired t-tests. Inset graphs indicated the dose-response relationship.

Abbrev. PDEs, patient-derived explants; AI, apoptotic index; PI, proliferative index; ATPD, appendiceal tumour peritoneal disease; HG, high grade; Ctl, control.

5.3.3.2 Treatment of PDEs with novel strategies

a) Mucolytic strategies

Mucolytic therapies using bromelain and N-acetylcysteine have been explored preclinically using patient-derived mucin and xenografts^{262, 271} and clinically as intraperitoneal therapy.¹¹¹ In Chapter 3, these agents were evaluated in the single cell dissociation protocols to assist with mucin digestion. While not suitable for use in these protocols, these agents may have mucolytic effects on the PDEs. If activity is observed, then future treatment strategies could include combinations of mucolytics and anti-cancer agents. Therefore, a pilot study was performed in a PDE from one patient case with LG ATPD treated with N-acetylcysteine and bromelain at various doses for 24 to 72 hours. The PDE, which was treated with increasing doses of N-acetylcysteine, had reduced proportion of mucin for both 24- and 48-hour timepoints compared to vehicle control treatment (Figure 5.11A). There did not appear to be any dose-response relationship for bromelain-treated PDE tissues and proportion of mucin (Figure 5.11B).

b) CDK4/6 inhibition

Increased cell cycle progression was implicated in the transcriptomic analysis of ATPD (Chapter 3) suggesting that that CDK4/6 inhibition had the potential for therapeutic efficacy. Therefore, a pilot study was performed consisting of PDEs from two patients with HG ATPD and one patient with LG ATPD, that were treated with palbociclib at various doses for 24 hours. The LG ATPD case did not yield any tumour cells and was excluded from further analysis. The PDEs from HG ATPD exhibited higher apoptotic index compared to control treated PDEs (except for the highest 1µM dose; Figure 5.11C). Paradoxically, palbociclib-treated PDEs had increasing proliferative index as the dose increased (Figure 5.11D). All cases tested with palbociclib exhibited positive Rb staining, which was important as Rb loss is associated with resistance to CDK4/6 inhibitors (Figure 5.11E,F).⁵⁷⁷

Novel *ex vivo* treatment strategies of ATPD PDEs

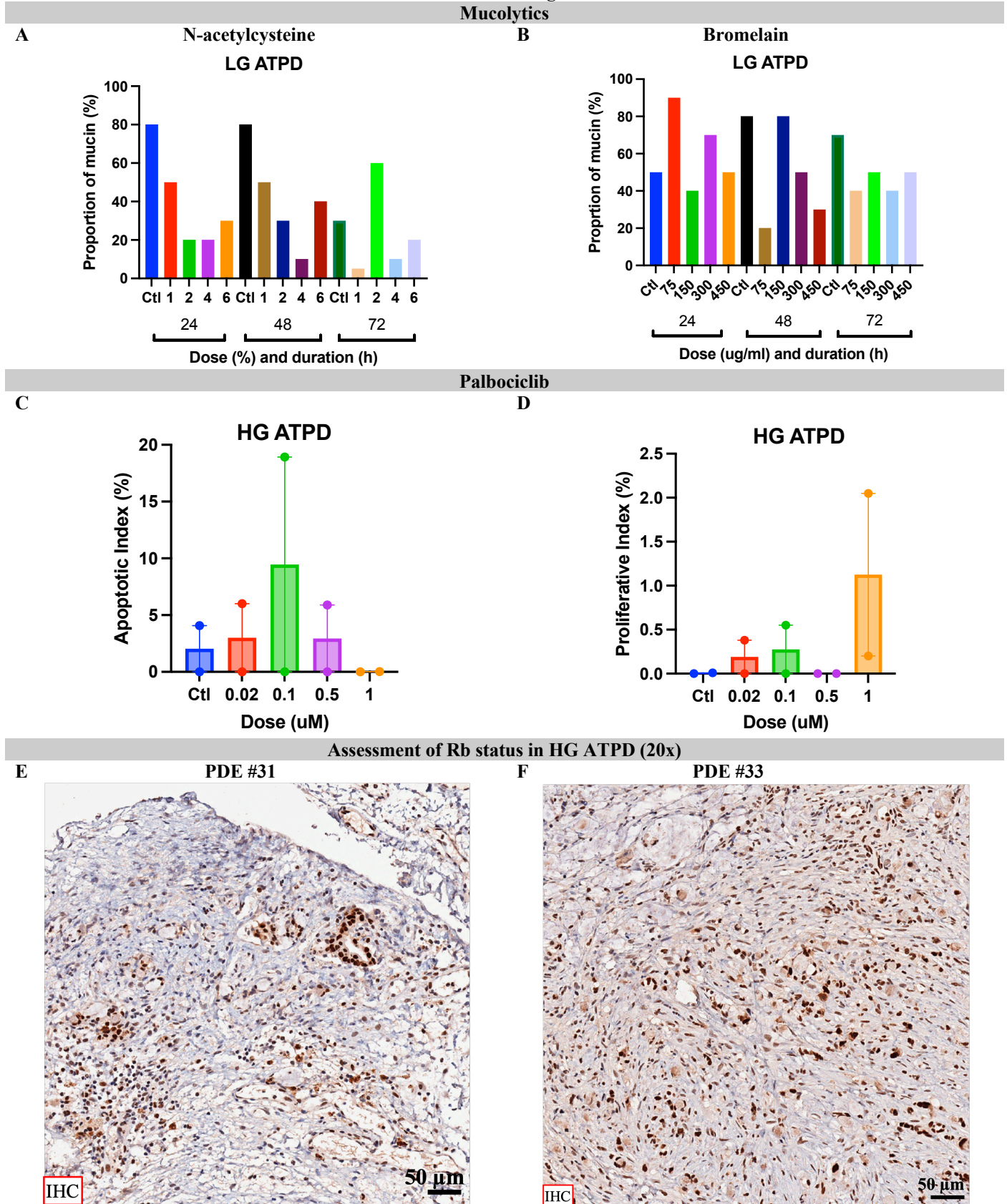


Figure 5.11 | Response assessment of novel *ex vivo* treatment strategies of PDEs

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Novel treatment strategies included mucolytic treatment with N-acetylcysteine (**A**) and bromelain (**B**), and CDK4/6 inhibition with palbociclib (**C,D**). Plots show the proportion of mucin at 24–72 hours (**A,B**), apoptotic index (**C**) and proliferative index (**D**) at 24-hour culture of various doses of the treatment and vehicle control on PDEs from LG (**A,B**) and HG ATPD (**C,D**). Columns represent the proportion of mucin (**A,B**) or the mean $\pm$ standard error of the mean and data points represent values of individual samples (**C,D**). Histological section show tissue (20x) from HG ATPD (PDE #31 and #33) immunostained with anti-Rb (brown). Abbrev. PDEs, patient-derived explants; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; Ctl, control; CDK, cyclin-dependent-kinase; Rb, retinoblastoma protein.

5.4 Discussion

This study is the first to report the development of a novel human preclinical model of ATPD that recapitulates the cell type, architecture and quality of patient-derived tumours. This optimised model demonstrated that these ATPDs PDEs have utility for *ex vivo* anti-cancer drug testing. The clinical implications of this study include the potential to incorporate this preclinical model into clinical studies where it could be used as a parallel translational platform.

The biological understanding of ATPD has lagged behind that of other solid tumours due to its rarity and challenging mucinous tissue type with often sparse distribution of tumour cells. Cell lines, patient-derived organoids (PDOs) from and xenograft (PDX) models have been challenging to generate from ATPD, especially mucinous lower-grade disease.^{269, 341, 342, 345, 586} Thus, preclinical models are biased to reflect more aggressive higher-grade disease. While these models have provided some important insights into the biology of the disease including identification of candidate genes differentiating LG ATPD from peritoneal metastases from colorectal cancer,²⁶⁹ potential repurposed treatment strategies from drug screens,³⁴¹ and the effectiveness of mucolytic therapy on reducing the quantity of mucin,²⁶² these techniques have not allowed the study of the surrounding tumour microenvironment (TME).

PDX models can provide important information on tumour growth kinetics in response to *ex vivo* drug testing and remain the gold standard for evaluation of preclinical novel therapeutics.⁵⁸⁷ However, they take a long time to generate, are resource intensive, use sacrificial animals, suffer from ingrowth of mouse supporting cells into the human tumour and do not reflect any host immune response. The PDE model overcomes many of these limitations and importantly is efficient with clinically relevant implications for informing personalised treatment strategies.³²⁷

Tumour and microenvironment viability has been preserved for more than 48 hours in other studies using PDEs for other solid tumours. One study demonstrated that PDEs retained stromal and tumoural architecture, proliferative capacity and viability up to 12 days³⁵² in pancreatic cancer PDEs, noting that the media was changed at least daily. Interestingly, cases with immune infiltrates maintained immune cells for up to five days of culture.³⁵² Incubation with *ex vivo* anti-cancer agents of 24 to 72 hours is important to allow for adequate exposure of cells to drug therapies including cytotoxic and targeted agents while retaining tissue architecture which can be influenced by both the anti-cancer agent and the culture conditions.³²⁷ Consistent with a similar method used for HG ATPD, tissues maintained moderate-low quality for duration up to 7 days, although there was a decline in the assigned quality score.³⁴⁴ The longer duration cultures have implications for testing of immunotherapies where different immune cell types (tissue resident) may be recruited as part of an adaptive immune response.^{327, 344} Voabil *et. al.* revealed immunological responses within 24 to 48 hours of immunotherapy using a patient-derived tumour fragment method for melanoma, lung, breast, ovarian and renal cell cancer.³⁵⁶ Pragmatically, the shorter the *in vitro* drug culture, the more efficient the turnaround time is, which has implications for these models being used to inform patient-specific personalised treatment approaches.^{327, 588}

The size of the tissue pieces forming the PDE influences the tissue quality, as a larger size of tissue reduces the perfusion of nutrients and oxygen, and increases the chance of tissue hypoxia, thus impacting viability.^{331, 344, 349}

Additionally, the size needs to allow infiltration of the *ex vivo* drugs.⁵⁸³ Finally, the size of the PDE tissue samples may influence the method chosen for analysis and vice versa. For example, confocal imaging resolution was lost in tissue pieces larger than 150 to 300 μm .³⁴⁴ If the pieces are too small, then the TME can be disrupted. ATPD often has large areas of tumour mucin and despite manual dissection to remove soft mucin, the harder mucin components were frequently included as part of the PDE. Furthermore, LG ATPD particularly is renowned for sparse tumour cellularity with some patients diagnosed with acellular mucin (no tumour cells observed).

A suitable tissue size was essential for this tumour type to compensate for large mucinous areas and sparse tumour cells with minimal disruption to the tumour architecture. Typically, PDEs are dissected with either a scalpel or 1 mm³ punch biopsies, or automated slicers to generate 150 to 300 μm thickness tissue pieces.^{327, 344, 349} Advantages of manual dissection include its simplicity as it is easy to implement and efficiency retains tissue integrity whereas disadvantages include non-uniform tissue pieces, which may risk hypoxic areas.³³¹ Advantages of using automated slicers, such as a vibratome (microtome with a vibrating blade), includes uniform slices reduced hypoxic areas but the longer set up time may impact on tissue integrity.³²⁷ The size of the optimised PDE model was 2 mm thick, slightly larger than suggested in the literature. This was chosen as it was deemed suitable for ATPD tissue using a manual dissection technique to maximise tumour cell yield while maintaining nutrient perfusion, minimising hypoxia and facilitating *ex vivo* drug delivery. The variable factor evaluated was width and length, with the small/medium dimensions associated with high quality PDEs and no increase in the apoptotic index.

A more recently published study of a similar model of ATPD used a 'slice' culture method where the tumour was solidified in agarose gel, sliced to 150 μm slices using a vibratome and cultured using a matrix of a polyester membrane, maintained viability over seven-day culture (20% loss) with an increase in tumour proliferation observed after 24 hours peaking at day four and five when measured by 5-ethynyl-2'-deoxyuridine (EdU, a nucleoside analogue similar to BrdU) incorporation and flow cytometry.³⁴⁴ Moreover, epithelial (pan-cytokeratin; excludes tumour marker CK20), immune (CD45) and stromal compartments (αSMA) were evaluated by immunostaining and the proportion of cell types on matched areas of sequential slices were similar.³⁴⁴ This study, while showing good preservation of cellular architecture with the PDE model, demonstrated the intra-patient variability in histological components with some PDEs exhibiting a broad range of tumour cell and mucin proportion, related to tissue sampling and characteristics of ATPD.

It was not unexpected that untreated PDEs had increased cell death compared to uncultured tissues, which in this study was shown for both LG and HG ATPD subtypes, although these data were not statistically significant. In the optimisation cohort, the maximal proportion of tumour cell death increased by about 30% to almost 40% at 48 hours, although this reduced between four and seven-day cultures. Second daily media changes was performed for the extended duration PDEs which may explain the return to baseline tumour cell death, noting this was a small cohort. This is consistent with another recent study where baseline viability of 60% with about a 20% loss in viability over the culture duration.³⁴⁴

This study revealed that LG tissues had increased apoptotic index compared to HG tissues. This was unexpected, as it would generally be expected based on clinical experience that HG ATPD would demonstrate higher rates of spontaneous tumour cell death compared to more indolent LG ATPD and that this would be reflected in the PDE tissues. LG tissues had technical challenges for generating PDEs including low cellularity and difficulties related to culturing mucin may have translated to increased tumour cell death. It is also possible that HG ATPD were more resilient to the varied PDE conditions.

Negligible proliferation index was demonstrated using Ki67 in LG ATPD and so restricted assessment of the proliferative index to HG ATPD and peritoneal metastases from colorectal cancer. An alternative method was assessed, using BrdU incorporation, but there was positive staining of any of the ATPD cases compared to positive control. The Weitz *et. al.* study used a similar method to assess proliferation using EdU incorporation.³⁴⁴ Proliferation was found to than using either Ki67 or BrdU for these tissues, noting that the majority of the fixation time-points were less than five days. Thus, the peak proliferation rate may have been missed. However, it should be noted that it is unclear why proliferation is peaking at the later time point and in fact this may be less representative of the proliferation index used clinically on fresh Day 0 samples. The only study that has looked at this clinically demonstrated average Ki67 of 15% with hotspot elevated proliferation of 50% in areas of dense cellularity and distorted architecture.³⁵⁴

These results are consistent with the literature where *ex vivo* responses to anti-cancer agents exist but are variable. Votanopolous *et. al.* demonstrated viability ranged from 38% to 90% for folinic acid/5-fluorouracil, 40% to 100% for oxaliplatin, 18% to 85% for folinic acid/5-fluorouracil and oxaliplatin combination, and 25% to 75% for folinic acid/5-fluorouracil and irinotecan treatment.³⁴² This study also found reduced responses to single agent oxaliplatin and may indicate secondary chemoresistance induced by prior treatment (three ATPD cases). Pilot studies on novel strategies including mucolytics and CDK4/6 inhibition were performed. Assessment of activity is limited by small numbers and may need ongoing optimisation of optimal culture conditions (such as extended duration of CDK4/6 inhibition). However, they are proof-of-concept studies that novel strategies identified in parallel translational studies can inform preclinical *ex vivo* drug testing.

5.5 Conclusion

PDE models are well-suited for studying the TME of ATPD despite tissue-related challenges of low tumour cell and high mucin content. ATPD PDEs were confirmed to maintain high-quality cellular architecture that could be used for *ex vivo* drug testing, which was performed with conventional anti-cancer agents used clinically for treating ATPD. Limitations of this study included a lack of positive control for *ex vivo* drug testing responses and challenges assessing tumour cell proliferation, especially in LG ATPD. ATPD studies are a robust companion model that could be incorporated in future clinical trials for this tumour type with significant utility for personalising drug treatment.

Chapter 6. Discussion, Conclusions and Future Directions

Peritoneal disease from appendiceal tumours causes significant morbidity and death from complications including bowel obstruction and malnutrition.³⁴ The contribution of HIPEC to the benefit of CRS has been challenging to disentangle and there is a dearth of effective systemic anticancer treatments to improve cure rates with conflicting data for any role in the curative perioperative setting. In the advanced setting, current systemic therapies have a limited efficacy. There is mounting evidence peritoneal metastases from appendiceal tumours are clinically and genomically different to those from colorectal cancer.¹¹⁸ Thus, treatment strategies following colorectal cancer paradigms are no longer rational and novel strategies are needed.

The rarity of this tumour type poses specific challenges for understanding of the natural history of LG and HG ATPD. Complex sequencing and combinations of different modalities of treatments (i.e. non-CRS surgery, CRS, HIPEC and systemic chemotherapy) makes distinguishing the role of any one therapy difficult. Multiple changes to the histological nomenclature over the years has also made the literature confusing and interpretation challenging.³ Retrospective outcomes are highly biased with selection of the highest risk patients for administration of systemic chemotherapy, leading to seemingly worse outcomes. Systemic chemotherapy is only modestly effective in HG ATPD and generally ineffective in LG ATPD. Thus, novel strategies are urgently required.

In order to gain deeper understanding of the biology of ATPD, evaluation of the TME was conducted to provide insights into the immune response. The peritoneal microenvironment was confirmed to be immunologically active²⁸⁴ and populations of mesothelial cells and CAFs were observed to play a potentially essential role in disease pathogenesis. Mucin overproduction is a hallmark of mucinous ATPD and aberrant mucin signalling via endoplasmic reticulum stress, the unfolded protein response and Wnt/ β -Catenin pathways were indicated in both single cell and spatial transcriptomic analysis (Chapter 3). Altered cell cycle pathways are implicated in ATPD tumour promotion and present targets for potential therapeutic strategies (Chapter 5). ATPD was found to exhibit an immunosuppressive microenvironment in both LG and HG disease (Chapter 4).

Mucinous tumours like ATPD present unique tissue processing challenges that require special attention for tissue processing protocols. Despite this, a single cell dissociation protocol incorporating a mucolytic pre-digestion strategy was developed (Chapter 3 and Chapter 4), and the optimal model for PDEs from ATPD was established. This PDE model was demonstrated to have utility for *ex vivo* drug testing (Chapter 5).

The overarching goal of this thesis was to examine the role of systemic chemotherapy, the clinical outcomes and the broad biology of ATPD in order to generate hypotheses that could lead to rationally derived treatment strategies for testing in the *ex vivo* setting.

6.1 The role of systemic chemotherapy in appendiceal tumours

The role of systemic chemotherapy is not well defined in treating ATPD. The literature suggests an extremely limited role for conventional cytotoxic agents in LG ATPD. Treatment patterns and clinical outcomes of appendiceal tumours were examined for two parallel cohorts of patients both from peritoneal specialist centres, one in Sydney, Australia (Chapter 2) and one in Manchester, UK ([Appendix 1.2](#)). Analysis of the Australian cohort confirmed prolonged survival outcomes for patients with LG ATPD, making any impact of potentially effective therapies challenging to demonstrate. Systemic chemotherapy did not provide any additional benefit in patients with HG ATPD who had no residual disease (CC0) after CRS, acknowledging these patients are highly selected for the most aggressive features. Study of the UK cohort represented a detailed evaluation of long-term outcomes, prognostic factors, pathology re-review for historically classified classes and variables to help improve selection for systemic chemotherapy in a homogenous cohort of patients with mucinous and non-mucinous appendiceal adenocarcinomas.

In both cohorts, systemic chemotherapy had most impact in HG ATPD after curative intent CRS/HIPEC for patients with positive lymph nodes. This finding is consistent with existing literature^{113, 171} and supports current clinical practice where adjuvant (post-CRS) combination chemotherapy with fluoropyrimidine and oxaliplatin is recommended. In the preoperative setting, where downstaging is often the goal, irinotecan may be added into a triplet regimen (FOLFOXIRI) for suitable candidates. Given the increasing use of this regimen on both the Australian and UK campus (n=9), a descriptive case series is planned to document the early experience. Once appropriate duration of follow-up after CRS/HIPEC has been reached, 6-month peritoneal disease-free recurrence will be evaluated as the primary outcome. In addition, a single arm prospective phase 2 trial compared to historical control was designed to evaluate neo/adjuvant chemotherapy for patients with HG ATPD who have high-risk features (positive lymph nodes, poorly differentiated, presence of signet ring cells), including FOLFOXIRI for suitable patients (Figure 6.1).

The challenges of performing a single centre study, even at high-volume peritoneal centres was illustrated in an open-label randomised crossover trial performed at a high-volume comprehensive cancer centre in the US.⁵⁸⁹ It took eight years to recruit 24 patients with LG ATPD who were randomised after CRS/HIPEC to six months of systemic chemotherapy then six months of observation or vice versa. Importantly this study demonstrated similar rates of tumour growth in both groups, further supporting a lack of benefit of systemic chemotherapy in this LG population. However, for timely recruitment and outcome assessment of clinical trials in rare cancers it is imperative to develop an international network to facilitate analysis and result dissemination.⁵⁹⁰ To facilitate this, it would be helpful to develop a network of subspeciality medical oncologists from specialised peritonectomy centres internationally, with the goal of generating research ideas including clinical trial concepts and fostering funding networks to support this initiative. Rare cancer trial designs need to be tailored to the key clinical questions addressed and guidance has been developed for credible trial approaches that are not reliant on high powered randomised trial designs.⁵⁹¹

Study design for phase 2 prospective chemotherapy trial in ATPD

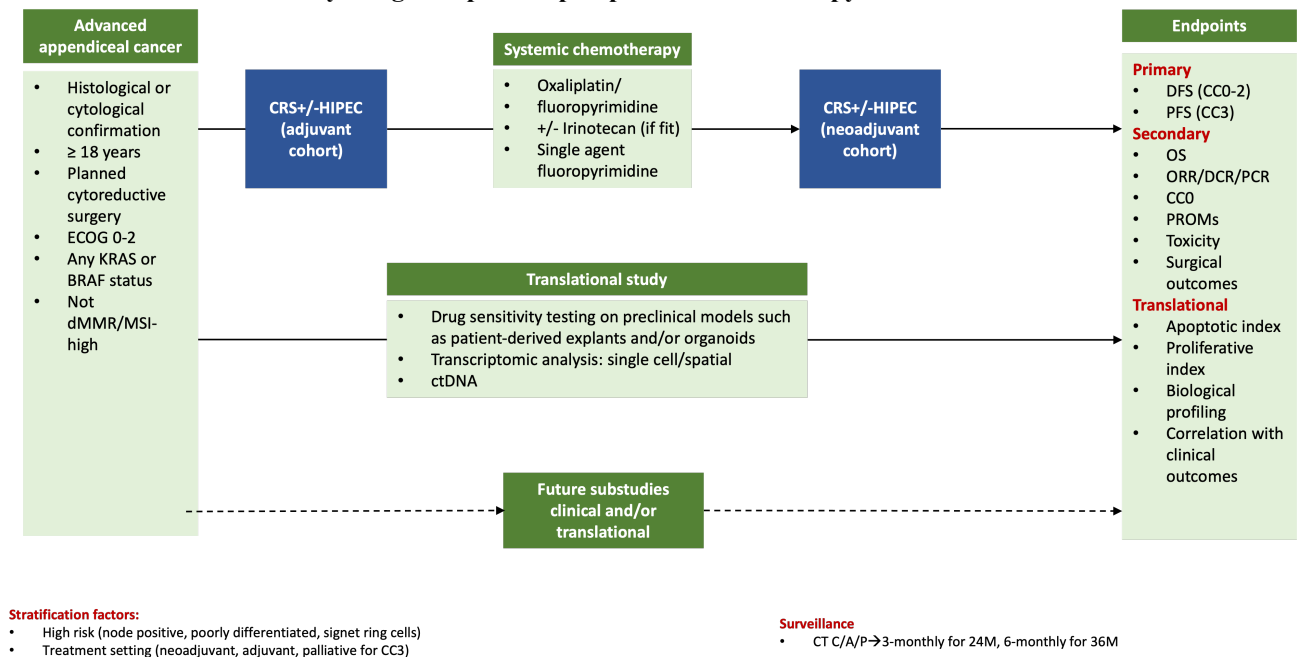


Figure 6.1 | Schema for phase 2 prospective adjuvant or neoadjuvant systemic chemotherapy study

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This study design was conceptualised for discussion at the Australia and New Zealand Peritoneal Malignancy Research Planning Meeting and for review by the collaborative research group Australian Gastro-intestinal Trials Group (AGITG). Competitive funding sources are being sought. International collaboration with peritoneal centres in the UK (Basingstoke and The Christie) and the US (MD Anderson) is planned.

Precision medicine approaches are essential for rare cancers. A high proportion of patients with ATPD have a genomic alteration.^{118, 119, 214, 226} In recent years, personalised molecular profiling has been introduced as part of the clinical pathway, capturing the range of genomic alterations in a subset of patients in both retrospective cohorts. This should be audited periodically to assess the longer-term clinical outcomes. Additionally, the development of an in-house molecular tumour board comprising expert oncologists, scientists, bioinformaticians, health-care providers and geneticists to directly discuss tumour profiling results, evaluate the validity of the platform used, assess pathogenicity of alterations, evaluate actionability, predict magnitude of benefit and provide a strength of recommendation for guidance to relevant early phase clinical trials or drug access pathways, is crucial in rare tumour clinical pathways.⁵⁹²⁻⁵⁹⁴ A molecular tumour board designed specifically for rare tumours like ATPD needs to address the issue of sporadic molecular alterations where evidence for pathogenicity, actionability and magnitude of benefit from any matched therapies is lacking.⁵⁹⁵

6.2 Molecular biology of appendiceal tumour peritoneal disease and future directions

6.2.1 Overview of transcriptomic findings

The single cell atlas of ATPD demonstrates that the key cell types contributing to the TME are mesothelial cells, CAFs, T cells and myeloid cells. There were transcriptomic differences between LG and HG tumour cells as well as between different microenvironments from the spatial transcriptomic analysis. The transcriptomic profile of LG compared to HG ATPD suggested a critical role for abnormal mucin synthesis (*AGR2*) and glycosylation pathways (*GALNT*). In contrast, transmembrane mucins (*MUC16*) appeared to have a greater role in mediating microenvironmental signalling in HG ATPD. Several pro-tumoural pathways were active in LG ATPD, including TNF α , NF- κ B, TGF- β , Wnt/ β -catenin and MAPK signalling. These also appeared to be influenced by the mucinous microenvironment and likely contribute to the reduced effectiveness of conventional anticancer treatments. The aggressive nature of HG ATPD arises from the activity of cell cycle progression pathways (MYC, E2F, G2M), metabolic reprogramming (upregulated oxidative phosphorylation) and epithelial mesenchymal transition that facilitates peritoneal dissemination. The mesothelial cells and CAFs displayed transcriptomic similarities with activity suggesting a role in coordinating innate immunity and facilitating tumour invasion and dissemination via cell adhesion and extracellular matrix remodelling. These interactions lend support to the hypothesis that mucin overproduction is an aberrant and self-perpetuating repair response.

6.2.2 Implications of biological findings and rational therapeutic options

Salient findings from the transcriptomic analysis are displayed in Table 6.1. This outlines the observed pathway and/or cell type profiles and the implications for relevant potential therapeutics.

Table 6.1 | Summary of key pathways and implicated cell types identified from transcriptomic findings and therapeutics

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Biological process and pathways	Evidence from transcriptomic analysis	Potential therapeutics
LG and HG tumour cells		
O-linked glycosylation		
↳ Wnt/β-catenin	- Overexpression of transmembrane mucins (<i>MUC1</i>, <i>MUC16</i>) - Upregulation of GALNTs especially in peri-mucinous tumour cells→ abnormal O-linked glycosylation→truncated mucins - Glandular tumour structure (<i>TEX10</i>→Wnt regulation & stemness) - HG tumour (<i>XACT</i>)	TFF inhibitors Wnt inhibitors Notch/γ-secretase inhibitors Hedgehog/SMO inhibitors
↳ EGFR/Src/PI3KCA/MAPK	<i>KRAS</i>→Upregulated <i>AGR2</i> and - Upregulated <i>TFFs</i>→excess and abnormal mucin synthesis - Abnormal O-linked glycosylation (<i>GALNTs</i>)→Truncated mucins - HG tumour (<i>XACT</i>)	EGFR inhibitors Dasatinib PI3KCA inhibitors KRAS inhibitors
↳ NF-κB	→ MUC2 transcription - Peri-mucinous tumour cells - LG tumour (<i>COL17A</i>)	Aspirin/NSAIDs Corticosteroids Thalidomide Proteasome inhibitors Tacrolimus
LG tumour cells		
TGF-β	Enriched compared to HG tumour cells	TGF-β inhibitors Integrin inhibitors
P53	- Enriched compared to HG tumour cells - Enriched in peri-mucinous tumour cells compared to per-inflammatory tumour cells	P53 antagonists
HG tumour cells		
Cell cycle	Enriched in peri-inflammatory tumour cells (<i>MYC</i>, <i>E2F</i>, <i>G2M</i>) compared to peri-mucinous tumour cells	CDK4/6 inhibitors CDK2 and CDK7 inhibitors
Metabolic reprogramming	Oxidative phosphorylation	Metformin Atovaquone Arsenic Trioxide
Non-tumour cells		
Mesothelial cell	Contributes to innate immunity	Anti-mesothelin antibody
CAF	Peri-mucinous stroma cells (=invasion profile: ECM remodelling, integrins)	Anti-fibroblast antibody
↳ epithelial mesenchymal transition		TGF-β inhibitors
↳ TGF-β	Peri-tumoural stromal cells (=pro-tumoural profile: <i>TGFBR2</i>, <i>IL6ST</i>, <i>CCL14</i>)	Integrin inhibitors

Abbrev. LG, low grade; HG, high grade; GALNTs, N-acetylgalactosaminyltransferase; EGFR, epidermal growth factor receptor; PI3KCA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β, transforming-growth factor beta; NSAID, non-steroidal anti-inflammatory drug; CAF, cancer-associated fibroblasts; CDK, cyclin-dependent kinase.

6.2.2.1 Wnt/B-Catenin pathway inhibitors

The transcriptomic analysis implicated the Wnt/β-catenin pathway in aberrant mucin production (Chapter 3).

Evidence suggests a role for over-expressed transmembrane mucins (MUC1, MUC4, MUC16) in activating canonical Wnt/β-catenin signalling.^{474, 596-598} Upregulation of GALNTs is involved in tumour cell proliferation, invasion and migration thought to be mediated by Wnt/β-catenin induced EMT.^{469, 596, 598}

There are multiple strategies for inhibition of the Wnt/β-catenin pathway, including strengthening the effect of immunotherapies, with multiple agents at various stages of investigation.⁴³⁴ E7386 is an oral CBP/β-catenin inhibitor

that has shown safety in the phase 1 dose escalation (and a partial response in APC mutant small bowel adenocarcinoma) and is currently being investigated in expansion cohorts.⁵⁹⁹

Evidence of crosstalk between Notch, Hedgehog and Wnt pathway signalling opens additional avenues for rational therapeutic targeting.⁶⁰⁰ γ -secretase inhibitors block Notch cell-cell signalling by selective inhibition of γ -secretase-mediated release of the Notch intracellular domain.^{601, 602} Nirogacestat is a γ -secretase inhibitor recently food and drug administration (FDA)-approved for the treatment of desmoid tumours.⁶⁰³ Vismodegib is an oral inhibitor of SMO, part of the Hedgehog pathway, which has a well-established role in treating advanced basal cell carcinoma.⁶⁰⁴ NOTCH3 and SMO inhibition have both been shown to reduce in vitro production of MUC5AC in airway epithelial cells from patients with asthma.^{605, 606} Inhibition of Hedgehog signalling also modulates the TME and can reverse stromal remodelling and cancer-stem-cell like phenotypes.^{607, 608} Thus, γ -secretase and Hedgehog pathway/SMO inhibition may be rational therapeutics to evaluate in preclinical models of ATPD.

6.2.2.2 *SRC/MAPK signalling pathway*

SRC was the most highly differentially expressed single gene on the spatial transcriptomic analysis in LG compared to HG ATPD (Chapter 3). *Src* is a tyrosine kinase with downstream signalling via PI3K and RAS pathways. *SRC* is downstream to the EGFR, which supports mechanistic use of EGFR inhibitors (cetuximab, panitumumab) in KRAS wild type tumours. However, there is limited data evaluating the role of EGFR-inhibitors in ATPD, and no clear evidence of benefit exists.^{119, 163, 165, 177, 178} NF- κ B activation is one of the downstream effects, suppression of which may be a therapeutic opportunity.

Dasatinib is an *Src* inhibitor that has been shown in preclinical models to reduce peritoneal metastasis from ovarian cancer.⁶⁰⁹ However, phase 2 clinical trials have not revealed a signal of activity as a single agent.⁶¹⁰ Dasatinib in combination with Quercetin (a flavonoid from plants) has been established as a method for eliminating senescent cells via inhibition of pro-survival pathways.^{611, 612} Dasatinib, as an *Src* inhibitor and senolytic drug, appears to be a rational therapeutic option for evaluation in ATPD. Given the high proportion of activating KRAS mutations in ATPD, combination with downstream inhibitors could be considered.

An alternative pathway for activating MAPK signalling is by the interaction of TFF proteins with human epidermal growth factor receptor 2 (HER2).⁴⁵⁸ In this analysis, *TFF* genes were identified in the top 10 upregulated genes in ATPD (Chapter 3). This suggests that oncogenic signalling via this pathway may be of relevance. TFF inhibition also presents another strategy to target mucin overproduction. A small-molecule TFF3 inhibitor resulted in reduced tumour growth *in vivo* in colorectal cancer models as well as demonstrating synergy with fluoropyrimidine treatment in cell lines.⁴⁵⁸

6.2.2.3 *Suppression of NF- κ B signalling pathway*

NF- κ B activation was observed in the gene profiles in LG ATPD and peri-mucinous tumour segments (Chapter 3). NF- κ B activation maintains cell survival and in the setting of overactivation is tumour promoting and mediates drug resistance.⁶¹³ It is the culmination of multiple pathways of which TNF- α signalling is one of the canonical

pathways.⁶¹³ Multiple therapies that can suppress NF- κ B signalling including ‘anti-inflammatories’ (aspirin, NSAIDs and dexamethasone), thalidomide, proteasome inhibitors (Bortezomib) and Tacrolimus.⁶¹³ Thalidomide was studied in the adjuvant setting after CRS/HIPEC for CRPM and HG ATPD; but the primary endpoint was not met.⁶¹⁴ Proteasome inhibitors block I κ Ba degradation and suppress NF- κ B⁶¹³ but they also target the unfolded protein response which causes accumulation of misfolded proteins and triggers cell death.⁶¹⁵ As the unfolded protein response in ATPD was shown to be a potential mechanism of mucin production, it is unclear what the impact of proteasome inhibition would be.

6.2.2.4 Cell cycle inhibitors

HG ATPD was associated with cell cycle progression pathways involving *MYC*, *E2F* and *G2M*. Cell cycle progression requires complex orchestration of multiple cellular signalling processes that balance the level of proliferation.^{439, 616} Hyperproliferation of cancer cells was one of the six original Hallmarks of Cancer¹⁶ and evasion of growth suppressors is one of the new generation Hallmarks of Cancer.⁴⁴⁰ Misregulation of the cell cycle is either pro-tumoural, resulting in sustained hyperproliferation or can generate significant genomic instability triggering cell death, the latter a mechanism that can be exploited in therapeutics.⁴³⁹ The mitogenic pathways (*MYC*, *E2F*) promote S phase entry and prevent the cell exiting the cell cycle.^{439, 617} E2F pathway activity has been used to enhance treatments using oncolytic viruses to increase tumour cell lysis or to induce antitumour immunity.^{618, 619}

CDK activity is the main driver of cell cycle progression and CDK inhibitors are associated with cell cycle exit and preventing cell cycle entry.⁴³⁹ Cell cycle inhibition is a promising rationale therapeutic option for further evaluation. *Ex vivo* drug testing experiments were performed on LG and HG ATPD with palbociclib, a CDK4/6 inhibitor (Chapter 5). It was expected that ATPD PDEs treated with CDK4/6 inhibitors would reduce tumour cell proliferation and increase tumour cell death.⁶¹⁶ The results indicated increased tumour cell death for one PDE but paradoxically demonstrated increased tumour cell proliferation (Chapter 5). It is well recognised that cell cycle regulation is complex and the interplay with oncogenic signalling pathways may impact responses.^{616, 620, 621} The interaction between MAPK pathway signalling and CDK4/6 inhibition is highly relevant. Studies in prostate cancer cell lines revealed that acquired CDK4/6 resistance was associated with activated MAPK signalling, although mitogen-activated extracellular signal-regulated kinase (MEK) inhibition could overcome this.^{620, 621}

A single case report described prolonged stable disease (at least 57 months) to palbociclib treatment for a patient with LG ATPD who was identified to have a *CCND1* amplification on blood cell-free DNA.⁴⁹⁴ A recent study presented at an international conference, reported using an *ex vivo* tumour slice model to test CDK4/6 inhibition on tissue from 18 patients with *GNAS* mutant peritoneal disease from gastrointestinal tumours (the primary tumour type was not reported) with findings of a reduction in tumour cell proliferation comparing mucinous to non-mucinous tumours.⁴⁹⁵ Furthermore, they designed a single arm prospective study that treated a similar cohort of patients with single agent palbociclib and 81% had at least a 10% reduction in CEA which, although not a typical endpoint, suggests a possible signal that warrants further exploration.⁴⁹⁵

This highlights that an ‘avatar’ PDE preclinical platform should be informed by tumour mutation testing to guide rational selection of *ex vivo* anticancer agents. ATPD provides a unique opportunity for neoadjuvant study of candidate drugs to evaluate the clinical, radiological and pathological response. A PDE preclinical platform may also provide biological *in vivo* endpoints that provide pharmacodynamic effects and identify potential biomarkers to predict treatment response. A schema for a proposed neoadjuvant study of CDK4/6 inhibition is shown in Figure 6.2. This could be adapted to be the design for other novel treatments of interest.

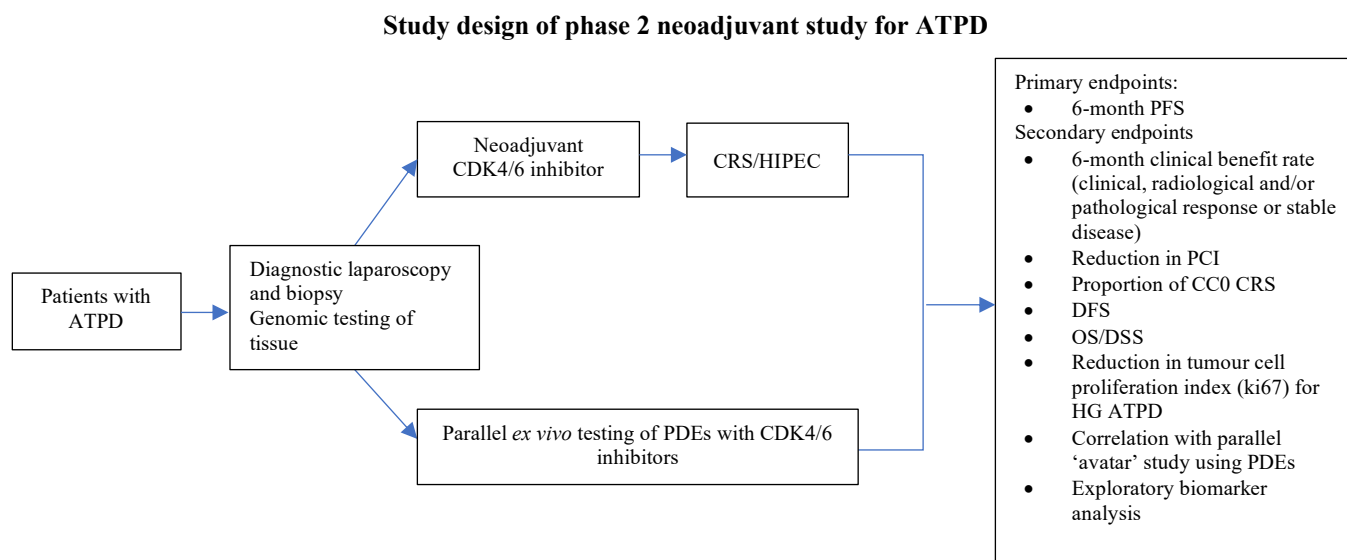


Figure 6.2 | Study schema for phase 2 single arm prospective neoadjuvant study of CDK4/6 inhibitor in ATPD

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Abbrev. ATPD, appendiceal tumour peritoneal disease; CDK, cyclin-dependent kinase; CRS, cytoreductive surgery; HIPEC, heated intraperitoneal chemotherapy; PDE, patient-derived explant; PFS, progression-free survival; PCI, peritoneal cancer index; CC, completeness of reduction score; DFS, disease free survival; OS, overall survival; DSS, disease-specific survival; HG, high grade.

6.2.2.5 Mesothelial cell modulation

Mesothelin is a protein found on normal mesothelial cells, but has also been found on a number of solid tumours.^{622, 623} It belongs to a group of proteins though to be involved in cell signalling and cell adhesion.^{242, 624, 625} It commonly interacts with MUC16 (CA125) and is expressed in the mesothelial populations in ATPD.^{242, 625} It is implicated as a potential mechanism of peritoneal dissemination via the Wnt pathway.^{242, 625} The resulting activation of Wnt signalling was implicated in secreted mucin production via upregulation of *MUC1* in LG ATPD tumour cells (Chapter 3). Furthermore, studies have demonstrated constitutive Wnt signalling is associated with expression of mesothelin.⁶²⁶

Several mesothelin-specific immunotherapies have been developed.⁶²⁷ While these therapies are aimed at targeting tumour cells, targeting mesothelial cells in the peritoneal microenvironment could have ATPD-modulating effects. Anti-mesothelin therapies are another rationally-derived therapeutic option which may have a more unique role in targeting the microenvironment rather than the tumour. Therapeutic targeting of the non-immune elements of the TME include therapies also able to target CAFs, integrin signalling, fibrosis and the extracellular matrix.⁶²⁸

CAFs were identified in the TME of LG and HG ATPD and shared transcriptomic similarities with the mesothelial cells. They exhibited similar gene profiles suggesting extracellular matrix remodelling that may foster tumoural

invasion and dissemination.⁶²⁸ This suggests that the mesothelium plays a similar role to CAFs in the ATPD TME. The enrichment of CAFs with genes associated with epithelial mesenchymal transition suggests the presence of mesothelial-mesenchymal transition. Sandoval *et. al.* proposed a theory that this phenomenon in the peritoneum creates a receptive TME for tumour cell attachment and promotes growth by vascularisation.⁴⁹⁹ A similar mechanism has been described for promotion of ovarian tumour peritoneal dissemination.⁵⁰¹ An analysis of pancreatic cancer CAFs described a mechanism of immune evasion where antigen-presenting CAFs which induced Treg formation.⁶²⁹ Mesothelial cells from LG ATPD expressed an array of transmembrane mucin genes along with upregulation of intracellular signalling pathways indicating a role in tumour-stromal crosstalk.⁶³⁰ A future direction of research would be to evaluate these mesothelial and CAF targeting therapies that modulate the TME in the ATPD PDE models.

The potential role of TGF- β in ATPD is complex when considering how TGF- β inhibition may impact on mucin production. Data exists in the asthma literature which can provide some guidance. The putative link between ATPD and asthma disease processes is the Th2 allergic phenotype where IL-13 upregulates MUC5AC in airway cells via MAPK/MEK/ERK/STAT6 signalling pathways.^{631, 632} These pathways are often constitutively active in ATPD due to frequent *KRAS* mutations.^{118, 119, 214, 226} Increased TGF- β levels have been observed in bronchoalveolar lavage fluid from patients with asthma. One study found that exogenous TGF- β 2 reduced MUC5AC and MUC5B production in primary bronchial epithelial cells as a model for asthma.⁶³³ However, another study found that TGF- β 3 promotes increased MUC5AC secretion by an autophagy pathway.⁶³⁴

Autophagy is a process of cellular recycling critical for homeostasis. The process involves a membrane called a phagophore which encloses cytoplasm into a double membrane vacuole called the autophagosome.^{43, 635} Autophagy has been implicated as a pathway resulting in mucin secretion in airway and intestinal goblet cells.^{43, 636} ATPD could be explained by deregulation of autophagy pathways causing abnormal accumulation of mucin. Evidence from this analysis was upregulated tumoural TGF- β and MAPK signalling as well as activation of extracellular matrix remodelling by CAFs (Chapter 3). TGF- β signalling has pleiotropic tumour-promoting effects on the TME, specifically in the development of CAFs.^{637, 638} CAFs in turn generate TGF- β to manipulate the extracellular matrix and facilitate tumour cell migration.^{637, 638} The excessive TGF- β signalling as a result could trigger autophagy, potentiating the ongoing accumulation of mucin. Furthermore, this autophagy processes may be an important mechanism of resistance to *KRAS* inhibitors, relevant for any potential studies in ATPD.^{639, 640}

6.2.3 Immunological states in ATPD

ATs are not conventionally thought of as ‘hot’ tumours. The transcriptomic analysis of the immune microenvironment provided key insights into the disease. Tumour immunoediting is a concept that explains the interaction between the developing tumour and the immune defence.^{517, 641} The three phases of elimination, equilibrium and evasion describe the how the immune system shapes a pro-tumoural environment and tumour immune evasion.^{517, 641} ATPD was identified to have an immunosuppressive immune microenvironment, representing a state of immune evasion, with the presence of pro-tumoural CD4 Th2 T cells, Tregs and immune-suppressive peritoneal myeloid subsets (M2 macrophages, tissue resident macrophages, neutrophils and myeloid-derived suppressor cells; Chapter 4).

6.2.3.1 Targeting M2-polarised tumour associated macrophages

M2 macrophages were present in LG and HG ATPD (Chapter 4). M2 macrophages are associated with a tumour promoting microenvironment with ample evidence of mediating drug resistance through a variety of mechanisms.⁶⁴² Two of these mechanisms include Notch signalling and NF-κB pathway. Inhibition of these pathways may counteract M2 induced tumour promotion and drug resistance. Methods of directly targeting M2 macrophages include: (1) inhibition of macrophage recruiting chemokines including colony stimulating factor 1 (CSF-1), (2) Macrophage-mediated drug delivery of nanodrugs whereby the tumour-associated macrophages are recruited to the tumour sites and release nanodrugs into the tumour, and (3) repolarisation to M1 macrophages by inhibition of the CSF-1 receptor.⁶⁴²

6.2.3.1 Tertiary lymphoid structures

Infiltrating T cells, myeloid cells and B cells have all been associated with modulating responses to immunotherapy, with development of multi-specific antibodies designed to manipulate this.⁶⁴³ Specifically, B cells in the form of tertiary lymphoid structures (TLS) have capacity for *in situ* humoral anti-tumoural immunity. Thus, the presence of mature TLS in ATPD tissues, particularly in LG disease (Chapter 4), is promising for immunotherapeutic strategies.³¹¹ A future avenue for further research will be to correlate clinical outcomes with the presence of these infiltrates.

6.3 Patient-derived explants as an *ex vivo* model of the appendiceal tumour peritoneal disease microenvironment for application of rational therapeutic drug evaluation

Translational research platforms using patient-derived tumour models facilitate rare cancer research.³²⁵ They provide a mechanism for personalised therapy and the basis to drive novel drug development and biomarkers to better select patients for existing therapies. They help to focus clinical trial efforts on the most rationally derived drugs i.e. those that exhibit robust mechanistic and preclinical responses. PDEs have key advantages compared to other 3D models. These include: (1) maintenance of the cell types and spatial architecture of an intact TME, (2) representation of tumour heterogeneity, and (3) resource-efficient set up and timely turn-around relevant for patient management (Chapter 5). The optimised PDE model for studying the biology and *ex vivo* anticancer drug responses of ATPD has considerable utility as an ‘avatar’ platform. This is where PDEs are generated from patients at clinical decision-making time-points (e.g. at the time of tumour progression) and are exposed to a drug screen to help inform the choice, sequence and combination of anticancer drugs. The schema illustrated previously in Figure 6.1 shows a parallel translational ‘avatar’ study.

6.3.1 Future directions

CRS provides a good opportunity for evaluating intra-tumour heterogeneity of ATPD. Tumour tissue can be selected from spatially separated tissue samples from primary and metastatic sites and evaluated using immunohistochemistry,

mutation and transcriptomic analysis. Furthermore, neoadjuvant studies can be performed with biological and clinical endpoints to evaluate the therapeutic strategy (Figure 6.2).

While the optimised ATPD PDE model has significant utility for performing *ex vivo* drug testing and multi-omic interrogation, there are a number of innovations in biotechnology that strive to even more closely replicate the TME.⁶⁴⁴⁻⁶⁴⁶ Bioprinting techniques allow fabrication of tumour models using cell-imbued biomaterials that deposit 'bioink' in a pre-designed structure.⁶⁴⁴ They were demonstrated to self-organise and interact as well as being amenable to drug testing.⁶⁴⁴ Organ-on-a-chip methods combine microfluidics and 3D models into a device designed to better simulate the dynamic TME, used for drug screening and investigation of mechanisms of tumour dissemination.⁶⁴⁵ The organ-on-a-chip method was taken further by some to develop multi-organ systems such as cancer cells, healthy cells, intestinal and connective tissue models.⁶⁴⁶ A similar platform could be designed to circulate heated topical chemotherapy to mimic HIPEC administration.

6.4 Conclusion

This multifaceted research project studying appendiceal tumours provides key insights into a unique and complex disease. It encompassed (1) the literature assessing systemic chemotherapy (Chapter 1 and [Appendix 1.1](#)), (1) the clinical outcomes from patients in two parallel cohorts in Australia (Chapter 2) and in the UK ([Appendix 1.2](#)), (3) a comprehensive single cell atlas and spatial transcriptomic profile of TME of ATPD (Chapter 3 and Chapter 4), and (4) optimised a PDE model of ATPD for *ex vivo* drug testing, and (5) discussed the implications of the key findings for a rational therapeutic approach (Chapter 5). Findings from this study advance the understanding of the biology and identify further avenues for research including: (1) clinical study design for phase 2 systemic chemotherapy trials in neoadjuvant and advanced disease settings, (2) establishing dedicated precision oncology programs to facilitate management of patients with ATPD and (3) hypotheses regarding the role of abnormal mucin production, mesothelial cell influence and immunological components for further functional testing. Overall, these findings may be used to identify clinically relevant biomarkers and new therapeutic targets. Rationally derived treatment strategies based on these findings include Wnt/ β -catenin, SRC/MAPK, NF- κ B, TGF- β and cell cycle inhibitors. Furthermore, therapies modulating mesothelial cells and immune modulators targeting T cells and myeloid populations are of interest. 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 are justified. Ultimately, this research project yielded several rational directions for future research to improve outcomes for patients who have ATPD.

Chapter 7. Reference List

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