Chapter 8. Appendices

8.1 Appendix 1: Reprints of published papers

Appendix 1.1

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

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Review

The role of chemotherapy in the treatment of advanced appendiceal cancers: summary of the literature and future directions

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***Abstract*:** Appendiceal cancer is rare and encompasses a diverse group of tumours ranging from low-grade appendiceal mucinous neoplasms to high-grade adenocarcinomas. Appendiceal cancers often spread to the peritoneal cavity causing extensive mucinous dissemination and peritoneal metastases. Prognosis varies with histological subtype. Cytoreductive surgery and heated intraperitoneal chemotherapy is well-established as the most effective treatment achieving long-term survival in some patients. Chemotherapy regimens used to treat appendiceal cancer are extrapolated from the colorectal cancer setting, but disease biology differs and outcomes are inferior. The role of chemotherapy in the treatment of appendiceal cancer remains poorly defined. There is an urgent need to develop novel tailored treatment strategies in the perioperative and unresectable setting. This review aims to evaluate the literature for patients who received intraperitoneal and systemic chemotherapy for appendiceal cancers.

Keywords: appendiceal cancer, appendix cancer, chemotherapy, HIPEC, perioperative treatment, treatment outcomes

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Introduction

Appendiceal cancer is rare with an incidence of 1–2 per million and is listed by the National Organisation for Rare Diseases.[1–3] Appendiceal epithelial neoplasms encompass a diverse group of tumours ranging from low-grade appendiceal mucinous neoplasms (LAMNs) to high-grade adenocarcinomas.[4,5] The most common pattern of spread for appendiceal cancers is the peritoneal cavity causing extensive mucinous dissemination and peritoneal metastases.[4]

Cytoreductive surgery and heated intraperitoneal chemotherapy (CRS-HIPEC) are the most effective treatments, but prognosis varies with histological subtype. Five-year survival ranges from 96% for low-grade disease to 23% with high-grade disease.[6–12] Although low-grade disease confers a better 5-year survival, long term it still recurs in up to 30% of patients, many of them will die from the disease.[13]

It is imperative in all disease settings to define therapeutic strategies that can meaningfully improve outcomes. The role of perioperative chemotherapy in the multidisciplinary setting is a dilemma. Chemotherapy regimens are extrapolated from the colorectal cancer (CRC) literature. Although some data are within CRC-directed studies, there has been increasingly homogenous data published specifically for appendiceal cancer populations over recent years.[14,15] Furthermore, distinctions have emerged between appendiceal and CRC with regard to the biological understanding of peritoneal disease and subsequent outcomes.[16–21]

This review aims to evaluate the literature for patients who received chemotherapy for

appendiceal cancers with peritoneal disease, with previous reviews focused primarily on surgical outcomes or were written prior to the publication of more recent studies using the updated 2019 WHO classification with limited discussion regarding the specific role of systemic chemotherapy.[22–24] We have excluded goblet-cell adenocarcinomas as treatment paradigms are generally similar to CRC, and the subgroup with neuroendocrine features is beyond the scope of this review. We have discussed evidence for the specific contribution of HIPEC in addition to CRS, the role of perioperative systemic chemotherapy and the outcomes of chemotherapy in the unresectable setting.

Although there is a need for prospective randomised trials paired with translational studies to improve biomarker and therapeutic advances, we demonstrated that despite significant limitations and challenges that exist in interpreting this literature, an evidence-based and rational therapeutic approach can still be employed. We emphasised that treatment decisions should be made by a multidisciplinary team at centralised, high-volume treatment centres.

Classification of appendiceal cancers

Over many years, the nomenclature of appendiceal cancer has been adapted to homogenise a complex and inconsistent histopathological classification.[5,25–27] A further challenge is the discordance between the primary and peritoneal tumour grade.[7,28,29] It is now convention to describe the primary and peritoneal disease separately.

The term 'pseudomyxoma peritonei' or 'PMP' describes the clinical syndrome of abdominal mucinous disease that can arise from both benign and malignant conditions.[4] It is a broad term that fails to capture the heterogeneous biology of the underlying histopathological diagnosis and associated tumour biology.[28]

The classification systems developed sequentially by Ronnett *et al.*,[26] Misdraji *et al.*[28] and Bradley *et al.*[30] are summarised in Table 1. These proposed classifications systems are used inconsistently,[31,32] causing confusion for clinicians and making it impossible to compare clinical outcomes.[4] The Peritoneal Surface Oncology Group International (PSOGI) 2016 classification was achieved by international consensus in a modified Delphi process at the world congress in Berlin, 2012.[27] This nomenclature is summarised in Table 2 and classifies peritoneal disease as low or high grade.

Table 1. Historical classification of appendiceal epithelial neoplasms.

Ronnett *et al.*[26]	Misdraji *et al.*[28]	Bradley *et al.*[30]
DPAM	LAMN	Low-grade
PMCA I/A PMCA	Mucinous adenocarcinoma	High-grade

DPAM, disseminated peritoneal adenomucinosis; LAMN, low-grade appendiceal mucinous neoplasm; PMCA, peritoneal mucinous carcinomatosis; PMCA-I/D, PMCA with intermediate or discordant features.

The current gold standard World Health Organisation (WHO) 2019 fifth edition refines this further (Table 3), combining the PSOGI nomenclature and including the tiered-grading used in the American Joint Committee on Cancer of G1, well-differentiated; G2, moderately differentiated and G3, poorly differentiated.[24,33,34]

We reconcile the heterogeneous classification used throughout the literature by describing the primary and peritoneal disease in both contemporary and historic nomenclature (i.e. that originally employed by the author) for clarity of data interpretation. We will also use the terms low-grade disease to refer to primary appendiceal mucinous neoplasms (AMNs) and high-grade disease to refer to primary appendiceal adenocarcinomas.

Methods

Key clinical databases (PubMed/MEDLINE, Scopus, Science Direct, OVID and Google Scholar) were extensively searched through 9 September 2021 with the search terms: appendix/appendiceal, cancer/carcinoma/neoplasm/malignancy and chemotherapy (systemic/intravenous, intraperitoneal/HIPEC, neo/adjuvant, peri/pre/postoperative).

Table 2. Peritoneal Surface Oncology Group International (PSOGI) 2016 classification of appendiceal epithelial neoplasms.[27]

Histological type	Features
LAMN HAMN	Mucinous neoplasm without infiltrative invasion but with any of the following: loss of muscularis mucosae, fibrosis of submucosa 'Pushing invasion' (expansile or diverticulum-like growth), dissection of acellular mucin in the wall, undulating or flattened epithelial growth, rupture of appendix, mucin and/or cells outside appendix.
Mucinous adenocarcinoma	Mucinous neoplasm with infiltrative invasion (40% of all appendiceal adenocarcinomas).
Poorly differentiated (mucinous) adenocarcinoma with signet ring cells	Signet ring cells present <50% of the cells in adenocarcinoma.
Mucinous signet ring cell carcinoma	Signet ring cells present >50% of the cells in adenocarcinoma.
Nonmucinous adenocarcinoma	Nonmucinous adenocarcinoma resembling usual colorectal type.

HAMN, high-grade appendiceal mucinous neoplasm; LAMN, low-grade appendiceal mucinous neoplasm.

Table 3. 2019 WHO classification of appendiceal epithelial neoplasms.[33]

Histological type	Definition	Subtype
Appendiceal mucinous neoplasms	Mucinous neoplasms are characterised by mucinous epithelial proliferation with extracellular mucin and pushing tumour margins.	None
Appendiceal adenocarcinoma	Malignant glandular neoplasms characterised by invasion.	A – signet-ring cell adenocarcinoma, B – mucinous adenocarcinoma, C – carcinoma, undifferentiated, not otherwise specified.
Appendiceal goblet cell adenocarcinoma	These are an amphicrine tumour composed of goblet-like mucinous cells, as well as variable numbers of endocrine cells and paneth-like cells, typically arranged as tubules resembling intestinal crypts.	None
Appendiceal neuroendocrine neoplasms	Neoplasms with neuroendocrine differentiation.	A – neuroendocrine tumours, B – neuroendocrine carcinomas.

LAMN, low-grade appendiceal mucinous neoplasm; HAMN, high-grade appendiceal mucinous neoplasm.

Results

A total of 65 articles were found to be relevant for this review: 33 evaluating the role of HIPEC (Table 4) and 42 evaluating systemic chemotherapy (Table 5), each including 10 articles that reported on both.[6,8–10,12,14–16,21,26,35–91] Table 6 is a summary of the characteristics of the literature included in this review.

Table 4. Literature review of outcomes of HIPEC in addition to CRS for appendiceal cancer with peritoneal disease.

#	Author	Study design	Primary tumour	Population	Agents/techniques	mOS	mPFS/DFS	Comments
Survival outcomes reported								
1	Kusamura *et al.*[35]	Retrospective registry study with propensity weighted analysis, 1993–2017	PMP (LG, HG)	1924 (1548 CRS/HIPEC, 376 CRS) Propensity adjusted (300 CRS-HIPEC, 305 CRS)	MMC Ox + 5FU/LV Cisplatin + MMC Coliseum *versus* closed ±EPIC	5 years OS CRS/HIPEC 58% CRS 46% CRS-HIPEC *versus* CRS HR 0.65 (0.50–0.83)	NR	Subgroup analysis no benefit for MMC HIPEC
2	Garach *et al.*[36]	Retrospective study of prospective database, 1996–2020	Mucinous *versus* no mucinous	315 (preop) Acellular mucin 28 (4) LG AMN 209 (33) HG/SRC AMN 55 931) MAC 23 (16) Nonmucinous ANOS 12 GCA 9 MANEC 2	MMC Cis + MMC Cis Cis + Dox	Nonmucinous 24 m (5 years 14%) Mucinous 160 m (5 years 74%)	Nonmucinous 11 m (5 years 13.6%) Mucinous 41.2 (5 years 40%)	Mucinous *versus* nonmucinous $p < 0.001$ Univariate analysis cis + MMC *versus* MMC *versus* other, no difference
3	Byrne *et al.*[37]	Retrospective database, 2004–2014	Appendiceal cancers	18,055 CRS/HIPEC 7% Surgery + CT 32% CT only 3%	NR	5 years Mucinous WD 69% MD 55% PD 28% Nonmucinous WD 79% MD 58% PD 32% 5 years OS CRS/HIPEC: 66% Surgery alone: 52% ($p < 0.01$)	NR	Missing data for grade Assumptions re surgery + HIPEC
4	Levine *et al.*[38]	Multicentre randomised control trial, 2009–2015	Mucinous appendiceal neoplasms	121 MMC (61) HG 32%; LG 68% Ox (60) HG 26%, LG 74%	MMC Ox	3 years OS MMC 84% Ox 87%	3 years DFS MMC 67% Ox 65%	QoL improved Ox > MMC
5	Gupta *et al.*[39]	Retrospective cohort, 2013–2015	Colorectal and appendiceal peritoneal metastasis	33 – appendix (13)	Dox + MMC + 5FU Ox + 5FU + EPIC	4 years OS CRS/HIPEC (26) 58% CRS alone (3) 33% ($p = 0.30$)	NR	CRS alone group abandoned HIPEC due to >CC1
6	Wu *et al.*[40]	Retrospective review of consecutive cohort, 2008–2015	GI and Gyne Ca	100 (13 PMP)	Lobaplatin + docetaxel ± adjuvant FOLFOX or FOLFIRI	24 m (15–33) 5 years: 19.8%	NR	PMP median OS not reached

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Table 4. (Continued)

#	Author	Study design	Primary tumour	Population	Agents/techniques	mOS	mPFS/DFS	Comments
7	Ihemelandu and Sugarbaker[41]	Retrospective of prospective database, 1989–2012	PMCA	494 IP chemo PMCA 2010 PMCA-S 45 PMCA-A 30	MMC IV 5FU (2010 + Dox)	Median (5 years) PMCA: 45.4 (38%) PMCA-S: 22 (18.9%) PMCA-A: 26.8 (15%)	NR	Cox regression no IP chemo *versus* IP chemo HR 1.4 [1.0–2.0] ($p = 0.015$)
8	Shaib *et al.*[42]	Retrospective database, multicentre, 1990–2010	AMN	163 (60 DPAM, 88 PMCA, 15 PMCA I/D) Complete CRS 76 HIPEC 79	MMC	HIPEC 77 m No HIPEC 25 m ($p < 0.01$)	NR	Multivariable analysis HIPEC independent predictor of improved OS
9	Glockzin *et al.*[43]	Retrospective database, 2007–2010	Colorectal and appendiceal adenocarcinoma	32 CC0/1	Ox (20) Iri (12)	3 years All 56.3% Ox 65% Iri 41.7% ($p = 0.295$)	NR	
10	Marcotte *et al.*[44] Marcotte *et al.*[45]	Prospective database, 2003–2011	PMP	78 (DPAM 24%, PMCA I 53%, PMCA 23%) 58 HIPEC	Ox	5 years 66% HIPEC 5 years 77%	HIPEC 5 years 50%	
11	Austin *et al.*[46]	Retrospective review, 2001–2010	Peritoneal carcinomatosis from appendiceal adenocarcinoma	282 (36% HG)	MMC	6.7 years 5 years: 52.7%	1.8 year 5 years 45.1%	
12	Chua *et al.*[47]	Retrospective review of prospective database, 1997–2010	Appendiceal adenoca (21 WD, 19 MD, 6 PD)	46 (38 HIPEC, 40 EPIC, 34 both)	MMC @42C 5FU	56 m HIPEC 65 EPIC 56 Both 65	mDFS 21 m HIPEC 25 EPIC 23 Both 26	EPIC and HIPEC influence on OS on univariate analysis, not on multivariate
13	Youssef *et al.*[8]	Retrospective review of prospective database, 1994–2009	PMP	465	MMC	Mean OS 11.8 years 5 years: 70%	Mean DFS 9.2 years	
14	Elias *et al.*[6]	Retrospective review, 1989–2007	Peritoneal carcinomatosis	615 (41 appendix)	MMC ± Cis Ox + 5FU/LV ±Iri	Appendix: 89 m 5 years: 63%	5 years DFS:18%	
15	Chua *et al.*[48]	Retrospective review of prospective database, 1997–2008	PMP	106 (73 DPAM, 22 PMCA-I, 11 PMCA)	MMC + 5FU	104 m 5 years: 75%	40 m 5 years: 38%	

(Continued)

Table 4. (Continued)

#	Author	Study design	Primary tumour	Population	Agents/techniques	mOS	mPFS/DFS	Comments
16	Baratti *et al.*[12]	Retrospective review of case series, 1996–2007	PMP	104 (95 CRS/HIPEC)	MMC + Cis	5 years: 71.9% CRS/HIPEC: 79.8%	5 years: 38.8% CRS/HIPEC: 42.6%	
17	Gusani *et al.*[49]	Retrospective review of case series, 2002–2005	Peritoneal malignancies	122 (appendiceal/PMP 39%, DPAM 36%, PMCA I/D 4.3%, PMCA 60%)	MMC	26.2 m 2 years: 51.1% Appendix: median not reached LG 2 years: 85.7% HG 2 years: 51.8%	NR	Morbidity G3/4 30%
18	Levine *et al.*[16]	Retrospective review of prospective database, 1991–2006	Peritoneal malignancies	460 (45 appendix)	MMC	22.2 m 5 years: 27.8% HIPEC improved OS $p = 0.006$	NR	
19	Smeenk *et al.*[50]	Retrospective review, 1996–2004	PMP	103 (DPAM 66, PMCA I/D 29, PMCA 7)	MMC	5 years: 59.5%	5 years DFS: 37.4% Recurrence rate 44%	
20	Stewart *et al.*[51]	Retrospective review of prospective database, 1993–2004	Appendiceal neoplasms with peritoneal disease	110 (55 DPAM, 18 PMCA-I, 29 PMCA, 8 HG nonmucinous lesions)	MMC	5 years: 53%	NR	
21	Hadi *et al.*[52]	Retrospective review of prospective data, 1996–2004	Peritoneal malignancy	60 (23 appendiceal) 34 HIPEC	MMC Ox + 5FU +EPIC @41–42C	3 years OS HIPEC 71% No HIPEC 28% ($p = 0.03$) 3 years OS appendiceal ca 74%	NR	Mortality 6.7% Morbidity 39% IV MMC changed to IP MMC in December 2001
22	Moran *et al.*[53]	Retrospective review of prospective database, 1994–2002	Peritoneal malignancy	100 (85 appendix) 65 complete cytoreduction (48 LAMN, 6 appendix adenoca) 28 debulking (25 appendix) 7 inoperable (6 LAMN)	MMC + 5FU	Median not reached 5 years: 72% Complete cytoreduction Palliative debulking: 25 m	NR	Mortality 6.2%
23	Güner *et al.*[54]	Retrospective review, 1995–2003	PMP	28 Cytoreduction Complete (11) Incomplete (17) Low volume (6) High volume (22)	Cis (15) MMC (6) 5FU (7)	51 m 5 years: 80% Cytoreduction - Complete: 73 m Incomplete: 26 m Unresected: 12 m Low volume: 78 m High volume: 37 m ($p = 0.05$)	NR	No OS difference MMC *versus* Cis ($p = 0.23$) Low volume = PCI < 15

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Table 4. (Continued)

#	Author	Study design	Primary tumour	Population	Agents/techniques	mOS	mPFS/DFS	Comments
24	Deraco *et al.*[10]	Prospective phase 2 trial, 1996–2003	PMP	33 (28 DPAM, 5 PMCA-I)	Cis + MMC	5 years: 96%	5 years: 43%	Excluded 4 PMCA, 1 DPAM for high volume disease
25	Van Ruth *et al.*[55]	Retrospective review of prospective case series, 1996–2002	PMP	62 (38 DPAM, 24 PMCA-I/D)	MMC +adj 5FU/LV for PMCA-I/D	48 m 3 years:67% 5 years: 38% DPAM 3 years: 89% PMCA I/D 3 years: 37% ($p = 0.0002$) Atypia: minimal *versus* moderate/marked ($p = 0.026$) Focal proliferation ⩽1 *versus* >1% ($p = 0.0008$)	DFS 33 m 3 years: 43% DPAM 3 years: 44% PMCA-I/D 3 years: 41% ($p = 0.22$)	Mortality 3% 2 acellular mucin = DPAM No DFS difference in cellularity, atypia or proliferation No OS diff in cellularity
26	Witkamp *et al.*[56]	Prospective case series, 1996–2000	PMP	46	MMC Malignant PMP +adj 5FU/LV (22)	3 years: 81%	Mean DFS 13 m	
27	Ronnett *et al.*[57] Ronnett *et al.*[26]	Retrospective case series, 1983–1993	PMP or mucinous adenocarcinoma	109	MMC EPIC MMC/5FU Adj IV MMC/IP 5FU	DPAM 5 years 75% 10 years 68% PMCA I/A 5 years 50% 10 years 21% PMCA 5 years 15% 10 years 3%	NR	
28	Sugarbaker and Chang[58]	Retrospective case series, 1989–1999	Appendiceal peritoneal disease	385 (224 adenomucinosis, 161 hybrid + mucinous adenoca)	205 MMC (>1997) 3x12xadj IP 5FU 21 EPIC + 3x IP 5FU + MMC 156 EIPC + 3x IP 5FU + IV MMC	5 years: complete cytoreduction + adenomucinosis: 86% hybrid + mucinous adenoca: 50% ($p = 0.0001$) Incomplete cytoreduction: 20%	NR	Mortality 2.7%
29	Gough *et al.*[59]	Retrospective review, 1957–1983	PMP (appendix 52%, ovary 34%)	56 IP chemo 13% IV chemo 27%	5FU Cyc	5.9 years 5 years 53%	IP chemo *versus* no IP chemo ($p = 0.059$) Incomplete surgery IP chemo *versus* no IP chemo ($p = 0.009$) Recurrence rate 76%	Also used intracavitary radiation

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Table 4. (Continued)

#	Author	Study design	Primary tumour	Population	Agents/techniques	mOS	mPFS/DFS	Comments
Survival outcomes not reported								
30	Cotte *et al.*[60]	Phase 1 Prospective single arm, 2008–2010	Peritoneal carcinomatosis (6 PMP, 2 appendiceal adenoca)	12	Iri + MMC MTD Iri 100 mg/m^2	NR	NR	5 dose levels planned, 3 DLTS at DLT, MTD level 1
31	Elias *et al.*[61]	Prospective phase 2, 2003–2005	Peritoneal malignancies	106 (5 appendix, 41 PMP)	Ox + Iri + 5FU @43C	NR	NR	Morbidity 66% Mortality 4%
32	Kusamura *et al.*[62]	Prospective phase 2, 1995–2004	Peritoneal malignancy	205 (49 PMP)	Cis + MMC Cis + Dox @42.5C Closed abdo	NR	NR	Major morbidity 12% Mortality 0.9%
33	Sugarbaker *et al.*[63]	Retrospective review of prospective database, 1998–2004	Appendiceal cancer	356 (DPAM 59%, PMCA 41%)	MMC @41.5C +EPIC PMCA postop 5FU	NR	NR	Morbidity (G3/4) 40% Mortality 2%

AMN, appendiceal mucinous neoplasm; CC, cytoreductive score; CRS, cytoreductive surgery; CT, chemotherapy; Cyc, cyclophosphamide; DFS, disease-free survival; DPAM, disseminated peritoneal adenomucinosis; EPIC, early postoperative intraperitoneal chemotherapy; 5FU, 5-fluorouracil; HG, high grade; HIPEC, heated intraperitoneal chemotherapy; IP, intraperitoneal; Iri, irinotecan; LAMN, low-grade appendiceal mucinous neoplasm; LG, low grade; LV, leucovorin; M, median; MD, moderately differentiated; MMC, mitomycin-C; NR, not reached; OS, overall survival; Ox, oxaliplatin; PCI, peritoneal cancer index; PD, poorly differentiated; PFS, progression-free survival; PMCA, peritoneal mucinous carcinomatosis; PMCA-I/D, PMCA with intermediate or discordant features; PMP, pseudomyxoma peritonei; adenoca, adenocarcinoma; QoL, quality of life; WD, well-differentiated; y, year.

Table 5. Literature review of systemic chemotherapy for appendiceal cancer with peritoneal disease.

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
Prospective trials								
1	Ramanathan *et al.*[91]	Prospective phase 2	Appendiceal, colorectal, mesothelioma	46 (24 appendiceal) LAMN 7, Mucinous adenoca: MD 8 PD 8	αDC1 vaccine Celecoxib Interferon-α Rintatolimod	Median not reached	LAMN 50.4 m Mucinous adenoca MD 34.2 m PD 8.9 m	Early termination due to futility, slow accrual, grade disparity Poor cell yield to create adequate vaccine dose
2	Raimondi *et al.*[64]	Prospective, 2015–2017	PMP	23 unresectable, relapsed	Metronomic Cap Cyc	1 year 74%	9.5 m	Disease control 27% >12 m
3	Glockzin *et al.*[65]	Prospective phase 2 multicentre single arm, 2010–2014	High-grade appendiceal or colorectal	25 preop (10 appendiceal)	5FU OX Iri CTX	23 m	14.9 m	Early termination due to poor recruitment Preop chemo feasible

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
4	Levine *et al.*[38]	Randomised multicentre control trial, 2009–2015	Mucinous appendiceal neoplasm	121 HIPEC MMC 61 (10% preop) Ox 60 (20% preop) Preop HG 38%, LG 6%	NR	3 years-OS MMC 84% Ox 87%	3 years DFS MMC 67% Ox 65%	Systemic chemo not evaluated
5	Pietrantonio *et al.*[66]	Prospective single arm, single institution, 2014–2015	PMP	Relapsed unresectable 15 (33% HG, 67% LG)	CAP Bev	Not reached 1 year OS 91%	8.2 m (95% CI 5.3–NA)	Noninferior to historical control ORR 20% GNAS 60% assoc with reduced PFS
6	Pietrantonio *et al.*[67]	Single arm prospective observational study, 2011–2013	PMP	Unresectable/ recurrent 20	5FU + Ox	26 m	8 m	ORR 20% (PR) 5 prior cape (3 + MMC) Chemo activity
7	Shen *et al.*[68]	Prospective phase 2 single arm, 2002–2006	Appendiceal and colorectal cancer	27 (14 appendiceal, 13 colorectal)	Postop oral thalidomide	43 m Appendiceal: not reached Colorectal: 30 m	9.3 m Appendiceal: 29 m Colorectal: 7 m	No ORR 18 SD Not conclusive
8	Bijelic *et al.*[69] Sugarbaker *et al.*[70]	Retrospective review of prospective database 2005–2009 Prospective study 2005–2009	PMCA	58 (34 preop, 24 no preop) 34 preop	FOLFOX CAPOX +Bev FOLFOX	preop: 37 m No preop: 51 m ($p = 0.56$) 65% SD on CT 50% progressed intraop 7 PR and 3 CR on path response	NR	Preop showed improved OS in those with complete response ($p = 0.032$)
9	Verwaal *et al.*[71] Verwaal *et al.*[72]	Randomised control trial, 1998–2001	Peritoneal carcinomatosis (appendix 17%, CRC 83%)	105	5FU Iri	Chemo: 12.6 m Chemo + CRS/ HIPEC: 22.2 m ($p = 0.028$)	Chemo: 7.7 m Chemo + CRS/ HIPEC: 12.6 m ($p = 0.02$)	Benefit of CRS/HIPEC in addition to chemo
10	Farquharson *et al.*[73]	Prospective phase 2 single-arm study, 2003–2006	PMP unresectable	40	MMC + CAP	1 year: 84% 2 years: 61%	Not reported	6 tumour reduction 17 stable disease Chemo activity
Retrospective reviews								
11	Kusamura *et al.*[35]	Retrospective registry study, multicentre 1993–2017	PMP	1924 CRS-HIPEC 529 preop, 1019 no chemo CRS 198 preop, 178 no chemo Propensity weighted CRS-HIPEC 149 preop, 152 no chemo CRS 149 NAC T 156 no chemo	NR	5 years OS CRS-HIPEC 58% CRS 46%	NR	Prior chemo HR 1.58 (1.23–2.03, $p < 0.01$)

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Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
12	Garach *et al.*[36]	Retrospective study of prospective database 1996–2020	Mucinous *versus* no mucinous	315 (preop) Acellular mucin 28 (4) LG AMN 209 (33) HG/SRC AMN 55 931) MAC 23 (16) Nonmucinous ANOS 12 GCA 9 MANEC 2	Nonmucinous (23) Ox-based Ox-based + bev Ox-based + panitumumab Iri-based Iri-based + bev	Nonmucinous 24 m (5 years 14%) Mucinous 160 m (5 years 74%)	Nonmucinous 11 m (5 years 13.6%) Mucinous 41.2 (5 years 40%)	Mucinous *versus* nonmucinous $p < 0.001$ Prior systemic chemo no significant on multivariate analysis
13	Kolla *et al.*[74]	Retrospective review single institution 2006–2015	Appendiceal Neoplasms	103 (68 complete CRS, 26/68 Adj chemo)	CAP CAPOX 5FU + OX	OS Non-LG/WD Postop chemo: 9.0 years no chemo: 2.9 years ($p = 0.02$) LG/WD – no difference	RFS Non-LG/WD Postop chemo: 2.6 years No chemo: 1.2 year ($p = 0.09$) LG/WD – no difference	Benefit of postop chemo in high grade
14	Chen *et al.*[75]	Retrospective review 12 centres (US HIPEC Collaborative) 2000–2017	Appendiceal cancer (WD 56%, MD 24%, PD 20%)	803 (225 preop, 578 SF) 24% postop chemo Multivariate analysis $N = 186$ 50% preop	5FU/CAP Ox Iri	Unmatched Preop: 19 m SF: 29 m ($p < 0.001$)	RFS Preop: 12 m SF:20 m ($p < 0.001$)	Preop worse OS: HR 1.81 [95% CI 1.03–3.18], $p = 0.04$ RFS: HR1.93 [95% CI 1.25–2.99] $p = 0.003$
15	Levinsky *et al.*[76]	Retrospective review 12 centres (US HIPEC Collaborative) 1999–2018	Appendiceal adenoca	514 (125 SRC present) Preop: SRC 45.6% Non-SRC 19.2% Postop SRC 52% Non-SRC 15.8%	NR	SRC:32 m Non-SRC: 91 m ($p < 0.001$) (Fig 2d – SRC) Postop ~50 m Preop ~30 m Pre + postop ~25 m No chemo ~20 m ($p = 0.71$)	RFS SRC: 18 m Non-SRC:32 m ($p < 0.001$)	Similar OS regardless of timing of chemo ($p = 0.71$) Multivariate analysis of whole cohort Systemic chemo assoc with worse survival HR 1.98 [1.23–3.19], $p < 0.01$, not signif in SRC
16	Lu *et al.*[77]	Retrospective review of NCDB 2004–2015	Mucinous low-grade appendiceal cancer	639 (431 Chemo)	NR	No association with OS		Excluded HIPEC patients Confounders No difference
17	Munoz-Zuluaga *et al.*[78]	Retrospective review of prospective single institution database 1998–2017	High grade mucinous adenocarcinoma	140 (64 preop, 76 no chemo	Preop 46% FOLFOX 48% FOLFOX-Bev 23% FOLFIRI 3% FOLFOX/ FOLFIRI-BEV 6% 5FU/LV 5% Other 14%	Chemo: 40 m No chemo: 86 m ($p = 0.006$) 3 years 53 *versus* 79% 5 years 38 *versus* 59% 10 years 17 *versus* 38%	Chemo: 19 m No chemo:43 m ($p = 0.007$) 3 years 38 *versus* 56% 5 years 20 *versus* 45% 10 years 10 *versus* 41%	Chemo worse

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
18	Byrne *et al.*[37]	Retrospective NCDB, 2004–2014	Appendiceal cancers	18,055 CRS/HIPEC 7% Surgery + chemo 32% Chemo only 3%	NR	5 years Mucinous WD 69% MD 55% PD 28% Nonmucinous WD 79% MD 58% PD 32%	NR	Signif missing data for grade Assumptions re surgery + HIPEC
19	Grotz *et al.*[79]	Retrospective review of prospective database 2004–2014	Appendiceal adenocarcinoma (MD + PD)	178 (preop chemo) Nonsurgical at DL (70%) 116 CRS + HIPEC (73%)	FOLFOX±Bev	48 m 5 years 41%	mDFS CC0/1 23 m 5 years 16.8%	No difference with periop chemo
20	Cummins *et al.*[92]	Retrospective review of prospective database 1991–2015	High-grade appendiceal or colorectal	165 (110 appendiceal; 92 preop)	NR	18 m Preop: 14.4 m No preop: 20.4 m ($p = 0.01$)	mDFS: 14.4 m ($p = 0.34$)	Predictors of OS: Resection status, LN involvement Chemo worse
21	Wu *et al.*[40]	Retrospective review of consecutive cohort, 2008–2015	GI and Gyne Ca	100 (13 PMP) Preop 47 No preop 53 Adj chemo (30) <6 cycles 49 ⩾6 cycles 51	FOLFIRI FOLFOX	24 m (15–33) Preop 21 m No preop 31 m ($p = 0.13$) Postop chemo <6 cycles 14 m ⩾6 cycles 32 m ($p < 0.001$)	NR	PMP median OS not reached
22	Asare *et al.*[80]	Retrospective, NCDB 1985–2006	Appendiceal cancer	11,871 (stage IV 5049; chemo 51.8% mucinous, 39.8% nonmucinous, 63.9% signet ring 9.2%)	NR	Chemo *versus* no chemo HR [95% CI] Stage IV Nonmucinous all grades 0.73 [0.65–0.83] ($p = 0.0001$) Mucinous WD 6.4 *versus* 6.5 years (p = NS) MD 3 *versus* 1.6 year ($p = 0.0005$) PD 1.6 *versus* 1 year ($p = 0.0007$)	NR	No code for HIPEC, used surgical resection as surrogate. 32% missing grade for mucinous tumours. Benefit of chemo in nonmucinous and mucinous MD and PD
23	Ihemelandu and Sugarbaker[41]	Retrospective of prospective database 1989–2012	PMCA	494 Preop chemo PMCA 152 PMCA-S 38 PMCA-A 35	5FU/cap + OX ±Bev	Median (5 years) PMCA: 45.4 (38%) PMCA-S: 22 (18.9%) PMCA-A: 26.8 (15%)	NR	Cox regression no preop chemo *versus* preop chemo HR 0.7 [0.5–1.1] ($p = 0.17$)

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
24	Milovanov *et al.*[81]	Retrospective or prospective database, single institution 1998–2014	PMCA (≤4 m of dx)	72 (30 preop, 42 no preop) Adj chemo Preop 82% No preop 77% SRC (18 preop, 10 no preop)	FOLFOX 25 CAPOX 2 FOLFIRI 3 +Bev 8 #Cycles Mean 4.4 (3–8)	1 year OS: preop 93% No preop 82% 2 years OS: preop 68% No preop 64% 3 years OS: preop 51% No preop 60% SRC 1 year OS: preop 94% No preop 43% 2 years OS: preop 67% No preop 14%	1 year PFS: preop 78% No preop 67% 2 years PFS: preop 49% No preop 53% 3 years PFS: preop 36% No preop 53%	OS $p = 0.74$ PFS $p = 0.46$ (no significance comparing chemo HG, LG) SRC OS $p = 0.028$
25	Choe *et al.*[82]	Retrospective registry review 2000–2007	Appendiceal epithelial neoplasm	130 unresectable WD (29%) MD (33%) PD (39%) Signet ring (25%)	135 chemo 58 Biologic 5FU (22%) FOLFOX/CAPOX (54%) FOLFIRI/CAPIRI (14%) Bev + chemo (91%) CTX + chemo (8%)	49 m (37–60) WD 68 m MD 56 m PD 27 m PD 29 m (17–37), HR 2.28 (1.02–5.1, $p = 0.04$) Bev 76 m No Bev 42 m HR 0.49 (0.25–0.04, $p = 0.03$)	WD 3 m MD 9 m PD 7 m Bev 9 m No bev 4 m HR 0.69 (0.47–0.997, $p = 0.047$)	20% prior CRS + HIPEC Benefit of bev + combo chemo
26	Votanopoulos *et al.*[83]	Retrospective review of prospective database 1991–2013	Appendiceal epithelial neoplasm	481 procedures (430 patients) LG 77% HG 23%	NR	HG Preop: 17 m No preop 30 m ($p = 0.02$) Adj chemo: 32 m No postop chemo: 6 m ($p = 0.001$) LG Preop: $p = 0.003$ Adj chemo: no effect ($p = 0.88$)	NR	Multivariate preop was predictor of poor OS HG: HR 2.5, $p = 0.006$ LG: HR 2.2, $p = 0.05$ Preop worse Benefit of postop chemo in HG
26	Baumgartner *et al.*[15]	Single-centre, retrospective review 2007–2013	High-grade appendiceal and colorectal adenocarcinoma	70 Appendiceal 19 (41%) Preop 59 (84%) Adj chemo 74% of 46 points	NR	NR	9.7 m Univariate analysis preop: HR 1.67 (95% CI 0.65–4.3, $p = 0.29$) Adj chemo: HR 0.98 (95% CI 0.46–2.12, $p = 0.96$)	46 data for postop chemo Preop/Adj chemo no difference

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
27	Shaib *et al.*[42]	Retrospective database, multicentre, 1990–2010	AMN	163 Periop chemo 78 No chemo 85	5FU based	Chemo *versus* no chemo HR 1.92 (1.14–3.23, $p=0.013$)	NR	Univariate analysis
28	Tejani *et al.*[93]	Retrospective review of NCCN database, 2005–2012	Appendiceal adenoca (44% mucinous, 48% nonmucinous)	99 Metastatic/recurrent (21% WD, 17% MD, 46% PD)	5FU/cap Ox Bev Iri	2.1 years Non-bev *versus* bev HR 1.24 [0.75–2.04] ($p=0.41$)	1.2 year Non-bev *versus* bev HR 1.91 [1.17–3.14] ($p=0.01$)	ORR 39% Worse outcomes: Mucinous PD No debulking Excluded 1L intraperitoneal chemo
29	Kuijpers *et al.*[14]	Retrospective review of prospective database, 2004–2012	pmCRC	73 Appendiceal 4 (5%) Preop 14 Adj 32 Pre and post 9 No chemo 16		Chemo *versus* no chemo 30 *versus* 14, $p=0.015$	Chemo *versus* no chemo 15 *versus* 4, p 0.024	Chemo benefit
30	Blackham *et al.*[84]	Retrospective study, 1997–2011	Appendiceal mucinous carcinoma	393 284 LG Preop: 13 Adj chemo 9 109 HG Preop: 37 Adj chemo: 22 Both: 11	5FU 5FU + Ox 5FU + Iri +Bev +Cetux	LG Periop chemo: 107 m No periop chemo: 72 m ($p=0.46$) HG Preop: 16 m Adj chemo: 36.4 m ($p=0.07$) Both: 17.8 m HIPEC only: 19.6 m ($p=0.14$)	LG Periop chemo: 29.5 m No periop chemo: 37 m ($p=0.18$) HG preop: 6.8 m Adj chemo: 13.6 m ($p<0.01$) Both: 12.9 m HIPEC only: 7 m ($p=0.03$)	LG chemo no diff Postop chemo improved PFS *versus* preop and HIPEC only OS no diff
31	Marcotte *et al.*[44] Marcotte *et al.*[45]	Retrospective review of prospective database, 2003–2011	PMP	78 (DPAM 24%, PMCA I 53%, PMCA 23%) 18 PMCA = chemo	5FU +Iri +Ox	5 years 66% DPMA: 100% PMCA-I: 40% PMCA: 20%	DPMA: 100% PMCA-I:40% PMCA: 20%	Chemo no impact on survival
32	Turner *et al.*[85]	Retrospective review from prospective database, 2005 to 2011	High-grade appendiceal adenocarcinoma	45 (26 preop, 29 postop chemo)	5FU + Ox CAPOX 5FU + IRI +Bev	39 m preop: 22 m No chemo: not reached ($p=0.12$)	NR	Chemo no diff
33	Raghav *et al.*[21]	Retrospective review, 2002–2010	Appendiceal adenocarcinoma	149 (64 G1, 31 G2, 54 G3)	Celecoxib (10) CTX/ panitumumab (29)	53.9 m	NR	No difference in OS in KRAS or COX-2 subgroups treated with CTX/pan or celecoxib

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
34	Jimenez *et al.*[86]	Retrospective study of prospective database 2010–2012	Appendiceal cancer	89 (47 VEGF high expressor)	Postop bev (12/47)	High expressor 1 year: 91% 3 years: 60% 5 years: 47% Low expressor 1, 3, and 5 years: 92% ($p = 0.13$)	High expressor 1 year: 87% 3 years: 42% 5 years: 34% Low expressor 1 year: 100% 3 years: 90% 5 years: NA ($p = 0.11$)	Use of bev no difference between high and low expression
35	Chua *et al.*[9]	Retrospective multi-institutional registry, 1993–2011	Appendiceal PMP	2298 (377 preop, 963 no preop)	Not reported	16 years Preop 5 years: 52% 10 years: 34% No preop 5 years: 77% 10 years: 62% ($p < 0.001$)	8.2 years Preop: not reported	Multivariate preop predicts poor OS (HR 1.7, $p = 0.001$) and PFS (HR 1.91, $p < 0.001$)
36	Lieu *et al.*[87] Kabbani *et al.*[88]	Retrospective review, 1992–2010 Retrospective review, 1995–2000	Poorly differentiated and signet ring appendiceal adenocarcinoma Appendiceal carcinoma (23 mucinous adenoca, 7 nonmucinous adenoca)	442 (106 stage IV, 78 1L chemo) 26 complete CRS (5 preop, 12 postop chemo) 30 (25 chemotherapy)	5FU Ox Iri Bev CTX Carboplatin Cisplatin Paclitaxel doxorubicin	1L Chemo: 20.4 m Periop chemo: HR 0.12 [0.01–1.59] ($p = 0.11$) Mean OS Mucinous 26 m Nonmucinous 13 m ($p = 0.0002$)	1L Chemo: 6.9 m RFS Periop chemo: HR 0.22 [004–1.25] ($p = 0.09$) Mean DFS Mucinous 18 m Nonmucinous 7 m ($p = 0.04$)	ORR 1L chemo 44% (correlates with improved PFS on multivariate analysis $p = 0.02$)
37	Shapiro *et al.*[89]	Retrospective review, 2000–2005	Appendiceal neoplasm suboptimal for surgery (24 WD, 11 MD, 15 PD)	54	5FU CAP Platinum Iri Gefitinib Bev CTX	55.6 m	7.6 m	ORR 24% Activity of chemo
38	Chua *et al.*[47]	Retrospective review of prospective database, 1997–2010	Appendiceal cancer (21 WD, 19 MD, 6 PD)	46 (24 preop, 22 no preop)	5FU Ox Iri Bev	56 m Preop: 34 m No preop: not reached	mDFS 21 m Preop: 11 m No preop: 38 m	Multivariate no chemotherapy showed longer DFS ($p = 0.021$)

(Continued)

Table 5. (Continued)

#	Author	Study design	Primary tumour	Population	Agents	mOS	mPFS	Comments
39	Baratti *et al.*[12]	Retrospective review of prospective database, 1996–2007	PMP	104 (26 preop, 78 no preop)	NR	5 years: 72% ($p = 0.0067$)	5 years: 39% ($p = 0.0026$)	Multivariate preop poor predictor of OS (HR 2.72, $p = 0.034$) and PFS (HR 2.04, $p = 0.045$)
40	Smeenk *et al.*[50]	Retrospective review, 1996–2004	PMP	103 (postop chemo 30, no chemo 73) 12 preop	5FU/LV	5 years: 60%	5 years DFS: 37.4% Recurrence rate 44%	No ORR to chemo
41	Gough *et al.*[59]	Retrospective review, 1957–1983	PMP (appendix 52%, ovary 34%)	56 IP chemo 13% IV chemo 27%	5FU Cyc Melphalan doxorubicin	5.9 years 5 years 53%	Recurrence rate 76%	Pre modern day chemo Systemic chemotherapy predicted worse OS $p = 0.005$
42	Smith *et al.*[90]	Retrospective review, single institution, 1952–1989	PMP	34 (17 appendiceal) Recurrence Chemo 10 (6 IV, 4 IP)	MOF-Strep 3 5FU 1 Melphalan 1 Cyc 1	75 m 5 years 75% Chemo *versus* no chemo $p = 0.48$	Recurrence rate 59%	Pre modern day chemo

Adenoca, adenocarcinoma; bev, bevacizumab; CAP, capecitabine; CAPOX, capecitabine + oxaliplatin; 95% CI, 95% confidence interval; COX-2, cyclooxygenase-2; CR, complete response; CRC, colorectal cancer; CRS, cytoreductive surgery; CT, chemotherapy; CTX, cetuximab; Cyc, cyclophosphamide; DFS, disease-free survival; DL, diagnostic laparoscopy; DPAM, disseminated peritoneal adenomucinosis; 5FU, 5-fluoruracil; HE, high expressor; HG, high-grade; HIPEC, heated intraperitoneal chemotherapy; HR, hazard ratio; Iri, Irinotecan; LE, low expressor; LN, Lymph node; MD, moderately differentiated; MMC, mitomycin-C; MOF-Strep, semustine + 5FU + vincristine + streptozotocin; LG, low-grade; N, number of patients; NCCN, National Comprehensive Cancer Network; NCDB, National Cancer Database; NR, not reported; OS, overall survival; Ox, oxaliplatin; PD, poorly differentiated; PFS, progression-free survival; PMP, pseudomyxoma peritonei; preop, preoperative; PR, partial response; PSM, propensity score matching; SF, surgery first; VEGF, vascular endothelial growth factor; PMCA, peritoneal mucinous carcinomatosis; PMCA-I/D, PMCA with intermediate or discordant features; RFS, recurrence-free survival; SRC, signet ring cell; WD, well-differentiated; y, years.

Intraperitoneal chemotherapy

Cytoreductive surgery and HIPEC

The combination of CRS-HIPEC is the standard of care for appendiceal cancer with peritoneal disease.[63,94] These therapies evolved together, with neither alone demonstrating success.[95] The concept of radical debulking followed by intraperitoneal chemotherapy was first described in 1969 and was followed by case series showing

Table 6. Characteristics of literature review for chemotherapy in appendiceal cancer.

N (%)	HIPEC	Systemic chemotherapy
N		
Articles*	33[a]	42[a]
Total participants[e]	23,969	33,205
Received chemotherapy	20,304	13,135
Year		
Published	1994–2021	1992–2021
Data	1957–2020	1952–2020
Sample size		
Median (range)	104 (12–18,055)	104 (10–18,055)
Study design		
Randomised control trial	1	2
Prospective cohort	6	8
Retrospective cohort	26	32
Chemotherapy agent		
5FU/cap[d]	3	18
Oxaliplatin	7	5
5FU + oxaliplatin		18
MMC	19	Na
Irinotecan	1	7
5FU + irinotecan		8
Bevacizumab	Na	15
Other[b,c]	14	10
Survival results		
Median (range)		
DFS	5 years 28% (18–37%)	–
PFS		
Chemo	5 years 40% (14–50%)	14 (7–98 months)

(Continued)

Table 6. (Continued)

N (%)	HIPEC	Systemic chemotherapy
No Chemo	–	14 (4–43 months)
OS		
Chemo	5 years 58% (15–96%)	33 (14–160 months)
No Chemo	5 years 50% (48–52%)	30.5 (6–86 months)

*Studies that are updates of previous literature are only counted once i.e. the most recent.
[a]10 studies included assessment of both HIPEC and systemic chemotherapy but are counted in each category for the purpose of analysis.
[b]Other HIPEC agent: lobaplatin and docetaxel, doxorubicin/MMC/5FU, melphalan, cisplatin ± MMC, cisplatin ± doxorubicin, oxaliplatin + irinotecan, cyclophosphamide.
[c]Other systemic chemotherapy agents: cyclophosphamide, thalidomide, panitimumab, cetuximab, gefitinib, celecoxib, carboplatin, paclitaxel, melphalan, MOF-strep (semustine, 5FU, vincristine, streptozotocin), doxorubicin, αDC1 vaccine, interferon-α, rintatolimod.
[d]Intravenous 5FU at the time of intraperitoneal oxaliplatin is not considered separately, this is grouped as HIPEC or intraperitoneal chemotherapy.
[e]Numbers are just appendix cancer patients where this is known.
cap, capecitabine; DFS, disease-free survival; DPAM, disseminated peritoneal adenomucinosis; EPIC, early postoperative intraperitoneal chemotherapy; 5FU, 5-fluorouracil; HG, high grade; HIPEC, heated intraperitoneal chemotherapy; LG, low grade; MCP-H, high-grade mucinous carcinomatosis peritonei; MCP-L, low-grade mucinous carcinomatosis peritonei; MMC, mitomycin-C; OS, overall survival; PFS, progression-free survival; PMP, pseudomyxoma peritonei; LAMN, low-grade appendiceal mucinous neoplasm; adenoca, adenocarcinoma; PMCA, peritoneal mucinous carcinomatosis; PMCA-I/D, PMCA with intermediate or discordant features.

further benefit.[6,8,11,12,58,59,96] The CRS or peritonectomy technique (surgical intention is for no residual macroscopic disease) was pioneered by Mr Sugarbaker *et al.*[95,97] with the goal of complete cytoreduction. Compared to debulking procedures alone (surgical intention is limited removal of macroscopic disease), CRS-HIPEC has been shown to improve 5-year survival rates from about 50% to 76 to 96%.[6,8,10–12,46,48,50,56,58,95,98] One randomised study by Verwaal *et al.*[71,72] has evaluated CRS-HIPEC versus systemic chemotherapy, but only a small proportion of appendiceal cancers were included (17%) and the specific contribution of HIPEC to the improved outcomes is unclear.

It is difficult to determine the role of complete cytoreduction with or without HIPEC and disentangling the specific contributions of each therapy is the purpose of this review. Furthermore, the definitions of complete and incomplete cytoreduction vary in the literature, therefore we have adopted complete cytoreduction to include cytoreductive score (CC) 0 and 1 (no or less than 0.25 cm residual disease), and incomplete to include CC 2 and 3 (more than 0.25 cm residual disease). However, we acknowledge that further stratification may refine selection of patients for potential treatment options.

Mechanism of action and rationale of intraperitoneal chemotherapy

The aim of intraperitoneal chemotherapy after cytoreduction is to sterilise the peritoneal cavity of occult tumour cells. Intraperitoneal delivery of the chemotherapy intends to improve drug exposure to the peritoneal surface, which is limited with systemic administration.[75] Active drug concentrates in tissue to a few millimetres; therefore, resection to a minimal volume is required for definitive treatment.[12,61,99,100] Further studies confirm immediate chemotherapy is needed so that tumour cells do not seed in surgical adhesions.[61,101] Factors associated with altered HIPEC clearance include the extent of resection, contracted peritoneal space and completeness of cytoreduction.[102,103]

Evidence for benefit of HIPEC in addition to CRS

The specific question of whether HIPEC contributes to improved outcomes compared to CRS alone has not been directly addressed. However, there is reasonable evidence in the literature of case series to suggest independent benefit in both appendiceal adenocarcinoma and mucinous neoplasms.[35,37,39,42,47,52,55,57]

Shaib *et al.*[42] attempted to answer this by evaluating their institutional registry data from multiple

centres including one which did not use HIPEC. Of the 163 patients included, 65 had complete cytoreduction with most (78%) receiving HIPEC. A clear improvement in overall survival (OS) was seen in patients who received HIPEC even after adjustment for HIPEC-treating centre and extent of surgical resection [hazard ratio (HR) 0.42, 95% confidence interval (CI) 0.24–0.73, $p = 0.002$].

Another study inadvertently resulted in a control group (no HIPEC) as HIPEC was introduced midway through the review period.[52] This enabled a comparison between 60 patients (23 appendiceal cancer) treated with CRS with or without HIPEC and demonstrated an improved survival in those patients who received HIPEC. This study is limited by a mixed tumour population and so the specific contribution of the role of HIPEC for appendiceal cancer is less clear.

Most single-centre retrospective studies lack a comparable control arm. For example, an Indian study evaluating their experience of 33 cases of appendiceal and CRC peritoneal metastases reported improved 4-year OS for CRS-HIPEC compared to CRS alone.[39] However, the CRS alone group represented the poorer prognostic group where HIPEC delivery was abandoned due to high burden disease, which would not achieve complete cytoreduction.

Large database analyses, such as that of the National Cancer Database (NCDB), can only bluntly evaluate the role of HIPEC.[37,77,80] Although these analyses suggest a survival benefit of the addition of HIPEC to surgery, the comparison of CRS-HIPEC compared to surgery alone is flawed as the surgery alone group likely represents patients who undergo debulking procedures rather than complete cytoreduction, which is known to be suboptimal for disease control.[9,95]

A recent retrospective study evaluating outcomes of CRS-HIPEC compared to CRS-alone is the best evidence to date of the efficacy of HIPEC. This study evaluated 1924 patients with PMP from more than 20 centres over 24 years.[35] The addition of HIPEC to CRS in PMP was associated with a 35% reduction in the risk of death (HR 0.65, 95% CI 0.50–0.83). This was a statistically robust cohort study with propensity matched analysis and inverse probability treatment weighting. Subgroup analysis further confirmed the benefit of HIPEC in both low and high-grade disease and those with complete and incomplete cytoreduction. However, there were several limitations of this study. The reasons the CRS-alone group did not receive HIPEC were not available.[104] Additionally, the registry itself was set up for prospective data collection in 2010, yet the period captured was from 1993 requiring a long period of retrospective data collection with incomplete data in 45% of the patients in the registry.

The only randomised trial evaluating HIPEC compared mitomycin-C (MMC) to oxaliplatin in 121 patients with appendiceal cancer with peritoneal disease, but given outcomes are similar in each arm the ultimate benefit of HIPEC in addition to CRS is unable to be evaluated.[38]

A systematic review and meta-analysis compiled from single case reports and case series demonstrated most studies had 5-year OS similar to expected.[23] Therefore, mortality was minimally affected by the different treatment regimens. This analysis is flawed by the quality of the studies evaluated making the assessment of the role of HIPEC compared to no HIPEC challenging.

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

Evidence for HIPEC after debulking surgery

Historically, most patients who underwent CRS or debulking received concomitant intraperitoneal chemotherapy. However, the poor outcomes of patients who undergo debulking despite also receiving HIPEC questions its utility. The addition of HIPEC to debulking surgery appears not to improve OS in one Swedish study comparing 110 patients who had CRS-HIPEC compared to 40 patients who had debulking and HIPEC.[94] However, recent literature contradicts this suggesting the even those patients with incomplete cytoreduction benefit.[35,42]

The challenge in this setting is that a proportion of patients who have a surgical intention of debulking (anticipated macroscopic residual disease) can still achieve surgical outcomes of no residual macroscopic disease. This occurred in

25% of the debulking group in the study by Andreasson *et al*.[94] It is this subgroup of patients that may still benefit from provision of HIPEC.

Therefore, the literature currently supports current practice of abandoning HIPEC in the setting of gross macroscopic residual disease and continuing its use in the setting of achieving complete cytoreduction (regardless of the initial surgical intention).

Hyperthermia. Hyperthermic infusion of chemotherapy was first clinically demonstrated after cytoreduction in 1980.[105] Hyperthermia alone may confer a therapeutic benefit by inducing heat stress in tumour cells, denaturing proteins and impairing DNA repair mechanisms, especially important after a cytotoxic insult.[106,107] Heat improves delivery of the chemotherapy into tissues and has a synergistic effect with particular cytotoxic agents.[108–113]

The optimal temperature was established initially by *in vitro* studies showing 42.5°C was more effective than 39°C.[114] Elias *et al.*[61] evaluated HIPEC at 43°C, the maximal tolerable temperature in animals, but found higher morbidity and mortality compared to other published literature.

Mitomycin-C HIPEC. MMC is the most ubiquitous of agents used for HIPEC with most of the evidence for the combination of CRS-HIPEC historically using this agent.[6,8,16,35,36,38,41,42,46,47,49–52,54–57,63]

In the randomised control trial that compared MMC- to oxaliplatin-HIPEC, no significant difference was found in survival rates consistent with prior data.[38,54] In a follow-up study, patients reported improved physical and functional well-being from oxaliplatin-HIPEC.[115,116]

The recent PSOGI registry analysis did not show survival benefit in the group receiving MMC-HIPEC compared to no HIPEC (HR 0.93, 95% CI 0.65–1.34) noting that this was not the most common agent in this cohort and has flaws of retrospective analysis.[35]

Other clinical considerations in choice of HIPEC agent include prior receipt and intended sequencing of perioperative chemotherapy. This has not been clearly evaluated in the literature, and current clinical practice at most institutions favour initial MMC-HIPEC to facilitate initial oxaliplatin-based systemic chemotherapy to reduce the dose-limiting risk of neurotoxicity.[115,117]

Oxaliplatin HIPEC. The use of oxaliplatin-HIPEC has been controversial in the CRC setting.[118] In appendiceal cancer, some studies show similar outcomes compared to MMC-HIPEC.[38,119,120] In the recent PSOGI registry study, oxaliplatin-HIPEC was shown to reduce the risk of death compared to no HIPEC (HR 0.42, 95% CI 0.19–0.93).[35] However, only two patients in the HIPEC group are evaluable at 5-years compared to 47 in the no HIPEC group, due to significant attrition in this group or short follow-up.[104] Oxaliplatin-HIPEC has also been shown to have survival benefit when compared to irinotecan-HIPEC.[43]

Cisplatin and mitomycin HIPEC. This regimen was shown to be an efficacious regimen in the recent PSOGI registry study with HR 0.57 (95% CI 0.42–0.78).[35] This regimen was originally developed for treatment of gastric cancer peritoneal metastases based on preclinical data showing the synergistic effect of MMC enhancing intracellular accumulation of platinum adducts.[121,122] The benefits shown in this study are consistent with the effect demonstrated in ovarian cancer.[123] Further randomised study is needed to compare the benefit of this regimen to that of MMC- and oxaliplatin-HIPEC.

Other HIPEC regimens. Data concerning other HIPEC regimens with comparable safety profiles, but no meaningful improvement in efficacy are summarised in Table 4.

Perioperative systemic chemotherapy

The literature evaluating the role of perioperative chemotherapy is conflicting with some studies showing benefit and others suggesting worse outcomes in patients with appendiceal cancer (Table 5). We use the terms pre- and postoperative chemotherapy, as the terms neoadjuvant and adjuvant used in this literature as descriptors imply a 'curative/definitive' treatment intent, which is not always applicable. The term perioperative is used when both pre- and postoperative chemotherapy is evaluated, but does not imply that patients have received both pre- and postoperative chemotherapy.

The decision-making process regarding selection of patients for upfront surgery is beyond the scope of this review but involves an assessment of the

patients' histological subtype, rate of disease progression, burden of disease and fitness for surgery. Multidisciplinary team assessment at an expert peritoneal tumour service is essential. Ultimately this decision is based on the likelihood of the CRS-HIPEC procedure to result in a complete cytoreduction with no or minimal residual disease. This is an important concept to bear in mind when reading this review, as patients selected for upfront 'preoperative' chemotherapy usually have disease or patient-related factors that suggest complete cytoreduction may not be achieved. This creates bias by selecting patients with poorer prognoses.

Studies suggesting benefit of perioperative chemotherapy

There have been two prospective trials in the perioperative setting. The COMBATAC trial was a prospective phase 2 single-arm study, which reported on 25 patients of whom 10 had appendiceal cancer.[65,124] Patients received 3 months of pre- and postoperative chemotherapy using FOLFOX or FOLFIRI with cetuximab. The median progression-free survival (PFS) was 14.9 months, which met the target threshold and is comparable to the literature.[14,69,125] Although this study supports the role of systemic chemotherapy, there is no control arm and no subgroup analysis of the appendix cancer cohort so further conclusions about the role of systemic chemotherapy is limited.

The other prospective trial of perioperative chemotherapy assessed the role of postoperative thalidomide following CRS-HIPEC in 27 participants of whom 14 had appendiceal cancer.[68] The median PFS was 9.3 months and failed to meet the prespecified threshold. Given the limited role of anti-angiogenic agents in the unresectable setting, it is unlikely that this treatment will be pursued further in the perioperative setting.[66,82,86]

Asare *et al.*[80] interrogated the NCDB and reported on 5049 patients with stage IV appendiceal adenocarcinoma. In the nonmucinous group, there was an improvement in survival with chemotherapy compared to no chemotherapy (HR 0.73, 95% CI 0.65–0.83). In the mucinous group, there appeared to be no benefit from chemotherapy (HR 0.95, 95% CI 0.86–1.04). This effect is driven by the lack of benefit in the well-differentiated group (median OS 6.4 years *versus* 6.5 years, $p > 0.05$), but with survival improvement in the moderate and poorly differentiated groups (3.0 year *versus* 1.6 year, $p = 0.0005$; 1.6 year *versus* 1.0 year, $p = 0.0007$).[80] A flaw with this study is that HIPEC may have been included as systemic chemotherapy, especially as the 5-fluorouracil (5FU) bolus given with oxaliplatin-HIPEC is administered intravenously. Furthermore, the NCDB has no specific code for CRS-HIPEC, which amplifies the potential inaccuracies of this analysis.

Studies suggesting no benefit of perioperative chemotherapy

The subsequent study of the NCDB by Lu *et al.*[77] focused on the role of chemotherapy in 639 patients with stage IV well-differentiated mucinous appendiceal adenocarcinomas. The majority (90%) of this patient population underwent a surgical resection. Patients who had chemotherapy had improved 5-year OS (61% *versus* 53%), but after multivariate analysis including surgical resection, there was no association of benefit with chemotherapy (HR 1.1, 95% CI 0.82–1.4). This study further supports the concept that well-differentiated mucinous adenocarcinomas remain relatively chemoresistant despite the increasing use of combination chemotherapy regimens.[80,93]

Further evidence of the lack of benefit of perioperative systemic chemotherapy is demonstrated in a retrospective study of 393 (72% low-grade) patients from two high-volume centres.[84] Very few had perioperative chemotherapy (13 pre- and 9 postoperative) making conclusions difficult, but lends support for a lack of benefit in low-grade disease (median PFS 30 months *versus* 37 months, $p = 0.18$; median OS 109 months *versus* 72 months $p = 0.46$). Low-grade appendiceal tumours are thought to be more resistant to conventional cytotoxic agents.[126] Although it should be noted with prolonged survival in low-grade disease, any absolute benefit of perioperative chemotherapy will need to be of sufficient magnitude to provide a clinically meaningful benefit.[9,38]

Studies that have not been conclusive about the role of perioperative chemotherapy have been those with mixed tumour populations.[14,15] For example, one retrospective review included high-grade (moderate or poorly differentiated) peritoneal carcinomatosis from 19 (41%) appendiceal cancer patients in a cohort of CRC. The perioperative chemotherapy status was not known for all of the cohort, and not described specifically for the appendix compared to the colorectal primary subgroups. The median PFS of 9.5 months seems slightly optimistic for this high-grade cohort

where expected PFS is around 6 months for both colorectal and appendiceal cancers with peritoneal metastases. Furthermore, this study is likely underpowered for follow-up as median follow-up is shorter than median PFS (with no CI described and many early censored cases).

A study demonstrating survival benefits in OS and PFS regardless of timing of systemic chemotherapy only had a small proportion of appendix origin tumours (5%) compared to the remaining cohort of CRC.[14] Therefore, the true benefit of perioperative chemotherapy for the appendix cancer cohort is difficult to extrapolate.

Studies suggesting worse outcomes with perioperative chemotherapy

Some studies, albeit with significant limitations, suggest that perioperative chemotherapy could in fact cause harm.[35,83,92] Thus, assessing which patient groups may be at increased risk of harm from a proposed treatment is essential.

High-grade disease is expected to derive potential benefit from perioperative chemotherapy due to biological aggressiveness and is recommended before and/or after CRS at multiple institutions, regardless of data suggesting otherwise.[83,126] However, the literature does not necessarily support this. For example, Cummins *et al.*[92] evaluated 165 patients, 110 with high-grade appendiceal cancer, which compared outcomes to 55 CRC with peritoneal metastases. Most of the cohort received perioperative chemotherapy. Preoperative chemotherapy was associated with poorer OS (14 months *versus* 20 months, $p = 0.01$) and postoperative chemotherapy with improved OS outcomes (5 months *versus* 35 months, $p < 0.0001$).

Furthermore, Levinsky *et al.*[76] evaluated prognostic factors of a subgroup of 125 patients with appendiceal adenocarcinomas with signet ring cells from the same study population described by Chen *et al.*[75] They have not described the proportion of signet rings cells within the tumour specimen. so it is unclear if these meet the current classification for signet ring adenocarcinoma (more than 50% signet ring cells). Multivariate analysis of the entire cohort suggested worse OS in those receiving systemic chemotherapy (HR 1.98, 95% CI 1.23–3.19, $p < 0.01$).

This propensity for high-grade ACs to have worse OS after perioperative chemotherapy could be explained by selection bias, given both these studies are retrospective. However, in the second study described by Levinsky *et al.*,[76] the majority of the non-signet ring cell subgroup included well-differentiated adenocarcinomas. These well-differentiated adenocarcinomas are conversely, less chemosensitive, which could also account for worse outcomes with systemic chemotherapy. This argument is strengthened by the study by Shaib *et al.* who in their multicentre study demonstrated worse survival outcomes in patients with LAMN who received perioperative chemotherapy compared to those who did not (HR 1.92, 95% CI 1.14–3.23, $p = 0.013$).[42] This demonstrates the selection bias that although lower-grade and well-differentiated tumours may have more indolent biology, at the time clinicians select patients for systemic chemotherapy, the burden of the disease is such that it is more imminently life-threatening. Thereby, if systemic agents do not achieve response, these patients will have worse overall outcomes.

Chemotherapy regimens

Further limitations affecting retrospective studies evaluating systemic chemotherapy is the lack of detail regarding the systemic agents used which is either not recorded in the large databases or difficult to attain from centralised records when delivered in the community.[15,77,80] Studies that provide a more detailed insight into treatment regimens confirm that perioperative chemotherapy choice was predominantly fluoropyrimidine and oxaliplatin especially in high-grade disease.[40,41,65,74,75,78,79,81,84,85,93]

Perioperative chemotherapy in the presence of signet ring cells

Signet ring cells, present in the tumour in any proportion, have been shown to predict for poor survival outcomes.[41,76,127–129] Despite small patient numbers, Milovanov *et al.*[81] showed a large difference in OS from their cohort of 28 patients with signet ring cells out of 70 patients with peritoneal mucinous carcinomatosis (PMCA) (high-grade peritoneal disease) with 1-year OS with preoperative chemotherapy 94% *versus* 43% in those without chemotherapy ($p = 0.028$). However, there was no difference seen when a similar analysis was done in a larger cohort of high-grade histology, suggesting that this result may occur from confounding, or chance. In this study, subgroup analysis of those with signet ring cells found a nonsignificant shorter median survival of 25 months in those

who received preoperative chemotherapy compared to 39 months for those who did not receive chemotherapy ($p = 0.18$).[78]

One study specifically looked at prognostic variables in 514 patients with appendiceal adenocarcinoma of whom 125 (24%) had signet ring cells.[76] Multivariate analysis of the entire cohort suggested worse OS in those receiving systemic chemotherapy (HR 1.98, 95% CI 1.23–3.19, $p < 0.01$). However, after multivariate analysis of the signet ring cell subgroup, there was no statistical difference between those who received perioperative chemotherapy compared to those who did not (HR 1.69, 95% CI 0.50–5.68, $p = 0.4$).[76]

Another study of 142 patients with poorly differentiated or signet ring cell appendiceal adenocarcinoma of whom 19 (13%) had signet ring adenocarcinoma and 9 (6%) well or moderately differentiated with focal signet ring cells revealed a 44% response rate in 78 patients who received first-line chemotherapy.[87] One-fifth (20%) of this cohort proceeded to CRS with or without HIPEC. In the group of patients who had complete cytoreduction, 5 had preoperative and 12 had postoperative chemotherapy, but with the assessment of outcomes challenging due to small numbers (recurrence-free survival (RFS), HR 0.22, 95% CI 0.04–1.25; OS HR 0.12, 95% CI 0.01–1.59).

Outcomes for poorly differentiated and signet ring cell appendiceal adenocarcinomas appear to be more comparable to that of CRC than low-grade disease.[87,92] This provides some support for extrapolation of treatment regimens for this histological subtype.

Duration of perioperative chemotherapy

The optimal duration of perioperative chemotherapy is not clearly defined. This is most challenging to assess in the preoperative setting, given the conflicting efficacy results.

The approach in most studies is 3 months of chemotherapy followed by clinical and/or surgical reassessment and then a decision for another 3 months or to proceed to surgery. Following surgery, a further 3 to 6 months of postoperative chemotherapy is often considered.[70,85,126] For patients with borderline disease, 6 months of treatment might be optimal, if there is an initial response to ensure maintenance of the response. However, for clearly resectable disease upfront, 3 months may be preferable to reduce the risk that chemoresistant disease may progress to be unresectable.[70]

One study evaluated the duration of postoperative chemotherapy and demonstrated an OS benefit for six or more cycles of chemotherapy compared to less than six cycles.[40] Unfortunately, this retrospective study was a heterogeneous mix of different cancer types including CRC and gynaecological malignancy and is confounded by the likelihood that patients with prolonged survival will receive more treatment.

Preoperative chemotherapy

Timing of perioperative chemotherapy is important and forms an important discussion point in multidisciplinary meetings. Reasons to consider preoperative or neoadjuvant chemotherapy are summarised in Table 7. Unfortunately, the current literature does not clearly guide the specific role of preoperative chemotherapy. Therefore, recommendation for preoperative chemotherapy remains a case-by-case discussion with individualised treatment decisions.

A number of studies suggest that preoperative chemotherapy is associated with worse survival outcomes.[12,13,35,58,75,78,83,92] Often the intent is for systemic control of disease, potential downstaging and for observation of disease biology.[73,75,85] Therefore, even those who remain or become candidates for CRS-HIPEC will likely have additional disease-related factors that portend a poor prognosis, confounding outcomes.

A large multicentre cohort of 803 patients with appendiceal peritoneal metastases of whom 225 (28%) had preoperative chemotherapy.[75] Following propensity scored matching and multivariable analysis, preoperative chemotherapy was associated with worse outcomes (RFS HR 1.93, 95% CI 1.25–2.99; OS HR 1.81, 95% CI 1.02–3.118).

Another large retrospective multicentre registry study also confirmed that preoperative systemic chemotherapy independently predicted poor survival in a cohort of patients with appendiceal peritoneal disease (30% high-grade).[9] After multivariate analysis, chemotherapy was not associated with detriment in the low-grade group, but predicted poor OS in the high-grade group (HR 1.75, 95% CI 1.2–2.6, $p = 0.005$). In both of these studies, unmeasured variables leading to selection bias must be considered in addition to a true detri-

ment of administering preoperative chemotherapy, potentially from delays to definitive surgery.

Worse outcomes are not confined to the high-grade group where they might be expected. A recent registry study demonstrated that in a cohort of patients with low- and high-grade PMP receiving CRS, prior systemic chemotherapy was significantly associated with increased risk of death (HR 1.58, 95% CI 1.23–2.03, $p < 0.001$).[35] This persisted after propensity matching and sensitivity analyses to control for selection bias.

A study by Votanopoulos *et al.*[83] in 2015 evaluated 481 patients with both low- and high-grade disease. Preoperative chemotherapy predicted for worse OS in both low-grade (HR 2.2, $p = 0.05$) and high-grade tumours (median OS 17 months *versus* 32 months, $p = 0.02$; HR 2.5, $p = 0.006$) on multivariate analysis.

Baratti *et al.*[12] evaluated 104 patients with PMP (78 low-grade DPAM, 26 high-grade PMCA). Five-year OS of 72% is consistent with the predominant histology of LAMN compared to adenocarcinoma. Previous chemotherapy was associated with worse OS (HR 2.72, $p = 0.033$) and PFS after multivariate analysis (HR 2.04, $p = 0.045$). This is consistent with chemoresistance in the low-grade subgroup.

There are a number of different explanations why preoperative chemotherapy predicts for worse survival outcomes. The role of selection bias was interrogated in the recent PSOGI registry study.[35] Analysis of 1571 excluded patients (192 preoperative chemotherapy, but 958 missing data) revealed similar balance of prognostic factors, except for increased early postoperative intraperitoneal chemotherapy (EPIC) use in the excluded group, but improved rates of 10-year OS and lower severe morbidity suggest the presence of unmeasured confounders. A number of studies also comment that the decision for preoperative chemotherapy sometimes occurs externally to the centralised referral institution.[83,84] This results in suboptimal selection of patients for preoperative chemotherapy with functional deterioration in addition to potential delay in definitive CRS.[84] Another theory postulated is the potential for selection pressure of the chemotherapy on chemoresistant clones.[9,81]

Despite some literature suggesting worse outcomes with preoperative chemotherapy, there are some intriguing aspects that emerge suggesting further biological insights. A prospective consecutive cohort of 34 patients with high-grade PMCA were treated with preoperative 5FU + oxaliplatin.[69,70] Most patients (65%) had the full treatment course of 6 months. Although there was no improvement in OS in patients who received preoperative chemotherapy compared to those who did not (median OS 51 months *versus* 37 months, $p = 0.56$), there were 10 (29%) patients who had a histological response. These patients did not reach median OS compared to 29.5 months in those who did not achieve histological response ($p = 0.032$). There were no clinical variables identified that could help predict histological response, but this is compelling data and contrasts to data in the unresectable setting, which suggests mucinous tumours may have poorer outcomes with systemic chemotherapy.[93]

Further data points to potential short-term benefits of preoperative chemotherapy in appropriately selected patients including evaluation of 45 high-grade mucinous adenocarcinoma patients.[85] There was no difference in OS based on their primary analysis, but calculation of OS from date of initial therapeutic intervention showed a nonsignificant trend to worse OS in those who had preoperative chemotherapy consistent with other literature suggesting this association.[9,12,35,75,78,83,92] However, in this study, there was a high response rate to chemotherapy of 58%, and no patient experienced disease progression. This is consistent with other data also suggesting high rates of stable disease following preoperative chemotherapy.[84] This raises the possibility that ongoing postoperative chemotherapy in those demonstrated to have initial response and tolerability may be warranted in this high-risk group to maintain suppression of disease beyond CRS.

These studies outline the challenges in the literature assessing accurate response to chemotherapy. Mucinous peritoneal disease can be notoriously hard to visualise. Intraoperative assessment of response was shown to be discordant with radiographic assessment, particularly concerning is the increased rate of true progression shown intraoperatively (50%) compared to only 20% by imaging.[70] Other literature evaluating chemotherapy by different response criteria have attempted to compensate for this issue with some investigators recommending a 'modified peritoneal RECIST criteria'.[64,73,89,131]

Table 7. Summary of reasons to consider preoperative or postoperative chemotherapy in patients with appendiceal cancer with peritoneal disease.

Reason	Description
Preoperative or neoadjuvant chemotherapy	
Biological information	Histologic response provides direct biological information regarding chemosensitivity, which may help select future regimens in the event of tumour recurrence.[85] This is of additional importance in the setting of appendiceal cancers with peritoneal metastases as both clinical and radiological assessments of response are challenging and are not always concordant with operative and histopathological findings.[70]
Facilitates surgical planning	Embarking on chemotherapy can provide more immediate treatment if there are logistical delays being seen at a high-volume centre and also allows time for a patient to adjust to their diagnosis and prepare for their surgical intervention.[70,78]
Natural history of disease	Preoperative chemotherapy provides valuable insight into the biology and natural history of the disease.[75,130]
Optimal performance status	Preoperative chemotherapy means that patients start this at their optimal performance status and are more likely to receive it rather than needing to wait for recovery from their surgery.[75,85]
Better disease control	Earlier chemotherapy theoretically should have more impact on eradicating occult metastatic disease.
Downstaging	In 'borderline resectable' cases to provide an opportunity for downstaging for potential definitive management in a small number of patients.[73,75] Downstaging may facilitate less extensive surgery.[69]
Postoperative or adjuvant chemotherapy	
Avoids unnecessary disease progression	In patients with immediately resectable disease in which any delay risks disease progression that may yield unresectable disease.[70]
Avoids unnecessary toxicity	Toxicity from preoperative chemotherapy may cause functional deterioration in the patient, which may impact on surgical decision-making, recovery and morbidity.[83] Although studies demonstrate similar perioperative morbidity in patients who receive preoperative chemotherapy compared to those who do not.[65,69,83]
Allows uninterrupted tissue collection for translational research	Untreated tissue may be obtained for future laboratory and molecular testing, important in the current era of personalised medicine, particularly in rare cancer types for requiring molecular testing for clinical trials.

Decision-making around preoperative chemotherapy for this rare and specialised cancer should be reserved for expert multidisciplinary meeting and early referral to these services essential. It also emphasises the importance that reference centres continue to audit outcomes and improve data retention to minimise the loss that comes when patients are treated at external locations.[15]

Postoperative chemotherapy

In contrast to the literature evaluating preoperative chemotherapy, outcomes after postoperative chemotherapy appear to be more favourable.[83,84] This likely reflects refined patient selection of a fit population, pathological characteristics, post CRS-HIPEC and also the lack of control arm, as the comparator is those who receive preoperative chemotherapy, which overall show worse outcomes. Principles that favour postoperative chemotherapy are summarised in Table 7.

The theme that emerges from the literature is a trend for benefit of postoperative systemic chemotherapy in high-grade tumours.[69,74,84] Kolla *et al.*[74] evaluated the role of postoperative chemotherapy in a retrospective cohort of 103 appendiceal cancer patients. There was a benefit of chemotherapy for non-low-grade tumours with a median OS 9 years compared to 3 years for the

low-grade group ($p = 0.02$) in those who had complete cytoreduction. However, the analysis grouped chemoresistant LAMNs with well-differentiated adenocarcinomas, so any signal for benefit in the adenocarcinoma group will be lost.

In another retrospective review of 430 patients with mostly low-grade disease, postoperative chemotherapy showed no survival benefit for low-grade tumours on univariate analysis ($p = 0.88$) but conferred a significant benefit for high-grade tumours (median OS 32 months *versus* 6 months, $p < 0.001$).[83]

Further evidence for a benefit of postoperative chemotherapy in high-grade tumours is shown by a retrospective study by Blackham *et al.*[84] Of 109 patients with high-grade tumours, those who received postoperative chemotherapy had prolonged median PFS compared to preoperative chemotherapy and HIPEC alone (14 months *versus* 7 months *versus* 7 months, $p < 0.001$). A similar trend to improved OS was not statistically significant (36 months *versus* 16 months *versus* 20 months, $p = 0.07$).

Although systemic chemotherapy was associated with worse survival outcomes in a particularly poor prognostic cohort of appendiceal adenocarcinoma with signet ring cells, this study demonstrated no statistical difference between the timing of pre-, post- or perioperative treatment ($p = 0.71$).[76]

The timing of postoperative chemotherapy also allows selection of high-risk patients based on pathological factors such as positive lymph nodes. Lymph node status is not routinely assessed as part of the patients' diagnostic work-up prior to CRS; however, there is literature demonstrating lymph node involvement portends worse prognosis and in high-grade disease may select patients who benefit from systemic chemotherapy.

Lymph node involvement may counter-intuitively be a later phase in appendix cancer progression as peritoneal disease can occur directly due to transcoelomic spread without spread to lymph nodes as an intermediary.[4] Baumgartner *et al.*[15] raised the question of focusing chemotherapy strategies on the lymph node positive group, which was a strong predictor of OS and had short PFS. Kuijpers *et al.*[14] also suggested the benefit of systemic chemotherapy in lymph node positive disease in those with peritoneal carcinomatosis, but there were questions raised regarding the influence of surgical complications. This was also a predominant CRC population making conclusions difficult for the appendix cancer subgroup.

Cummins *et al.*[92] concluded that all high-grade appendiceal tumours with positive lymph nodes should have systemic chemotherapy. However, both positive lymph nodes and chemotherapy were associated with poorer survival, and their data do not interrogate if there is any difference in lymph node positive patients who received chemotherapy compared to those who did not. Another study evaluated positive lymph node status as a predictor of improved survival from perioperative chemotherapy but revealed no advantage.[84] While Votanopoulos *et al.*[83] commented on their institutional approach to lymph node positive patients, they did not explore in their data the proportion and outcomes of chemotherapy in this group. It is also worth noting that lymph node status as a possible selection tool is likely limited to higher-grade disease as a recent study after propensity matching did not demonstrate positive lymph nodes to be prognostic for OS in patients with low-grade disease.[35]

Patients who have incomplete cytoreduction (CC 2/3) are a specific subgroup worthy of discussion. Firstly, they have a known volume of residual disease and so could bear similarity to patients who have not had any form of CRS. Therefore, chemotherapy decisions should be based on evidence from the unresectable studies discussed in detail below. This fits with a trend to improved PFS being demonstrated in this patient subgroup without an OS benefit.[84]

The discrepancy in outcomes between pre- and postoperative chemotherapy is consistent with the ultimate need to improve selection of patients for these therapies. The literature clearly defines the contrast between those patients who achieve favourable outcomes compared to those who do not with existing treatments. Lymph node involvement in high-grade cases may be a strategy to help select patients who might benefit from systemic chemotherapy, but more evidence is needed to support this hypothesis. While the search for improved therapeutic strategies is important, delineating predictive biomarkers is also critical.

Systemic anticancer agents for unresectable disease

There is limited literature demonstrating the activity of systemic chemotherapy in the unresectable

setting, with few prospective trials.[21,64,66,67,73,82,86,89] These are summarised in Table 5.

The rare nature and centralised speciality care pathways for treating this disease in most countries leads to some degree of referral bias in the literature. Firstly, more severe cases are likely to be referred for treatment at speciality centres, leading a bias towards poorer outcomes. Conversely, patients with advanced disease whom local physicians pre-empt a pathway of best supportive care may never refer their patient for specialty centre management. Fitter, motivated patients are more likely to be seen at specialised centres, which would lean a possible bias to improved outcomes in patients that are reported from these centres. Most of the literature arises from centralised referral centres, so meaningful chemotherapy data is often missed from patients treated in the community.

5-Fluorouracil or capecitabine

The chemotherapy backbone in appendiceal cancer is 5FU or capecitabine as in CRC. In a single institutional retrospective analysis of chemotherapy agents, 30% of the participants were treated with single-agent 5FU or capecitabine.[89] It is likely that single-agent treatment was given in the advanced setting to those less fit for combination chemotherapy.

The benefit of capecitabine in combination with cyclophosphamide and mitomycin has been demonstrated by two studies.[64,73] These were both small cohorts, but demonstrated activity of these agents with disease control rate of 27% in the first by Raimondi *et al.* and a clinical benefit rate of 38% in the second by Farquharson *et al.* The Raimondi study of low-grade disease included those with progressing disease on consecutive scans, whereas the Farquharson study of both low and high-grade PMP did not require this. The Farquharson study demonstrated 1-year OS of 84% and 2-year OS of 61%.[73] Interestingly, two patients originally deemed unresectable achieved CRS following treatment with this regimen. This study attempted to overcome the challenges of measuring radiological responses by systematic application of disease volume assessment, discrete deposit measurement and compressive effects on intraperitoneal organs assessed by experienced radiologists.

Oxaliplatin-based combinations

Oxaliplatin and fluoropyrimidine chemotherapy regimens have been the mainstay of CRC chemotherapy for many decades.[132–136] Its use has been extrapolated to the treatment of settings of appendiceal cancer and is often the preferential first-line regimen.[87,89,93]

The best evidence for this doublet treatment is from a single-arm prospective study by Pietrantonio *et al.*[67] This study evaluated survival outcomes in 20 consecutive patients with unresectable or recurrent PMP (low- and high-grade) treated with FOLFOX-4. This was a high-burden disease group and 45% patients achieved stable disease. This compares to a 24% partial response rate in a retrospective study by Shapiro *et al.*,[89] which did not use RECIST and defined partial response as any degree of response. Median PFS was 8 months and median OS 26 months in the Pietrantonio *et al.*[67] study. This contrasts to longer median OS of 56 months likely driven by a mostly well-differentiated tumour population in the retrospective analysis by Shapiro *et al.*[89] The poorer survival in this prospective study is not inconsistent with a high-burden disease population, some of whom have high-grade disease. Two of the six initially unresectable patients underwent laparotomy with one achieving complete CRS.

Tejani *et al.*[93] demonstrated activity and similar survival outcomes (response rate 39%, median PFS 1.2 years and median OS 2.1 years) in a select group of 112 appendiceal adenocarcinomas from the National Comprehensive Cancer Network (NCCN) database. The majority of this cohort (71%) received combination chemotherapy of 5FU or capecitabine and oxaliplatin. Worse survival outcomes were shown in those with mucinous and poorly differentiated tumours. Whether this is due to chemotherapy being less efficacious for these subgroups or poorer disease biology is difficult to tell without a comparator arm.[80]

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

Irinotecan-based combinations

There are small numbers of appendiceal cancer patients treated with irinotecan-based combinations.[82,89] No further details are provided in these studies on the outcomes of this subgroup due to low patient numbers. In the retrospective analysis by Lieu *et al.*,[87] there was a trend towards improved PFS in patients who had the first-line irinotecan (1.0 year *versus* 0.5 year, $p = 0.07$). In the control arm of the Verwaal *et al.*[71,72] randomised control trial, a small number of patients were given single-agent irinotecan as the second-line chemotherapy agent. No further detail is available about these patients to draw any conclusions.

Biologic therapy

Anti-angiogenic agents. Vascular endothelial growth factor (VEGF) expression has been shown to be associated with poor OS in appendiceal cancer.[137–139] Studies that have evaluated the role of anti-VEGF therapy with bevacizumab are conflicting, confounded by the chemotherapy regimen.[66,82,86,87,93] The most recent was a prospective phase 2 study that evaluated 15 patients with PMP (mostly low-grade) who relapsed after prior CRS-HIPEC and received capecitabine and bevacizumab.[66] Median PFS was 8.2 months (95% CI 5.3–not assessable) which met the pre-specified non-inferiority threshold of 5 months, and median OS was not reached, with 1-year OS 91%. Three of the 15 patients had a partial response (20%).

Choe *et al.*[82] evaluated the role of biological therapy through analysis of 130 of 353 patients with appendiceal cancers. Most patients (91%) received bevacizumab in addition to combination chemotherapy, so that the comparison is to those who received single-agent chemotherapy. Median PFS was improved with bevacizumab compared to no bevacizumab (9 months *versus* 4 months, HR 0.69, 95% CI 0.470–0.995). Median OS was improved by 34 months for patients receiving additional bevacizumab, but this finding could be confounded by the use of combination chemotherapy.

Jimenez *et al.*[86] evaluated differential VEGFR-2 gene expression in a cohort of 59 of 89 patients with peritoneal carcinomatosis from appendiceal cancer. Twelve of the 47 high expressors received adjuvant bevacizumab. There was no statistical difference between these groups, yet there was a trend to better outcomes in VEGFR-2 low expressors, noting that this comparison is underpowered.

Conversely, a subgroup analysis of 112 patients with appendiceal adenocarcinoma (51% received bevacizumab) suggested worse PFS in those who received bevacizumab (HR 1.91, 95% CI 1.17–3.14, $p = 0.01$) and no advantage to OS.[93] Most patients who received bevacizumab had combination chemotherapy, likely reflecting selection of more aggressive disease biology; however, this study does not support the use of bevacizumab in treatment of advanced appendiceal adenocarcinoma.

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

Molecular-directed therapy

EGFR-inhibitors. In the study by Shapiro *et al.*,[89] 11 (20%) of the patients studied received biologic therapy alone or in combination with chemotherapy. Five patients were treated with gefitinib alone, which is interesting as this is not an extrapolated CRC regimen.

Another study by Choe *et al.*[82] also included a small subgroup of patients treated with anti-EGFR monoclonal antibodies in addition to systemic chemotherapy. In this group, OS outcomes were worse, (18 months *versus* 20 months; HR 3.83 95% CI 1.04–14.14). The authors have not described RAS testing and the study period included the time prior to knowledge of the lack of benefit from EGFR-antibodies in RAS-mutant CRC.[140]

In the COMBATAC trial, perioperative systemic chemotherapy (48% 5FU + oxaliplatin; 48% 5FU + irinotecan) and cetuximab was given to KRAS wild-type appendiceal cancer patients in a cohort of CRC.[65,124] No additional conclusions for the role of cetuximab could be made from this study as the appendiceal cancer subgroup was not evaluated independently.

There was a trend to prolonged OS in 20 of 49 KRAS wild type patients who received cetuximab or panitumumab. However, in another study of 149 of 600 patients who had molecular testing, there was no statistical difference compared to those who did not receive an EGFR inhibitor (median OS 68.4 m *versus* 51.7 m, $p = 0.83$).[21]

Cox inhibitors. In one study evaluating molecular markers, 30% of the patients found to have cyclooxygenase-2 (COX-2) expressing tumours received selective COX-2 inhibition with celecoxib.[21] Median OS was not statistically different between those receiving celecoxib compared to those who did not (57.6 months *versus* 55.7 months, $p = 0.84$).

Immunotherapy

In the current era of immunotherapeutics, appendiceal cancers are considered 'cold' tumours as they lack the ability to initiate an effective immune response, and few are MMR-deficient or high tumour mutation burden.[20,141,142]

Given the recency in advances for the role of immunotherapy in MSI-high cancers, there is sparing literature on appendiceal cancer patients receiving this treatment. Lu *et al.*[77] reported in their cohort of stage IV well-differentiated mucinous adenocarcinomas that 5% received immunotherapy.

A novel approach of a dendritic vaccine (αDC1) as an adjuvant treatment in combination with immunomodulators celecoxib, interferon-α and rintatolimod was evaluated in a phase 2 study including 24 patients with appendiceal cancer following CRS-HIPEC (7 LAMN, 16 mucinous adenocarcinoma).[91] This study was hampered by technical issues with difficulty isolating adequate cells to achieve the target dose and ultimately the trial was stopped prematurely for futility, slow accrual and grade disparity in the appendiceal cancer group with PFS for low- and high-grade tumours 50.4 and 8.9 months respectively.

Conclusion and future directions

This review reconciles the evidence for the role of intraperitoneal and systemic chemotherapy for the treatment of appendiceal cancer with peritoneal disease. Challenges to reaching definite conclusions include retrospective study designs and broad study populations due to disease rarity and inconsistent use of tumour nomenclature due to changing classification systems. Heterogeneous chemotherapy regimens, inadequate chemotherapy data due to the centralised nature of the surgical service with local delivery of chemotherapy and difficulty accurately measuring radiological treatment responses further complicate interpretation.

It is clear from the literature that the addition of HIPEC to complete cytoreduction has survival benefits with a more limited role of HIPEC in the setting of incomplete cytoreduction.

There is contradictory evidence as to the benefit of perioperative chemotherapy in the setting of appendiceal cancer, especially of any additional benefit to that of complete CRS-HIPEC. Studies of preoperative chemotherapy generally appear to be associated with worse survival outcomes, although this group will be most influenced by selection bias. Studies of postoperative chemotherapy generally show some degree of benefit, especially in high-grade disease with presence of signet ring cells and lymph node involvement as possible selection tools. Based on evidence from this review there is a minimal role of perioperative systemic chemotherapy in addition to complete CRS-HIPEC for low-grade mucinous peritoneal disease (from AMNs) and that use of systemic agents should be reserved for use within clinical trials.

Systemic chemotherapy agents demonstrate some activity in the treatment of unresectable disease, but it remains unclear the optimal way to refine selection of patients who can benefit. This review also highlights the necessity of thorough collection of information about systemic agents and ensuring accuracy of clinical outcomes. Furthermore, there are a number of unstudied treatment strategies such as maintenance chemotherapy, intermittent-dosing or histology-tailored therapy that should be the focus of future prospective study.

Ultimately, further studies of the same agents are unlikely to yield more meaningful or convincing information. There is an urgent need for novel treatment agents and strategies. Preclinical and translational research models that interrogate the biological nature of this rare and unpredictable malignancy are needed to help postulate rational therapeutic development. We propose a translational medicine platform where we can interrogate the true biology of each individual patients' tumour and microenvironment. By establishing robust preclinical models and evaluating a multiomic profile using cutting edge technology such as single-cell RNA sequencing and spatial transcriptomic analysis of these tumours and their microenvironment, our research group is attempting to rationally identify targets and pathways of novel and repurposed therapeutic strategies. In

the meantime, individualised treatment decisions should be made in the setting of a multidisciplinary discussion at a high-volume appendiceal cancer treatment centre and international collaboration is vital for the design of feasible prospective studies that can evaluate these clinical dilemmas more definitively.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Author contribution(s)
Madeleine C Strach: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing.

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Competing Interests
MS has received personal fees from Specialised Therapeutics for participation in an advisory board. The other authors declare that there are no conflict of interests.

Availability of data and materials
Data used in writing this review is available and provided in Tables 4 and 5.

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Appendix 1.2

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

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ORIGINAL RESEARCH

Defining a role for systemic chemotherapy in local and advanced appendix adenocarcinoma☆

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Background: Appendix adenocarcinomas (AAs) are rare tumours that often present late, with a propensity for peritoneal metastases (PMs). This study aimed to evaluate outcomes of AA patients undergoing cytoreductive surgery (CRS) with curative intent and determine the role of systemic chemotherapy.

Materials and methods: Data were collected from a prospective database and classified according to World Health Organization (WHO) 2019 classification. Tumour clearance from CRS was described using a completeness of cytoreduction (CC) score ranging from 0 [no residual disease (RD)] to 3 (>2.5 cm RD). Patients with CC0-2 CRS received hyperthermic intraperitoneal chemotherapy (HIPEC). Systemic chemotherapy was categorised as 'prior' (>6 months before), 'neoadjuvant' (<6 months before), 'adjuvant' (<6 months after CC0-1 CRS) or 'palliative' (after CC2-3 CRS). Analyses used Kaplan–Meier and Cox regression methods.

Results: Between January 2005 and August 2021, 216 AA patients were identified for inclusion. Median age was 59 years (21-81 years). CRS/HIPEC was carried out in 182 (84%) patients, of whom 164/182 (76%) had mitomycin C HIPEC. CC0-1 was achieved in 172 (80%) patients. Systemic chemotherapy was given to 97 (45%) patients from the whole cohort and to 37/46 (80%) patients with positive nodes. Median overall survival (OS) was 122 months (95% confidence interval 61-182 months). After multivariate analysis, patients with acellular and lower-grade PM had similar OS to those with localised (M0) disease ($P = 0.59$ and $P = 0.19$). For patients with positive nodes, systemic chemotherapy was associated with reduced risk of death compared to no chemotherapy ($P < 0.0019$).

Conclusion: This study identifies AA patients with positive lymph nodes derive the most benefit from systemic chemotherapy. We confirm the prognostic importance of stage and peritoneal grade, with excellent outcomes in patients with acellular mucin and lower-grade PM.

Key words: appendix adenocarcinoma, appendix cancer, peritoneal metastases, chemotherapy, treatment outcomes

INTRODUCTION

Appendix adenocarcinomas (AAs) are rare, heterogeneous and aggressive tumours often presenting with peritoneal metastases (PMs).[1-4] The incidence is estimated to be 1-2 per million per year.[1,5] Prognosis from small retrospective and heterogeneous cohorts is poor, with a 5-year survival ranging from 14% to 59%.[6-11] Due to changing nomenclature over the years, these cohorts have been mixed including indolent appendiceal mucinous neoplasms (AMNs).[3,12] The current World Health Organization (WHO) 2019 classification provides stricter guidance for classification of primary AAs as mucinous adenocarcinomas (MACs), adenocarcinomas not otherwise specified (ANOS) and signet ring adenocarcinomas (SRCs); goblet cell adenocarcinomas (GCAs) are classified separately.[13] This also

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incorporates the American Joint Committee on Cancer (AJCC) tiered grading.[14] PMs are recommended to be graded separately to that of primary tumours due to potential for discordant grading.[3,15] The WHO 2019 terminology for PM has equivalence to the Peritoneal Surface Oncology Group International (PSOGI) 2016 consensus.[15] We describe both these systems in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Cytoreductive surgery and heated intraperitoneal chemotherapy (CRS/HIPEC) is a potentially curative treatment for AA, either for patients with existing PMs or for those at high risk of developing PMs. During CRS, the surgeon aims to remove all macroscopic disease, after which, HIPEC at 42°C is instilled intraperitoneally to eradicate occult tumour cells.[16,17] The peritoneal cancer index (PCI) is an operative scoring system to quantify the volume and distribution of PM (range 0-39) and the completeness of cytoreduction (CC) score records the volume of disease at the end of surgery (range 0-3).[18,19] A lower PCI and CC score has been shown to be prognostic with longer survival outcomes in AA.[6,20-22] The addition of HIPEC cannot be conclusively said to improve outcomes over CRS alone in AA, particularly in the setting of complete cytoreduction, and supportive data are based on heterogeneous pathology cohorts.[6,23,24] Systemic chemotherapy can be considered, and in the palliative setting regimens are extrapolated from colorectal cancer treatments, but the role is yet to be defined in the perioperative setting.[25-28]

This study aimed to analyse patients with AAs who underwent curative intent CRS/HIPEC, and to evaluate the role of systemic chemotherapy. The primary endpoint of this study was overall survival (OS) and secondary endpoints progression-free survival (PFS), radiological and pathological response rates to previous systemic chemotherapy. This study was approved by the institutional clinical audit committee (reference 3091).

MATERIALS AND METHODS

Population

Between January 2005 and August 2021, patients with histologically confirmed AAs were identified from a prospectively collected database at a peritoneal tumour centre in the UK. All who were intended for curative CRS/HIPEC were included. Patients were discussed in a specialised peritoneal tumour multidisciplinary team (MDT) and all pathology slides reported at external institutions were imported and re-reported by specialist pathologists.

Operative technique

A standardised operative technique was used and has been described in detail previously.[22] The PCI was calculated by the surgeons at the time of CRS and scored 0-39.[18] The CC score was calculated after maximal resection and defined as: CC0, no residual disease (RD); CC1, <0.25 cm RD; CC2, 0.25-2.5 cm RD; and CC3, >2.5 cm RD.[19]

Chemotherapy

Treatments with intraperitoneal and intravenous chemotherapies were given following standard institutional protocols (Supplementary Appendix S1, available at https://doi.org/10.1016/j.esmoop.2023.101619). Chemotherapy was grouped as: 'prior' chemotherapy >6 months before CRS, 'neoadjuvant' chemotherapy as <6 months before CRS, 'adjuvant' chemotherapy defined as <6 months after CC0-1 CRS and 'palliative' chemotherapy as that given after CC2-3 CRS or for those with recurrent unresectable disease.

Data collection and pathological classification

Data including demographics, clinicopathological variables, treatment characteristics and survival status were extracted from the database. All patients had pathology reports reviewed (MCS, BC); missing data were referenced from the hospital record. Diagnoses were strictly documented based on the WHO 2019 classification defined in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2023.101619, with the exception of retaining the classification of peritoneal acellular mucin (AM) from the PSOGI classification.[13,15] Classification according to the prior PSOGI 2016 consensus was recorded which formed the basis for grade reclassification of the ANOS subgroup based on tumour differentiation: 'well' as grade 1, 'moderate' as grade 2 and 'poor' as grade 3.[15]

Follow-up

Patients were routinely followed up from the date of CRS/HIPEC clinically, biochemically with tumour markers [including carcinoembryonic antigen (CEA)] and with computed tomography (CT) of chest/abdomen/pelvis every 6 months for 2 years until 5 years and then at years 8 and 10. Patients who received systemic chemotherapy had more frequent follow-up while receiving treatments.

Outcome measures

The primary outcome of OS was defined as the time from diagnosis to death of any cause, censored for the time of last known follow-up. The secondary outcome of PFS was defined as the time from diagnosis to first of disease recurrence, progression or death, censored for the time of last known follow-up. Response rates and all suspected recurrence and progression were confirmed radiologically using RECIST1.1 criteria,[29] discussed and documented at MDT and the date of first event used even if observed in retrospect. Pathological response was defined using the tumour regression grade (TRG): grade 0, complete response with no remaining viable tumour cells; grade 1, moderate response with only small cluster or single cells remaining; grade 2, minimal response with residual cancer remaining, but with predominant fibrosis; grade 3, poor response with minimal or no tumour death with extensive residual cancer.[30]

Statistical analysis

Descriptive epidemiological methods were used to describe the cohort. Tumour marker analysis used the clinical threshold of CEA ≤5 as normal. Survival analysis was carried out using the Kaplan–Meier method and the log-rank test was used to assess statistical significance. Univariate and multivariate analysis of prognostic variables was carried out using Cox regression. Statistical analysis was carried out using IBM SPSS (IBM SPSS Statistics, version 28.0, Armonk, NY) and R 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria) (23 June 2022).

RESULTS

Baseline characteristics

We identified 1077 patients with appendix tumours referred to our UK centre between 2005 and 2021. Of these, 704 AMNs, 146 GCAs and 11 non-appendix tumours were excluded, resulting in 216 AA patients: 141 MAC, 71 ANOS and 4 SRC (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2023.101619). Baseline demographics and clinical characteristics are summarised in Table 1.

At initial diagnosis, 82 (38%) patients had localised disease (M0) on CT imaging and preoperative pathology, 134 (62%) with metastatic disease (M1) and 132 (61%) with PM (M1b). Subsequently, 12 (15%) patients were found to have PM (M1b) confirmed pathologically at CRS and 1 patient had confirmed extraperitoneal metastasis (M1c). In this confirmed M0 group, 48/69 (70%) patients had CRS within 6 months of their initial diagnostic procedure. This was 92/147 (63%) for the M1 group.

Ninety-seven patients had systemic chemotherapy; 28 had neoadjuvant or adjuvant chemotherapy (N/ACT). Reasons for systemic chemotherapy recommendations are presented in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Survival outcomes related to clinicopathological factors

At a median follow-up of 56 months (range 1-286 months), the median OS was 122 months [95% confidence interval (CI) 62-182 months] with 5-year and 10-year OS of 63% and 51%, respectively (Figure 1). The median PFS was 41 months (95% CI 28-54 months) with 5-year and 10-year PFS of 43% and 40%, respectively (Figure 1). Beyond 5 years, we found two progression events. Results of univariate and multivariate analysis of prognostic factors for OS are presented in Table 2 and for PFS in Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Univariate analysis. Variables associated with prognosis on univariate analysis included CEA, PCI, CC score, nodal stage, metastasis stage, peritoneal grade and the presence of signet ring cells (srcs) (Supplementary Figures S2 and S3, available at https://doi.org/10.1016/j.esmoop.2023.101619). Patients with lower-grade PM or AM demonstrated similar OS and PFS compared to patients with no PM [median OS not reached (NR), $P = 0.4$ and median PFS NR versus 194 months, $P = 0.68$]; Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2023.101619. The prognostic impact of positive compared to negative lymph nodes on OS and PFS was maintained even in the presence of confirmed metastatic (M1) disease (median OS 29 versus 70 months, $P = 0.0011$ and median PFS 18 versus 30 months, $P < 0.0001$); Supplementary Figure S5, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Similar OS and PFS outcomes were seen following analysis of AA subtype (Figure 1), presence of mucin (including ANOS with 1%-50% mucin; Supplementary Figure S6, available at https://doi.org/10.1016/j.esmoop.2023.101619) and primary tumour grade (Supplementary Figure S7, available at https://doi.org/10.1016/j.esmoop.2023.101619). The latter remained so when the localised disease (M0) cohort was analysed separately for OS and PFS ($P = 0.68$ and $P = 0.88$; Supplementary Figure S7, available at https://doi.org/10.1016/j.esmoop.2023.101619).

Multivariate analysis. After multivariate analysis for impact on OS (Table 2), variables that retained significance for poorer prognosis included SRC subtype ($P < 0.001$), CC2/3 cytoreduction ($P < 0.001$), positive lymph nodes ($P < 0.001$) and stage M1b/c ($P = 0.004$). Multivariate analysis revealed that female sex predicted increased risk of death compared to male sex ($P < 0.001$), and confirmed that age, CC1 cytoreduction and stage M1a (AM) were not associated with worse OS outcomes. After multivariate analysis for impact on PFS (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2023.101619), variables that retained significance for increased risk of recurrence or progression included CC2/3 cytoreduction ($P = 0.002$), elevated CEA ($P = 0.032$), positive lymph nodes ($P = 0.004$) and stage M1b/c ($P < 0.001$). While PCI score was prognostic in univariate analysis, even the highest PCI score group did not remain prognostic for OS or PFS after multivariate analysis including cytoreductive status in the model.

Outcomes for patients treated with systemic chemotherapy

Whole cohort. Unselected patients who received chemotherapy had worse OS and PFS compared to those not who did not ($P < 0.0001$); Table 2, Figure 2 and Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Lymph node-positive patients. On multivariate analysis, N/ACT, prior and palliative chemotherapy settings were associated with reduced risk of death compared to no chemotherapy ($P = 0.005$, $P = 0.011$ and $P < 0.001$, respectively); Table 2 and Figure 2. On multivariate analysis for PFS, palliative chemotherapy was associated with a lower risk of recurrence ($P = 0.01$). There was no statistical difference for prior ($P = 0.055$) and N/ACT ($P = 0.08$); Figure 2 and Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Patients with peritoneal signet ring cells. The presence of peritoneal srcs did not impact outcomes of prior ($P = 0.22$)

Table 1. Baseline demographics and clinical characteristics of patients with appendix adenocarcinoma

Variable	Whole cohort *n* (%)	Localised (M0) *n* (%)	Metastatic (M1) *n* (%)
	216 (100)	**69 (100)**	**147 (100)**
Age (median, range)	59 (21-80)	60 (29-79)	58 (21-80)
Sex			
Male	90 (42)	24 (48)	57 (39)
Female	126 (58)	36 (52)	90 (61)
Subtype			
MAC	141 (65)	36 (52)	105 (71)
ANOS	71 (33)	32 (46)	39 (27)
SRC	4 (2)	1 (1)	3 (2)
CEA (mean, range)	22 (<3-990)	7 (<3-446)	25 (<3-990)
<6	139 (65)	58 (84)	81 (55)
≥6	55 (26)	5 (7)	50 (34)
Missing	20 (9)	6 (9)	16 (11)
PCI (median, range)	8 (0-39)	2 (0-27)	14 (0-39)
Missing	7 (3)	0	7 (5)
CRS[a]	214 (99)	66 (96)	146 (99)
HIPEC	182 (84) [100]	66 (96) [100]	116 (79) [100]
Mitomycin C	164 (76) [90]	61 (88) [92]	103 (70) [89]
Oxaliplatin	16 (7) [9]	5 (7) [8]	11 (5) [9]
Missing	2 (1) [1]	0	2 (1) [2]
CC score			
0	133 (62)	68 (99)	65 (44)
1	39 (18)	1 (1)	38 (26)
2-3	44 (20)	0	44 (30)
Stage at CRS			
N0/X	168 (79)	57 (83)	113 (77)
N1/2	46 (21)	12 (17)	34 (23)
M0[b]	69 (32)	69 (100)	0
M1a[c]	25 (12)	0	25 (17)
M1b/c[d]	122 (56)	0	122 (83)
Primary tumour grade[e]			
MAC	141 (65) [100]	36 (52) [100]	105 (71) [100]
Grade 2	108 (50) [77]	27 (39) [75]	81 (55) [77]
Grade 3[f]	30 (14) [21]	9 (13) [25]	21 (14) [20]
Missing	3 (1) [2]	0	3 (2) [3]
ANOS	71 (38) [100]	32 (46) [100]	39 (27) [100]
Low grade	53 (25) [75]	26 (38) [81]	27 (18) [69]
High grade	18 (8) [25]	6 (9) [19]	12 (8) [31]
Primary tumour differentiation[g]			
Well	68 (32)	21 (30)	49 (33)
Moderate	131 (61)	43 (62)	87 (59)
Poor	11 (5)	5 (7)	7 (5)
Missing	4 (2)	0	4 (3)
Peritoneal tumour grade[h]	116 (54)	0	116 (79)
Mucinous carcinoma peritonei	83 (38)		83 (56)
Grade 1	7 (3)		7 (5)
Grade 2	13 (6)		13 (9)
Grade 3	23 (11)		23 (16)
Non-mucinous metastases	33 (15)		33 (22)
Low grade	3 (1)		3 (2)
High grade	30 (14)		30 (20)
Mucinous[i]			
Yes	182 (84)	47 (68)	135 (92)
No	34 (16)	22 (32)	12 (8)
Signet ring cells[j]			
Yes	46 (22)	10 (15)	37 (25)
No	167 (78)	59 (86)	109 (74)
Missing	1 (0.5)	0	1 (1)
Radiotherapy	10 (4)	1 (1)	9 (6)
Systemic chemotherapy	97 (45)	20 (29)	77 (52)
None	119 (55)	49 (71)	70 (48)
Neoadjuvant or adjuvant[k]	28 (13)	11 (16)	17 (12)
Neoadjuvant	12 (6)	2 (3)	10 (7)
Adjuvant	16 (7)	9 (13)	7 (5)
Both	0	0	0
Prior (>6 months before CRS)	18 (8)	4 (6)	14 (10)
Palliative	51 (24)	5 (7)	46 (31)

Continued

Table 1. Continued

Variable	Whole cohort *n* (%)	Localised (M0) *n* (%)	Metastatic (M1) *n* (%)
	216 (100)	69 (100)	147 (100)
First-line chemotherapy agents			
Oxaliplatin + fluoropyrimidine[l]	63 (29)	13 (19)	50 (34)
Fluoropyrimidine	13 (7)	6 (9)	7 (5)
Irinotecan + fluoropyrimidine	10 (5)	0	10 (7)
Mitomycin + capecitabine	5 (2)	1	4 (3)
+Bevacizumab	2 (1)	0	2 (1)
+Cetuximab/panitumumab[m]	2 (1)	0	2 (1)
Other[n]	1 (0.5)	0	1
Missing	5 (2)		

ANOS, adenocarcinoma not otherwise specified; CRS/HIPEC, cytoreductive surgery and heated intraperitoneal chemotherapy; MAC, mucinous adenocarcinoma; SRC, signet ring cell carcinoma; WT, wild type.
[a]Of 34 cases that had initial debulking, 3 proceeded to have subsequent CRS/HIPEC, CC1 ($n = 2$), CC2 ($n = 1$). Two cases did not proceed with CRS and had caecectomy ($n = 1$) and diagnostic biopsy ($n = 1$).
[b]M1 at subsequent CRS ($n = 13$).
[c]Staged as M1c due to acellular mucin and visceral metastasis ($n = 1$).
[d]Five patients had M1c disease only (i.e. no peritoneal metastasis).
[e]Primary tumour grading based on World Health Organization (WHO) 2019 classification, grade 1 MAC is a low-grade AMN which is considered a different histological subtype of appendiceal tumour.
[f]This group is defined as presence of ≤50% signet ring cells.
[g]Includes all subtypes of mucinous and non-mucinous adenocarcinoma.
[h]This group categorises patients with cellular peritoneal metastases and excludes patients with M1c only extraperitoneal metastases ($n = 5$) and a case with peritoneal acellular mucin and visceral metastasis ($n = 1$).
[i]Any proportion of mucin, includes presence of mucin in ANOS/non-mucinous metastasis ≤50%.
[j]In either primary or peritoneal disease.
[k]±6 months of CRS.
[l]Changed from 5-fluorouracil to raltitrexed chemotherapy for cardiotoxicity ($n = 2$).
[m]RAS WT ($n = 1$), RAS status unknown ($n = 1$).
[n]First-line carboplatin/paclitaxel pre-CRS for initial diagnosis of ovarian cancer ($n = 1$).

and N/ACT ($P = 0.89$) on OS, Table 2, noting the small numbers in this group.

Response rate to systemic chemotherapy

Radiological response. There were 77/97 (79%) patients in whom disease was potentially evaluable during the first chemotherapy regimen. This excludes those who received adjuvant chemotherapy and includes patients post-CC2-3 CRS with RD evident on imaging. The objective response rate was 13% and disease control rate 30% (Table 3).

Pathological response. There were 15/97 (15%) patients who had preoperative chemotherapy which allowed assessment for pathological response on the resected specimen. The pathological response rate (TRG 0-1) was 20% (Table 3).

Exploratory pathological analyses

Discordant primary and peritoneal grade. We identified 75 patients with discordant grade (40 higher-grade primary tumour to lower-grade PM, 35 lower-grade primary tumour to higher-grade PM); Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2023.101619. Thirty-one (41%) patients in this group received chemotherapy, 13 before CRS (6 prior and 7 neoadjuvant).

When this subgroup was classified by primary tumour grade, there was no significant difference on OS ($P = 0.42$) although a difference was noted for PFS ($P = 0.014$); Supplementary Figure S8, available at https://doi.org/10.1016/j.esmoop.2023.101619. When classified by PM grade, there was a significant difference in both OS and PFS outcomes ($P = 0.0075$ and $P < 0.0001$); Supplementary Figure S8, available at https://doi.org/10.1016/j.esmoop.2023.101619. Patients with higher-grade primary tumour and lower-grade PM had longer OS and PFS compared to patients with lower-grade primary tumour and higher-grade PM (median OS 122 versus 48 months, $P = 0.00094$ and median PFS NR versus 15 months, $P < 0.0001$); Supplementary Figure S9, available at https://doi.org/10.1016/j.esmoop.2023.101619.

Discordant primary and peritoneal signet ring cell status. We identified 18 patients from our cohort who had discordant srcs (10 had srcs in primary tumour and not in PM; 8 had srcs in PM and not in primary tumour); Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2023.101619. Analysis of the impact of srcs in different disease settings revealed that despite the presence of srcs in the primary tumour, a small cohort of patients ($n = 12$) with peritoneal AM or no disease relapse had excellent OS and PFS outcomes; Supplementary Figure S10, available at https://doi.org/10.1016/j.esmoop.2023.101619.

ANOS three-tier grade reclassification. Seventy ANOS cases were available for three-tier grade reclassification, which was predictive for OS ($P = 0.028$); Supplementary Table S5 and Figure S11, available at https://doi.org/10.1016/j.esmoop.2023.101619. This is mostly driven by the difference in OS between grade 1 and 3 reclassification (median 94 versus 49 months, $P = 0.016$). Reclassification of the ANOS subgroup into three-tiered grading also suggests

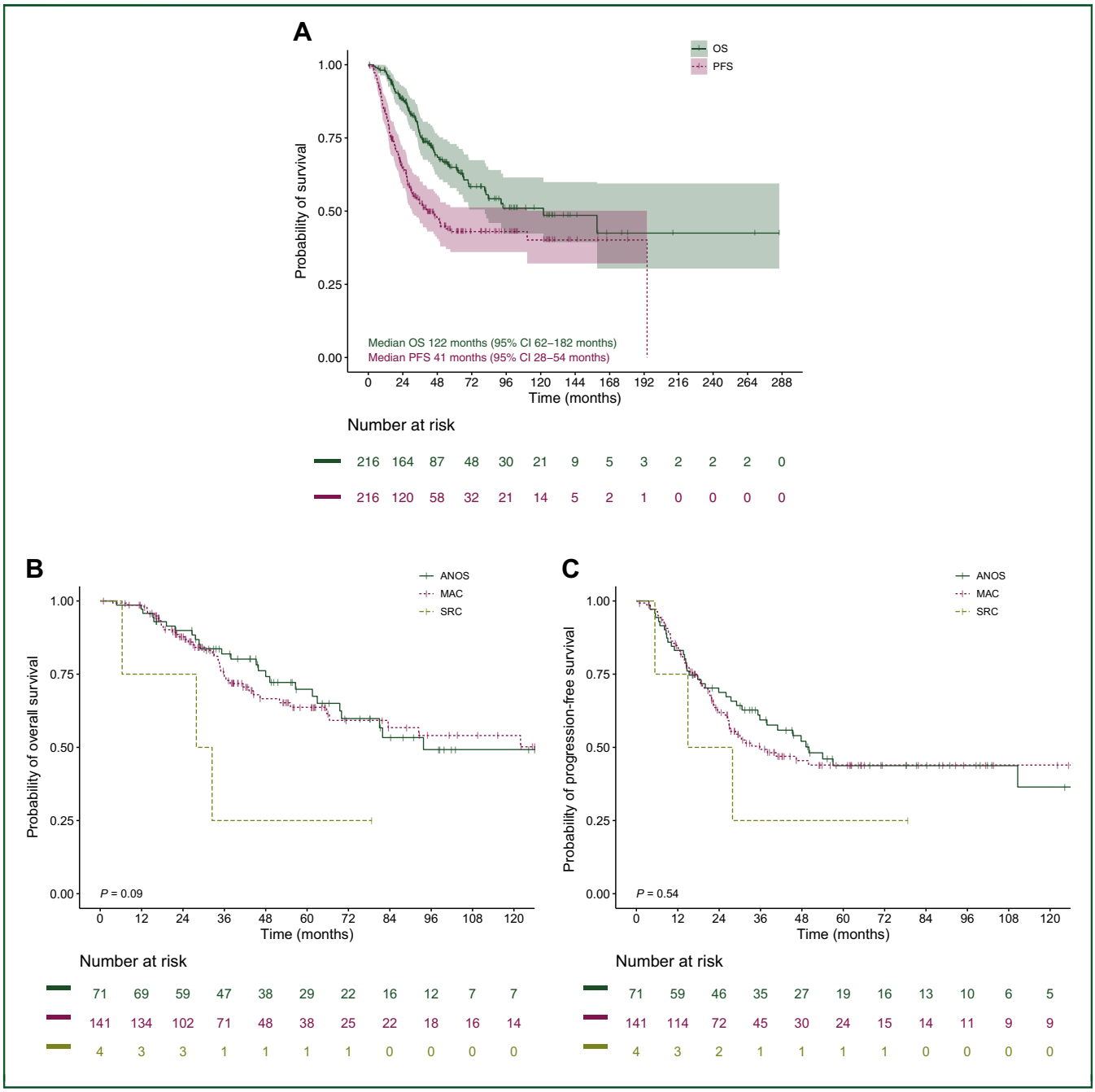

Figure 1. Survival outcomes of patients with appendix adenocarcinoma. (A) Whole cohort. (B) Overall survival by histological subtype. (C) Progression-free survival by histological subtype.
ANOS, adenocarcinoma not otherwise specified; CI, confidence interval; MAC, mucinous adenocarcinoma; OS, overall survival; PFS, progression-free survival; SRC, signet ring cell adenocarcinoma.

improved stratification for predicting PFS for the whole cohort ($P = 0.0077$) and for those with PM ($P = 0.041$); Supplementary Table S5 and Figure S11, available at https://doi.org/10.1016/j.esmoop.2023.101619.

DISCUSSION

Summary

Our study is the largest homogeneous cohort of patients with AA classified according to the WHO 2019 system to evaluate the impact of systemic chemotherapy on survival. We have demonstrated that in patients with positive lymph nodes, chemotherapy was associated with longer survival compared to no chemotherapy. Other key findings from this study include prolonged survival for patients with peritoneal AM and lower-grade PM alongside confirmation of the prognostic impact of PM grading in those with discordant disease, and preliminary validation of a three-tier grade classification for the ANOS subtype.

Survival outcomes

This cohort demonstrated better survival outcomes than expected from previous studies in this aggressive

Table 2. Univariate and multivariate analysis of prognostic factors for overall survival

Variable	Univariate, *n* = 216						Multivariate, *n* = 208			
	n	Median OS, months	95% CI	HR	95% CI	*P* value	*n*	HR	95% CI	*P* value
Subtype										
MAC	141	NR	—	Reference			135	Reference		
ANOS	71	94	55-132	1.0	0.6-1.6	0.88	70	1.6	0.8-2.9	0.17
SRC	4	28	2-53	3.3	1.0-10.8	0.044*	4	20.7	4.5-94.6	<0.001*
Age at Dx, years										
<60	116	122	54-190	Reference			112	Reference		
≥60	100	84	—	1.1	0.7-1.7	0.71	97	1.2	0.7-2.2	0.43
Sex										
Male	90	NR		Reference			85	Reference		
Female	126	82	55-108	1.4	0.9-2.3	0.16	124	3.9	1.9-7.9	<0.001*
PCI										
<10	110	NR	—	Reference			110	Reference		
10-19	49	70	48-92	2.5	1.3-4.8	0.005*	49	0.8	0.3-1.7	0.49
≥20	50	33	24-41	7.3	4.1-13.1	<0.001*	50	0.5	0.1-1.6	0.23
Cytoreduction										
CC0	134	NR		Reference			133	Reference		
CC1	38	82	—	2.0	1.0-4.0	0.058	38	1.9	0.8-4.5	0.17
CC2/3	44	25	14-35	11.6	6.8-20.0	<0.001*	38	29.2	8.3-102.6	<0.001*
CEA										
≤5	139	NR		Reference			137	Reference		
>5	55	38	25-51	3.6	2.2-6.0	<0.001*	52	1.5	0.8-2.8	0.24
Missing	22	49	40-58				20	1.4	0.6-3.4	0.44
N stage										
N0/X	170	159	56-262	Reference			164	Reference		
N1/2	46	48	28-68	2.1	1.4-3.2	0.002*	45	19.1	5.8-62.4	<0.001*
M stage										
M0	69	NR		Reference			69	Reference		
M1a	25	NR		2.2	0.5-10.1	0.29	24	1.4	0.2-8.7	0.70
M1b/c	122	49	32-66	13.7	5.0-37.8	<0.001*	116	5.4	1.7-16.9	0.004*
Primary tumour grade										
ANOS LG	53	NR		Reference			—	—	—	—
MAC G2	108	NR		1.1	0.6-2.0	0.75	—	—	—	—
ANOS HG	18	62	15-108	1.9	0.9-4.3	0.10	—	—	—	—
MAC G3	34	66	21-111	1.9	0.9-3.8	0.076	—	—	—	—
Peritoneal tumour grade										
No disease	74	NR	—	Reference			—	—	—	—
AM[a]	26	NR	—	1.5	0.4-5.8	0.59	—	—	—	—
MCP G1[b]	6	92	—	2.9	0.6-14.2	0.19	—	—	—	—
ANOS LG[b]	4	NR								
MCP G2	53	66	42-89	7.3	2.9-18.2	<0.001*	—	—	—	—
ANOS HG	30	57	35-78	9.8	4.0-14.3	<0.001*	—	—	—	—
MCP G3	23	30	20-41	15.5	6.2-38.9	<0.001*	—	—	—	—
Mucinous										
No	34	122	55-189	Reference			—	—	—	—
Yes	182	82	—	1.1	0.6-2.1	0.76	—	—	—	—
Signet ring cells (peritoneal)										
No	190	159	—	Reference			—	—	—	—
Yes	25	32	22-43	3.9	2.3-6.5	<0.001*	—	—	—	—
Systemic chemotherapy										
None	119	NR	—	Reference			118	Reference		
Neoadjuvant or adjuvant	28	NR	—	1.0	0.4-2.5	1.0	28	1.5	0.4-5.8	0.58
Prior	18	82	78-86	2.3	1.0-5.0	0.038*	18	1.9	0.5-6.7	0.33
Palliative	51	36	30-42	3.9	6.2-3.6	<0.001*	45	3.8	1.8-8.2	<0.001*
Systemic chemotherapy for N+[c]										
None	9	27	4-49	Reference			9	Reference		
Neoadjuvant or adjuvant	15	NR	—	0.6	0.2-1.7	0.37	15	0.04	0.01-0.40	0.005*
Prior	9	81	23-139	1.4	0.6-3.4	0.41	9	0.09	0.01-0.57	0.011*
Palliative	13	28	12-43	5.5	3.1-9.6	<0.001*	13	0.06	0.02-0.25	<0.001*
Systemic chemotherapy for presence of peritoneal signet ring cells										
None	9	41	15-68	Reference			—	—	—	—
Neoadjuvant or adjuvant	2	28	—	1.1	0.4-2.7	0.89	—	—	—	—

Continued

Table 2. Continued

Variable	Univariate, $n = 216$						Multivariate, $n = 208$			
	n	Median OS, months	95% CI	HR	95% CI	*P* value	*n*	HR	95% CI	*P* value
Prior	3	81	0-169	1.7	0.7-3.9	0.22	—	—	—	—
Palliative	10	21	13-29	5.5	3.2-9.7	<0.001*	—	—	—	—

Only age, sex and variables that had a *P* value of significance <0.10 in the univariate model were introduced in the multivariate Cox model. Peritoneal grade was excluded from the multivariate Cox model due to overlap with M stage variables which introduced instability to the model.
95% CI, 95% confidence interval; AM, acellular mucin; ANOS, adenocarcinoma not otherwise specified; CC, cytoreductive score; Dx, diagnosis; G1, grade 1; G2, grade 2; G3, grade 3; HG, high grade; HR, hazard ratio; LG, low grade; MAC, mucinous adenocarcinoma; MCP, mucinous carcinoma peritonei; N+, node positive; OS, overall survival; PCI, peritoneal cancer index; SRC, signet ring cell carcinoma.
[a]This group of AM includes a case of M1c disease (peritoneal AM and visceral metastasis).
[b]These groups were combined for analysis due to small patient numbers.
[c]This variable is included in the multivariate model as an interaction factor between systemic chemotherapy group × nodal status.
**P* value <0.05 is significant.

cancer.[6-11] In the localised M0 cohort, the high 5-year OS of 93% can be explained by early CRS/HIPEC with 70% receiving this procedure within 6 months of their initial diagnosis. Importantly, 15% of initially M0 patients had PM confirming a low threshold for MDT recommendation for CRS/HIPEC in this population. A similar approach was demonstrated in a small cohort of 39 patients who had CRS/HIPEC at a median of 4 months from diagnosis, resulting in median OS of 110 months and 5-year OS of 83%.[31] Despite higher proportions of lower-grade primary tumours in this cohort (85%), there was no difference in survival on univariate analysis.

In the metastatic cohort with a 5-year OS of 52%, the improved outcomes compared to the literature is influenced by selection bias at our institution, where patients with higher-risk disease that impacts resectability (such as small bowel involvement) are usually not deemed suitable for CRS/HIPEC. Thus, patients who proceed to CRS are thought to have a better prognosis.

While these improved survival outcomes are also reflected in the PFS data, there remain a small number of patients who have progression after 5 years. This supports our current surveillance schedule incorporating follow-up to 10 years and we support other peritonectomy centres taking this approach.

We emphasise the importance of complete cytoreduction (CC0) regardless of tumour burden. PCI is prognostic in the univariate analysis but after adjusting for covariates this is not retained. This means that even for patients with high PCI scores (>20), complete CC0 CRS is associated with more favourable outcomes compared to CC2-3 CRS.

A major strength of this cohort is the adherence to the WHO 2019 classification. This was to reconcile the challenges of interpreting survival outcomes from the existing literature where AA patients are included within the broad 'pseudomyxoma peritonei' group of appendix tumours that also includes AMNs.[3,12] We have analysed the ANOS subgroup by reclassification of tumour grade from two-tier into three tier. Analysis suggests that three-tiered grading was more discriminatory, with grade 1 (well differentiated) tumours associated with the best prognosis. We also show discrimination between grade 2 and grade 3 tumours in predicting both OS and PFS. Further validation in a larger cohort is needed.

Discordant cohort

Discordant peritoneal grade compared to primary tumour grade has been previously documented in appendiceal tumours but the prognostic implication for AA specifically has been suggested but not confirmed.[12,26,32] A recent study evaluated 37 cases of discordant grade.[32] These patients had no deaths and three recurrences, but this smaller cohort had a lower PCI and a higher rate of CC0-1 CRS favouring good outcomes. We confirm these findings for the first time in a larger cohort of 75 patients with pure AA with discordant grade and demonstrate the improved outcomes of those with lower peritoneal grade and higher primary grade compared to patients with higher peritoneal grade and lower primary grade. This confirms that peritoneal and primary grade should continue to be reported separately and for those with discordance, the peritoneal grade is most prognostic. The excellent survival outcomes of AM and lower-grade PM seen in our study have previously been demonstrated in mixed cohorts of primary AMNs, and this study confirms these outcomes in a larger cohort of pure AA.[32-34] The implication of this finding is that patients with AA primary diagnosis who experience AM or lower-grade PM can be reassured of expected good longer-term outcomes.

The presence of srcs is associated with poor prognosis and some authors have questioned the role of CRS/HIPEC for patients with AA who have evidence of these.[6,8,27,35] Our study confirms that the presence of srcs was most relevant in the PM setting and that patients who have srcs in the primary tumour can still have a good outcome.

Lymph node status

Our cohort demonstrates that positive nodes are predictive of survival even in the metastatic setting. On multivariate analysis, patients with positive lymph nodes received benefit from chemotherapy. Other authors have suggested focusing chemotherapy on this positive node group, which has been shown in some studies to predict outcomes.[36,37] There are also studies that have shown that lymph node status is not a

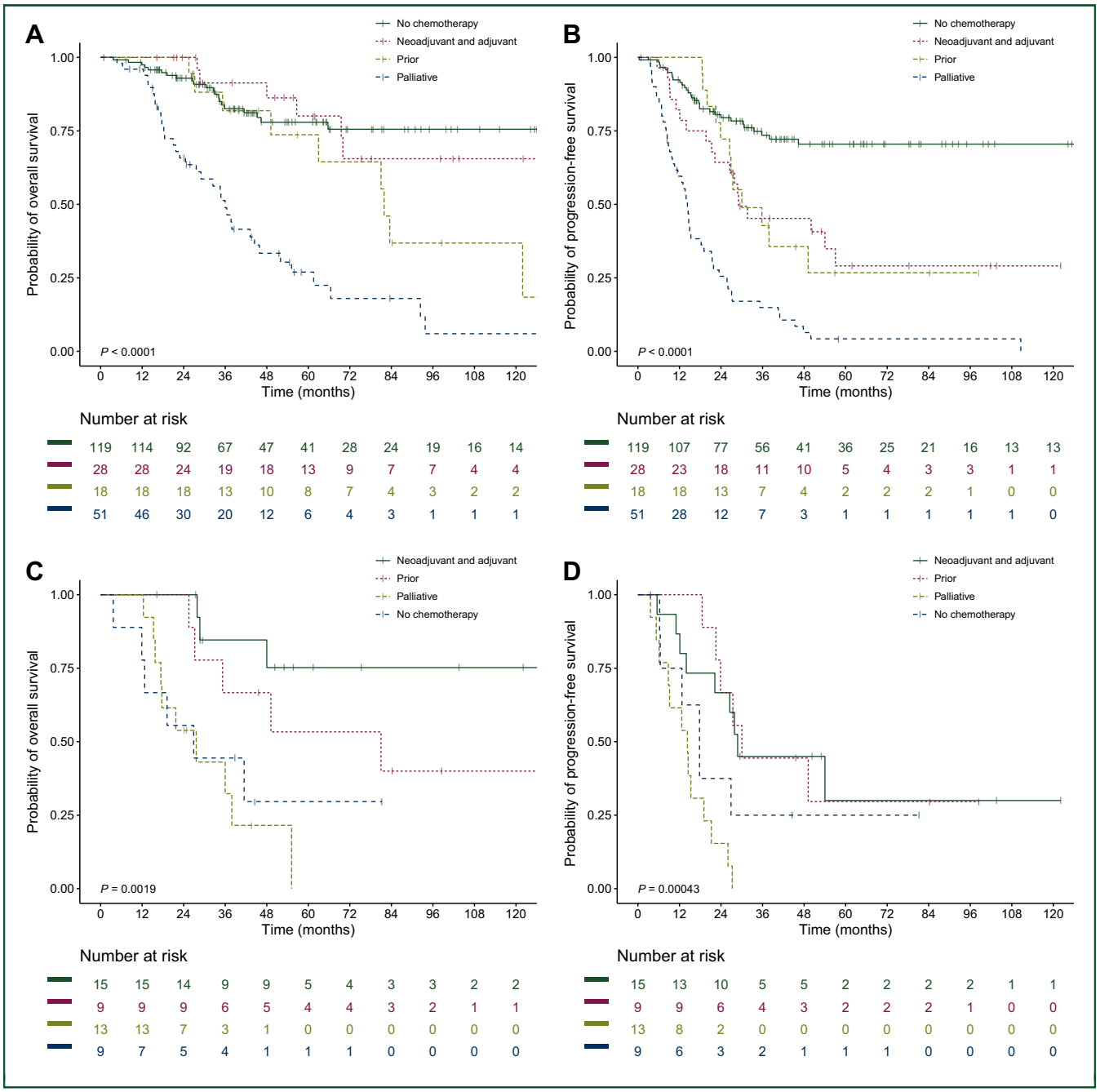

Figure 2. Outcomes of patients with appendix adenocarcinoma receiving chemotherapy. (A) Overall survival. (B) Progression-free survival. (C) Overall survival in patients with positive lymph nodes. (D) Progression-free survival in patients with positive lymph nodes.

significant predictor of survival after CRS/HIPEC.[38,39] For lymph nodes to be fully assessed, patients require a nodal resection, such as during right hemicolectomy. However, data suggest that for MAC with peritoneal seeding and low-risk ANOS (T1, low grade, no lymphovascular invasion) there is no survival advantage for including right hemicolectomy over appendicectomy or partial colectomy.[38,40,41] Our institution has a selective strategy for right hemicolectomy based on tumour grade, radiological findings and patient factors. This is appropriate, given the 21% rate of lymph node involvement in our current cohort is consistent with the literature.

Systemic chemotherapy

There have been few prospective studies and several retrospective reviews attempting to evaluate the role of systemic chemotherapy in AA.[42] Much of the literature has focused on the poor outcomes of those receiving perioperative chemotherapy, but this is in mixed disease cohorts, with those selected for chemotherapy having the worst risk factors, which we have seen in our results on univariate analysis.[28,43] When these groups are further interrogated, patients with non-mucinous, poorly differentiated, positive lymph nodes and srcs may derive benefit from

Table 3. Radiological and pathological responses to systemic chemotherapy[a] in patients with appendix adenocarcinoma

	All (n = 97)	%	95% CI
Evaluable for radiological response (RECIST1.1)	77	100	
PD	22	29	19-39
SD	13	17	9-25
ORR	10	13	5-21
PR	9	12	—
CR	1	1	—
Missing	32	42	—
Pathological response[b]	15	100	
TRG 0-1	3	20	4-48
TRG 0	1[c]	7	—
TRG 1	2	13	—
TRG 2-3	12	80	52-95
TRG 2	1	7	—
TRG 3	11	73	—

CI, confidence interval; CR, complete response; ORR, overall response rate; PD, progressive disease; PR, partial response; SD, stable disease; TRG, tumour regression grade.
[a]This assessment is for first systemic chemotherapy regimen received.
[b]For patients who had preoperative chemotherapy.
[c]One case had acellular mucin only with no neoplastic component.

chemotherapy.[26,27,36,44] We are unable to draw conclusions from our analysis of the role of systemic chemotherapy in patients with peritoneal srcs due to small numbers, but cannot exclude a potential benefit in the N/ACT or prior chemotherapy groups.

Oxaliplatin and fluoropyrimidine chemotherapy has been the preferred first-line systemic regimen used worldwide and this is reflected in our cohort.[25,45-47] We demonstrated lower chemotherapy response rates compared to the literature where one study demonstrated response rates to neoadjuvant chemotherapy up to 58%, another with up to 45% stable disease rate and 24% partial response rate.[25,46,48] However, given the retrospective nature of these studies and differing referral patterns, it is difficult to compare these results. Furthermore, radiological response is challenging to assess in PM and these studies do not always use RECIST, with some authors suggesting that a modified peritoneal RECIST should be developed.[49] A limitation to our study is that we have not carried out centralised review of response imaging; however, most of our patients have their imaging either at our institution or the images are re-reported by our expert radiologists.

There are limited data on pathological response to neoadjuvant chemotherapy and our cohort of 15 patients who had this approach provides further insights. One prospective cohort of 34 patients with high-grade PM were treated with preoperative oxaliplatin and 5-fluorouracil (FOLFOX4).[50,51] Although there was no improvement in OS in patients who received preoperative chemotherapy compared to those who did not (median 51 versus 37 months, $P = 0.56$), 10 (29%) patients had a pathological response, slightly higher than our pathological response rate of 20%. It is unclear what the impact of the TRG score for the pathological response is on predicting outcomes as this has been validated for response to chemoradiotherapy in rectal cancer and the application to non-rectal cancer adenocarcinoma is unclear.[30]

There is some literature that suggests in AA that regression in grade (from high to low) could be driven by systemic chemotherapy.[51] In our cohort, 13 patients in the discordant grade cohort received systemic chemotherapy before CRS which is challenging to draw conclusions from. The biology of this potential phenomenon is poorly understood, and theories include cancer cell heterogeneity, grade regression and stimulation of mucin production as a chemotherapy resistance mechanism.[51-53]

There have been studies that demonstrate a benefit of adjuvant chemotherapy for patients with AA PM following CRS/HIPEC.[28,54,55] The rationale for chemotherapy referral has not been systematically evaluated in these retrospective studies. One of the key reasons for referral for perioperative chemotherapy that emerged from our study was the presence of positive lymph nodes. Our findings confirm that selection of these patients for chemotherapy is appropriate. There were also a number of patients who historically had an external decision for chemotherapy to be given after their initial local procedure before referral to our specialist centre. Some of these patients have been selected by potential referrers as unresectable due to a high burden of disease and this reinforces the need for early referral to a specialist MDT.

Limitations

A limitation of our study was that despite the large total cohort, we still had small numbers of patients in all examined subgroups. As such, the evaluation of neoadjuvant versus adjuvant chemotherapy could not be carried out. Advantages of neoadjuvant chemotherapy include assessment of biological response to help select for future CRS and which could inform choice of future treatments; it deals with occult metastases earlier and there is improved deliverability of systemic therapy as there is no waiting period for operative recovery. Benefits of adjuvant chemotherapy include improved knowledge of disease histology, staging and amount of RD; it minimises the risk of progression through a treatment window and maintains patients' fitness for CRS. Furthermore, it needs to be emphasised that there is heavy selection bias to improved outcomes for patients selected for multimodality treatments and an aggressive chemotherapeutic approach.

Selection bias also influences patients who are referred to specialist centres and subsequent MDTs. Patients with rapidly progressing, higher-grade disease are more likely to be managed in the community without subsequent referral for specialist MDT opinion as they present with advanced and likely inoperable disease. Therefore, the biology of disease that is referred to specialist centres likely biases to improved outcomes.

Another limitation is our study is not representative of all patients with advanced disease. Patients who undergo debulking surgery with higher burden RD (CC2-3) may still derive benefit from a reduced burden of disease. However, literature evaluating AA regarding these questions is sparse and further study is needed on all patients with advanced disease.

Patients who undergo, or planning for, chemotherapy may have more frequent intervals for radiological imaging. These patients are more likely to have worse PFS outcomes because of detection bias from more frequent imaging, meaning events are picked up earlier.

Finally, loss to follow-up is an inherent limitation of retrospective cohorts. The median follow-up of our cohort was almost 5 years; therefore, the 5-year data are robust. However, the longer-term outcomes are less accurate, which is depicted by the widening CI. Regardless, these results remain highly informative to ongoing clinical practice and raise the possibility of generating AA-specific society guidelines.

Conclusions

This study represents the single largest series of pure AA with long-term follow-up data that evaluates the role of systemic chemotherapy in different disease settings and confirms that positive lymph node status identifies a subgroup of patients with AA who derive the most benefit from systemic chemotherapy. Patients with high-grade disease, peritoneal srcs and positive lymph nodes had the poorest outcomes and should be the focus for clinical trials and development of novel therapies. Finally, we demonstrate the importance of prognostication based on disease stage and PM grade, and, the relevance of continuing to report primary tumour and PM grade separately. In particular, we can reassure patients with AM and lower-grade PM of excellent outcomes, akin to those with localised (M0) disease.

GLOSSARY

Cytoreduction score—a score of residual peritoneal disease calculated after maximal resection during cytoreductive surgery defined as CC0 (no residual disease), CC1 (<0.25 cm residual disease), CC2 (0.25-2.5 cm residual disease) and CC3 (>2.5 cm residual disease).

Cytoreductive surgery—a radical surgical procedure where the surgeon resects the peritoneal lining and if needed associated internal organs of the abdomen.

Heated intraperitoneal chemotherapy—cytotoxic chemotherapy agents that are heated to 42°C and instilled into the abdominal cavity after surgical cytoreduction to treat occult cancer cells.

Peritoneal cancer index—the total score of individually scored regions of peritoneal cancer by the surgeons at the start of cytoreductive surgery (scored 0-39).

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DISCLOSURE

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DATA SHARING

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix 1.3

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

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Serum Tumor Markers and Outcomes in Patients With Appendiceal Adenocarcinoma

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Abstract

IMPORTANCE Serum tumor markers carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and cancer antigen 125 (CA125) have been useful in the management of gastrointestinal and gynecological cancers; however, there is limited information regarding their utility in patients with appendiceal adenocarcinoma.

OBJECTIVE To assess the association of serum tumor markers (CEA, CA19-9, and CA125) with clinical outcomes and pathologic and molecular features in patients with appendiceal adenocarcinoma.

DESIGN, SETTING, AND PARTICIPANTS This is a retrospective cohort study at a single tertiary care comprehensive cancer center. The median (IQR) follow-up time was 52 (21-101) months. Software was used to query the MD Anderson internal patient database to identify patients with a diagnosis of appendiceal adenocarcinoma and at least 1 tumor marker measured at MD Anderson between March 2016 and May 2023. Data were analyzed from January to December 2023.

MAIN OUTCOMES AND MEASURES Association of serum tumor markers with survival in patients with appendiceal adenocarcinoma. Cox proportional hazards regression analyses were also performed to assess associations between clinical factors (serum tumor marker levels, demographics, and patient and disease characteristics) and patient outcomes (overall survival).

RESULTS A total of 1338 patients with appendiceal adenocarcinoma were included, with a median (range) age at diagnosis of 56.5 (22.3-89.6) years. The majority of the patients had metastatic disease (1080 patients [80.7%]). CEA was elevated in 742 of the patients tested (56%), while CA19-9 and CA125 were elevated in 381 patients (34%) and 312 patients (27%), respectively. Individually, elevation of CEA, CA19-9, or CA125 were associated with worse 5-year survival; elevated vs normal was 81% vs 95% for CEA (hazard ratio [HR], 4.0; 95% CI, 2.9-5.6), 84% vs 92% for CA19-9 (HR, 2.2; 95% CI, 1.4-3.4), and 69% vs 93% for CA125 (HR, 4.6; 95% CI, 2.7-7.8) ($P < .001$ for all). Quantitative evaluation of tumor markers was associated with outcomes. Patients with highly elevated (top 10th percentile) CEA, CA19-9, or CA125 had markedly worse survival, with 5-year survival rates of 59% for CEA (HR, 9.8; 95% CI, 5.3-18.0), 64% for CA19-9 (HR, 6.0; 95% CI, 3.0-11.7), and 57% for CA125 (HR, 7.6; 95% CI, 3.5-16.5) ($P < .001$ for all). Although metastatic tumors had higher levels of all tumor markers, when restricting survival analysis to 1080 patients with metastatic disease, elevated CEA, CA19-9, or CA125 were all still associated worse survival (HR for CEA, 3.4; 95% CI, 2.5-4.8; $P < .001$; HR for CA19-9, 1.8; 95% CI, 1.2-2.7; $P = .002$; and HR for CA125, 3.9; 95% CI, 2.4-6.4; $P < .001$). Interestingly, tumor grade was not associated with CEA or CA19-9 level, while CA-125 was slightly higher in high-grade tumors relative to low-grade tumors (mean value, 18.3 vs 15.0; difference, 3.3;

(continued)

Key Points

Question Are serum tumor markers carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), or cancer antigen 125 (CA125) associated with outcomes in patients with appendiceal adenocarcinoma?

Findings In this cohort study of 1338 patients, elevation of CEA, CA19-9, or CA125 was associated with significantly worse 5-year survival; 81% vs 95%, 84% vs 92%, and 69% vs 93% elevated vs normal, respectively. Moreover, quantitative evaluation of tumor markers was associated with outcomes.

Meaning These findings suggest elevated levels of CEA, CA19-9, and CA125 are associated with overall survival in appendiceal adenocarcinoma, highlighting the importance of including all 3 biomarkers in the initial workup of patients with this disease.

+ Supplemental content

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Abstract (continued)

95% CI, 0.9-3.7; *P* < .001). Multivariable analysis identified an incremental increase in the risk of death with an increase in the number of elevated tumor markers, with an 11-fold increased risk of death in patients with all 3 tumor markers elevated relative to those with none elevated. Somatic mutations in *KRAS* and *GNAS* were associated with significantly higher levels of CEA and CA19-9.

CONCLUSIONS AND RELEVANCE In this retrospective study of serum tumor markers in patients with appendiceal adenocarcinoma, CEA, CA19-9, and CA125 were associated with overall survival in appendiceal adenocarcinoma. Given their value, all 3 biomarkers should be included in the initial workup of patients with a diagnosis of appendiceal adenocarcinoma.

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Introduction

Appendiceal adenocarcinoma (AA) is a heterogenous disease, with marked contrast in the natural history of low-grade and high-grade tumors (5-year overall survival [OS] 68% for low-grade vs 7% for high-grade).[1-4] Unfortunately, for the majority of patients, there is already metastatic disease at the time of diagnosis.[5,6] Metastatic spread of AA is almost exclusively limited to the peritoneal cavity, causing the clinical syndrome pseudomyxoma peritonei (PMP), in which the peritoneal surfaces and omentum are involved with diffuse gelatinous, mucinous implants.[7,8] However, PMP progression is difficult to measure with traditional cross-sectional imaging as it frequently exists as a contiguous, erratically shaped area in the peritoneal cavity. In addition, current Response Evaluation Criteria in Solid Tumor (RECIST) criteria do not consider mucinous or cystic disease as measurable. For these reasons, standard RECIST criteria are poorly applicable to AA.[9] Moreover, AA is a slowly progressive disease, and classically defined thresholds for determining changes in disease extent (typically ≥20% increase) may take years to occur. For these reasons, having a more dependable method to distinguish patients with AA who are more or less likely to have favorable disease outcomes would be highly advantageous for guiding patient discussions and treatment options.

Serum tumor markers (TMs) are commonly used for aid in evaluating diagnosis, prognosis, and treatment response in different types of malignant neoplasms.[10,11] Three TMs have been well established in gastrointestinal cancers: carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and cancer antigen 125 (CA125).[12-14] These TMs have been associated with metastatic dissemination of tumor cells.[15-17] While serum markers have been useful in detecting gastrointestinal tumors, there is a lack of information regarding their efficacy in patients with AA. Researchers have suggested using CEA as a potential TM based on its utility in detection and management of colon cancer.[12] However, studies conducted thus far have not established a definitive and consistent correlation between any of the TMs and AA outcomes.[18-21]

The objectives of this study were to investigate the association between serum TM levels and clinical outcomes, as well as pathologic and molecular features across the spectrum of AA. We hypothesized that elevation in any of the 3 TM levels would be associated with a decline in 5-year survival, independent of other factors.[10,11,22-24]

Methods

Under approved institutional review board (IRB) protocol from MD Anderson Cancer Center (MDACC), the Palantir Foundry software system (Palantir)[25-28] was used to query the MDACC internal patient database to identify patients with a diagnosis of AA and at least 1 TM measured at MDACC between 2016 to 2023 for inclusion in this retrospective study. The platform aids in the integration, analysis, extraction, and transformation of clinical data, allowing the many elements of

the electronic health record to be merged into a dataset amenable to research analyses. The data cutoff point was May 12, 2023. The IRB waived consent under 45 CFR 46.116(F) as the research involves no more than minimal risk of harm to the participants. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for cohort studies.

Statistical Analysis

The value of each TM measured was categorized as normal, elevated, or highly elevated. TM levels below the laboratory upper limit of normal (CEA ≥3 ng/mL, CA19-9 ≥35 U/mL, and CA125 ≥35 U/mL from March 2016 to March 2018, and CEA >3.8 ng/mL, CA19-9 >35 U/mL, and CA125 >38 U/mL from April 2018 to May 2023, as the TM panels changed in 2018) were defined as normal. TM levels above the laboratory upper limit of normal were defined as elevated; while levels in the top tenth percentile for each respective TM (>99.8 ng/mL for CEA, >338.6 U/mL for CA19-9, and >99.0 U/mL for CA125) were defined as highly elevated. For patients with more than 1 measurement for each TM, the highest measurement was considered for analysis. Other clinical information collected through the platform included patient demographics, histopathology, tumor grade, surgical history, and tumor somatic mutation profiles for patients who had next-generation sequencing (NGS) performed at MDACC. Patients' race and ethnicity were self-reported and were assessed to better understand if race is associated with survival or disease outcomes. Histologic classification and grade were collected from patients' pathology records. Pathologic diagnosis was determined by a team of expert pathologists (including M.W.T. and W.C.F.) using a 3-tier classification.[29] Well-differentiated and well- to moderately differentiated tumors were considered low-grade tumors, while moderately differentiated, moderately to poorly differentiated, and poorly differentiated tumors were considered high-grade tumors. Low-grade tumors lacked high cellularity, invasive implants, or significant cytologic atypia. High-grade tumors exhibited invasive implants, cytologic atypia, and signet ring cells. OS was defined as the time from initial diagnosis with appendiceal cancer until death. From the main cohort, a subcohort of patients who received chemotherapy at MDACC, had CEA measured 30 days before chemotherapy start date, and had CEA measured again within 180 days after chemotherapy were identified to evaluate the utility of TM measurement in assessing response to treatment; OS was assessed from chemotherapy start date until death. Significance was set at a *P* value of .05. All statistical analysis was performed using GraphPad Prism software version 9.0.0 for Windows (GraphPad Software) and RStudio (RStudio Team). Data were analyzed from January to December 2023. For additional details regarding methods, see eAppendix in Supplement 1.

Results

Patient Characteristics

Between 2016 to 2023, a total of 1338 patients who had a diagnosis of AA and at least 1 TM (CEA, CA19-9, or CA125) measured at MD Anderson were identified (eFigure 1 in Supplement 1). The median (IQR) follow-up time from AA diagnosis was 52 (21-101) months and median (range) age of the patients at diagnosis was 56.5 (22.3-89.6) years (**Table**). The median OS was not yet reached, while 5-year and 10-year OS were 85% and 75%, respectively. The study population included slightly more female patients (753 patients [56.3%]), and 37 self-reported reported Asian race (2.8%), 81 self-reported Black race (6.1%), and 1067 patients self-reported White race (79.7%). Most of the patients in our study had stage IV metastatic disease (1080 patients [80.7%]). A total of 693 patients (52.4%) had mucinous tumors; 130 had colonic (9.8%), 93 had goblet cell (7.0%), 221 had signet ring cell (16.7%), and 147 had a mix of signet ring and goblet cell (11.1%) histopathology. Overall, 766 patients (57.2%) had high-grade tumors (moderately or poorly differentiated) (Table; eFigure 2 in Supplement 1).

Table. Patient Characteristics of MD Anderson Cohort

Patient characteristics	Patients, No. (%) (N = 1338)
Age at diagnosis, median (range), y	56.5 (22.3-89.6)
Race and ethnicity	
Asian	37 (2.8)
Black or African	81 (6.1)
Hispanic	127 (9.5)
White	1067 (79.7)
Other[a]	26 (1.9)
Sex	
Female	753 (56.3)
Male	585 (43.7)
Smoking status	
Never	897 (67.0)
Smoker	53 (4.0)
Former	299 (22.3)
Missing	89 (6.7)
Alcohol use status	
Yes	743 (55.5)
No	478 (35.7)
Missing	117 (8.7)
Histologic grade	
Well differentiated	465 (34.8)
Moderately differentiated	410 (30.6)
Poorly differentiated	412 (30.8)
Missing	51 (3.8)
Histologic grade binary	
Low grade	521 (38.9)
High grade	766 (57.2)
Missing	51 (3.8)
Histopathology	
Mucinous	693 (52.4)
Colonic	130 (9.8)
Goblet cell	93 (7.0)
Signet ring cell	221 (16.7)
Mix of goblet and signet	147 (11.1)
Missing	54 (3.0)
Disease metastatic state	
Localized disease (stage I, II, III)	258 (19.3)
Metastatic disease (stage IV)	1080 (80.7)
No. of elevated tumor markers	
0	485 (36.2)
1	419 (31.3)
2	286 (21.4)
3	148 (11.1)
CEA	
Normal	589 (44.0)
Elevated	609 (45.5)
Highly elevated	133 (9.9)
Not tested	7 (0.5)
CA19-9	
Normal	751 (56.1)
Elevated	268 (20.0)

(continued)

Table. Patient Characteristics of MD Anderson Cohort (continued)

Patient characteristics	Patients, No. (%) (N = 1338)
Highly elevated	113 (8.4)
Not tested	206 (15.4)
CA125	
Normal	853 (63.8)
Elevated	196 (14.6)
Highly elevated	116 (8.7)
Not tested	173 (12.9)
Overall survival	
Median (range), mo	Not yet reached (0-272)

Abbreviations: CA125, cancer antigen 125; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

[a] Other race includes Native Hawaiian, Other Pacific Islander, American Indian, or Alaska Native.

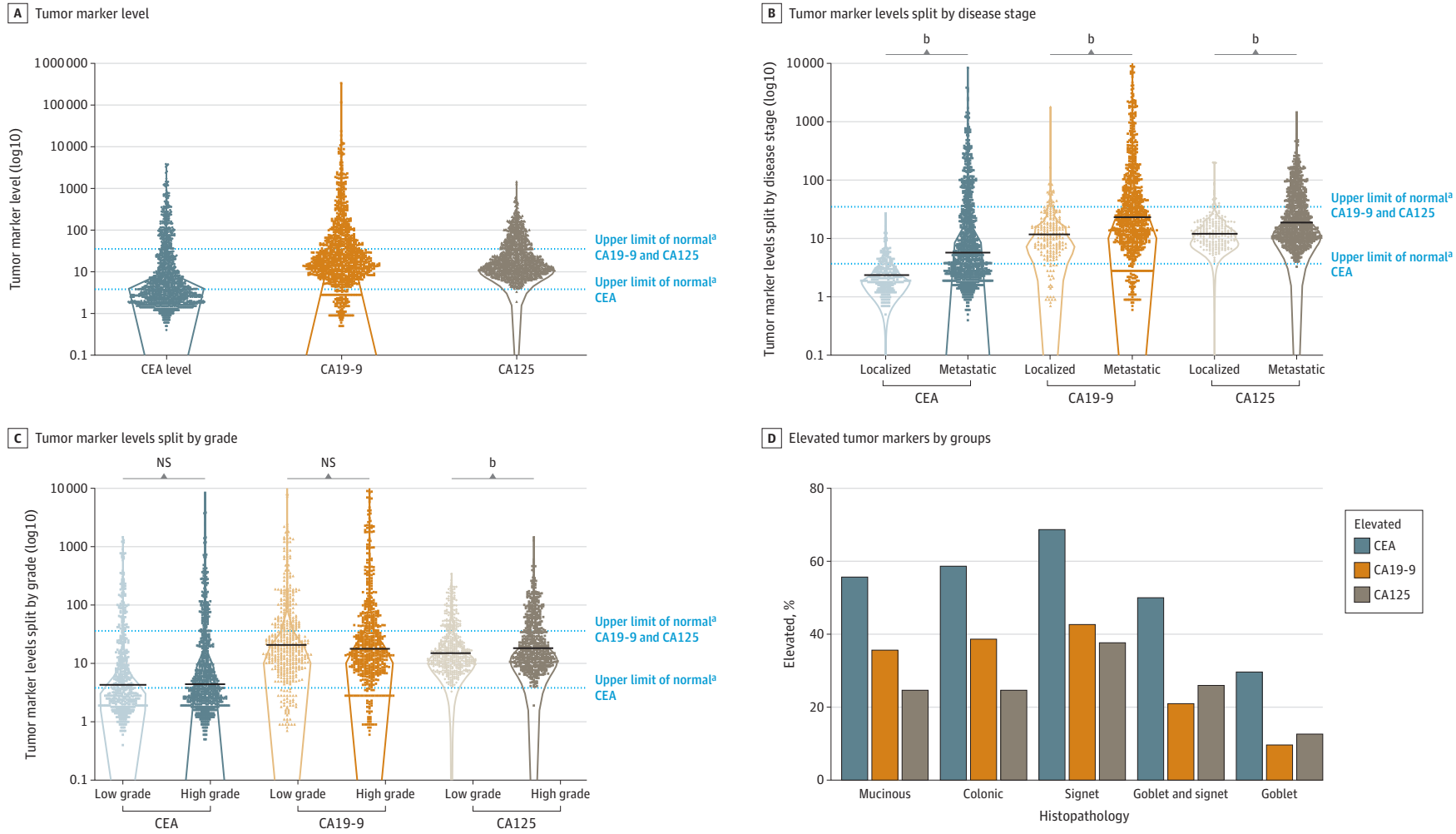
In the validation cohort (The Christie NHS Foundation Trust Cohort), 216 patients with AA were identified. A total of 141 had mucinous adenocarcinoma, 71 had adenocarcinoma not otherwise specified, and 4 had signet ring cell carcinoma.[30] Baseline demographics and clinical characteristics are summarized in eTable 1 in Supplement 1. At initial diagnosis, 69 patients (32%) had localized disease on CT imaging and preoperative pathology, and 147 (68%) had metastatic disease. At a median (range) follow-up of 56 (1-286) months, the median OS was 122 (95% CI, 62-182) months with 5-year and 10-year OS of 63% and 51%, respectively.

Tumor Marker Assessment

CEA was the most frequently tested TM (1331 patients); CA19-9 and CA125 were also evaluated in the majority of patients (1132 and 1165 patients, respectively) (eTable 2 in Supplement 1). CEA was elevated 742 of the patients tested (56%, including highly elevated levels), while CA19-9 was elevated in 381 patients (34%, including highly elevated levels) and CA125 in 312 of the patients tested (27%, including highly elevated levels); 148 patients (11%) had elevated levels of all 3 TMs (eTable 2, eTable 3, and eFigure 3 in Supplement 1). There has been a trend of increased testing of all 3 markers at time of diagnosis starting in 2018 (eFigure 4 in Supplement 1).

CA19-9 and CA125 levels were statistically equivalent between male and female patients (eFigure 5 in Supplement 1). CEA, CA19-9, and CA125 were significantly higher in patients with metastatic disease compared with patients with localized disease (median [IQR] of metastatic vs localized for CEA, 5.8 [2.6-26.9] vs 2.2 [1.6-3.4]; difference, 3.6; 95% CI, 2.5 to 4.3 ng/mL; for CA19-9, 23.3 [10.1-77.3] vs 11.6 [6.6-19.3]; difference, 11.7; 95% CI, 8.0 to 14.7 U/mL; and for CA125, 18.5 [10.5-50.0] vs 12.5 [8.8-18.5]; difference, 6.0; 95% CI, 4.1 to 8.3 U/mL) ($P < .001$ for all) (**Figure 1**B). CEA and CA19-9 were statistically equivalent for high- and low-grade tumors (CEA difference, 0.1; 95% CI, −0.2 to 0.6; $P = .40$; CA19-9 difference, −3.0; 95% CI, −3.0 to 1.5; $P = .55$), CA125 was slightly higher in high-grade tumors (median [IQR] of high grade vs low grade, 18.3 [10.5-48.8] vs 15.0 [9.8-31.3]; difference, 3.25; 95% CI, 0.9 to 3.7; $P < .001$) (Figure 1C; eTable 4 in Supplement 1). Comparing across histologic findings, all 3 TMs were most often elevated in patients with signet ring cell histologic findings (CEA, 153 patients [69%]; CA19-9, 77 patients [43%]; and CA125, 73 patients [38%]; Kruskal-Wallis statistic, 47.4 for CEA, 34.0 for CA19-9, and 29.9 for CA125; $P < .001$ for each) and least likely to be elevated in Goblet cell adenocarcinoma (Figure 1D; eFigure 6, eTable 5, eTable 6, and eTable 7 in Supplement 1). When measured on the same day, CEA and CA19-9 levels were highly correlated ($r = 0.63$; $P < .001$), but there was a subset of patients with highly elevated CEA but normal CA19-9, consistent with the fact that the carbohydrate CA19-9 cannot be produced in patients who genetically lack the Lewis antigen A.[31] Both CEA and CA19-9 were correlated with CA125 but to a lesser degree (CEA $r = 0.29$; CA19-9 $r = 0.25$; $P < .001$ for each) (eFigure 7 in Supplement 1). In

Figure 1. Distribution of Carcinoembryonic Antigen (CEA), Carbohydrate Antigen 19-9 (CA19-9), and Cancer Antigen (CA125) Tumor Marker Levels



A, Distribution of all patients CEA, CA19-9, and CA125 tumor markers levels; each point represents one patient. B, Distribution of all patients CEA, CA19-9, and CA125 tumor markers levels split by disease stage (metastatic vs localized); lines represent median levels. C, Distribution of all patients CEA, CA19-9, and CA125 tumor markers levels split by grade; lines represent median levels. D, Percentage of elevated tumor markers in different histopathological groups.

[a] Laboratory upper limit of normal (CEA 3.8 ng/mL, CA19-9 35 U/mL, and CA125 38 U/mL).

[b] $P < .001$.

patients who had all 3 markers measured (1112 patients), isolated CEA elevation was more common (207 patients [18.6%]) than CA19-9 (40 patients [3.6%]) or CA125 (43 patients [3.9%]); all 3 TMs were elevated in 148 patients (13.3%) (eFigure 3 in Supplement 1).[32]

Outcomes

Kaplan-Meier survival analysis of OS by TM marker level (normal, elevated, or highly elevated) demonstrated that CEA, CA19-9, and CA125 were all associated with OS (eFigure 8 in Supplement 1). Compared with 5-year OS of 92% to 95% in patients with normal values for each TM, 5-year OS with elevated CEA was 81% (hazard ratio, [HR], 4.0; 95% CI, 2.9-5.6), CA19-9 was 84% (HR, 2.2; 95% CI, 1.4-3.4), and CA125 was 69% (HR, 4.6; 95% CI, 2.7-7.8) ($P < .001$ for all). Moreover, 5-year OS for those with highly elevated CEA was 59% (HR, 9.8; 95% CI, 5.3-18.0), CA19-9 was 64% (HR, 6.0; 95% CI, 3.0-11.7), and CA125 was 57% (HR, 7.6; 95% CI, 3.5-16.5) ($P < .001$ for all). Given the association of metastasis with increased TM levels, the survival analysis for each TM was repeated, restricting to only patients with metastatic disease (1080 patients). Elevated levels of all TMs remained associated with OS (HR elevated vs normal for CEA, 3.4; 95% CI, 2.5-4.8; $P < .001$; HR for CA19-9, 1.8; 95% CI, 1.2-2.7; $P = .002$; HR for CA125, 3.9; 95% CI, 2.4-6.4; $P < .001$) (**Figure 2**A, Figure 2B, and Figure 2C) as well as highly elevated levels (HR highly elevated vs normal for CEA, 7.4; 95% CI, 4.2-12.8; HR for CA19-9, 4.7; 95% CI, 2.6-8.5; HR for CA125, 6.4; 95% CI, 3.1-13.1) ($P < .001$ for all). Survival analysis for each TM was again repeated controlling for tumor grade. Elevated levels of all TMs remained associated with OS in both low-grade and high-grade tumor subgroups (**Figure 3**A and Figure 3B; eFigure 9 in Supplement 1). As a further control, analysis was restricted to those patients with TMs measured within the initial 6 months from the date of diagnosis (560 for CEA, 461 for CA19-9, and 475 for CA125) to allow for assessment of TMs at time of diagnosis. Again, survival analysis by TM level (normal, elevated, or highly elevated) demonstrated that CEA, CA19-9, and CA125 were all associated with OS (HR, 2.4; 95% CI, 1.4-3.9 for elevated CEA; HR, 3.6; 95% CI, 1.4-9.3 for highly elevated CEA; HR, 1.8; 95% CI, 0.9-3.7 for elevated CA19-9; HR, 4.5; 95% CI, 2.1-9.8 for highly elevated CA19-9; HR, 2.4; 95% CI, 1.2-4.6 for elevated CA125; and HR, 4.0; 95% CI, 2.0-7.8 for highly elevated CA125) ($P < .001$ for all) (eFigure 10 in Supplement 1).

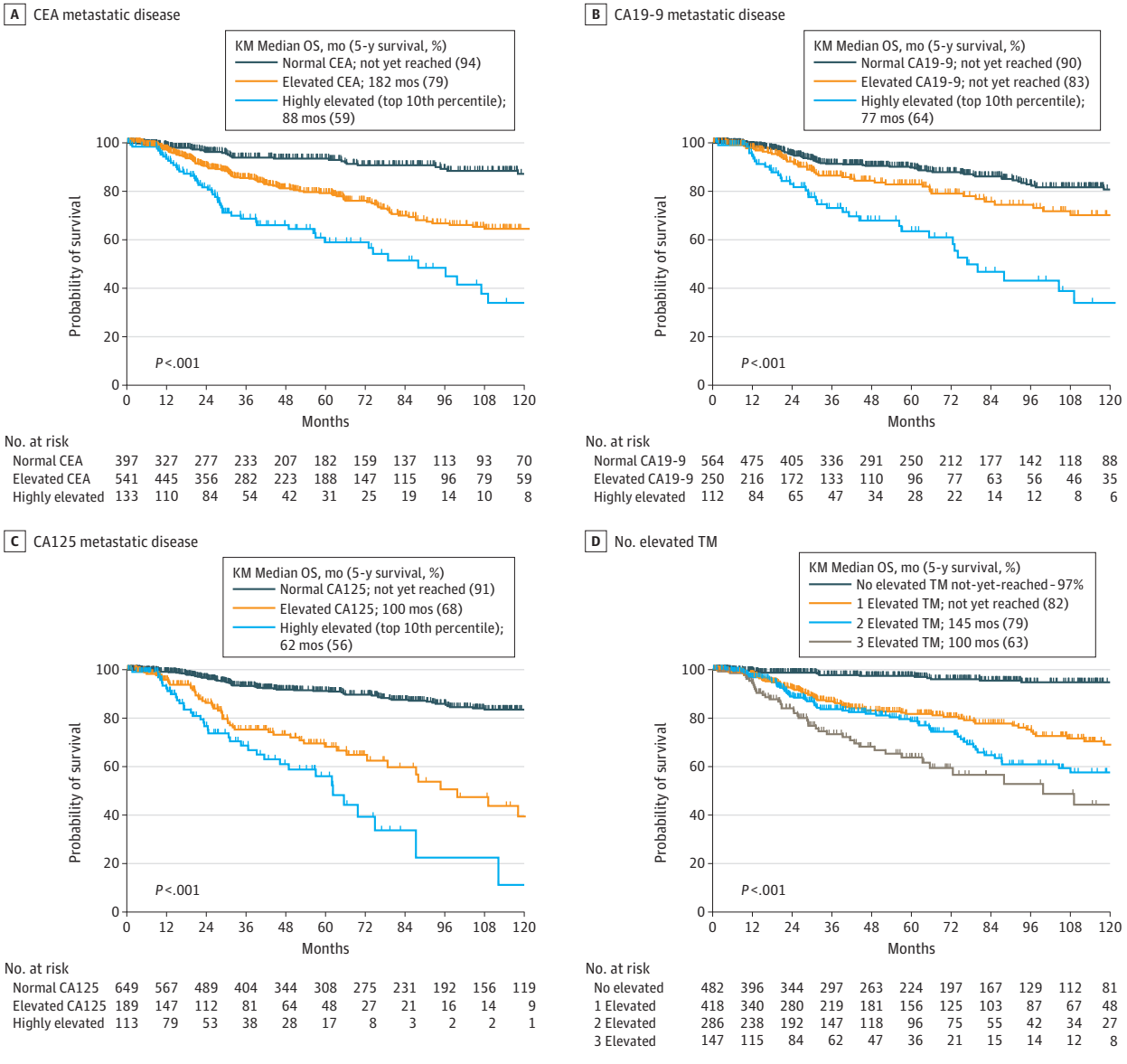
In the validation cohort, similar to MD Anderson cohort, Kaplan-Meier survival analysis of OS by TM level (normal, elevated, or highly elevated) demonstrated that CEA, CA19-9, and CA125 were all associated with OS; however, only the HR for highly elevated CA19-9 achieved statistical significance (eFigure 11 in Supplement 1). Compared with 5-year OS of 75% to 84% in the patients with normal values for each TM, 5-year OS with elevated CEA was 43% (HR, 3.5; 95% CI, 2.0-6.3; $P < .001$), CA19-9 was 59% (HR, 1.6; 95% CI, 0.9-3.0; $P = .09$), and CA125 was 57% (HR, 2.1; 95% CI, 1.1-4.2; $P = .006$). Moreover, 5-year OS for those with highly elevated CEA was 28% (HR, 5.8; 95% CI, 2.1-15.8), CA19-9 was 33% (HR, 4.9; 95% CI, 1.7-14.0), and CA125 was 29% (HR, 4.1; 95% CI, 1.6-10.0) ($P < .001$ for all). (eFigure 11 in Supplement 1). Given the association of metastasis with increased TM levels, the survival analysis for each TM was repeated restricting to the 147 patients with metastatic disease. Elevated levels of CEA remained associated with OS (HR, 2.4; 95% CI, 1.4-4.1; $P = .001$), while OS was not associated with CA19-9 and CA125 (HR for CA19-9, 1.3; 95% CI, 0.6-2.8; $P = .49$; HR for CA125, 1.5; 95% CI, 0.8-2.8; $P = .18$). However, highly elevated levels for all remained associated with OS (HR highly elevated vs normal, CEA, 3.3; 95% CI, 1.4-7.8; $P < .001$; HR for CA19-9, 3.0; 95% CI, 1.3-7.0; $P < .001$; HR for CA125, 2.6; 95% CI, 1.2-5.6; $P = .001$) (eFigure 11 in Supplement 1).

In multivariable analysis, after controlling for race and ethnicity, goblet or signet histologic findings, tumor grade, and tumor stage, elevated CEA (HR, 2.8; 95% CI, 1.7-4.9; $P < .001$), elevated CA19-9 (HR, 1.5; 95% CI 1.0-2.2; $P = .03$), and elevated CA125 (HR, 3.2; 95% CI 2.2-4.7; $P < .001$) remained significantly associated with decreased OS (eTable 8 in Supplement 1). Notably, when the same variables were modeled together with the number of elevated TMs, the incremental increase in the number of elevated TMs continued to worsen survival when the number of elevated TMs were compared with one another (HR, 1.7; 95% CI, 1.1-2.5; $P = .01$, for 3 vs 2 TMs elevated) (Figure 3C;

eTable 9 in Supplement 1). Similar findings were observed in the subset of 560 patients who had their TMs measured within the first 6 months from diagnosis (eFigure 10 in Supplement 1).

In the subset of 121 patients who received chemotherapy at MDACC, 32 had a 2 ng/mL or more increase in CEA levels, and 89 had stable or decreased levels of CEA. There was a significant difference in median OS between the 2 groups; median OS for patients who had 2 ng/mL or less elevation in CEA was 10 vs 49 months for patients who had CEA levels that decreased or remained stable (HR, 3.0; 95% CI, 1.6-5.6; $P < .001$) (eFigure 12 in Supplement 1). These data suggest a possible role for serial TM measurement while receiving chemotherapy to assess for therapeutic response, important in appendiceal cancer given the difficulty in quantitating response by traditional imaging methods.[33]

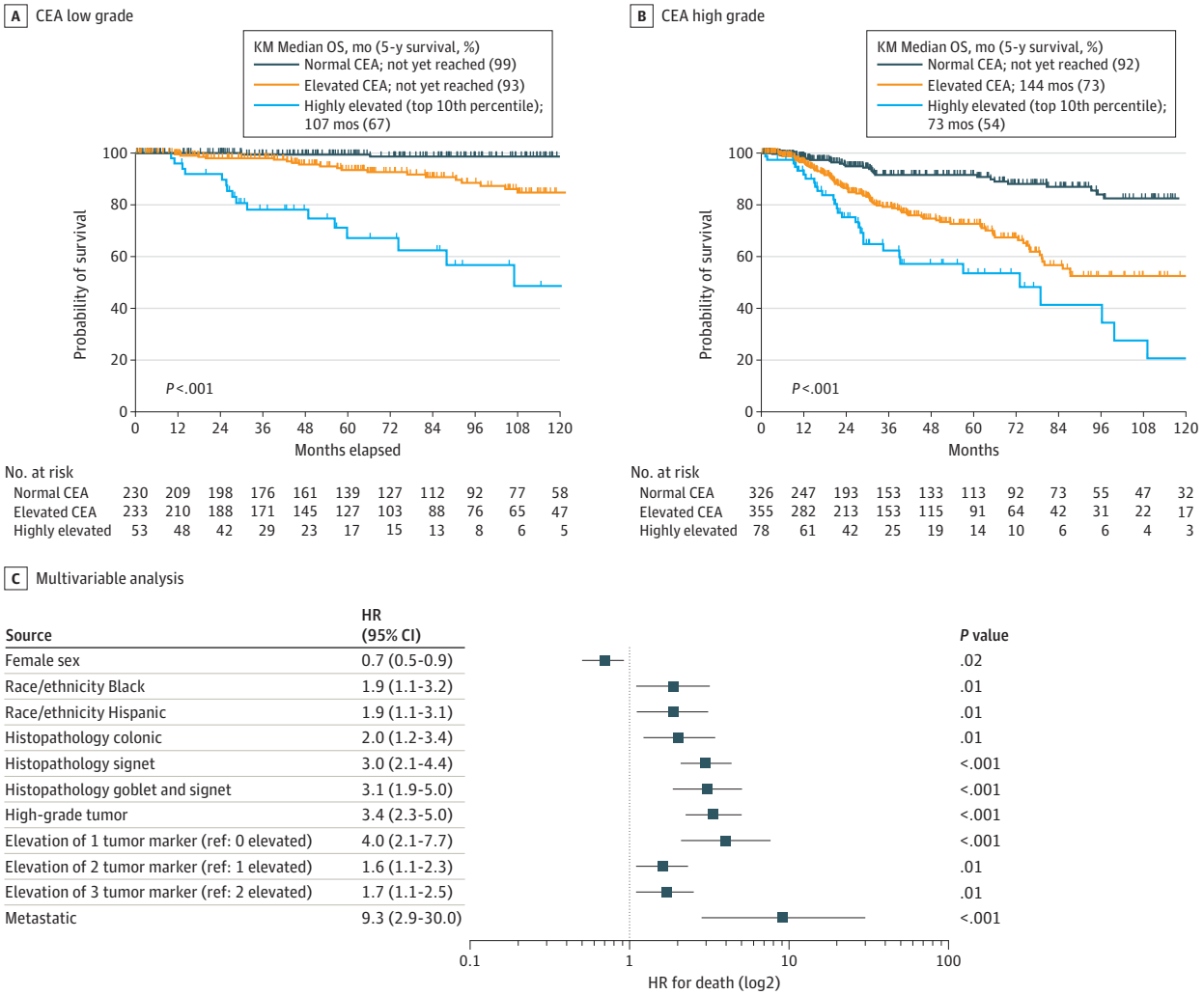
Figure 2. Survival Plots of Patients With Metastatic Disease by Tumor Markers



A, Stage IV metastatic disease patients with normal, elevated, and highly elevated levels of carcinoembryonic antigen (CEA). B, Stage IV metastatic disease patients with normal, elevated, and highly elevated levels of carbohydrate antigen 19-9 (CA19-9). C, Stage IV metastatic disease patients with normal, elevated, and highly elevated levels of cancer antigen 125 (CA125). D, All patients with number of elevated tumor markers. KM indicates Kaplan-Meier; OS, overall survival.

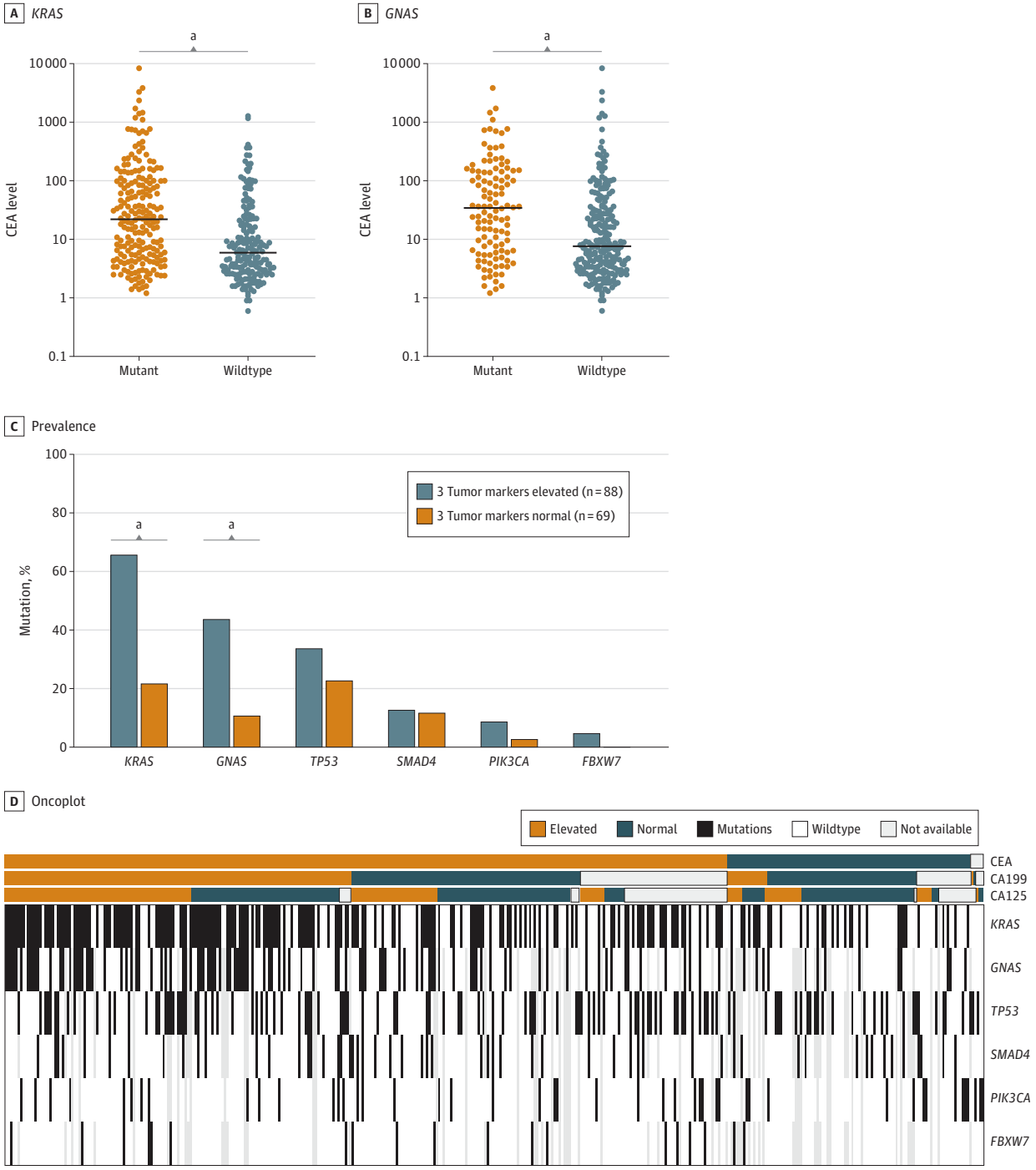
A subset of 398 tumors were also profiled with a targeted somatic mutation panel; CEA and CA19-9 were more frequently elevated in patients with *KRAS* somatic mutation (79% vs 64% for CEA; odds ratio [OR], 2.0; 95% CI, 1.3-3.4; *P* = .003; 64% vs 33% for CA19-9; OR, 3.6; 95% CI, 2.2-5.7; *P* < .001) (eFigure 13 in Supplement 1). Similarly, both CEA and CA19-9 were more frequently elevated in patients with tumors with *GNAS* somatic mutations (88% vs 72% for CEA; OR, 2.8; 95% CI, 1.5-5.1; *P* = .001; 68% vs 43% for CA19-9; OR, 2.8; 95% CI, 1.7-4.7; *P* < .001) (eFigure 13 in Supplement 1). CEA and CA19-9 levels were higher in patients with tumors with *KRAS* somatic mutation (median [IQR], 22.0 [5.4-99] vs 6.0 [2.8-19.5]; *P* < .001 for CEA; and 80.4 [16.9-480.0] vs 18.9 [10.0-55.2]; *P* < .001 for CA19-9) (**Figure 4**A; eFigure 13 in Supplement 1) and tumors with *GNAS* somatic mutations (median [IQR], 34.3 [6.4-143.6] vs 7.6 [3.4-31.4]; *P* < .001 for CEA; and 94.0 [23.5-326.7] vs 26.2 [11.2-118.8]; *P* < .001 for CA19-9) (Figure 4B; eFigure 13 in Supplement 1). *TP53* somatic mutation was not associated with differences in TM level (eFigure 13 in Supplement 1). CA-125 levels were statistically equivalent regardless of somatic mutation status for *KRAS*, *GNAS*, and *TP53*. A total of 88 patients had elevated values for all 3 TMs (pan-elevated TM group), and 69 patients had normal values for all 3 TMs (the pan-normal TM group). Prevalence of *KRAS* and *GNAS* somatic mutations was significantly higher in the pan-elevated group than in the pan-normal group (66% vs 22% for

Figure 3. Survival Plots for Patients With Low and High Tumor Grades



A, Patients with low grade tumor for normal, elevated, and highly elevated levels of carcinoembryonic antigen (CEA). B, Patients with high grade tumor for normal, elevated, and highly elevated levels of CEA. C, Multivariable analysis showing HR for death in all patients on a log2 axis. KM indicates Kaplan-Meier; OS, overall survival.

Figure 4. Somatic Mutation Landscape and Association With Tumor Markers



A, *KRAS* somatic mutation vs wildtype with carcinoembryonic antigen (CEA) levels; lines represent the median levels. B, *GNAS* somatic mutation vs wildtype with CEA levels; lines represent the median levels. C, Prevalence of *KRAS, GNAS, TP53, SMAD4, PIK3CA,* and *FBXW7* (most common genes with somatic mutations in our cohort) somatic mutations in patients with the 3 tumor markers elevated (pan-elevated) vs patients with normal levels of the 3 tumor markers (pan-normal). D, 3 Tumor markers elevated levels on the left and normal levels on the right, and the somatic mutation status of *KRAS, GNAS, TP53, SMAD4, PIK3CA*, and *FBXW7.*

[a] $P < .001$.

KRAS; OR, 7.0; 95% CI, 3.3-14.4; and 44% vs 11% for *GNAS*; OR, 6.3; 95% CI, 2.6-15.9) ($P < .001$ for both) (Figure 4C). Finally, Figure 4D displays significant concurrent elevation between CEA and CA19-9 (OR, 9.4; 95% CI, 6.8-12.9; $P < .001$), CEA and CA125 (OR, 4.9; 95% CI, 3.6-6.7; $P < .001$), and CA19-9 and CA125 (OR, 4.1; 95% CI, 3.1-5.4; $P < .001$). In our cohort, *KRAS* and *GNAS* somatic mutations were the most common (52% and 33%, respectively).

Discussion

This study represents the first comprehensive evaluation we know of the clinical utility of the tumor markers CEA, CA19-9, and CA125 in more than a thousand patients with appendiceal adenocarcinoma, establishing that each of the 3 are biomarkers associated with outcomes. In 2018 the Chicago Consensus Working Group for the first time developed guidelines for the treatment of appendiceal cancer; these endorsed the measurement of CEA, CA19-9, and CA125 in all patients with metastatic appendiceal cancer.[34] However, in practice, even at this National Comprehensive Cancer Network–designated tertiary referral center, testing of all 3 tumor markers was not universal (eFigure 4 in Supplement 1). Here we show that using the combination of CEA, CA19-9, and CA125 can stratify patients with appendiceal adenocarcinoma into groups with 5-year survival ranging from 97% for those with no tumor markers elevated to 63% for those with all 3 markers elevated (Figure 2D). Our results are consistent with and expand upon multiple prior retrospective analyses in small cohorts[35,36] and prior studies restricted to patients undergoing cytoreductive surgery[19,37] which have suggested prognostic value of these tumor markers. Most prior studies of TMs in appendiceal cancer dichotomized each TM into elevated and normal categories; however, we find that treating each as a continuous variable retains important information. The distribution of values for each of CEA, CA19-9, and CA-125 were positively skewed but unimodal, so an arbitrary cutoff of the highest 10% was chosen to evaluate the survival association of highly elevated TM. We found that highly elevated CEA, CA19-9, and CA-125 had a greater association with poor survival, similar in magnitude to stage.

To assess the clinical utility of TMs in both operable and inoperable patients, we first evaluated their association with tumor stage, grade,[38,39] histopathology, and somatic mutation profile. Although metastatic tumors had higher levels of all 3 TMs, the association of elevated TM with survival was independent of stage, sex, race, tumor grade, tumor histopathology. The analyses by tumor grade also highlighted the unique observation that patients with low-grade AA and normal CEA levels had a particularly good prognosis (99% survival at 5 years and 94% survival at 5 years in metastatic disease). This observation could be explained by early diagnosis in these patients when the tumor volume is insufficient to cause elevation of the TMs, which would be associated with an especially favorable outcome. This should be studied prospectively, but there could be a role for treatment deescalation in these patients, particularly considering recent prospective data showing 5-fluorouracil–based chemotherapy is ineffective in this patient population.[33] Conversely, for patients with highly elevated tumor markers, the poor prognoses seen with current treatments, which have historically been chemotherapy designed for CRC, suggests that these patients be prioritized for clinical studies testing appendiceal cancer–specific therapies. The association with *KRAS* and *GNAS* somatic mutations and elevated levels of both CEA and CA19-9 suggests that high TM expression tumors may have intrinsically distinct biology compared with nonexpressing tumors, which may be indicative of different therapeutic vulnerabilities and should be explored in future studies.

Despite the notable distinctions between AA and CRC,[2,38] the current American Joint Committee on Cancer (AJCC) staging system treats them similarly[40] and does not provide meaningful stratification of survival outcome for AA. Specifically, under the current AJCC stage I to III, all have excellent survival, and there is wide variation in survival among patients classified as stage IV. The greater survival association seen with CEA, CA19-9, and CA125 suggests that these tumor markers should be included in appendiceal adenocarcinoma staging to stratify patients with

metastatic disease in a similar manner to human chorionic gonadotropin, α fetoprotein, and lactate dyhydrogenase in germ cell tumors.[41]

Limitations

The study is subject to several limitations. First, the retrospective design introduces inherent limitations in data collection and potential biases. Additionally, the low number of patients undergoing NGS analysis (only 30% of the cohort) limits the comprehensive assessment of genetic somatic mutations. Additionally, the selective approach in ordering tumor markers by different physicians may have led to the exclusion of certain patients from the cohort, potentially overrepresenting those with a more advanced disease stage. Another limitation is the lack of consideration for patients' chemotherapy and cytoreductive surgery history. This omission is primarily due to the fact that many patients receive initial treatment at local hospitals before seeking care at our institution. Furthermore, the analysis did not investigate the differences in tumor marker levels among patients with localized disease (stage I, II, and III) due to incomplete stage data in our cohort. Despite these limitations, our study benefited from the ability to assemble a substantial cohort of 1338 patients with AA, accompanied by comprehensive clinical data and outcomes. To our knowledge, this represents the largest and most comprehensive patient cohort with AA ever assembled at a single institution, providing valuable insights into the disease and its management and enabled us to control for potential variations in clinical practice, which is particularly relevant in rare diseases such as AA.

Conclusions

In conclusion, these data demonstrate the practical value of CEA, CA19-9, and CA125 in management of AA. These biomarkers are associated with overall survival for patients with AA. We suggest incorporating the measurement of these 3 TMs as a standard part of AA's clinical management. Additionally, it is important to explore the potential role of TMs in AA's tumor cell adhesion and disease progression to enhance our comprehension of the disease's biological behavior.

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SUPPLEMENT 1.

eAppendix. Supplemental Methods
eTable 1. Validation Cohort Patient Characteristics
eTable 2. Tumor Marker Levels and Cutoffs for the Main Cohort
eTable 3. Number of Tests Performed for Each TM per Patient
eTable 4. Tumor Marker Levels and Cutoffs Split by Tumor Grade
eTable 5. CEA Level and Cutoff Split by Histopathology
eTable 6. CA19-9 Level and Cutoff Split by Histopathology
eTable 7. CA125 Level and Cutoff Split by Histopathology

Supplemental Online Content

Yousef A, Yousef M, Zeineddine MA, et al. Serum tumor markers and outcomes in patients with appendiceal adenocarcinoma. *JAMA Netw Open*. 2024;7(2):e240260. doi:10.1001/jamanetworkopen.2024.0260

This supplemental material has been provided by the authors to give readers additional information about their work

eAppendix. Supplemental Methods

Survival analyses were performed using the Kaplan-Meier (KM) method with log-rank test. Univariate and multivariable Cox-proportional hazards (CPH) regression analyses were performed to assess associations between clinical factors (serum TM levels, demographics, and patient and disease characteristics) and patient outcomes (OS). KM and CPH analyses modeled TMs individually (model A) as well as in a single aggregate variable indicating the number of elevated TMs (model B). In model B, for TMs, the covariate was an ordinal variable of 0 biomarkers elevated, 1 biomarker elevated, 2 biomarkers elevated, or 3 biomarkers elevated. In both models, sex, race, smoking status, alcohol use, age at diagnosis, tumor histologic findings, tumor grade, and tumor stage were also considered. All variables with $P < .05$ on univariate analysis were included in the multivariable analysis. Differences in tumor histopathological types between patients with elevated vs normal TM levels were assessed using Fisher exact test. Differences in TM levels between tumor grade and gender were assessed using Mann-Whitney U test, while differences in TM levels between tumor histopathology were assessed using Kruskal-Wallis test as TM levels didn't follow Gaussian distribution. Correlation between the different TMs were assessed using Spearman correlation. Frequency of mutations among patients with elevated vs normal TM levels was assessed using Fisher exact test.

A cohort from The Colorectal and Peritoneal Oncology Center, The Christie National Health Service (NHS) Foundation Trust, Manchester, United Kingdom, was used for validation. Between January 2005 and August 2021, patients with histologically confirmed AAs were identified from a prospectively collected database. Patients were discussed in a specialized peritoneal tumor multidisciplinary team (MDT) and all pathology slides reported at external institutions were imported and re-reported by specialist pathologists. Data including demographics, clinicopathological variables, TMs, and survival status were extracted from the database. All patients had pathology reports reviewed; missing data were referenced from the hospital record.

eTable 1. Validation Cohort Patient Characteristics

Patient Characteristics		All N (100%)
All		216 (100%)
Age at diagnosis - median (min, max)		59 (21, 80)
Sex		
	Female	126 (58%)
	Male	90 (42%)
Histopathology		
	Mucinous	141 (65%)
	Adenocarcinoma not otherwise specified	71 (33%)
	Signet ring carcinoma	4 (2%)
Disease Metastatic State		
	Localized Disease (Stage I, II, III)	69 (32%)
	Metastatic Disease (Stage IV)	147 (68%)
CEA		
	Normal	116 (54%)
	Elevated	67 (31%)
	Highly Elevated	21 (9%)
	Not tested	12 (6%)
CA19-9		
	Normal	137 (63%)
	Elevated	48 (22%)
	Highly Elevated	19 (9%)
	Not tested	12 (6%)
CA125		
	Normal	137 (63%)
	Elevated	46 (21%)
	Highly Elevated	21 (10%)
	Not tested	12 (6%)
Overall Survival (months)		
	Median (min, max)	122 [1, 286]

eTable 2. Tumor Marker Levels and Cutoffs for the Main Cohort

	CEA	CA 19-9	CA 125
Number of Patients (n)	1331	1132	1165
Cut off Value	> 3.8 ng/mL	> 35 U/mL	> 38 U/mL
Percentage Elevated (%)	46%	24%	17%
Number Elevated	609	268	196
Highly Elevated cut off (top 10th percentile)	> 99.8 ng/mL	> 338.6 U/mL	> 99.0 U/mL
Percentage Highly Elevated (%)	10%	10%	10%
Number Highly Elevated	133	113	116

eTable 3. Number of Test Performed for Each TM per Patient

	CEA	CA 19-9	CA 125
Mode	1	1	1
Approximate Median	4	3	3
Approximate Mean	7	5	5
Min	1	1	1
Max	98	60	36

eTable 4. Tumor Marker Levels and Cutoffs Split by Tumor Grade

	CEA		CA 19-9		CA 125	
Grade	Low Grade	High Grade	Low Grade	High Grade	Low Grade	High Grade
Number of Patients (n)	519	761	486	607	487	636
Cut off Value	> 3.8 ng/mL		> 35 U/mL		> 38 U/mL	
Percentage Elevated (%)	45%	44%	27%	22%	15%	18%
Number Elevated	235	355	129	132	75	113
Highly Elevated cut off (top 10th percentile)	> 99.8 ng/mL		> 338.6 U/mL		> 99.0 U/mL	
Percentage Highly Elevated (%)	10%		10%		10%	
Number Highly Elevated	53	78	41	70	37	76

eTable 5. CEA Level and Cutoff Split by Histopathology

	CEA				
Grade	**Mucinous**	**Colonic**	**Goblet**	**Signet**	**Goblet & Signet**
Number of Patients (n)	693	130	93	221	147
Cut off Value			> 3.8 ng/mL		
Percentage Elevated (%)	56%	59%	30%	69%	50%
Number Elevated	391	77	28	153	73
Mean	64.3	172.8	12.2	44.6	10.5
Std. Deviation	244	593	62	126	28
Median	4.3	5.1	2.7	6.7	3.6

eTable 6. CA19-9 Level and Cutoff Split by Histopathology

	CA19-9				
Grade	**Mucinous**	**Colonic**	**Goblet**	**Signet**	**Goblet & Signet**
Number of Patients (n)	630	90	77	180	118
Cut off Value			> 35 U/mL		
Percentage Elevated (%)	36%	39%	10%	43%	21%
Number Elevated	224	35	8	77	25
Mean	272	5829	35	408	72
Std. Deviation	1431	37295	130	1413	390
Median	20	21	13	27	14

eTable 7. CA125 Level and Cutoff Split by Histopathology

	CA125				
Grade	**Mucinous**	**Colonic**	**Goblet**	**Signet**	**Goblet & Signet**
Number of Patients (n)	631	84	79	190	130
Cut off Value			> 38 U/mL		
Percentage Elevated (%)	25%	25%	13%	38%	26%
Number Elevated	157	21	10	73	34
Mean	34.0	59.0	21.5	59.2	44.5
Std. Deviation	49.6	168.4	26.7	89.8	74.5
Median	15.7	15.4	12.9	21.6	17.7

eTable 8. Cox proportional hazards regression model A for overall survival (n=1338)

	Univariate Analysis				Multivariate Analysis			
Clinical Characteristics	HR	P value	95 CI%		HR	P value	95 CI%	
Male gender		reference				reference		
Female gender	0.6589	0.0036	0.4977	0.8722	0.78	0.16	0.54	1.1
Race white		reference				Reference		
Race Black or African American	1.9	0.0072	1.19	3.033	2.9	0.0005	1.6	5.1
Race Hispanic/ Latino	1.264	0.3156	0.7999	1.997	2.5	0.0017	1.4	4.5
Race Asian	1.531	0.2717	0.7164	3.272				
Race Others	0.7099	0.6305	0.1758	2.867				
Smoking status Never		reference				reference		
Smoking status Former	1.294	0.1215	0.9338	1.794				
Smoking status Smoker	0.9791	0.9566	0.4575	2.095				
Alcohol use status Never		reference				reference		
Alcohol use status Yes	0.7444	0.0462	0.5569	0.995	1.2	0.45	0.8	1.7
Age at diagnosis	1.015	0.0218	1.002	1.028	1	0.28	0.99	1
Tumor Histopathology Mucinous		reference				reference		
Tumor Histopathology Colonic	2.855	9.32E-06	1.795	4.539	1.9	0.063	0.97	3.7
Tumor Histopathology Goblet	0.2172	0.1297	0.03014	1.565				
Tumor Histopathology Signet	4.278	5.24E-17	3.045	6.009	3.1	1.8E-07	2	4.8
Tumor Histopathology Goblet/Signet	3.228	6.13E-08	2.112	4.933	3.3	4.0E-05	1.9	5.8
Low Grade tumor		reference				reference		
High Grade Tumor	3.782	1.61E-14	2.693	5.31	3.5	1.70E-08	2.3	5.4
Normal CEA		reference				reference		
Elevated CEA	4.997	6.52E-16	3.382	7.382	2.8	0.0001	1.7	4.9
Normal CA19-9		reference				reference		
Elevated CA19-9	3.088	1.16E-11	2.23	4.277	1.5	0.028	1	2.2
Normal CA125		reference				reference		
Elevated CA125	5.936	2.97E-27	4.298	8.198	3.2	1.70E-09	2.2	4.7
Localized Disease		reference				reference		
Metastatic Disease	11.51	1.33E-6	4.275	30.99	9.8	0.0017	2.4	41

eTable 9. Cox proportional hazards regression model B for overall survival (n=1338)

	Univariate Analysis				Multivariate Analysis			
Clinical Characteristics	**HR**	**P value**	**95 CI%**		**HR**	**P value**	**95 CI%**	
Male gender	reference				reference			
Female gender	0.6589	0.0036	0.4977	0.8722	0.69	0.017	0.51	0.94
Race white	reference				reference			
Race Black or African American	1.9	0.0072	1.19	3.033	1.9	0.013	1.1	3.2
Race Hispanic/ Latino	1.264	0.3156	0.7999	1.997	1.9	0.013	1.1	3.1
Race Asian	1.531	0.2717	0.7164	3.272	1.2	0.64	0.5	3.1
Race Others	0.7099	0.6305	0.1758	2.867	1	0.98	0.25	4.2
Smoking status Never	reference				reference			
Smoking status Former	1.294	0.1215	0.9338	1.794				
Smoking status Smoker	0.9791	0.9566	0.4575	2.095				
Alcohol use status Never	reference				reference			
Alcohol use status Yes	0.7444	0.0462	0.5569	0.995	1	0.99	0.73	1.4
Age at diagnosis	1.016	0.0127	1.003	1.029	1	0.5	0.99	1
Tumor Histopathology Mucinous	reference				reference			
Tumor Histopathology Colonic	2.855	9.32E-06	1.795	4.539	2	0.0071	1.2	3.4
Tumor Histopathology Goblet	0.2172	0.1297	0.03014	1.565				
Tumor Histopathology Signet	4.278	5.24E-17	3.045	6.009	3	7.60E-09	2.1	4.4
Tumor Histopathology Goblet/Signet	3.228	6.13E-08	2.112	4.933	3.1	6.10E-06	1.9	5
Low Grade tumor	reference				reference			
High Grade Tumor	3.782	1.61E-14	2.693	5.31	3.4	1.10E-09	2.3	5
Normal level of all the 3 TMs	reference				reference			
Elevated one TM (Ref: 0 elevated)	6.831	5.13E-11	3.85	12.12	4	3.6E-05	2.1	7.7
Elevated two TM (Ref: 1 elevated)	1.484	0.02	1.1	2.1	1.6	0.012	1.1	2.3
Elevated three TM (Ref: 2 elevated)	1.575	0.02	1.1	2.3	1.7	0.011	1.1	2.5
Elevated three TM (Ref: 0 elevated)	15.97	3.30E-19	8.709	29.27	11	1.0E-11	5.4	21
Localized Disease	reference				reference			
Metastatic Disease	11.51	1.33E-06	4.275	30.99	9.3	0.0002	2.9	30

Figure Legends

eFigure 1. Flowchart diagram showing cohort patients selection. Abbreviations include MDACC (MD Anderson Cancer Center), HER (Electronic Health Records).

eFigure 2. (A) Distribution of all patients in our cohort by year of diagnosis. (B) Distribution of all patients in our cohort by tumor histopathological grade. (C) Distribution of all patients in our cohort by tumor histopathological grade binary.

eFigure 3. (A) Bar plot showing number of patients with normal, elevated, and highly elevated levels of CEA, CA19-9, and CA125. (B) Proportionate Venn diagram showing the overlapping of elevated (highly elevated included) levels of the three tumor markers for all patients tested for the 3 TMs. (C) Proportionate Venn diagram showing the overlapping of elevated (highly elevated included) levels of the three tumor markers for patients with metastatic disease tested for the 3 TMs.

eFigure 4. Spider plot showing % of patients tested for each tumor marker at the time of diagnosis over the last 8 years.

eFigure 5. Violin plot showing the distribution of all patients CEA, CA19-9, and CA125 tumor markers levels split by patients sex, lines represent median levels

eFigure 6. (A) Violin plot showing the distribution of all patients CEA tumor markers levels split by tumor histopathology, lines represent median levels. (B) Violin plot showing the distribution of all patients CA19-9 tumor markers levels split by tumor histopathology, lines represent median levels. (C) Violin plot showing the distribution of all patients CA125 tumor markers levels split by tumor histopathology, lines represent median levels.

eFigure 7. (A) Scattered plot for patients who had CEA (on X-axis) and CA19-9 (on Y-axis) measured on the same day showing the correlation between both tumor markers. (B) Scattered plot for patients who had CEA (on X-axis) and CA125 (on Y-axis) measured on the same day showing the correlation between both tumor markers (C) Scattered plot for patients who had CA19-9 (on X-axis) and CA125 (on Y-axis) measured on the same day showing the correlation between both tumor markers. Each point represents one measurement on one day for one patient. Non parametric Spearman's correlation was used to measure the degree of association, (all $p<0.0001$)

eFigure 8. (A) KM survival plot of all patients with normal, elevated, and highly elevated levels of CEA. (B) KM survival plot of all patients with normal, elevated, and highly elevated levels of CA19-9. (C) KM survival plot of all patients with normal, elevated, and highly elevated levels of CA125.

eFigure 9. (A) KM survival plot of patients with low grade tumor for normal, elevated, and highly elevated levels of CA19-9. (B) KM survival plot of patients with high grade tumor for normal, elevated, and highly elevated levels of CA19-9. (C) KM survival plot of patients with low grade tumor for normal, elevated, and highly elevated levels of CA125. (D) KM survival plot of patients with high grade tumor for normal, elevated, and highly elevated levels of CA125.

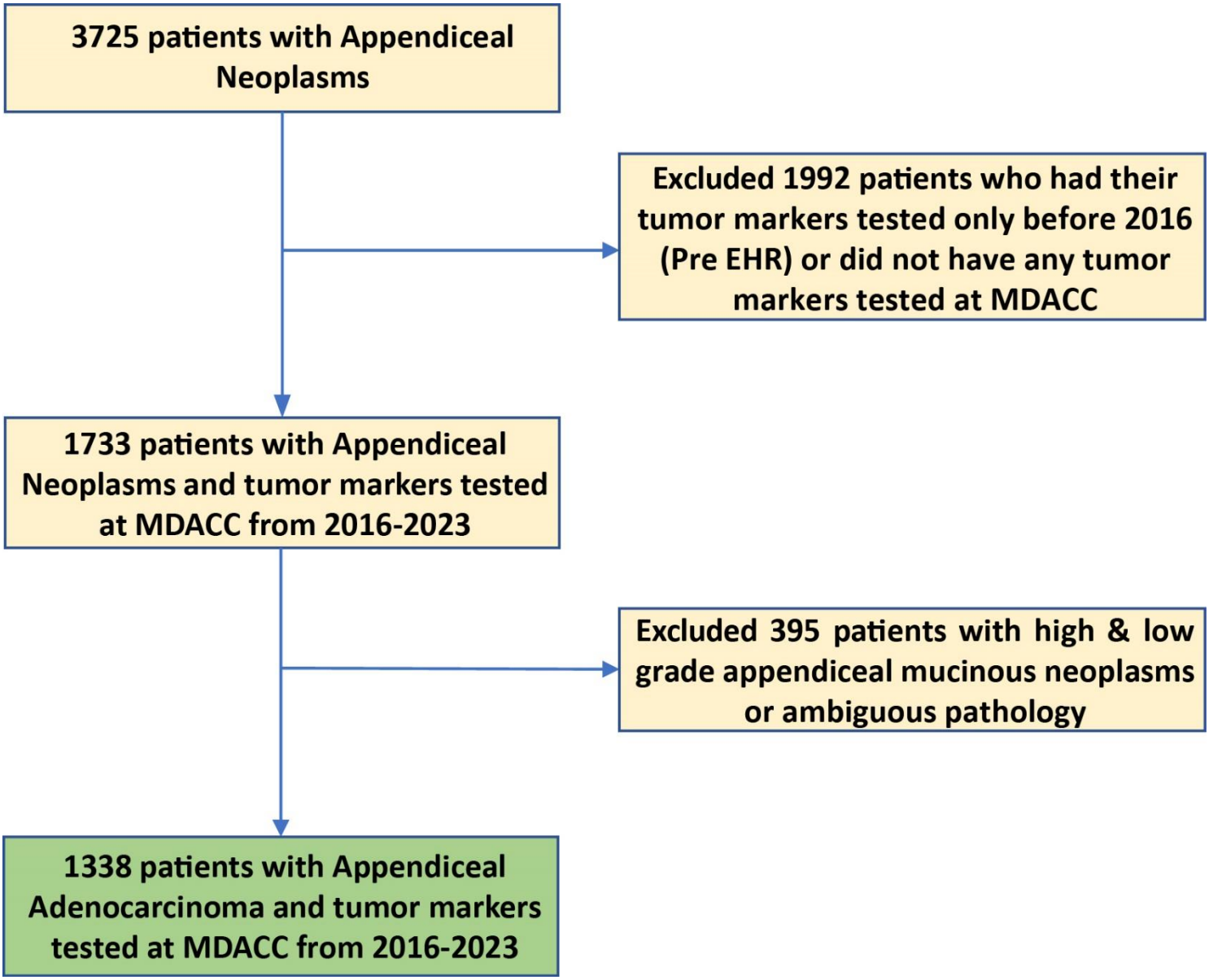
eFigure 10. (A) KM survival plot of all patients with normal, elevated, and highly elevated levels of CEA measured within the initial six months from the date of diagnosis. (B) KM

survival plot of all patients with normal, elevated, and highly elevated levels of CA19-9 measured within the initial six months from the date of diagnosis. (C) KM survival plot of all patients with normal, elevated, and highly elevated levels of CA125 measured within the initial six months from the date of diagnosis. (D) KM survival plot of all patients with number of elevated tumor markers measured within the initial six months from the date of diagnosis. (E) Forest plot for multivariable analysis showing HR for death in the subset of patients who had their tumor markers measured within the initial six months from the date of diagnosis.

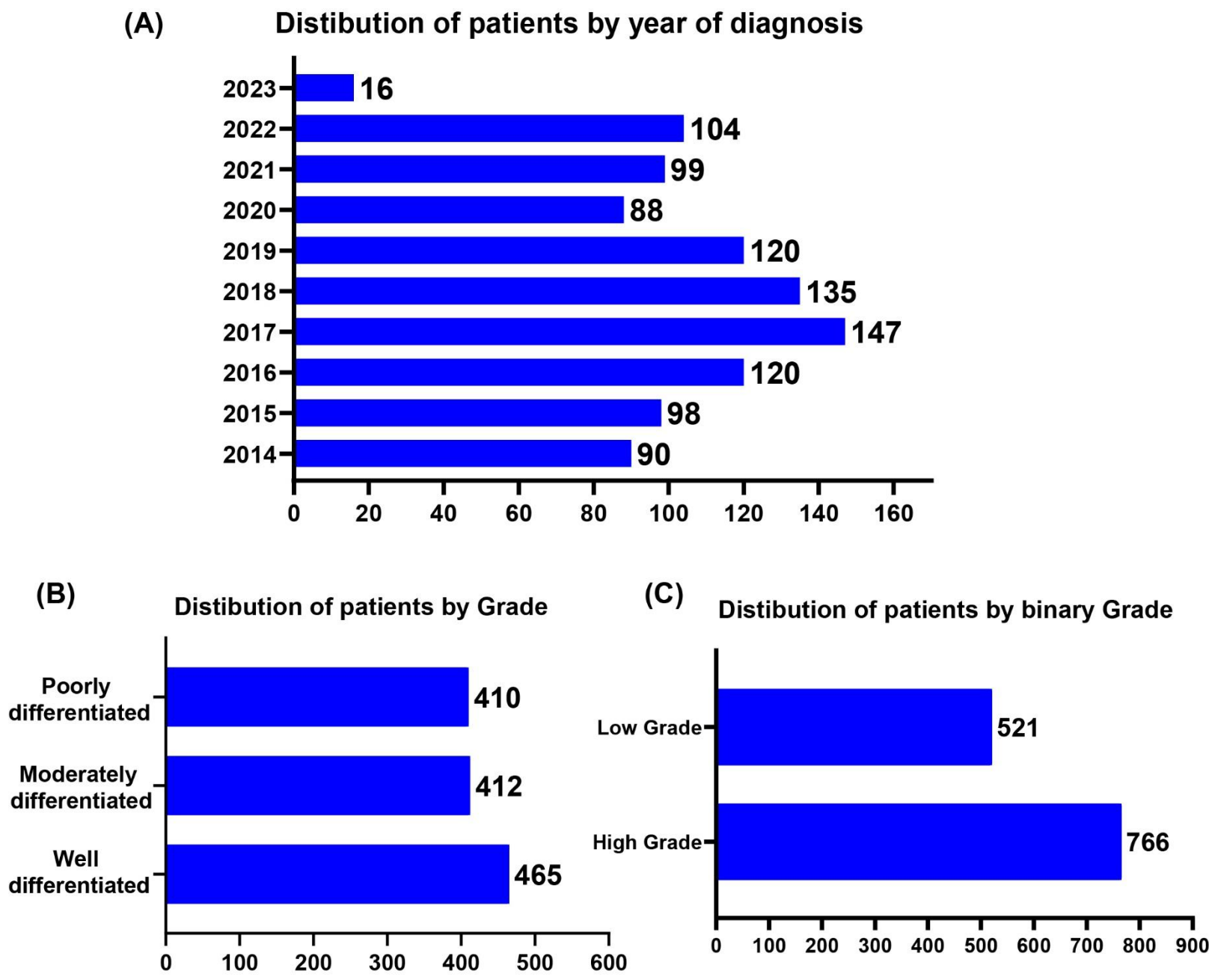
eFigure 11. The validation cohort (The Christie NHS Foundation Trust Cohort) (A) KM survival plot of all patients with normal, elevated, and highly elevated levels of CEA. (B) KM survival plot of metastatic disease patients with normal, elevated, and highly elevated levels of CEA (C) KM survival plot of all patients with normal, elevated, and highly elevated levels of CA19-9. (D) KM survival plot of metastatic disease patients with normal, elevated, and highly elevated levels of CA19-9. (E) KM survival plot of all patients with normal, elevated, and highly elevated levels of CA125. (F) KM survival plot of metastatic disease patients with normal, elevated, and highly elevated levels of CA125.

eFigure 12. KM survival plot for the subset of patients (n=121) who received chemotherapy at MDACC showing the difference in OS between patients who had $\geq$ 2ng/mL elevation in CEA and patients who had CEA levels normalized or remained stable.

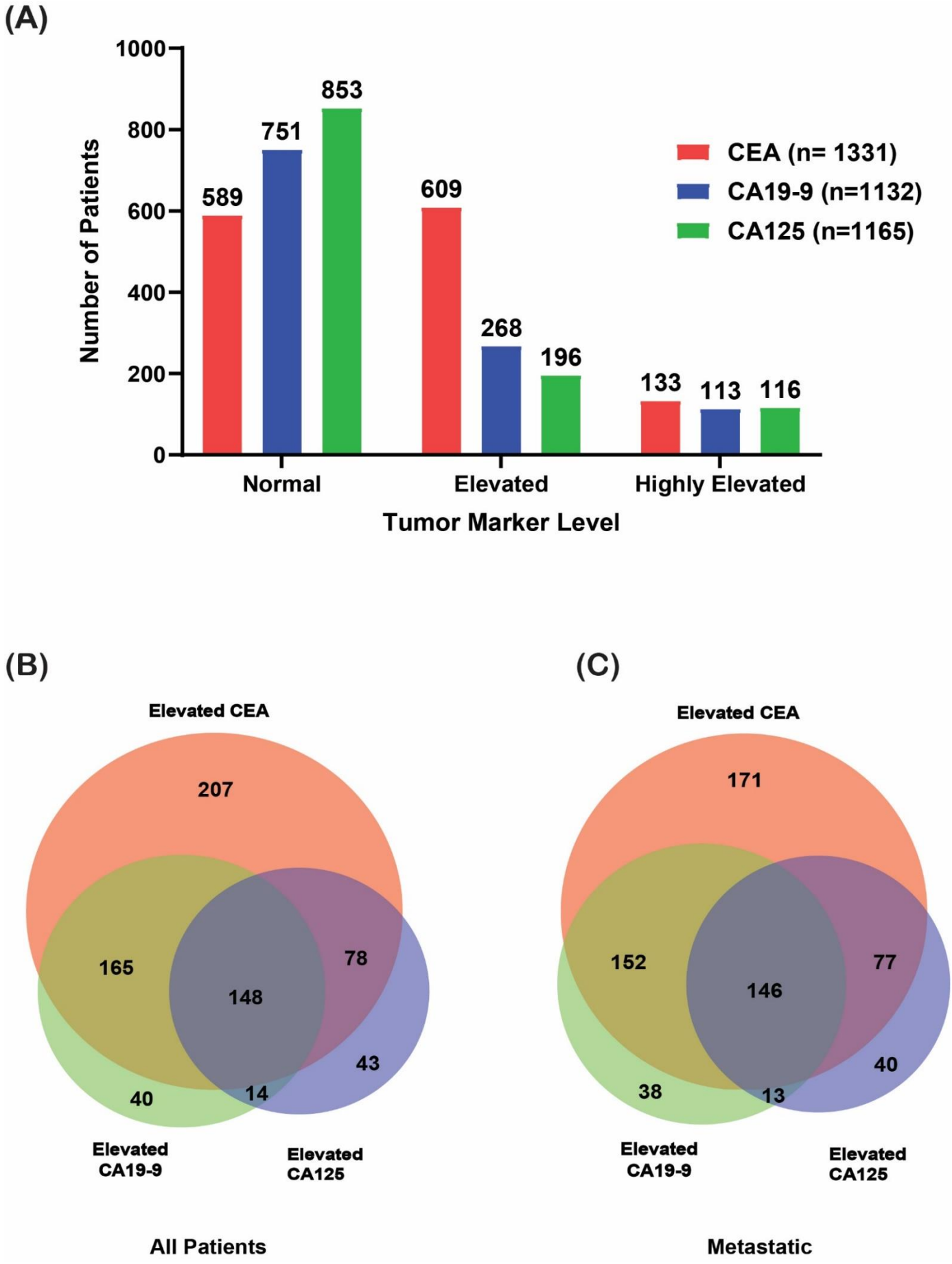
eFigure 13. (A-C) Bar graphs showing the percentage of patients with elevated CEA (red), CA19-9 (blue), and CA125 (green) in mutant and wildtype *KRAS*, *GNAS*, and *TP53*. (D) Scattered plots for *KRAS* mutant vs wildtype with CA19-9 and CA125 levels, lines represents the median levels. (E) Scattered plots for *GNAS* mutant vs wildtype with CA19-9 and CA125 levels, lines represents the median levels.



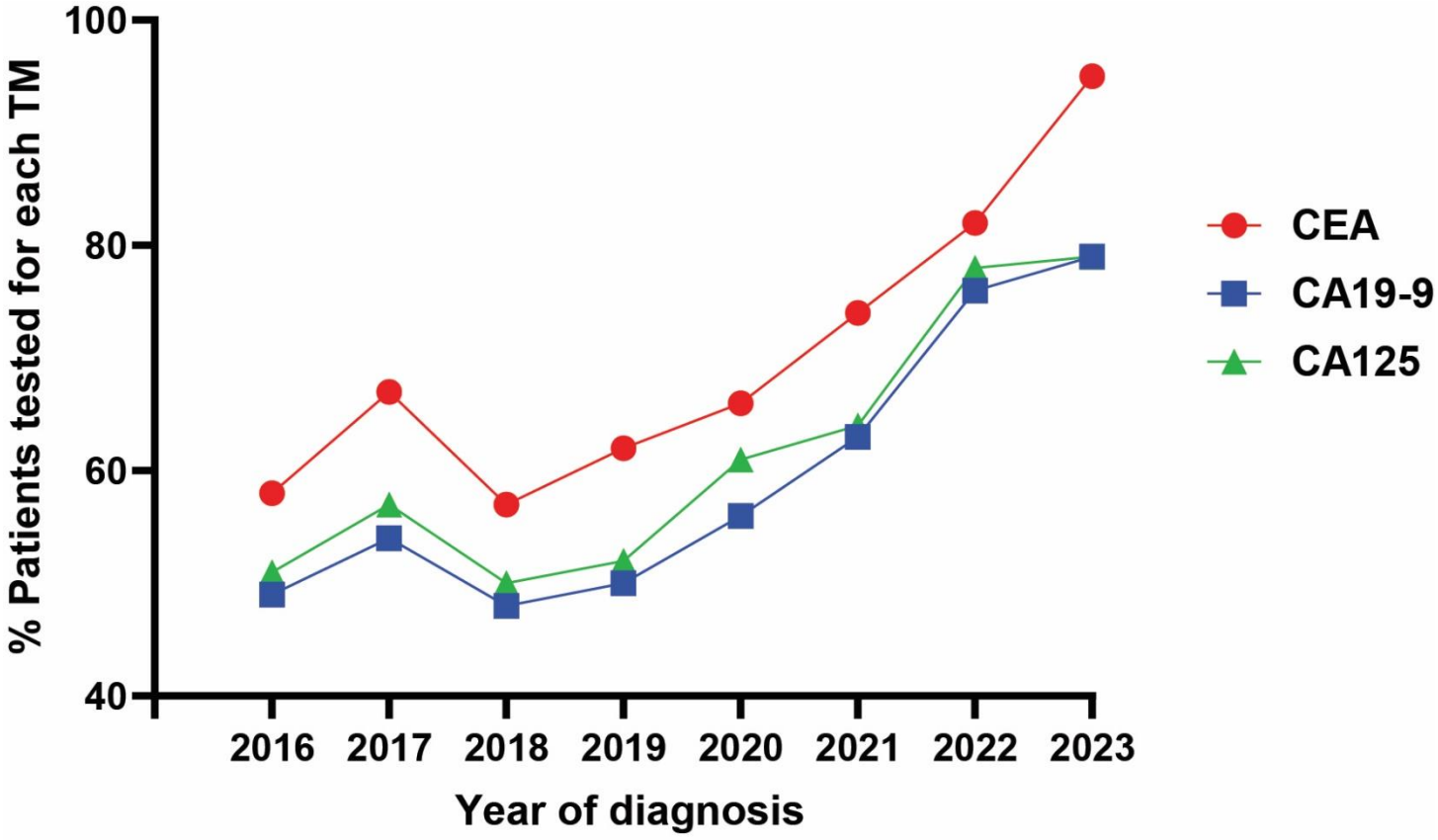
eFigure 1. Study Flow Chart



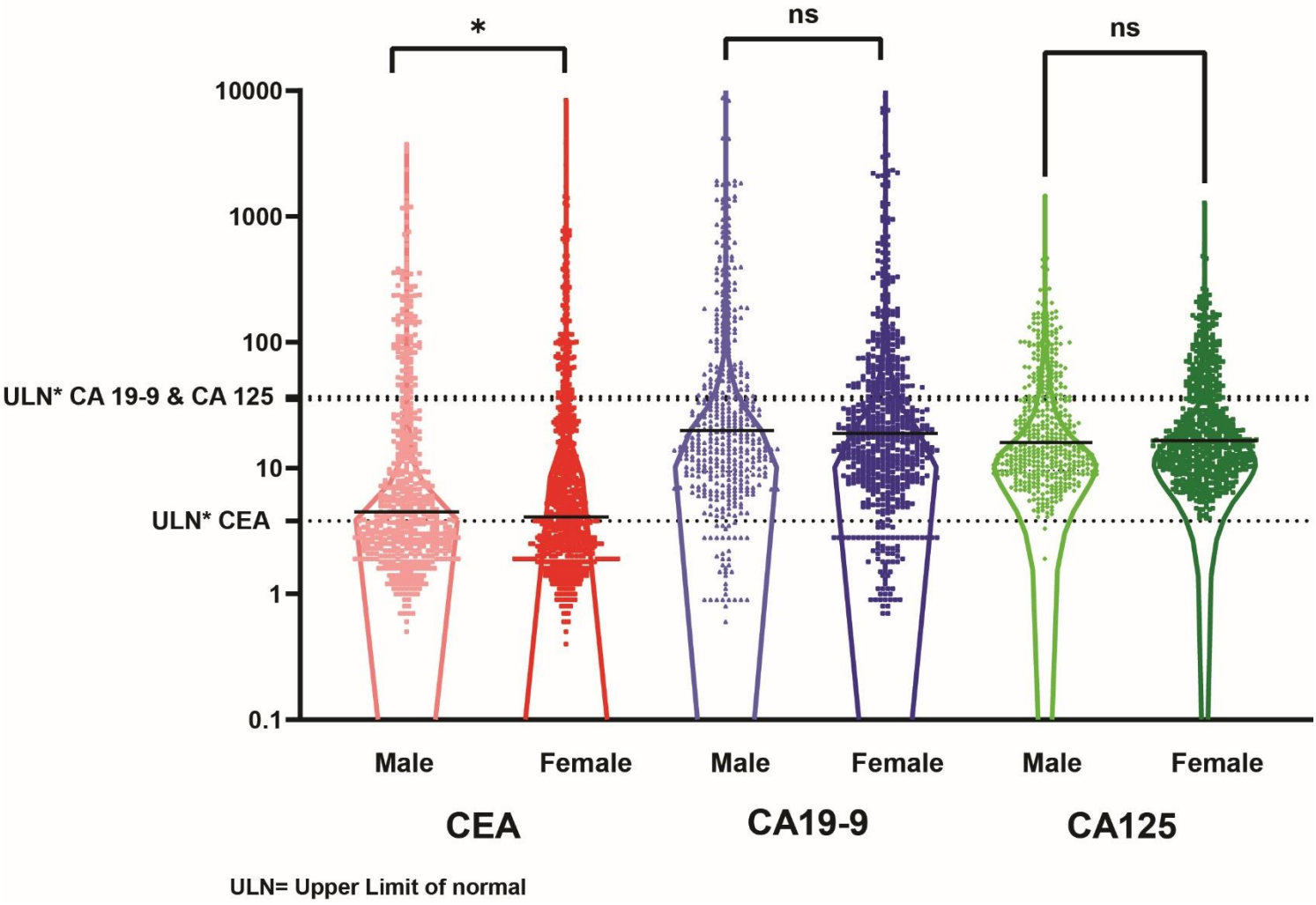
eFigure 2. Distribution of Patients



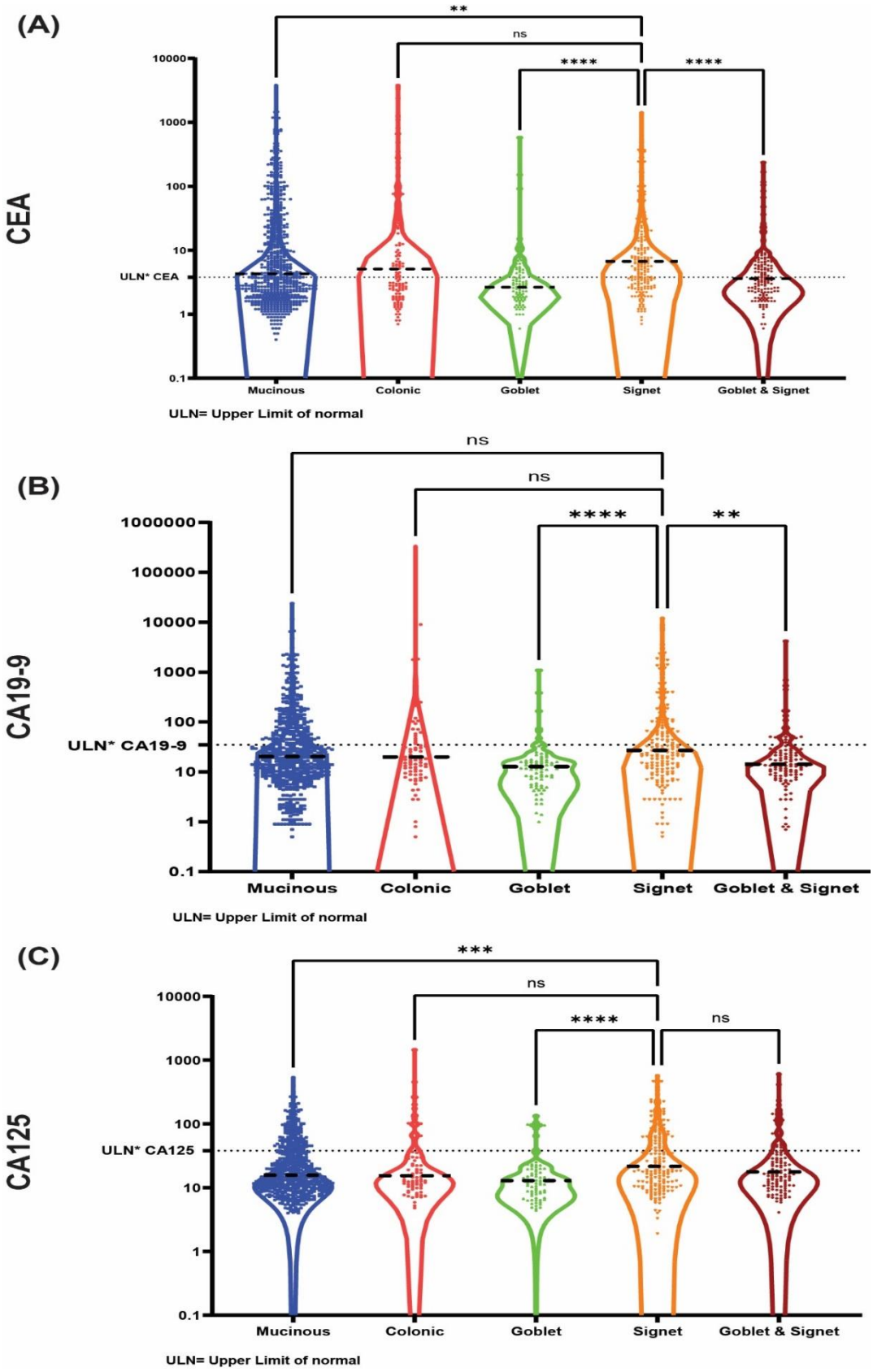
eFigure 3. Tumor Markers



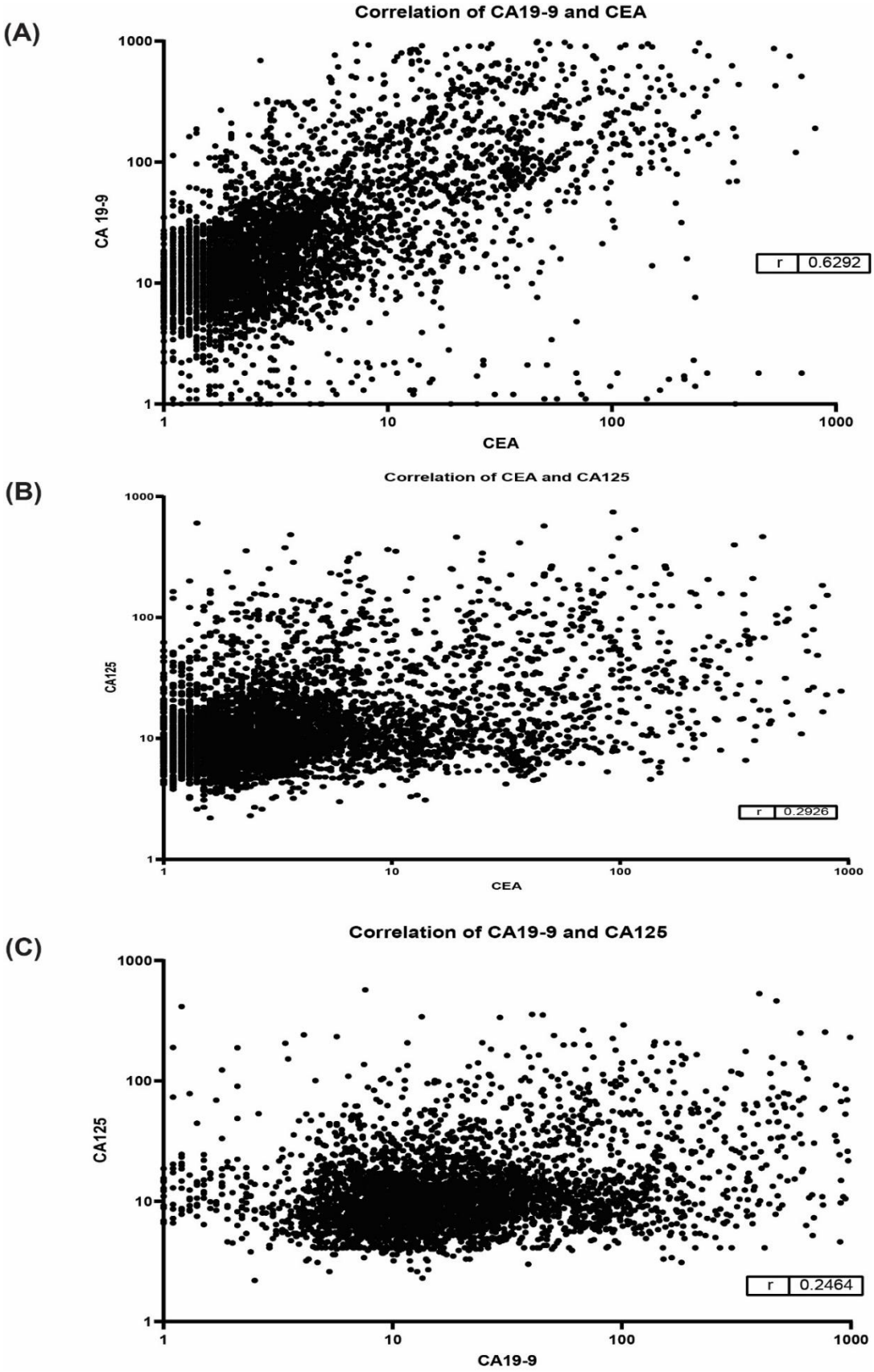
eFigure 4. Percentage of Patients Tested by Year of Diagnosis For Each Tumor Marker



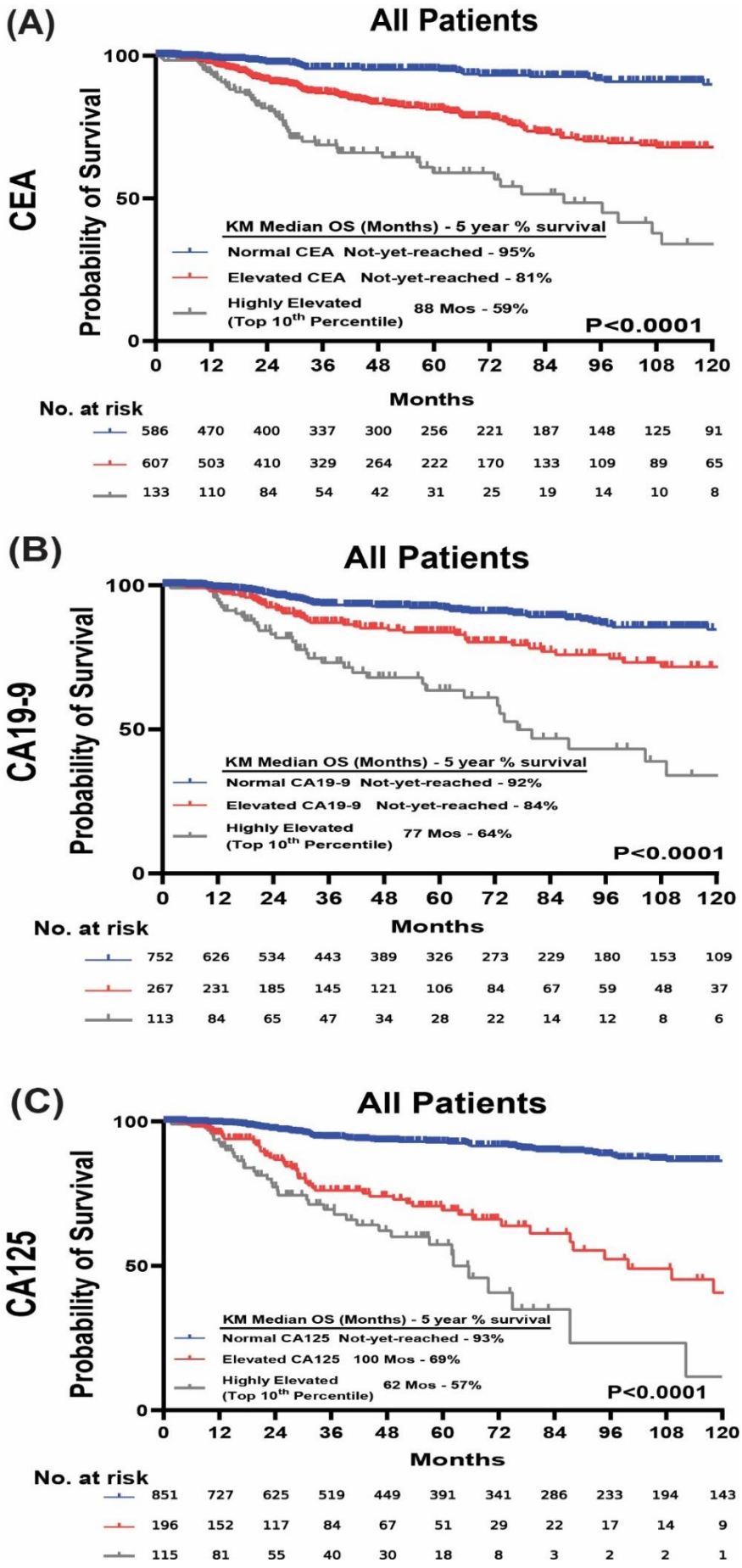
eFigure 5. CEA, CA19-9, and CA125 by Sex



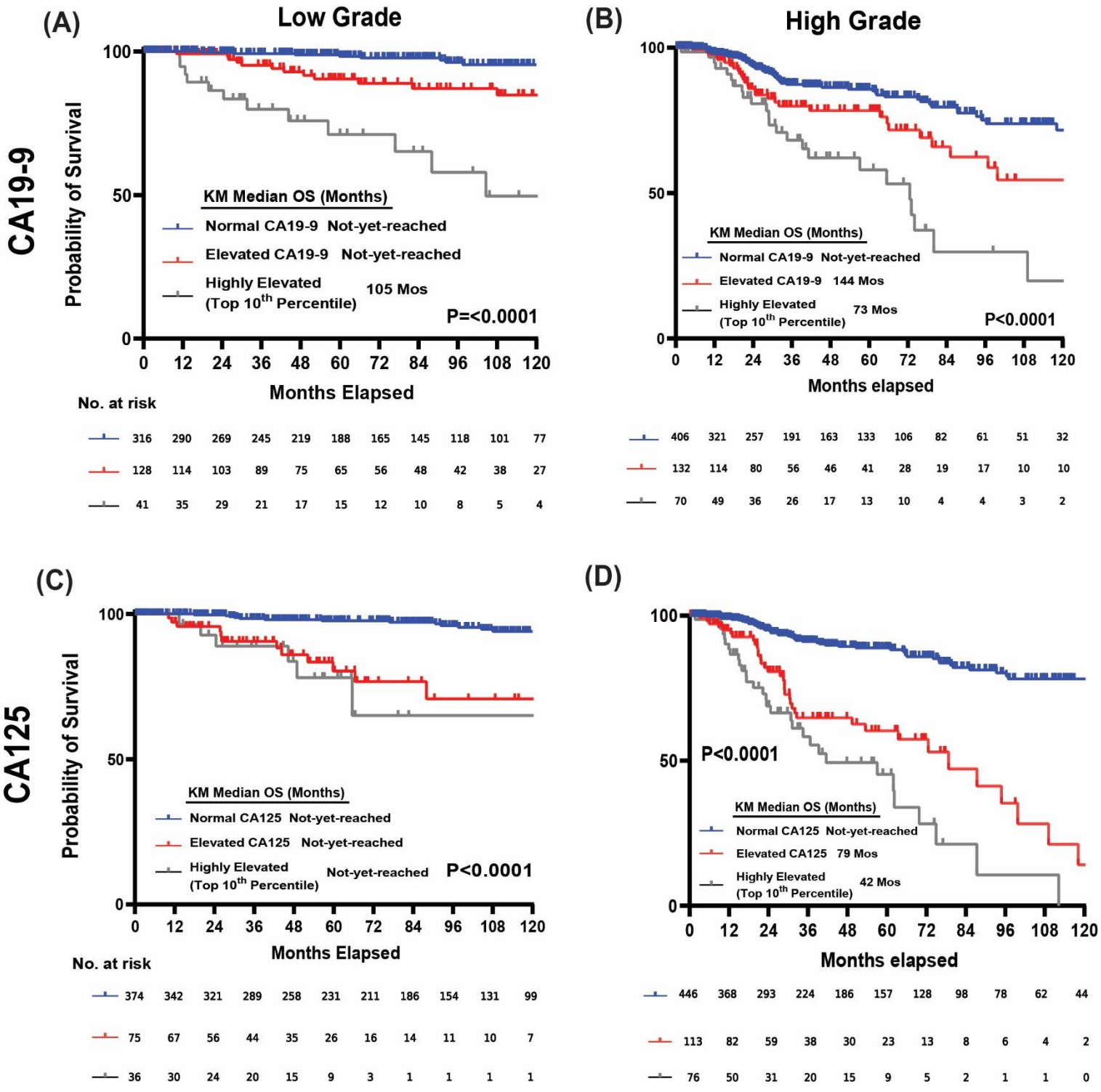
eFigure 6. CEA, CA19-9, and CA125 by histopathology



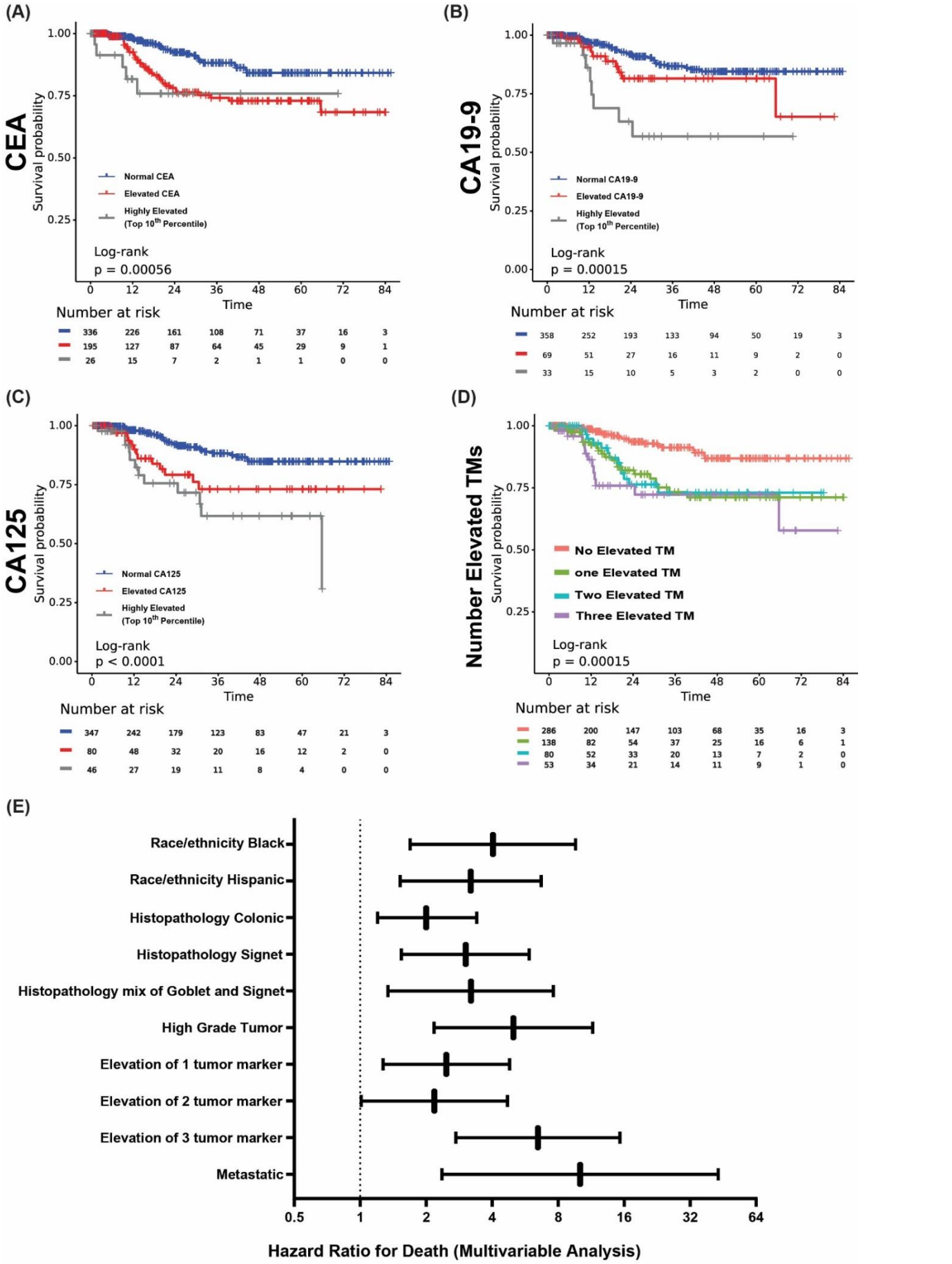
eFigure 7. Correlation of CEA, CA19-9, and CA125



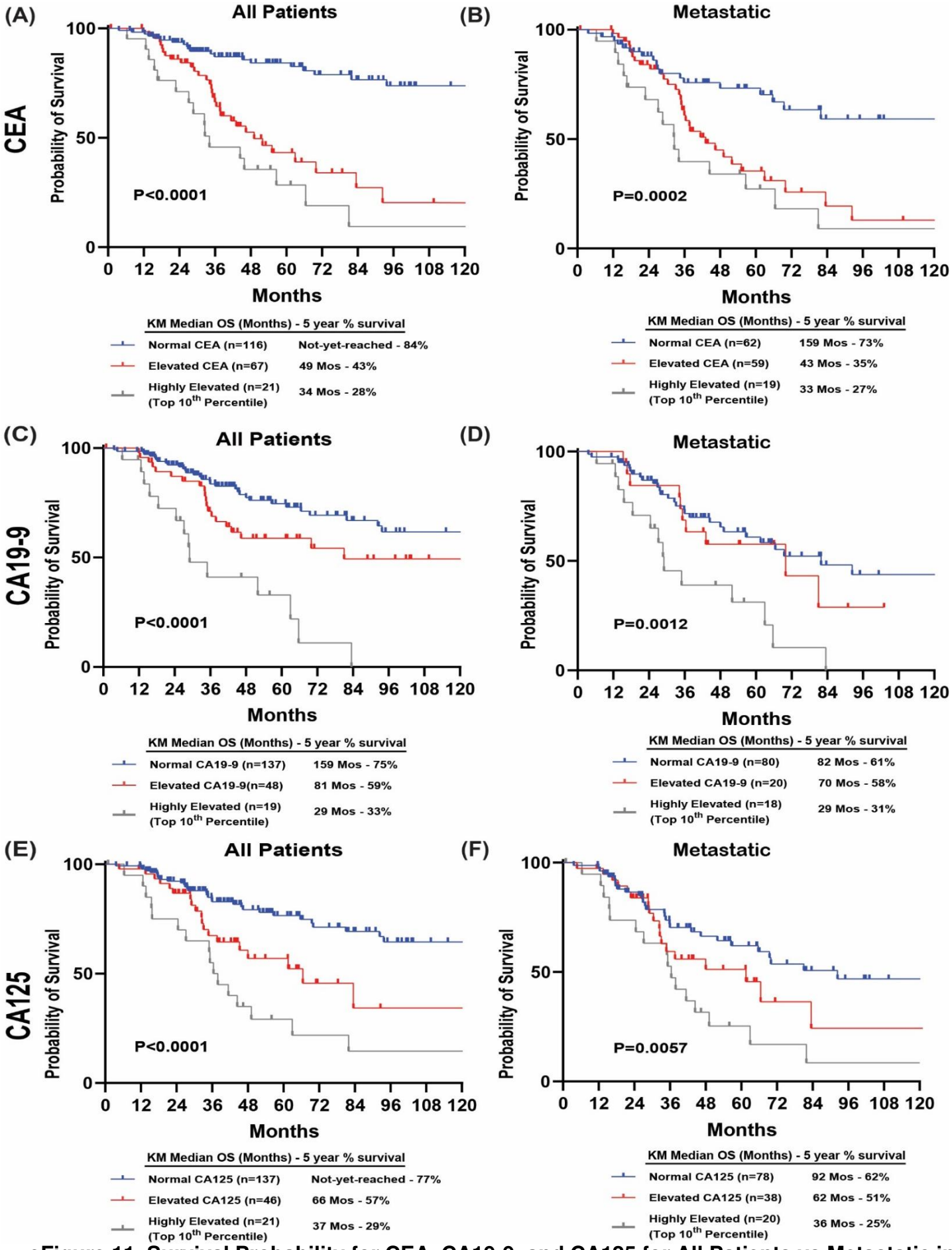
eFigure 8. Survival Probability for all patients stratified by tumor markers



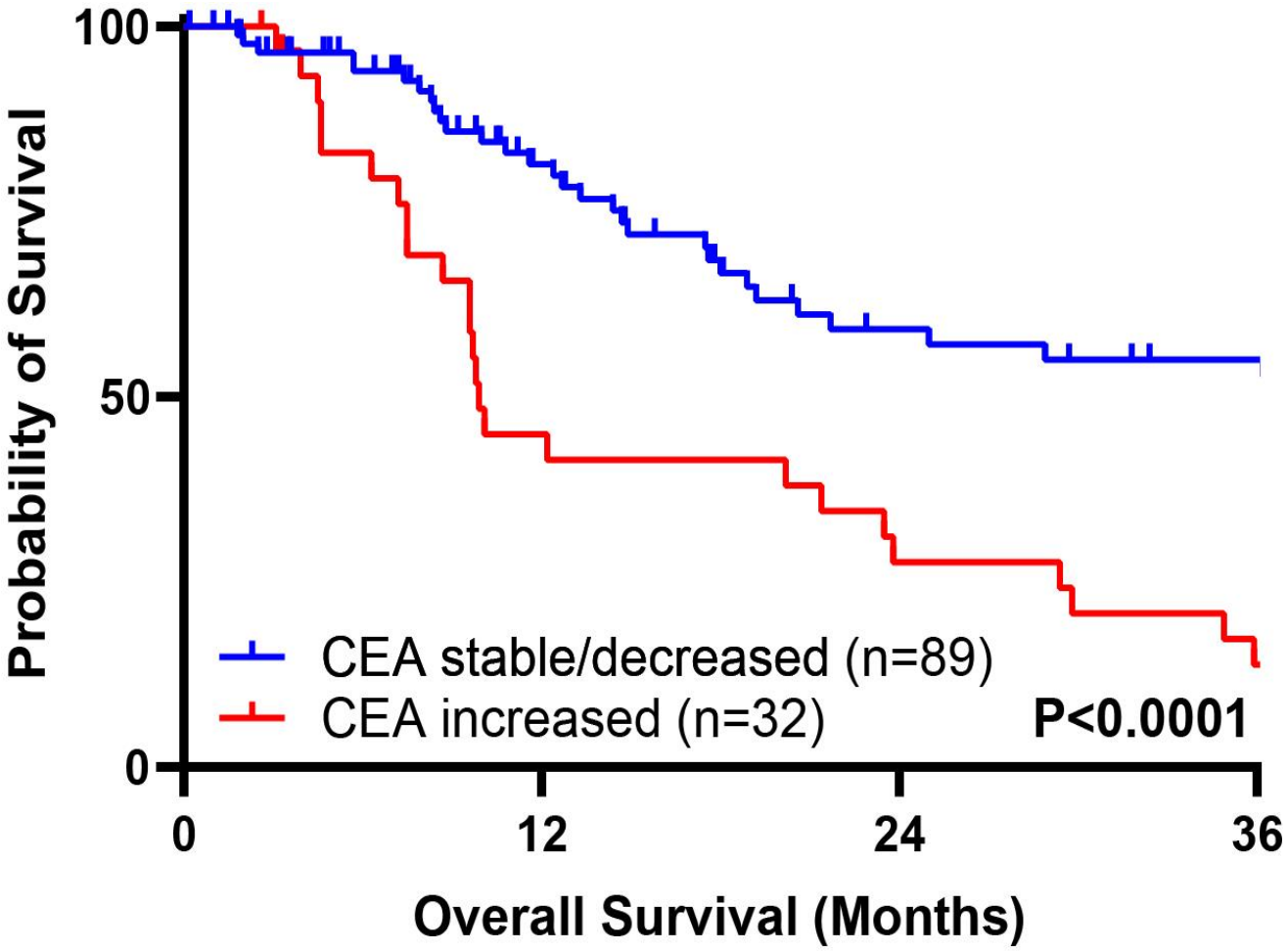
eFigure 9. Survival Probability by Grade



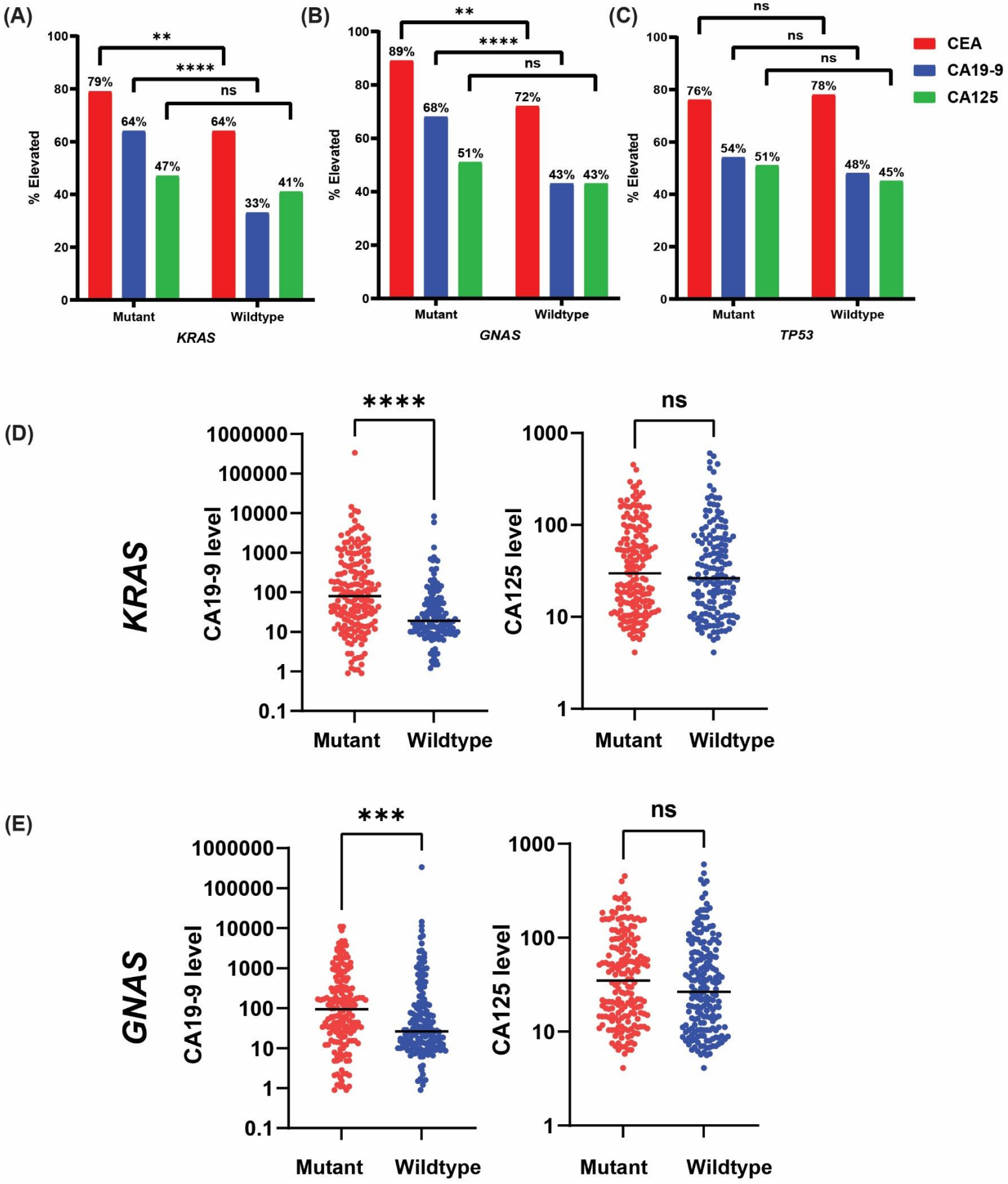
eFigure 10. Survival Probability for CEA, CA19-9, and CA125 for patients tested within the First 6 months from diagnosis



eFigure 11. Survival Probability for CEA, CA19-9, and CA125 for All Patients vs Metastatic in the validation cohort



eFigure 12. Survival probability after receiving chemotherapy stratified by CEA



eFigure 13. Association of *GNAS* and *KRAS* Somatic Mutations With Tumor Markers

Data Sharing Statement

Yousef. Serum Tumor Markers and Outcomes in Patients With Appendiceal Adenocarcinoma. *JAMA Netw Open*. Published February 23, 2024. doi:10.1001/jamanetworkopen.2024.0260

Data
Data available: Yes
Data types: Deidentified participant data
How to access data: The data generated in this study are included in the supplementary tables or otherwise available upon request to the corresponding author
When available: With publication

Supporting Documents
Document types: None

Additional Information
Who can access the data: The data generated in this study are included in the supplementary tables or otherwise available upon request to the corresponding author
Types of analyses: any purpose
Mechanisms of data availability: after approval of a proposal

Appendix 1.4

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

Genomic Subtypes of Appendiceal Adenocarcinoma: Enough to Guide Clinical Decision Making?

TO THE EDITOR:

Foote et al[1] have reported on the molecular classification of 273 patients with appendiceal adenocarcinoma (AC) who had next-generation sequencing through the MSK-IMPACT study. We applaud the authors of this study for investigating the outcomes of these patients with such a rare and aggressive disease where so little is understood regarding the biology of the tumor and its microenvironment. The investigators have classified AC into four molecular subtypes: (1) RAS-mutant (mut) predominant, (2) GNAS-mut predominant, (3) TP53-predominant, and (4) triple-negative (*RAS*/*GNAS*/*TP53* wildtype). The first RAS-mut predominant group demonstrated improved overall survival (OS) compared with the other subtypes with median OS not reached. This group also demonstrated signals of chemoresistance with the investigators suggesting implications on clinical decision making regarding recommendations for cytoreductive surgery (CRS) and use of systemic chemotherapy.

We would like to raise some additional discussion points not included in this manuscript that are important for readers interpretation of the data provided. Although the investigators have documented the surgical status of the patient cohort studied (whether CRS was performed or palliative debulking) and described the burden of disease on the basis of the standard scoring according to the Peritoneal Cancer Index, there has not been presentation of outcomes according to the completeness of cytoreduction (CC) for those patients, where CC0 is no residual disease (RD), CC1 <0.25 cm RD, CC2 0.25-2.5 cm RD, and CC3 >2.5 cm RD.[2] Interestingly, 180 (66%) patients did not receive intraperitoneal chemotherapy, potentially indicating a significant cohort of patients who received suboptimal debulking operations rather than complete cytoreduction. This suggests a group of patients with either high burden of disease or with unfavorable location of the disease (eg, implants affecting the small bowel). This is also supported by the fact that only 15 samples had <10% tumor purity. Given that CRS can be curative, especially when complete cytoreduction is achieved (CC0), we feel this would be an important confounder to include in any analysis of prognostic variables. Additionally, nodal staging has not been included as a covariate, with previous data demonstrating prognostic value.[3] It is, therefore, important to take these factors into consideration before any conclusions regarding guidance for current clinical decision making are drawn.

To conclude, this study provides important insights to this neglected disease; we congratulate the investigators on their in-depth analysis and eagerly await external validation in other cohorts before any change in clinical decision making. In addition, although understanding the genomic classification of AC in different populations is important, we need more dedicated study of the tumor and the peritoneal microenvironment to gain further understanding of the mechanisms of chemotherapy response and resistance to ultimately drive rational development of novel drug and treatment strategies.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at DOI https://doi.org/10.1200/JCO.22.02895.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Genomic Subtypes of Appendiceal Adenocarcinoma: Enough to Guide Clinical Decision Making?

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Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

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No other potential conflicts of interest were reported.

8.2 Appendix 2: Ethics

8.3 Appendix 3: Supplementary material for chapters 3 and 4

Supplementary Table ST3.1 | Top 10 differentially expressed genes per scRNAseq cluster for LG ATPD

Clusters are compared against all other clusters using the MAST method (FindAllMarkers) with a log-scale fold change threshold of 0.25, detected in at least 0.25 of cells in either cluster. Statistical significance for differential gene expression in MAST was determined using the Wilcoxon test, with all p-values adjusted using the Bonferroni correction.

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.05	0.933	0.36	<0.0001	0	*IL7R*
<0.0001	1.95	0.741	0.179	<0.0001	0	*KLRB1*
<0.0001	1.70	0.92	0.405	<0.0001	0	*STAT4*
<0.0001	1.69	0.606	0.127	<0.0001	0	*LINC01871*
<0.0001	1.63	0.6	0.158	<0.0001	0	*ANK3*
<0.0001	1.58	0.954	0.454	<0.0001	0	*CD69*
<0.0001	1.46	0.895	0.416	<0.0001	0	*PARP8*
<0.0001	1.41	0.762	0.309	<0.0001	0	*RORA*
<0.0001	1.40	0.695	0.283	<0.0001	0	*CDC14A*
<0.0001	1.36	0.777	0.242	<0.0001	0	*PBX4*
<0.0001	4.44	0.987	0.113	<0.0001	1	*SLPI*
<0.0001	4.13	0.932	0.093	<0.0001	1	*HP*
<0.0001	3.90	0.968	0.126	<0.0001	1	*PLA2G2A*
<0.0001	3.46	0.997	0.167	<0.0001	1	*CCDC80*
<0.0001	3.45	0.886	0.048	<0.0001	1	*PRG4*
<0.0001	3.29	0.997	0.242	<0.0001	1	*C3*
<0.0001	3.20	0.578	0.056	<0.0001	1	*ITLN1*
<0.0001	3.09	0.977	0.064	<0.0001	1	*CALB2*
<0.0001	2.97	0.992	0.197	<0.0001	1	*SERPING1*
<0.0001	2.87	0.988	0.144	<0.0001	1	*RARRES2*
<0.0001	3.81	0.944	0.17	<0.0001	2	*HMOX1*
<0.0001	3.05	0.651	0.099	<0.0001	2	*MT1G*
<0.0001	2.93	0.971	0.127	<0.0001	2	*C1QB*
<0.0001	2.92	0.985	0.134	<0.0001	2	*C1QA*
<0.0001	2.85	0.893	0.058	<0.0001	2	*LYVE1*
<0.0001	2.85	0.876	0.273	<0.0001	2	*FN1*
<0.0001	2.84	0.963	0.181	<0.0001	2	*SLC16A10*
<0.0001	2.75	0.884	0.117	<0.0001	2	*RNASE1*
<0.0001	2.68	0.938	0.134	<0.0001	2	*FMNL2*
<0.0001	2.62	0.915	0.089	<0.0001	2	*MARCO*
<0.0001	2.31	0.706	0.207	<0.0001	3	*TAF4B*
<0.0001	1.96	0.88	0.392	<0.0001	3	*RAPGEF6*
<0.0001	1.91	0.72	0.077	<0.0001	3	*LEF1*
<0.0001	1.65	0.778	0.207	<0.0001	3	*INPP4B*
<0.0001	1.63	0.876	0.25	<0.0001	3	*ITK*
<0.0001	1.56	0.592	0.102	<0.0001	3	*TXK*
<0.0001	1.56	0.865	0.518	<0.0001	3	*NDFIP1*
<0.0001	1.55	0.646	0.091	<0.0001	3	*PRKCQ-AS1*
<0.0001	1.55	0.79	0.293	<0.0001	3	*PRKCA*
<0.0001	1.49	0.783	0.28	<0.0001	3	*AP001011.1*
<0.0001	3.03	0.744	0.064	<0.0001	4	*CRTAM*
<0.0001	2.66	0.603	0.143	<0.0001	4	*CCL4L2*
<0.0001	2.59	0.915	0.319	<0.0001	4	*CCL4*
<0.0001	2.36	0.49	0.061	<0.0001	4	*IFNG*
<0.0001	2.34	0.96	0.29	<0.0001	4	*CCL5*
<0.0001	2.30	0.877	0.159	<0.0001	4	*GZMK*
<0.0001	2.16	0.857	0.243	<0.0001	4	*PPP1R16B*
<0.0001	2.14	0.888	0.446	<0.0001	4	*GNG2*
<0.0001	2.09	0.986	0.562	<0.0001	4	*FYN*
<0.0001	2.09	0.84	0.291	<0.0001	4	*BICDL1*
<0.0001	1.63	0.835	0.387	<0.0001	5	*IL7R*
<0.0001	1.53	0.928	0.844	<0.0001	5	*TSC22D3*
<0.0001	1.43	0.997	0.951	<0.0001	5	*RPS27*
<0.0001	1.33	0.99	0.919	<0.0001	5	*RPS29*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	1.20	0.712	0.679	<0.0001	5	*SRSF2*
<0.0001	1.19	0.987	0.922	<0.0001	5	*RPL39*
<0.0001	1.15	0.996	0.939	<0.0001	5	*RPL28*
<0.0001	1.13	0.996	0.927	<0.0001	5	*RPS12*
<0.0001	1.12	0.998	0.944	<0.0001	5	*RPLP1*
<0.0001	1.07	0.987	0.905	<0.0001	5	*RPS15A*
<0.0001	3.83	0.978	0.188	<0.0001	6	*VCAN*
<0.0001	3.12	0.955	0.276	<0.0001	6	*THBS1*
<0.0001	2.98	0.992	0.467	<0.0001	6	*JARID2*
<0.0001	2.96	0.844	0.193	<0.0001	6	*SLC16A10*
<0.0001	2.89	0.995	0.256	<0.0001	6	*LYZ*
<0.0001	2.81	0.988	0.152	<0.0001	6	*KYNU*
<0.0001	2.74	0.837	0.104	<0.0001	6	*EREG*
<0.0001	2.68	0.816	0.074	<0.0001	6	*PID1*
<0.0001	2.62	0.998	0.5	<0.0001	6	*SIPA1L1*
<0.0001	2.45	0.774	0.109	<0.0001	6	*PPARG*
<0.0001	4.37	0.922	0.18	<0.0001	7	*AQP9*
<0.0001	4.05	0.81	0.165	<0.0001	7	*IL1R2*
<0.0001	3.99	0.866	0.353	<0.0001	7	*CPD*
<0.0001	3.84	0.698	0.16	<0.0001	7	*LUCAT1*
<0.0001	3.81	0.711	0.159	<0.0001	7	*LIMK2*
<0.0001	3.73	0.402	0.087	<0.0001	7	*AZIN1-AS1*
<0.0001	3.69	0.819	0.25	<0.0001	7	*DOCK4*
<0.0001	3.68	0.994	0.717	<0.0001	7	*NAMPT*
<0.0001	3.60	0.962	0.592	<0.0001	7	*SAMSN1*
<0.0001	3.58	0.864	0.353	<0.0001	7	*LCP2*
<0.0001	3.04	0.579	0.11	<0.0001	8	*C1QC*
<0.0001	3.04	0.664	0.164	<0.0001	8	*C1QA*
<0.0001	3.00	0.624	0.158	<0.0001	8	*C1QB*
<0.0001	2.96	0.48	0.217	<0.0001	8	*PLTP*
<0.0001	2.93	0.899	0.289	<0.0001	8	*HLA-DRB5*
<0.0001	2.86	0.97	0.596	<0.0001	8	*CD74*
<0.0001	2.67	0.821	0.197	<0.0001	8	*CD14*
<0.0001	2.67	0.98	0.451	<0.0001	8	*HLA-DRA*
<0.0001	2.46	0.622	0.021	<0.0001	8	*HLA-DQA2*
<0.0001	2.41	0.941	0.266	<0.0001	8	*LYZ*
<0.0001	2.86	0.752	0.178	<0.0001	9	*NKG7*
<0.0001	2.84	0.822	0.333	<0.0001	9	*CCL4*
<0.0001	2.38	0.495	0.123	<0.0001	9	*KLRD1*
<0.0001	2.35	0.878	0.304	<0.0001	9	*CCL5*
<0.0001	2.13	0.717	0.274	<0.0001	9	*CST7*
<0.0001	2.11	0.811	0.477	<0.0001	9	*DUSP2*
<0.0001	1.62	0.765	0.531	<0.0001	9	*HCST*
<0.0001	1.52	0.771	0.676	<0.0001	9	*SRSF2*
<0.0001	1.23	0.392	0.342	<0.0001	9	*ABHD17A*
<0.0001	1.94	0.675	0.395	<0.0001	9	*TUBA4A*
<0.0001	4.30	0.984	0.244	<0.0001	10	*COL1A1*
<0.0001	4.13	0.957	0.123	<0.0001	10	*FBLN1*
<0.0001	4.11	0.98	0.232	<0.0001	10	*COL1A2*
<0.0001	3.96	0.969	0.229	<0.0001	10	*COL3A1*
<0.0001	3.87	0.955	0.187	<0.0001	10	*MGP*
<0.0001	3.59	0.913	0.116	<0.0001	10	*BGN*
<0.0001	3.58	0.779	0.082	<0.0001	10	*SPARCL1*
<0.0001	3.44	0.983	0.178	<0.0001	10	*DCN*
<0.0001	3.37	0.953	0.201	<0.0001	10	*SPARC*
<0.0001	3.37	0.948	0.176	<0.0001	10	*IGFBP7*
<0.0001	2.67	0.698	0.107	<0.0001	11	*FAAH2*
<0.0001	2.60	0.515	0.124	<0.0001	11	*PTPN13*
<0.0001	2.39	0.77	0.269	<0.0001	11	*THADA*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.27	0.957	0.386	<0.0001	11	*LDLRAD4*
<0.0001	2.16	0.729	0.139	<0.0001	11	*TOX*
<0.0001	2.05	0.976	0.516	<0.0001	11	*RNF19A*
<0.0001	2.01	0.921	0.315	<0.0001	11	*GPRIN3*
<0.0001	1.93	0.933	0.403	<0.0001	11	*RAPGEF6*
<0.0001	1.91	0.76	0.073	<0.0001	11	*CTLA4*
<0.0001	1.86	0.905	0.373	<0.0001	11	*ABCC1*
<0.0001	3.44	0.94	0.037	<0.0001	12	*BANK1*
<0.0001	3.23	0.934	0.113	<0.0001	12	*AFF3*
<0.0001	2.85	0.883	0.043	<0.0001	12	*EBF1*
<0.0001	2.59	0.899	0.018	<0.0001	12	*MS4A1*
<0.0001	2.57	0.771	0.047	<0.0001	12	*IGHM*
<0.0001	2.55	0.926	0.034	<0.0001	12	*CD79A*
<0.0001	2.50	0.921	0.352	<0.0001	12	*BACH2*
<0.0001	2.45	0.759	0.009	<0.0001	12	*FCRL1*
<0.0001	2.44	0.816	0.036	<0.0001	12	*AC120193.1*
<0.0001	2.43	0.839	0.092	<0.0001	12	*ADAM28*
<0.0001	3.36	0.932	0.097	<0.0001	13	*ALDH1A3*
<0.0001	3.27	0.577	0.047	<0.0001	13	*PTX3*
<0.0001	3.13	0.986	0.174	<0.0001	13	*APOE*
<0.0001	3.09	0.992	0.33	<0.0001	13	*MT1E*
<0.0001	3.05	0.889	0.043	<0.0001	13	*CCN1*
<0.0001	3.05	0.677	0.047	<0.0001	13	*GPC6*
<0.0001	3.04	0.843	0.146	<0.0001	13	*SERPINE1*
<0.0001	3.04	0.972	0.137	<0.0001	13	*IGFBP2*
<0.0001	3.02	0.942	0.181	<0.0001	13	*MT1M*
<0.0001	2.94	0.895	0.184	<0.0001	13	*CCDC71L*
<0.0001	2.46	0.707	0.075	<0.0001	14	*IKZF2*
<0.0001	2.32	0.855	0.237	<0.0001	14	*BATF*
<0.0001	2.28	0.68	0.118	<0.0001	14	*IL2RA*
<0.0001	2.17	0.672	0.026	<0.0001	14	*RTKN2*
<0.0001	2.07	0.739	0.077	<0.0001	14	*CTLA4*
<0.0001	1.72	0.692	0.207	<0.0001	14	*STAM*
<0.0001	1.04	0.375	0.005	<0.0001	14	*FOXP3*
<0.0001	1.56	0.648	0.13	<0.0001	14	*TBC1D4*
<0.0001	1.64	0.578	0.07	<0.0001	14	*TIGIT*
<0.0001	1.80	0.872	0.297	<0.0001	14	*LTB*
<0.0001	4.65	0.977	0.312	<0.0001	15	*S100A9*
<0.0001	4.64	0.915	0.346	<0.0001	15	*CXCL8*
<0.0001	4.41	0.931	0.242	<0.0001	15	*S100A8*
<0.0001	3.84	0.873	0.391	<0.0001	15	*ALOX5AP*
<0.0001	3.76	0.975	0.718	<0.0001	15	*IFITM2*
<0.0001	3.50	0.662	0.277	<0.0001	15	*NCF1*
<0.0001	3.49	0.812	0.181	<0.0001	15	*IL1R2*
<0.0001	3.34	0.77	0.224	<0.0001	15	*CSF3R*
<0.0001	3.33	0.506	0.168	<0.0001	15	*MNDA*
<0.0001	3.29	0.832	0.342	<0.0001	15	*BCL2A1*
<0.0001	1.42	1	0.958	<0.0001	16	*MT-ND3*
<0.0001	1.28	1	0.966	<0.0001	16	*MT-CO2*
<0.0001	1.33	0.991	0.951	<0.0001	16	*MT-CYB*
<0.0001	1.26	0.391	0.549	<0.0001	16	*BRD2*
<0.0001	1.26	0.822	0.844	<0.0001	16	*MT-ND5*
<0.0001	1.36	0.535	0.662	<0.0001	16	*HNRNPH1*
<0.0001	1.08	0.512	0.696	<0.0001	16	*FUS*
<0.0001	1.34	0.968	0.934	<0.0001	16	*MT-ND2*
<0.0001	1.17	0.391	0.616	<0.0001	16	*TUBB4B*
<0.0001	1.02	0.498	0.676	<0.0001	16	*SLC38A2*
<0.0001	2.43	0.876	0.124	<0.0001	17	*KLRD1*
<0.0001	2.04	0.622	0.021	<0.0001	17	*KLRC1*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.56	0.777	0.249	<0.0001	17	*CMC1*
<0.0001	2.83	0.706	0.049	<0.0001	17	*XCL1*
<0.0001	2.84	0.779	0.075	<0.0001	17	*XCL2*
<0.0001	2.63	0.895	0.188	<0.0001	17	*NKG7*
<0.0001	1.96	0.832	0.13	<0.0001	17	*CTSW*
<0.0001	1.59	0.511	0.015	<0.0001	17	*KLRC3*
<0.0001	1.55	0.55	0.028	<0.0001	17	*TRDC*
<0.0001	1.04	0.426	0.009	<0.0001	17	*NCR1*
<0.0001	3.37	0.995	0.19	<0.0001	18	*KRT18*
<0.0001	3.33	1	0.339	<0.0001	18	*MT1E*
<0.0001	3.12	0.995	0.138	<0.0001	18	*CFB*
<0.0001	3.11	0.983	0.191	<0.0001	18	*MT1M*
<0.0001	2.88	0.99	0.184	<0.0001	18	*TM4SF1*
<0.0001	2.82	0.99	0.178	<0.0001	18	*ADIRF*
<0.0001	2.77	0.998	0.163	<0.0001	18	*KRT19*
<0.0001	2.58	0.985	0.167	<0.0001	18	*CAV1*
<0.0001	2.37	0.998	0.182	<0.0001	18	*KRT8*
<0.0001	2.26	0.998	0.501	<0.0001	18	*SNHG29*
<0.0001	8.53	0.959	0.555	<0.0001	19	*IGKC*
<0.0001	8.45	0.864	0.325	<0.0001	19	*IGLC2*
<0.0001	7.58	0.918	0.207	<0.0001	19	*IGHG1*
<0.0001	7.56	0.862	0.225	<0.0001	19	*IGHGP*
<0.0001	7.23	0.831	0.179	<0.0001	19	*IGHG3*
<0.0001	7.05	0.806	0.197	<0.0001	19	*IGHG4*
<0.0001	5.40	0.877	0.071	<0.0001	19	*JCHAIN*
<0.0001	3.82	0.949	0.033	<0.0001	19	*MZB1*
<0.0001	3.32	0.908	0.449	<0.0001	19	*ANKRD28*
<0.0001	2.71	0.973	0.685	<0.0001	19	*SSR4*
<0.0001	6.50	0.983	0.125	<0.0001	20	*TFF3*
<0.0001	6.25	0.994	0.065	<0.0001	20	*TFF1*
<0.0001	5.70	0.836	0.076	<0.0001	20	*SPINK4*
<0.0001	5.61	0.898	0.038	<0.0001	20	*REG4*
<0.0001	5.47	0.924	0.038	<0.0001	20	*TFF2*
<0.0001	5.45	0.963	0.067	<0.0001	20	*MUC2*
<0.0001	4.91	0.986	0.031	<0.0001	20	*TSPAN8*
<0.0001	4.74	0.989	0.021	<0.0001	20	*AGR2*
<0.0001	4.61	0.926	0.012	<0.0001	20	*CEACAM5*
<0.0001	4.23	0.975	0.026	<0.0001	20	*CLDN4*
<0.0001	1.47	0.328	0.429	<0.0001	21	*SNHG25*
<0.0001	1.14	0.379	0.595	<0.0001	21	*CALR*
<0.0001	2.10	0.836	0.284	<0.0001	21	*C3*
<0.0001	1.72	0.819	0.718	<0.0001	21	*FOSB*
<0.0001	1.01	0.394	0.605	<0.0001	21	*SEC61G*
<0.0001	1.01	0.339	0.536	<0.0001	21	*B4GALT1*
<0.0001	2.00	0.638	0.163	<0.0001	21	*SLPI*
<0.0001	1.32	0.463	0.496	<0.0001	21	*HSPB1*
<0.0001	1.34	0.943	0.819	<0.0001	21	*S100A6*
<0.0001	1.03	0.201	0.309	<0.0001	21	*PPP1R14B*
<0.0001	3.47	0.997	0.387	<0.0001	22	*HLA-DPB1*
<0.0001	3.31	0.997	0.376	<0.0001	22	*HLA-DPA1*
<0.0001	3.23	0.997	0.278	<0.0001	22	*HLA-DQA1*
<0.0001	1.79	0.801	0.02	<0.0001	22	*FCER1A*
<0.0001	3.10	1	0.412	<0.0001	22	*HLA-DRB1*
<0.0001	2.31	0.943	0.117	<0.0001	22	*CLEC10A*
<0.0001	2.73	0.997	0.351	<0.0001	22	*HLA-DQB1*
<0.0001	3.01	1	0.468	<0.0001	22	*HLA-DRA*
<0.0001	1.94	0.958	0.162	<0.0001	22	*CSF2RA*
<0.0001	1.11	0.604	0.015	<0.0001	22	*CD1C*
<0.0001	4.11	0.89	0.125	<0.0001	23	*APOC1*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.62	0.932	0.114	<0.0001	23	*KCNMA1*
<0.0001	2.48	0.997	0.339	<0.0001	23	*CTSZ*
<0.0001	3.03	0.981	0.449	<0.0001	23	*CTSD*
<0.0001	2.87	0.997	0.431	<0.0001	23	*CTSB*
<0.0001	2.71	1	0.943	<0.0001	23	*FTL*
<0.0001	1.07	0.739	0.027	<0.0001	23	*MMP2-AS1*
<0.0001	4.34	0.897	0.191	<0.0001	23	*APOE*
<0.0001	2.96	1	0.412	<0.0001	23	*HLA-DRB1*
<0.0001	2.83	0.99	0.279	<0.0001	23	*HLA-DQA1*
<0.0001	6.72	0.957	0.013	<0.0001	24	*TPSB2*
<0.0001	5.23	0.94	0.006	<0.0001	24	*TPSAB1*
<0.0001	5.20	0.965	0.004	<0.0001	24	*CPA3*
<0.0001	5.02	0.855	0.004	<0.0001	24	*CTSG*
<0.0001	4.62	0.915	0.102	<0.0001	24	*HPGD*
<0.0001	4.20	0.837	0.044	<0.0001	24	*SLC24A3*
<0.0001	3.68	0.918	0.013	<0.0001	24	*HPGDS*
<0.0001	3.54	0.95	0.027	<0.0001	24	*IL1RL1*
<0.0001	3.35	0.635	0.013	<0.0001	24	*ADCYAP1*
<0.0001	3.21	0.823	0.009	<0.0001	24	*KIT*
<0.0001	4.74	0.821	0.265	<0.0001	25	*PLCG2*
<0.0001	5.62	0.94	0.072	<0.0001	25	*MUC2*
<0.0001	4.39	0.821	0.03	<0.0001	25	*ELF3*
<0.0001	4.61	0.908	0.13	<0.0001	25	*TFF3*
<0.0001	2.46	0.408	0.112	<0.0001	25	*INF2*
<0.0001	4.43	0.739	0.024	<0.0001	25	*PHGR1*
<0.0001	3.90	0.876	0.072	<0.0001	25	*TFF1*
<0.0001	3.10	0.734	0.314	<0.0001	25	*GSN*
<0.0001	3.37	0.743	0.107	<0.0001	25	*C19orf33*
<0.0001	3.68	0.633	0.106	<0.0001	25	*HES1*
<0.0001	2.03	0.748	0.018	<0.0001	26	*PCLAF*
<0.0001	1.71	0.704	0.024	<0.0001	26	*TYMS*
<0.0001	1.71	0.675	0.01	<0.0001	26	*MKI67*
<0.0001	2.55	0.845	0.211	<0.0001	26	*STMN1*
<0.0001	1.60	0.646	0.013	<0.0001	26	*RRM2*
<0.0001	1.75	0.694	0.021	<0.0001	26	*TOP2A*
<0.0001	1.10	0.597	0.014	<0.0001	26	*TPX2*
<0.0001	1.21	0.587	0.013	<0.0001	26	*ASPM*
<0.0001	1.01	0.524	0.007	<0.0001	26	*UBE2C*
<0.0001	1.96	0.947	0.577	<0.0001	26	*HMGN2*
<0.0001	3.57	0.969	0.127	<0.0001	27	*COL5A2*
<0.0001	3.97	0.888	0.048	<0.0001	27	*CCN2*
<0.0001	3.82	0.99	0.059	<0.0001	27	*GPC6*
<0.0001	2.27	0.908	0.026	<0.0001	27	*LTBP1*
<0.0001	2.42	0.811	0.009	<0.0001	27	*COL11A1*
<0.0001	2.44	0.949	0.056	<0.0001	27	*FAP*
<0.0001	4.08	0.893	0.044	<0.0001	27	*POSTN*
<0.0001	2.72	0.944	0.063	<0.0001	27	*COL12A1*
<0.0001	2.54	0.847	0.022	<0.0001	27	*KIF26B*
<0.0001	3.01	0.964	0.079	<0.0001	27	*PRKG1*
<0.0001	2.96	0.772	0.057	<0.0001	28	*CD79A*
<0.0001	2.45	0.598	0.046	<0.0001	28	*TNFRSF13C*
<0.0001	2.07	0.87	0.528	<0.0001	28	*CD37*
<0.0001	2.35	0.511	0.04	<0.0001	28	*LINC00926*
<0.0001	1.58	0.549	0.471	<0.0001	28	*LSM7*
<0.0001	2.36	0.587	0.066	<0.0001	28	*IGHM*
<0.0001	2.21	0.576	0.042	<0.0001	28	*MS4A1*
<0.0001	1.85	0.989	0.61	<0.0001	28	*CD74*
<0.0001	2.03	0.533	0.044	<0.0001	28	*HLA-DQA2*
<0.0001	1.67	0.386	0.083	<0.0001	28	*P2RX5*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	4.26	0.858	0.007	<0.0001	29	*VWF*
<0.0001	2.62	0.811	0.063	<0.0001	29	*HYAL2*
<0.0001	2.80	0.831	0.062	<0.0001	29	*RAMP2*
<0.0001	2.61	0.872	0.008	<0.0001	29	*ECSCR*
<0.0001	3.59	0.791	0.004	<0.0001	29	*CLDN5*
<0.0001	2.48	0.777	0.003	<0.0001	29	*ADGRL4*
<0.0001	2.11	0.764	0.002	<0.0001	29	*EMCN*
<0.0001	1.89	0.757	0.002	<0.0001	29	*PCAT19*
<0.0001	1.75	0.703	0.002	<0.0001	29	*CDH5*
<0.0001	2.77	0.811	0.04	<0.0001	29	*CALCRL*
<0.0001	2.58	0.96	0.615	<0.0001	30	*SEC61B*
<0.0001	2.38	0.919	0.022	<0.0001	30	*PLD4*
<0.0001	2.22	0.847	0.006	<0.0001	30	*SCT*
<0.0001	3.34	0.96	0.135	<0.0001	30	*IRF4*
<0.0001	4.81	0.968	0.106	<0.0001	30	*GZMB*
<0.0001	3.06	0.968	0.102	<0.0001	30	*IL3RA*
<0.0001	2.77	0.919	0.304	<0.0001	30	*PPP1R14B*
<0.0001	1.61	0.774	0.006	<0.0001	30	*LILRA4*
<0.0001	2.42	0.903	0.059	<0.0001	30	*TSPAN13*
<0.0001	2.90	0.935	0.237	<0.0001	30	*SEL1L3*
<0.0001	3.25	0.966	0.033	<0.0001	31	*AC023590.1*
<0.0001	3.11	0.977	0.173	<0.0001	31	*LRMP*
<0.0001	2.92	0.885	0.019	<0.0001	31	*RAPGEF5*
<0.0001	2.48	0.931	0.055	<0.0001	31	*LMO2*
<0.0001	2.98	0.92	0.11	<0.0001	31	*PRPSAP2*
<0.0001	2.81	0.92	0.018	<0.0001	31	*TCL1A*
<0.0001	2.68	0.966	0.041	<0.0001	31	*CD22*
<0.0001	2.17	0.874	0.047	<0.0001	31	*BCL7A*
<0.0001	2.52	0.943	0.024	<0.0001	31	*PAX5*
<0.0001	3.27	0.977	0.042	<0.0001	31	*MS4A1*
<0.0001	5.48	0.758	0.22	<0.0001	32	*CFD*
<0.0001	5.28	0.758	0.031	<0.0001	32	*APOD*
<0.0001	3.28	0.955	0.037	<0.0001	32	*COL4A1*
<0.0001	3.15	0.939	0.062	<0.0001	32	*COL4A2*
<0.0001	2.89	0.773	0.029	<0.0001	32	*NEGR1*
<0.0001	3.49	0.742	0.025	<0.0001	32	*LAMA2*
<0.0001	4.28	0.848	0.134	<0.0001	32	*MT1A*
<0.0001	2.38	0.773	0.072	<0.0001	32	*FBLN5*
<0.0001	3.32	0.833	0.09	<0.0001	32	*GPX3*
<0.0001	2.85	0.97	0.138	<0.0001	32	*LHFPL6*
<0.0001	3.02	0.963	0.013	<0.0001	33	*LAMP3*
<0.0001	3.03	0.889	0.008	<0.0001	33	*GPC5*
<0.0001	2.74	0.852	0.045	<0.0001	33	*ENOX1*
<0.0001	3.38	0.963	0.547	<0.0001	33	*TXN*
<0.0001	2.33	0.852	0.07	<0.0001	33	*SLC22A23*
<0.0001	2.87	0.667	0.003	<0.0001	33	*CCL19*
<0.0001	1.91	0.815	0.009	<0.0001	33	*IDO2*
<0.0001	2.83	1	0.289	<0.0001	33	*UVRAG*
<0.0001	1.92	0.815	0.025	<0.0001	33	*SLCO5A1*
<0.0001	2.88	0.778	0.014	<0.0001	33	*IDO1*
<0.0001	3.10	1	0.19	<0.0001	34	*IGHG3*
<0.0001	1.10	0.941	0.017	<0.0001	34	*TPSAB1*
<0.0001	1.38	0.941	0.01	<0.0001	34	*C2CD4B*
<0.0001	1.03	0.824	0.009	<0.0001	34	*CCL14*
<0.0001	1.29	0.941	0.023	<0.0001	34	*TPSB2*
<0.0001	1.96	0.941	0.043	<0.0001	34	*ACKR1*
<0.0001	1.57	1	0.059	<0.0001	34	*LYPD2*
<0.0001	1.49	0.941	0.045	<0.0001	34	*MT1H*
<0.0001	1.30	0.765	0.021	<0.0001	34	*S100B*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.34	1	0.132	<0.0001	34	*MT1G*

Abbrev. Log2FC, log2 fold change; scRNAseq, single cell RNA sequencing; LG ATPD, low grade appendiceal tumour peritoneal disease.

Supplementary Table ST3.2 | Top 10 differentially expressed genes per scRNAseq cluster for HG ATPD

Clusters are compared against all other clusters using the MAST method (FindAllMarkers) with a log-scale fold change threshold of 0.25, detected in at least 0.25 of cells in either cluster. Statistical significance for differential gene expression in MAST was determined using the Wilcoxon test, with all p-values adjusted using the Bonferroni correction.

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.84	0.89	0.19	<0.0001	0	*CCL5*
<0.0001	2.64	0.57	0.21	<0.0001	0	*CCL4*
<0.0001	2.45	0.69	0.07	<0.0001	0	*GZMK*
<0.0001	2.20	0.96	0.52	<0.0001	0	*FYN*
<0.0001	2.04	0.85	0.54	<0.0001	0	*RNF19A*
<0.0001	2.02	0.90	0.49	<0.0001	0	*CNOT6L*
<0.0001	1.99	0.80	0.21	<0.0001	0	*BCL11B*
<0.0001	1.91	0.74	0.20	<0.0001	0	*CST7*
<0.0001	1.90	0.69	0.24	<0.0001	0	*TC2N*
<0.0001	1.89	0.61	0.09	<0.0001	0	*GZMA*
<0.0001	3.86	0.95	0.14	<0.0001	1	*KRT17*
<0.0001	3.76	0.93	0.12	<0.0001	1	*AGR2*
<0.0001	3.51	0.95	0.18	<0.0001	1	*TFF1*
<0.0001	3.42	0.97	0.14	<0.0001	1	*C19orf33*
<0.0001	3.31	0.95	0.11	<0.0001	1	*CEACAM5*
<0.0001	3.30	0.97	0.16	<0.0001	1	*CLDN4*
<0.0001	3.26	0.91	0.09	<0.0001	1	*CEACAM6*
<0.0001	3.25	0.96	0.20	<0.0001	1	*KRT19*
<0.0001	3.20	0.96	0.11	<0.0001	1	*ELF3*
<0.0001	3.13	0.92	0.10	<0.0001	1	*CLDN3*
<0.0001	2.65	0.73	0.13	<0.0001	2	*FAAH2*
<0.0001	1.98	0.82	0.18	<0.0001	2	*LTB*
<0.0001	1.96	0.60	0.20	<0.0001	2	*PGAP1*
<0.0001	1.92	0.89	0.35	<0.0001	2	*BACH2*
<0.0001	1.85	0.94	0.26	<0.0001	2	*IL7R*
<0.0001	1.80	0.54	0.19	<0.0001	2	*SESN3*
<0.0001	1.78	0.80	0.22	<0.0001	2	*ANK3*
<0.0001	1.77	0.90	0.25	<0.0001	2	*BICDL1*
<0.0001	1.76	0.72	0.39	<0.0001	2	*CMSS1*
<0.0001	1.72	0.70	0.09	<0.0001	2	*CCR7*
<0.0001	3.23	0.88	0.35	<0.0001	3	*LUCAT1*
<0.0001	3.17	0.90	0.50	<0.0001	3	*DENND5A*
<0.0001	3.02	0.92	0.31	<0.0001	3	*AQP9*
<0.0001	3.00	0.87	0.34	<0.0001	3	*ITGAX*
<0.0001	2.92	1.00	0.82	<0.0001	3	*NAMPT*
<0.0001	2.89	0.92	0.45	<0.0001	3	*LCP2*
<0.0001	2.85	0.76	0.29	<0.0001	3	*LIMK2*
<0.0001	2.85	0.59	0.11	<0.0001	3	*KCNJ15*
<0.0001	2.80	0.86	0.62	<0.0001	3	*NEDD9*
<0.0001	2.79	0.88	0.30	<0.0001	3	*CSF3R*
<0.0001	3.61	0.86	0.26	<0.0001	4	*APOE*
<0.0001	3.52	0.78	0.30	<0.0001	4	*SPP1*
<0.0001	3.31	0.83	0.16	<0.0001	4	*APOC1*
<0.0001	3.14	0.85	0.12	<0.0001	4	*C1QB*
<0.0001	3.03	0.87	0.13	<0.0001	4	*C1QA*
<0.0001	2.97	0.99	0.57	<0.0001	4	*CD74*
<0.0001	2.93	0.99	0.44	<0.0001	4	*HLA-DRA*
<0.0001	2.93	0.95	0.19	<0.0001	4	*SLC16A10*
<0.0001	2.70	0.85	0.08	<0.0001	4	*C1QC*
<0.0001	2.70	0.99	0.49	<0.0001	4	*CTSB*
<0.0001	3.32	0.98	0.30	<0.0001	5	*VCAN*
<0.0001	3.27	0.99	0.39	<0.0001	5	*LYZ*
<0.0001	2.73	0.82	0.15	<0.0001	5	*EREG*
<0.0001	2.71	0.97	0.10	<0.0001	5	*FCN1*
<0.0001	2.66	0.99	0.63	<0.0001	5	*JARID2*
<0.0001	2.31	0.92	0.10	<0.0001	5	*CD300E*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.20	0.99	0.43	<0.0001	5	*S100A9*
<0.0001	2.20	0.98	0.49	<0.0001	5	*CTSS*
<0.0001	2.16	0.93	0.45	<0.0001	5	*TRPS1*
<0.0001	2.13	0.68	0.21	<0.0001	5	*CRADD*
<0.0001	3.50	1.00	0.36	<0.0001	6	*COL1A1*
<0.0001	3.42	0.98	0.21	<0.0001	6	*SERPINE1*
<0.0001	3.36	1.00	0.33	<0.0001	6	*COL3A1*
<0.0001	3.35	1.00	0.31	<0.0001	6	*COL1A2*
<0.0001	3.30	0.93	0.12	<0.0001	6	*IGFBP2*
<0.0001	3.13	0.99	0.14	<0.0001	6	*CCDC80*
<0.0001	3.09	0.99	0.14	<0.0001	6	*CALD1*
<0.0001	3.04	0.83	0.17	<0.0001	6	*CCL2*
<0.0001	3.01	0.88	0.10	<0.0001	6	*ACTA2*
<0.0001	3.00	0.97	0.25	<0.0001	6	*FN1*
<0.0001	4.05	0.99	0.33	<0.0001	7	*COL3A1*
<0.0001	3.99	0.99	0.36	<0.0001	7	*COL1A1*
<0.0001	3.98	0.94	0.07	<0.0001	7	*SFRP2*
<0.0001	3.97	0.99	0.32	<0.0001	7	*COL1A2*
<0.0001	3.83	0.98	0.27	<0.0001	7	*SPARC*
<0.0001	3.76	0.98	0.21	<0.0001	7	*MGP*
<0.0001	3.76	0.96	0.13	<0.0001	7	*LUM*
<0.0001	3.64	0.98	0.16	<0.0001	7	*DCN*
<0.0001	3.00	0.97	0.15	<0.0001	7	*SERPINF1*
<0.0001	2.95	0.83	0.04	<0.0001	7	*SFRP4*
<0.0001	4.11	0.98	0.33	<0.0001	8	*S100A8*
<0.0001	3.83	0.99	0.43	<0.0001	8	*S100A9*
<0.0001	3.74	0.84	0.12	<0.0001	8	*S100A12*
<0.0001	3.63	0.78	0.08	<0.0001	8	*PROK2*
<0.0001	2.94	0.93	0.74	<0.0001	8	*IFITM2*
<0.0001	2.84	0.95	0.56	<0.0001	8	*CXCL8*
<0.0001	2.69	0.72	0.29	<0.0001	8	*PTGS2*
<0.0001	2.64	0.59	0.05	<0.0001	8	*CMTM2*
<0.0001	2.59	0.97	0.48	<0.0001	8	*BASP1*
<0.0001	2.47	0.91	0.39	<0.0001	8	*G0S2*
<0.0001	3.56	0.90	0.30	<0.0001	9	*SPP1*
<0.0001	2.88	0.79	0.14	<0.0001	9	*C1QA*
<0.0001	2.76	0.73	0.14	<0.0001	9	*C1QB*
<0.0001	2.76	0.95	0.21	<0.0001	9	*SLC16A10*
<0.0001	2.53	0.71	0.18	<0.0001	9	*APOC1*
<0.0001	2.47	0.99	0.45	<0.0001	9	*HLA-DRB1*
<0.0001	2.46	0.96	0.15	<0.0001	9	*CD163*
<0.0001	2.40	0.88	0.10	<0.0001	9	*MRC1*
<0.0001	2.40	0.69	0.10	<0.0001	9	*C1QC*
<0.0001	2.37	1.00	0.94	<0.0001	9	*FTL*
<0.0001	3.25	0.80	0.33	<0.0001	10	*IRAK3*
<0.0001	3.18	0.54	0.08	<0.0001	10	*AC005050.3*
<0.0001	3.12	0.89	0.47	<0.0001	10	*ALOX5AP*
<0.0001	3.11	0.76	0.30	<0.0001	10	*OLR1*
<0.0001	3.01	0.39	0.06	<0.0001	10	*CCL3L1*
<0.0001	2.90	0.60	0.34	<0.0001	10	*C15orf48*
<0.0001	2.72	0.71	0.63	<0.0001	10	*TANK*
<0.0001	2.69	0.90	0.75	<0.0001	10	*ELL2*
<0.0001	2.69	0.59	0.15	<0.0001	10	*FPR2*
<0.0001	2.65	0.35	0.13	<0.0001	10	*SATB1-AS1*
<0.0001	4.17	0.75	0.02	<0.0001	11	*XACT*
<0.0001	3.76	0.92	0.25	<0.0001	11	*TFF3*
<0.0001	3.49	0.96	0.20	<0.0001	11	*MUC2*
<0.0001	3.30	0.94	0.21	<0.0001	11	*LMO7*
<0.0001	3.24	0.88	0.13	<0.0001	11	*ITGA2*
<0.0001	3.17	0.80	0.14	<0.0001	11	*PHGR1*
<0.0001	3.15	0.90	0.22	<0.0001	11	*TFF1*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.99	0.89	0.10	<0.0001	11	*MUC4*
<0.0001	2.98	0.86	0.15	<0.0001	11	*LAMC2*
<0.0001	2.90	0.91	0.15	<0.0001	11	*CEACAM5*
<0.0001	4.91	0.69	0.05	<0.0001	12	*GNLY*
<0.0001	3.46	0.88	0.16	<0.0001	12	*NKG7*
<0.0001	2.75	0.93	0.23	<0.0001	12	*CCL5*
<0.0001	2.73	0.68	0.06	<0.0001	12	*GZMB*
<0.0001	2.37	0.40	0.01	<0.0001	12	*XCL2*
<0.0001	2.23	0.76	0.12	<0.0001	12	*GZMA*
<0.0001	2.17	0.33	0.01	<0.0001	12	*XCL1*
<0.0001	2.10	0.58	0.05	<0.0001	12	*GZMH*
<0.0001	1.94	0.61	0.10	<0.0001	12	*KLRD1*
<0.0001	1.89	0.72	0.10	<0.0001	12	*CTSW*
<0.0001	2.73	0.71	0.09	<0.0001	13	*KLRB1*
<0.0001	2.44	0.98	0.30	<0.0001	13	*IL7R*
<0.0001	2.06	0.91	0.34	<0.0001	13	*RORA*
<0.0001	2.05	0.83	0.28	<0.0001	13	*TTC39C*
<0.0001	1.53	0.55	0.05	<0.0001	13	*CCR6*
<0.0001	1.63	0.89	0.23	<0.0001	13	*CD3D*
<0.0001	1.99	0.83	0.21	<0.0001	13	*LTB*
<0.0001	1.63	0.85	0.23	<0.0001	13	*CD2*
<0.0001	1.57	0.83	0.22	<0.0001	13	*CD96*
<0.0001	1.54	0.82	0.20	<0.0001	13	*CAMK4*
<0.0001	4.34	0.81	0.02	<0.0001	14	*CCL14*
<0.0001	4.22	0.69	0.05	<0.0001	14	*FABP4*
<0.0001	4.00	0.92	0.03	<0.0001	14	*VWF*
<0.0001	3.95	0.71	0.02	<0.0001	14	*ACKR1*
<0.0001	3.73	0.88	0.04	<0.0001	14	*ZNF385D*
<0.0001	3.71	0.92	0.10	<0.0001	14	*ADAMTS9*
<0.0001	3.59	0.93	0.14	<0.0001	14	*SPARCL1*
<0.0001	3.56	0.93	0.05	<0.0001	14	*CALCRL*
<0.0001	3.14	0.59	0.01	<0.0001	14	*SELE*
<0.0001	2.98	0.83	0.04	<0.0001	14	*AQP1*
<0.0001	3.51	0.99	0.40	<0.0001	15	*HLA-DPB1*
<0.0001	3.47	0.99	0.21	<0.0001	15	*HLA-DQA1*
<0.0001	3.41	0.99	0.38	<0.0001	15	*HLA-DPA1*
<0.0001	3.38	1.00	0.47	<0.0001	15	*HLA-DRA*
<0.0001	3.17	1.00	0.46	<0.0001	15	*HLA-DRB1*
<0.0001	2.99	0.98	0.27	<0.0001	15	*HLA-DQB1*
<0.0001	2.76	1.00	0.59	<0.0001	15	*CD74*
<0.0001	2.31	0.98	0.54	<0.0001	15	*CST3*
<0.0001	2.16	0.92	0.18	<0.0001	15	*CD86*
<0.0001	1.86	0.97	0.26	<0.0001	15	*HLA-DMA*
<0.0001	3.17	0.73	0.45	<0.0001	16	*ISG15*
<0.0001	2.89	0.53	0.07	<0.0001	16	*IFIT3*
<0.0001	2.77	0.95	0.48	<0.0001	16	*ALOX5AP*
<0.0001	2.73	0.82	0.61	<0.0001	16	*PELI1*
<0.0001	2.50	0.94	0.80	<0.0001	16	*DUSP1*
<0.0001	2.26	0.33	0.05	<0.0001	16	*IFIT1*
<0.0001	3.02	0.75	0.15	<0.0001	16	*FPR2*
<0.0001	2.50	0.94	0.66	<0.0001	16	*SAMSN1*
<0.0001	2.34	0.35	0.05	<0.0001	16	*RSAD2*
<0.0001	2.57	0.94	0.46	<0.0001	16	*BCL2A1*
<0.0001	4.16	0.86	0.09	<0.0001	17	*PLA2G2A*
<0.0001	4.15	0.90	0.05	<0.0001	17	*CXCL14*
<0.0001	4.07	1.00	0.24	<0.0001	17	*MGP*
<0.0001	3.55	0.82	0.06	<0.0001	17	*FST*
<0.0001	3.47	0.99	0.19	<0.0001	17	*DCN*
<0.0001	3.35	0.85	0.07	<0.0001	17	*MT1A*
<0.0001	3.34	0.99	0.12	<0.0001	17	*MEDAG*
<0.0001	3.28	1.00	0.68	<0.0001	17	*MT2A*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	3.24	0.97	0.20	<0.0001	17	*GLIS3*
<0.0001	3.12	0.98	0.16	<0.0001	17	*C1R*
<0.0001	1.82	1.00	0.93	<0.0001	18	*MT-ND2*
<0.0001	1.97	1.00	0.92	<0.0001	18	*MT-CYB*
<0.0001	2.01	1.00	0.93	<0.0001	18	*MT-ND3*
<0.0001	1.71	1.00	0.93	<0.0001	18	*MT-ND1*
<0.0001	1.88	1.00	0.96	<0.0001	18	*MT-ATP6*
<0.0001	1.75	1.00	0.94	<0.0001	18	*MT-CO3*
<0.0001	1.63	0.98	0.83	<0.0001	18	*MT-ND5*
<0.0001	1.47	1.00	0.94	<0.0001	18	*MT-ND4*
<0.0001	1.58	1.00	0.96	<0.0001	18	*MT-CO2*
<0.0001	1.46	0.85	0.72	<0.0001	18	*MT-ATP8*
<0.0001	2.97	0.85	0.11	<0.0001	19	*IL2RA*
<0.0001	2.40	0.70	0.06	<0.0001	19	*RTKN2*
<0.0001	2.72	0.83	0.16	<0.0001	19	*BATF*
<0.0001	2.67	0.73	0.09	<0.0001	19	*IKZF2*
<0.0001	2.08	0.70	0.04	<0.0001	19	*CTLA4*
<0.0001	1.24	0.51	0.01	<0.0001	19	*FOXP3*
<0.0001	2.74	0.67	0.08	<0.0001	19	*TNFRSF4*
<0.0001	2.04	0.71	0.06	<0.0001	19	*TIGIT*
<0.0001	2.55	0.91	0.45	<0.0001	19	*IL32*
<0.0001	2.90	0.62	0.05	<0.0001	19	*AL136456.1*
<0.0001	2.68	0.99	0.18	<0.0001	20	*HLA-DRB5*
<0.0001	2.62	0.93	0.13	<0.0001	20	*MRC1*
<0.0001	2.63	0.97	0.34	<0.0001	20	*FABP5*
<0.0001	1.59	0.99	0.71	<0.0001	20	*YBX1*
<0.0001	1.52	0.99	0.45	<0.0001	20	*AP2S1*
<0.0001	2.39	0.91	0.18	<0.0001	20	*FBP1*
<0.0001	1.90	0.99	0.42	<0.0001	20	*CTSZ*
<0.0001	1.12	0.72	0.04	<0.0001	20	*CLEC10A*
<0.0001	1.77	1.00	0.82	<0.0001	20	*SH3BGRL3*
<0.0001	1.65	0.95	0.37	<0.0001	20	*BLVRB*
<0.0001	4.86	0.97	0.04	<0.0001	21	*HP*
<0.0001	4.82	1.00	0.15	<0.0001	21	*SLPI*
<0.0001	3.84	1.00	0.15	<0.0001	21	*CFB*
<0.0001	3.43	0.94	0.02	<0.0001	21	*PRG4*
<0.0001	2.58	0.94	0.04	<0.0001	21	*CXCL6*
<0.0001	2.56	0.99	0.05	<0.0001	21	*CALB2*
<0.0001	2.11	0.99	0.11	<0.0001	21	*CLDN1*
<0.0001	1.73	0.89	0.01	<0.0001	21	*UPK3B*
<0.0001	1.17	0.95	0.03	<0.0001	21	*AC245041.2*
<0.0001	1.01	0.89	0.02	<0.0001	21	*ANXA8*
<0.0001	3.14	0.92	0.02	<0.0001	22	*CD79A*
<0.0001	3.05	0.83	0.04	<0.0001	22	*BANK1*
<0.0001	2.38	0.83	0.01	<0.0001	22	*MS4A1*
<0.0001	1.99	0.69	0.03	<0.0001	22	*LINC00926*
<0.0001	2.39	0.76	0.05	<0.0001	22	*AC120193.1*
<0.0001	1.95	0.72	0.01	<0.0001	22	*TNFRSF13C*
<0.0001	2.46	0.69	0.01	<0.0001	22	*IGHM*
<0.0001	2.96	0.84	0.09	<0.0001	22	*EBF1*
<0.0001	3.00	0.82	0.07	<0.0001	22	*AFF3*
<0.0001	2.15	0.94	0.40	<0.0001	22	*CD37*
<0.0001	2.78	0.98	0.20	<0.0001	23	*STMN1*
<0.0001	2.44	1.00	0.56	<0.0001	23	*HMGN2*
<0.0001	1.93	0.89	0.02	<0.0001	23	*MKI67*
<0.0001	2.03	0.88	0.04	<0.0001	23	*PCLAF*
<0.0001	1.66	0.84	0.04	<0.0001	23	*TYMS*
<0.0001	1.10	0.83	0.02	<0.0001	23	*BIRC5*
<0.0001	1.94	0.88	0.03	<0.0001	23	*TOP2A*
<0.0001	2.42	0.99	0.63	<0.0001	23	*TUBA1B*
<0.0001	1.86	0.82	0.03	<0.0001	23	*RRM2*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	2.30	0.99	0.44	<0.0001	23	*TUBB*
<0.0001	3.73	0.99	0.22	<0.0001	24	*SEL1L3*
<0.0001	3.00	0.97	0.21	<0.0001	24	*SLC15A4*
<0.0001	3.10	0.95	0.01	<0.0001	24	*RHEX*
<0.0001	2.53	0.90	0.04	<0.0001	24	*P2RY6*
<0.0001	2.66	0.93	0.17	<0.0001	24	*RUBCN*
<0.0001	3.54	0.96	0.09	<0.0001	24	*IRF4*
<0.0001	5.05	0.98	0.07	<0.0001	24	*GZMB*
<0.0001	2.46	0.95	0.61	<0.0001	24	*SEC61B*
<0.0001	2.74	0.92	0.05	<0.0001	24	*AC023590.1*
<0.0001	1.94	0.92	0.03	<0.0001	24	*PLD4*
<0.0001	5.06	0.99	0.15	<0.0001	25	*ADIRF*
<0.0001	4.43	0.99	0.17	<0.0001	25	*MYL9*
<0.0001	4.26	0.85	0.04	<0.0001	25	*MYH11*
<0.0001	2.61	0.93	0.07	<0.0001	25	*MYLK*
<0.0001	2.22	0.91	0.06	<0.0001	25	*NOTCH3*
<0.0001	4.48	0.85	0.01	<0.0001	25	*MUSTN1*
<0.0001	4.41	0.99	0.19	<0.0001	25	*TPM2*
<0.0001	2.86	0.94	0.10	<0.0001	25	*SOD3*
<0.0001	5.24	1.00	0.20	<0.0001	25	*TAGLN*
<0.0001	3.11	0.94	0.03	<0.0001	25	*PPP1R14A*
<0.0001	10.48	0.89	0.22	<0.0001	26	*IGKC*
<0.0001	7.87	0.71	0.06	<0.0001	26	*IGHG3*
<0.0001	8.31	0.72	0.05	<0.0001	26	*IGHG1*
<0.0001	6.39	0.92	0.04	<0.0001	26	*JCHAIN*
<0.0001	3.84	0.96	0.02	<0.0001	26	*MZB1*
<0.0001	2.92	0.96	0.65	<0.0001	26	*SSR4*
<0.0001	2.78	0.94	0.25	<0.0001	26	*TXNDC5*
<0.0001	6.55	0.62	0.04	<0.0001	26	*IGHG4*
<0.0001	1.69	0.84	0.00	<0.0001	26	*FCRL5*
<0.0001	9.35	0.40	0.08	<0.0001	26	*IGLC2*
<0.0001	6.20	0.90	0.00	<0.0001	27	*TPSB2*
<0.0001	6.31	0.90	0.00	<0.0001	27	*TPSAB1*
<0.0001	4.32	0.91	0.10	<0.0001	27	*HPGD*
<0.0001	4.83	0.89	0.00	<0.0001	27	*CPA3*
<0.0001	3.23	0.90	0.00	<0.0001	27	*HDC*
<0.0001	4.13	0.81	0.07	<0.0001	27	*SLC24A3*
<0.0001	3.19	0.83	0.03	<0.0001	27	*HPGDS*
<0.0001	4.33	0.68	0.00	<0.0001	27	*CTSG*
<0.0001	2.07	0.66	0.00	<0.0001	27	*MS4A2*
<0.0001	2.22	0.59	0.03	<0.0001	27	*GATA2*
<0.0001	5.27	1.00	0.32	<0.0001	28	*MT1X*
<0.0001	2.67	0.79	0.14	<0.0001	28	*MT1F*
<0.0001	4.79	0.90	0.27	<0.0001	28	*MT1E*
<0.0001	3.59	1.00	0.68	<0.0001	28	*MT2A*
<0.0001	3.90	0.51	0.08	<0.0001	28	*MT1G*
<0.0001	1.79	0.97	0.26	<0.0001	28	*CCL5*
<0.0001	1.41	0.93	0.60	<0.0001	28	*SOD1*
<0.0001	1.53	0.91	0.32	<0.0001	28	*RUNX3*
<0.0001	1.59	0.86	0.26	<0.0001	28	*CST7*
<0.0001	1.62	1.00	0.82	<0.0001	28	*HSP90AB1*
<0.0001	5.11	1.00	0.11	<0.0001	29	*ACP5*
<0.0001	5.16	1.00	0.13	<0.0001	29	*CTSK*
<0.0001	4.05	0.96	0.16	<0.0001	29	*CKB*
<0.0001	5.46	1.00	0.14	<0.0001	29	*MMP9*
<0.0001	2.68	0.98	0.14	<0.0001	29	*SLC9B2*
<0.0001	1.43	0.95	0.01	<0.0001	29	*SIGLEC15*
<0.0001	2.09	0.89	0.01	<0.0001	29	*AK5*
<0.0001	2.62	1.00	0.46	<0.0001	29	*RGS10*
<0.0001	2.62	0.89	0.02	<0.0001	29	*LINC02725*
<0.0001	3.67	1.00	0.55	<0.0001	29	*CST3*

p-value	Average log2FC	% cells gene detected in the cluster	% cells gene detected on average in other clusters	Adjusted p-value	Cluster	Gene
<0.0001	5.12	1.00	0.07	<0.0001	30	*ST18*
<0.0001	5.79	0.97	0.52	<0.0001	30	*PDE4D*
<0.0001	3.59	1.00	0.31	<0.0001	30	*ANXA4*
<0.0001	4.47	1.00	0.03	<0.0001	30	*NCKAP5*
<0.0001	4.81	1.00	0.00	<0.0001	30	*ITPRID1*
<0.0001	5.48	0.93	0.01	<0.0001	30	*KCNB2*
<0.0001	4.01	0.97	0.00	<0.0001	30	*SH2D6*
<0.0001	4.00	0.97	0.04	<0.0001	30	*RGS13*
<0.0001	2.83	1.00	0.04	<0.0001	30	*CORO2B*
<0.0001	3.44	0.93	0.04	<0.0001	30	*LINC01091*

Abbrev. Log2FC, log2 fold change; scRNAseq, single cell RNA sequencing; HG ATPD, high grade appendiceal tumour peritoneal disease.

Supplementary Table ST3.3 | Significantly differentially expressed genes (FDR≤0.05) comparing HG to LG ATPD tumour segments from spatial transcriptomic analysis

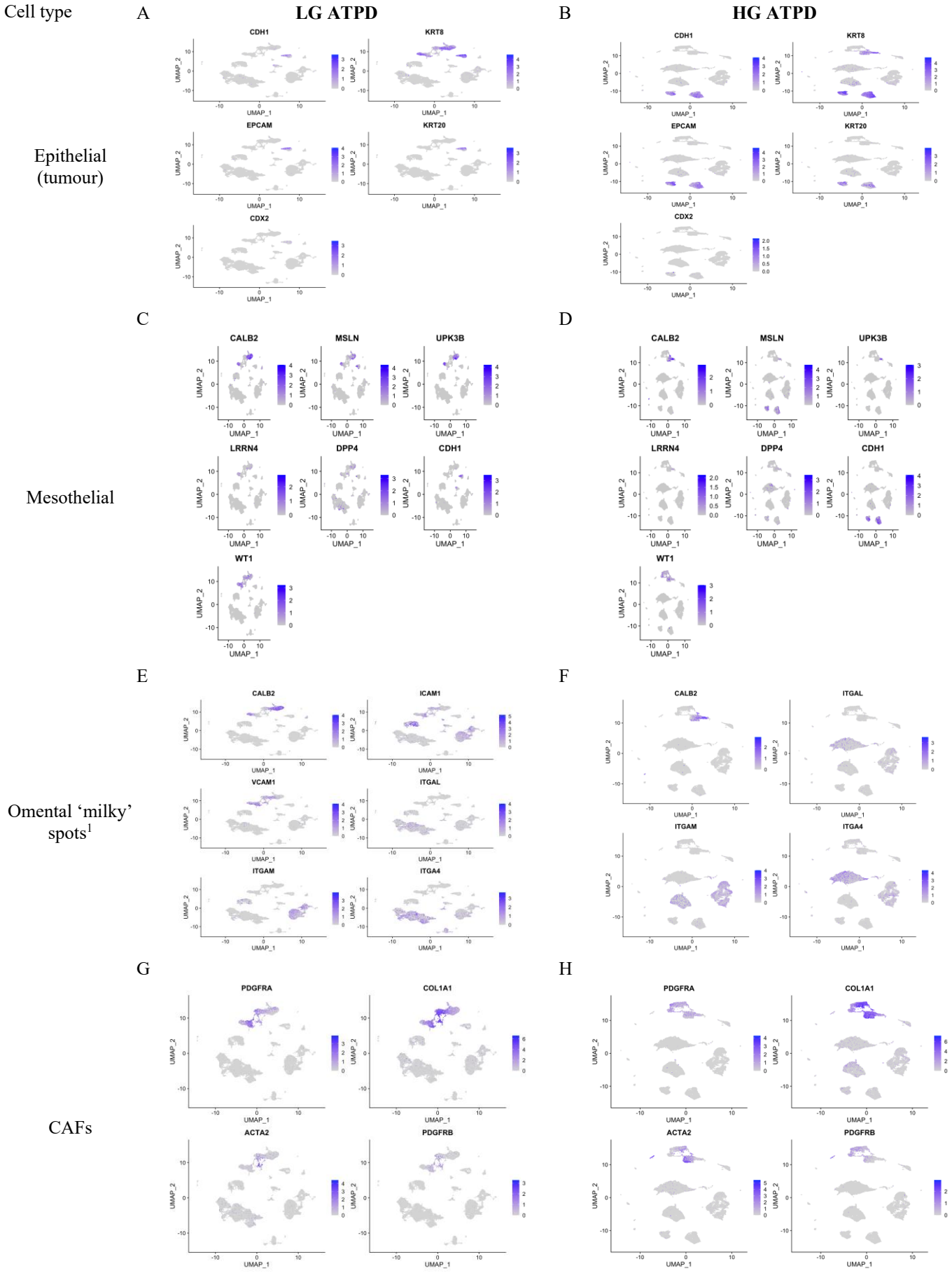
Differentially expressed genes across the tumour segments. Areas are compared using linear mixed-effect modelling using case number to control for tissue subsampling and Benjamini-Hochberg multiple test correction to generate a FDR p-value. Genes were considered differentially express if FDR≤0.05 and log fold change >0.5. Negative Log2FC are the values for the contrasting factor i.e. LG ATPD

Gene	Contrast	Log2FC	Raw p-value	FDR
PLA2G2A	HG - LG	2.63	<0.001	<0.001
TM6SF2	HG - LG	-0.97	<0.001	0.001
FHL2	HG - LG	-1.23	<0.001	0.013
DBNDD2	HG - LG	-1.08	<0.001	0.047
DBI	HG - LG	1.06	<0.001	0.040
CALHM3	HG - LG	-0.58	<0.001	0.035
SP9	HG - LG	-0.54	<0.001	0.035
CT47B1	HG - LG	1.21	<0.001	0.032
CDHR1	HG - LG	2.03	<0.001	0.001
PLS3	HG - LG	-0.97	<0.001	0.031
ZNF549	HG - LG	-0.71	<0.001	0.016
FABP1	HG - LG	2.53	<0.001	0.010
DCN	HG - LG	1.39	<0.001	0.047
DSC3	HG - LG	-0.72	<0.001	0.036
TK1	HG - LG	0.87	<0.001	0.023
YBX3	HG - LG	-1.55	<0.001	0.001
BHLHE40	HG - LG	-1.93	<0.001	0.020
EPS8	HG - LG	-0.75	<0.001	0.001
GALNT2	HG - LG	0.93	<0.001	0.001
NDUFB10	HG - LG	0.93	<0.001	0.047
SERPINB6	HG - LG	-0.68	<0.001	0.023
GRK3	HG - LG	1.08	<0.001	0.003
SRC	HG - LG	-1.11	<0.001	<0.001
TFF2	HG - LG	-2.74	<0.001	0.039
HMGCS2	HG - LG	3.25	<0.001	0.009
ST3GAL4	HG - LG	-1.97	<0.001	0.038
ZNF346	HG - LG	0.83	<0.001	0.035
KDM2A	HG - LG	-0.60	<0.001	0.038
TMA7	HG - LG	1.05	<0.001	0.003
PRPF39	HG - LG	1.10	<0.001	0.024
REG4	HG - LG	-3.41	<0.001	0.034
NAA38	HG - LG	0.77	<0.001	0.029
C19orf33	HG - LG	-1.09	<0.001	0.020
SLFNL1	HG - LG	0.93	<0.001	0.010
ZNRF2	HG - LG	0.74	<0.001	0.035
TC2N	HG - LG	-1.30	<0.001	0.016
ZNF781	HG - LG	-0.65	<0.001	0.038
PARP15	HG - LG	-0.59	<0.001	0.032
C10orf99	HG - LG	1.59	<0.001	0.002
ATL3	HG - LG	-1.19	<0.001	0.006
CCND2	HG - LG	1.72	<0.001	0.020
COL17A1	HG - LG	-2.43	<0.001	0.001
COX7B	HG - LG	0.96	<0.001	0.019
DGKA	HG - LG	-0.60	<0.001	0.050
DST	HG - LG	-1.39	<0.001	0.005
FOXP1	HG - LG	-0.72	<0.001	0.035
H2BC17	HG - LG	1.25	<0.001	0.027
H2BC3	HG - LG	1.55	<0.001	0.022
MAOA	HG - LG	1.15	<0.001	0.006
MGP	HG - LG	2.38	<0.001	0.003
PRKACB	HG - LG	-0.72	<0.001	0.050
SLC25A5	HG - LG	1.29	<0.001	<0.001
NBPF26	HG - LG	0.60	<0.001	0.038
UGT2B15	HG - LG	1.92	<0.001	<0.001

Gene	Contrast	Log2FC	Raw p-value	FDR
TIMM13	HG - LG	1.04	<0.001	<0.001
SERPINB1	HG - LG	-1.37	<0.001	<0.001
H3C10	HG - LG	1.65	<0.001	<0.001

Abbrev. FDR, false discovery rate; Log2FC, log2 fold change; HG, high grade; LG, low grade; ATPD, appendiceal tumour peritoneal disease

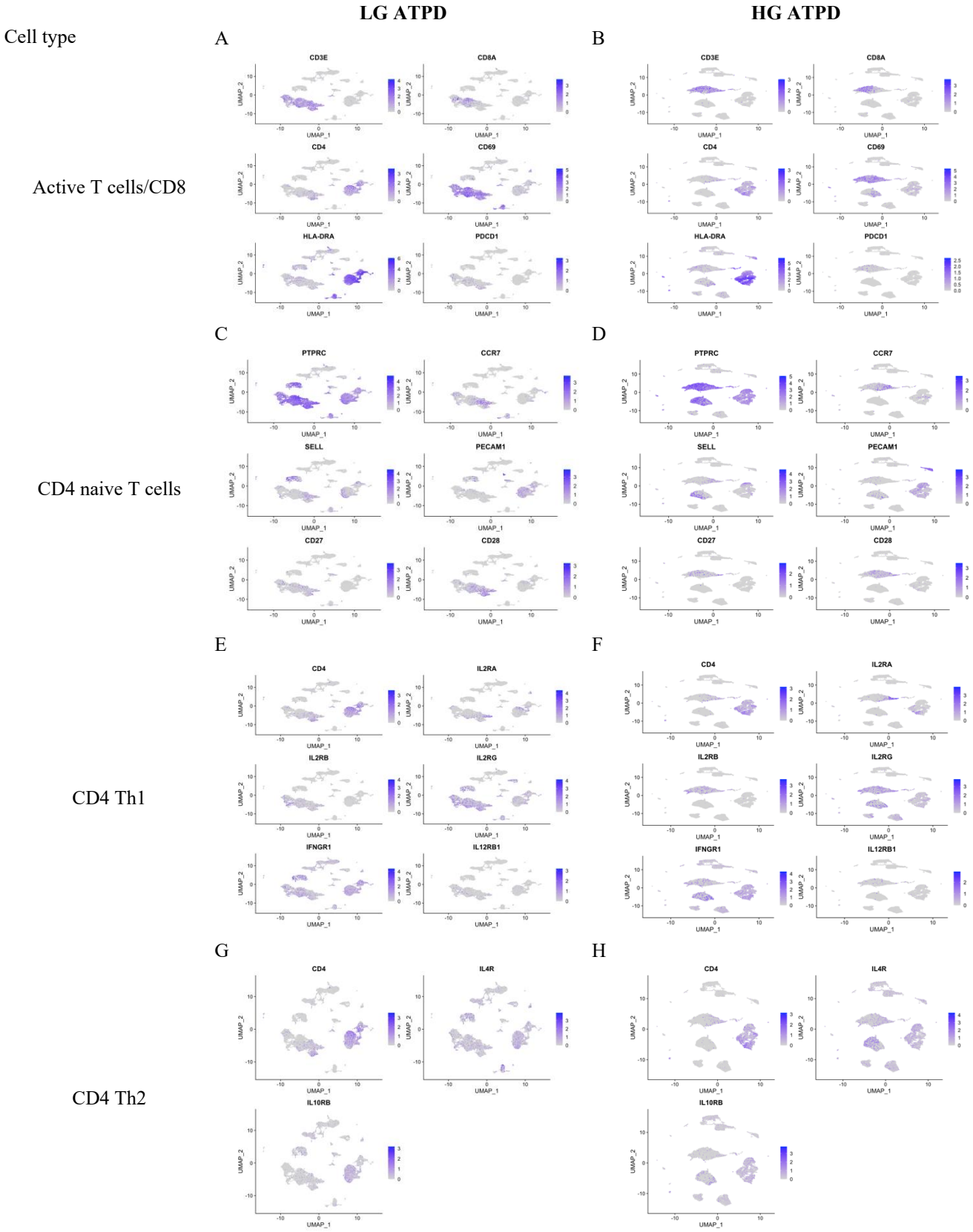
8.3.1 Supplementary Figures



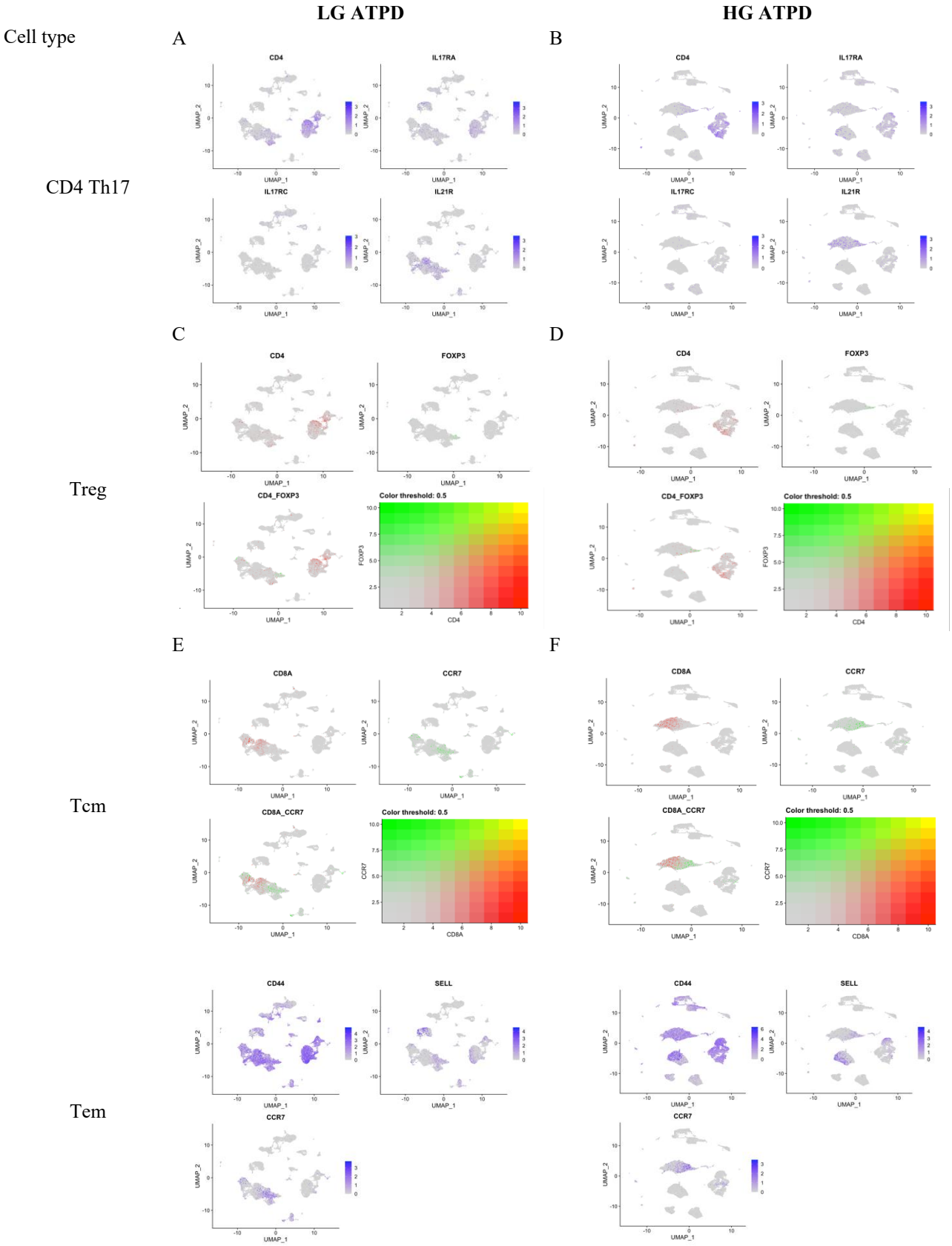
Supplementary Figure SF3.1 | Canonical marker genes for tumour, mesothelial cells, 'milky' spots and CAFs

Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: epithelial (tumour; **A, B**), mesothelial cells (**C, D**), omental 'milky' spots (**E, F**) and CAFs (**G, H**) in LG (**A, C, E, G**) and HG (**B, D, F, H**) ATPD. [1]Cui L, Johkura K, Liang Y, Teng R, *et al*. Biodefense function of omental milky spots through cell adhesion molecules and leukocyte proliferation. Cell Tissue Res. 2002;310(3):321–30.

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



Supplementary Figure SF3.2 | Canonical marker genes for T cell subsets (part A)
Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: Activated T cells/CD8 T cells (**A, B**), Naïve CD4 T cells (**C, D**), Helper T cells type 1 (Th1) (**E, F**) and Helper T cells type 2 (Th2) (**G, H**) in LG (**A, C, E, G**) and HG (**B, D, F, H**) ATPD.
Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease



Supplementary Figure SF3.3 | Canonical marker genes for T cell subsets (part B)
Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: Helper T cells type 17 (Th17) (**A, B**), regulatory T cells (Treg) (**C, D**), Central memory T cells (Tcm) (**E, F**) and effector memory T cells (Tem) (**G, H**) in LG (**A, C, E, G**) and HG (**B, D, F, H**) ATPD. Plots **C-F** additionally show 'blend' where cells co-expressing the genes of interest are displayed in yellow.
Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease

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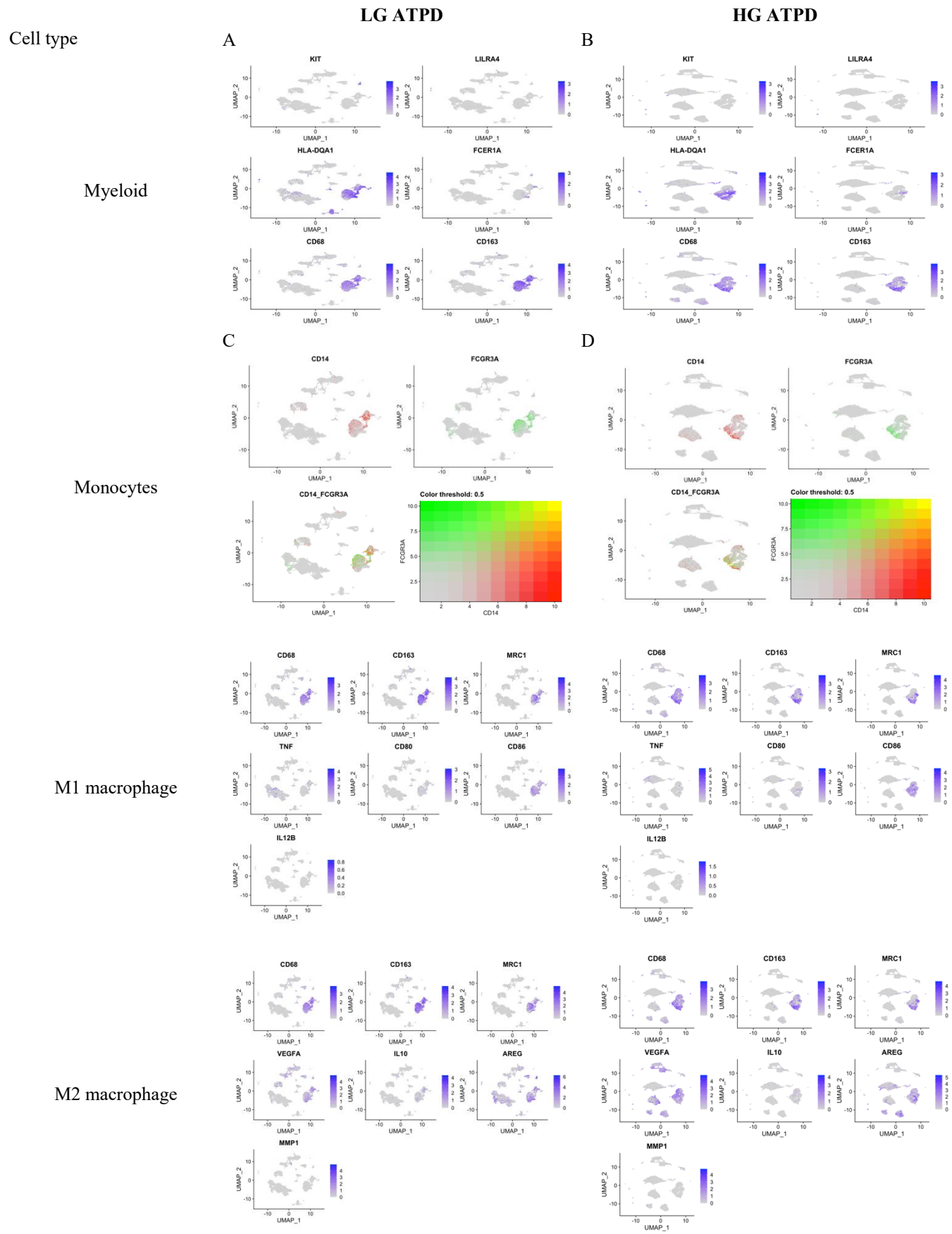
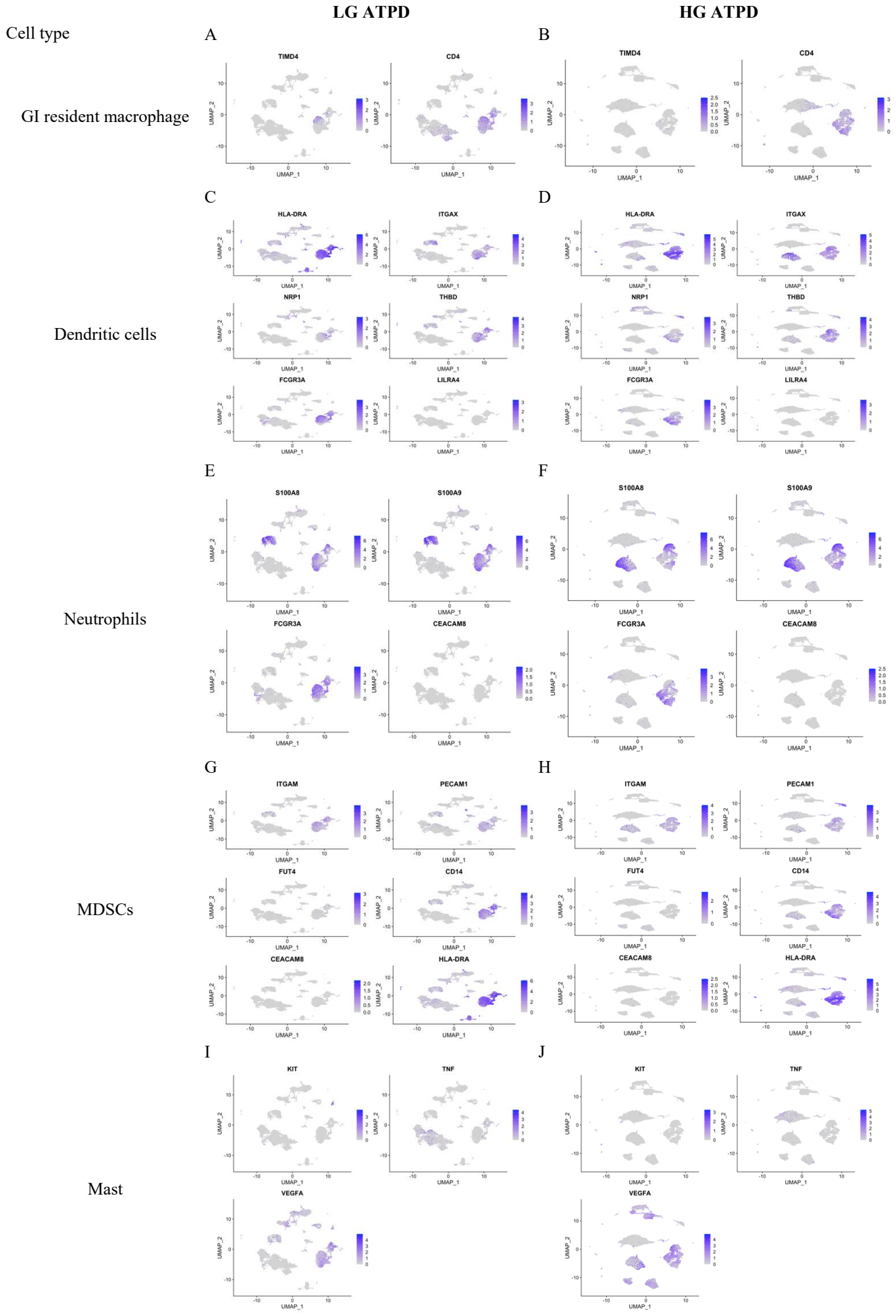
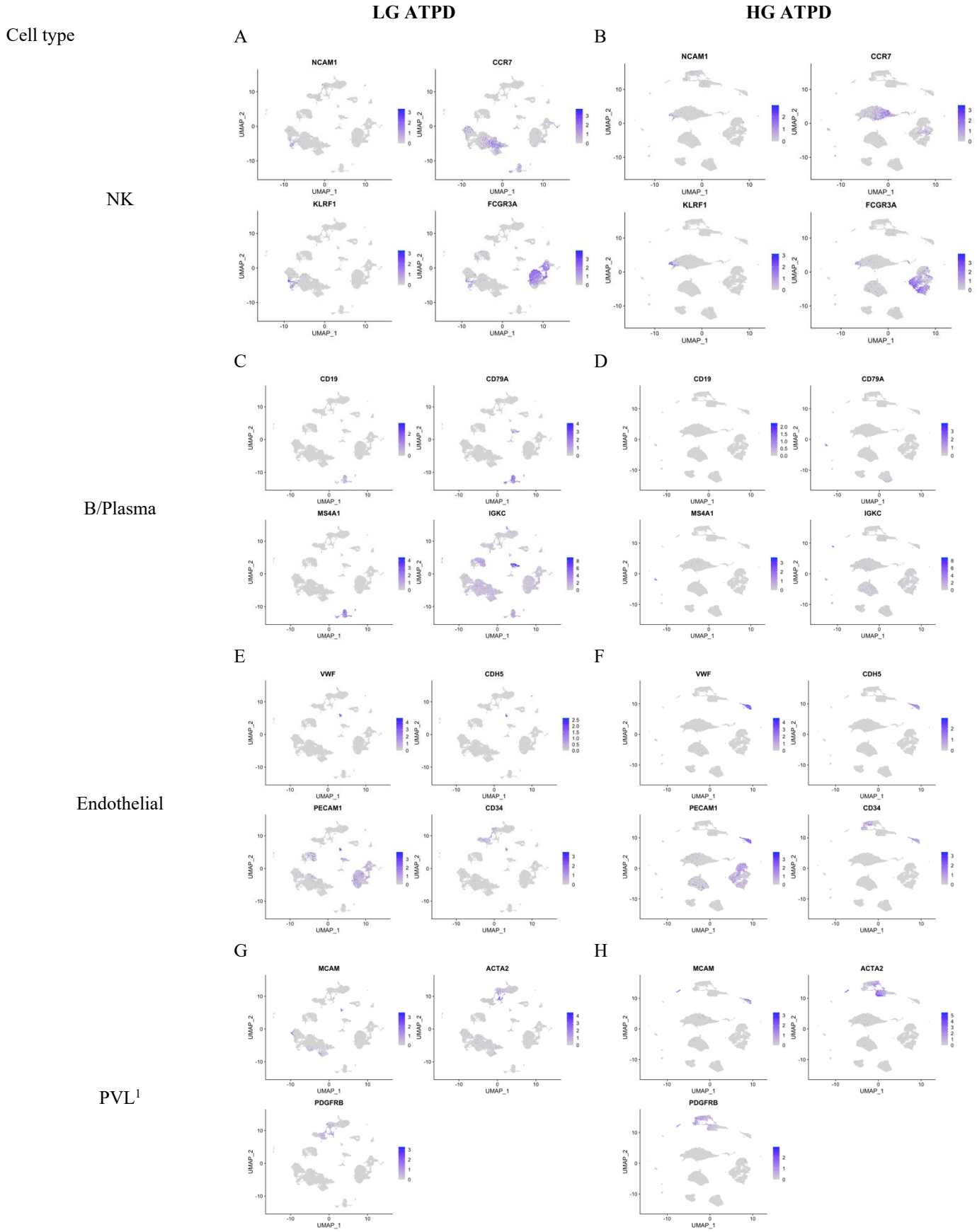


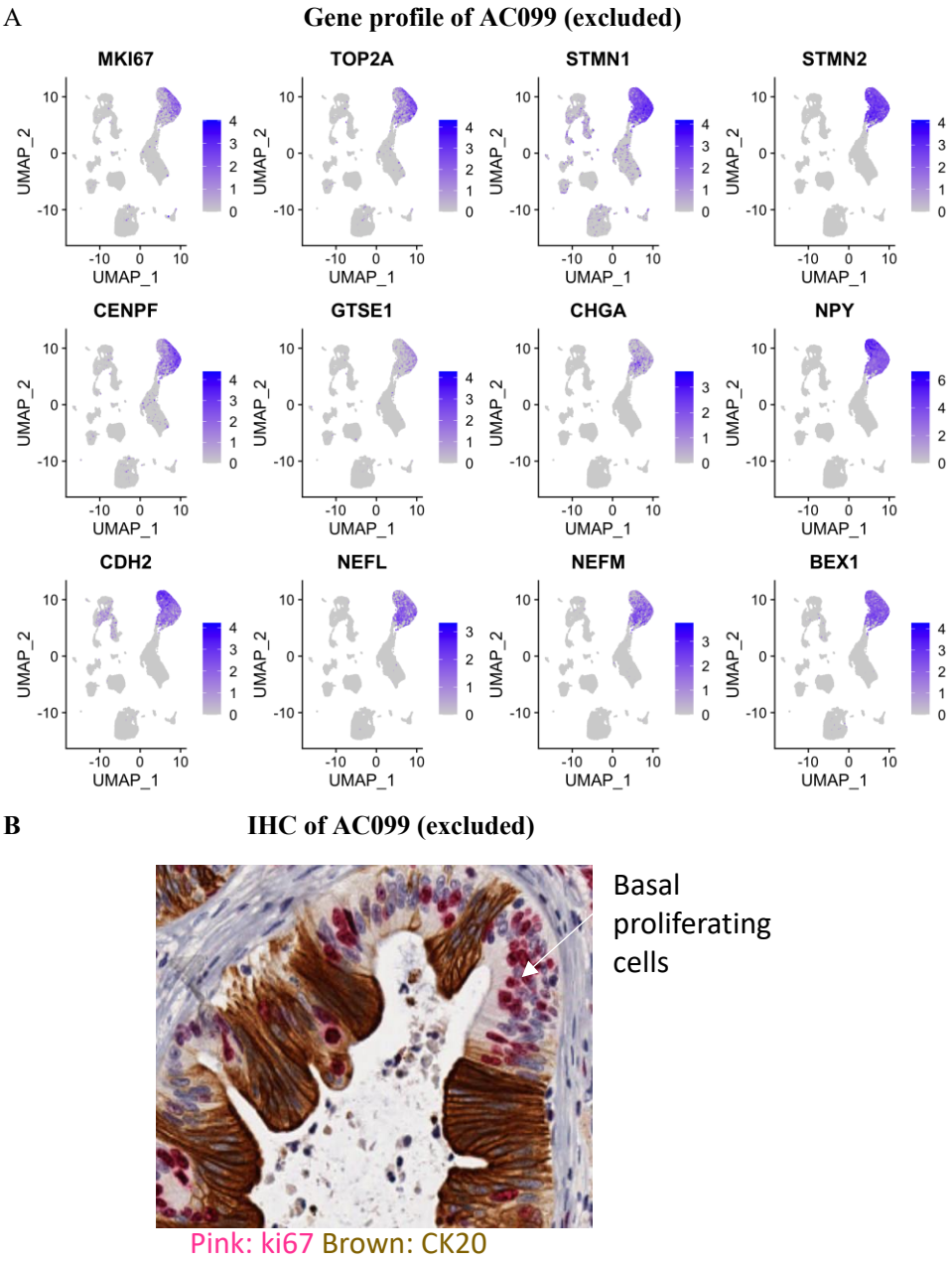
Supplementary Figure SF3.4 | Canonical marker genes for myeloid subsets (part A)
Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: Myeloid cells (**A, B**), Monocytes (**C, D**), M1 macrophage (**E, F**) and M2 macrophage (**G, H**) in LG (**A, C, E, G**) and HG (**B, D, F, H**) ATPD. Plots **C-D** additionally show 'blend' where cells co-expressing the genes of interest are displayed in yellow.
Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease


LG ATPD
HG ATPD
Cell type
A
B
TIMD4
CD4
GI resident macrophage
C
D
HLA-DRA
ITGAX
NRP1
THBD
FCGR3A
LILRA4
Dendritic cells
E
F
S100A8
S100A9
FCGR3A
CEACAM8
Neutrophils
G
H
ITGAM
PECAM1
FUT4
CD14
CEACAM8
HLA-DRA
MDSCs
I
J
KIT
TNF
VEGFA
Mast
UMAP_1
UMAP_2

Supplementary Figure SF3.5 | Canonical marker genes for myeloid subsets (Part B)
Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: GI resident macrophage (**A, B**), dendritic cells (**C, D**), neutrophils (**E, F**), MDSCs (**G, H**) and mast cells **(I, J**) in LG (**A, C, E, G, I**) and HG (**B, D, F, H, J**) ATPD.
Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; GI, gastrointestinal; MDSCs, myeloid derived suppressor cells



Supplementary Figure SF3.6 | Canonical marker genes for NK cells, B/Plasma cells, endothelial cells and PVL cells
Cluster feature plots showing distribution of canonical marker genes for known cell types of the tumour microenvironment: NK cells (**A, B**), B/Plasma cells (**C, D**), endothelial cells (**E, F**), and PVL cells (**G**, **H**) in LG (**A, C, E, G**) and HG (**B, D, F, H**) ATPD. [1]Wu SZ, Al-Eryani G, Roden DL, Junankar S, Harvey K, Andersson A, et al. A single-cell and spatially resolved atlas of human breast cancers. Nat Genet. 2021;53(9):1334–47.
Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; NK, natural killer; PVL, perivascular-like cells

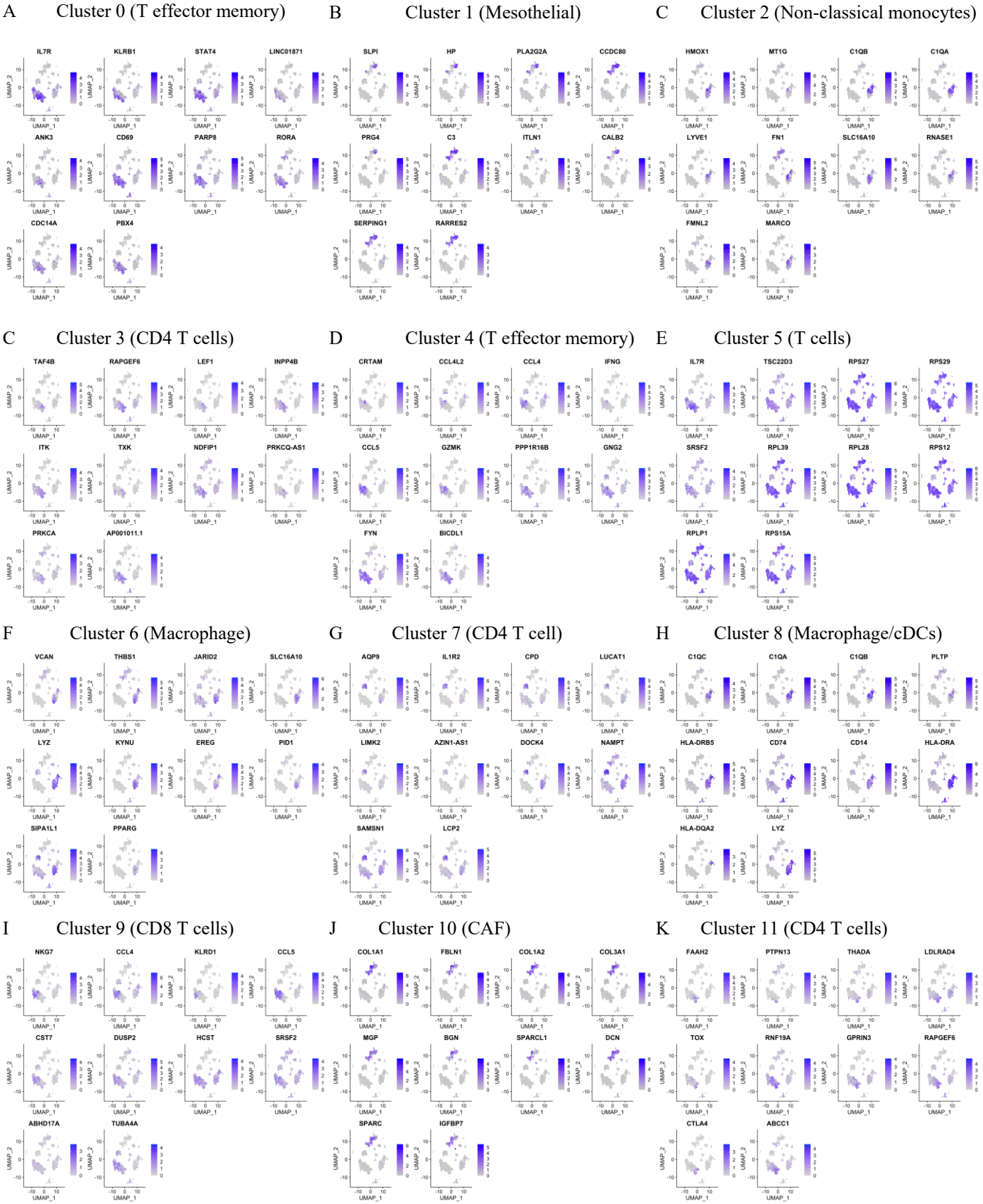


Supplementary Figure SF3.7 | Genetic and phenotypic features of excluded HG ATPD case (AC099/AA99) from scRNAseq and STP analysis

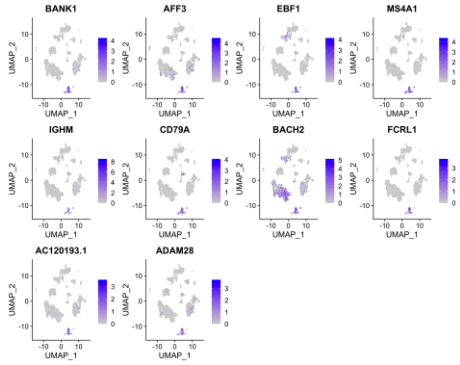
Cluster feature plots showing the localisation of genes for basal proliferating cells (*MK67, TOP2A, STMN1, STMN2, CENPF, GTSE1)*, neuroendocrine *(CHGA, NPY)* and neuronal (*NEFL, NEFM, BEX1*) differentiation and EMT (*CDH2)* (A) and IHC-stained section at 40X showing alternating CK20+ tumour cells with CK20-ki67+ basal proliferating cells (B).

Abbrev. HG, high grade; ATPD, appendiceal tumour peritoneal disease; scRNAseq, single cell RNA sequencing; STP, spatial transcriptomic analysis; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry.

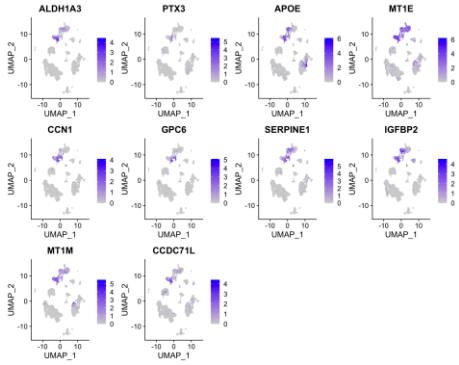
Supplementary Figure SF3.8 | Top ten genes per scRNAseq cluster for LG ATPD



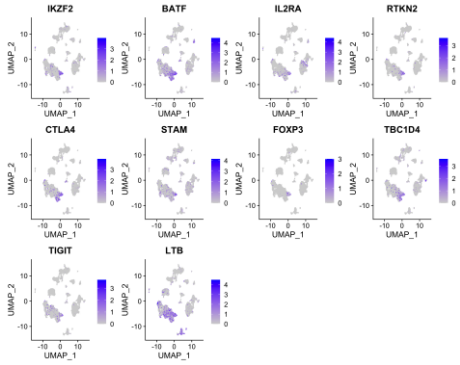
L Cluster 12 (B cells)



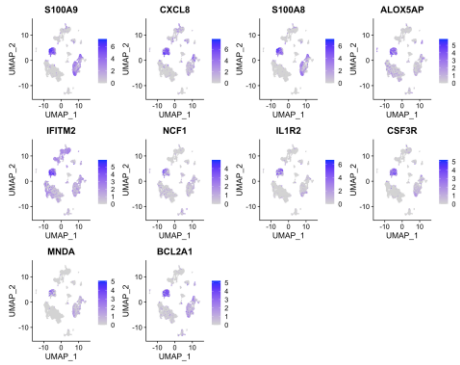
M Cluster 13 (CAF)



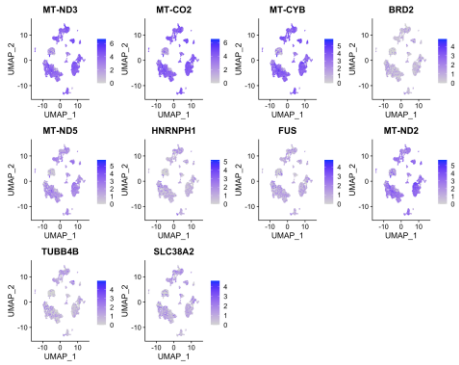
N Cluster 14 (CD4 T cells)



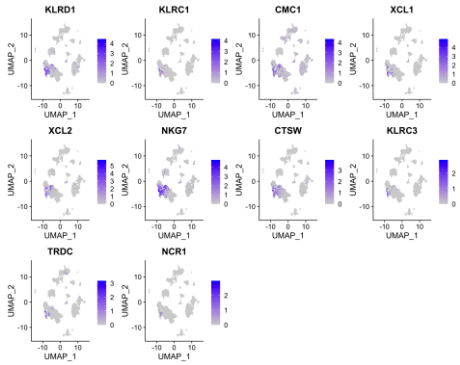
O Cluster 15 (Myeloid cells)



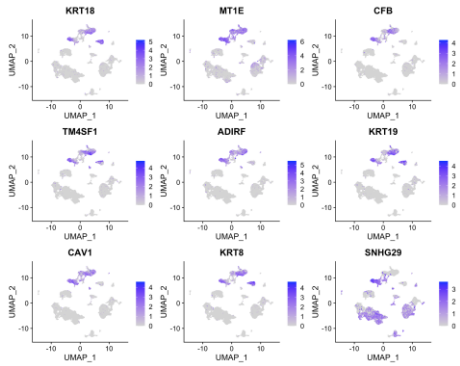
P Cluster 16 (CD8 T cells)



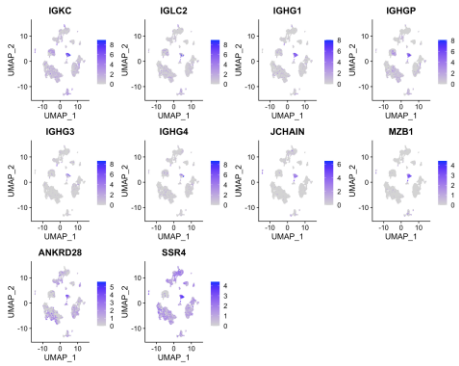
Q Cluster 17 (NK cells)



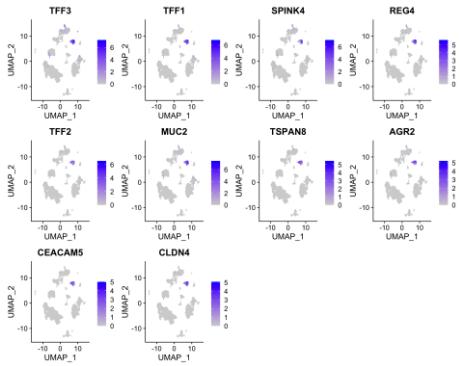
R Cluster 18 (Mesothelial cells)



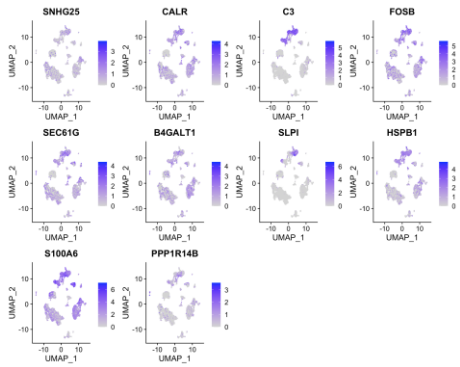
S Cluster 19 (Plasma cells)



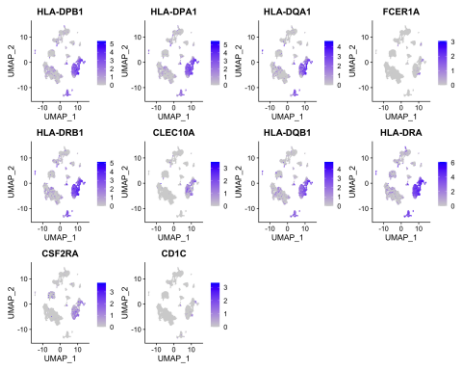
T Cluster 20 (Tumour)



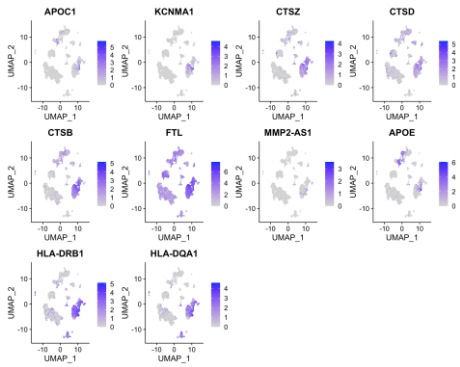
U Cluster 21 (CAF)



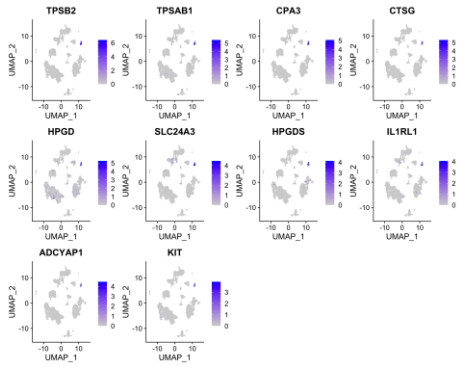
V Cluster 22 (cDCs)



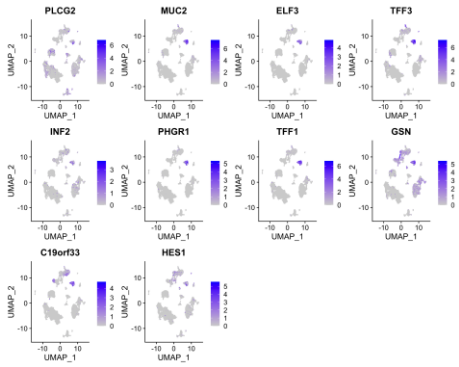
W Cluster 23 (Macrophage)



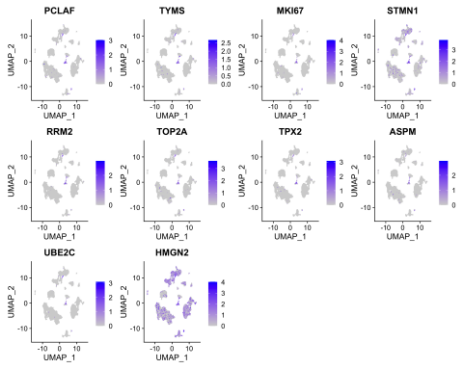
X Cluster 24 (Mast cells)



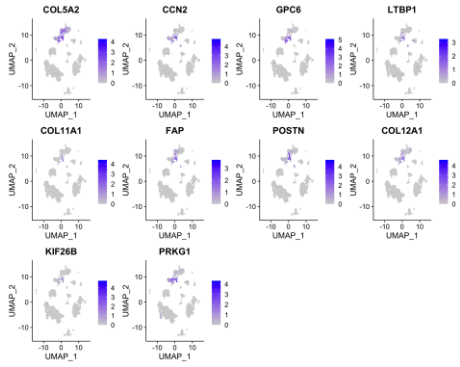
Y Cluster 25 (Tumour)



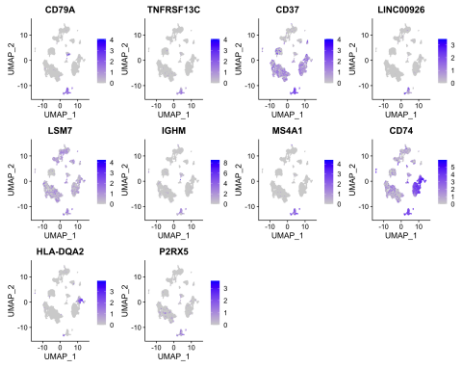
Z Cluster 26 (Cycling cells)



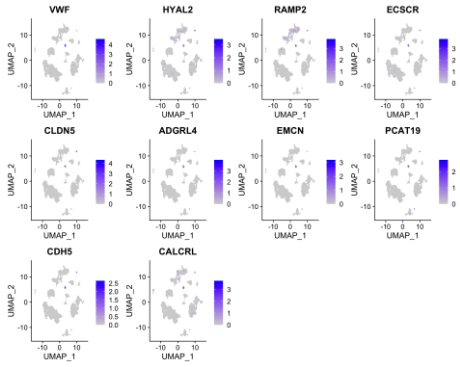
AA Cluster 27 (CAF)



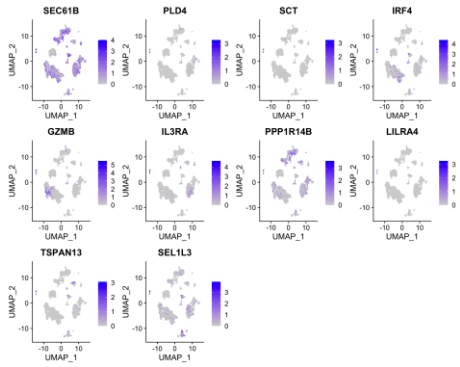
AB Cluster 28 (B cells)



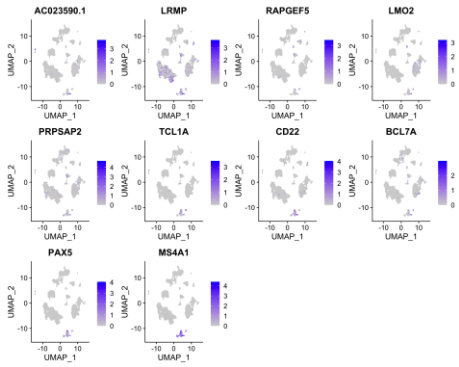
AC Cluster 29 (Endothelial cells)



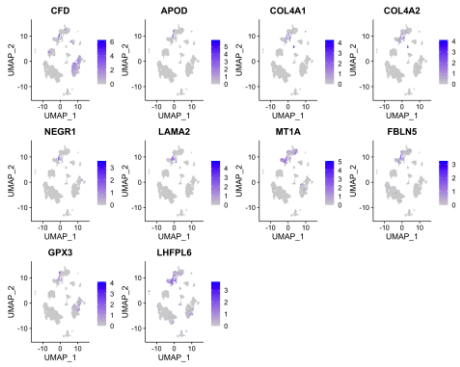
AD Cluster 30 (pDCs)



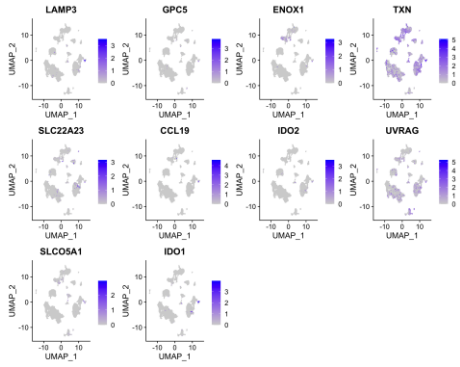
AE Cluster 31 (B cells)



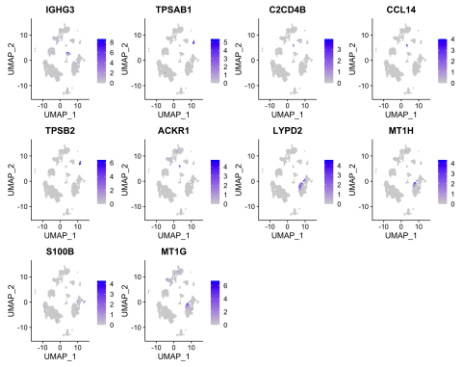
AF Cluster 32 (CAF)



AG Cluster 33 (Mature DCs)



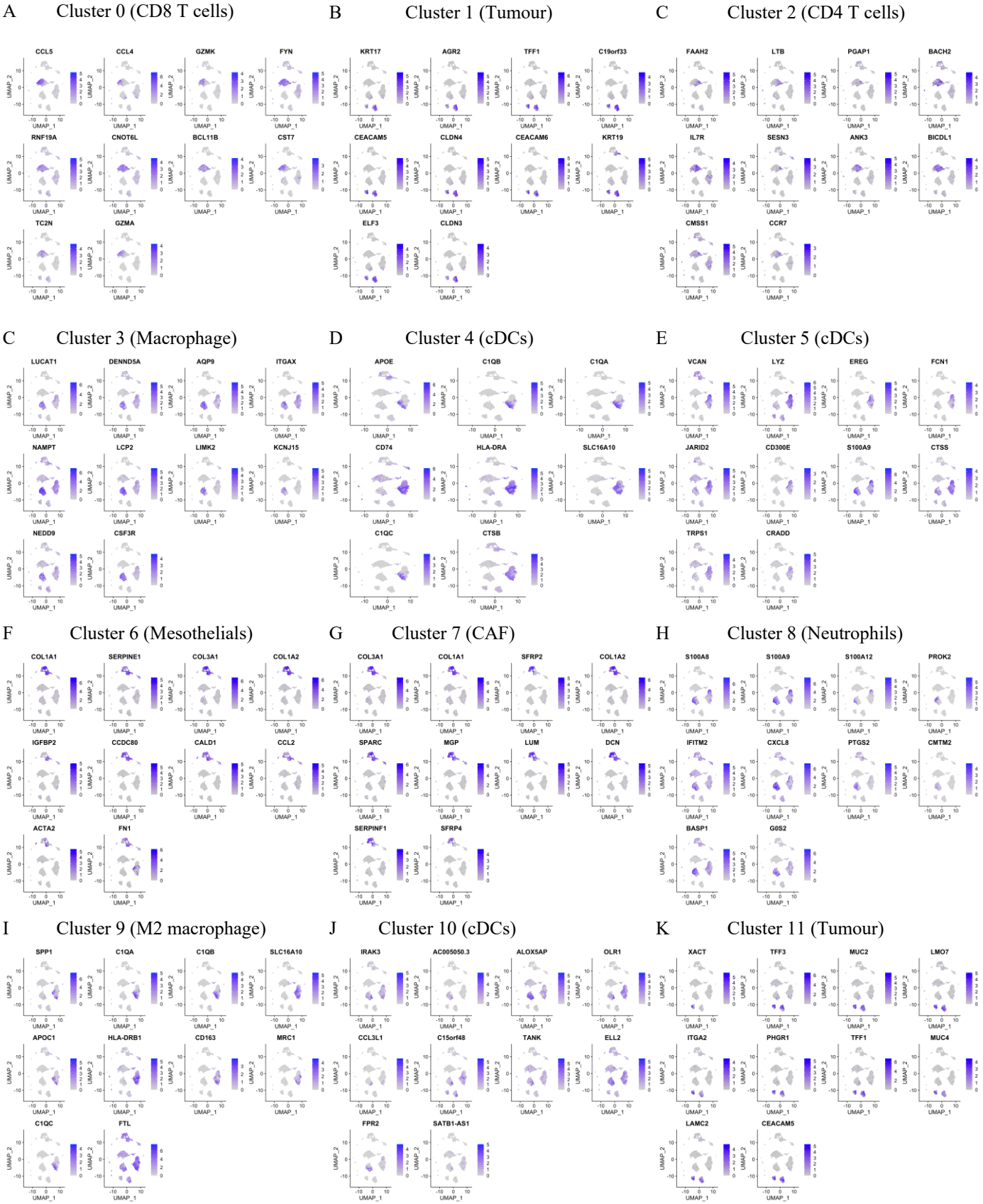
AH Cluster 34 (CAFs)



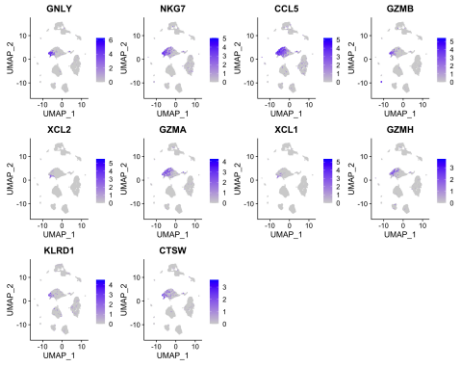
Cluster feature plots (**A-AH**) show the top ten differentially expressed genes for each cell cluster informing the annotation of LG ATPD.

Abbrev. DCs, dendritic cells; pDCs, plasmacytoid DCs; cDCs, conventional DCs; CAFs, cancer-associated fibroblasts; LG ATPD, low grade appendiceal tumour peritoneal disease.

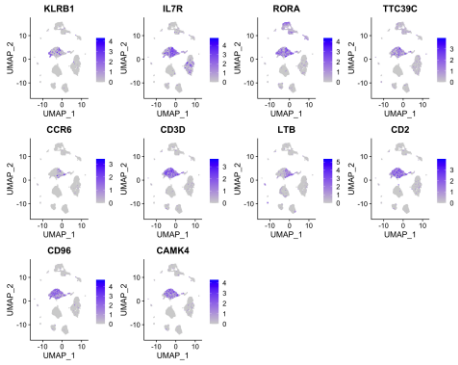
Supplementary Figure SF3.9 | Top ten genes for scRNAseq cluster for HG ATPD



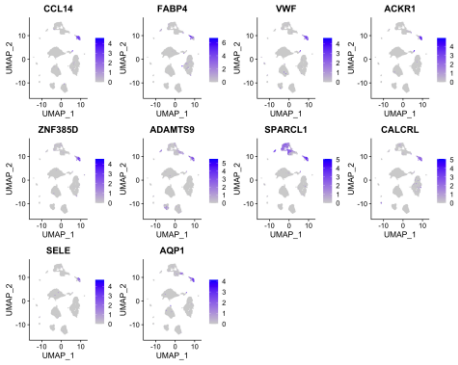
L **Cluster 12 (NK cells)**



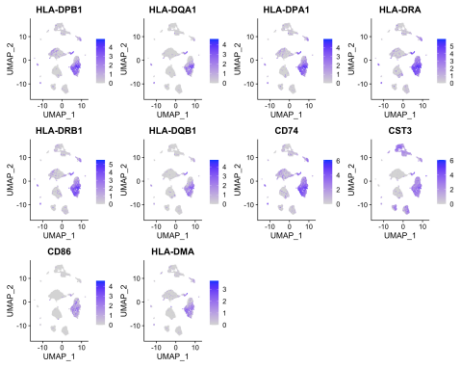
M **Cluster 13 (CD4 T cells)**



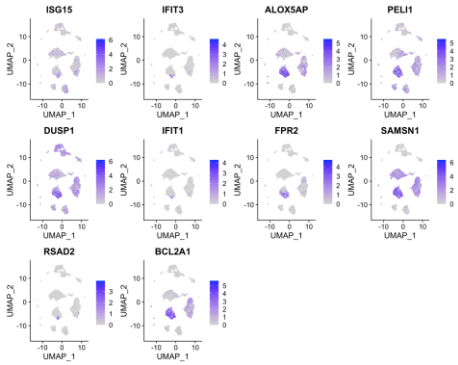
N **Cluster 14 (Endothelial cells)**



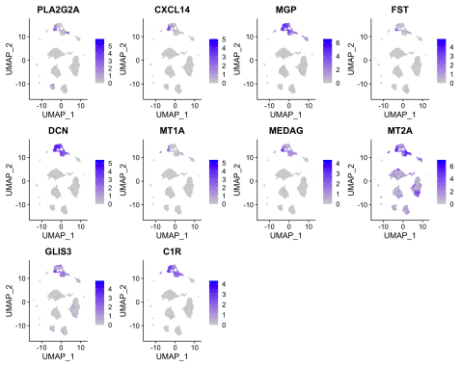
O **Cluster 15 (M2 macrophage)**



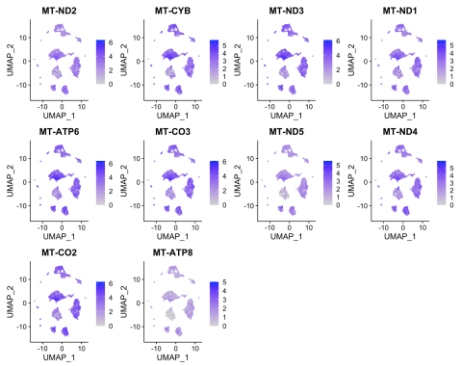
P **Cluster 16 (Neutrophils)**



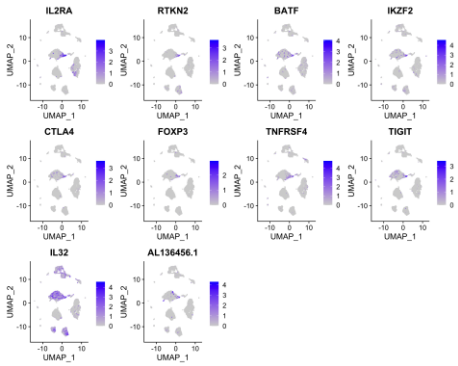
Q **Cluster 17 (CAF)**



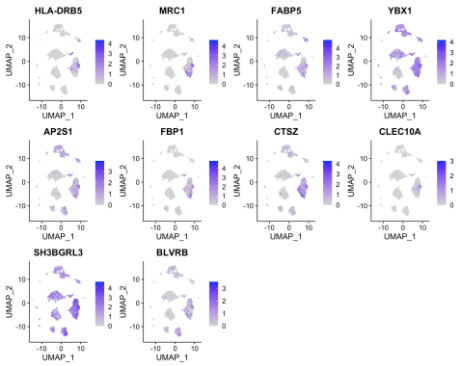
R **Cluster 18 (CD8 T cells)**



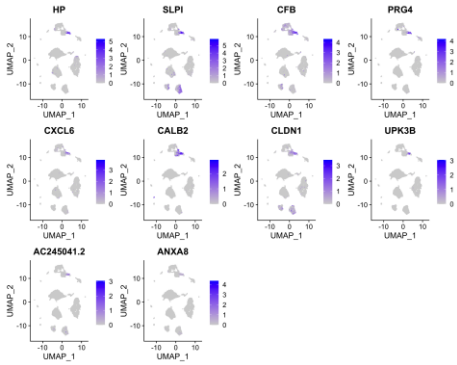
S **Cluster 19 (T regs)**



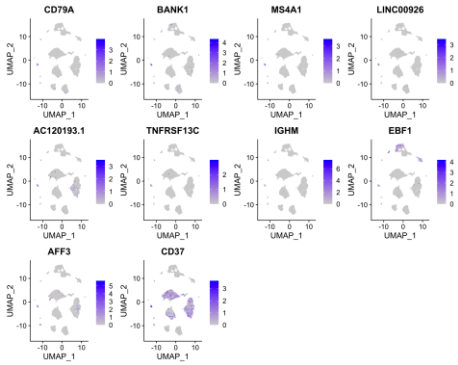
T **Cluster 20 (pDCs)**



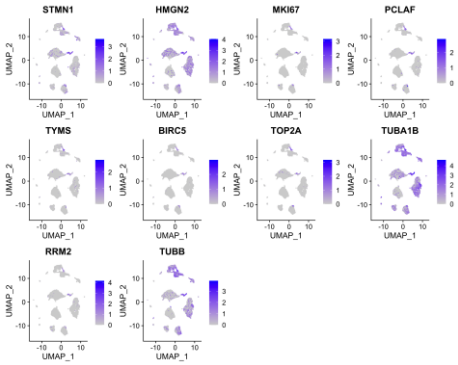
U **Cluster 21 (Mesothelial cells)**

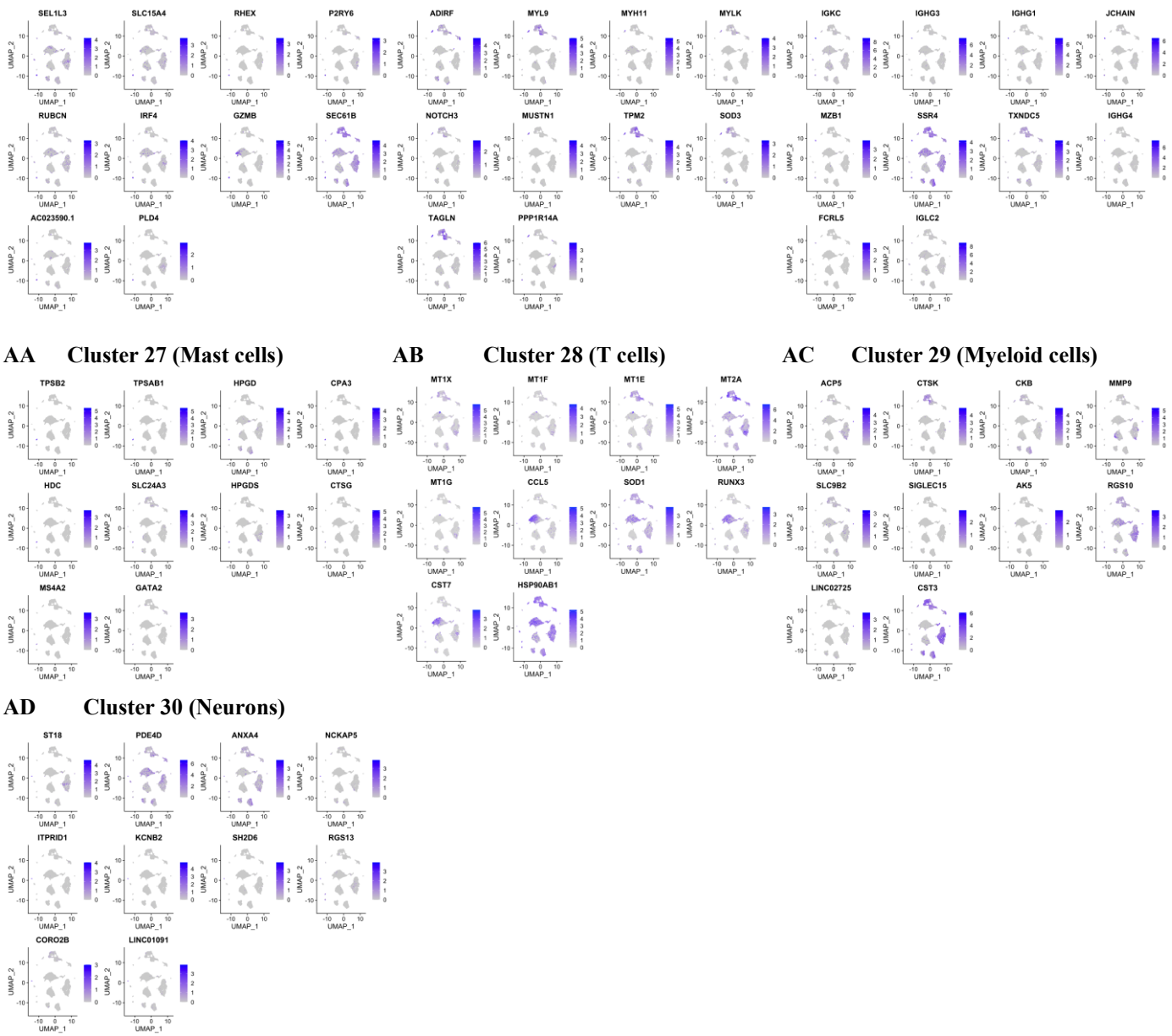


V **Cluster 22 (B cells)**



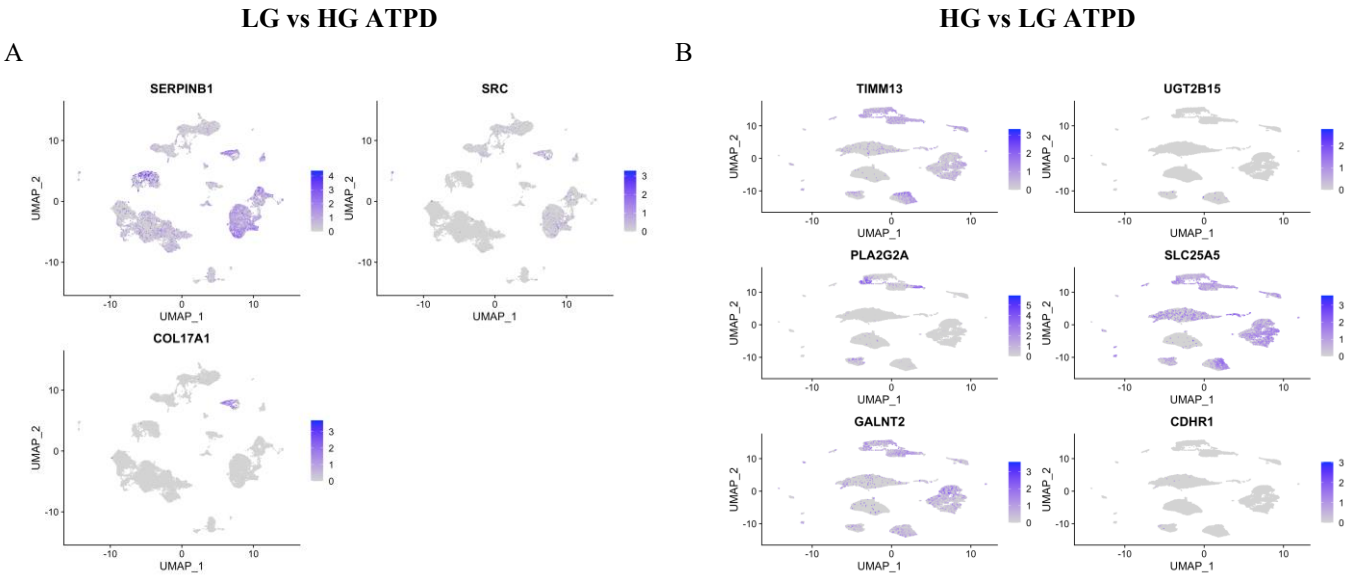
W **Cluster 23 (Cycling cells)**





Cluster feature plots (**A-AD**) show the top ten differentially expressed genes for each cell cluster informing the annotation of LG ATPD.

Abbrev. DCs, dendritic cells; pDCs, plasmacytoid DCs; cDCs, conventional DCs; CAFs, cancer-associated fibroblasts; LG ATPD, low grade appendiceal tumour peritoneal disease.



Supplementary Figure SF3.10 | Expression in scRNAseq data of differentially expressed genes from spatial transcriptomic profiling of tumour segments

Cluster feature plots showing distribution of top differentially expressed genes (FDR≤0.0001) in scRNAseq data from analysis of spatial profiling of tumour segments: LG compared to HG ATPD (**A**) and HG compared to LG ATPD (**B**).

Abbrev. scRNAseq, single cell RNA sequencing; LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease; FDR, false discovery rate.

8.4 Appendix 4: R code for chapter 3

8.4.1 Single cell RNA sequencing analysis

```
# author: Madeleine Strach
# date: 2023-09-15
# last edit: 2024-05-08

# Adapted from Script by
# author: Eva Apostolov
# date: 2023-05-19
# last edit:

#based on Seurat - Guided Clustering Tutorial • Seurat (satijalab.org)

#Seurat for scRNA-seq
#packageVersion("Seurat")
#[1] '4.3.0.1'

BiocManager::install("Seurat")
install.packages('Seurat')


library(dplyr)
library(ggplot2)
library(viridis)
library(Seurat)
library(cowplot)
library(tidyverse)
library(clustree)
library(data.table)
library(patchwork)
library(clustree)
library(MAST)
library(knitr)

set.seed(42)
```

a) # Load the dataset

```
#AC.data <- Read10X(data.dir =
"/Users/madeleine/PB164_summary/filtered_feature_bc_matrix/")
#Main
AC.data <- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB190_summary/filtered_feature_bc_m
atrix/")
#pilot
#092
AC.data <- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Results of Pilot
study/211124_A00152_0502_AHJ7W2DSX2_summary/211124_A00152_0502
_AHJ7W2DSX2/GE/PB092/summary/sample_feature_bc_matrix")
#099
AC.data <- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Results of Pilot
study/211124_A00152_0502_AHJ7W2DSX2_summary/211124_A00152_0502
_AHJ7W2DSX2/GE/PB099/summary/sample_feature_bc_matrix")


#setwd("/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/")

#restore previously saved object
#Restore the object

#AAcomb cm file
cluster.markers<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/AAcomb_cm_file.rds")

#AMNcomb cm file
cluster.markers<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/AMNcomb_cm_file.rds")

#AMNcomb_int2 (use for feature cluster graphs)
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/AMNcomb_int2.rds")

#AAcomb_int2
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/AAcomb_int2.rds")

#ACall_int1
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single Cell/Objects/ACall_int1.rds")
#explants

#180res1.2
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AC180_tissue_pde_int2.rds")

#190res0.2
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AC190_tissue_pde_int2_res0.2_seeddifferent.rds")

#190cm file
cluster.markers<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AC190_tissue_pde_cm_file_top200_2.rds")

#180cm file
cluster.markers<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AC180_tissue_pde_cm_file.rds")
```

b) #individual objects post QC

```
#AMN_092
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/Maddy_ApCa/results/20230517_scRNA_prelim_analysis/01_p
reprocess_seurat_individual/PB092/rObjects/PB092jackstraw_100_
0.01_clusters.rds")

#AMN_164
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/Maddy_ApCa/results/20230517_scRNA_prelim_analysis/01_p
reprocess_seurat_individual/PB164/rObjects/PB164jackstraw_100_
0.01_clusters.rds")

#AMN_190
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
```

```
Methods/Transcriptomics/scRNAseq/Main
project/Maddy_ApCa/results/20230517_scRNA_prelim_analysis/01_p
reprocess_seurat_individual/PB190/rObjects/PB190jackstraw_100_
0.01_clusters.rds")


#AA176
AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/Maddy_ApCa/results/20230517_scRNA_prelim_analysis/01_p
reprocess_seurat_individual/PB176/rObjects/PB176jackstraw_100_
0.01_clusters.rds")


AC<-AC.data

#QC raw data
# Initialize the Seurat object with the raw (non-normalized
data).
AC <- CreateSeuratObject(counts = AC, project = "AC",
min.cells = 3, min.features = 200)
#Check object to check how many total genes and total cells

#AC<-AMN.combined
#AC<-ACall.combined
#AC

#QC Metrics
# calculate % mitochondrial reads per cell; genes starting
with MT-
AC[["percent.mt"]] <- PercentageFeatureSet(AC, pattern = "^MT-
")

# calculate % ribosomal reads per cell; genes starting with
RPS or RPL
AC[["percent.ribo"]] <- PercentageFeatureSet(AC, pattern =
"^RP[SL]")

#Number of UMMI's per cell
AC$log10GenesPerUMI <- log10(AC$nFeature_RNA) /
log10(AC$nCount_RNA)

# Show QC metrics for the first 5 cells
head(AC@meta.data, 5)

# initial QC plot as violin plot
resfactor=4
png("AC190_tissue_pde_VP_QC1.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p1 <- VlnPlot(AC, features = c("nFeature_RNA", "nCount_RNA"),
ncol = 2, log=TRUE)
p2 <- VlnPlot(AC, features = c("percent.mt", "percent.ribo"),
ncol = 2, log=FALSE)
# combine plots
p <- plot_grid(p1,p2, ncol=1)
print(p)
dev.off()

resfactor=4
png("AC190_tissue_pde_VP_UMI.png", res=72*resfactor,
height=320*resfactor, width=320*resfactor)
p<-VlnPlot(AC, features = c("log10GenesPerUMI"), ncol = 2,
log=TRUE)
print(p)
dev.off()


# feature scatters
resfactor=4
png("AC190_tissue_pde_FS_QC1.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p1 <- FeatureScatter(AC, feature1 = "nCount_RNA", feature2 =
"nFeature_RNA") + NoLegend()
p2 <- FeatureScatter(AC, feature1 = "nCount_RNA", feature2 =
"percent.mt")
# combine and plot
p <- plot_grid(p1,p2, ncol=2)
print(p)
dev.off()


# filter based on QC metrics
# this can be done more stringently, but for a first pass
# we're just going to filter out cells with > 20%
mitochondrial reads
# as well as cells with low number of genes per cell (<300)

AC <- subset(AC, subset = nFeature_RNA > 300 & percent.mt <
20)

#post QC object number of cells and genes
AC

# replot QC metrics - VP
resfactor=4
png("AC190_tissue_pde_VP_QC2.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p1 <- VlnPlot(AC, features = c("nFeature_RNA", "nCount_RNA"),
ncol = 2, log=TRUE)
p2 <- VlnPlot(AC, features = c("percent.mt", "percent.ribo"),
ncol = 2, log=FALSE)
# combine plots
p <- plot_grid(p1,p2, ncol=1)
print(p)
dev.off()

resfactor=4
png("ACall_VP_UMI2.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p<-VlnPlot(AC, features = c("log10GenesPerUMI"), ncol = 1,
log=TRUE)
print(p)
dev.off()

# feature scatters
resfactor=4
png("AC190_tissue_pde_FS_QC2.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p1 <- FeatureScatter(AC, feature1 = "nCount_RNA", feature2 =
"nFeature_RNA") + NoLegend()
p2 <- FeatureScatter(AC, feature1 = "nCount_RNA", feature2 =
"percent.mt")
# combine and plot
p <- plot_grid(p1,p2, ncol=2)
print(p)
dev.off()

#Normalise the data (Scale 10,000 is default)
AC <- NormalizeData(AC, normalization.method = "LogNormalize",
scale.factor = 10000)

#Identify highly variable features (focus on these genes for
downstream analysis)
AC <- FindVariableFeatures(AC, selection.method = "vst",
nfeatures = 2000)

# Identify the 10 most highly variable genes
top10 <- head(VariableFeatures(AC), 10)

# plot variable features with and without labels
resfactor=4
png("AC190_tissue_pde_variablefeatures.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p1 <- VariableFeaturePlot(AC)
p2 <- LabelPoints(plot = p1, points = top10, repel = TRUE)
p<-plot_grid(p1,p2, ncol=2)
print(p)
dev.off()

#Scale the data (mean expression is 0, variance across cells
is 1, gives equal weight so highly expressed genes don't
dominate)
#Scale all genes as heatmap needs all genes, but PCA doesn't -
default is to scale the 2000 highly variable genes
all.genes <- rownames(AC)
#AC164 <- ScaleData(AC164, features = all.genes)
#If variables are provided in vars.to.regress, they are
individually regressed against each feature, and the resulting
residuals are then scaled and centered.
#we could ‘regress out’ heterogeneity associated with (for
example) cell cycle stage, or mitochondrial contamination.
#NB/the function SCTransform() also includes a vars.to.regress
parameter.
AC <- ScaleData(AC, verbose = TRUE, vars.to.regress =
"percent.mt")

#Run PCA
```

```
#By default, only the previously determined variable features
are used as input, but can be defined using features argument
if you wish to choose a different subset.
AC <- RunPCA(AC, features = VariableFeatures(object = AC))

# Examine and visualize PCA results a few different ways
print(AC[["pca"]], dims = 1:5, nfeatures = 5)

resfactor=4
png("AC190_tissue_pde_VizDimRed.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-VizDimLoadings(AC, dims = 1:2, reduction = "pca")
print(p)
dev.off()


resfactor=4
png("AC190_tissue_pde_DimPlot.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(AC, reduction = "pca")
print(p)
dev.off()


resfactor=4
png("AC190_tissue_pde_DimHeatmap.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimHeatmap(AC, dims = 1, cells = 500, balanced = TRUE)
print(p)
dev.off()

resfactor=4
png("AC190_tissue_pde_DimHeatmap2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimHeatmap(AC, dims = 1:22, cells = 500, balanced = TRUE)
print(p)
dev.off()

#define number of principle components
nPCs=100
AC <- RunPCA(AC, verbose = TRUE, npcs = nPCs)

#Determine dimensionality using JackStraw
# NOTE: This process can take a long time for big datasets,
comment out for expediency. More
# approximate techniques such as those implemented in
ElbowPlot() can be used to reduce
# computation time

#AC <- JackStraw(AC, num.replicate = 100)
#AC <- ScoreJackStraw(AC, dims = 1:20)

AC <- JackStraw(AC, dims = nPCs, num.replicate=100, verbose =
TRUE)
AC <- ScoreJackStraw(AC, dims = 1:nPCs)

png("AC190_tissue_pde_Jackstraw.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-JackStrawPlot(AC, dims = 1:20)
print(p)
dev.off()

png("AC190_tissue_pde_Elbow.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-ElbowPlot(AC)
print(p)
dev.off()

saveRDS(AC, file = "AC190_tissue_pde_int1.rds")
```

c) #Cluster Cells

```
# choose sig PCs
pcsData <-
as.data.frame(AC@reductions$pca@jackstraw@overall.p.values)
pcs <- pcsData[pcsData$Score<0.01,"PC"]
AC <- FindNeighbors(AC, reduction = "pca", dims = pcs, verbose
= TRUE)

#AC <- FindNeighbors(AC, dims = 1:20)
#AC<- FindClusters(AC, resolution = 0.5)

# use multiple clustering resolutions, from 0.2 to 1.2, by 0.2
#AC <- FindClusters(AC, resolution=c(seq(0.2, 1.2, 0.2)))
#AC <- FindClusters(AC, resolution=1.2)
#AC <- FindClusters(AC, resolution=0.8)
AC <- FindClusters(AC, resolution=0.2)
AC <- RunUMAP(AC, reduction = "pca", dims = pcs)

resfactor=4
png("PB190explant_UMAP_0.2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(AC, reduction = "umap",
           label=TRUE
           #label=FALSE
)
print(p)
dev.off()

saveRDS(AC, file = "AC190_tissue_pde_int2_res0.2.rds")


#metadata$sample -> AC[["sample"]]

# get annotations stored in metadata
#annotations <- AC$seurat_annotations



#Identify original samples
resfactor=4
png("AMNcomb_UMAP_subset.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(AC, reduction = "umap", group.by = "orig.ident",
           #label=TRUE
           label=FALSE
)
print(p)
dev.off()

#split umaps by sample
meta_columns <- grep("orig.ident", names(AC@meta.data), value
= TRUE)
plot_list <- list()
for(column in meta_columns){
  plot_list[[column]] <- DimPlot(AC, group.by =
"RNA_snn_res.1.2", reduction = "umap", split.by = column,
label = TRUE) + ggtitle(column) + NoLegend()
}

#x2
resfactor=4
png("AMNcomb_UMAP_splitby.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p <- plot_grid(plotlist = plot_list, ncol = 1)
print(p)
dev.off()

#x3
resfactor=4
png("AMNcomb_UMAP_splitby.png", res=72*resfactor,
height=640*resfactor, width=1200*resfactor)
p <- plot_grid(plotlist = plot_list, ncol = 1)
print(p)
dev.off()
```

d) #Differential Expression of genes

```
#“MAST” : GLM-framework that treats cellular detection rate as
a covariate (Finak et al, Genome Biology, 2015)
#MAST_1.25.1.

cluster.markers <- FindAllMarkers(AC, only.pos = TRUE, min.pct
= 0.25, logfc.threshold = 0.25, test.use='MAST')
top10 <- cluster.markers %>% group_by(cluster) %>% top_n(n =
10, wt = avg_log2FC)
top10_ident <- cluster.markers %>% group_by(orig.ident) %>%
top_n(n = 10, wt = avg_log2FC)
top50 <- cluster.markers %>% group_by(cluster) %>% top_n(n =
50, wt = avg_log2FC)
top200 <- cluster.markers %>% group_by(cluster) %>% top_n(n =
200, wt = avg_log2FC)

head(cluster.markers)

Idents(AC) <- "orig.ident"
C6 <- FindAllMarkers(AC, ident.1="AC190pde", ident.2="AC190",
min.pct = 0.25, logfc.threshold = 0.25, test.use='MAST')
top50 <- cluster.markers %>% group_by(cluster) %>% top_n(n =
50, wt = avg_log2FC)
```

```
top200 <- cluster.markers %>% group_by(cluster) %>% top_n(n =
200, wt = avg_log2FC)

write.csv(top200, file="AC190_tissue_pde_top200_deg_2.csv")
write.csv(top10, file="AC180_tissue_pde_top10_deg.csv")
write.csv(top10_ident,
file="AC190_tissue_pde_top10_deg_2_ident.csv")
saveRDS(cluster.markers,
file="AC190_tissue_pde_cm_file_top200_2_sample.rds")
```

e) #Heatmaps

```
# generate heatmaps per unannotaed seurat clusters
DefaultAssay(AC) <- "RNA"
AC <- ScaleData(AC, features = rownames(AC))

cluster.markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC > 1) %>%
  slice_head(n = 10) %>%
  ungroup() -> top10

resfactor=4
png("X.png", res=72*resfactor, height=2560*resfactor,
width=4000*resfactor)
p<-DoHeatmap(AC, group.by = "clusters", features = top10$gene)
+ NoLegend()
print(p)
dev.off()

#this works
resfactor=4
png("190tissuepde_heatmap_top10_C30.png", res=72*resfactor,
height=2560*resfactor, width=4000*resfactor)
p<-DoHeatmap(AC, cells=cells.30, features = top10$gene) +
NoLegend()
print(p)
dev.off()

# generate heatmaps by cell list
DefaultAssay(AC) <- "RNA"
AC <- ScaleData(AC, features = rownames(AC))

cluster.markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC > 1) %>%
  slice_head(n = 10) %>%
  ungroup() -> top10



# generate heatmaps per annotated seurat clusters
DefaultAssay(ACanno) <- "RNA"
ACanno <- ScaleData(ACanno, features = rownames(ACanno))

cluster.markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC > 1) %>%
  slice_head(n = 10) %>%
  ungroup() -> top10

resfactor=4
png("AAcomb_heatmap_top10_anno.png", res=72*resfactor,
height=2560*resfactor, width=4000*resfactor)
p<-DoHeatmap(ACanno, features = top10$gene) + NoLegend()
print(p)
dev.off()

kable(top10)
```

f) #UMAP annotation

```
#AA annotation
#Assign cell type identity to clusters
new.cluster.ids <- c("CD8 T cells",
                     "Tumour",
                     "CD4 T cells",
                     "Macrophage",
                     "cDC",
                     "cDC",
                     "Mesothelial cells",
                     "CAF",
                     "Neutrophils",
                     "M2 macrophage",
                     "cDC",
                     "Tumour",
                     "NK cells",
                     "CD4 T cells",
                     "Endothelial cells",
                     "M2 macrophage",
                     "Neutrophils",
                     "CAF",
                     "CD8 T cells",
                     "Tregs",
                     "pDC",
                     "Mesothelial cells",
                     "B cells",
                     "Cycling cells",
                     "cDCs/pDCs",
                     "Smooth muscle",
                     "Plasma cells",
                     "Mast cell",
                     "T cells",
                     "Myeloid cells",
                     "Neurons")
names(new.cluster.ids) <- levels(AC)
ACanno <- RenameIdents(AC, new.cluster.ids)

resfactor=4
png("AAcomb_UMAP_annotated.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(ACanno, reduction = "umap", label = TRUE, pt.size =
0.2) + NoLegend()
print(p)
dev.off()


#AMN annotation
#Assign cell type identity to clusters
new.cluster.ids <- c("Tem",
                     "Mesothelial cells",
                     "Non-classical monocytes",
                     "CD4 T cells",
                     "Tem",
                     "T cells",
                     "Macrophage",
                     "CD4 T (naive/Th2)",
                     "Macrophage/cDCs",
                     "CD8 T cells",
                     "CAF",
                     "CD4 T cells",
                     "B cells",
                     "CAF",
                     "CD4 T cells",
                     "Myeloid cells",
                     "CD8 T cells",
                     "NK T cells",
                     "Mesothelial cells",
                     "Plasma cells",
                     "Tumour",
                     "CAF",
                     "cDCs",
                     "Macrophage",
                     "Mast cells",
                     "Tumour",
                     "Cycling cells",
                     "CAF",
                     "B cells",
                     "Endothelial cells",
                     "pDCs",
                     "B cells",
                     "CAF",
                     "Mature DCs",
                     "Mast cells")
names(new.cluster.ids) <- levels(AC)
ACanno <- RenameIdents(AC, new.cluster.ids)

resfactor=4
png("AMNcomb_UMAP_annotated.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(ACanno, reduction = "umap", label = TRUE, pt.size =
0.2) + NoLegend()
print(p)
dev.off()

#HG_180_PDE_annotation
#Assign cell type identity to clusters
new.cluster.ids <- c("CD4 T cells",
                     "CD8 T / NK cells",
                     "Neutrophils",
                     "CAF",
                     "Neutrophils",
```

```
                    "CAF",
                    "Myeloid",
                    "Myeloid CD4+",
                    "Neutrophils",
                    "Myeloid CD4+/DCs/M2",
                    "Endothelial",
                    "CAF",
                    "CAF",
                    "Endothelial",
                    "CAF",
                    "Mesothelial",
                    "CAF",
                    "CAF",
                    "Endothelial",
                    "NK cells",
                    "Plasma cells",
                    "B cells",
                    "Cycling cells",
                    "Mesothelial",
                    "pDCs",
                    "Myeloid",
                    "NK cells",
                    " ")
names(new.cluster.ids) <- levels(AC)
ACanno <- RenameIdents(AC, new.cluster.ids)

resfactor=4
png("HG_180_PDE_UMAP_annotated.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(ACanno, reduction = "umap", label = TRUE, pt.size =
0.2) + NoLegend()
print(p)
dev.off()

#LG190_pde_annotated

#Assign cell type identity to clusters
new.cluster.ids <- c("T cell CD4/Treg/activated",
                    "Myeloid M1/M2",
                    "T cell CD8/NK cell",
                    "Neutrophils",
                    "CAF",
                    "CAF",
                    "B cell",
                    "Plasma cell",
                    "Endothelial/PVL",
                    "T cell",
                    "Myeloid CD4+/M2",
                    "Mesothelial",
                    "CAF",
                    "T cell/Cycling",
                    "Plasma cell",
                    "Mast cells",
                    "CAF",
                    "CAF",
                    "CAF",
                    "pDCs",
                    "CAF",
                    "CAF",
                    "CAF",
                    "CAF",
                    "CAF",
                    "Myeloid",
                    "CAF",
                    "CAF",
                    "CAF",
                    "CAF",
                    "CAF",
                    "CAF")
names(new.cluster.ids) <- levels(AC)
ACanno <- RenameIdents(AC, new.cluster.ids)

resfactor=4
png("LG_190_PDE_UMAP_annotated.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DimPlot(ACanno, reduction = "umap", label = TRUE, pt.size =
0.2) + NoLegend()
print(p)
dev.off()


#Read in raw data for each case
#read in other data set(s)

#AMN cases = 92, 164, 190

AC92.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Results of Pilot
study/211124_A00152_0502_AHJ7W2DSX2_summary/211124_A00152_0502
_AHJ7W2DSX2/GE/PB092/summary/sample_feature_bc_matrix/")
AC92<- CreateSeuratObject(counts = AC92.data, project =
"AC92")
AC92

AC164.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB164_summary/filtered_feature_bc_m
atrix/")
AC164<- CreateSeuratObject(counts = AC164.data, project =
"AC164")
AC164

AC190.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB190_summary/filtered_feature_bc_m
atrix/")
AC190<- CreateSeuratObject(counts = AC190.data, project =
"AC190")
AC190

#AA cases 176, 180, 188
AC176.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB176_summary/filtered_feature_bc_m
atrix/")
AC176<- CreateSeuratObject(counts = AC176.data, project =
"AC176")
AC176

AC180.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB180_summary/filtered_feature_bc_m
atrix/")
AC180<- CreateSeuratObject(counts = AC180.data, project =
"AC180")
AC180

AC188.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB188_summary/filtered_feature_bc_m
atrix/")
AC188<- CreateSeuratObject(counts = AC188.data, project =
"AC188")
AC188


#AC99 includes PB66 and 70
AC99.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Results of Pilot
study/211124_A00152_0502_AHJ7W2DSX2_summary/211124_A00152_0502
_AHJ7W2DSX2/GE/PB099/summary/sample_feature_bc_matrix/")
AC99<- CreateSeuratObject(counts = AC99.data, project =
"AC99")
AC99

#Explant 180 and 190
AC180pde.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
```

```
project/scRNAseq_summaries/PB180_explant_summary/filtered_feat
ure_bc_matrix/")
AC180pde<- CreateSeuratObject(counts = AC180pde.data, project
= "AC180pde")
AC180pde

AC190pde.data<- Read10X(data.dir =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD
Peritonectomy/Projects/Peritoneal Lab Project/Experimental
Methods/Transcriptomics/scRNAseq/Main
project/scRNAseq_summaries/PB190_explant_summary/filtered_feat
ure_bc_matrix/")
AC190pde<- CreateSeuratObject(counts = AC190pde.data, project
= "AC190pde")
AC190pde

#combine cases for analysis
#Merge 2 seurat objects
AC.combined <- merge(AC164, y = AC176, add.cell.ids =
c("PB164", "PB176"), project = "AC2merged")
AC.combined

#Merge AMN cases
AMN.combined <- merge(AC92, y = c(AC164, AC190), add.cell.ids
= c("PB92", "PB164", "PB190"), project = "AMNmerged")
AMN.combined
saveRDS(AMN.combined, file = "AMNcombined.rds")

#Merge AA cases
AA.combined <- merge(AC176, y = c(AC180, AC188), add.cell.ids
= c("PB176", "PB180", "PB188"), project = "AAmerged")
AA.combined
saveRDS(AA.combined, file = "AAcombined.rds")

#Merge all exclude explants - Merge all add 66, 70 (AMN) and
99 (AA) processed together
ACall.combined <- merge(AC92, y = c(AC99, AC164, AC176, AC180,
AC188, AC190), add.cell.ids = c("PB92", "PB99", "PB164",
"PB176", "PB180", "PB188", "PB190"), project = "ACallmerged")
ACall.combined
saveRDS(ACall.combined, file = "ACall.rds")

#Merge Explants
ACpde.combined <- merge(AC180pde, y = AC190pde, add.cell.ids =
c("PB180explant", "PB190explant"), project = "ACexplant")
ACpde.combined
saveRDS(ACpde.combined, file = "ACpde.rds")

#Merge PB180 and PB180explant
AC180_tissue_pde.combined <- merge(AC180pde, y = AC180,
add.cell.ids = c("PB180explant", "PB180"), project =
"AC180_Tissue_pde")
AC180_tissue_pde.combined
saveRDS(ACpde.combined, file = "AC180_tissue_pde.rds")

#Merge PB190 and PB190explant
AC190_tissue_pde.combined <- merge(AC190pde, y = AC190,
add.cell.ids = c("PB190explant", "PB190"), project =
"AC190_Tissue_pde")
AC190_tissue_pde.combined
saveRDS(ACpde.combined, file = "AC190_tissue_pde.rds")


table(ACall.combined$orig.ident)


# let's replot sample id, patient id, explant id
meta_columns <- grep("sample_id|patient_id",
names(seurat@meta.data), value = TRUE)
#plot_list <- list()
for(column in meta_columns){

  pdf(paste0(figDir, "UMAP_", column, ".pdf"), height = 9,
width = 12)
  p <- DimPlot(seurat, group.by = "RNA_snn_res.0.2", reduction
= "umap", split.by = column, label = TRUE, ncol=3) +
ggtitle(column) + NoLegend()
  print(p)
  dev.off()
}

# plot by sample type
pdf(paste0(figDir, "UMAP_sample_type.pdf"), height=6, width=9)
p <- DimPlot(seurat, group.by = "RNA_snn_res.0.2", reduction =
"umap", split.by = "sample_type", label = TRUE, ncol=2) +
ggtitle("sample_type") + NoLegend()
print(p)
dev.off()

#featureplots - canonical
#10 types of immune cells including Th2 cells, mast cells,
DCs, NK.CD56dim cells, Tregs, Tgd, Th17 cells, Neutrophils,
aDCs and T helper cells.

#Wu Nature Coms 2021 (lung) Endothelial cells (CLDN5, VWF,
PECAM1), Epithelial cells (CAPS, SNTN), Alveolar cells
(CLDN18, AQP4, FLOR1), Fibroblasts (COL1A1, COL1A2, DCN), T
cells (CD2, CD3D, CD3E, CD3G), B cells (CD79A, CD79B), Myeloid
cells (CD14, LYZ), Neutrophils (CSF3R, S100A8, S100A9),
Follicular dendritic cells (FDCSP), Mast cells (GATA2, TPSAB1,
TPSB2).

#Summary of cells:tumour KRT20, Mesothelial, CALB2, T=CD3D,
NK=KLRF1 B/Plasma CD79A, CAFs COL1A1, endothelial - PECAM1,
pDCs-LILRA4, Mast-TPSAB1, Myeloid LYZ
resfactor=4
png("190_tissue_pde_CF_summary.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KRT20", "CALB2", "CD3D",
"CD4", "CD8A", "KLRF1", "CD79A", "COL1A1", "PECAM1", "LILRA4",
"TPSAB1", "LYZ"))
print(p)
dev.off()

#B Cells, IGKC = plasma cell marker Gentles 2015
resfactor=4
png("AMNcomb_Bcells3.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD19", "CD79A", "MS4A1",
"IGKC"))
print(p)
dev.off()

#endothelial Cells
resfactor=4
png("180_tissue_pde_CF_endothelialcells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VWF", "CDH5", "PECAM1",
"CD34"))
print(p)
dev.off()

#endothelial Cells/Platelets=PF4
resfactor=4
png("AAcomb_CF_endothelialcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VWF", "CDH5", "PECAM1",
"PF4"))
print(p)
dev.off()

#Epithelial Cells
resfactor=4
png("180_tissue_pde_CF_epithelialcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CDH1", "KRT8", "EPCAM",
"KRT20", "CDX2"))
print(p)
dev.off()

#Fibroblasts
resfactor=4
png("180_tissue_pde_CF_Fibroblastscells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PDGFRB", "DCN"))
print(p)
dev.off()

##############################################################
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#Myeloid
# From Cheng Cell 2021: KIT (Mast), LILRA4 (pDC), HLA-DQA1
(cDC), FCER1a (cDC), CD68 (mono/mac),  CD163 (mono/mac)
resfactor=4
png("180_tissue_pde_CF_myeloidcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KIT", "LILRA4", "HLA-DQA1",
"FCER1A", "CD68", "CD163"))
print(p)
```

```
dev.off()

resfactor=4
png("180_tissue_pde_CF_myeloidcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("FCGR2A", "CD163", "MRC1",
"CD68", "ARG1", "CD206"))
print(p)
dev.off()

#M2 Macrophages CD206 = MRC1
resfactor=4
png("180_tissue_pde_CF_M2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD68", "CD163", "MRC1",
"VEGFA", "IL10", "AREG", "MMP1" ))
print(p)
dev.off()

#FOLR1=folate receptor (A. Rodriguez-Garcia 2021)
resfactor=4
png("180_tissue_pde_CF_M3.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD68", "CD163", "FOLR1",
"VEGFA", "IL10", "AREG", "MMP1" ))
print(p)
dev.off()

#M1 Macrophages
resfactor=4
png("180_tissue_pde_CF_M1.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD68", "CD163", "MRC1",
"TNF", "CD80", "CD86", "IL12B" ))
print(p)
dev.off()

#RTM=resident tissue macrophages = LYVE1 Chakarov 2019, ZHang
2020, Cao 2024
resfactor=4
png("AMNcomb_CF_RTM.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD68", "CD163", "CD14",
"LYVE1", "FOLR2"))
print(p)
dev.off()


#GI resident macrophages - Shaw 2018 = TIM4+CD4+ TIM4 = TIMD4
gene
resfactor=4
png("180_tissue_pde_CF_GI_macrophage.png", res=72*resfactor,
height=320*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TIMD4", "CD4"))
print(p)
dev.off()

#Dendritic cells cDCs 1 XCR1 CADM1 2 CDA CD172A (Mellman 2013)
pDCs
resfactor=4
png("180_tissue_pde_CF_DCs.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DQA1", "FCER1A" ))
print(p)
dev.off()

#CD11=ITAGX, CD304=plasmacytoid DCs, CD141 = CD14+DCs that
cross present
resfactor=4
png("AAcomb_CF_DCs.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DRA", "ITGAX", "NRP1",
"THBD", "FCGR3A", "LILRA4"))
print(p)
dev.off()

#Monocytes, CD16=FCGR3A, Classical (CD14+CD16-), non-classical
(CD14dimCD16+), intermediate (CD14+CD16+) - Fendle 2023
resfactor=4
png("180_tissue_pde_CF_monocytes_blend.png", res=72*resfactor,
height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("CD14", "FCGR3A"), blend=T)
print(p)
dev.off()

#MDSCs PMN= ITGAM+, CD14-, FUT4+/CEACAM8+ M-MDSCs = + HLADR-
/low
resfactor=4
png("180_tissue_pde_CF_MDSCs.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ITGAM", "PECAM1",
"FUT4","CD14", "CEACAM8", "HLA-DRA"),)
print(p)
dev.off()

#Neuts
resfactor=4
png("180_tissue_pde_CF_Neuts.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("S100A8", "S100A9", "FCGR3A",
"CEACAM8"),)
print(p)
dev.off()

#MAst cells:
resfactor=4
png("180_tissue_pde_CF_mast.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KIT", "TNF", "VEGFA"),)
print(p)
dev.off()
############################################################
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#Activated immune cells
resfactor=4
png("180_tissue_pde_CF_activated_immmune_cells.png",
res=72*resfactor, height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("IL2RA", "TFRC"), blend=T)
print(p)
dev.off()

#Secreted mucins Cells
resfactor=4
png("A180_tissue_pde_CF_secretedmucinscells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MUC2", "MUC5A", "MUC5B",
"MUC6", "MUC19", "MUC7", "MUC20"))
print(p)
dev.off()

#Smooth muscle Cells
resfactor=4
png("180_tissue_pde_CF_smoothmusclecells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("RGS5", "ACTA2"))
print(p)
dev.off()

############################################################
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#T Cells
resfactor=4
png("180_tissue_pde_CF_Tcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TRBC2", "CD2", "CD3E",
"IL7R", "CD8A", "NKG7"))
print(p)
dev.off()

resfactor=4
png("180_tissue_pde_CF_Tcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD3D", "CD3E", "TRBC2",
"CD2"))
print(p)
dev.off()

#NK T cells NCAM1=CD56bright, FCGR3A = CD16 = CD56 dim =
Kurioka 2018
resfactor=4
png("180_tissue_pde_CF_TcellsNK.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("NCAM1", "FCGR3A", "CD3E",
"CD3D"))
print(p)
dev.off()

#NK T CD56 bright v dim =Kurioka 2018
resfactor=4
```

```
png("180_tissue_pde_CF_TcellsNK_blend.png", res=72*resfactor,
height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("NCAM1", "FCGR3A"),
blend=TRUE)
print(p)
dev.off()

#Naive CD4 T cells - Van den Broek 2018
resfactor=4
png("180_tissue_pde_CF_CD4_naive.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PTPRC", "CCR7", "SELL",
"PECAM1", "CD27", "CD28"))
print(p)
dev.off()

#CD4 Th1 T cells - Mitchell 2015
resfactor=4
png("180_tissue_pde_CF_CD4_Th1.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD4", "IL2RA", "IL2RB",
"IL2RG", "IFNGR1", "IL12RB1"))
print(p)
dev.off()

#CD4 Th2 T cells - Mitchell 2015
resfactor=4
png("180_tissue_pde_CF_CD4_Th2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD4", "IL4R", "IL10RB"))
print(p)
dev.off()

#Th17 T cells - Mitchell 2015
resfactor=4
png("180_tissue_pde_CF_CD4_Th17.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD4", "IL17RA", "IL17RC",
"IL21R"))
print(p)
dev.off()


#T reg cells
resfactor=4
png("180_tissue_pde_CF_Treg.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD4", "FOXP3", "CD25"))
print(p)
dev.off()

resfactor=4
png("180_tissue_pde_CF_Treg_blend.png", res=72*resfactor,
height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("CD4", "FOXP3"), blend=TRUE)
print(p)
dev.off()

#MAIT T Cells
resfactor=4
png("180_tissue_pde_CF_C0_MAITcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TRAV1-2", "TRAJ33", "TRBV20-
1", "TRBV6"))
print(p)
dev.off()

#SLEC

#MPEC

#Tem=effector memory T cells - Sallusto 2004
resfactor=4
png("180_tissue_pde_CF_Tem.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD8A", "CX3CR1", "CD127",
"TBET", "BLIMP1", "ID2", "STAT4"))
print(p)
dev.off()

#CD62L=L-selectin SELL gene
resfactor=4
png("180_tissue_pde_CF_Tem2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD44", "SELL", "CCR7"))
print(p)
dev.off()

#Tpm

#Tcm= central memory T cells (CCR7-=Tem) - Sallusto 2004
resfactor=4
png("180_tissue_pde_CF_Tcm_blend.png", res=72*resfactor,
height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("CD8A", "CCR7"), blend=TRUE)
print(p)
dev.off()

#T cell activation, CD69=early activation, HLADRA-late
activation, PD1=-nihbitory
resfactor=4
png("AAcomb_CF_Tcellactive.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD3E", "CD8A", "CD4", "CD69",
"HLA-DRA", "PDCD1"))
print(p)
dev.off()

#############################################################
##########################

#NK CD56=NCAM1
resfactor=4
png("180_tissue_pde_CF_NK.png", res=72*resfactor,
height=320*resfactor, width=1600*resfactor)
p<-FeaturePlot(AC, features = c("CD8A", "CCR7"), blend=TRUE)
print(p)
dev.off()

#NK CD56=NCAM1
resfactor=4
png("AAcomb_CF_NK2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("NCAM1", "CCR7", "KLRF1",
"FCGR3A"))
print(p)
dev.off()

#Transmembrane mucins
resfactor=4
png("180_tissue_pde_CF_transmembranemucinscells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MUC1", "MUC3A", "MUC4",
"MUC12", "MUC13", "MUC15", "MUC16", "MUC17", "MUC21",
"MUC22"))
print(p)
dev.off()

#Mesothelial cells
resfactor=4
png("180_tissue_pde_CF_mesothelialcells.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CALB2", "ADGRD1", "CDH1",
"DPP4", "FGF1"))
print(p)
dev.off()

#Mesothelial cells (Lugli 2003, Kjiyama 2007, Yoon 2021, ,
Zhao 2012, Mutsaers 2015). (HMBE1 binds mesothelin)
resfactor=4
png("PB190_pde_CF_mesothelialcells2.png", res=72*resfactor,
height=640*resfactor, width=600*resfactor)
p<-FeaturePlot(AC, features = c("CALB2", "MSLN", "DPP4",
"CDH1", "WT1"))
print(p)
dev.off()

#"Milky spots = immune mesothelial cells", ITGAL=LFA-1,
ITGAM=MAC-1, ITGA4=VLA-4, NOT "ICAM1", "ITGAL", "ITGAM",
"ITGA4"
resfactor=4
png("180_tissue_pde_CF_milky.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CALB2",  "VCAM1" ))
print(p)
dev.off()

#"Milky spots = immune mesothelial cells", ITGAL=LFA-1,
ITGAM=MAC-1, ITGA4=VLA-4, NOT "ICAM1", "ITGAL", "ITGAM",
"ITGA4"
resfactor=4
```

```
png("AAcomb_CF_milky2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CALB2",  "ITGAL", "ITGAM",
"ITGA4" ))
print(p)
dev.off()

#Peritoneal attachment, MUC16=CA125 protein, beta ig-h3=TGFBI,
Not "CX3CL1", "CXCR4", "TGFBI", "CD44"
resfactor=4
png("180_tissue_pde_CF_peritonealattachment3.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CALB2", "MSLN", "MUC16",
"VTN"))
print(p)
dev.off()

#cycling cells
resfactor=4
png("180_tissue_pde_CF_cyclingcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MKI67"))
print(p)
dev.off()

#Cancer associated fibroblasts; CXCL12 Recruits M2
resfactor=4
png("180_tissue_pde_CF_CAFcells2.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PDGFRA", "COL1A1", "ACTA2",
"PDGFRB", "CXCL12"))
print(p)
dev.off()

#Epithelial-Mesenchymal transition
resfactor=4
png("180_tissue_pde_CF_EMTcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VIM", "ACTA2"))
print(p)
dev.off()

#Perivascular cells"
resfactor=4
png("180_tissue_pde_PVLcells.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MCAM", "ACTA2", "PDGFRB"))
print(p)
dev.off()

#cell stress genes (Wu 2021)= heat shock proteins and pathways
resfactor=4
png("AMNcomb_CF_cellstress.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HSPA1A", "HSPA1B",
"HSP90AA1"))
print(p)
dev.off()
-------

# SplitDotPlotGG has been replaced with the `split.by`
parameter for DotPlot



resfactor=4
png("AAcomb_GSEA_C11_GO.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-DotPlot(AC, features = features) + RotatedAxis()
print(p)
dev.off()

features <- c("TFF3", "MUC2", "TFF1", "MUC4", "MUC13", "TFF2")

resfactor=4
png("AAcomb_GSEA_C11_GO.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = features) + RotatedAxis()
print(p)
dev.off()

#secreted mucins
features <- c("MUC2", "MUC5AC", "MUC5B", "MUC6", "MUC7",
"MUC19")

#transmembrane mucins
features <- c("MUC1", "MUC3A", "MUC4", "MUC12", "MUC13",
"MUC15", "MUC16", "MUC17", "MUC20", "MUC21", "MUC22")

#all mucins
features <- c("MUC2", "MUC5AC", "MUC5B", "MUC6", "MUC7",
"MUC19", "MUC1", "MUC3A", "MUC4", "MUC12", "MUC13", "MUC15",
"MUC16", "MUC17", "MUC20", "MUC21", "MUC22")

#mucins(no MUC21)
features <- c("MUC2", "MUC5AC", "MUC5B", "MUC6", "MUC7",
"MUC19", "MUC1", "MUC3A", "MUC4", "MUC12", "MUC13", "MUC15",
"MUC16", "MUC17", "MUC20", "MUC22")

resfactor=4
png("AMNcomb_vln_mucins.png", res=72*resfactor,
height=1280*resfactor, width=2000*resfactor)
p<-VlnPlot(AC, features = features)
print(p)
dev.off()

#annotated
resfactor=4
png("AMNcomb_vln_allmucins_noMUC21.png", res=72*resfactor,
height=1280*resfactor, width=2000*resfactor)
p<-VlnPlot(ACanno, features = features)
print(p)
dev.off()

p1 <- VariableFeaturePlot(AC)
p2 <- LabelPoints(plot = p1, points = top10, repel = TRUE)
p<-plot_grid(p1,p2, ncol=2)
print(p)
dev.off()

#HG Feature plots top 10 genes per cluster
#Featureplots-AAcomb Top 10/GSEA
#C0
resfactor=4
png("AAcomb_CF_C0_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CCL5", "CCL4", "GZMK", "FYN",
"RNF19A", "CNOT6L", "BCL11B", "CST7", "TC2N", "GZMA"))
print(p)
dev.off()

#C0-inflammatory response
resfactor=4
png("AAcomb_CF_C0_GSEA_Inflamresponse.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CCL5", "FYN", "CST7", "IFNG",
"AOAH", "NKG7", "ETS1", "AKNA", "PTPRC"))
print(p)
dev.off()


#C1
resfactor=4
png("AAcomb_CF_C1_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KRT17", "AGR2", "TFF1",
"C19orf33", "CEACAM5", "CLDN4", "CEACAM6", "KRT19", "ELF3",
"CLDN3"))
print(p)
dev.off()

#C1 hallmark-early estrogen response
resfactor=4
png("AAcomb_CF_C1_Hestrogenresponse.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TFF1", "KRT19", "ELF3",
"TFF3", "KRT8", "KLK10", "KRT18"))
print(p)
dev.off()


#C2
resfactor=4
png("AAcomb_CF_C2_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("FAAH2", "LTB", "PGAP1",
"BACH2", "IL7R", "SESN3", "ANK3", "BICDL1", "CMSS1", "CCR7"))
print(p)
dev.off()
```

```
#C3
resfactor=4
png("AAcomb_CF_C3_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("LUCAT1", "DENND5A", "AQP9",
"ITGAX", "NAMPT", "LCP2", "LIMK2", "KCNJ15", "NEDD9",
"CSF3R"))
print(p)
dev.off()


#C4
resfactor=4
png("AAcomb_CF_C4_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("APOE", "APOE", "APOC1 ",
"C1QB", "C1QA", "CD74", "HLA-DRA", "SLC16A10", "C1QC",
"CTSB"))
print(p)
dev.off()

#C5
resfactor=4
png("AAcomb_CF_C5_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VCAN", "LYZ", "EREG", "FCN1",
"JARID2", "CD300E", "S100A9", "CTSS", "TRPS1", "CRADD"))
print(p)
dev.off()

#C5 GSEA R ECM organisation
png("AAcomb_CF_C5_GSEA_ECMorganisation.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VCAN", "CTSS", "TIMP1",
"SDC2", "EMILIN2", "THBS1", "CD44"))
print(p)
dev.off()


#C6
resfactor=4
png("AAcomb_CF_C6_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("COL1A1", "SERPINE1",
"COL3A1", "COL1A2", "IGFBP2", "CCDC80", "CALD1", "CCL2",
"ACTA2", "FN1"))
print(p)
dev.off()


#C7
resfactor=4
png("AAcomb_CF_C7_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("COL3A1", "COL1A1", "SFRP2",
"COL1A2", "SPARC", "MGP", "LUM", "DCN", "SERPINF1", "SFRP4"))
print(p)
dev.off()

#C8
resfactor=4
png("AAcomb_CF_C8_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("S100A8", "S100A9", "S100A12",
"PROK2", "IFITM2", "CXCL8", "PTGS2", "CMTM2", "BASP1",
"G0S2"))
print(p)
dev.off()


#C9
resfactor=4
png("AAcomb_CF_C9_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("SPP1", "C1QA", "C1QB",
"SLC16A10", "APOC1", "HLA-DRB1", "CD163", "MRC1", "C1QC",
"FTL"))
print(p)
dev.off()


#C9 GSEA R - IFN SIGNALLING
resfactor=4
png("AAcomb_CF_C9_GSEA_IFNsignalling.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DRB1", "IFI6", "HLA-DRA",
"IFI30", "HLA-DPA1", "HLA-DQA1", "HLA-DQB1", "MT2A", "HLA-
DPB1"))
print(p)
dev.off()

#C10
resfactor=4
png("AAcomb_CF_C10_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IRAK3", "AC005050.3",
"ALOX5AP", "OLR1", "CCL3L1", "C15orf48", "TANK", "ELL2",
"FPR2", "SATB1-AS1"))
print(p)
dev.off()


#C11
resfactor=4
png("AAcomb_CF_C11_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("XACT", "TFF3", "MUC2",
"LMO7", "ITGA2", "PHGR1", "TFF1", "MUC4", "LAMC2", "CEACAM5"))
print(p)
dev.off()


#C12
resfactor=4
png("AAcomb_CF_C12_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("GNLY", "NKG7", "CCL5",
"GZMB", "XCL2", "GZMA", "XCL1", "GZMH", "KLRD1", "CTSW"))
print(p)
dev.off()

#C13
resfactor=4
png("AAcomb_CF_C13_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KLRB1", "IL7R", "RORA",
"TTC39C", "CCR6", "CD3D", "LTB", "CD2", "CD96", "CAMK4"))
print(p)
dev.off()

#C14
resfactor=4
png("AAcomb_CF_C14_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CCL14", "FABP4", "VWF",
"ACKR1", "ZNF385D", "ADAMTS9", "SPARCL1", "CALCRL", "SELE",
"AQP1"))
print(p)
dev.off()

#C15
resfactor=4
png("AAcomb_CF_C15_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DPB1", "HLA-DQA1", "HLA-
DPA1", "HLA-DRA", "HLA-DRB1", "HLA-DQB1", "CD74", "CST3",
"CD86", "HLA-DMA"))
print(p)
dev.off()


#C16
resfactor=4
png("AAcomb_CF_C16_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ISG15", "IFIT3", "ALOX5AP",
"PELI1", "DUSP1", "IFIT1", "FPR2", "SAMSN1", "RSAD2",
"BCL2A1"))
print(p)
dev.off()


#C17
resfactor=4
png("AAcomb_CF_C17_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PLA2G2A", "CXCL14", "MGP",
"FST", "DCN", "MT1A", "MEDAG", "MT2A", "GLIS3", "C1R"))
print(p)
dev.off()
```

```
#C18
resfactor=4
png("AAcomb_CF_C18_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MT-ND2", "MT-CYB", "MT-ND3",
"MT-ND1", "MT-ATP6", "MT-CO3", "MT-ND5", "MT-ND4", "MT-CO2",
"MT-ATP8"))
print(p)
dev.off()


#C19
resfactor=4
png("AAcomb_CF_C19_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IL2RA", "RTKN2", "BATF",
"IKZF2", "CTLA4", "FOXP3", "TNFRSF4", "TIGIT", "IL32",
"AL136456.1"))
print(p)
dev.off()


#C20
resfactor=4
png("AAcomb_CF_C20_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DRB5", "MRC1", "FABP5",
"YBX1", "AP2S1", "FBP1", "CTSZ", "CLEC10A", "SH3BGRL3",
"BLVRB"))
print(p)
dev.off()

#C21
resfactor=4
png("AAcomb_CF_C21_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HP", "SLPI", "CFB", "PRG4",
"CXCL6", "CALB2", "CLDN1", "UPK3B", "AC245041.2", "ANXA8"))
print(p)
dev.off()

#C22
resfactor=4
png("AAcomb_CF_C22_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD79A", "BANK1", "MS4A1",
"LINC00926", "AC120193.1", "TNFRSF13C", "IGHM", "EBF1",
"AFF3", "CD37"))
print(p)
dev.off()

#C23
resfactor=4
png("AAcomb_CF_C23_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("STMN1", "HMGN2", "MKI67",
"PCLAF", "TYMS", "BIRC5", "TOP2A", "TUBA1B", "RRM2", "TUBB"))
print(p)
dev.off()


#C24
resfactor=4
png("AAcomb_CF_C24_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("SEL1L3", "SLC15A4", "RHEX",
"P2RY6", "RUBCN", "IRF4", "GZMB", "SEC61B", "AC023590.1",
"PLD4"))
print(p)
dev.off()


#C25
resfactor=4
png("AAcomb_CF_C25_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ADIRF", "MYL9", "MYH11",
"MYLK", "NOTCH3", "MUSTN1", "TPM2", "SOD3", "TAGLN",
"PPP1R14A"))
print(p)
dev.off()

#C26
resfactor=4
png("AAcomb_CF_C26_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IGKC", "IGHG3", "IGHG1",
"JCHAIN", "MZB1", "SSR4", "TXNDC5", "IGHG4", "FCRL5",
"IGLC2"))
print(p)
dev.off()


resfactor=4
png("AAcomb_CF_C27_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TPSB2", "TPSAB1", "HPGD",
"CPA3", "HDC", "SLC24A3", "HPGDS", "CTSG", "MS4A2", "GATA2"))
print(p)
dev.off()


#C28
resfactor=4
png("AAcomb_CF_C28_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MT1X", "MT1F", "MT1E",
"MT2A", "MT1G", "CCL5", "SOD1", "RUNX3", "CST7", "HSP90AB1"))
print(p)
dev.off()


#C29
resfactor=4
png("AAcomb_CF_C29_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ACP5", "CTSK", "CKB", "MMP9",
"SLC9B2", "SIGLEC15", "AK5", "RGS10", "LINC02725", "CST3"))
print(p)
dev.off()


#C30
resfactor=4
png("AAcomb_CF_C30_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ST18", "PDE4D", "ANXA4",
"NCKAP5", "ITPRID1", "KCNB2", "SH2D6", "RGS13", "CORO2B",
"LINC01091"))
print(p)
dev.off()
```

g) #LG Feature plots – top 10 genes per cluster

```
#Featureplots-AMNcomb Top 10/GSEA
#C0
resfactor=4
png("AMNcomb_CF_C0_top10_blend.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IL7R", "KLRB1", "STAT4",
"LINC01871", "ANK3", "CD69", "PARP8", "RORA", "CDC14A",
"PBX4"))
print(p)
dev.off()

#C0-interleukin signalling
resfactor=4
png("AMNcomb_CF_C0_GSEA_ILsignalling.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IL7R", "STAT4", "RORA",
"IL32", "CCL5", "FYN"))
print(p)
dev.off()

#C1
resfactor=4
png("AMNcomb_CF_C1_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("SLPI", "HP", "PLA2G2A",
"CCDC80", "PRG4", "C3", "ITLN1", "CALB2", "SERPING1",
"RARRES2"))
print(p)
dev.off()


#C2
resfactor=4
png("AMNcomb_CF_C2_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HMOX1", "MT1G", "C1QB",
"C1QA", "LYVE1", "FN1", "SLC16A10", "RNASE1", "FMNL2",
"MARCO"))
```

```
print(p)
dev.off()

#C3
resfactor=4
png("AMNcomb_CF_C3_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TAF4B", "RAPGEF6", "LEF1",
"INPP4B", "ITK", "TXK", "NDFIP1", "PRKCQ-AS1", "PRKCA",
"AP001011.1"))
print(p)
dev.off()

#C4
resfactor=4
png("AMNcomb_CF_C4_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CRTAM", "CCL4L2", "CCL4",
"IFNG", "CCL5", "GZMK", "PPP1R16B", "GNG2", "FYN", "BICDL1"))
print(p)
dev.off()

#C5
resfactor=4
png("AMNcomb_CF_C5_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IL7R", "TSC22D3", "RPS27",
"RPS29", "SRSF2", "RPL39", "RPL28", "RPS12", "RPLP1",
"RPS15A"))
print(p)
dev.off()

#C5-GSEA CD8 T cells
resfactor=4
png("AMNcomb_CF_C5_GSEA_CD8T.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IL7R", "KLRB1", "TSC22D3",
"CD7", "ZFP36L2", "LINC01871", "LEPROTL1", "TRBC2", "CXCR4",
"IL32", "CD52", "DUSP2"))
print(p)
dev.off()

#C6
resfactor=4
png("AMNcomb_CF_C6_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VCAN", "THBS1", "JARID2",
"SLC16A10", "LYZ", "KYNU", "EREG", "PID1", "SIPA1L1",
"PPARG"))
print(p)
dev.off()

#C7
resfactor=4
png("AMNcomb_CF_C7_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("AQP9", "IL1R2", "CPD",
"LUCAT1", "LIMK2", "AZIN1-AS1", "DOCK4", "NAMPT", "SAMSN1",
"LCP2"))
print(p)
dev.off()

#C8
resfactor=4
png("AMNcomb_CF_C8_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("C1QC", "C1QA", "C1QB",
"PLTP", "HLA-DRB5", "CD74", "CD14", "HLA-DRA", "HLA-DQA2",
"LYZ"))
print(p)
dev.off()


#C9
resfactor=4
png("AMNcomb_CF_C9_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("NKG7", "CCL4", "KLRD1",
"CCL5", "CST7", "DUSP2", "HCST", "SRSF2", "ABHD17A",
"TUBA4A"))
print(p)
dev.off()

#C10
resfactor=4
png("AMNcomb_CF_C10_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("COL1A1", "FBLN1", "COL1A2",
"COL3A1", "MGP", "BGN", "SPARCL1", "DCN", "SPARC", "IGFBP7"))
print(p)
dev.off()

#C11
resfactor=4
png("AMNcomb_CF_C11_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("FAAH2", "PTPN13", "THADA",
"LDLRAD4", "TOX", "RNF19A", "GPRIN3", "RAPGEF6", "CTLA4",
"ABCC1"))
print(p)
dev.off()

  #C12
  resfactor=4
png("AMNcomb_CF_C12_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("BANK1", "AFF3", "EBF1",
"MS4A1", "IGHM", "CD79A", "BACH2", "FCRL1", "AC120193.1",
"ADAM28"))
print(p)
dev.off()

  #C13
  resfactor=4
png("AMNcomb_CF_C13_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("ALDH1A3", "PTX3", "APOE",
"MT1E", "CCN1", "GPC6", "SERPINE1", "IGFBP2", "MT1M",
"CCDC71L"))
print(p)
dev.off()

  #C14
  resfactor=4
png("AMNcomb_CF_C14_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IKZF2", "BATF", "IL2RA",
"RTKN2", "CTLA4", "STAM", "FOXP3", "TBC1D4", "TIGIT", "LTB"))
print(p)
dev.off()

  #C15
  resfactor=4
png("AMNcomb_CF_C15_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("S100A9", "CXCL8", "S100A8",
"ALOX5AP", "IFITM2", "NCF1", "IL1R2", "CSF3R", "MNDA",
"BCL2A1"))
print(p)
dev.off()

  #C16
  resfactor=4
png("AMNcomb_CF_C16_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("MT-ND3", "MT-CO2", "MT-CYB",
"BRD2", "MT-ND5", "HNRNPH1", "FUS", "MT-ND2", "TUBB4B",
"SLC38A2"))
print(p)
dev.off()

  #C17
  resfactor=4
png("AMNcomb_CF_C17_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KLRD1", "KLRC1", "CMC1",
"XCL1", "XCL2", "NKG7", "CTSW", "KLRC3", "TRDC", "NCR1"))
print(p)
dev.off()

#C18
resfactor=4
png("AMNcomb_CF_C18_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("KRT18", "MT1E", "CFB", "MT1M
", "TM4SF1", "ADIRF", "KRT19", "CAV1", "KRT8", "SNHG29"))
print(p)
dev.off()

#C19
resfactor=4
```

```
png("AMNcomb_CF_C19_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IGKC", "IGLC2", "IGHG1",
"IGHGP", "IGHG3", "IGHG4", "JCHAIN", "MZB1", "ANKRD28",
"SSR4"))
print(p)
dev.off()

  #C20
  resfactor=4
png("AMNcomb_CF_C20_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TFF3", "TFF1", "SPINK4",
"REG4", "TFF2", "MUC2", "TSPAN8", "AGR2", "CEACAM5", "CLDN4"))
print(p)
dev.off()

  #C21
  resfactor=4
png("AMNcomb_CF_C21_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("SNHG25", "CALR", "C3",
"FOSB", "SEC61G", "B4GALT1", "SLPI", "HSPB1", "S100A6",
"PPP1R14B"))
print(p)
dev.off()

  #C22
  resfactor=4
png("AMNcomb_CF_C22_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("HLA-DPB1", "HLA-DPA1", "HLA-
DQA1", "FCER1A", "HLA-DRB1", "CLEC10A", "HLA-DQB1", "HLA-DRA",
"CSF2RA", "CD1C"))
print(p)
dev.off()

  #C23
  resfactor=4
png("AMNcomb_CF_C23_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("APOC1", "KCNMA1", "CTSZ",
"CTSD", "CTSB", "FTL", "MMP2-AS1", "APOE", "HLA-DRB1", "HLA-
DQA1"))
print(p)
dev.off()

  #C24
  resfactor=4
png("AMNcomb_CF_C24_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("TPSB2", "TPSAB1", "CPA3",
"CTSG", "HPGD", "SLC24A3", "HPGDS", "IL1RL1", "ADCYAP1",
"KIT"))
print(p)
dev.off()

  #C25
  resfactor=4
png("AMNcomb_CF_C25_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PLCG2", "MUC2", "ELF3",
"TFF3", "INF2", "PHGR1", "TFF1", "GSN", "C19orf33", "HES1"))
print(p)
dev.off()

  #C26
  resfactor=4
png("AMNcomb_CF_C26_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("PCLAF", "TYMS", "MKI67",
"STMN1", "RRM2", "TOP2A", "TPX2", "ASPM", "UBE2C", "HMGN2"))
print(p)
dev.off()

  resfactor=4
png("AMNcomb_CF_C27_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("COL5A2", "CCN2", "GPC6",
"LTBP1", "COL11A1", "FAP", "POSTN", "COL12A1", "KIF26B",
"PRKG1"))
print(p)
dev.off()

  #C28
  resfactor=4
png("AMNcomb_CF_C28_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CD79A", "TNFRSF13C", "CD37",
"LINC00926", "LSM7", "IGHM", "MS4A1", "CD74", "HLA-DQA2",
"P2RX5"))
print(p)
dev.off()

  #C29
  resfactor=4
png("AMNcomb_CF_C29_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("VWF", "HYAL2", "RAMP2",
"ECSCR", "CLDN5", "ADGRL4", "EMCN", "PCAT19", "CDH5",
"CALCRL"))
print(p)
dev.off()

#C30
resfactor=4
png("AMNcomb_CF_C30_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("SEC61B", "PLD4", "SCT",
"IRF4", "GZMB", "IL3RA", "PPP1R14B", "LILRA4", "TSPAN13",
"SEL1L3"))
print(p)
dev.off()


  #C31
  resfactor=4
png("AMNcomb_CF_C31_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("AC023590.1", "LRMP",
"RAPGEF5", "LMO2", "PRPSAP2", "TCL1A", "CD22", "BCL7A",
"PAX5", "MS4A1"))
print(p)
dev.off()

  #C32
  resfactor=4
png("AMNcomb_CF_C32_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("CFD", "APOD", "COL4A1",
"COL4A2", "NEGR1", "LAMA2", "MT1A", "FBLN5", "GPX3",
"LHFPL6"))
print(p)
dev.off()

  #C33
  resfactor=4
png("AMNcomb_CF_C33_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("LAMP3", "GPC5", "ENOX1",
"TXN", "SLC22A23", "CCL19", "IDO2", "UVRAG", "SLCO5A1",
"IDO1"))
print(p)
dev.off()

  #C34
  resfactor=4
png("AMNcomb_CF_C34_top10.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-FeaturePlot(AC, features = c("IGHG3", "TPSAB1", "C2CD4B",
"CCL14", "TPSB2", "ACKR1", "LYPD2", "MT1H", "S100B", "MT1G"))
print(p)
dev.off()
```

h) #Automated annotation – single R – Human Primary Cell Atlas

```
#Single R

if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

BiocManager::install("SingleR")

BiocManager::install("celldex")
BiocManager::install("scuttle")
BiocManager::install("scater")
BiocManager::install("scran")
```

```
nlibrary(SingleR)
#https://bioconductor.org/packages/release/bioc/vignettes/Sing
leR/inst/doc/SingleR.html
library(celldex)
library(scRNAseq)
#https://bioconductor.org/packages/3.18/data/experiment/vignet
tes/scRNAseq/inst/doc/scRNAseq.html
library(scuttle)
library(scater)
library(scran)
library(sctransform)
library(ggrepel)

#load previous
pred.AC<=readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AC180tissuepde_predicted_celldex.rds")

AC<-readRDS(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Single
Cell/Objects/AAcomb_singleR_HCA_labelled.rds")


hpca.se <- HumanPrimaryCellAtlasData()
hpca.se

pred.AC <- SingleR(test = GetAssayData(AC), ref = hpca.se,
assay.type.test=1,
                   labels = hpca.se$label.main)
pred.AC

table(pred.AC$labels)

saveRDS(pred.AC, file = "190tissuepde_predicted_celldex.rds")

write.csv(pred.AC, file = "190tissuepde_predicted_celldex.csv"
)


resfactor=4
png("190tissuepde_predicted_plotscoreheatmap.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-plotScoreHeatmap(pred.AC)
print(p)
dev.off()


resfactor=4
png("190tissuepde_predicted_deltadistribution.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-plotDeltaDistribution(pred.AC, ncol = 3)
print(p)
dev.off()


summary(is.na(pred.AC$pruned.labels))




#Add metadata to seurat object
AC[["HCA"]] <- pred.AC$labels


resfactor=4
png("190tissuepde_predAC_UMAP_annotated_nosplit.png",
res=72*resfactor, height=640*resfactor, width=800*resfactor)
p<-DimPlot(AC, group.by = c("HCA"), reduction = "umap",label
=T, label.size = 3, repel=TRUE)
print(p)
dev.off()

head(AC)

resfactor=4
png("190tissuepde_predAC_UMAP_annotated_redo.png",
res=72*resfactor, height=640*resfactor, width=1600*resfactor)
p<-DimPlot(AC, group.by = c("HCA","ident"), reduction =
"umap",label =T, label.size = 3, repel = TRUE,)
print(p)
dev.off()

#split umaps by meta
meta_columns <- grep("orig.ident", names(AC@meta.data), value
= TRUE)
plot_list <- list()
for(column in meta_columns){
  plot_list[[column]] <- DimPlot(AC, group.by =
c("HCA","ident"), reduction = "umap", split.by = column, label
= TRUE, label.size=3, repel=TRUE) + ggtitle(column) +
NoLegend()
}

#x4
resfactor=4
png("190tissuepde_UMAP_splitby.png", res=72*resfactor,
height=800*resfactor, width=1000*resfactor)
p <- plot_grid(plotlist = plot_list, ncol = 1)
print(p)
dev.off()

#x3
resfactor=4
png("AMNcomb_UMAP_splitby.png", res=72*resfactor,
height=640*resfactor, width=1200*resfactor)
p <- plot_grid(plotlist = plot_list, ncol = 1)
print(p)
dev.off()

saveRDS(AC, file = "180tissuepde_singleR_HCA_labelled.rds")
```

i) #Automated Annotation – Prostate cancer – script courtesy of Ms Eva Apostolov

```
# SCRIPT INFORMATION ------------------------------------------
--------------

# author: Eva Apostolov (from Beata Kiedik)
# date: 2022-08-18
# last edit: 2023-05-22

# script name: 03_SCN_annotate_major.R

# NOTES -------------------------------------------------------
---------------

# This script use SCN for preliminary annotation of Maddy's
ApCa scRNA-seq data
# with the classifier trained on Eva's PCa atlas scRNA-seq
data

# annotating only cell type major
# this helps ensure that cell type major and minor annotation
match
# cell type minor gets annotated with another script

# Input:
# Output:

# TODO --------------------------------------------------------
--------------

# LIBS --------------------------------------------------------
--------------

suppressPackageStartupMessages(expr = {
    library(singleCellNet)
    library(Seurat)
})

# ARGS --------------------------------------------------------
-----------
# and directory structure

temp_start_time <- Sys.time()
temp_args <- commandArgs(trailingOnly = TRUE)

# 01 PROJECT
projectName <- temp_args[1]
# 02 EXP CODE
exp <- temp_args[2]
# 03 ANALYSIS
analysis <- temp_args[3]
# 04 REACTION ID (sample id here)
data_id <- temp_args[4]
```

```
# directory structure
projectDir=paste0("/share/ScratchGeneral/nicyeu/",projectName,
"/")
resultDir=paste0(projectDir,"results/", exp, "/", analysis,
"/", data_id, "/")

OutputDir = resultDir
FigureOutDir = paste0(OutputDir, "figures/")
ScoreOutDir = paste0(OutputDir, "score_matrices/")
ObjectOutDir = paste0(OutputDir, "rObjects/")

# ensure output directories exist
system(paste0("mkdir -p ",OutputDir))
system(paste0("mkdir -p ",FigureOutDir))
system(paste0("mkdir -p ",ScoreOutDir))
system(paste0("mkdir -p ",ObjectOutDir))

# RELEVANT FILE PATHS ----------------------------------------
# and info on annotation

TrainingDataPath =
"/share/ScratchGeneral/evaapo/projects/PCa/results/20221004_at
las_annotation_filtered/06_merge_recluster_filtered_updateJan2
3/merged/POOLED_CTP/rObjects/PCa_atlas_annotated_v2_obj_merged
_filtered_js_100_0.01_clusters.rds"
QueryDataPath =
paste0("/share/ScratchGeneral/nicyeu/Maddy_ApCa/results/202305
17_scRNA_prelim_analysis/02_merge_seurat_preprocess/MERGED_ApC
a/rObjects/MERGED_ApCa_jackstraw_100_0.01_clusters.rds")

idents = "orig.ident"
annotation_level <- c("celltype_major_v2")

# START ------------------------------------------------------
-----------

# read Seurat object for training
train_obj <- readRDS(TrainingDataPath)
Seurat::DefaultAssay(object = train_obj) <- "RNA"

## merge normal and cancer epithelial cells into epithelial
#epithelial_all <- gsub("Cancer Epithelial|Normal Epithelial",
"Epithelial", train_obj@meta.data$celltype_major)
#train_obj@meta.data$celltype_major <- epithelial_all

# read Seurat object for training
query_obj <- readRDS(QueryDataPath)
Seurat::DefaultAssay(object = query_obj) <- "RNA"

Idents(query_obj) <- idents

for(celltype in annotation_level){
  annotation_per_sample_SCN_raw <- list()
  for (sample in unique(Idents(query_obj))){
    print(sample)
    sample_query_obj <- subset(x = query_obj, idents = sample)


#################################################################

                            SCN

#################################################################

    # load training data
    st_train <- train_obj@meta.data
    st_train <- droplevels(st_train)
    st_train$cell <- rownames(st_train)
    exp_train <- train_obj@assays$RNA@counts

    # load test data
    st_query <- sample_query_obj@meta.data
    st_query <- droplevels(st_query)
    st_query$cell <- rownames(st_query)
    exp_query <- sample_query_obj@assays$RNA@counts

    #rm(expPark)
    gc()

    # find common genes
    commonGenes = intersect(rownames(exp_train),
rownames(exp_query))
    length(commonGenes)

    exp_train = exp_train[commonGenes,]
    exp_query <- exp_query[commonGenes,]

    # split train set into train & validation
    set.seed(100) #can be any random seed number
    stList = splitCommon(sampTab=st_train, ncells=100,
dLevel=celltype)

    # get train set
    stTrain = stList[[1]]
    # get matched expression data
    expTrain = exp_train[,rownames(stTrain)]

    # get validation set
    stVal <- stList[[2]]
    # get matched expression data
    expVal <- exp_train[,rownames(stVal)]

 ############## train classifier ##################
    system.time(class_info <- scn_train(stTrain = stTrain,
expTrain = expTrain, nTopGenes = 50, nRand = 70, nTrees =
1000, nTopGenePairs = 25, dLevel = celltype, colName_samp =
"cell"))

###########validate classifier ####################

    classRes_val_all <- scn_predict(class_info[['cnProc']],
expVal, nrand = 70)

    ##################### annotate cells in query data
####################
    prediction <- scn_predict(class_info[['cnProc']],
exp_query, nrand = 70)
    prediction_scores_t <- t(prediction)

    stQuery <- assign_cate(classRes =
prediction[,1:nrow(st_query)], sampTab = st_query, cThresh =
0.5)

    match_cells <- match(rownames(stQuery),
rownames(prediction_scores_t))
    prediction_scores_t <- prediction_scores_t[match_cells,]
    stQuery <- cbind(stQuery, prediction_scores_t)

    # add metadata column to the object
    match_cells <- match(rownames(sample_query_obj@meta.data),
rownames(stQuery))
    stQuery <- stQuery[match_cells,]
    index <- grep("category", colnames(stQuery))
    sample_query_obj@meta.data <-
cbind(sample_query_obj@meta.data,
stQuery[index:ncol(stQuery)])

    annotation_per_sample_SCN_raw[[sample]] <- stQuery
  }
  saveRDS(annotation_per_sample_SCN_raw, paste0(ScoreOutDir,
"prediction_tables_", celltype, "_all_samples.rds"))
}


# SCRIPT INFORMATION -----------------------------------------

# author: Eva Apostolov (adapted from Beata Kiedik)
# date: 2023-05-29
# last edit: 2023-

# script name: 04_SCN_annotate_minor.R

# NOTES ------------------------------------------------------
---------------

# This script use SCN for preliminary annotation of Maddy's
ApCa scRNA-seq data
# with the classifier trained on Eva's PCa atlas scRNA-seq
data
# this is an updated script where cell type minor annotations
will only be done
# within a cell type major class to ensure major & minor match
# the input (query) object needs to contain cell type major
annotations

# Input:
# Output:

# TODO -------------------------------------------------------
--------------
```

```
# LIBS ----------------------------------------------------------
--------------

suppressPackageStartupMessages(expr = {
    library(singleCellNet)
    library(Seurat)
})

# ARGS ----------------------------------------------------------
-----------
# and directory structure

temp_start_time <- Sys.time()
temp_args <- commandArgs(trailingOnly = TRUE)

# 01 PROJECT
projectName <- temp_args[1]
# 02 EXP CODE
exp <- temp_args[2]
# 03 ANALYSIS
analysis <- temp_args[3]
# 04 REACTION ID (sample id here)
data_id <- temp_args[4]

# directory structure
projectDir=paste0("/share/ScratchGeneral/nicyeu/",projectName,
"/")
resultDir=paste0(projectDir,"results/", exp, "/", analysis,
"/", data_id, "/")
OutputDir = resultDir
FigureOutDir = paste0(OutputDir, "figures/")
ScoreOutDir = paste0(OutputDir, "score_matrices/")
ObjectOutDir = paste0(OutputDir, "rObjects/")

# ensure output directories exist
system(paste0("mkdir -p ",OutputDir))
system(paste0("mkdir -p ",FigureOutDir))
system(paste0("mkdir -p ",ScoreOutDir))
system(paste0("mkdir -p ",ObjectOutDir))

# RELEVANT FILES ------------------------------------------------
# dataset used to train SCN
TrainingDataPath =
"/share/ScratchGeneral/evaapo/projects/PCa/results/20221004_at
las_annotation_filtered/06_merge_recluster_filtered_updateJan2
3/merged/POOLED_CTP/rObjects/PCa_atlas_annotated_v2_obj_merged
_filtered_js_100_0.01_clusters.rds"
# dataset needing annotation
QueryDataPath =
paste0("/share/ScratchGeneral/nicyeu/Maddy_ApCa/results/202305
17_scRNA_prelim_analysis/02_merge_seurat_preprocess/MERGED_ApC
a/rObjects/MERGED_ApCa_jackstraw_100_0.01_clusters.rds")
# SCN major annotation (run previously with SCN)
SCN_major_inFile <- paste0(projectDir,
"results/20230517_scRNA_prelim_analysis/03_SCN_annotate_major/
", data_id,
"/score_matrices/prediction_tables_celltype_major_v2_all_sampl
es.rds")

# START ---------------------------------------------------------

# read Seurat object for training SCN
train_obj <- readRDS(TrainingDataPath)
Seurat::DefaultAssay(object = train_obj) <- "RNA"

# read Seurat object for annotation
query_obj <- readRDS(QueryDataPath)
Seurat::DefaultAssay(object = query_obj) <- "RNA"

# add SCN major annotation to query object (raw SCN calls)
for(tier in c("celltype_major_v2")){

  print(tier)

  SCN_raw_annotation <- readRDS(SCN_major_inFile)

  SCN_annotation_df <- do.call("rbind", SCN_raw_annotation)
  rownames(SCN_annotation_df) <- gsub(".*\\.", "",
rownames(SCN_annotation_df))

  # extract the assigned name and scores only
  # different annotation tiers (and on different data) will
have different column names
  # one thing they have in common is that the scores are
usually given after the column 'category'
  # select only columns that are found after the column
'category'
  SCN_scores <- SCN_annotation_df %>%
dplyr::select(category:last_col(), - category)

  # select SCN 'category' column in case it is needed later on
  SCN_raw_category <- SCN_annotation_df %>%
dplyr::select(category)

  # rename
  names(SCN_raw_category) <- paste0(tier)

  ## add straight to object
  query_obj <- AddMetaData(query_obj, SCN_raw_category)
}

# > table(query_obj$celltype_major_v2)
#
#      B-cells         CAFs      Cycling  Endothelial
Epithelial      Myeloid
#         1956         9438         1210         1683
5955        20235
# Plasmablasts    PNS_glial         rand         SMCs      T-
cells   Unassigned
#          604           12           16          535
21196         8001


# if SCN was used for annotations, change 'rand' to 'unknown'
query_obj@meta.data$celltype_major_v2 <- gsub("rand",
"unknown", query_obj@meta.data$celltype_major_v2)
# note, I'm still unsure what Unassigned cells are... it might
be a EMT-like phenotype??

# r = getOption("repos")
# r["CRAN"] = "https://cran.csiro.au/"
# options(repos = r)

query_obj@meta.data[["celltype_minor_new"]] <-
query_obj@meta.data[["celltype_major_v2"]]

Idents(query_obj) <- "celltype_major_v2"
Idents(train_obj) <- "celltype_major_v2"

for (celltype in unique(Idents(query_obj))) {


  # Only 12 PNS glial cells are detected in Maddy's data
  # so add them all to unknown category

  # Check if the celltype contains "unknown" or "PNS_glial"

  if (grepl("unknown", celltype) | grepl("PNS_glial",
celltype)) {

    # Get the indices of cells with the specified celltype
    cells <- which(query_obj@meta.data$celltype_major_v2 ==
celltype)

    # Set the value of "celltype_minor_new" for these cells to
"unknown"
    query_obj@meta.data$celltype_minor_new[cells] <- "unknown"

  } else {

    query_obj_SCN <- subset(x = query_obj, idents = celltype)
    train_obj_SCN <- subset(x = train_obj, idents = celltype)


############################################################
SCN
############################################################

    # load training data
    st_train <- train_obj_SCN@meta.data
    st_train <- droplevels(st_train)
    st_train$cell <- rownames(st_train)
    exp_train <- train_obj_SCN@assays$RNA@counts

    # load test data
    st_query <- query_obj_SCN@meta.data
    st_query <- droplevels(st_query)
    st_query$cell <- rownames(st_query)
    exp_query <- query_obj_SCN@assays$RNA@counts

    #rm(expPark)
```

```
    gc()

    # find common genes
    commonGenes = intersect(rownames(exp_train),
rownames(exp_query))
    length(commonGenes)

    exp_train <-  exp_train[commonGenes,]
    exp_query <- exp_query[commonGenes,]

    # split train set into train & validation
    set.seed(100) #can be any random seed number
    stList = splitCommon(sampTab=st_train, ncells=100,
dLevel="celltype_minor_v2")

    # get train set
    stTrain = stList[[1]]
    # get matched expression data
    expTrain = exp_train[,rownames(stTrain)]

    # get validation set
    stVal <- stList[[2]]
    # get matched expression data
    expVal <- exp_train[,rownames(stVal)]

##############train classifier ########################
    class_info <- scn_train(stTrain = stTrain, expTrain =
expTrain, nTopGenes = 50, nRand = 70, nTrees = 1000,
nTopGenePairs = 25, dLevel = "celltype_minor_v2", colName_samp
= "cell")

########### annotate cells in query data ################
    prediction <- scn_predict(class_info[['cnProc']],
exp_query, nrand = 70)
    #saveRDS(prediction, paste0(OutputDir, "score_matrix_",
celltype, ".rds"))

    stQuery <- assign_cate(classRes =
prediction[,1:nrow(st_query)], sampTab = st_query, cThresh =
0.5)
    # save query data with new annotation (in category column)
    #saveRDS(stQuery, paste0(outputDir, celltype, ".rds"))

    # match new annotations to cells in main object
    cells <- match(rownames(stQuery),
rownames(query_obj@meta.data))
    query_obj@meta.data[["celltype_minor_new"]][cells] <-
stQuery[,"category"]
  }
}

query_obj@meta.data[["celltype_minor_new"]] <- gsub("rand",
"unknown", query_obj@meta.data[["celltype_minor_new"]])
query_obj@meta.data[["celltype_minor_v2"]] <-
query_obj@meta.data[["celltype_minor_new"]]

table(query_obj@meta.data[["celltype_minor_v2"]],
query_obj@meta.data[["celltype_major_v2"]])

# save meta data
SCN_minor <- query_obj@meta.data[, "celltype_minor_v2",
drop=FALSE]
saveRDS(SCN_minor, paste0(ScoreOutDir ,
"prediction_tables_celltype_minor_v2_all_samples.rds"))

# save sessionInfo
writeLines(capture.output(sessionInfo()), paste0(OutputDir,
"sessionInfo.txt"))
```

8.4.2 Spatial transcriptomic analysis

```
# author: Madeleine Strach
# date: 2022-09-27
# last edit: 2024-05-16

#adapted from Analyzing GeoMx-NGS RNA Expression Data with GeomxTools
(bioconductor.org)

#load libraries
library(NanoStringNCTools)
library(GeomxTools)
library(GeoMxWorkflows)
library(knitr)
library(dplyr)
library(ggforce)
library(ggplot2)
library(scales)
library(reshape2) # for melt
library(cowplot) # for plot_grid
library(umap)
library(Rtsne)
library(pheatmap)
library(ggrepel)
library(EnvStats)
library(ggiraph)
```

a) Set up files

```
#set files
setwd("/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory")

#datadir <- file.path("~/Folder/SubFolder/DataLocation")

datadir <-
file.path("/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Pilot")

DCCFiles <- dir(file.path(datadir, "dccs"), pattern = ".dcc$",
                full.names = TRUE, recursive = TRUE)

PKCFiles <- dir(file.path(datadir, "pkcs"), pattern = ".pkc$",
                full.names = TRUE, recursive = TRUE)

SampleAnnotationFile <-
  dir(file.path(datadir, "annotation"), pattern = ".xlsx$",
      full.names = TRUE, recursive = TRUE)

#Data sheets:
#NY_WTA_1_20220524T0315_LabWorks
#AA_99_rest
#Slides124_exCRC_tons (7/3/23)
#Slides124_exAA99norm_CRCtons (10/3/23)
#Slides124_exAA99_CRCtons (16/2/24) - normal included

demoData <- readNanoStringGeoMxSet(dccFiles = DCCFiles,
pkcFiles = PKCFiles, phenoDataFile = SampleAnnotationFile,
phenoDataSheet = "Slides124_exAA99_CRCtons",
phenoDataDccColName = "Sample_ID", protocolDataColNames =
c("aoi", "roi"), experimentDataColNames = c("panel"))

pkcs <- annotation(demoData)
modules <- gsub(".pkc", "", pkcs)
kable(data.frame(PKCs = pkcs, modules = modules))
```

b) #QC raw data

```
#QC
demoData <- shiftCountsOne(demoData, useDALogic = TRUE)

QC_params <-
  list(minSegmentReads = 1000,
# Minimum number of reads (1000)
       percentTrimmed = 80,
# Minimum % of reads trimmed (80%)
       percentStitched = 80,
# Minimum % of reads stitched (80%)
       percentAligned = 80,
# Minimum % of reads aligned (80%)
       percentSaturation = 50,
# Minimum sequencing saturation (50%)
       minNegativeCount = 1,
# Minimum negative control counts (1)
       maxNTCCount = 1000,
# Maximum counts observed in NTC well (1000)
       minNuclei = 20,
# Minimum # of nuclei estimated (20)
       minArea = 1000)
# Minimum segment area (1000)

demoData <- setSegmentQCFlags(demoData, qcCutoffs = QC_params)

QCResults <- protocolData(demoData)[["QCFlags"]]
flag_columns <- colnames(QCResults)
QC_Summary <- data.frame(Pass = colSums(!QCResults[,
flag_columns]),
                         Warning = colSums(QCResults[,
flag_columns]))
QCResults$QCStatus <- apply(QCResults, 1L, function(x) {
```

```
  ifelse(sum(x) == 0L, "PASS", "WARNING")
})
QC_Summary["TOTAL FLAGS", ] <-
  c(sum(QCResults[, "QCStatus"] == "PASS"),
    sum(QCResults[, "QCStatus"] == "WARNING"))

#Visualise QC
col_by <- "segment"

QC_histogram <- function(assay_data = NULL,
                         annotation = NULL,
                         fill_by = NULL,
                         thr = NULL,
                         scale_trans = NULL) {
  plt <- ggplot(assay_data,
                aes_string(x = paste0("unlist(`", annotation,
"`)"),
                           fill = fill_by)) +
    geom_histogram(bins = 50) +
    geom_vline(xintercept = thr, lty = "dashed", color =
"black") +
    theme_bw() + guides(fill = "none") +
    facet_wrap(as.formula(paste("~", fill_by)), nrow = 4) +
    labs(x = annotation, y = "Segments, #", title =
annotation)
  if(!is.null(scale_trans)) {
    plt <- plt +
      scale_x_continuous(trans = scale_trans)
  }
  plt
}


resfactor=4
png("QC_raw.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
QC_histogram(sData(demoData), "Raw", col_by, 1000)
dev.off()

resfactor=4
png("QC_trimmed.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
QC_histogram(sData(demoData), "Trimmed (%)", col_by, 80)
dev.off()

resfactor=4
png("QC_stitched.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
QC_histogram(sData(demoData), "Stitched (%)", col_by, 80)
dev.off()

resfactor=4
png("QC_aligned.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
QC_histogram(sData(demoData), "Aligned (%)", col_by, 80)
dev.off()

resfactor=4
png("QC_saturated.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
QC_histogram(sData(demoData), "Saturated (%)", col_by, 50) +
labs(title = "Sequencing Saturation (%)", x = "Sequencing
Saturation (%)")
dev.off()

resfactor=4
png("QC_area.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
QC_histogram(sData(demoData), "area", col_by, 1000,
scale_trans = "log10")
dev.off()

#negative geometric means
negativeGeoMeans <-
  esBy(negativeControlSubset(demoData),
       GROUP = "Module",
       FUN = function(x) {
         assayDataApply(x, MARGIN = 2, FUN = ngeoMean, elt =
"exprs")
       })


protocolData(demoData)[["NegGeoMean"]] <- negativeGeoMeans

negCols <- paste0("NegGeoMean_", modules)
pData(demoData)[, negCols] <- sData(demoData)[["NegGeoMean"]]
for(ann in negCols) {
  plt <- QC_histogram(pData(demoData), ann, col_by, 2,
scale_trans = "log10")
  print(plt)
}

resfactor=4
png("QC_negativemeans.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
plt
dev.off()

pData(demoData) <- pData(demoData)[,
!colnames(pData(demoData)) %in% negCols]

kable(table(NTC_Count = sData(demoData)$NTC), col.names =
c("NTC Count", "# of Segments"))

#QC summary
kable(QC_Summary, caption = "QC Summary Table for each
Segment")

#Remove flagged segments
demoData <- demoData[, QCResults$QCStatus == "PASS"]
dim(demoData)

#Probe QC
demoData <- setBioProbeQCFlags(demoData,
                               qcCutoffs = list(minProbeRatio
= 0.1,

percentFailGrubbs = 20),
                               removeLocalOutliers = TRUE)
ProbeQCResults <- fData(demoData)[["QCFlags"]]


qc_df <- data.frame(Passed = sum(rowSums(ProbeQCResults[, -1])
== 0), Global = sum(ProbeQCResults$GlobalGrubbsOutlier), Local
= sum(rowSums(ProbeQCResults[, -2:-1]) > 0 &
!ProbeQCResults$GlobalGrubbsOutlier))

kable(qc_df)

#Exclude outlier probes
ProbeQCPassed <-
  subset(demoData,
         fData(demoData)[["QCFlags"]][,c("LowProbeRatio")] ==
FALSE &

fData(demoData)[["QCFlags"]][,c("GlobalGrubbsOutlier")] ==
FALSE)

dim(ProbeQCPassed)

demoData <- ProbeQCPassed

#Gene level count data
length(unique(featureData(demoData)[["TargetName"]]))

target_demoData <- aggregateCounts(demoData)
dim(target_demoData)

exprs(target_demoData)[1:5, 1:2]

#Limit of quantification (LOQ) per segment
cutoff <- 2
minLOQ <- 2

LOQ <- data.frame(row.names = colnames(target_demoData))
for(module in modules) {
  vars <- paste0(c("NegGeoMean_", "NegGeoSD_"), module)
  if(all(vars[1:2] %in% colnames(pData(target_demoData)))) {
    LOQ[, module] <-
      pmax(minLOQ,
           pData(target_demoData)[, vars[1]] *
             pData(target_demoData)[, vars[2]] ^ cutoff)
  }
}
pData(target_demoData)$LOQ <- LOQ

#filtering
LOQ_Mat <- c()
for(module in modules) {
  ind <- fData(target_demoData)$Module == module
  Mat_i <- t(esApply(target_demoData[ind, ], MARGIN = 1,
                     FUN = function(x) {
```

```
                      x > LOQ[, module]
                    }))
  LOQ_Mat <- rbind(LOQ_Mat, Mat_i)
}

LOQ_Mat <- LOQ_Mat[fData(target_demoData)$TargetName, ]


pData(target_demoData)$GenesDetected <-
  colSums(LOQ_Mat, na.rm = TRUE)

pData(target_demoData)$GeneDetectionRate <-
  pData(target_demoData)$GenesDetected / nrow(target_demoData)

pData(target_demoData)$DetectionThreshold <-
  cut(pData(target_demoData)$GeneDetectionRate,
      breaks = c(0, 0.01, 0.05, 0.1, 0.15, 1),
      labels = c("<1%", "1-5%", "5-10%", "10-15%", ">15%"))

p<-ggplot(pData(target_demoData),
       aes(x = DetectionThreshold)) +
  geom_bar(aes(fill = region)) +
  geom_text(stat = "count", aes(label = ..count..), vjust = -
0.5) +
  theme_bw() +
  scale_y_continuous(expand = expansion(mult = c(0, 0.1))) +
  labs(x = "Gene Detection Rate",
       y = "Segments, #",
       fill = "Segment Type")

resfactor=4
png("QC_genedetectionrate.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p
dev.off()

kable(table(pData(target_demoData)$DetectionThreshold,
pData(target_demoData)$class))

target_demoData <- target_demoData[,
pData(target_demoData)$GeneDetectionRate >= .01]
dim(target_demoData)

#Gene detection rate
LOQ_Mat <- LOQ_Mat[, colnames(target_demoData)]
fData(target_demoData)$DetectedSegments <- rowSums(LOQ_Mat,
na.rm = TRUE)
fData(target_demoData)$DetectionRate <-
  fData(target_demoData)$DetectedSegments /
nrow(pData(target_demoData))


goi <- c("PDCD1", "CD274", "IFNG", "CD8A", "CD68", "EPCAM",
         "KRT20", "MUC2", "MUC5A", "CALB2", "CLDN8")

goi_df <- data.frame( Gene = goi, Number =
fData(target_demoData)[goi, "DetectedSegments"], DetectionRate
= percent(fData(target_demoData)[goi, "DetectionRate"]))

kable(goi_df)

plot_detect <- data.frame(Freq = c(1, 5, 10, 20, 30, 50))
plot_detect$Number <-
  unlist(lapply(c(0.01, 0.05, 0.1, 0.2, 0.3, 0.5),
                function(x)
{sum(fData(target_demoData)$DetectionRate >= x)}))
plot_detect$Rate <- plot_detect$Number /
nrow(fData(target_demoData))
rownames(plot_detect) <- plot_detect$Freq


p2<-ggplot(plot_detect, aes(x = as.factor(Freq), y = Rate,
fill = Rate)) + geom_bar(stat = "identity") +
geom_text(aes(label = formatC(Number, format = "d", big.mark =
",")), vjust = 1.6, color = "black", size = 4) +
scale_fill_gradient2(low = "orange2", mid = "lightblue", high
= "dodgerblue3", midpoint = 0.65, limits = c(0,1), labels =
scales::percent) + theme_bw() + scale_y_continuous(labels =
scales::percent, limits = c(0,1), expand = expansion(mult =
c(0, 0))) + labs(x = "% of Segments", y = "Genes Detected, %
of Panel > LOQ")

resfactor=4
png("QC_genespersegment.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
p2
dev.off()

negativeProbefData <- subset(fData(target_demoData), CodeClass
== "Negative")
neg_probes <- unique(negativeProbefData$TargetName)
target_demoData <-
  target_demoData[fData(target_demoData)$DetectionRate >= 0.01
|
                    fData(target_demoData)$TargetName %in%
neg_probes, ]

goi <- goi[goi %in% rownames(target_demoData)]


#Normalisation

ann_of_interest <- "segment"

Stat_data <- data.frame(row.names =
colnames(exprs(target_demoData)), Segment =
colnames(exprs(target_demoData)), Annotation =
pData(target_demoData)[, ann_of_interest], Q3 =
unlist(apply(exprs(target_demoData), 2, quantile, 0.75, na.rm
= TRUE)), NegProbe = exprs(target_demoData)[neg_probes, ])

Stat_data_m <- melt(Stat_data, measure.vars = c("Q3",
"NegProbe"), variable.name = "Statistic", value.name =
"Value")

plt1 <- ggplot(Stat_data_m, aes(x = Value, fill = Statistic))
+ geom_histogram(bins = 40) + theme_bw() +
scale_x_continuous(trans = "log2") + facet_wrap(~Annotation,
nrow = 1) + scale_fill_brewer(palette = 3, type = "qual") +
labs(x = "Counts", y = "Segments, #")

plt2 <- ggplot(Stat_data, aes(x = NegProbe, y = Q3, color =
Annotation)) + geom_abline(intercept = 0, slope = 1, lty =
"dashed", color = "darkgray") + geom_point() + guides(color =
"none") + theme_bw() + scale_x_continuous(trans = "log2") +
scale_y_continuous(trans = "log2") + theme(aspect.ratio = 1) +
labs(x = "Negative Probe GeoMean, Counts", y = "Q3 Value,
Counts")

plt3 <- ggplot(Stat_data, aes(x = NegProbe, y = Q3 / NegProbe,
color = Annotation)) + geom_hline(yintercept = 1, lty =
"dashed", color = "darkgray") + geom_point() + theme_bw() +
scale_x_continuous(trans = "log2") + scale_y_continuous(trans
= "log2") + theme(aspect.ratio = 1) + labs(x = "Negative Probe
GeoMean, Counts", y = "Q3/NegProbe Value, Counts")

btm_row <- plot_grid(plt2, plt3, nrow = 1, labels = c("B",
""), rel_widths = c(0.43,0.57))

resfactor=4
png("QC_q3.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
plot_grid(plt1, btm_row, ncol = 1, labels = c("A", ""))
dev.off()


target_demoData <- normalize(target_demoData , norm_method =
"quant", desiredQuantile = .75, toElt = "q_norm")

target_demoData <- normalize(target_demoData , norm_method =
"neg", fromElt = "exprs", toElt = "neg_norm")

#Visualise normalisation
resfactor=4
png("QC_normalisation_raw.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
boxplot(exprs(target_demoData)[,1:10], col = "#9EDAE5", main =
"Raw Counts", log = "y", names = 1:10, xlab = "Segment", ylab
= "Counts, Raw")
dev.off()

resfactor=4
png("QC_normalisation_Q3.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
boxplot(assayDataElement(target_demoData[,1:10], elt =
"q_norm"), col = "#2CA02C", main = "Q3 Norm Counts", log =
"y", names = 1:10, xlab = "Segment", ylab = "Counts, Q3
Normalized")
dev.off()

resfactor=4
```

```
png("QC_normalisation_neg.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
boxplot(assayDataElement(target_demoData[,1:10], elt =
"neg_norm"), col = "#FF7F0E", main = "Neg Norm Counts", log =
"y", names = 1:10, xlab = "Segment", ylab = "Counts, Neg.
Normalized")
dev.off()
```

c) #UMAP analysis

```
#UMAP
custom_umap <- umap::umap.defaults

custom_umap$random_state <- 42
set.seed(42)

umap_out <- umap(t(log2(assayDataElement(target_demoData , elt
= "q_norm"))), config = custom_umap)

pData(target_demoData)[, c("UMAP1", "UMAP2")] <-
umap_out$layout[, c(1,2)]

umapplot<-ggplot(pData(target_demoData), aes(x = UMAP1, y =
UMAP2, color = segment, shape = class)) + geom_point(size = 3)
+ theme_bw()

resfactor=4
png("UMAP.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
umapplot
dev.off()

umapplot2<-ggplot(pData(target_demoData), aes(x = UMAP1, y =
UMAP2, color = class, shape = segment)) + geom_point(size = 3)
+ theme_bw()

resfactor=4
png("UMAP2.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
umapplot2
dev.off()

#Add Sample-ID = as rownames
#ggplot(pData(target_demoData), aes(x = UMAP1, y = UMAP2,
color = segment, shape = class)) + geom_point(size = 3) +
theme_bw() + geom_text(label=rownames(pData(target_demoData)))
#ggplot(pData(target_demoData), aes(x = UMAP1, y = UMAP2,
color = segment, shape = class)) + geom_point(size = 3) +
theme_bw() +
geom_text_repel(label=rownames(pData(target_demoData)),
size=1, max.overlaps=Inf)

#pData(target_demoData)[["slide"]] <-
factor(pData(target_demoData)[["slide name"]])

#TSNE
set.seed(42)

tsne_out <- Rtsne(t(log2(assayDataElement(target_demoData ,
elt = "q_norm"))), perplexity = ncol(target_demoData)*.15)

pData(target_demoData)[, c("tSNE1", "tSNE2")] <- tsne_out$Y[,
c(1,2)]

tsneplot<-ggplot(pData(target_demoData), aes(x = tSNE1, y =
tSNE2, color = class, shape = segment)) + geom_point(size = 3)
+ theme_bw()

resfactor=4
png("tsne.png", res=72*resfactor, height=640*resfactor,
width=640*resfactor)
tsneplot
dev.off()

#Clustering highly CV genes
assayDataElement(object = target_demoData, elt = "log_q") <-
assayDataApply(target_demoData, 2, FUN = log, base = 2, elt =
"q_norm")

calc_CV <- function(x) {sd(x) / mean(x)}
CV_dat <- assayDataApply(target_demoData, elt = "log_q",
MARGIN = 1, calc_CV)

sort(CV_dat, decreasing = TRUE)[1:5]

#Create heatmap of CV genes

# Identify genes in the top 3rd of the CV values
GOI <- names(CV_dat)[CV_dat > quantile(CV_dat, 0.8)]

pheatmap(assayDataElement(target_demoData[GOI, ], elt =
"log_q"), scale = "row", show_rownames = FALSE, show_colnames
= FALSE, border_color = NA, clustering_method = "average",
clustering_distance_rows = "correlation",
clustering_distance_cols = "correlation", breaks = seq(-3, 3,
0.05), color = colorRampPalette(c("purple3", "black",
"yellow2"))(120), annotation_col = pData(target_demoData)[,
c("class", "segment")])
```

d) #differential gene expression using Linear mixed method model

```
library(NanoStringNCTools)
library(GeomxTools)
library(GeoMxWorkflows)
library(knitr)
library(dplyr)
library(ggforce)
library(ggplot2)
library(scales)
library(reshape2) # for melt
library(cowplot) # for plot_grid
library(umap)
library(Rtsne)
library(pheatmap)
library(ggrepel)
library(EnvStats)
library(ggiraph)

#load data
target_demoData<-load(file =
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring objects/ACexplant.RData")

#check number of genes in object
dim(target_demoData)

#Differential Gene Expression using Linear Mixed Models
pData(target_demoData)[["case"]] <-
factor(pData(target_demoData)[["case no"]])

assayDataElement(object = target_demoData, elt = "log_q") <-
assayDataApply(target_demoData, 2, FUN = log, base = 2, elt =
"q_norm")

#CLASS
#AAvAMN
#pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("AA", "AMN"))
#CRCvAA
#pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("CRC", "AA"))
#AMNvCRC
#pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("AMN", "CRC"))

#AAvnormal
pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("AA", "Normal"))

#AMNvnormal
#pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("AMN", "Normal"))

#Explant
pData(target_demoData)$testClass <-
factor(pData(target_demoData)$class, c("control", "chemo"))

#SEGMENT
pData(target_demoData)$testSegment <-
factor(pData(target_demoData)$segment, c("ck20", "stroma"))

#REGION
#pData(target_demoData)$testRegion <-
factor(pData(target_demoData)$region, c("1", "2"))

#pData(target_demoData)$testRegion <-
factor(pData(target_demoData)$region, c("1", "3"))

#pData(target_demoData)$testRegion <-
factor(pData(target_demoData)$region, c("1", "4"))
```

```
# etc.

#STRUCTURE
#pData(target_demoData)$testStructure <-
factor(pData(target_demoData)$structure, c("1", "2", "3",
"4"))


#DEG TABLE
#LMM ith random slope
results <- c()
#no ck20 in normal no tumour areas
for(status in c("ck20", "stroma")) {
  ind <- pData(target_demoData)$segment == status
  mixedOutmc <-
    mixedModelDE(target_demoData[, ind],
                 elt = "log_q",
                 modelFormula = ~ testClass + (1 + testClass |
case),
                 groupVar = "testClass",
                 nCores = parallel::detectCores(),
                 multiCore = FALSE)

# format results as data.frame
  r_test <- do.call(rbind, mixedOutmc["lsmeans", ])
  tests <- rownames(r_test)
  r_test <- as.data.frame(r_test)
  r_test$Contrast <- tests
# use lapply in case you have multiple levels of your test
factor to
# correctly associate gene name with it's row in the results
table
  r_test$Gene <-
    unlist(lapply(colnames(mixedOutmc),
                  rep, nrow(mixedOutmc["lsmeans", ][[1]])))
  r_test$Subset <- status
  r_test$FDR <- p.adjust(r_test$`Pr(>|t|)`, method = "fdr")
  r_test <- r_test[, c("Gene", "Subset", "Contrast",
"Estimate",
                       "Pr(>|t|)", "FDR")]
  results <- rbind(results, r_test)
}

#LMM without random slope
results <- c()
#no ck20 in normal no tumour areas
for(segment in c("cd45", "stroma")) {
  ind <- pData(target_demoData)$segment == segment
  mixedOutmc <-
    mixedModelDE(target_demoData[, ind],
                 elt = "log_q",
                 modelFormula = ~ testClass + (1 | case),
                 groupVar = "testClass",
                 nCores = parallel::detectCores(),
                 multiCore = FALSE)

  r_test <- do.call(rbind, mixedOutmc["lsmeans", ])
  tests <- rownames(r_test)
  r_test <- as.data.frame(r_test)
  r_test$Contrast <- tests
  r_test$Gene <-
    unlist(lapply(colnames(mixedOutmc),
                  rep, nrow(mixedOutmc["lsmeans", ][[1]])))
  r_test$Subset <- segment
  r_test$FDR <- p.adjust(r_test$`Pr(>|t|)`, method = "fdr")
  r_test <- r_test[, c("Gene", "Subset", "Contrast",
"Estimate",
                       "Pr(>|t|)", "FDR")]
  results <- rbind(results, r_test)
}


#Create table of DGE
#kable(subset(results), digits = 3,       align = "lc",
row.names = FALSE)

#Save DGE table (a=with random slope, b=without random slope)
write.csv(results, "results_PDEctlvchemo.csv")

#Volcano Plot
results$Color <- "NS or FC < 0.5"
results$Color[results$`Pr(>|t|)` < 0.05] <- "P < 0.05"
results$Color[results$FDR < 0.05] <- "FDR < 0.05"
results$Color[results$FDR < 0.001] <- "FDR < 0.001"
results$Color[abs(results$Estimate) < 0.5] <- "NS or FC < 0.5"
results$Color <- factor(results$Color,
                        levels = c("NS or FC < 0.5", "P <
0.05",
                                   "FDR < 0.05", "FDR <
0.001"))
results$invert_P <- (-log10(results$`Pr(>|t|)`)) *
sign(results$Estimate)

top_g <- c()
#no ck20 in normal
for(cond in c("ck20", "stroma")) {
  ind <- results$Subset == cond
  top_g <- c(top_g,
             results[ind, 'Gene'][
               order(results[ind, 'invert_P'], decreasing =
TRUE)[1:15]],
             results[ind, 'Gene'][
               order(results[ind, 'invert_P'], decreasing =
FALSE)[1:15]])
}

top_g <- unique(top_g)
results <- results[, -1*ncol(results)]

#Show and/or print plot
resfactor=4
png("results_PDEctlvchemo.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
ggplot(results,
       aes(x = Estimate, y = -log10(`Pr(>|t|)`),
           color = Color, label = Gene)) +
  geom_vline(xintercept = c(0.5, -0.5), lty = "dashed") +
  geom_hline(yintercept = -log10(0.05), lty = "dashed") +
  geom_point() +
  labs(x = "Enriched in PDE chemo <- log2(FC) -> Enriched in
PDE control",
       y = "Significance, -log10(P)",
       color = "Significance") +
  scale_color_manual(values = c(`FDR < 0.001` = "dodgerblue",
                                `FDR < 0.05` = "lightblue",
                                `P < 0.05` = "orange2",
                                `NS or FC < 0.5` = "gray"),
                     guide = guide_legend(override.aes =
list(size = 4))) +
  scale_y_continuous(expand = expansion(mult = c(0,0.05))) +
  geom_text_repel(data = subset(results,
                                Gene %in% top_g & FDR < 0.05),
                  size = 4, point.padding = 0.15, color =
"black",
                  min.segment.length = .1, box.padding = .2,
lwd = 2,
                  max.overlaps = 50) +
  theme_bw(base_size = 16) +
  theme(legend.position = "bottom") +
  facet_wrap(~Subset, scales = "free_y")
dev.off()
```

e) #Heatmap

```
library(pheatmap)
library(dplyr)

#load object
datadir <-
load("/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring
objects/Nanostring20230310.RData")

#Heatmap after DGE/volcano plots
#GOI <- unique(subset(results, `FDR` <0.001)$Gene)
#GOI<-c()
ann_colors<- list(region = c("1" = "red", "2"="green",
"3"="orange", "4" = "blue", "5"="violet", "6"="white",
"7"="darkgreen"))
GOI<-c("IRF4", "HSP90AA1", "IL2RG", "JUNB", "RORC", "ICAM1",
"STAT1", "MUC1", "ANXA1", "FSCN1", "IL6", "IL1B", "FOS",
"TGFB1", "CXCL8", "IL6R", "VIM", "TWIST1", "OSM", "PIM1",
"MMP2", "VCAM1", "IL18", "ALOX5", "ITGB2", "LIF", "IL1A",
"COL1A2", "VEGFA", "TIMP1", "CCL2", "FN1")
pheatmap(log2(assayDataElement(target_demoData[GOI, ], elt =
"q_norm")),
         scale = "row",
         show_rownames = TRUE, show_colnames = FALSE,
         border_color = NA,
         clustering_method = "average",
```

```
         clustering_distance_rows = "correlation",
         clustering_distance_cols = "correlation",
         cutree_cols = 3,
         breaks = seq(-3, 3, 0.05),
         color = colorRampPalette(c("purple3", "black",
"yellow2"))(120),
         annotation_col = pData(target_demoData)[, c("region",
"segment")],
         annotation_colors = ann_colors)


#annotation_col = pData(target_demoData)[, c("region",
"segment")])

head(pData(target_demoData))
heatmap<-pData(target_demoData)

pData(target_demoData)$testRegion <-
factor(pData(target_demoData)$region, c("1", "2"))
```

8.4.3 Gene set enrichment analysis

```
#author: Madeleine Strach
# date: 2022-10-24
# last edit: 2024-02-16

#adapted from 📖 Introduction | Biomedical Knowledge Mining using GOSemSim and
clusterProfiler (yulab-smu.top)

if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

BiocManager::install("viridis")
a
Yes
force=TRUE

install.packages("devtools")
devtools::install_github("junjunlab/GseaVis")

install.packages("stringi")

browseVignettes("clusterProfiler")

#GSEA

library(clusterProfiler)
library (DOSE)
library(knitr)
library(AnnotationHub)
library(GOSemSim)
library(enrichplot)
library(org.Hs.eg.db)
library (GOplot)
library(dplyr)
library(ggstance)
library(ggupset)
library(msigdbr)
library(BiocParallel)
register(SerialParam())
library("pathview")
library("GseaVis")
library("stringi")
library("viridis")

#Read in datafile by choosing file
df <- read.csv(file.choose())
head(df)
```

a) #Set file

```
#Ensure all spaces are deleted in gene name column
#Sort by L2FC
original_gene_list <- df$L2FC
names(original_gene_list) <- df$Gene
gene_list<-na.omit(original_gene_list)
gene_list = sort(gene_list, decreasing = TRUE)

#single cell  is lower case gene
#names(original_gene_list) <- df$gene

#If need entrezid
gene_list<-bitr(gene_list , fromType="SYMBOL",
toType="ENTREZID", OrgDb="org.Hs.eg.db")

head(keys(org.Hs.eg.db))
#[1] "1"  "2"  "3"  "9"  "10" "11"

AnnotationDbi::select(org.Hs.eg.db,
                      keys = "1",
                      columns = "SYMBOL")
#  ENTREZID

#1        1   A1BG
```

b) #GSEA

```
#Gene ontology ALL
GO <- gseGO(geneList=gene_list,
            ont ="ALL",
            keyType = "SYMBOL",
            minGSSize = 3,
            maxGSSize = 800,
            pvalueCutoff = 0.05,
            verbose = TRUE,
            OrgDb = org.Hs.eg.db,
            pAdjustMethod = "BH")

#BP
GO_BP <- gseGO(geneList=gene_list,
            ont ="BP",
            keyType = "SYMBOL",
            minGSSize = 3,
            maxGSSize = 800,
            pvalueCutoff = 0.05,
            verbose = TRUE,
            OrgDb = org.Hs.eg.db,
            pAdjustMethod = "BH")

#CC
GO_CC <- gseGO(geneList=gene_list,
               ont ="CC",
               keyType = "SYMBOL",
               minGSSize = 3,
               maxGSSize = 800,
               pvalueCutoff = 0.05,
               verbose = TRUE,
               OrgDb = org.Hs.eg.db,
               pAdjustMethod = "BH")

#MF
GO_MF <- gseGO(geneList=gene_list,
               ont ="MF",
               keyType = "SYMBOL",
               minGSSize = 3,
               maxGSSize = 800,
               pvalueCutoff = 0.05,
               verbose = TRUE,
               OrgDb = org.Hs.eg.db,
               pAdjustMethod = "BH")

#MsigDB
m_df<-msigdbr(species ="Homo sapiens")
head(m_df, 2)%>%as.data.frame

#Hallmark
H_t2g<-msigdbr(species ="Homo sapiens", category ="H")%>%
  dplyr::select(gs_name, gene_symbol)

#C2Kegg
C2kegg_t2g<-msigdbr(species ="Homo sapiens", category ="C2",
subcategory = "CP:KEGG")%>%
  dplyr::select(gs_name, gene_symbol)

#C2Reactome
C2reactome_t2g<-msigdbr(species ="Homo sapiens", category
="C2", subcategory = "CP:REACTOME")%>%
  dplyr::select(gs_name, gene_symbol)

#C8 Cell type signature gene sets
C8_t2g<-msigdbr(species ="Homo sapiens", category ="C8")%>%
  dplyr::select(gs_name, gene_symbol)

#GSEA msigDB
H <- GSEA(gene_list, TERM2GENE = H_t2g, verbose=FALSE)
K <- GSEA(gene_list, TERM2GENE = C2kegg_t2g, verbose=FALSE)
R <- GSEA(gene_list, TERM2GENE = C2reactome_t2g,
verbose=FALSE)


#C <- GSEA(gene_list, TERM2GENE = C8_t2g, verbose=FALSE)
```

c) #tables

```
write.csv(GO, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial
Analyses/Analysis_20240512_redoexplantGSEA/GSEA/Tables/Ctl_che
mo_ck20_GO.csv")
write.csv(H, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial
Analyses/Analysis_20240512_redoexplantGSEA/GSEA/Tables/Ctl_che
mo_ck20_H.csv")
write.csv(K, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial
Analyses/Analysis_20240512_redoexplantGSEA/GSEA/Tables/Ctl_che
mo_ck20_K.csv")
write.csv(R, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial
Analyses/Analysis_20240512_redoexplantGSEA/GSEA/Tables/Ctl_che
mo_ck20_R.csv")


#write.csv(GO,
"/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial Analyses/Analysis
20230412_GSEA_structure/Structure/Lead
Edge/2v3/67a_CK20_2v3_GO.csv")
#write.csv(H, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial Analyses/Analysis
20230412_GSEA_structure/Structure/Lead
Edge/2v3/67a_CK20_2v3_H.csv")
#write.csv(K, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial Analyses/Analysis
20230412_GSEA_structure/Structure/Lead
Edge/2v3/67a_CK20_2v3_K.csv")
#write.csv(R, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial Analyses/Analysis
20230412_GSEA_structure/Structure/Lead
Edge/2v3/67a_CK20_2v3_R.csv")

#write.csv(C, "/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring Spatial Analyses/Analysis
20230318_GSEA/Region Analysis/GSEA/1v3/48a_CK20_1v3_H.csv")

kable(C)
```

d) #Dotplots

```
#resfactor=4
#png("resultsAAcomb_C28_C.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
#dotplot(C, showCategory=10, label_format = 50, split=".sign")
+ facet_grid(.~.sign) + ggtitle("Dotplot for AA C28 cell type
signature")
#dev.off()

resfactor=4
png("Ctl_chemo_ck20_GO.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(GO, showCategory=10, label_format = 50, split=".sign")
+ facet_grid(.~.sign) + aes(shape = I(16)) + aes(color =
p.adjust) + set_enrichplot_color(type = "color", name =
"p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_GOBP.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(GO_BP, showCategory=10, label_format = 50,
split=".sign") + facet_grid(.~.sign) + aes(shape = I(16)) +
aes(color = p.adjust) + set_enrichplot_color(type = "color",
name = "p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_GOCC.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(GO_CC, showCategory=10, label_format = 50,
split=".sign") + facet_grid(.~.sign) + aes(shape = I(16)) +
aes(color = p.adjust) + set_enrichplot_color(type = "color",
name = "p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_GOMF.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(GO_MF, showCategory=10, label_format = 50,
split=".sign") + facet_grid(.~.sign) + aes(shape = I(16)) +
aes(color = p.adjust) + set_enrichplot_color(type = "color",
name = "p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_H.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(H, showCategory=10, label_format = 50, split=".sign")
+ facet_grid(.~.sign) + aes(shape = I(16)) + aes(color =
p.adjust) + set_enrichplot_color(type = "color", name =
"p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_K.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(K, showCategory=10, label_format = 50, split=".sign")
+ facet_grid(.~.sign) + aes(shape = I(16)) + aes(color =
p.adjust) + set_enrichplot_color(type = "color", name =
"p.adjust")
dev.off()

resfactor=4
png("Ctl_chemo_ck20_R.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
options(enrichplot.colours = c("red","blue"))
dotplot(R, showCategory=10, label_format = 50, split=".sign")
+ facet_grid(.~.sign) + aes(shape = I(16)) + aes(color =
p.adjust) + set_enrichplot_color(type = "color", name =
"p.adjust")
dev.off()

labels<- as_labeller(c(`activated` = "2", `suppressed` = "3"))
```

e) #Other plots

```
#GSEAplots
resfactor=4
png("resultsAMNcomb_C22_R_IFNGSIGNALLING.png",
res=72*resfactor, height=640*resfactor, width=640*resfactor)
gseaNb(object = R,
       geneSetID = 'REACTOME_INTERFERON_GAMMA_SIGNALING')
dev.off()
R


#Barplot
data(geneList)
de <- names(geneList)[abs(geneList) > 2]
edo <- enrichDGN(de)

resfactor=4
png("resultsAMNcomb_C22_G0BP_barplot.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
barplot(edo, showCategory=20)
dev.off()

#GGplot
png("resultsAMNcomb_C22_G0BP_barplot_ggplot.png",
res=72*resfactor, height=640*resfactor, width=640*resfactor)
ggplot(GO, showCategory=10,
       aes(NES, fct_reorder(Description, NES), fill=qvalue)) +
  geom_col() +
  scale_fill_gradientn(colours=c("#b3eebe", "#46bac2",
"#371ea3"),
                       guide=guide_colorbar(reverse=TRUE)) +
```

```
  theme_dose(12) +
  xlab("Normalized Enrichment Score") +
  ylab(NULL) +
  ggtitle("C22 Gene ontology")
dev.off()

#Heatplot
resfactor=4
png("resultsAMNcomb_C0_R_heatplot.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
heatplot(R, foldChange=geneList, showCategory=10)
dev.off()

#Heatmap
data(geneList)
data(ID)
gene <- names(geneList)[abs(geneList) > 1]
geneID <- ID[abs(geneList) > 1]
gene.df <- data.frame(gene, geneID)
gene.df$gene <- factor(gene.df$gene, levels = gene)
gene.df$geneID <- factor(gene.df$geneID, levels = geneID)

png("resultsAMNcomb_C22_R_heatplot.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
pheatmap(gene.df, cluster_rows = TRUE, cluster_cols = TRUE,
show_rownames = FALSE, show_colnames = FALSE, color =
colorRampPalette(c("white", "blue"))(100))
dev.off()


#Treeplots
GO2<-pairwise_termsim(H)
emapplotGO2, showCategory=5)
treeplot(GO2, hclust_method ="average", fontsize=2)

H2<-pairwise_termsim(H)
emapplot(H2, showCategory=5)
treeplot(H2, hclust_method ="average", fontsize=2)

K2<-pairwise_termsim(H)
emapplot(K2, showCategory=5)
treeplot(K2, hclust_method ="average", fontsize=2)

R2<-pairwise_termsim(H)
emapplot(R2, showCategory=5)
treeplot(R2, hclust_method ="average", fontsize=2)


resfactor=4
png("results68_2_AAvAMN_CK20_GO_tp.png", res=72*resfactor,
height=640*resfactor, width=640*resfactor)
treeplot(GO2, hclust_method ="average", fontsize=2)
#treeplot(H2, hclust_method ="average", fontsize=2)
#treeplot(K2, hclust_method ="average", fontsize=2)
#treeplot(R2, hclust_method ="average", fontsize=2)
dev.off()

#network plots
#cnetplot(GO, categorySize="pvalue", foldChange=gene_list)
cnetplot.enrich(H,
                colorparams=list(foldChange="pvalue"),
                foldChange=gene_list,
                cex_params=list(gene_label=0.4,
category_label=1)
#cnetplot(K, categorySize="pvalue", foldChange=gene_list)
#cnetplot(R, categorySize="pvalue", foldChange=gene_list)

#cnetplot(GO, foldChange=geneList, circular = TRUE, colorEdge
= TRUE)

cnetplot(H, categorySize="pvalue", foldChange=gene_list)
cnetplot(K, categorySize="pvalue", foldChange=gene_list)
cnetplot(R, categorySize="pvalue", foldChange=gene_list)
cnetplot(C, categorySize="pvalue", foldChange=gene_list)

#upset plot
upsetplot(H)

#Heatplot
heatplot(C, foldChange=gene_list, showCategory=6)
```

8.4.3.1 Spatial deconvolution

```
#author: Madeleine Strach
# date: 2024-03-28
# last edit: 2024-03-28

#adapted from Bioconductor - SpatialDecon
#Spatial decon

BiocManager::install("SpatialDecon")

library(SpatialDecon)
library(GeomxTools)


load("/Users/madeleine/Library/CloudStorage/OneDrive-
TheUniversityofSydney(Students)/PhD Peritonectomy/Projects/R
Working Directory/Nanostring
objects/Nanostring20230310.RData")

dim(target_demoData)

head(pData(target_demoData))

target_demoData@assayData$exprs[seq_len(5), seq_len(5)]

sampleNames(target_demoData) <-
  paste0(target_demoData$ROI, target_demoData$AOI.name)

featureType(target_demoData) <- "Target"

bg = derive_GeoMx_background(norm =
target_demoData@assayData$exprs_norm,
                             probepool =
fData(target_demoData)$Module,
                             negnames = c("NegProbe-CTP01",
"NegProbe-Kilo"))

data("safeTME")
data("safeTME.matches")

signif(safeTME[seq_len(3), seq_len(3)], 2)

resfactor=4
png("safeTME_heatmap.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-heatmap(sweep(safeTME, 1, apply(safeTME, 1, max), "/"),
           labRow = NA, margins = c(10, 5))
print(p)
dev.off()


res = runspatialdecon(object = target_demoData,
                      norm_elt = "q_norm",
                      raw_elt = "exprs",
                      X = safeTME,
                      align_genes = TRUE)

str(pData(res))

#Error: no slot of name "assaydata" for this object of class
"NanoStringGeoMxSet"
names(res@assaydata)


resfactor=4
png("estimated cell abundances.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-heatmap(t(res$beta), cexCol = 0.5, cexRow = 0.7, margins =
c(10,7))
print(p)
dev.off()


target_demoData$istumor = target_demoData$segment.name ==
"ck20"

restils = runspatialdecon(object = target_demoData,
                          norm_elt = "q_norm",          #
normalized data
                          raw_elt = "exprs",             #
expected background counts for every data point in norm
                          X = safeTME,
# safeTME matrix, used by default
                          cellmerges = safeTME.matches,
# safeTME.matches object, used by default
                          cell_counts =target_demoData$nuclei,
# nuclei counts, used to estimate total cells
```

```
                          is_pure_tumor =
target_demoData$istumor,  # identities of the Tumor
segments/observations
                          n_tumor_clusters = 1)           #
how many distinct tumor profiles to append to safeTME



resfactor=4
png("complete decon.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-
heatmap(sweep(restils@experimentData@other$SpatialDeconMatrix,
1, apply(restils@experimentData@other$SpatialDeconMatrix, 1,
max), "/"),
           labRow = NA, margins = c(10, 5))
print(p)
dev.off()

str(pData(restils))




rdecon = runReverseDecon(object = target_demoData,
                          norm_elt = "q_norm",
                          beta = res$beta)

resfactor=4
png("reversedecon.png", res=72*resfactor,
height=640*resfactor, width=800*resfactor)
p<-heatmap(pmax(pmin(rdecon@assayData$resids, 2), -2))
print(p)
dev.off()



#TIL barplot
data("cellcols")
cellcols

o = hclust(dist(t(restils$cell.counts$cell.counts)))$order
layout(mat = (matrix(c(1, 2), 1)), widths = c(7, 3))
TIL_barplot(t(restils$cell.counts$cell.counts[, o]),
draw_legend = TRUE,
            cex.names = 0.5)

resfactor=4
png("TIL_plot.png", res=72*resfactor, height=640*resfactor,
width=800*resfactor)
p<-TIL_barplot(t(restils$cell.counts$cell.counts[, o]),
draw_legend = TRUE,
               cex.names = 0.5)
print(p)
dev.off()


temp = replace(restils$prop_of_nontumor,
is.na(restils$prop_of_nontumor), 0)
o = hclust(dist(temp[restils$segment.name == "cd45",]))$order

TIL_barplot(t(restils$prop_of_nontumor[restils$AOI.name ==
"cd45",])[, o],
            draw_legend = TRUE, cex.names = 0.
```