Colorectal peritoneal metastases: Current status in treatment, evaluation of the immune landscape and development of a novel platform for personalised medicine

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Abstract

Peritoneal metastases from colorectal cancer confer the worst survival in patients with metastatic colorectal cancer. Historical survival from peritoneal metastases was dismal, with the condition generally viewed with nihilism.

The adoption of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) can offer selected patients with colorectal peritoneal metastases (CRPM) a favorable survival. However, its uptake is variable due to skepticism about its efficacy. Additionally, most patients have inoperable disease, and are treated with systemic chemotherapy, fraught with high rates of treatment failures. With limited advances in management of peritoneal disease, there is an urgent need to explore newer avenues of treatment.

In this thesis, I firstly confirm that there remains skepticism among surgeons regarding the utilisation of CRS and HIPEC for CRPM. Most recognise the role of cytoreductive surgery, however there remains deficiencies in awareness and knowledge regarding its indications and efficacy.

To explore the efficacy of surgery, I evaluated outcomes from CRS and HIPEC for all peritoneal surface malignancies at a statewide referral centre. CRS and HIPEC was safe, and offered favorable survival. With CRPM specifically, median survival was 32 months, with a relapse free survival of 13 months following CRS and HIPEC. Incomplete cytoreduction and mucinous histology were key factors influencing survival. The choice of mitomycin C or oxaliplatin as HIPEC agent did not influence survival. In a meta-analysis, apart from well known factors such as PCI, completeness of cytoreduction and lymph node involvement, the use of adjuvant chemotherapy, a rectal primary and grade III/IV morbidity were significant prognostic factors influencing survival in patients undergoing CRS and HIPEC.

The immune landscape of CRPM, a previously unchartered area, was then explored as a means to exploring newer treatment avenues. CRPM do have an immune infiltrate, albeit largely stromal, with a prominence of T cells, with over a fifth expressing PD-1. In an *in-vitro* tumouroid-TIL co-culture platform, we demonstrated that T cells in CRPM are functional, and the use of checkpoint antibodies can significantly improve T cell cytotoxicity in selected patients, offering this platform to personalise use of checkpoint antibodies in patients with CRPM. Additionally, gene expression analysis revealed most CRPM to be part of the worst prognostic CMS 4 subtype, with up regulation of immunosuppressive pathways.

Another avenue garnering interest is personalised medicine, wherein drugs can be assigned based on unique molecular features of each cancer. Lastly, I led a multicentre, prospective study wherein a novel *in-vitro* patient derived tumouroid platform was established, that can integrate functional drug testing with genomic profiling to identify suitable therapeutic options in a clinically timely manner. Additionally, our team showed that results from this *in-vitro* platform successfully mirror patient drug responses *in-vivo*, and can help identify novel therapeutic options in patients with treatment refractory disease with no genomic-guided biomarkers.

While current treatment of CRPM does offer highly selected patients a favourable survival, much remains to be improved for the remainder. Exploring a tumouroid based precision model of care has true potential in offering novel therapeutic options to an otherwise poor prognostic cohort.

This declaration is to certify that:

- This thesis comprises my original work towards the degree of Doctor of Philosophy (PhD) except where indicated in the preface

- Due acknowledgement has been made in the text to all other material used

- The thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies and appendices

Vignesh Narasimhan

Preface

This thesis includes nine accepted/published first author original papers, and six contributing author papers, with an additional one first author paper currently under peer review. I wish to acknowledge all my co-authors for their contribution. In addition, I wish to very gratefully acknowledge the following people who have made direct contributions to this thesis:

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Chapter 8

1) Kong J, Guerra G, Millen R, Roth S, Xu H, Neeson P, Darcy P, Kershaw M, Sampurno S, Malaterre J, Liu D, Pham T, **Narasimhan V**, Wang M, Huang YK, Visvanathan K, McCormick J, Lynch A, Warrier S, Michael M, Desai J, Murray W, Mitchell C, Ngan S, Phillips W, Heriot A, Ramsay R. Tumor Infiltrating Lymphocyte Function Predicts Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer. *JCO Precision Oncology* 2018. DOI: 10.1200/PO.18.00075

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3) Ceelan W, Ramsay R, **Narasimhan V**, Heriot A, De Wever O. Targeting the tumor microenvironment in colorectal peritoneal metastases. *Trends Cancer* 2020, DOI: 10.1016/j.trecan.2019.12.008

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List of Abbreviations

5-FU	5-Fluorouracil
7-AAD	7-Aminoactinomycin D
ACK buffer	Ammonium Chloride Potassium buffer
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of Variance
APC	Antigen presenting cell
ASA	American Society of Anesthesiology
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
CAF	Cancer associated fibroblast
CAPOX	Capecitabine/Oxaliplatin
CAR-T cell	Chimeric antigen receptor T cell
CBA	Cytokine bead analysis
CC	Completeness of cytoreduction
CDX2	Colorectal specific nuclear marker caudal type homeobox 2
CEA	Carcinoembryonic antigen
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK20	Cytokeratin 20
CLIA	Clinical Laboratory Improvement Amendments
CM	Conditioned media
CMS	Consensus molecular subtype
CNV	Copy number variation
CRC	Colorectal cancer
CRC media	Colorectal media
CRLM	Colorectal liver metastases
CRPM	Colorectal peritoneal metastases
CRS	Cytoreductive surgery

CSSANZ	Colorectal Society of Australia and New Zealand
CT	Computed tomography
CTC	Circulating tumour cell
ctDNA	Circulating tumour DNA
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated protein-4
DC	Dendritic cell
DEGs	Differentially expressed genes
DFS	Disease free survival
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	Dehydropyrimidine dehydrogenase
DVT	Deep vein thrombosis
DW-MRI	Diffusion-weighted magnetic resonance imaging
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transformation
EPIC	Early post-operative intraperitoneal chemotherapy
ERAS	Enhanced recovery after surgery
ETOH	Ethanol
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FDG	Fluorodeoxyglucose
FFPE	Fixed in formalin, paraffin embedded
FOLFIRI	5-FU/Leucovorin/Irinotecan
FOLFOX	5-FU/Leucovorin/Oxaliplatin
FoxP3	Forkhead/winged-helix transcription factor box P3
FVS	Fixable viability control
GM-CSF	Granulocyte monocyte colony stimulating factor
GSEA	Gene set enrichment analysis

H&E	Haematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HIPEC	Hyperthermic intraperitoneal chemotherapy
HMGB1	High mobility group box 1 protein
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
HREC	Human Research Ethics Committee
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
IM	Invasive margin
IP	Intraperitoneal
IPC	Intraperitoneal chemotherapy
ISO	Isotype control
LAG-3	Lymphocyte activation gene-3
LGR5	Leucine-rich repeating G-protein-coupled receptor-5
LN	Liquid Nitrogen
LOS	Length of stay
LT	Lymphotoxin
MDSC	Myeloid derived suppressor cell
MDT	Multidisciplinary meeting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mIHC	Multiplex immunohistochemistry
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinase
MMT	Mesenchymal to mesenchymal transition
MSI-H	Microsatellite unstable
MSS	Microsatellite stable
MΦ	Macrophage

NAC	Neo-adjuvant chemotherapy
NBF	Neutral buffered formalin
NGS	Next Generation Sequencing
NGT	Nasogastric tube
NK	Natural killer cell
NKT	Natural killer T cell
NP	Normal peritoneum
NSG	Nod scid gamma
OBM	Organoid basal media
Opal	Multiplex immunohistochemistry
OR	Odds ratio
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCI	Peritoneal carcinoma index
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PDGFα/β	Platelet derived growth factor alpha/beta
PDT	Patient derived tumouroid
PDX	Patient derived xenograft
PECAM	Platelet endothelial cell adhesion molecule
PET	Positron emission tomography
PFS	Progression free survival
PI	Propidium Iodide
PIPAC	Pressurised aerosolised intraperitoneal chemotherapy
PM	Peritoneal metastases
PMCC	Peter MacCallum Cancer Centre
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
PMP	Pseudomyxoma peritonei
PSM	Peritoneal surface malignancy
PSOGI	Peritoneal Surface Oncology Group International

RANTES	Regulated on activation, normal T-cell expressed and secreted
RCT	Randomised controlled trial
RNASeq	RNA sequencing
RT	Room temperature
SAHMRI	South Australia Health and Medical Research Institute
SEM	Standard error of the mean
SM	Staining media
SNP	Single nucleotide polymorphism
SRC	Signet ring cell
STAIN	Stained control
STR	Short tandem repeat
TAM	Tumour-associated macrophage
TBS	Tri-Phosphate Buffer
TC	Tumour centre
TCGA	The Cancer Genome Atlas
TCR	T cell repertoire
TF	Transcription factor
TGF-β	Transforming growth factor beta
T_h	T helper cell
TIL	Tumour infiltrating lymphocyte
TMB	Tumour mutational burden
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
TNM	Tumour/Node/Metastasis
TPN	Total parenteral nutrition
Treg	T regulatory cell
US	Unstained control
VAF	Variant allele frequency
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFRi	Vascular endothelial growth factor receptor inhibitor
WES	Whole exome sequencing

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1 Literature Review

Material from this chapter has been published/submitted as:

1. **Narasimhan V**, Flood, M, Ramsay R, Warrier S, Heriot A. Hyperthermic intraperitoneal chemotherapy (HIPEC) for colorectal peritoneal metastases: Still a necessity? *ANZ J Surg* (Under Review)

2. **Narasimhan V**, Ooi G, Michael M, Ramsay R, Lynch C, Heriot A. Colorectal peritoneal metastases-Pathogenesis, diagnosis and treatment options: an evidence based update. *ANZ J Surg* (Under Review)

3. **Narasimhan V**, Pham T, Ramsay R, Heriot A, Warrier S. Colorectal peritoneal metastases: still a nihilistic outlook*? ANZ J Surg.* 2019; 89(9): 996-997

4. **Narasimhan V**, Das A, Pham T, Wilson K, Kong J, Ramsay R, Heriot A. Organoids: the new kid in cancer research. *ANZ J Surg.* 2019; 89(10):1189-1190

5. Wilson K, **Narasimhan V**, Pham T, Das A, Ramsay R, Heriot A. Precision medicine in colorectal surgery: coming to a hospital near you. *ANZ J Surg.* 2019; 89(9): 995-996

6. Pham T, **Narasimhan V**, Guerra G, Kong J, Desai J, Ramsay R, Heriot A. Wither Surgical Oncology? *ANZ J Surg.* 2019; 89 (1-2): 10-11

7. Pham T, Roth S, Kong J, Guerra G, **Narasimhan V**, Pereira L, Desai J, Heriot A, Ramsay R. An update on immunotherapy for solid tumors: A Review. *Ann Surg Oncol*. 2018; 25(11):3404-3412

1.1 Colorectal cancer

1.1.1 Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and second leading cause of cancer related mortality in Australia and worldwide.[1, 2] There is significant geographical variation in incidence worldwide, with Australia and New Zealand having among the highest estimated rates of CRC (age standardised rate of 44.8 and 32.2 per 100,000 men and women respectively), with similar geographical patterns in both sexes.[2, 3] Based on current rates, approximately six percent of people will develop CRC during their lifetime. Among those diagnosed with CRC, approximately 56% will die of their cancer.[4] The overall five-year survival for patients with CRC (all stages) is 69.9%, which lags well behind other common cancers such as breast, melanoma and prostate with survival rates of 90-98%.[1, 5]

Similar to many cancers, the prognosis of CRC is largely based on stage at time of diagnosis. Prognosis is highly favourable when diagnosed at an early stage, with a five-year survival of 82-91% for localised disease, representing approximately 40% of cases. Five-year survival drops to 70% for loco-regional disease,[6] representing 35-40% of cases. In stark contrast, the five-year survival is only 12% for Stage IV cancer,[7, 8] which represent approximately 20-25% of patients at time of diagnosis.[9] The majority of deaths from CRC occur as a result of metastases that are resistant to standard therapies, even if these therapies may have been successful in the treatment of the primary cancer.[10]

1.1.2 Current treatment of CRC

Colon cancer accounts for approximately two thirds of all CRC, with rectal cancer accounting for the remainder.[1]

1.1.2.1 Stage I-III disease

Complete macroscopic surgical resection is the gold standard for early stage colon cancer (Stage I and most Stage II). For high risk Stage II and Stage III colon cancer, the standard of care remains surgery followed by adjuvant systemic chemotherapy for three to six months.[11, 12] The role of neoadjuvant therapy is still undefined in resectable Stage II/III colon cancer. The recently completed FOxTROT randomised controlled trial[13] (RCT) evaluated the role of neoadjuvant chemotherapy in the treatment of locally advanced resectable colon cancer. This trial reported a two-year disease recurrence rate of 13.6% in the neoadjuvant therapy arm compared to 17.2% in the standard of care arm, translating to a hazard ratio of 0.75 (95% CI: 0.55-1.04, p=0.08). While not statistically significant, this trial has raised interest among the oncologic community for neoadjuvant therapy to be incorporated into the management of locally advanced colon cancer.

Similarly, surgical resection is the gold standard for early stage disease in rectal cancer. In the case of locally advanced rectal cancer, management involves a combination of neoadjuvant chemo-radiation followed by surgery.[14] In recent years, there has been an increased interest in exploring a longer neoadjuvant treatment approach incorporating neoadjuvant chemo-radiation, with induction/consolidation systemic chemotherapy (total neo-adjuvant therapy). This approach has been shown to improve complete pathological response rates from 20% with chemo-radiation alone to up to 38% with total neo-adjuvant therapy, with an associated improved disease free survival.[15, 16] However, this has yet to be confirmed in Phase III trials.

1.1.2.2 Stage IV disease

Stage IV disease is defined by the presence of distant metastases. The most common sites for CRC metastases are the liver, lung and the peritoneum.[17, 18] At presentation, one third of patients will have regional nodal metastases, and 10-15% will have distant metastases, with most of them involving the liver.[19] Anatomically, all colonic blood

drains via the portal circulation through the liver, making the liver the first and most common site for metastatic disease. Metastatic disease is managed on a case-by-case basis, with unique considerations and options available for treatment of metastases to specific organs.

Advances in loco-regional, systemic therapies and surgical techniques have led to dramatic improvement in survival in patients with colorectal liver metastases (CRLM). The advent of various treatments such as ablative therapies, intra-arterial treatment, staged hepatectomy, modern day systemic therapies (oxaliplatin, irinotecan and anti-VEGR and anti-EGFR monoclonal antibodies), neoadjuvant chemotherapy or conversion chemotherapy to downsize and render lesions resectable now offer clinicians more options in treating patients with CRLM. With current treatment options, selected patients with CRLM can now achieve a five-year relapse free survival of 30%, with a five-year overall survival of up to 58%.[20, 21]

Similar to liver metastases, there have been notable advances in the treatment of colorectal lung metastases. In selected patients, resection of isolated pulmonary metastases can increase survival rates up to 40-54% at five years.[22, 23]

Peritoneal metastases however, confer the worst survival amongst all sites in patients with metastatic CRC.[24] They are less responsive to standard systemic chemotherapy than liver and lung metastases.[25] The advent and adoption of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) can offer selected patients with isolated colorectal peritoneal metastases (CRPM) a favourable 30-58 months median survival, with a 27-46% five-year survival.[26, 27] However, the majority of patients with CRPM are not surgical candidates for CRS and HIPEC owing to inoperable disease. Systemic chemotherapy thus becomes the mainstay of treatment for these patients. With limited chemotherapy options, treatment failures and disease progression are very common. This is clearly one area in CRC where further investments in research are required to develop newer strategies to improve outcomes.

1.1.3 Present and evolving classifications of colorectal cancer

1.1.3.1 TNM classification

The American Joint Committee on Cancer (AJCC) established a standardised method for communicating cancer diagnoses called the TNM staging system, incorporating characteristics of the tumour (T), lymph node involvement (N) and distant metastases (M). With regard to the presence of metastases (M), the AJCC 7th edition in 2009 subdivided M into M1a and M1b.[28] M1a referred to metastases confined to one organ or site (not peritoneum). M1b referred to metastases in more than one organ/site or involvement of the peritoneum; recognising peritoneal metastases as a poor prognostic factor. The updated AJCC 8th edition in 2018 went one step further.[29, 30] Here, M stage has been redefined as M1a, M1b and M1c. Metastasis to a single site (nonregional lymph node or organ excluding peritoneum) is considered M1a, whereas involvement of multiple organs is considered M1b. Peritoneal metastases, irrespective of the involvement of other sites is classified as M1c, clearly reflecting the significantly worse outcome with peritoneal metastases compared to other sites (Table 1.1).[24] Consequently, Stage IV-C is reserved for the presence of peritoneal metastases, clearly stratifying patients with peritoneal metastases as having the worst prognosis (Table 1.2).

Table 1.1 Updates in the TNM classification, 8th edition (Weiser et al)[29]

Category	Update/Clarification
T category	(1) Intramucosal adenocarcinoma but not high-grade dysplasia is categorized as Tis; definition of intramucosal adenocarcinoma has been clarified
	(2) T1: prognostic parameters in T1 cancers arising in polyps are discussed
	(3) T4a: definition has been clarified
N category	(1) Definition of TD has been clarified
	(2) ITCs will continue to be designated as N0. Micrometastasis will be categorized as node positive; specific designation of micrometastasis not necessary
M category	(1) Definitions of M1a, M1b have been updated
	(2) Separate category of M1c has been created for peritoneal metastasis

Table 1.2. Updates in the TNM classification, 8th edition. Presence of peritoneal metastases is now designated Stage IV-C (Weiser et al)[29]

Stage 0:	Tis	N0	M0
Stage I:	T1 - T2	N0	M0
Stage IIA:	T3	N0	M0
Stage IIB:	T4a	N0	M0
Stage IIC:	T4b	N0	M0
Stage IIIA:	T1 - T2	N1 / N1c	M0
	T1	N2a	M0
Stage IIIB:	T3 - T4a	N1 / N1c	M0
	T2 - T3	N2a	M0
	T1 - T2	N2b	M0
Stage IIIC:	T4a	N2a	M0
	T3 - T4a	N2b	M0
	T4b	N1 - N2	M0
Stage IVA:	any T	any N	M1a
Stage IVB:	any T	any N	M1b
Stage IVC:	any T	any N	M1c

1.1.3.2 Consensus molecular subtype (CMS)

In recent years, CRCs have begun to be seen as a group of molecularly heterogenous diseases characterised by a variety of genomic and epigenetic alterations. This has improved our understanding of the transcriptional landscape of CRCs, allowing CRC to be classified into biologically and clinically relevant subtypes that would permit better use of personalised therapies.

While many studies initially conducted gene expression profiling to group CRCs into subtypes, the results did not show concordance with each other, failing to lead to a common classification.[31, 32] As a result, members of the Colorectal Cancer Subtyping Consortium used their combined genomic datasets of over 4000 samples to generate what is now accepted as the consensus molecular subtyping of CRC.[33]

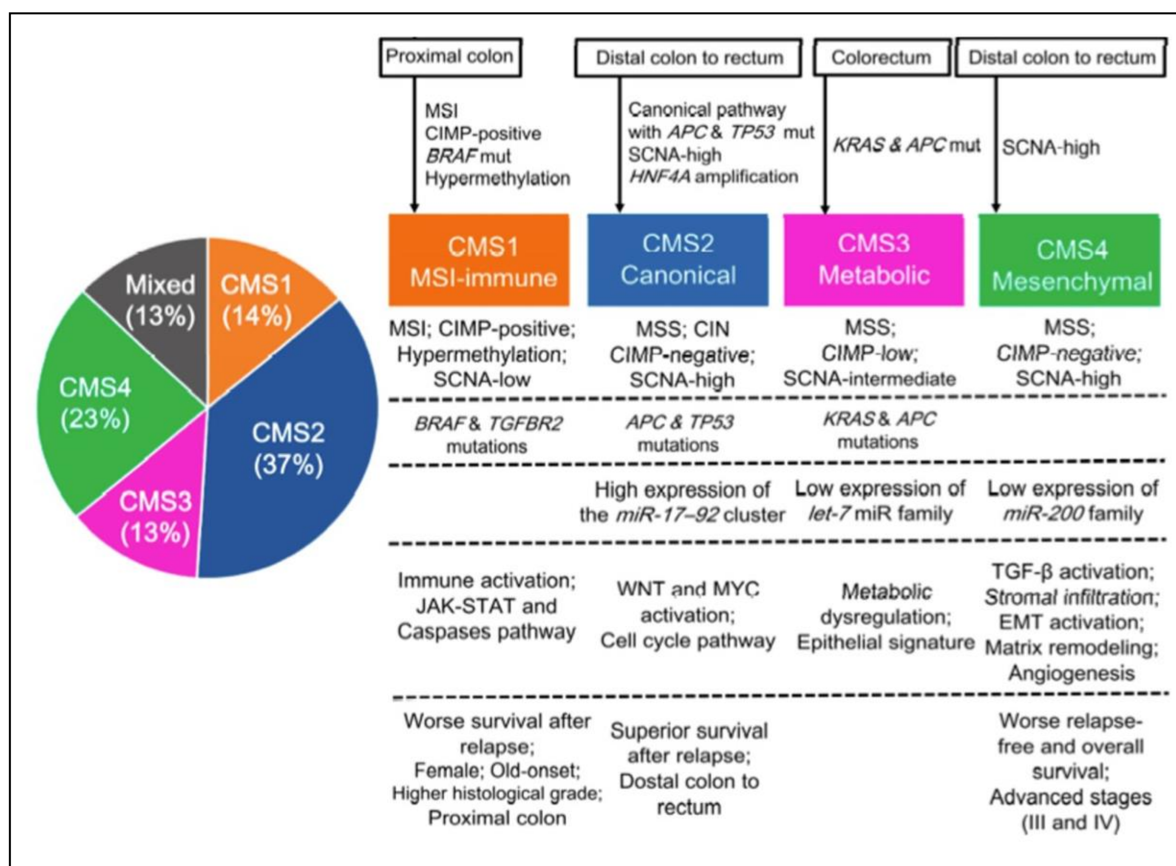


Figure 1.1. Consensus Molecular Subtypes of colorectal cancer, reflecting biological differences in gene expression pathways (Guinney et al)[34]

Four consensus molecular subtypes (CMS) were established (Figure 1.1). CMS 1 is typically characterised by microsatellite unstable (MSI-H) cancers with immune activation; CMS 2 are microsatellite stable (MSS), with chromosomal instability (CIN) and Wnt/MYC pathway activation; CMS 3 are MSS, with the presence of KRAS and APC mutations, exhibiting an epithelial signature and metabolic dysregulation. CMS 4 are MSS, occurring at advanced stages, and associated with poor overall survival. CMS 4 is further characterised by predominant signatures of TGF-β activation, stromal infiltration, epithelial-mesenchymal transition (EMT), matrix remodeling and angiogenesis. Mixed features (13%) account for the remainder and are thought to be due to transition phenotypes or intratumoural heterogeneity.

Overall, CMS is a transcriptomic-based, gene expression guided classification that helps to stratify the key pathways at play in each of the four subtypes. This can help both as a prognostic and research tool to develop drugs tailored to each subtype: a precision model of care based on gene expression, rather than generic models of care that are fraught with potential failure. CMS was developed based on early stage cancers, with its applicability in the metastatic setting still being evaluated.

Recent studies have started exploring the impact of the CMS classification as a prognostic factor and its predictive utility in optimal drug choice in metastatic CRC. For instance, Mooi et al[35] explored the benefit of bevacizumab in metastatic CRC based on the CMS subtypes in patients recruited as part of the AGITG MAX clinical trial. The MAX RCT evaluated the role of mitomycin C, bevacizumab and capecitabine in patients with untreated metastatic CRC and found that bevacizumab in addition to chemotherapy offered an improvement in progression free survival (PFS). In this molecular study of 237 patients from the MAX study, primary archival tumours were classified based on CMS subtypes and correlated with survival. The distribution of CMS subtypes was similar to that reported in the sentinel study by Guinney et al.[33] CMS was found to have prognostic value, with CMS 2 having the best overall survival (OS) (median 24.2 months, 95% CI 19.1-27.4), while CMS 1 had the worst (8.8 months, 95% CI 6.5-16.0). CMS 3 and CMS 4 had intermediate survival. CMS also had value in predicting response to bevacizumab, with significant improvements seen in progression free survival for CMS 2 (HR 0.50; 95% CI 0.33-0.76), but not for CMS 1 (HR 0.83; 95% CI 0.43-1.62) or CMS 4 (HR 1.24, 95% CI 0.68-2.25). While the specific mechanisms underlying the interactions between CMS groups and bevacizumab are unclear, it was notable from the study that CMS 1 and CMS 4, which are characterised by high immune and stromal infiltration respectively, appeared to derive minimal benefit from bevacizumab compared to CMS 2. Immunotherapy, which may have benefitted the MSI-H CMS 1 subtype, was not considered in the study.

Another trial called the CALHB/SWOG 80405 RCT[36] evaluated the role of cetuximab or bevacizumab in addition to first line FOLFOX or FOLRIRI in patients with metastatic

CRC. This study found no improvement in overall survival (OS) with the addition of cetuximab or bevacizumab. In the associated molecular study, Lenz et al[37] evaluated the impact of CMS on survival in 581 patients who were part of the CALHB/SWOG 80405 clinical trial. CMS classification was found to be a significant prognostic marker for OS ($p<0.001$) with a median OS of 15, 40.2, 24.3 and 31.4 months for CMS 1, CMS 2, CMS 3 and CMS 4 respectively. Similarly, in those receiving Cetuximab, CMS was again prognostic for OS, with CMS 2 having the best OS in this study. CMS was however, not found to independently influence survival in multivariate analysis in those receiving bevacizumab ($p=0.246$) in this study. This study, however, failed to report sites of metastases for each CMS subtype, making it challenging to comment on which sites of metastases are more difficult or resistant to treatment.

Presently, the CMS classification system is still in its infancy and is not yet incorporated into routine clinical practice. It is slowly being recognised by clinicians as a classification that potentially can offer prognostic information, along with better selection and development of drugs tailored to each individual cancer.

1.1.3.3 Immunoscore

The Immunoscore is another evolving classification system that serves to complement the existing TNM staging system. The current TNM classification parameters rely solely on tumour cell characteristics. A number of studies have investigated the host immune response to cancer and demonstrated the prognostic impact of the *in situ* immune cell infiltrate in the tumour and shown that the intra-tumoural immune cell infiltrate is an important determinant of outcome.[38-40] The Immunoscore is a method used to formally quantify the *in situ* immune infiltrate and has been shown in CRC to be a superior prognostic factor to the TNM classification.[40-42]

The Immunoscore is an immunohistochemistry based scoring system that quantifies the $CD3^+$ and $CD8^+$ T cells populations within the tumour centre (TC) and its invasive margin (IM).[43-45]. It provides a scoring based system ranging from low immune cell

densities in the TC and IM (Immunoscore 0) to high densities (Immunoscore 4), with increasing scores correlating with increased patient survival. In 2018, an international consortium validated the use of the Immunoscore in patients with Stage I-III CRC as a reliable measure of predicting disease recurrence,[42] showing that those with a high Immunoscore had a significantly better overall and disease free survival. Notably, its role in Stage IV CRC remains to be established.[42]

1.2 Colorectal peritoneal metastases (CRPM)

1.2.1 Incidence of colorectal peritoneal metastases

Approximately 30-40% of patients with CRC develop peritoneal metastases (PM) at some point during their surveillance and follow-up.[18, 46-49] At the time of CRC diagnosis, synchronous peritoneal metastases are seen in 4-13% of patients,[18, 47, 48] whilst metachronous PM develop in up to 19% of patients.[50] Of these, the peritoneum is the only site of metastases in 41%.[50]

Unlike visceral metastases, which are more readily detectable using various imaging modalities, peritoneal metastases are poorly detected on CT or PET/CT imaging modalities. Therefore, the true incidence of peritoneal metastases is difficult to ascertain. Some of the better evidence on the incidence of peritoneal metastases comes from autopsy studies such as one from the Netherlands,[51] where autopsies conducted on 5817 patients with CRC revealed an incidence of CRPM in 25%. Of these, 30% had isolated peritoneal metastases, with the rest also having other organ involvement. It is likely that all estimates on the incidence of peritoneal metastases may be disproportionately lower than the true incidence.

1.2.2 Risk factors for peritoneal recurrence

At index operation for primary CRC, there are a number of factors that contribute to an increased risk of peritoneal recurrence (Table 1.3). The site of cancer confers prognostic impact with right-sided cancers associated with a higher risk for peritoneal recurrence than left-sided cancers.[52] Honore et al[53, 54] reported in two systematic reviews that ovarian metastases and synchronous peritoneal disease resected at index operation carry the greatest risk of peritoneal recurrence. Completely resected synchronous peritoneal metastases at time of index surgery carry a 54-71% risk of

peritoneal recurrence at one year, while the presence of ovarian metastases carries a risk of peritoneal recurrence of 62-71% at one year.

Perforated tumours and T4 tumours carry an intermediate risk of peritoneal recurrence. The risk of peritoneal recurrence after resection of a perforated primary tumour is approximately 27% at one year. With T4 cancers, the risk at one year is 15.6% and 36.7% at 3 years.[54] While positive peritoneal cytology, obstructed tumour and lymph node positivity have been reported to increase the risk of peritoneal recurrence; there is insufficient data to quantify the risk of peritoneal recurrence posed by these factors. [53, 55]

Histological subtype is another important risk factor for peritoneal recurrence. Mucinous and signet ring cell (SRC) adenocarcinomas account for 3-15% and 0.1-2.4% of all colorectal cancers respectively,[53] with synchronous and metachronous PM more common with these subtypes. A Dutch autopsy study of 5817 patients found a 6% incidence of isolated PM in generic adenocarcinomas, with a 15% incidence of isolated PM in mucinous and SRC adenocarcinomas. Similarly, while the proportion of patients with any PM was 20% in those with generic adenocarcinomas, it was 48% with mucinous adenocarcinomas and 51% with SRC adenocarcinomas.[56] Riihimaki et al[17] performed a registry-based study in Sweden of 49096 patients and similarly found mucinous and SRC adenocarcinomas more frequently metastasised to the peritoneum (OR 3.8, 95% CI 3.2-4.5).

In another study, Segelman et al[57] explored the risk factors for metachronous peritoneal recurrence using a nation-wide registry study of more than 8000 patients with Stage I-III colorectal cancer. Metachronous PM were detected in 4.9% of patients with a colonic primary, particularly right sided, pT3, pT4, pN+, non-radical resection and emergency setting being risk factors for peritoneal recurrence. These findings were used to develop a risk prediction score for peritoneal recurrence at one, two and three years, that has been subsequently validated and is available online (http://www.imm.ki.se/biostatis-tics/calculators/pcrisk/)

Table 1.3. Summary of risk factors for peritoneal recurrence

Risk Factor	**Risk of peritoneal recurrence**
Isolated peritoneal metastases resected with primary tumour	54-71% at one year[25, 53]
Ovarian metastases	62-71% at one year[58, 59]
Perforated cancer	27% at one year[53, 59]
T4a cancer	15.6% at one year[53, 60]
Mucinous histology	16-22% at one year[53, 60, 61]
Positive cytology	0-36%[53, 55]
Obstructed tumour, lymph node positivity, tumour location, signet ring cell histology	Known to confer higher risk

1.2.3 Historical survival associated with peritoneal metastases

Historically, CRPM were viewed with a nihilistic attitude associated with an overall survival of six to eight months.[46, 62] The first prospective study evaluating patients with PM from non-gynaecologic malignancies found that patients with CRPM had a median survival of just 8.5 months.[46] Debilitating symptoms such as bowel obstruction, ascites, perforated viscus and enterocutaneous fistulae were common. Surgical procedures to alleviate these conditions were largely unsuccessful.

A subsequent study that explored prognostic factors in patients with PM reported an even lower median survival of 6.9 months in patients with CRPM.[62] This study described the extent and size of tumour deposits to be associated with OS (p=0.001), the first description of what we now universally refer to as the peritoneal carcinoma index (PCI).

1.2.4 Pathogenesis of Peritoneal metastases

The peritoneum is a fertile site for metastases to develop largely because of its extensive surface area. The development of peritoneal metastases is based on the "tumour cell entrapment" hypothesis (Figure 1.2).[63]

1.2.4.1 1) Cancer cells detach from the primary tumour.

The risk factors for primary tumours shedding cancer cells into the peritoneal cavity, and thus the development of peritoneal metastases includes risk factors such as serosal invasion (T4a), perforated cancers, and positive resection margins (Table 1.3). Shedding of cancer cells occur due to high interstitial fluid pressure within tumours together with inefficient lymphatic drainage.[64]

During this process, cancer cells undergo EMT, with loss of cell polarity, degradation of cell-cell junctions, changes in the cytoskeleton and loss of membrane glycoproteins, notably E-Cadherin (Figure 1.2a). E-Cadherin normally suppresses cell motility, invasion and metastasis. Other EMT changes in expression markers facilitate invasion, degradation of the basement membrane and movement in the surrounding microenvironment. EMT is considered to be a key step in initiation of local invasion and subsequent dissemination.[65]

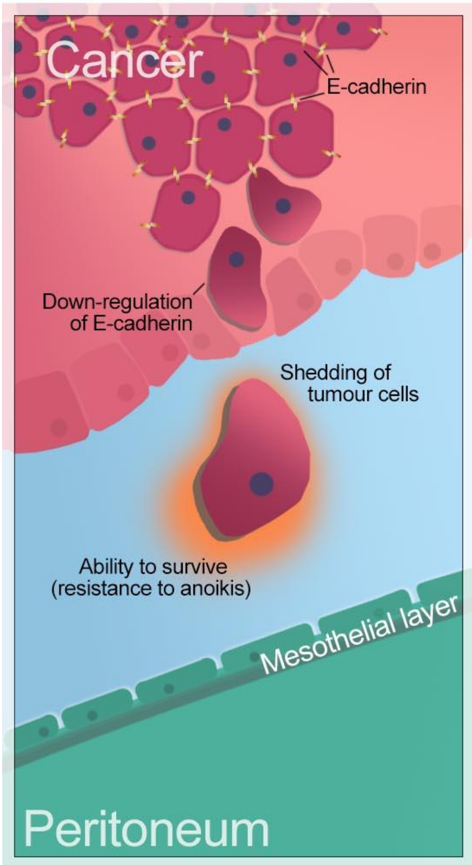
1.2.4.2 2) Cancer cells develop the ability to survive in the peritoneal environment.

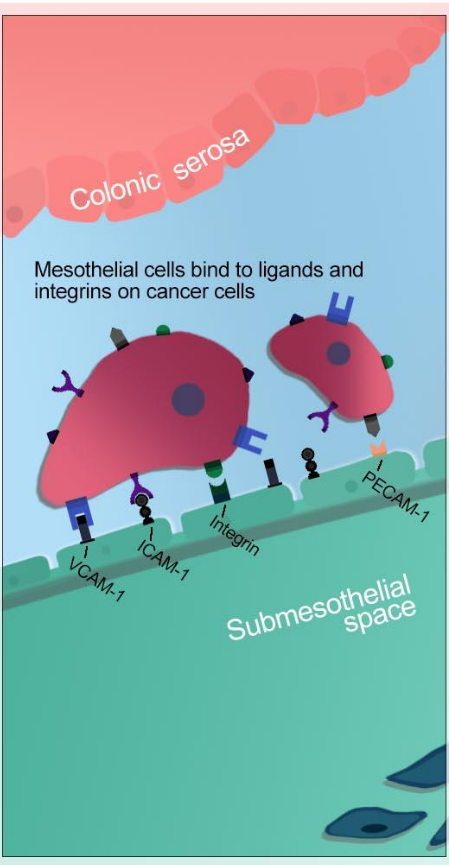
Normal epithelial cells undergo programmed cell death when they lose attachment to the extracellular matrix (ECM), a phenomenon called anoikis (Figure 1.2a). Tumour cells are resistant to anoikis, thereby being able to survive as circulating tumour cells (CTCs).[65] Anoikis resistance is driven by apoptosis modulators resulting in production of TrkB and AKT/PKB proteins.[66] Dissemination of cancer cells is facilitated by normal peristaltic movements of the bowel. Furthermore, cancer cells can increase motility through activating actin microfilaments on their membrane surface.[67]

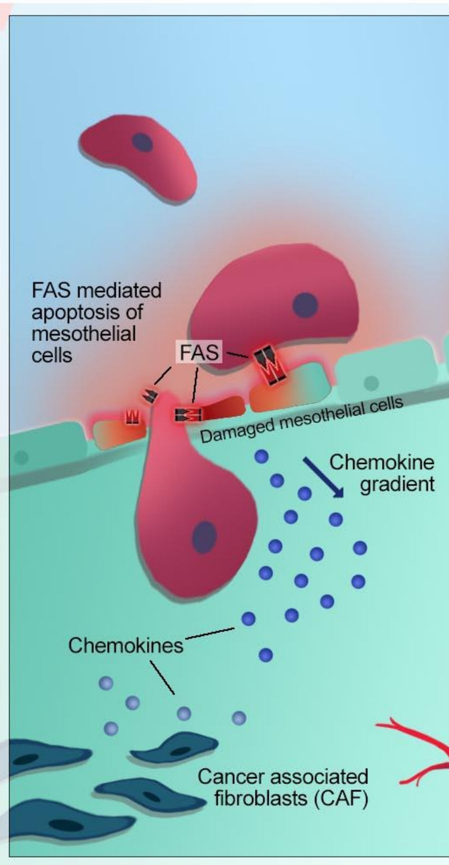
1.2.4.3 3) Cancer cells adhere to the peritoneum.

While CTCs can theoretically go to any site, they deposit preferentially in certain sites due to a pre-existing metastatic niche. Coined by Paget[68] as the "soil and seed hypothesis", it suggests that cancer cells can only grow in certain microenvironments. While the peritoneal cavity is large, the greater omentum is one of the most common sites for metastases. Gerber et al[69] showed this was because of the presence of 'milky spots'; aggregates of immune cells with large numbers of macrophages, with high vascularity and active angiogenesis, creating an environment highly favourable for tumour cell growth.

Cancer cells express surface proteins such as integrins, which can adhere to components of the extracellular matrix such as collagen, laminin, and fibronectin (Figure 1.2b). Mesothelial cells express a number of adhesion molecules such as intercellular (ICAM-1), vascular (VCAM-1) and platelet endothelial cell adhesion molecules (PECAM-1). Mesothelial cells assist adhesion of cancer cells to the peritoneum through interaction of CD44, a surface ligand on cancer cells with ICAM-1 on the surface of mesothelial cells.[70] The binding of mesothelial cells to integrins on cancer cells up regulates production of pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α, promoting binding to adhesion molecules.[71] Once cancer cells adhere, the submesothelial stroma provides necessary mediators that allow cell survival, proliferation and invasion.


(a) Shedding
Cancer
E-cadherin
Down-regulation of E-cadherin
Shedding of tumour cells
Ability to survive (resistance to anoikis)
Mesothelial layer
Peritoneum


(b) Binding
Colonic serosa
Mesothelial cells bind to ligands and integrins on cancer cells
VCAM-1
ICAM-1
Integrin
PECAM-1
Submesothelial space


(c) Migration
FAS mediated apoptosis of mesothelial cells
FAS
Damaged mesothelial cells
Chemokine gradient
Chemokines
Cancer associated fibroblasts (CAF)

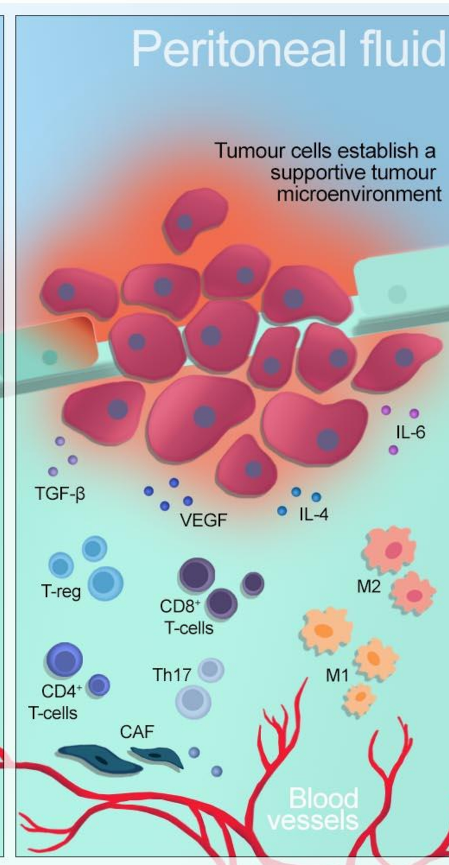

(d) Survival
Peritoneal fluid
Tumour cells establish a supportive tumour microenvironment
TGF-β
VEGF
IL-4
IL-6
T-reg
CD8+ T-cells
M2
M1
Th17
CD4+ T-cells
CAF
Blood vessels

Figure 1.2 a-d. Sequence of events leading from cancer cells being shed from the primary tumour, to establishment of peritoneal metastases

VCAM-1, ICAM-1, PECAM-1: vascular, intercellular and platelet endothelial cell adhesion molecules; TGF-β: Transforming growth factor beta; CAF: cancer associated fibroblasts; M_1: M_1 macrophages: that are tumour suppressive; M_2: M_2 macrophages that are pro-tumourogenic; IL-4, IL-6: Interleukin 4 and 6; VEGF: Vascular endothelial growth factor; T-reg: T- regulatory cell; Th_{17}: subtype of CD4

1.2.4.4 4) Cells migrate and gain access to the submesothelial space.

The submesothelial stroma is largely composed of fibroblasts that secrete structural proteins including collagen, fibronectin and elastin.[70] This tissue between the mesothelial cell layer and the submesothelial capillaries is also designated the peritoneal-blood barrier, which generally does not permit movement of nutrients and oxygen from the capillaries to the peritoneal cavity. This ensures that most cancer cells that attach to the mesothelial layer die off.[72] However, once cells adhere to the peritoneum with adhesion molecules like CD44, they can gain access to the submesothelial space via a chemotactic gradient generated by stimuli released by mesothelial cells, fibroblasts and adipocytes.[70] They can also gain entry to the submesothelial space in areas of peritoneal discontinuity from prior peritoneal injury or trauma.[64] Additionally, cancer cells themselves are able to induce mesothelial cell apoptosis through Fas-dependent mechanisms (Figure 1.2c).[73]

Once cancer cells establish themselves in the submesothelial space, cancer associated fibroblasts (CAFs) support tumour growth. Mesothelial cells can also undergo a mesenchymal to mesenchymal transition (MMT) switching to a CAF phenotype, rendering the peritoneum more receptive to tumour attachment, and invasion into the submesothelial space[74]. This transition may be dependent on TGF-β1[75]. Peritoneal macrophages also assist tumour development through production of angiogenesis factors including VEGF, MMP-1 and Amphiregulin.[70]

5) Cells must be able to establish a supportive microenvironment.

Cancer cells can release factors that disrupt the peritoneal blood barrier, leading to neo-angiogenesis and creation of an immunosuppressive tumour microenvironment (Figure 1.2d). Once cancer cells have invaded the submesothelial stroma, the endothelial lining of the lymphatics and blood vessels in the stroma promote further cancer growth through recruitment of monocytes and other immunosuppressive cells, supported by CAFs and peritoneal macrophages. Among their other functions, CAFs produce various cytokines, including VEGF and remodel the ECM to create a pro-tumourogenic environment facilitating tumour growth and proliferation. Cancer cells also release

chemokines such as HMGB1, which help neo-angiogenesis and recruit macrophages and myeloid derived suppressor cells (MDSCs). Overall, these factors promote further tumour growth.

While transcoelomic spread is a more commonly appreciated method of peritoneal spread, haematogenous or translymphatic spread is another important route. Factors such as lymph node positivity of the primary tumour are established risk factors for development of peritoneal metastases.[27, 76] Here, CTCs travel first via draining lymph nodes to eventually reach the circulation. CTCs can develop the ability to leave the circulation through activating platelets and secretion of adenine nucleotides, which induce opening of the endothelial barrier, allowing CTCs to gain direct entry into the submesothelial space.[77]

1.2.5 Detection and presentation of CRPM

There are no specific symptoms that suggest the presence of peritoneal metastases. Patients can be asymptomatic, while others may present with abdominal pain, distension, bowel obstruction, unintentional weight loss or cachexia, prompting further investigation. In many cases, PM are detected on staging or surveillance imaging or incidentally at the time of surgery.

Common imaging modalities used in the diagnosis of PM include contrast-enhanced multi-detector computed tomography (CT), ^{18}F-fluorodeoxyglucose positron emission tomography (PET-CT), and contrast-enhanced and diffusion-weighted magnetic resonance imaging (DW-MRI).

Classic imaging features in keeping with peritoneal disease include omental caking, scalloping of the liver or diaphragm, mucinous ascites and nodules >1 cm.[19] Studies have found that CT scanning has limited sensitivity in detecting peritoneal nodules <0.5cm, with improved sensitivity with increased lesion size.[78] Furthermore, peritoneal carcinomatosis index (PCI) was found to be significantly underestimated by radiological assessment compared to intraoperative PCI. The combination of CT and DW-MRI offers a more accurate assessment of the PCI than CT alone.[79] However, the overall accuracy of

dual modality imaging was again found to be dependent on the volume of disease. Similar to other modalities, PET/CT has low sensitivity in the detection of nodules <1cm. However, PET/CT scans are helpful when MRI or CT have been inconclusive. Two recent meta-analyses found an overall diagnostic accuracy of 87-92% with PET/CT[80,81], suggesting that a hot spot found on PET/CT can be reliably assumed to be a peritoneal metastasis.

Limitations in imaging modalities have led to the use of staging laparoscopy as an adjunct to help evaluate disease burden. Many centres perform staging laparoscopies routinely before CRS and HIPEC to evaluate resectability. Laparoscopy has been shown to be helpful in many studies[82,83] in identifying clearly unresectable patients, thereby avoiding a morbid laparotomy. Iversen et al[83] utilised laparoscopy in 45 patients to help better select cases for CRS and HIPEC. They found that the use of laparoscopy contributed to an increase in the completion rate of CRS and HIPEC from 56 to 70%. However, laparoscopic evaluation failed in 10 out of 27 patients, who still ended up with a laparotomy for unresectable disease. Use of laparoscopy decreased the rate of incomplete cytoreduction from 44% to 37%. However, despite laparoscopy and multi-modal imaging techniques, a proportion of patients are still found to have unresectable disease at operation. This underlines the overall challenge in accurately staging and detecting peritoneal metastases.

A recent survey of the Peritoneal Surface Oncology Group International (PSOGI) found that most experts recommended only a thoraco-abdominal CT scan as a routine part of the pre-operative staging, with MRI, PET/CT and laparoscopy utilised only when additional information was needed.[84]

1.2.5.1 Biomarkers

There are currently no biomarkers that can accurately predict the presence of peritoneal metastases or peritoneal recurrence.

One evolving tool that appears promising is circulating tumour DNA (ctDNA). A recent study by Baumgartner et al[85] demonstrated that a high pre-operative ctDNA in patients

undergoing CRS and HIPEC independently predicted a worse progression free survival. In another study using immunohistochemistry to stain for cancer stem cell markers, Nagata et al[86] reported that absence of CD133 expression was independently associated with a worse DFS after surgical resection for CRPM.

Recently, Nagata et al[87] evaluated whether a cancer stem cell marker leucine-rich repeating G-protein-coupled receptor-5 (LGR5) could help predict peritoneal recurrence after primary tumour resection. They found that lack of LGR5 expression was an independent risk factor for peritoneal recurrence (HR 2.79, 95% CI 1.37-5.67).

These are potentially promising biomarkers that need further validation before they can be utilised in regular clinical practice. Given the limitations in imaging modalities, the development and validation of a robust biomarker that could predict development of PM or recurrence after treatment would be an important addition to the current tools available. This is an area of ongoing research.

1.3 Current treatment options for colorectal peritoneal metastases (CRPM)

Current treatment options for patients with CRPM can be broadly divided into three distinct categories (Figure 1.3):

1) Resectable CRPM
2) Unresectable CRPM
3) High risk primary tumours with no evidence of CRPM on imaging

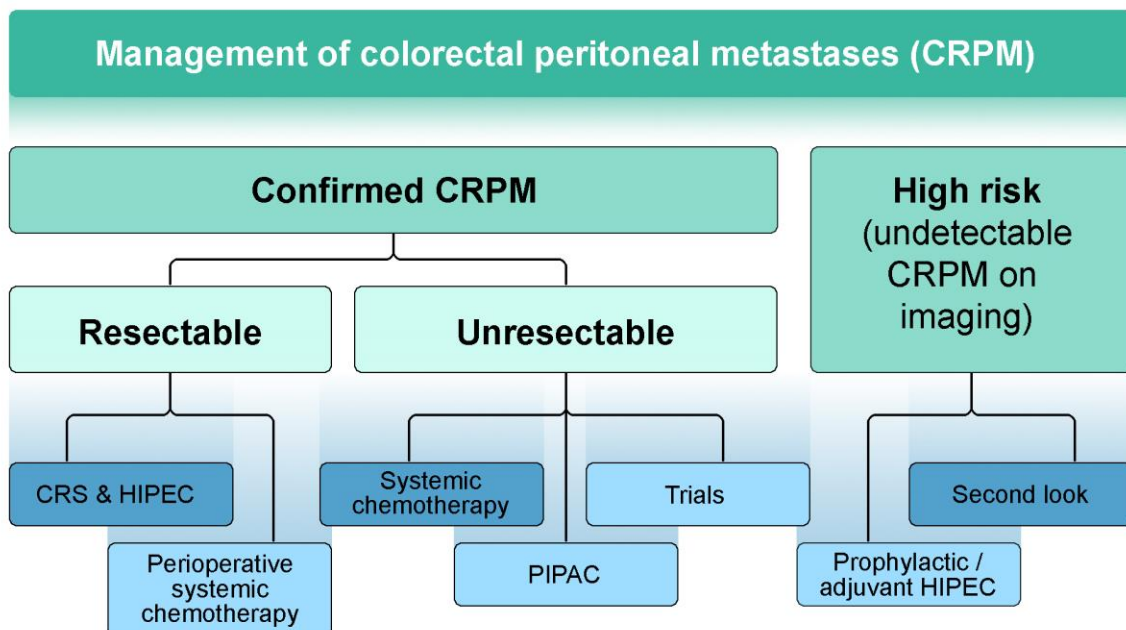


Figure 1.3. Treatment options for patients with colorectal peritoneal metastases

CRPM – colorectal peritoneal metastases; CRS – cytoreductive surgery; HIPEC – hyperthermic intraperitoneal chemotherapy; PIPAC – pressurised intraperitoneal aerosolised chemotherapy.

1.3.1 Resectable CRPM: Cytoreductive Surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC)

Over two decades ago, an aggressive treatment for peritoneal disease arose in the form of cytoreductive surgery (CRS) with heated intraperitoneal chemotherapy (HIPEC). Pioneered by Sugarbaker,[88, 89] it involves removal of all macroscopic deposits of tumour

within the peritoneal cavity. Chemotherapy, heated to 41-43°C is then administered to the peritoneal cavity. HIPEC aims at delivering a higher concentration of chemotherapy intraperitoneally, whilst minimising systemic distribution and therefore toxicity.[90] Hyperthermia works synergistically with chemotherapy to eradicate microscopic disease.[91] Since Sugarbaker demonstrated successful long term survival in his original series[89] of 47 patients with peritoneal carcinomatosis treated with CRS and HIPEC, multiple institutional programs across the world have evaluated this therapy and demonstrated favourable results.

The two key prognostic factors that influence survival following CRS and HIPEC are the peritoneal carcinoma index (PCI) and the completeness of cytoreduction (CC score). PCI is a numeric score from 0-39 that reflects the burden of disease. The CC score is a measure of residual disease after all tumour deposits have been removed. CC-0 implies no macroscopic residual disease. CC-1, 2 and 3 implies residual disease less than 2.5mm, 2.5mm-2.5cm, and greater than 2.5cm respectively. CC-0/1 is considered complete cytoreduction, with subsequent administration of HIPEC. CC-2/3 cases are deemed incomplete cytoreduction and are not given HIPEC, except in selected cases, such as intractable ascites.[92, 93]

To date, there have only been a handful of randomised trials (RCTs) in this field. In the only RCT comparing CRS and HIPEC with systemic therapy in the management of CRPM, Verwaal et al[94] showed that CRS and HIPEC nearly doubled the median survival compared to 5-FU based systemic therapy alone (22.3 months v 12.6 months, $p=0.032$). These survival differences remained significant at 8-year follow-up, with a median disease free and disease specific survival of 12.6 vs 7.7 months and 22.2 vs 12.6 months respectively with CRS and HIPEC compared to systemic chemotherapy. This trial emphasised that CRPM is a surgical entity, with patients undergoing successful complete cytoreduction having a 48 months median OS, with a 45% five-year survival.[95]

Elias et al[96] explored the role of early post-operative intraperitoneal chemotherapy (EPIC) with systemic chemotherapy to systemic chemotherapy alone following complete cytoreduction. While this RCT trial failed to show any benefit with EPIC due to

difficulties in patient accrual, it did demonstrate an impressive 60% two-year survival in both arms following successful complete cytoreduction.

Cashin et al[97] explored the role of CRS and intraperitoneal chemotherapy (IPC) with 5-Fluorouracil compared to FOLFOX systemic chemotherapy in patients with resectable CRPM. While this RCT was also terminated early due to recruitment difficulties, it reported a significant benefit in median OS of 25 months with a two-year survival of 54% for CRS and IPC compared to 18 months and two-year survival of 38% for systemic chemotherapy.

The majority of the evidence supporting the use of CRS and HIPEC has been in the form of non randomised and retrospective studies that have demonstrated a 19.2-34 months median OS with a 19-48% five-year survival in selected patients (Table 1.4).[27, 98-103] If complete cytoreduction is achieved, a 33-62.7 months median OS, with a 31-51% five-year survival can be attained.[100, 104-107] In recent years, CRS and HIPEC has slowly been integrated into treatment pathway guidelines in the management of low volume CRPM in many countries.[108-111]

CRS and HIPEC does however, carry significant risks of complications, with a 17.6-52.4% rate of Grade III/IV morbidity and 0-8.1% mortality.[112-118] This equates to an approximate one in four chance of suffering a Grade III/IV complication following CRS and HIPEC.

Table 1.4. Outcomes from large cohort studies

Author (year)	**Patients (n=)**	**Overall Survival (months)**
Glehen et al[98] (2004)	506	32
Elias et al[27] (2010)	523	33
Quenet et al[105] (2011)	146	41
Prada-Villaverde et al[107] (2014)	584	33
Faron et al[119] (2016)	173	41
Maillet et al[120] (2016)	231	43
Kozman et al[121] (2018)	260	35
Sluiter et al[122] (2018)	175	27

The recently completed PRODIGE 7 trial[106] has raised some doubts about the efficacy of HIPEC. This RCT evaluated the effect of oxaliplatin based HIPEC on overall survival following complete cytoreduction compared to complete cytoreduction alone. An impressive 41.2 months median OS was achieved following CRS alone, with the addition of HIPEC not offering any survival benefit (41.2 months v 41.7 months, HR 1.00; 95% CI: 0.73-1.37). The use of HIPEC however contributed to significantly more grade III-V complications (24.1% v 13.6%, p=0.03). Subgroup analysis however suggested a survival benefit with HIPEC in patients with a PCI 11-15.

While HIPEC offered potential survival benefit in those with a moderate PCI (PCI 11-15), the challenge however, is to accurately identify these patients with moderate PCI pre-operatively. The trial also questioned whether oxaliplatin is indeed the optimal agent for HIPEC. Verwaal et al[94] in the pivotal RCT used Mitomycin C as the intraperitoneal chemotherapy agent. Similarly, in many centres in Europe and the USA, Mitomycin C is the preferred drug for HIPEC.[123] Presently, there are no prospective trials evaluating Mitomycin C versus Oxaliplatin as the drug of choice for HIPEC. Whilst the PRODIGE 7 trial[106] is a landmark study reaffirming the importance of high quality cytoreductive surgery, it has raised a number of important issues for further study, particularly with regard to HIPEC.

Despite favourable survival after CRS and HIPEC, risk of recurrence remain high. Studies have reported peritoneal recurrence rates after CRS and HIPEC of 19.2-62.4%,[124-128] with a median disease free survival (DFS) of 9-23 months. All site recurrence rates are higher ranging from 22.5-82%.[27, 99, 124-130] The optimal treatment for those with isolated peritoneal recurrence following CRS and HIPEC remains undefined. Some centres have reported a favourable median OS of 20 to 39 months following iterative CRS and HIPEC. [131-134] Factors such as a longer disease free interval, lower PCI, and completeness of cytoreduction influence survival following iterative CRS and HIPEC.[135-140] For patients with extra-peritoneal recurrence, chemotherapy usually becomes the mainstay of treatment. Chemotherapy options remain limited, with treatment failures and recurrences common, with an urgent need to explore newer options for treatment.

1.3.1.1 Hyperthermic intraperitoneal chemotherapy (HIPEC)

Early studies demonstrated a significant advantage with intraperitoneal chemotherapy (IPC) compared to systemic chemotherapy. Dedrick et al[141] showed that the rate of clearance of a drug was slower after intraperitoneal delivery, compared to clearance from the blood, leading to a higher drug concentration in the peritoneal cavity in direct contact with PM compared to systemic delivery. Furthermore, systemic toxicity of the chemotherapy was lower with the use of IPC.[142] Speyer et al[143] identified 5-FU as a suitable drug for intraperitoneal delivery, demonstrating high concentrations within the peritoneal cavity, with low systemic absorption and low systemic toxicity. Further studies have since identified numerous drugs suitable for IPC that can be safely utilised in the treatment of CRPM. Pharmacokinetic studies demonstrated that due to the large molecular size and hydrophobic surface, certain chemotherapy agents have a slower absorption from the peritoneal cavity into the circulatory system. This in effect translates to a much higher therapeutic delivery to tumour deposits on the peritoneum, with reduced systemic toxicity.[144]

The addition of heat as an adjunct to cancer surgery has previously been explored in animal models.[145] Studies have shown the effects of chemotherapy could be enhanced through the addition of hyperthermia.[146] The first documented case of the use of heated intraperitoneal chemotherapy (HIPEC) however, was by Spratt et al[147] in 1980 when IPC was used at 42°C in a patient following resection of pseudomyxoma peritonei. The treatment was well tolerated and repeated some days later without incident.

Since then, IPC in the form of HIPEC has accompanied CRS for CRPM. Mitomycin C and Oxaliplatin are the two most widely used HIPEC agents for CRPM.

While the identification of newer drugs that can be utilised as HIPEC agents should be an area of research, a more rapid area for translational change could involve the potential use of other agents already used as HIPEC in other cancers. For example, doxorubicin and cisplatin are common HIPEC agents used in the treatment of PM from gastric or ovarian cancer. A rational approach would be to develop a method to functionally evaluate a tumour’s sensitivity to these various HIPEC agents, hence

ensuring patients receive the agents with the greatest possible efficacy against the specific tumour.

1.3.1.2 Peri-operative systemic chemotherapy

The use of peri-operative systemic chemotherapy in the form of neo-adjuvant or adjuvant or both in patients undergoing CRS and HIPEC is variable across the world. In countries such as Netherlands, peri-operative chemotherapy is not used and patients with resectable CRPM undergo upfront CRS and HIPEC.[148] In countries such as France, patients are given peri-operative systemic chemotherapy alongside CRS and HIPEC.[106]

There are merits to both approaches. Peritoneal metastases arise because of an advanced primary cancer, with a high risk of systemic spread. Furthermore, even after successful CRS and HIPEC, recurrence rates are high, with a significant number of recurrences occurring extra-peritoneally.[149] Risk factors for peritoneal recurrence such as lymph node positivity are well known risk factors for systemic failure as well.[27, 76] Peri-operative systemic chemotherapy may help eradicate micro-metastatic systemic disease and help decrease the risk of systemic failure. Additionally, the only RCTs evaluating the efficacy of CRS and HIPEC have utilised peri-operative chemotherapy alongside CRS and HIPEC.[94, 106]

Some studies have reported superior outcomes with CRS and HIPEC following neoadjuvant chemotherapy, including reporting morphological and histopathological responses in over 20% of patients undergoing neoadjuvant therapy before CRS and HIPEC.[150, 151] Kwakman et al[76] demonstrated in a meta-analysis that use of neo-adjuvant chemotherapy was independently associated with an improved survival in patients undergoing CRS and HIPEC. Neo-adjuvant chemotherapy may help decrease tumour burden before CRS and HIPEC, ensuring a greater likelihood of achieving a complete cytoreduction. Assessment of response could also improve patient selection for CRS and HIPEC, as well as choice of HIPEC agent.

Similarly, while the purpose of HIPEC is to eradicate any micro-metastatic disease in the peritoneum after CRS, the use of adjuvant systemic chemotherapy may help in

eradicating micro-metastatic disease systemically after CRS and HIPEC. A number of studies have demonstrated that use of adjuvant chemotherapy is independently associated with an improved survival after CRS and HIPEC.[27, 121]

Conversely, standard systemic chemotherapy in general is less effective for PM compared to other sites of disease.[24] Furthermore, systemic chemotherapy carries more toxicity than IPC. While systemic chemotherapy may delay rate of disease recurrence, it may not ultimately improve OS. In CRLM, Nordlinger et al showed that peri-operative chemotherapy with liver resection improved progression free, but had no effect on OS.[152, 153]

Based on current evidence, systemic chemotherapy is well established as peri-operative treatment in patients undergoing CRS and HIPEC. However, it has limited role as a stand-alone treatment in patients with resectable CRPM. No prospective trials to date have explored the role of CRS and HIPEC without systemic chemotherapy. The CAIRO 6 trial[148] (NCT 02758951) is a multi-centre RCT currently recruiting in Netherlands that is evaluating peri-operative systemic chemotherapy with CRS and HIPEC against upfront CRS and HIPEC. Results from this trial would greatly help better define the role of peri-operative systemic chemotherapy.

1.3.2 Unresectable CRPM: Systemic Chemotherapy

Patients are generally deemed inoperable based on unresectable sites or volume of disease. Studies have demonstrated that benefit from CRS and HIPEC diminishes with a higher PCI, with five-year survival less than 10% in those with a PCI > 20.[27] Similarly, Goere et al[154] showed that CRS and HIPEC offered no survival benefit over palliative treatment in patients with PCI > 17. While five-year survival with systemic chemotherapy is only 4.1% in the setting of unresectable CRPM,[155] its lower morbidity compared to CRS and HIPEC makes it the mainstay of treatment for patients with high volume disease. Not surprisingly, disease progression and treatment failures are exceedingly common.

Patients with CRPM are under-represented in clinical trials of metastatic colorectal cancer. Tseng et al[156] reported that only seven (9.7%) clinical trials from 2003-2016 reported specifically on the inclusion of patients with CRPM (Table 1.5), with the percentage of patients with peritoneal disease ranging from 7.9-21.5%. This equated to only 670 (1.5%) patients out of 45,783 patients evaluated in all 72 trials. Such under-representation has limited the evaluation of newer therapy options in patients with unresectable CRPM.

Table 1.5. Clinical trials in metastatic colorectal cancer involving patients with peritoneal metastases (72 clinical trials; 45,783 patients) (adapted from Tseng et al)[156]

Author, year	**Number of patients in treatment groups**	**Number with peritoneal metastases (%)**
Seymour et al[157] (2007)	2135	288 (13.5%)
Jonker et al[158] (2007)	572	45 (7.9%)
Ducreux et al[159] (2011)	410	63 (15.4%)
Hong et al[160] (2012)	340	73 (21.5%)
Yoshino et al[161] (2012)	169	28 (16.6%)
Seymour et al[162] (2013)	460	99 (21.5%)
Tournigand et al[163] (2015)	700	83 (11.9%)

Early studies[46, 62] had demonstrated that patients with unresectable CRPM had a median survival of three to six months with supportive care only. Over with last few decades, there have been substantial advances in systemic treatment. With advances in systemic agents, with modulated 5-FU and the oral 5-FU pro-drug Capecitabine to combination therapy with Oxaliplatin (FOLFOX or CAPOX) or Irinotecan (FOLFIRI), chemotherapy along with biologic agents (EGFR-antibodies and VEGFR inhibitors) became a feasible option for these patients.

While systemic chemotherapy is the mainstay of treatment for patients with unresectable CRPM, their survival is worse than those with liver or lung metastases. In a pooled analysis of clinical trials of over 10,000 patients with metastatic CRC, Franko et al[24] demonstrated that patients with CRPM treated with chemotherapy have a median survival of 16.3 months, compared to 19.1 months for those with liver metastases and 24.6 months for those with lung metastases (Figure 1.4).

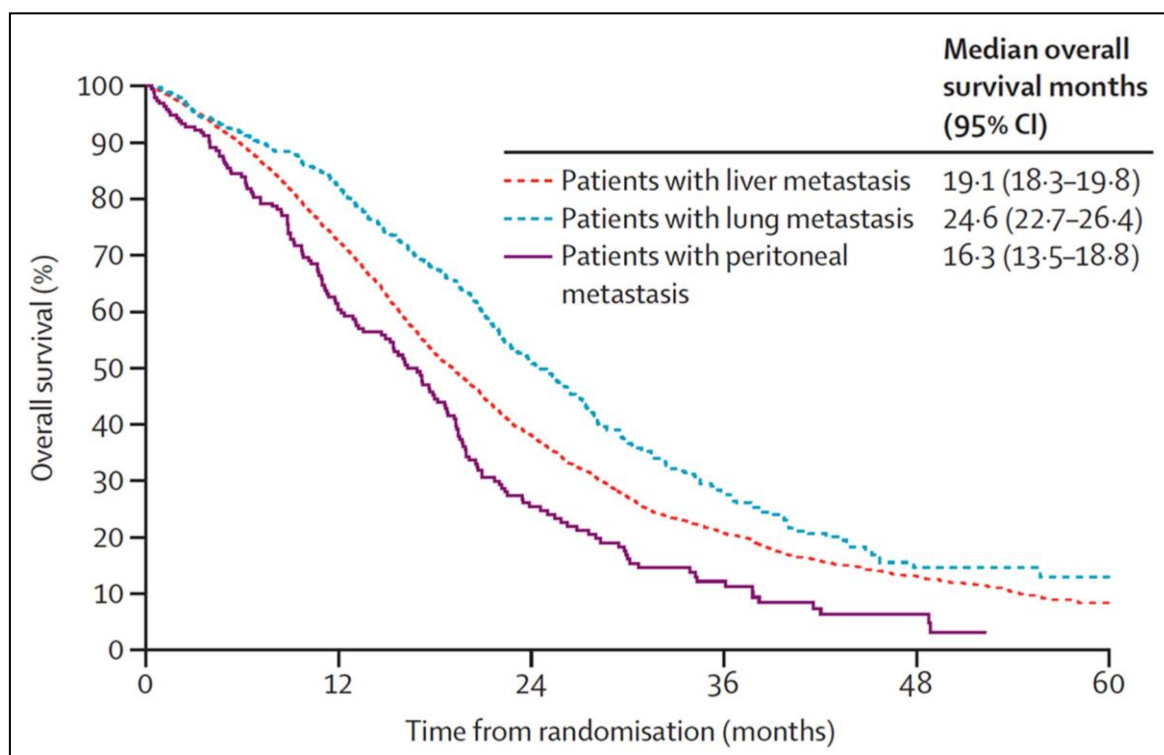


Figure 1.4. Patients with colorectal peritoneal metastases have a significantly worse survival compared to other sites of metastases (Franko et al)[24]

Klaver et al[164] combined pooled data from the CAIRO and CAIRO 2 trials evaluating survival in patients undergoing systemic therapy with biologic agents for metastatic CRC. In this study as well, patients with CRPM had a substantially worse survival than those with metastases to other sites (CAIRO: 10.4 months v 17.3 months; CAIRO 2: 15.2 months v 20.7 months). Other studies[165] have similarly demonstrated that unresectable CRPM do not respond well to systemic chemotherapy compared to other sites of metastases.

The exact reasons for the comparatively worse survival in patients with CRPM is unknown, but is postulated to be due to a combination of chemo-resistance, aggressive tumour biology and altered peritoneal tumour microenvironment.[166, 167]

Bevacizumab is a targeted agent against vascular endothelial growth factor receptor inhibitor (VEGFRi) that has been used with reasonable success in metastatic CRC. It inhibits angiogenesis and suppresses expression of FasL (CD95L) allowing for increased tumour suppressive $CD8^+$ T cell honing. However, it also inhibits dendritic cell

maturation and expression in the tumoural microenvironment, with the extent of dendritic cell suppression associated with survival in patients with CRC.[168, 169]

A Dutch study[170] of 182 patients explored the use of bevacizumab by comparing the benefit of chemotherapy and bevacizumab on non-operative patients with CRPM. Those receiving chemotherapy and bevacizumab had a median survival of 20.3 months compared to 13.0 and 3.4 months with chemotherapy alone and no treatment respectively. Similarly, In a population based study of 1235 patients receiving systemic therapy for CRPM, Razenberg et al[171] showed that the addition of bevacizumab to chemotherapy led to an improved median survival of 11 months compared to 7.5 months with chemotherapy alone. While promising, further prospective studies are needed to thoroughly evaluate the potential benefit of bevacizumab in the treatment of CRPM.

1.3.3 Unresectable CRPM: Emerging techniques

More recently, the development of pressurised intraperitoneal aerosolised chemotherapy (PIPAC) has been used in a number of centres as a palliative treatment in patients with unresectable CRPM. It aims to provide symptomatic palliation and improved quality of life to patients with unresectable CRPM

The premise behind the use of PIPAC is based on the aerosol delivery mechanism, ensuring a more homogenous drug distribution and improved tissue penetration compared to standard delivery of intraperitoneal chemotherapy.[172] Oxaliplatin is the standard chemotherapeutic agent used in PIPAC for CRPM. Early reports on the use of PIPAC are promising, showing it to be safe, with low toxicity, with objective tumour regression seen in selected patients.[172-174] Further Phase II clinical trials are underway worldwide to evaluate the safety and feasibility of PIPAC in CRPM (NCT03868228, NCT03246321).

1.3.4 High risk primary tumours with no evidence of CRPM on imaging: Role of early re-look, prophylactic HIPEC or adjuvant HIPEC

In approximately 25% of primary CRC resections, there are clinical or pathological findings that indicate a high risk[54] for future PM (Table 1.3). Given well-established factors that predict early peritoneal recurrence, studies have evaluated the role of a pro-active cytoreductive approach in patients at high risk for peritoneal recurrence.

Elias et al[175] demonstrated that asymptomatic PM were diagnosed in 55% of cases undergoing a "pro-active" second look laparotomy 13 months after resection of a high-risk primary cancer. These included cases of synchronous peritoneal metastases resected with the primary, synchronous ovarian metastases or perforated primary tumours. In a follow-up study using this "pro-active" approach, PM were found in 56% of patients undergoing second look surgery 12 months following high-risk primary tumour resection. Following second look CRS and HIPEC, five-year OS was 90% and five-year DFS was 44%.[59]

Sammartino et al[61] explored the use of adjuvant HIPEC in patients with clinical T3/T4 mucinous or SRC adenocarcinomas, and noted that those receiving adjuvant HIPEC had a better DFS (but not OS) compared to retrospective controls.

Two recently completed French RCTs have explored a pro-active approach of early relook with CRS and HIPEC in high-risk patients. The ProphyloChip trial (NCT01226394)[176] explored the role of systematic second look CRS with oxaliplatin HIPEC versus surveillance for asymptomatic patients at six months following a primary tumour at high risk for peritoneal recurrence. High risk was defined as minimal CRPM resected with the primary, ovarian metastases or perforated primary tumour. While PM were found in 52% of patients undergoing second look surgery, there was no difference in three-year DFS (44% v 51%, p=0.75) or OS between the two arms (79% v 80%).

With regard to adjuvant HIPEC use, the recently published COLOPEC trial (NCT02231086),[177] evaluated the role of adjuvant oxaliplatin HIPEC after high-risk primary tumour resection. These included pT4 or perforated cancers. Patients were randomised to receive adjuvant oxaliplatin HIPEC followed by systemic chemotherapy or adjuvant chemotherapy alone. This trial also failed to find a difference in peritoneal metastasis free survival (77% v 81%) by diagnostic laparoscopy at 18 months in the control and adjuvant HIPEC arms. Furthermore, there was no difference in DFS and OS at 18 months between the two arms.

The Spanish HIPECT4 trial (NCT02614534)[178] is an ongoing RCT exploring the efficacy of adjuvant Mitomycin C based HIPEC followed by systemic chemotherapy for T4 colorectal cancers versus systemic chemotherapy alone. The results of this trial are awaited.

Despite optimism for a pro-active approach to treating and preventing early peritoneal recurrence, recent RCTs have shown no benefit for prophylactic or adjuvant oxaliplatin based HIPEC. Based on current evidence, a pro-active approach of CRS with prophylactic or adjuvant oxaliplatin-based HIPEC does not appear to offer any survival advantage to standard surveillance.

1.3.5 Summary of current standard of care for patients with CRPM

The greatest advance in the management of CRPM in recent decades has been the advent and adoption of CRS and HIPEC. In selected patients with low volume isolated CRPM, CRS and HIPEC is the accepted standard of care. The additional benefit of peri-operative systemic chemotherapy is being investigated in prospective trials.

Recurrence after successful CRS and HIPEC is high, with a median DFS after CRS and HIPEC of 11-23 months.[179, 180] Following recurrence, systemic chemotherapy is the mainstay of treatment in most cases, with the role of iterative CRS and HIPEC still evolving. Compared to other sites, systemic chemotherapy is less effective for PM, with treatment resistance and failure common. Newer and more rationally selected therapeutic options are urgently needed.

In patients with unresectable CRPM, systemic chemotherapy is the mainstay of treatment. Systemic chemotherapy has poor efficacy in the treatment of PM, compared to other sites. Chemotherapy options for CRC are limited, with 5-FU, Oxaliplatin, Irinotecan, Trifluridine and tipiracil (lonsurf), VEGFRi (bevacizumab) and EGFR monoclonal antibodies (cetuximab, panitumumab) and the multi-targeted tyrosine kinase inhibitor regorafenib being available. Most patients often exhaust all options, without any new options on the horizon. There is an opportunity here to explore newer options and approaches that can help improve survival in these patients. Emerging procedures such as PIPAC are being investigated in clinical trials as a treatment option in the palliative setting.

In high-risk patients without evidence of peritoneal metastases on imaging, there appears to be no benefit from a pro-active approach involving early relook surgery with prophylactic HIPEC or adjuvant HIPEC. Close surveillance in keeping with national CRC guidelines is the accepted standard of care.

1.3.6 Genomic landscape of CRPM

Colorectal cancers are a heterogeneous group with different behaviours and varied responses to therapy. Most CRCs develop through a series of acquired mutations in driver genes such as *APC, KRAS, BRAF, PIK3CA*, and *TP53*. However, similar to the "seed and soil" concept in the development of metastases, it is possible that certain mutations may be seen more frequently at certain metastatic sites. This is important to appreciate as it can guide future use of targeted therapy that is more specific to certain sites.

Studies have explored concordance between the primary tumour and the matched metastases and found over 90% concordance in mutational profiles between the primary and matched metastases.[181-183] One of the drawbacks in almost all studies is that PM are under-represented, possibly because they are harder to biopsy compared to other sites, such as liver metastases.

Various studies have explored differences in mutational profiles at different sites in patients with metastatic CRC. Peritoneal metastases were found to have a similar

somatic mutational profile to the primary tumour in most studies. In an analysis of over 6,800 CRC samples, El-Deiry et al[184] showed that PM had a similar incidence of *KRAS, BRAF* and *PIK3CA* mutations to the primary tumour. Franko et al[24] similarly reported no difference in mutational profile in patients with PM.

In a comparative study, Sasaki et al[185] reported that the incidence of *BRAF*^{V600E} mutations was significantly higher in patients with CRPM. However, they found that the presence of *BRAF* mutations in their cohort was not associated with prognosis. The presence of *RAF/RAS* pathway mutations is known to be associated with a worse prognosis, as they predict resistance to anti-EGFR therapy, thereby limiting treatment options for patients with these mutations. Recently, Schneider et al[186] found that in patients with CRPM, presence of *RAS/RAF* mutations was an independent prognostic factor for worse overall survival.

Based on current evidence, most studies reporting on the mutational landscape in metastatic CRC do not demonstrate a significant difference in the mutational landscape in CRPM compared to the primary tumour.

1.4 Colorectal Cancer and Immunity

1.4.1.1 Colorectal cancer development

Despite the heterogeneity displayed in CRC, there are three main pathways through which CRC develops.[187] It is important to distinguish between these three, as they produce cancers of different biology, which have different clinical implications.

1) Chromosomal instability pathway (CIN) is characterised by imbalances in chromosome number and loss of heterozygosity. This is when one allele is lost by a chromosomal event, leading to an aneuploid tumour. The accumulation of mutations in specific oncogenes and tumour suppressor genes activate pathways in a sequential manner critical for CRC development and progression. Described by Vogelstein,[188, 189] and referred to as the classical pathway, CIN accounts for over 85% of CRC. It usually follows an initial *APC* gene mutation, and progresses through other driver mutations in genes such as *KRAS, SMAD4, TP53*, and *DCC*. CIN tumours are microsatellite stable (MSS) and CpG island methylator phenotype (CIMP) negative.

KRAS mutations are among the most common in sporadic CRC, occurring in 30-40% of all CRCs.[190] *KRAS* is an important proto-oncogene downstream of EGFR, with over 95% of mutations occurring in codon 12/13 of exon 2. Among the extended *RAS* family of oncogenes, *NRAS* mutations occur in 3-5% of CRCs. *BRAF* is a RAF kinase downstream of *KRAS*, and is mutated in 10% of CRCs. Mutations in *KRAS, NRAS* and *BRAF* all predict resistance to anti-EGFR therapy. [181] Apart from the RAS/RAF pathway, mutations in *PTEN* are seen in 5-10%, with mutations in *PIK3CA* seen in 15-20% of cases. Mutations in both these genes also render resistance to anti-EGFR therapy.[181]

Beyond the EGFR pathway, a number of other genes mutations occur with variable frequency in CRC, including *APC* in 51-81%, *TP53* in 20-60% and *SMAD4* in 10-20% of cases.[181] *APC* mutations are the most prevalent in the development of sporadic CRC. *TP53* mutations similarly, occur early in the carcinogenesis pathway. *SMAD4* encodes a

tumour suppressor gene that regulates activity downstream of TGF-β receptor signalling.

2) Microsatellite unstable (MSI-H) cancers account for approximately 15% of CRCs. They can occur through germline mutations in mismatch repair genes or sporadic hypermethylation of mismatch repair genes. Microsatellites are short repeating base sequences that are prone to damage or slippage during DNA replication. They occur throughout the genome, including in genes relevant to CRC. Damage to these sequences are normally repaired by mismatch repair genes. Failure to repair these due to defects in mismatch repair genes leads to MSI-H cancers. A germline mutation in mismatch repair genes is the hallmark of Lynch syndrome, or hereditary non-polyposis colorectal cancer (HNPCC), accounting for 2-3% of all CRCs. Additionally, frameshift mutations in MSI-H cancers lead to the development of potentially targetable neoantigens.[191]

3) Methylator tumours are the third pathway, arising due to epigenetic events. Epigenetic events are events that occur independent to the actual gene sequence, with the gene sequence left intact, while its function is altered. There is generally an associated driver mutation in *BRAF* or *KRAS* genes. DNA methylation, where methyl groups are added to the CpG promoter area, leads to silencing of gene expression and therefore loss of gene expression. This typically occurs in tumours of the CpG island methylator phenotype (CIMP-positive).

CIMP-positive tumours can be MSI-H, due to methylation of *MLH1*. There is thus an overlap between methylator tumours and MSI-H in a number of sporadic CRCs. The primary mechanism is often hypermethylation of one of the mismatch repair genes, leading to an acquired MSI-H phenotype. Overall, a small proportion of MSI-H cancers are due to Lynch syndrome, with the majority caused by epigenetic silencing of the promoter region of a mismatch repair gene (commonly *MLH1*).[192]

It is important to understand the various pathways, as they have different prognostic implications. MSI-H cancers confer a better prognosis than CIN cancers in stages I-III, with CIMP-positive cancers having a worse prognosis.[193] The improved prognosis with MSI-H cancers is believed to be due to an increased tumour mutation load, and presence

of increased neo-antigens, contributing to an amplified host immune cell response.[194] While MSI-H cancers do not respond well to 5-FU monotherapy, they do demonstrate a durable response to irinotecan and oxaliplatin based therapies.[195, 196] Recently, the advent of immunotherapy in the form of anti-PD-1 checkpoint blockade has shown improved efficacy in the treatment of Stage IV MSI-H cancers.[197, 198]

1.4.1.2 The immune system in cancer

In 1909, Ehrlich postulated that the immune system plays a pivotal role in the suppression of cancer.[199]

Many decades later, as we slowly gained an improved understanding of tumour immunobiology through transplantation immunology, Burnet[200, 201] postulated that tumour cell specific neo-antigens could provoke an effective immunologic reaction that could eliminate developing cancers. Thomas[202] similarly theorised that complex organisms such as humans possess innate mechanisms to protect against neoplasia, similar to those mediating organ transplant rejection. In transplantation models, tumours are rejected by the host, while normal tissue is accepted, confirming the concept of tumour-specific antigens. With further evidence in murine models of tumour specific antigens supporting the ideas of Burnet and Thomas, the concept of 'cancer immunosurveillance' developed. Despite surveillance, cancers do develop. Over time, it was recognised that immunosurveillance represents a small part of a highly complex relationship between the immune system and cancer.[199, 203] These observations slowly led to the development of the cancer immuno-editing hypothesis.

Cancer immuno-editing is a dynamic process that describes the interaction between the immune system and tumour. It comprises three phases: Elimination, Equilibrium and Escape (Figure 1.5).

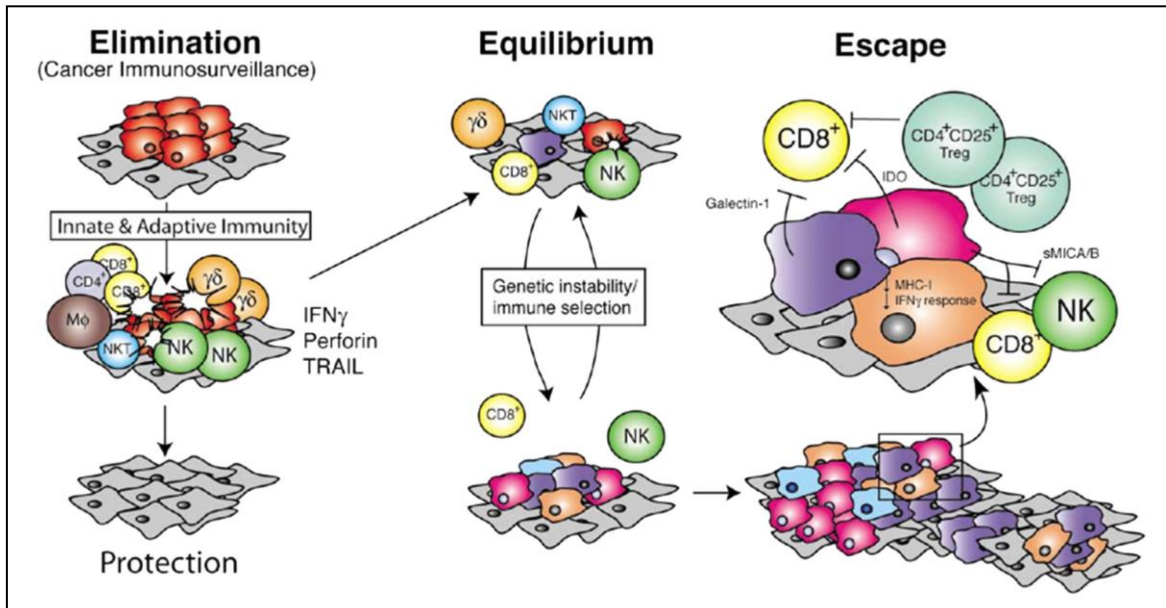


Figure 1.5. Cancer immuno-editing demonstrating the concept of the three E's; elimination, equilibrium and eventually escape (Dunn et al)[199]

'Elimination' represents the concept of cancer immunosurveillance, where the host immune system is able to eliminate any tumour cells through recognition of tumour antigen as foreign.

Some tumour cells can escape destruction by the immune system. This leads to the 'Equilibrium' phase, a period of immune latency, where the immune system is able to keep tumour cells at bay, but unable to completely eradicate them. Tumour cells are able to survive, but have yet to develop other mechanisms to outgrow the immune system completely.

The 'Escape' phase reflects the stage when tumour cells outgrow the immune system's ability to restrict them. This is when tumours become clinically detectable. Tumours can escape through a variety of evasion mechanisms, which may vary based on site.[199] Metastatic sites may experience the most significant immunologic sculpting with tumour cells having first survived the immune system at the primary site and then again at the site of metastasis.

Immune escape is postulated to occur as a consequence of genetic alterations in the tumour targets themselves. For example, some tumours may develop the ability to alter

antigen processing and presentation to evade the adaptive immune system. This would lead to tumours being able to down regulate expression of major histocompatibility complex (MHC) class I or II proteins.[199, 204] In melanoma for example, truncated mutations in the B_2M subunit of the MHC I complex allows melanoma cells to evade immune recognition through down regulating MHC class I.[205] Others tumours may adapt and become unresponsive to IFN-δ, rendering them resistant to $CD8^+$ mediated cytotoxicity.[206] More recently, it is becoming clear that tumours also establish a microenvironment that actively suppresses the immune response.[207, 208] Understanding the tumour microenvironment (TUME) is therefore an area of intense ongoing research.

The role of the immune system in the interplay between immunity and cancer can be divided into two broad systems: the innate and adaptive immune system

1.4.1.2.1 The Innate Immune system

While the two arms of the immune system have historically been viewed independently, recent evidence suggest that they work in concert. The innate immune system consists broadly of leucocytes such as Natural killer (NK) cells, Natural killer T (NKT) cells, as well as phagocytic cells such as Macrophages (MΦ), Dendritic cells (DC) and Neutrophils. The innate immune system offers immediate action against pathogens, with a non-specific defense mechanism where antigen presentation is not required.

Natural killer cells, first described in the 1970s,[209, 210] are lymphocytes that circulate through the blood stream and reside in all organs. They were first identified for their ability to kill tumour cells without deliberate activation or immunisation. During maturation, NK cells undergo education so that they remain tolerant to healthy host tissue. In 1986, Karre et al[211] demonstrated NK cells attack cells that lack expression of MHC class I. Further work demonstrated that NK cells carry an inhibitory receptor that specifically recognises MHC class I molecules and suppresses NK cell function, preventing attack against self. NK cells also kill cells infected with viruses, and are able to eliminate stressed, infected or transformed cells without prior sensitisation, minimising damage to normal host tissue.[212] They are activated *in-vivo* and *in-vitro* by various cytokines such as IL-2, IL-12, IL-15 and IFN-α/β.[213] Upon activation, NK cells

can secrete a variety of cytokines and chemokines including IL-4, IFN-γ, TNF-α, GM-CSF, IL-8, macrophage derived chemokine, macrophage inflammatory protein-1α (MIP-1α) and regulated on activation, normal T-cell expressed and secreted (RANTES).[212, 214] In addition, they can also produce cytolytic molecules like granzyme B and perforin without the need for transcription.[215]

1.4.1.2.2 The adaptive immune system

The adaptive immune system comprises of T cells, B cells, and their respective subsets. They are highly specific in their response to antigens. T cells mature in the thymus, where they gain the ability to differentiate between self and non-self. The two main subsets of T cells are $CD4^+$ T cells and $CD8^+$ cytotoxic T cells. $CD4^+$ and $CD8^+$ T cells recognise tumour antigen through expression of MHC class II and class I proteins on their cell surface respectively. $CD4^+$ T cells further comprise two distinct lineages: T Helper cells (Th), and $CD4^+$ $FoxP3^+$ T regulatory (Treg) cells.

Th cells recognise antigens as peptide epitopes 12-20 residues long, presented by MHC class II molecules found on antigen presenting cells (APC) such as DCs, MΦ, or B cells.[216] They are responsible for regulating various immune cells through release of cytokines that lead to promoting or suppressing anti-tumour responses. While there are many subgroups, the most commonly described ones are Th_1, Th_2 and Th_{17} (Figure 1.6).

Th_1 and Th_2 are the best described Th cell lineages. They are defined by their transcription factors *Tbx2 1* (T-bet) and *GATA-3*, respectively.[217]

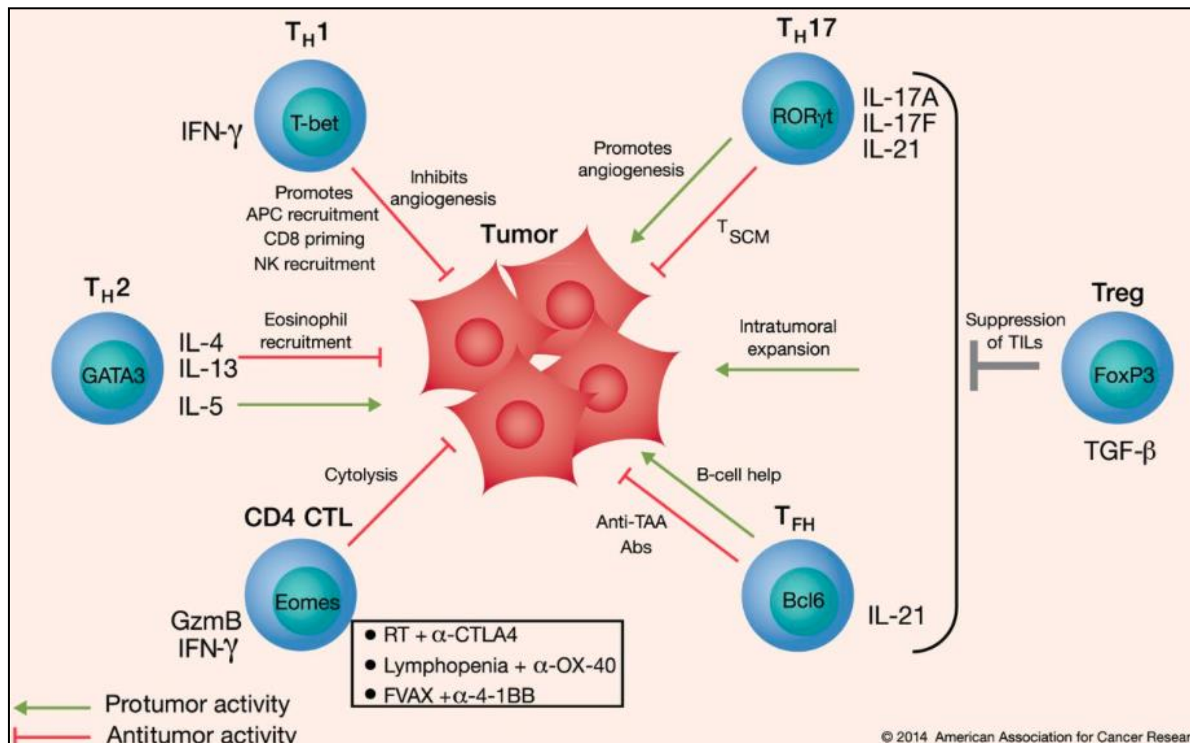


Figure 1.6. Contributions of CD4+ T cell subsets to anti-tumour immunity.
Th1 cells promote anti-tumour activity through APC and NK cell recruitment, priming of CD8+, and inhibiting angiogenesis. Th2 contribute to anti-tumour responses through IL-4 and IL-13 and pro-tumourogenic effects through IL-5. Tregs regulate immune homeostasis and self-tolerance. Intratumoural Tregs impair anti-cancer immunity, with high levels of Tregs correlating with poor survival in many cancers (Kim et al)[217]

Th1 cells secrete cytokines such as IL-2, IFN-γ, TNF-α, lymphotoxin (LT) and MIP-1α. They are responsible for cellular immunity by activating immune responses against intracellular pathogens by enhancing CD8+ T-cell responses or activating MΦ to undertake phagocytosis. Th2 on the other hand secrete cytokines such as IL-4, IL-5, IL-13 and regulate humoral immunity, eosinophilic inflammation and control allergic responses.[216] Th1 are broadly responsible for clearing intracellular pathogens, while Th2 are involved in clearing extracellular pathogens.

While both mediate anti-tumour immunity, Th1 responses are more potent, through production of large amounts of IFN-γ, along with chemokines that enhance priming and expansion of CD8+ cells. IL-2 production by Th1 cells serves to recruit and retain cytotoxic CD8+ T cells at the tumour site. IFN-γ production by Th1 cells leads to upregulation of MHC molecules on tumour cells, leading to enhanced T cell recognition.[216]

Th_1 cells also recruit NK cells and type I MΦ, to work in concert towards tumour elimination.[218]

The role of Th_2 is more complex, with its effects believed be to site dependent. IL-4 secreted by Th_2 has anti-tumour effects. However, Th_2 have also been shown to promote tumour growth in pancreatic cancer, with IL-5 production by Th_2 correlating with tumour progression in renal cell cancer and melanoma.[219]

Th_{17} are a further subset of regulatory $CD4^+$ T cells that secrete IL-17A, IL-17F, IL-21, IL-22, GM-CSF, TNF-α and IL-6.[220] They are differentiated by a combination of TGF-β1, IL-1 and IL-6, which induces *RORγt*, a transcription factor that characterises them. They are postulated to be critical drivers of autoimmune tissue inflammation and autoimmune diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis. Th_{17} cells have also been shown to be highly pathogenic.[221]

The exact factors governing pathogenicity of Th_{17} cells are not well understood. While generally induced by TGF-β1 and IL-6 from naïve $CD4^+$ T cells, interactions with IL-23 produced by other innate cells appear to be critical in stabilising Th_{17} cells, and transformation into acquiring pathogenic functions.[220, 221] Recent studies have confirmed the role of IL-23 in the induction of pathogenic Th_{17} cells, and found TGF-β3 to be the driving force behind the pathogenicity of Th_{17} cells.[222]

Tregs are characterised by expression of the forkhead/winged-helix transcription factor box P3 (*FoxP3*). Tregs help maintain immunologic homeostasis and self-tolerance. Identification of Tregs is somewhat problematic, as many of the recognised Treg markers ($CD25^+$, CTLA-4, LAG-3, $FoxP3^+$, CD127) represent general T-cell activation markers, and are not necessarily Treg specific.[223] The *FoxP3*$^+$ transcription factor is however considered the most reliable marker for Treg cells.[224]

The exact prognostic impact of Tregs is debated in the literature. In normal tissue, Tregs suppress cytotoxic $CD8^+$ T-cell function as a mechanism to minimise auto-immunity. This is through secretion of immunosuppressive factors including IL-10, TGF-β and CTLA-4.[225] These factors serve to suppress cytotoxic T lymphocyte (CTL) function and IFN-γ production.

There is growing evidence that tumours can promote $FoxP3^+$ Treg recruitment and expansion. While Treg proportions are normally 5-10% of $CD4^+$ T cells in peripheral lymphoid tissues, they may be substantially higher in tumours.[216, 226] Inflammatory cytokines and chemokines such as CCL2 and CCL21 promote accumulation of $FoxP3^+$Tregs and are associated with robust tumour growth.[227] Additionally, intratumoural Tregs can impede anti-cancer immunity, with high Treg/$CD8^+$ ratios in tumour infiltrates correlating with poor prognosis.[217]

Tregs also secrete TGF-β, which is strongly associated with pro-tumourogenic changes in advanced cancer development.[216]

A systematic review[228] found the presence of Tregs have a variable prognostic impact based on tumour type and location. In hepatocellular cancer, high levels of $FoxP3^+$ T cells were associated with a poor prognosis. However, in other cancers like CRC, ovarian cancer and breast cancer, Tregs appeared to have conflicting prognostic impact, largely due to heterogeneity between studies.

Recently, Saito et al[229] demonstrated that there are two types of $FoxP3^+$ cells in CRC, based on whether they were $FoxP3^{high}$ or $FoxP3^{lo}$. CRCs with a high infiltration of $FoxP3^{lo}$ were associated with a good prognosis, while those infiltrated with $FoxP3^{high}$ were associated with a poorer prognosis. Overall, it is likely that some of the discrepancies in the prognostic impact of Tregs is related to the variability in cell surface and nuclear markers used to identify Tregs, highlighting the need to standardise accepted surface markers for Tregs.

Cytotoxic $CD8^+$ T cells (CTLs) are key effector cells in the adaptive immune system, that detect peptides presented by MHC class I. $CD8^+$ T cell mediated cytotoxicity is based on two broad phases: priming and effector phases. In the priming phase, DCs present antigen to $CD8^+$ T cells in a receptor-ligand mediated fashion. Other immune cells such as NK cells and activated $CD4^+$ cells provide co-stimulus with the production of necessary cytokines such as IL-2. IL-2 also leads to rapid proliferation and expansion of $CD8^+$ T cells. Primed CTLs can then traffic to the target site to execute efficient $CD8^+$ T cell cytolytic activities.

CTLs kill target cells through a few key pathways. The first involves secretion of cytokines such as TNF-α and IFN-γ that induce cytotoxicity. The second involves exocytosis of granules such as granzyme A and B. When cancer cells are encountered, perforin is released by CTLs that make pores in the membrane of cancer cells, creating a pathway for granules to enter the cell and cleave the intracellular structures. Granzymes are proteinases that can cleave intracellular proteins eventually shutting down production of key proteins in the cell, causing the cell to undergo apoptosis. The third mechanism involves Fas ligand (FasL) mediated apoptosis.[207, 230] CTLs express FasL on their surface, which can bind to the Fas receptor on cancer cells. This leads to activation of caspases, eventually leading to cytochrome c release in the target cell and apoptosis of the cancer cell.[207]

Activated CTLs also express co-inhibitory receptors such as programmed death-1 receptor (PD-1) in response to IFN-γ in the environment. This can lead to suppression of T-cell function upon interaction with programmed death ligand-1 (PD-L1) expressed on tumour cells, and other immune cells such as macrophages.[207]

In recent years, numerous studies have demonstrated that high levels of tumour infiltrating lymphocytes (TILs), particularly $CD8^+$ T cells, correlate with a favorable prognosis.[231-234] However, merely the presence of TILs does not inform us of whether TILs recognise tumour antigen, whether they are activated, or even functional. Furthermore, for a T cell to be activated against an antigen, the antigen peptide needs to bind to MHC class I with sufficient affinity for adequate antigen presentation to induce T-cell activation. Therefore, while the presence of TILs is no doubt of great importance, it is possibly of greater importance to understand the expression and functional status of the TILs.[235]

1.4.1.3 The immune system and the tumour microenvironment in colorectal cancer

In recent decades, there has been intense interest in the role of the adaptive immune system in controlling cancer growth and recurrence. In 1986, lymphocytic infiltration was shown to be associated with survival in patients with rectal cancer.[236] Since then,

there has been an enormous body of research exploring the relationship between the immune system and prognosis in CRC.

Pages et al[38] demonstrated that the presence of an immune response in the form of a high proportion of $CD8^+$ T cells, ranging from early memory to effector memory cells correlated with a lack of metastases and improved survival. Galon et al[40] demonstrated in a seminal study that the type, density and location of tumoural immune cells in primary CRC were a better predictor of survival than the standard TNM staging system. In this study, a strong immune infiltration in the tumour center and invasive margin correlated with a favourable prognosis, regardless of stage and regional lymph node status. Conversely, a weak immune reaction in both tumour areas correlated with a poor prognosis, even in patients with stage I CRC. More specifically, density of $CD3^+$ cells and $CD45RO^+$ memory T cells in the tumour centre and invasive margin were predictive of DFS and OS (Figure 1.7).

In CRC, this study was one of the first to demonstrate that once CRCs become detectable, the adaptive immune system plays a governing role in preventing recurrence and determining OS. Subsequent studies[237, 238] have since also shown that an increased T cell infiltrate is associated with improved survival in CRC.

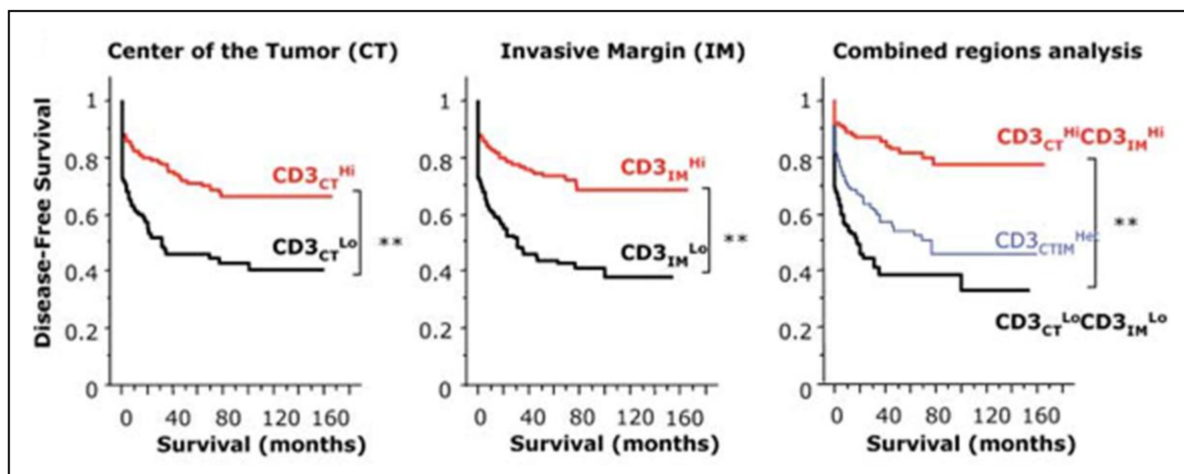


Figure 1.7. An increased presence of $CD3^+$ cells in the tumour centre, invasive margin and on combined analysis of both regions were consistently associated with a better disease free survival (Galon et al)[40]

The role of the tumour microenvironment (TUME) has gained significant attention in recent years, given its role in influencing the behaviour, recruitment and function of immune cells. The TUME can be broadly divided into two components: the tumour and stroma. With increasing recognition of the CMS classification,[33] it is becoming evident that the tumour establishes a microenvironment wherein the role of the stroma plays a critical role in influencing the immune milieu, and determining progression of CRC. These include cells such as tumour-associated macrophages (TAMs), cancer associated fibroblasts (CAFs), DCs and myeloid derived suppressor cells (MDSCs) (Figure 1.8).

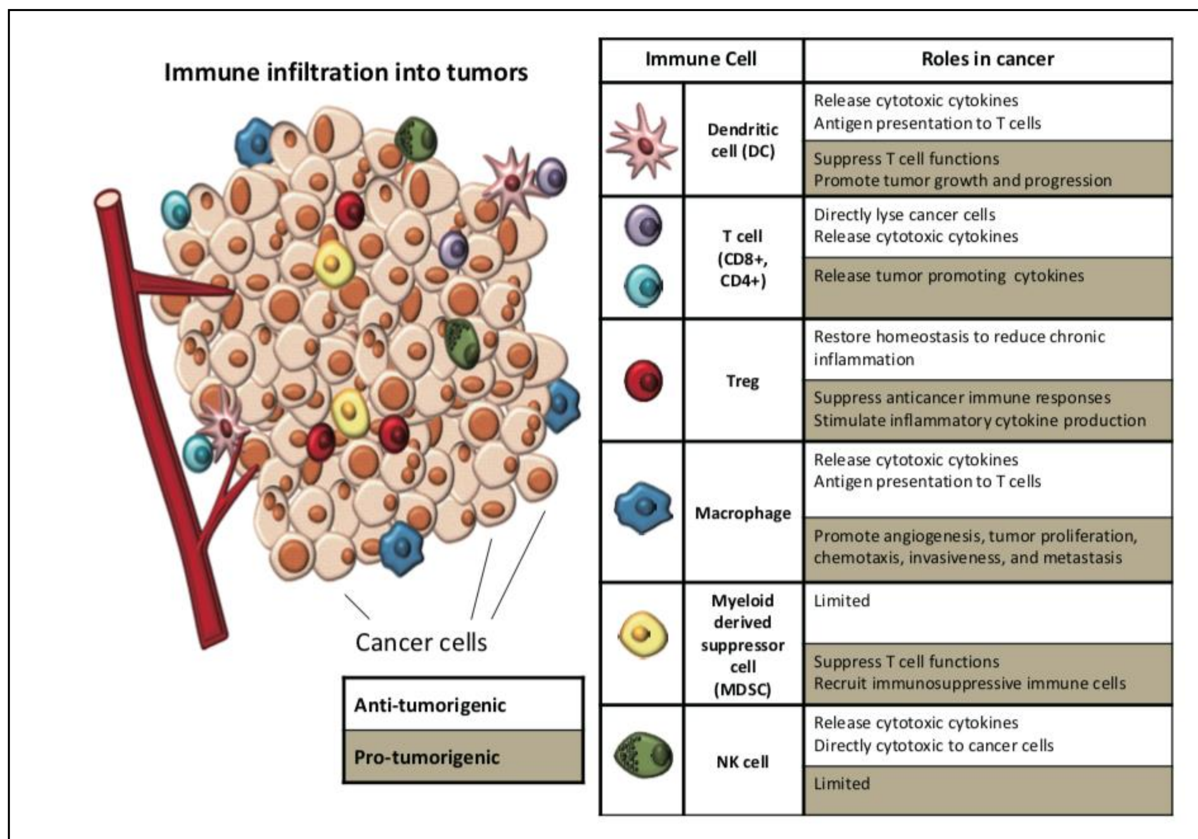


Figure 1.8. The common cell types involved in the tumour microenvironment. Many of the immune cells have pro and anti-tumourogenic effects, as demonstrated above. $CD4^+$ pro-tumourogenic effects reflect the role of Th_2 and Th_{17} cells (Markman et al)[239]

Tumours utilise various mechanisms to suppress the immune response within the TUME. One mechanism is by secreting potent immunosuppressive factors such as TGF-β to prevent CTLs from destroying the tumour. TGF-β acts on both tumour cells and the surrounding environment. Tumour and stromal cells secrete TGF-β through a self-sustaining manner, where there is increased protease mediated activation of latent

TGF-β, along with the self-induction of TGF-β gene activity, and recruitment of other TGF-β secreting cells such as Macrophages. Apart from its early growth inhibition of tumour cells, most TGF-β activities promote tumour growth and progression.[239]

TGF-β is an important cytokine associated with tumour progression, produced by many immunosuppressive immune and stromal cells (Figure 1.9). Chromosomal alterations involving TGF-β contribute to the CIN pathway in CRC development. In normal tissue and early CRC development, the TGF-β pathway acts as a tumour suppressor by maintaining cellular homeostasis, inhibiting cell proliferation, and inducing apoptosis. During advanced stages of CRC development, the TGF-β pathway becomes pro-tumourogenic and activating the pathway leads to cancer progression, invasion and metastatic growth and development.[240, 241]

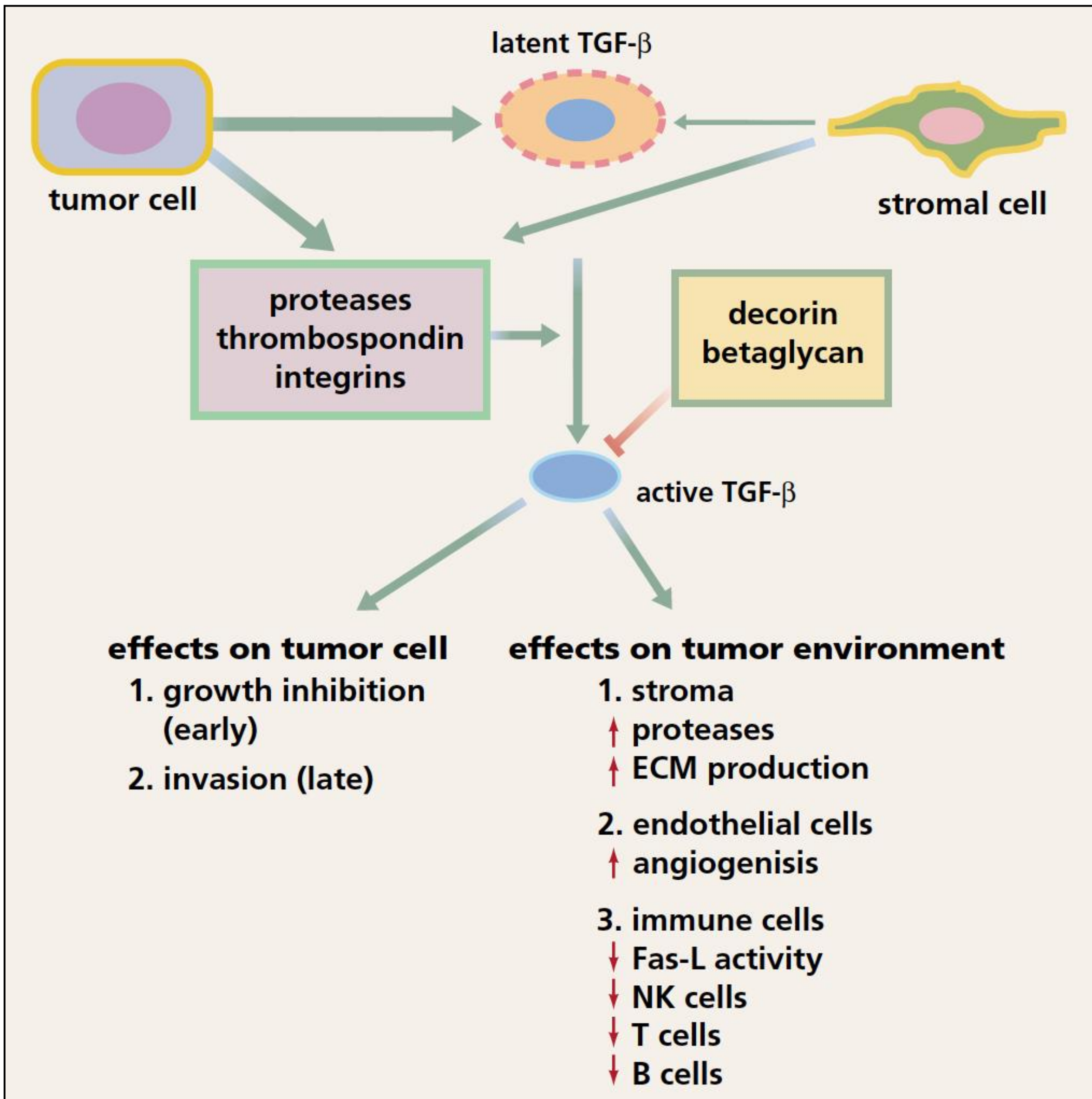


Figure 1.9. TGF-β acts on both the tumour and tumour microenvironment. Tumour and stromal cells both produce TGF-β, which is activated by protease activity. Apart from its action as a growth inhibitor in the early stages of tumour development, most TGF-β activity is pro-tumourogenic (Derynck et al)[241]

Tumours also recruit immunosuppressive immune cells such as Tregs and MDSCs. The prognostic impact of Tregs is complex in CRC, as described previously. In studies where Tregs are reported to be associated with a poor outcome, they function by suppressing production of cytokines, as well as reducing activation and proliferation of $CD4^+$ and $CD8^+$ T cells.

MDSCs are a heterogenous group of myeloid derived cells comprising myeloid progenitors, immature macrophages, immature dendritic cells and immature granulocytes that expand during cancer, inflammatory conditions and infection. They produce factors like arginase-1, which suppress T-cell activity,[242] and TGF-β that enhance Treg immunosuppressive activity. They are characterised by a range of markers including $CD11b^{+}$ $CD33^{+}$ or $HLA\text{-}DR^{-}$ $CD33^{+}$.

Macrophages may constitute more than half the immune cells in the stroma.[243] They can vary their response depending on the signals received from other stromal components. Broadly, M_1-macrophages are pro-inflammatory, activated macrophages that promote an anti-tumour immune response. M_2-macrophages on the other hand, are pro-tumourogenic and suppress the immune response. The presence of M_2-macrophages is associated with poor prognosis.[207] In the early stages of cancer, macrophages in the tumour display an M_1 phenotype. However, as the cancer progresses, there is a functional switch to M_2, which contributes to tumour progression.[70]

Tumour associated M_2-macrophages (TAMs) promote angiogenesis, tumour proliferation and invasive potential. They also secrete IL-10, TGF-β and arginase and cause T-cell dysfunction through TNF-α and IL-10 mediated induction of PD-L1.[70] Tumour cells undergoing necrotic cell death lead to recruitment of immune cells including macrophages, myeloid progenitors and triggering of pro-inflammatory cytokines such as IL-1 and HMGB1.[244, 245] Together, these factors lead to an angiogenic switch, with development of new vasculature. These pro-tumourogenic immune cells also produce factors such as NF-κB and STAT3 that further promote tumour cell proliferation and survival.[246] Macrophages also secrete metalloproteinases and cysteine cathespin proteases that break down extracellular matrix allowing cancer cell invasion. MMP-9 secreted by macrophages degrades type IV collagen, leading to disruption of the basement membrane, which subsequently contributes to allowing tumour invasion. High RNA expression levels of MMP-9 have been shown to be independently associated with a worse disease free and overall survival in patients with CRC.[247]

There are many proposed mechanisms by which cancers evade the TGF-β tumour suppressive pathway to proliferate, and consequently utilise this pathway for tumour

progression. One mechanism is through inactivating mutations in the TGF-β signalling pathway, the receptors and the *SMAD* genes. Loss of *SMAD4* results in the loss of the TGF-β tumour suppressive function, transforming it into a tumour promoter.[248] As tumours progress, epigenetic and genetic changes in the tumour cell and microenvironment may switch the TGF-β pathway from a tumour suppressor to pro-tumourogenic.[249] Mutations in other key genes such as *TP53* can also alter the TGF-β response. Phosphorylation of mutant p53 can lead to complex ligand interactions, eventually enabling TGF-β to promote EMT, invasion and metastasis.[250]

Fibroblasts constitute a significant proportion of the non-immune cell population in the TUME. Subsets of fibroblasts called cancer associated fibroblasts (CAFs) are strong contributors to the immunosuppressive TUME. They are made up of activated fibroblasts, characterised by α-smooth muscle actin (α-SMA)-positive myofibroblasts. CAFs are also characterised by a number of factors including platelet-derived growth factor–alpha/beta (PDGFα/β), vimentin and fibroblast activation protein.[251] Various factors such as cancer cell produced TGF-β, PDGF, IL-1β are believed to be responsible for conferring an activated CAF phenotype over a non-activated resident fibroblast.[252]

CAFs contribute to tumour progression by influencing cancer stemness, neo-angiogenesis, inducing drug resistance and promoting ECM stiffness. They promote tumour cell invasion based on signalling relevant to ECM modeling such as JAK1-STAT3 and Rho-associated protein kinase pathways.[251] CAFs produce TGF-β that favour Th_2 polarisation in tumours. They also produce CXC-chemokine ligand 8 (CXCL-8), IL-4 and IL-6 that support M_2-macrophage polarisation,[253] and secrete VEGF-A, and other factors to promote neo-angiogenesis. The tumour promoting role of CAFs are slowly being understood in recent years. The development of prognostic CAF markers and therapeutic approaches at targeting CAFs is still some years away.

Table 1.6. Summary of cytokines, cells of origin and effects on the tumour and tumour microenvironment.

Cytokine	**Cells responsible for production**	**Major effects**
TNF-α	CTL, M_1-macrophages, Th_{17}	Anti-tumour: stimulate activity of anti-tumour immune cells[216]; inhibit angiogenesis in cancers[230]; induces apoptosis in cancer cells[230] Pro-tumour: impair $CD8^+$ infiltration into tumour[70, 207]
IFN-γ	CTL, M_1-macrophages, Th_1, NK	Anti-tumour: induces MHC-I upregulation on cancer cells; cytotoxic to cancer cells[216]; promote NK cell activity[212, 213]; suppress angiogenesis in cancer cells[230]; induces macrophage polarity to M_1[70]; induces $CD8^+$ T cells into antigen specific CTLs[207]; induces $CD4^+$ differentiation into Th_1[216] Pro-tumour: continous exposure leads to T-cell exhaustion and tumour progression; leads to upregulation/release of PD-L1, IDO1, STAT3 that suppress T cells.[207]
IL-2	Th_1	Anti-tumour: activates several aspects of innate and adaptive immunity; promotes CTL and NK cell proliferation, induces M_1-macrophage polarisation[216, 217] Unknown effect on tumour: transition of Treg into functional $FoxP3^+$ Treg[216]
IL-4	Th_2	Anti-tumour: Th_2 growth, suppresses Th_1[219]
IL-5	Th_2	Pro-tumour: promotes tumour progression[219]
IL-17A	Th_{17}	Pro-tumour: promotes tumour growth, neo-angiogenesis.[220]
TGF-β	Tumour cells, CAFs, M_2-macrophages,	Anti-tumour: anti-tumour in early stages of cancer development[240, 241]

	MDSCs, Th_{17}, Treg	Pro-tumour: potent inducer of cancer progression; anti-inflammatory mediator for immune suppression[254]; resistance to immunotherapeutics; recruit immune suppressive cells, blocks IL-2 effect, CAF proliferation[216, 240, 241, 249, 254, 255]

1.4.1.4 Immune system in peritoneal metastases

The peritoneum is a single layer of mesothelial cells with a surface area of approximately 1.8m^2.[256] It is composed of two layers, the parietal and visceral peritoneum. The parietal peritoneum covers the abdomino-pelvic cavity, while the visceral peritoneum covers most of the visceral organs. Mesothelial cells arise from the mesoderm and have epithelial and mesenchymal features. They originate as fibroblasts from the mesoderm, but their function resembles epithelial cells. They express molecular features of both mesoderm (vimentin) and epithelium (cytokeratins). Under TGF-β influence, they adopt a more spindle shaped appearance typical of cells undergoing EMT.[70] Mesothelial cells are linked by intercellular junctions, with small openings present that provide access to the submesothelial lymphatic system. Under normal conditions, the peritoneal cavity contains 5-20mL of peritoneal fluid that is produced by peritoneal capillaries, and allows frictionless movement of the abdominal organs, which enables exchange of immune cells and other molecules with plasma. The fluid is renewed every one to two hours.[257]

Monocytes and macrophages normally make up 50-90% of the leucocytes in normal peritoneum.[258] Studies have shown that peritoneal macrophages predominantly express features such as CD206, highly characteristic of M_2-macrophages.[259] Therefore, while M_1-macrophages serve to dispose of debris and pathogen, the M_2-macrophages are capable of secreting Th_2 related anti-inflammatory cytokines including IL-4, IL-10, IL-13 and TGF-β, thereby suppressing immune and inflammatory responses. They also express Prostagladin E2, VEGF and ICAM-1, helping promote angiogenesis and tissue repair that promote tumour growth.[259] Overall, the potentially immunosuppressed environment provided by M_2-macrophages could be one of the reasons why PM can

grow rapidly once established. B1 lymphocytes are the second most common leucocyte in normal peritoneum, being a source of antibodies. Other innate and adaptive immune cells such as T-cells, DCs, NK cells are also present, albeit in small numbers.

In response to injury or peritoneal disruption, macrophages increase phagocytic activity, generate reactive oxygen species and recruit mesothelial cells and fibroblasts to aid in repair.[258] Similar signals that promote cell survival, proliferation, and neo-angiogenesis for tissue repair are released, which also help cancer cells gain footing and grow.

Inflammation, with repair and remodeling of the peritoneal surface is a common feature in PM. TAMs release proteases, and chemokines such as CCL-2 and CXCL-8, which promote tissue remodeling and recruitment of CAFs.[258, 260] During this process, further growth factors, inflammatory mediators and angiogenesis factors such as VEGF-A, NF-κB, TGF-β, EGF promote angiogenesis under hypoxic conditions. The very presence of ectopic tissue on peritoneum in the form of PM leads to an overall overproduction of prostaglandins, cytokines, inflammatory mediators and chemokines that sculpt the peritoneal environment to suit the cancer cells.

While the immune landscape of primary CRC has been explored extensively, the role of the immune system and TUME in CRPM remains poorly defined. There is very little data to even indicate whether T cells and the adaptive immune system are able to infiltrate CRPM. Understanding the role of the immune system in CRPM may provide more insight into why the immune system is unable to fight PM, and help develop new strategies in targeting this disease.

1.4.2 Emerging treatment options

1.4.2.1 Immunotherapy

The major obstacles we face in advancing anti-tumour immunity are central and peripheral tolerance, and tumour driven immunosuppression. Central tolerance refers to the concept of eradication of potential tumour reactive T cells during T cell

maturation in the thymus.[207] This limits the availability of the T cell repertoire. Peripheral tolerance refers to poor activation of DCs and other APCs or conversely, activation of immunosuppressive T cells by the tumour. It is imperative to overcome peripheral resistance to offer a strong anti-tumour T cell response. Tumour-associated immunosuppression is based on the impact of the tumour and the TUME in creating an immunosuppressive environment.

The earliest records of immunotherapy trace its roots to the work of William Coley, a New York surgeon who noted that toxins from bacterial infections were able to activate the immune system to eventually fight cancer.[261] Subsequent work evolved from the work of Old et al,[262] the Bacille Calmette-Guerin (BCG) vaccine and tumour necrosis factor. The next few decades led to the understanding of immune recognition, immunosurveillance, and roles of specific immune cells such as T cells, DCs and the tumour along with its microenvironment. Many have explored the use of other agents such as viruses, vaccines, and antibodies in their efforts to harness the immune system to battle malignancy.

Immunotherapy is now rapidly becoming the fourth pillar of cancer care, alongside chemotherapy, radiotherapy and surgery. Immunotherapy broadly refers to any therapeutic approach that harnesses the immune system to fight cancer. Immunotherapy aims to invigorate anti-tumour responses and generate a strong CTL response through a variety of methods including vaccination and checkpoint antibodies. Enhancing the response from CTLs is the first aim for targeting relatively non-immunogenic cancers like CRC. The use of immunomodulating antibodies in the form of anti-PD-1 and anti CTLA-4 antibodies has revolutionised the treatment landscape for various cancers in recent years.[263]

The focus of immunotherapy is to improve the priming of CTLs in lymphatic tissues, enhance the activity of CTLs within a tumour, and ensure durable anti-tumour immunity. Use of immunomodulatory monoclonal antibodies such as anti-PD-1 antibodies, has been one of the key breakthroughs in immunotherapy. These antibodies do not target cancer cells directly, but are directed towards membrane receptors that

regulate T-cell response. Treatment with such immune checkpoint antibodies reactivate potentially tumour specific CTLs.

Checkpoint antibodies block inhibitory interactions between checkpoint proteins on $CD8^+$ T cells and their associated ligands on tumour cells or other immunosuppressive cells, enabling T cell activation and clonal proliferation. The two main checkpoint antibodies approved for use in clinical practice are anti-PD-1 targeting the PD-1/PD-L1 axis and anti-CTLA-4 targeting CTLA-4/CD80/CD86.

The introduction of anti-CTLA-4 (ipilimumab) in advanced melanoma has led to improved survival, with a 10-year survival rate of 20%.[264] Similarly, in patients with metastatic non-small cell lung cancer, the use of anti-PD-1 therapy (pembrolizumab) led to a significantly improved OS compared to use of standard chemotherapy.[263] In CRC, use of immune checkpoint inhibitors has shown significant efficacy in MSI-H CRC. Le et al[198] first evaluated the use of anti-PD-1 therapy (pembrolizumab) in patients with metastatic MSI-H and MSS CRC in a phase II trial. They reported a significantly improved response rate and progression free survival in MSI-H CRC compared to MSS CRCs. Subsequently, Overman et al[197] demonstrated in a Phase II trial that anti-PD-1 (nivolumab) therapy was safe in metastatic MSI-H CRC and offered durable response and disease control with a 86% one year survival. More recently, the combination of anti-PD-1 (nivolumab) with anti-CTLA-4 (ipilimumab) showed high response rates, with encouraging progression free survival and OS of 85% at one year in patients with metastatic MSI-H CRC.[265]

Mutational load in cancer cells varies widely, from as few as 0.8 to over 1200 coding mutations per megabase. These variations in tumour mutational burden (TMB) have been studied in various cancers,[266] with skin and lung cancers characterised by the most mutations, and leukemia with the fewest. CRC in general, has an intermediate TMB, which is however much higher in the MSI-H cohort. The number of novel peptides resulting from mutations varies in proportion to the total TMB, with cancers having a higher TMB shown to respond better to immunotherapeutic approaches such as checkpoint blockade.[267]

Mlecnik et al[268] demonstrated that MSI-H CRCs showed significantly higher expression of immune genes on transcriptomic analysis compared to MSS cancers. They found a common frameshift mutation in the gene coding for TGFβR2. Such mutations led to increased $CD8^+$ T cell recognisable antigens. However, merely being an MSI-H cancer is not a reliable biomarker for predicting response to immunotherapy, as only 53% of patients with MSI-H cancers showed an objective response.[269]

Similarly, while PD-1/PD-L1 expression has been correlated with response to PD-1/PD-L1 blockade, there remain numerous instances where patients still benefit from PD-1/PD-L1 blockade despite little or no PD-L1 expression, rendering expression of PD-1/PD-L1 as poor biomarkers to predict response to immune checkpoint antibodies.[270] Recently, Chen et al[271] compared the use of durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) to supportive care in an RCT with MSS patients with refractory metastatic CRC. They reported that use of dual checkpoint antibodies led to an improved OS of 6.6 months compared to 4.4 months (HR 0.72; 90% CI: 0.54-0.97). Furthermore, patients with a TMB over 28 mutations per megabase had the greatest OS benefit from immunotherapy. This study reaffirms that MSI-H status alone may not be the only predictor for response to checkpoint blockade, and that other factors such as TMB may be important in predicting response to checkpoint blockade.

1.4.2.1.1 Immune based approaches in CRPM

There have been very few novel immunomodulatory approaches in the management of CRPM. The use of monoclonal antibodies in the form of anti-EGFR (cetuximab or panitumumab) has entered routine care for metastatic CRCs based on their RAS mutation status. Similarly, there are a number of other immunotherapeutic agents such as vaccines, gene therapy and adoptive T cell therapy under investigation for metastatic CRC. There have however, been very limited studies specifically exploring immunotherapeutic agents in CRPM.

Seimetz et al[272] evaluated the intraperitoneal use of catumaxomab, a trifunctional antibody to treat malignant ascites from EPCAM expressing cancers. While initial trials

were promising and demonstrated symptomatic improvement in ascites, the drug was taken off the market in 2014 owing to other concerns.

Carcinoembryonic antigen (CEA) is overexpressed by many CRCs, however it can also be expressed by normal colonic epithelium. Parkhurst et al[273] utilised a CAR-T cell therapy against CEA in three patients with metastatic CRC. All three had objective responses, demonstrating the efficacy and cytotoxic capacity of highly activated CAR-T cells. However, the therapy also led to significant systemic toxicity and colitis in all three patients. This highlights the potential benefit of CAR-T cell therapy, but also the need to identify more specific tumour associated antigens.

Katz et al[274] utilised a similar CAR-T cell therapy, but as regional treatment in murine models. They found that intraperitoneal (IP) CAR-T cell therapy resulted in better control of peritoneal CEA^+ tumours than systemic therapy. Furthermore, they also found IP therapy led to protection against tumour growth at distant sites along with development of memory phenotypes that protected against re-challenge with IP tumour. In addition, they also found high levels of MDSCs and Tregs in the IP tumours expressing PD-L1. This suggests that CAR-T cells may have better efficacy if given regionally, and raises the possibility of combination therapy with immune checkpoint blockade.

With growing interest in vaccine based immunotherapy, Alkayyal et al[275] utilised an oncolytic Maraba MG1 virus expressing IL-12 in the treatment of CRPM in a murine model. The use of IL-12 served to promote migration of activated IFN-γ secreting NK cells to the peritoneal cavity. Use of the vaccine was associated with reduced tumour burden and improved survival in this model.

Recently, Froysnes et al[276] explored the IP use of a novel MOC31PE immunotoxin against tumour-associated EPCAM in a Phase I trial. The immunotoxin was found to be safe, with low systemic concentrations. Further data from Phase II trials are awaited to evaluate its feasibility and efficacy in CRPM.

While immune checkpoint antibodies in particular have demonstrated benefit in MSI-H CRC, its role in CRPM remains unexplored. As previously described, patients with CRPM

are greatly underrepresented in clinical trials involving patients with metastatic CRC. Furthermore, merely MSI-H status or PD-1/PD-L1 expression are not highly reliable biomarkers to predict response to checkpoint antibodies. MSI-H cancers are generally right sided, and mucinous in nature. Right sided and mucinous cancers in general tend to have a predilection to metastasise to the peritoneum[53, 277,57] Therefore, given such similarities between MSI-H cancers and those that lead to peritoneal metastases, it is conceivable that there may be similarities in other aspects such as response to therapies. The evaluation of immune checkpoint antibodies in CRPM is an entirely unexplored field that would be an extremely valuable area for research.[278]

1.5 Personalised medicine

Personalised medicine in cancer is a rapidly evolving area in therapeutics aimed at customising treatment to patients based on unique features of each patients' tumour.

This approach to treatment for cancer is the ultimate goal, as it ensures a more precise diagnosis and risk evaluation, thereby maximising treatment effectiveness and minimising side effects and toxicities. Over the years, there have been numerous advances that help select patients who are more likely to respond to certain therapies, and conversely help identify those who are at higher risk for side effects or complications without benefit.

1.5.1 Current application of personalised medicine in colorectal cancer

In the last decade, there have been several advances in personalised medicine for colorectal cancer. By 2010, studies had demonstrated that harboring a *KRAS* mutation was predictive of resistance to anti-EGFR monoclonal antibodies.[279, 280] This was the first move towards a precision-based approach in treating metastatic CRC. Now, all metastatic CRCs are routinely tested for RAS status. In more recent years, we have recognised the impact of *BRAF*^{V600E} mutations in older patients, which are classically on the right side, with over 10% of them being MSI-H.[198, 281] Given a higher neo-antigen load in MSI-H cancers, immunotherapy can now be offered these patients. The role of immunotherapy in the setting of high tumour mutational syndromes such as those with DNA polymerase (*POLE*) mutated tumours are still under investigation. More recently, the identification of CMS subtypes has assisted in the identification of molecular and genetic drivers of these subsets of CRC at a more fundamental level. This can help understand the aetiology of these subsets to help prognosticate and aid development of newer targeted therapies.

With regard to minimising side effects, the ability to test for deficiency of dehydropyrimidine dehydrogenase (DPD) can now identify those at high risk for

toxicity from the use of fluoropyrimidines such as 5-FU or capecitabine. These agents are among the most commonly prescribed chemotherapies for CRC. However, severe toxicities are seen in 10-40% of patients, with death in 0.8%. Approximately 3-15% of patients have a partial deficiency in DHD, with 0.1-0.5% having a complete DPD deficiency.[282, 283] Avoiding 5-FU or significant dose reduction in such patients would be of paramount importance to avoid serious side effects. In countries like France, it is routine to test patients for DHD deficiency before prescribing 5-FU.[283] Similarly, in some centres, UGT1A1 genotyping is performed to detect certain polymorphisms can help tailor dosages to minimise toxicity associated with the use of irinotecan.[284]

Despite advances made in the management of metastatic CRC, less than 15% survive to five years.[285] This number is conceivably even lower for patients with CRPM. With various advances in treatment, we can now reliably identify those who will not respond to certain therapies, and likewise those who may be at higher risk for side effects without response. While RAS mutations predict resistance to EGFR based therapies, and CMS helps explain the biological drivers in the tumour and the microenvironment, they do not provide a real time functional assessment of whether a specific drug will work for a specific tumour with its unique mutational pattern, or deal with intra-patient tumoural heterogeneity. Therefore, we still cannot reliably predict who may respond to a specific therapy.

Studies such as the MOSCATO 01 trial have successfully demonstrated that by using high throughput genomic analyses, treatment can be directed to patients based on each tumour's specific mutation profile. Massard et al[286] demonstrated that such an approach can improve outcomes in patients with hard to treat tumours of various types. However, with the use of genomics to tailor treatment, objective responses were only seen in 11% (95% CI: 7-17%) of patients. The MOSCATO 01 trial (NCT01566019)[287] evaluated a genomics based precision approach to treatment in patients with advanced biliary cancers. This trial demonstrated that by using real time molecular analysis to drive targeted therapies, a matched therapy was identified in 53% of patients. The overall response rate to targeted therapies was 33%, with 37% having a PFS of ≥6 months. These studies suggest that while molecular genomic analysis may be useful, it alone does not predict response to treatment in a large of proportion of cases, and a more

functional assessment of response may be necessary to accurately predict response to therapy.

1.6 Research tools in personalised medicine

1.6.1 Pre-clinical models

To study cancer biology, understand tumourogeneis and evaluate new therapies, pre-clinical models that recapitulate the native tumour in its architecture, genomics, and cellular interactions are essential. Pre-clinical models can be divided into mouse or human derived models.

While murine models have greatly advanced our understanding of diseases over the years, there remains significant differences between human and mouse tumours, limiting their clinical applicability.[288] Human derived models include cell lines and patient derived tumour xenografts (PDXs) (Figure 1.10). In recent years, the development of organoids as a novel human derived pre-clinical model has changed the research landscape of many diseases including CRC.

1.6.1.1 Cell lines

Cell lines are two-dimensional models of cancer that can be readily grown in the laboratory. They are one of the oldest pre-clinical models of disease, with the first cell line developed from a patient with cervical carcinoma in 1951.[289] They are homogenous, relatively easy to grow and propagate, and have formed the integral basis for much of basic science research over the years. However, they do have several limitations. Culturing cancer cells *in-vitro* leads to genotypic drift and loss of tumour heterogeneity.[290] While cell lines have been generated from aggressive and metastatic cancers, slow growing cancers are greatly under-represented. Furthermore, control cell lines from normal tissue are exceedingly rare.[291] Therefore, cell lines are unable to adequately model conditions such as cancer development and progression.

1.6.1.2 Patient derived Xenografts (PDXs)

Patient derived xenografts (PDXs) overcome some of the deficiencies of cell lines. They are developed by implanting fresh tumour tissue subcutaneously or orthotopically into immunocompromised mice. PDXs are a reliable way to expand tumour tissue; they recapitulate the tumour microenvironment with stromal elements better than cell lines, with lesser genetic divergence. These benefits make PDXs a useful pre-clinical model for biological assays and drug screening. They can be grown with approximately 70% success rates.[292] However, they are expensive, time and resource intensive. Furthermore, with PDXs being immunocompromised and lacking a functional immune system limits any potential immunotherapeutic testing.[293] Growing tumours in mice can lead to mouse related changes in the TUME,[294] making them less representative of the native tumour over time.

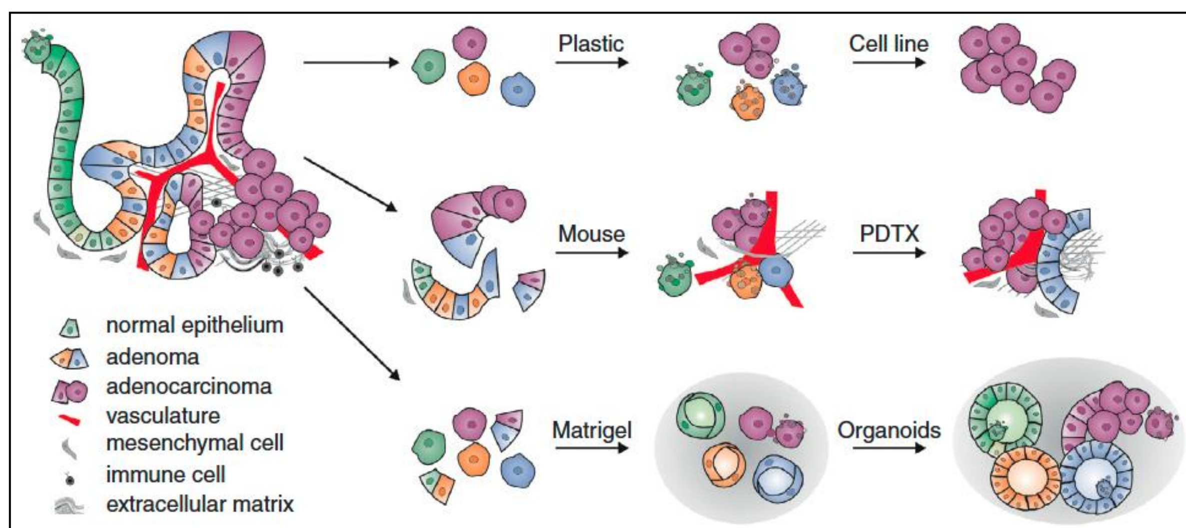


Figure 1.10. Existing pre-clinical models. Cell lines are two-dimensional models grown on a petri dish. Patient derived xenografts involve injecting a small tumour piece into an immunocompromised mouse. Organoids are three-dimensional clusters of cells embedded in a synthetic extracellular matrix (Sachs et al)[291]

1.6.1.3 Organoid models

Organoids are three-dimensional models of disease grown *in-vitro*. Pluripotent stem cells from the native tissue are embedded in a synthetic ECM and allowed to self

organise into clusters of cells that represent the native tissue. Organoids recapitulate tumour heterogeneity better than cell lines and murine models. Tumour heterogeneity is postulated to be one of the critical reasons for failure of many targeted therapies.[295] Therefore, development of a pre-clinical model that maintains intra-tumoural heterogeneity is vital to furthering accurate pre-clinical modeling of disease. Various studies have shown that organoids share over 90% of the genomic alternations seen in the native tumour, making them an ideal resource for pre-clinical studies.[290, 296]

Gastrointestinal organoids can be readily established from surgical resection specimens or percutaneous biopsies with high success rates. As organoids can be grown from normal colonic epithelium as well as cancer tissue, they provide highly reliable models to investigate cancer biology and tumourigenesis.

Organoids provide a valuable pre-clinical model for various diseases.[297] For example, human gastric organoids injected with *Helicobacter Pylori* reproduced typical features of *H.Pylori* infection.[298] This model is particularly relevant as species-specific stomach features make murine models unsuitable. Studies have shown that *H.Pylori* injected into stomachs of mice do not progress into ulceration and cancer as it can in humans.[299] An emerging application for organoids is the development of organoid-based biobanks for various diseases.[300] These biobanks can store organoids from various cancers that can be used in the future. In addition, organoid models have the potential to be used for throughput drug screening platforms, which can help personalise therapy.

While they have enormous benefits, organoids do have some limitations. Organoid modeling is more expensive than cell lines, largely due to the need for a laminin/collagen rich ECM called matrigel to embed the cells in. It is, however, far less time intensive than PDX models. Organoids are pure epithelial cultures that lack stromal elements and vasculature, limiting the study of tumour-stromal interactions and role of certain immunotherapeutic applications. However, more recently, organoid modeling has expanded to include immune aspects of the TUME.[301] Neal et al[301] developed a novel air-liquid interface method of growing organoids with endogenous syngeneic TILs as a cohesive unit. Such propagation of organoids with immune stroma may permit the assessment of immunotherapeutics on the TUME. The lack of

vasculature is an aspect that remains limited in organoid models. However, given the intense interest and advances in this field in recent years, it is likely that newer techniques will evolve to overcome this deficiency.

1.7 Deficiencies in treatment and research

Peritoneal metastases confer the worst survival in patients with metastatic CRC. Most present with inoperable disease, with systemic chemotherapy the mainstay of treatment for these patients. Given significant limitations with the efficacy of current chemotherapies, median survival for these patients is approximately 16 months.[24] At this point, there are no new therapeutic options for these patients on the horizon.

For those with resectable disease, CRS and HIPEC can offer a favourable survival. However, recurrence rates are high, with median DFS only 12-18 months. The role of iterative CRS and HIPEC is evolving. After recurrence, chemotherapy becomes the mainstay of treatment with a similar outlook as the inoperable cohort.

Despite advances in immunotherapy in various cancers including MSI-H CRC, there has been very limited research into the evaluation of the immune landscape in CRPM. To date, there have been no clinical studies published on the use of immune checkpoint antibodies in CRPM.

Overall, there is an urgent need to explore newer approaches and avenues of treatment in CRPM. The immune landscape and the role of checkpoint blockade is one such unchartered avenue that warrants research. Similarly, there is a strong need to explore newer cytotoxic and targeted drugs that can be used in CRPM. Given advances in genomic sequencing and pre-clinical modeling of disease, there is potential to explore personalised models of care that is unique to each patients' tumour characteristics.

1.8 Outline of this thesis and chapter content

1.8.1 Aims

The broad aims of this thesis are as follows:

1) To assess current perceptions among clinicians in the management of CRPM and evaluate outcomes from current management of peritoneal surface malignancies (PSM) and in particular, CRPM. Subsequently, this thesis also seeks to identify key prognostic factors impacting survival in patients undergoing CRS and HIPEC for isolated CRPM.

2) To establish and validate robust pre-clinical models that can help explore newer therapies in patients with CRPM.

3) To explore the immune landscape, using a multitude of technologies such as flow cytometry (FACS), multiplex immunohistochemistry (OPAL), RNA sequencing (RNASeq) and a functional immune based assay that allows evaluation of immunotherapy in the form of checkpoint blockade.

4) To develop a novel organoid based platform that can be used to deliver personalised care to patients with CRPM.

1.8.2 Chapter Outline

Chapter 2 provides an overview of all the methods utilised in the laboratory-based research during this project. In particular, there is a strong emphasis on the establishment and validation of pre-clinical models of CRPM.

Chapter 3 describes current perceptions and management strategies among colorectal surgeons in Australia and New Zealand in the management of CRPM. This is based on a

structured survey circulated to all colorectal surgeons affiliated with the Colorectal Society of Australia and New Zealand (CSSANZ).

Chapter 4 evaluates the outcomes from CRS and HIPEC for all PSM at a statewide peritoneal disease centre.

Chapter 5 focuses specifically on the outcomes from CRS and HIPEC for CRPM over an eight-year period at a statewide peritoneal disease centre.

Chapter 6 evaluates the difference in outcomes at a statewide peritoneal disease centre based on the use of Oxaliplatin and Mitomycin C, the two agents used in HIPEC in the treatment of CRPM.

Chapter 7 is a systematic review and meta-analysis evaluating key prognostic factors influencing survival in patients undergoing CRS and HIPEC for isolated CRPM.

Chapter 8 explores the immune landscape of CRPM, including the role of immune checkpoint antibodies to improve lymphocyte cytotoxicity against CRPM.

Chapter 9 describes the establishment and feasibility of a novel organoid based platform that can be used to offer personalised therapy to patients with CRPM.

Chapter 10 briefly summarises the pertinent findings in this thesis, and outlines a few key areas for further research that would potentially benefit the field of CRPM.

2 Chapter 2: Materials and Methods

2.1 Human Participants

2.1.1 Clinical studies

Detailed methods for all clinical studies are described within the methods for each clinical study.

2.1.2 Laboratory Studies

Patients undergoing surgical intervention in the form of CRS and HIPEC or staging laparoscopy for synchronous or metachronous CRPM or percutaneous biopsies at Peter MacCallum Cancer Centre (PMCC) were prospectively recruited for the study from February 2017 to December 2018. A total of 37 patients were recruited for the study. Fresh tissue was acquired from 31 at time of CRS and HIPEC, five from staging laparoscopies and one from percutaneous biopsy.

All work involving human participants was conducted in accordance with a protocol approved by the PMCC Human Research Ethics Committee (HREC) (PMCC 15/76).

2.2 Human Sample Processing

2.2.1 Blood collection for Peripheral Blood Mononuclear Cells

Forty millilitres of blood was collected from each recruited patient by venipuncture in BD Vacutainer® CPT™ Cell Preparation Tubes (BD Biosciences, New Jersey, USA), stored upright and processed within two hours of collection. Tubes were centrifuged for 20 minutes, at 1700 x g, at room temperature (RT) in a horizontal rotor (swing-out head). After centrifugation, whole plasma was removed by transfer pipette and stored at -80°C if required or discarded. The peripheral blood mononuclear cell (PBMC) layer was gently transferred into Falcon tubes (maximum 2 CPT tubes per Falcon) using a transfer pipette. The cell suspension was washed with 30 mL of phosphate buffered saline (PBS), inverted 5-10 times and centrifuged for 10 minutes, at 300 x g, RT. The supernatant was gently removed without disturbing the pellet. To remove any residual red blood cells, 1-2mL of Ammonium Chloride Potassium (ACK) buffer was added at RT for 2-3 minutes and the reaction stopped by adding 30 mL PBS and consequent centrifugation for 10 minutes, at 300 x g, RT. Supernatant was discarded, and one final wash step completed, by adding PBS and centrifuging again for 10 minutes, at 300 x g, RT. Cells were counted in 0.4% trypan blue (Gibco, Thermo fisher Scientific, Massachusetts, USA) using a haemocytometer and were cryopreserved in 10 million PBMC aliquots/cryovial for future use at -80°C.

2.2.2 Fresh Tissue Preparation and storage of samples

Fresh tumour tissue was collected in RPMI 1640 (Gibco, Thermo fisher Scientific, Massachusetts, USA) supplemented with 5% (v/v) fetal bovine serum (FBS) (Gibco, Thermo fisher Scientific, Massachusetts, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, Missouri, USA), and placed on ice.

Tissue was initially dissected to remove necrotic/fibrotic regions and adjacent peritoneum to expose a viable tumour margin and divided into multiple pieces. Small

amounts were snap frozen, cryopreserved, paraffin embedded (FFPE), with the remaining fresh tissue used for flow cytometry, establishment of tumouroids and enrichment and expansion of tumour infiltrating lymphocytes (TILs).

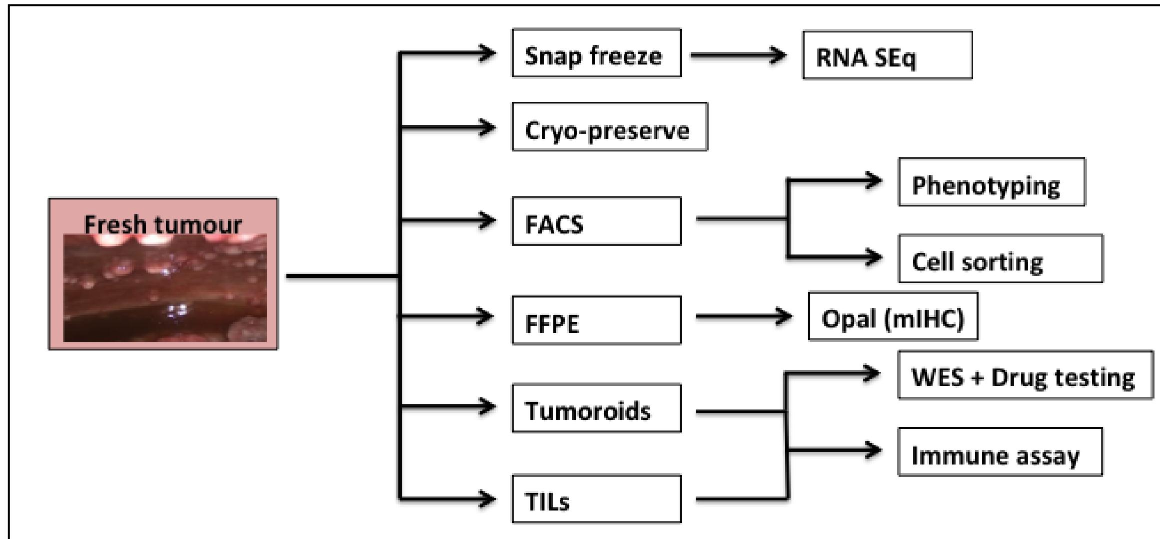


Figure 2.1. Use of fresh tissue for various purposes in this project. Samples were used for as many purposes as possible such as FACS, tumouroid development, OPAL, RNA Seq depending on quantity of tissue received.

FACS: florescence associated cell sorting; FFPE: Fixed in formalin paraffin embedded; TILs: tumour infiltrating lymphocytes; RNA Seq: RNA sequencing; OPAL/mIHC: Multiplex immunohistochemistry; WES: whole exome sequencing

2.2.2.1 Snap Freezing

Tissue was dissected and placed in a Cryotube (Thermo fisher Scientific, Massachusetts, USA) on dry ice and subsequently stored at -80°C.

2.2.2.2 Cryopreservation

Tissue was suspended in 10% Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Missouri, USA) in fetal bovine serum (FBS) (Gibco, Thermo fisher Scientific, Massachusetts, USA) and immediately transferred to -80°C in Mr Frosty containers (Thermo fisher Scientific, Massachusetts, USA). After 24 hours, vials were removed from the Mr Frosty containers and stored at -80°C. They were subsequently transferred to Liquid Nitrogen (LN) storage on a periodic basis.

2.2.2.3 Formalin Fixation (FFPE)

Tissue was placed in 10% Neutral-Buffered Formalin (NBF) (Australian Biostain, Traralgon, Australia). Formalin-fixed tissues were transferred to 70% Ethanol (EtOH) after 24 hours and subsequently embedded in paraffin. FFPE blocks were stored at RT and slides were cut fresh prior to use for Haematoxylin and Eosin (H&E) staining, Immunohistochemistry (IHC) and multiplex immunohistochemistry (mIHC or Opal).

2.2.2.4 Tumour Infiltrating Lymphocyte (TIL) Expansion

To expand TILs, small pieces of viable tumour (1 mm^3) were placed in a 48-well plate containing conditioned media (CM) (Table 2.1) supplemented with 6000 IU/mL recombinant human interleukin-2 (IL-2) (NCI, Charles River Laboratories, Massachusetts, USA). Media was changed twice weekly; by removing half the CM and replacement with fresh CM supplemented with 12,000 IU/mL IL-2. Once wells reached 100% confluency, TILs were transferred to a T25 flask and maintained at 1.5×10^6 cells/mL of CM supplemented with IL-2 (6000 IU/mL). When in culture, expanded TILs were used for assays and subsequently cryopreserved at 10 million TILs/vial for long-term storage at -80°C or liquid nitrogen.

Table 2.1. Conditioned Media for expansion of tumour infiltrating lymphocytes

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
RPMI 1640	Gibco #11875-093	1x	500 mL	1x
Human AB Serum Sterile Filtered Heat Inactivated, 0.22 µM filtered	Valley Biomedical, Inc. #HS1017	100%	50 mL	10%
HEPES	Media Kitchen (Life Technologies #15630080)	1M	12.5 mL	25 mM
Pen/Strep	Media Kitchen (Sigma #P3032-100MU, #S9137-100g)	200x (20.000 U/mL Pen, 20 mg/mL Strep)	2.5 mL	1x (100 U/mL Pen, 100 µg/mL Strep)
Glutamax	Media Kitchen Media Kitchen (Life Technologies #35050-061)	100x (200 mM)	5 mL	1x (2 mM)
Gentamycin	Pfizer, #A502	40 mg/mL	125 µL	10 µg/mL
Amphotericin B	Gibco, #15290018	250 µg/mL	500 µL	0.25 µg/mL

2.2.3 Establishment of pre-clinical models

2.2.3.1 Establishment of CRPM tumouroid cultures

Tumouroids refer to tumour-derived organoids. To establish CRPM tumouroids, viable tumour was first minced and enzymatically digested in organoid digestion media (Table 2.2) in a water bath at 37°C for 30-60 minutes.

Table 2.2. Organoid digestion media

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
DMEM	Gibco, #12634028	-	99 mL	1x
Pen/Strep	Media Kitchen (Sigma #P3032-100MU, #S9137-100g)	200x (20.000 U/mL Pen, 20 mg/mL Strep)	0.5 mL	1x (100 U/mL Pen, 100 µg/mL Strep)
Collagenase IV	CLS-4 Worthington #43N14578	225U/mg	30 mg	67.5U/mL
Dispase	GIBCO #17105-041	1.839U/mg	12.5 mg	0.23U/mL
Hyaluronidase	Sigma Aldrich #H3506-500MG	400-1000U/mg	2 mg	8-20U/mL
DNAse Type I	Sigma Aldrich #DN25-1G	10,000 Kunits/mL	500 uL	50 Kunits/mL

Table 2.3. Organoid basal media (OBM)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
Advanced DMEM/F12	Sigma #D6421	-	487.3 mL	1x
HEPES	Media Kitchen (Life Technologies #15630080)	1 M	5 mL	10 mM
Glutamax	Media Kitchen (Life Technologies #35050-061)	100x (200 mM)	5 mL	1x (2 mM)
Pen/Strep	Media Kitchen (Sigma #P3032-100MU, #S9137-100g)	200x (20.000 U/mL Pen, 20 mg/mL Strep)	2.5 mL	1x (100 U/mL Pen, 100 µg/mL Strep)
Gentamycin	Pfizer, #A502	40 mg/mL	100 µL	8 µg/mL
Amphotericin B	Gibco #15290018	250 µg/mL	500 µL	0.25 µg/mL

Tumour pieces were dispersed using the plunger of a 3mL syringe, washed with organoid basal media (OBM) (Table 2.3) and filtered through a 70 µM filter. Cells were centrifuged at 400 x g. The supernatant was gently removed, leaving a pellet of tumour cells. To remove any residual red blood cells, 1-2 mL of ACK buffer was added at RT for 2-3 minutes and the reaction was stopped by the addition of 8-10mL of OBM. Cells were centrifuged at 400 x g, with the supernatant discarded and the pellet then suspended in matrigel (BD Corning, USA). Approximately six to eight wells of a 24-well plate were

seeded with 50 µL of matrigel containing tumour cells and maintained with 500 µL/well of colorectal media (CRC media) (Table 6.4).

Tumouroids were grown in hypoxic conditions (37°C, 5% O_2, 5% CO_2) and CRC media was changed twice weekly, with growth monitored until passaging was required. In my experience, it was usually noticeable within 48-72 hours if tumouroids were starting to grow. Tumouroids that did not grow within 14 days were discarded.

Table 6.4. Colorectal media (CRC media)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
OBM	See Table 6.3	-	47.7 mL	1x
A 83-01	TOCRIS #2939-10mg	5 mM	5 µL	0.5 µM
B27	Thermo Fisher Sc. #12587010	50x	2000 µL	2x
EGF	Sigma Aldrich #SRP3027-500UG	500 µg/mL	25 µL	0.25 µg/ml
Gastrin	Sigma Aldrich #G9145-0.5MG	1 mg/mL (480.7 µM)	50 µL	1 µg/mL (480.7 nM)
N acetyl Cyst	Sigma Aldrich #A9165-5G	5g/50mL (612.8 mM)	100 µL	1.23 mM
SB202190	Sigma Aldrich #S7067-5MG	3.333 mg/ml = 10.1 mM	25 µL	5. 0 µM
Y27632	Sigma Aldrich #Y0503-5mg	10 mM	50 µL	10 µM

2.2.3.2 Establishment of normal colonic epithelium organoids

Establishment of normal colonic epithelium organoids followed the same protocol as the establishment of tumouroids, with the only difference being the media used. The media for normal colonic organoids also contained Wnt, R-Spondin and Noggin and was a commercially available media called Intesticult™ (Stem cell technologies).

2.2.3.3 Passaging of CRPM tumouroid cultures

Tumouroids were passaged when they were ≥ 100 µm in diameter. At each passage, a minimum of two wells were cryopreserved for future use and establishment of a CRPM tumouroid biobank.

The remaining wells were aspirated with cold OBM media, transferred to a Falcon tube and centrifuged at 300 x g for five minutes. The supernatant was then aspirated, and 1 mL of Tryple (Gibco, Thermo fisher Scientific, Massachusetts, USA) was added to the pellet and incubated in a water bath at 37°C for 10 minutes. The mixture was titurated with a p1000 pipette, 9 mL of OBM was added and centrifuged at 300 x g, for five minutes. The supernatant was aspirated and matrigel was added to cell pellet. The amount of matrigel added to the pellet was determined by cell pellet size, with the general aim to atleast double the number of wells compared to the previous passage. Similar to establishment of tumouroids, a 50 µL tumour cell/matrigel suspension was seeded in a 24-well plate and media added based on the number of wells used.

2.2.3.4 Establishment of tumouroid derived xenografts

To confirm tumourigenicity in mice, tumouroids (approximately 1×10^5 cells) were suspended in 1:1 matrigel with PBS mix at 4°C. Cells were subcutaneously injected into the right leg of Nod scid gamma (NSG) mice (4-6 weeks). The area over the site was shaved prior to injection. Injection was performed in a laminar flow operating room. Antiseptic in the form of 2% (v/v) chlorhexidine gluconate or 70% ethanol was applied and allowed to dry for skin asepsis prior to injection. Mice and tumour growth were monitored, with tumour measurements performed twice weekly. Mice were euthanised at ethical endpoint. The tumour was extracted, with fixation (10% NBF) and paraffin embedding for H & E staining, pathological examination and IHC.

2.2.3.5 Establishment of patient derived xenografts (PDXs)

Patient tumour derived xenografts (PDXs) were also established in addition to tumouroid models, ensuring a replenishable quantity of tumour would be available.

Tumour pieces were dissected into 1 mm^3 size and placed in 50 μL of matrigel in a 1:1 ratio on ice at 4°C until implantation. NSG mice (4-6 weeks) were appropriately anaesthetised with volatile anaesthetic, prepped with 2% chlorhexidine gluconate or 70% ethanol. A vertical incision was made along the dorsal midline of the mouse. The subcutaneous plane was dissected on the right side to expose the paravertebral musculature. A 4/0 Vicryl Rapide™ suture was placed into the musculature to facilitate creation of an intra-muscular pocket with dissecting tenotomy scissors. The matrigel/tumour mix was implanted into the intra-muscular pocket. The pocket was then closed with the pre-placed suture, followed by skin closure with 4/0 Vicryl Rapide™. The mouse was recovered and monitored, with tumour measurements performed twice weekly.

Mice were euthanised at ethical endpoint. The tumour was extracted, with fixation (10% NBF) and paraffin embedding for H & E staining, pathological examination and IHC.

2.2.4 Validation of pre-clinical models

2.2.4.1 Validation of CRPM tumouroids and normal colonic organoids

Tumouroids were validated with a number of techniques to confirm that they recapitulated the native tumour tissue. Using immunohistochemistry (IHC), tumouroids were shown to retain similar morphology to the native tumour, with consistent staining of colorectal specific nuclear marker caudal type homeobox 2 (CDX2) and cytokeratin 20 (CK20) between native tumour and tumouroids confirming colorectal origin (Figure 2.2 and 2.3). All IHC performed was reviewed by a specialist gastrointestinal pathologist to confirm concordance between the native tissue and tumouroid.

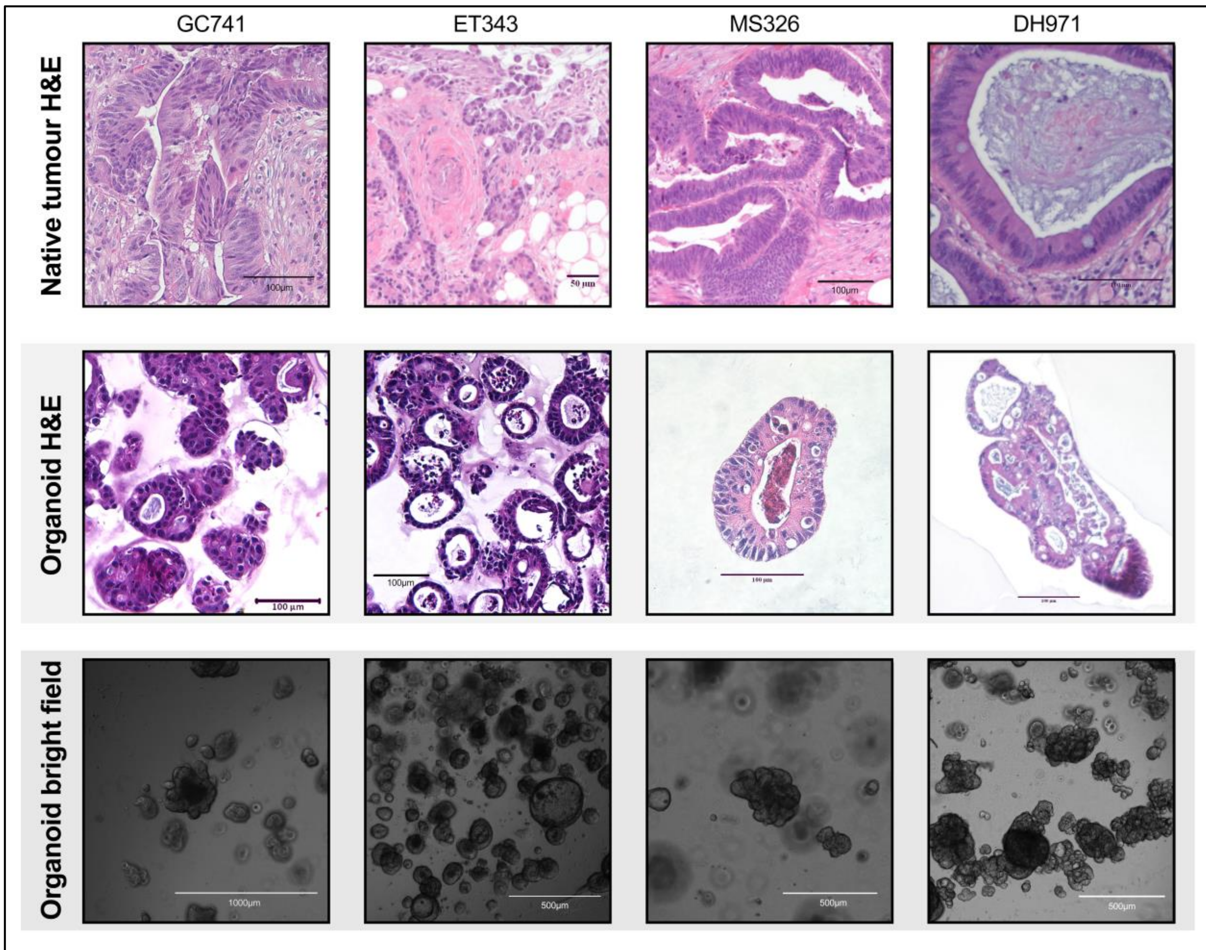


Figure 2.2. Tumour derived organoids retaining similar morphology to the native tumour tissue. Four examples demonstrated above, along with their appearance under bright field microscopy.

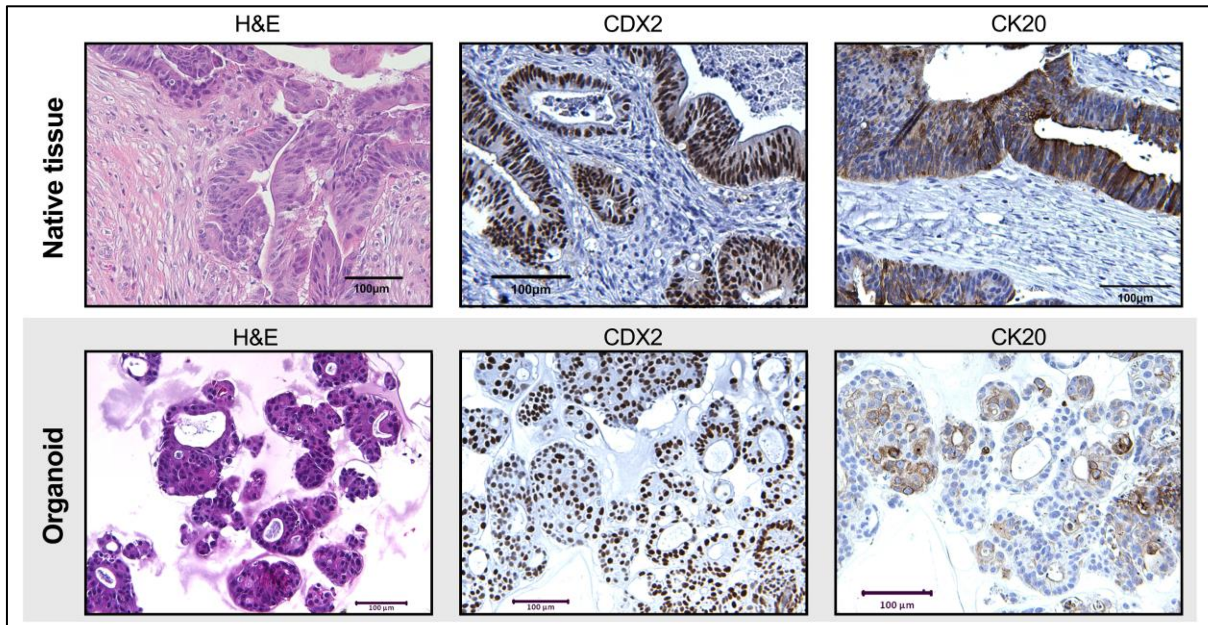


Figure 2.3. Tumour derived organoids retain similar morphology to the native tumour. Additionally, they retain CDX2 and CK20 positivity consistent with the native CRPM tumour.

2.2.4.2 Validation of tumouroid derived xenografts

Tumouroid derived xenografts were similarly validated with immunohistochemistry, with the addition of human anti-mitochondrial antibody, confirming tissue to be of human origin (Figure 2.4). The median time to growth of tumouroid-derived xenografts was 63 days (Range 36-196). A total of seven tumouroid-derived xenografts were validated with immunohistochemistry.

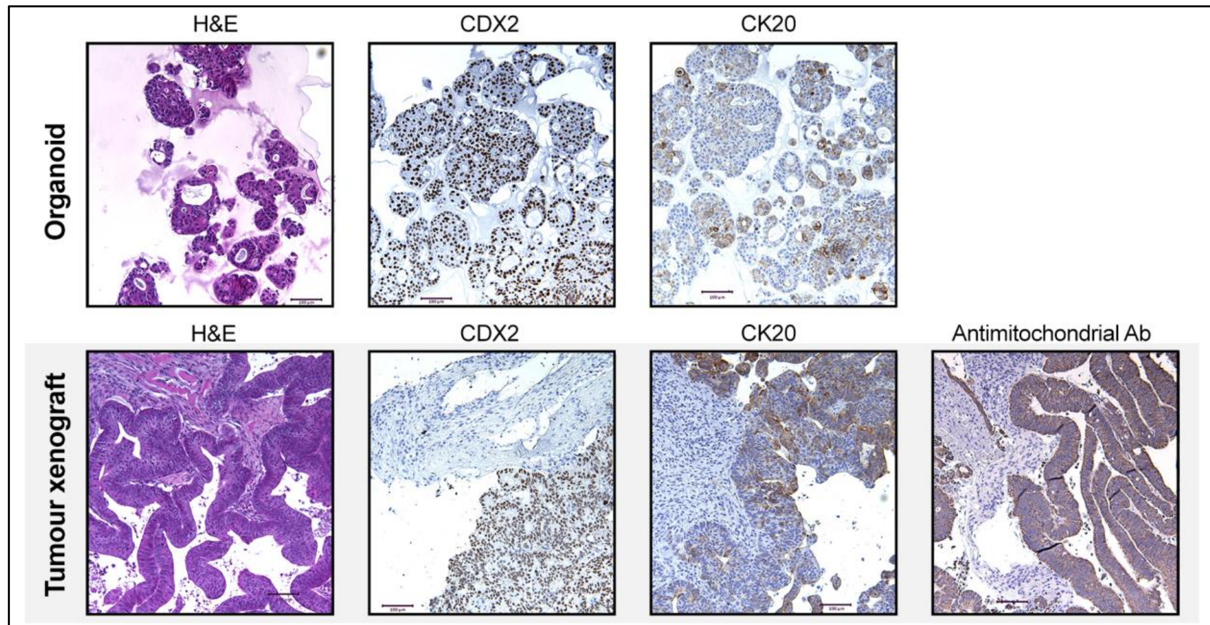


Figure 2.4. Organoid derived mouse xenograft to confirm tumourigenicity. CDX2 and CK20 antibodies remain positive, confirming colorectal origin, with anti-mitochondrial antibody positivity confirming human origin.

2.2.4.3 Validation of normal colonic epithelium organoids

As normal colon also stains CDX2 and CK20, a p53 stain was used to differentiate normal organoids from patient matched tumouroids. Normal p53 positivity stains in a patchy manner. A significant proportion of CRCs however, have p53 mutations resulting in either loss or overexpression of p53. This would result in either a lack of p53 staining, or an abundance of p53 staining (Figure 2.5). Additionally, normal colonic organoids require additional growth factors in the form of Wnt, R-Spondin and Noggin, while tumouroid CRC media does not have these additional growth factors. When Wnt, R-Spondin and Noggin are withdrawn from normal colonic organoid media, they cease to grow, demonstrating different growth factor dependence between normal colonic organoids and tumouroids.

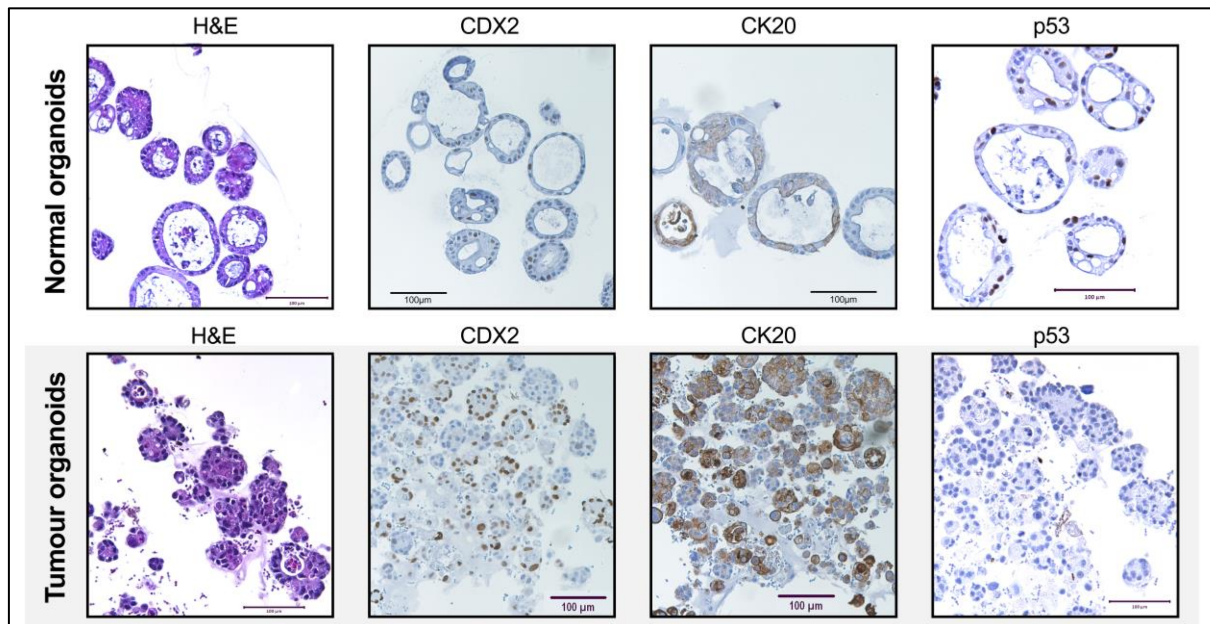


Figure 2.5. Normal colonic epithelium organoids and matched primary colorectal tumouroids from the same patient. While both are CDX2 and CK20 positive, only the normal colonic organoids are occasionally p53 positive, in keeping with patchy staining seen in normal tissue. The tumouroids are comparatively p53 negative, likely due to loss of p53. TP53 mutations are among the more common mutations seen in colorectal cancer. This demonstrates that normal colonic organoids are different to tumouroids.

2.2.4.4 Validation of Patient derived Xenografts (PDXs)

PDXs were similarly validated with immunohistochemistry, with the addition of human anti-mitochondrial antibody, confirming tissue to be of human origin (Figure 2.6). The median time to growth of PDXs was 120 days (Range 100-140). Three PDXs were established and validated. I stopped establishing PDXs once I started having greater success with establishing tumouroids.

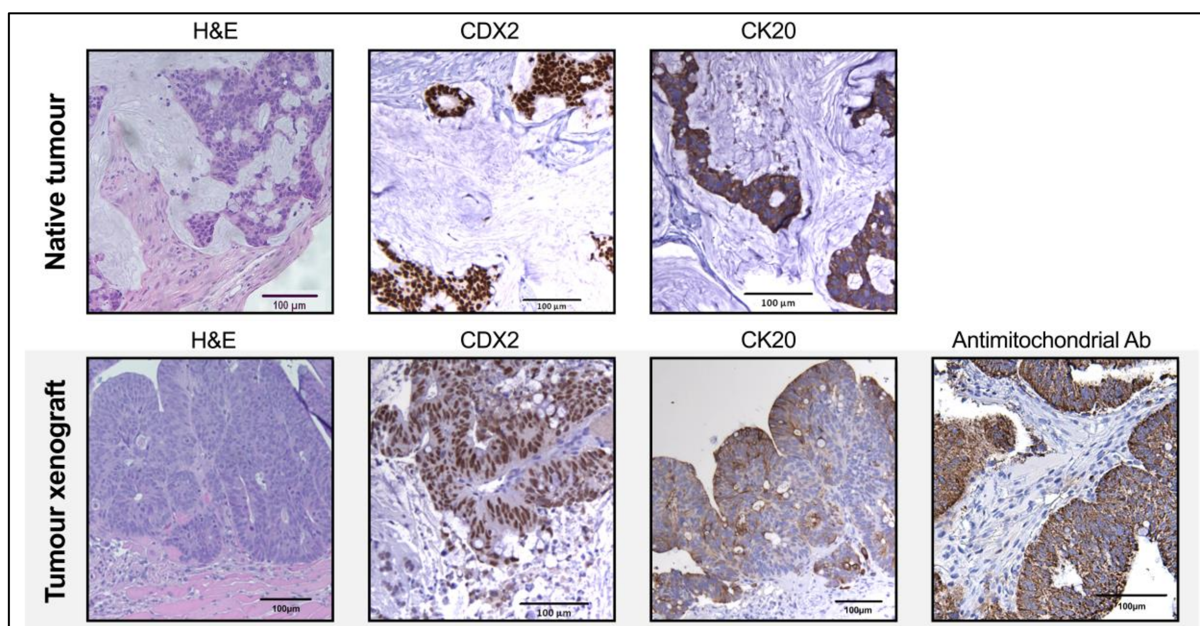


Figure 2.6. Patient derived xenograft demonstrating the tumour xenograft retains CDX2 and CK20 confirming colorectal origin. Additionally, the tumour xenograft is anti-mitochondrial antibody positive, confirming human origin.

2.2.4.5 Validation: Short tandem repeat (STR) analysis

Short tandem repeat (STR) analysis is a genomic-based approach for verifying and authenticating tumouroids to confirm that they have originated from the native tumour. Short tandem repeats are stable, repetitive genomic sequences three to seven base pairs long scattered throughout the human genome. Amplifying these polymorphic loci and comparing the resulting STR profile to that of the native tumour tissue can verify the origin of the tissue in question. By amplifying ten STR loci, the power of discrimination is very high, making it extremely unlikely that two DNA profiles will match at random.

STR analysis was performed on ten markers. These were AMEL, CSF1P0, D13S317, D16S539, D21S11, D5S818, D7S820, TH01, TPOX and vWA. A minimum of 80% concordance was deemed necessary for authentication. Sixteen tumouroid lines were tested with STR analysis. Two lines had less than 80% concordance and were excluded from functional experiments such as drug testing (Table 2.5)

Table 2.5. Short tandem repeat analysis results

Tumouroid code	Concordance with native tumour (%)
DH971, GB539, SP740P, LW928, MS326	100%
GC741, ET343, CR016	95%
DL246, DK743, SP740C	90%
MT117C, MT117P, PS835	85%
ET799C, ET799P	70%

2.2.4.6 Quality Control

All tumouroid lines were tested for mycoplasma as per laboratory guidelines. All lines were mycoplasma negative.

2.2.5 Summary of tumouroids established from patients

In total, 31 samples were utilised for establishment of tumouroids. Twenty-two tumouroids from 19 patients were successfully established. Three patients had synchronous resections of a primary tumour with PM, with tumouroids successfully grown from both the primary and PM. The overall success rate for tumouroid establishment was 71.0% (22 out of 31 samples). Seventeen out of 26 samples were established from operative CRS and HIPEC samples, four out of four were established from laparoscopies and one was established from percutaneous biopsy (Table 2.6). All four laparoscopic biopsies were performed on patients with unresectable high volume disease and therefore more tumour deposits available to biopsy. This possibly contributed to the relatively higher success rate in growing tumouroids from laparoscopic biopsies. This is however clinically relevant, as a laparoscopic approach carries low morbidity, and may serve as a suitable modality to biopsy patients with unresectable or treatment refractory disease in future studies.

Table 2.6. Characteristics of patients recruited to the study with successful tumouroid establishment

Patient Code	Patient No.	Sex	Age	Biopsy type*	Systemic Chemo	What chemo**
PS835	1	F	71	Operative	Yes	5FU monotherapy; FOLFOX, FOLFIRI + EGFRi
MS326	2	F	47	Operative	Yes	CAPOX/FOLFOX; FOLFIRI + EGFRi
DL246	3	F	60	Laparoscopy	Yes	FOLFIRI + VEGFRi
ET343	4	M	78	Operative	Yes	5-FU as part of neo-adjuvant chemo-radiation
GC741	5	M	80	Operative	Yes	FOLFOX + VEGFRi; FOLFIRI + VEGFRi; 5-FU with radiation
SP740C#	6	F	69	Operative	No	Chemo Naive
SP740P#	6	F	69	Operative	No	Chemo Naïve
MT117C#	7	F	60	Operative	No	Chemo Naïve
MT117P#	7	F	60	Operative	No	Chemo Naïve
GB539	8	F	34	Operative	Yes	5-FU as part of neo-adjuvant chemo-radiation
CR016	9	F	34	Percutaneous	Yes	FOLFOX; FOLFOX + VEGFRi; FOLFIRI + EGFRi; CAR-T cell trial; Lonsurf
DH971	10	F	59	Operative	Yes	FOLFOX
SQ258	11	M		Operative	Yes	FOLFOX
DK743	12	F	57	Laparoscopy	Yes	FOLFOX; 5-FU monotherapy
ET799C#	13	35	F	Operative	No	Chemo naïve
ET799P#	13	35	F	Operative	No	Chemo naïve
QEH020	14	67	F	Operative	Yes	CAPOX
LW928	15	52	F	Operative	Yes	FOLFOX
LR833	16	63	F	Operative	Yes	FOLFOX; CAPOX; FOLFIRI + EGFRi
MF004	17	50	F	Operative	Yes	FOLFOX + VEGFRi; CAPOX
IK899	18	71	M	Laparoscopy	Yes	FOLFOX
DG077	19	73	M	Laparoscopy	Yes	FOLFOX, FOLFIRI

** Operative: at time of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; Laparoscopy: at time of staging laparoscopy; #Synchronous resection of primary tumour with peritoneal metastases. C: colonic primary, P: synchronous peritoneal metastases; percutaneous: needle biopsy guided by ultrasound/computed tomography; **: FOLFOX: 5FU/Leucovorin+*

Oxaliplatin; FOLFIRI: 5FU/leucovorin + irinotecan; EGFRi: cetuximab; CAPOX: Capecitabine + Oxaliplatin; VEGFRi: bevacizumab; Lonsurf: Trifluridine and tipiracil

2.2.6 Immune cytotoxic assay (Tumouroid-TIL co-culture)

2.2.6.1 Immune Cytotoxic Assay: Preparation of tumouroids

Tumouroids were deemed suitable for cytotoxic co-culture assay by passage 2-3 (approximately 14-21 days post receiving sample) and were plated at least five to seven days prior to performing the assay. For optimal assay conditions, tumouroids were grown to ≥ 50μm and checked for morphological viability using a light microscope on the day of assay, before proceeding.

2.2.6.2 Immune Cytotoxic Assay: Passaging of tumouroids

By approximately passage 2-3, when tumouroids cultivated in 24-well plates were ≥ 100 μm in size, media was aspirated from each well. Wells were washed twice with ice-cold OBM to dissolve the matrigel, with the contents of the well then transferred to a Falcon tube and centrifuged at 300 x g for five minutes. The supernatant was then aspirated, and 1mL of TrypleE (Gibco, Thermo fisher Scientific, Massachusetts, USA) was added to the pellet and incubated in a water bath at 37°C for 10 minutes. The mixture was titurated with a p1000 pipette, 9 mL of OBM was added and centrifuged at 300 x g, for five minutes. To ensure tumouroids were dissociated to a single cell level, the cell suspension was checked under the light microscope and further incubation with fresh TrypleE was performed, if necessary.

The supernatant was aspirated and matrigel was added to the cell pellet. A small aliquot of the cell suspension in matrigel was visualised under a light microscope to evaluate density, with additional matrigel added if required. Tumouroids were plated on a pre-warmed μ-Plate 96 Well (ibidi, GmbH Germany), by seeding 15 μL of matrigel/cell suspension into each well, and placed to set at 37°C, incubated for 30-60 minutes.

Following incubation, 350 µL of pre-warmed CRC media was added to each well, and tumouroids were grown in hypoxic conditions (37°C, 5% O_2, 5% CO_2) for five to seven days prior to running the cytotoxic assay. A minimum of 20 tumouroids around the edges of the matrigel per well measuring ≥ 50 µm in size were needed for the assay to be run.

2.2.6.3 Immune Cytotoxic Assay: Preparation of TILs

On the day of the planned co-culture assay, if tumouroid number and size were adequate, expanded TILs were harvested from the 48-well plate and sequentially filtered through 70 µM and 45 µM filters. Viable TIL count was established using a haemocytometer. To calculate Effector: Target (E: T) ratio of TILs: tumouroid, tumouroids per well near the edge of the matrigel were counted. TIL concentration was determined for 5000 TILs (effector): one tumouroid (target), and did not exceed 250,000-300,000 TILs/well. TILs were then centrifuged, washed with CM, centrifuged again and re-suspended in the required volume of pre-warmed CRC media and kept at 37°C until ready for addition to the cytotoxic assay.

Approximately 1-1.5 x 10^6 TILs were also kept aside and stained for phenotyping on the same day as the immune cytotoxic assay using flow cytometry techniques described later in this chapter.

2.2.6.4 Preparing the Immune Cytotoxic Assay Media

Media from the cultivated tumouroids in the µ-Plate 96 was aspirated. Wells were set up in duplicate per condition:

1) Tumouroids alone (negative control)
2) Tumouroids + TILs
3) Tumouroids + TILs + anti-PD-1 antibody
4) Tumouroids + Triton X-100 (positive control)

The total media in each well was 350 μL, comprising 6 μL of Propidium Iodide (PI) (Sigma Aldrich, Missouri, USA), for a final concentration of 17.1 μg/mL, 2 μL (6,000 IU/mL) of rh-IL-2, with CRC media making up the remaining 342 μL. Wells containing anti-PD-1 antibody also had 2 μL of anti-PD-1 antibody (Keytruda, Merck, New Jersey, USA) (final anti-PD-1 antibody concentration of 142.86 μg/mL). In the anti-PD-1 antibody wells, only 340 μL of CRC media was added. In the positive control wells, 2.5 μL of 3.5% Triton X-100 was used, along with 6 μL PI, 2 μL IL-2 and 339.5 μL CRC. In experiments evaluating specificity of TILs, tumouroids were incubated with 50 μg/mL of MHC-I antibody for 30 minutes before adding in TILs (blocking antibody was present throughout the co-culture). The remaining empty wells within the plate were filled with PBS to prevent evaporation.

2.2.6.5 Imaging Acquisition

The μ-Plate (96 wells) was mounted on a heated stage in a temperature controlled chamber, maintained at 37°C, 5% CO_2. The CellSens software (Olympus) was used to image the plate on a 10x air objective, selecting 10 tumouroids at the periphery of the matrigel. Optical sections were acquired through sequential scans of bright field and PI (excitation 493nm) every 2 hours for 48 hours.

Imaging series were analysed using Image J software (NIH, Maryland, USA) to obtain the mean fluorescence intensity (MFI) of PI uptake in tumouroids over time. The mean of 10 tumouroids per well was taken to analyse kinetics of TIL-induced cytotoxicity over time. The maximum MFI per time point was also assessed to obtain the overall maximum killing threshold for each tested condition.

2.2.7 Flow Cytometry (FACS)

2.2.7.1 Tissue Processing for Flow Cytometry

Fresh CRPM tissue and matched normal peritoneum (> 3cm away from tumour deposits) were collected in RPMI 1640 (Gibco, Gibco, Thermo fisher Scientific,

Massachusetts, USA) supplemented with 5% FBS (Gibco, Thermo fisher Scientific, Massachusetts, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, Missouri, USA) herein referred to as wash medium and kept on ice until processed for FACS.

Viable tumour and matched normal peritoneum were weighed, finely minced with a scalpel and enzymatically digested in Organoid digestion media (Table 2.2) in separate Falcon tubes in a water bath at 37°C for 30-45 minutes. The tissue homogenate was initially passed through a 100 µm filter into a 50 mL Falcon tube, with the plunger of a 3 mL syringe used to grind any residual whole tissue. The filter was rinsed with wash medium and the single cell suspension sequentially filtered through a 70 µm and 40 µm filter. Cells were centrifuged at 330 x g, 10 minutes, 4°C. The supernatant was gently removed, leaving a pellet of cells. To remove any residual red blood cells, 1-2 mL of ACK buffer was added at RT for 2-3 minutes and the reaction was stopped by the addition of 25-30 mL of wash medium. The tubes were centrifuged at 300 x g, 10 minutes, 4°C. The supernatant was aspirated and cells re-suspended in 2-3 mL wash medium for counting using a haemocytometer. Cells were kept at 4°C until ready for FACS staining.

2.2.7.2 Cell Surface Staining for Flow Cytometry

Following cell counting, approximately 5.0×10^5 cells/well were plated into four wells of a 96-well v-bottom plate (Nunc, Thermo scientific, Massachusetts, USA). The four wells represented the following four conditions:

1) Unstained control (US)
2) Fixable Viability control (FVS)
3) Isotype control (ISO)
4) Stained control (STAIN)

Cells were washed twice with PBS and centrifuged at 400 x g, 4 minutes, 4°C. Fixable Viability Stain 575 (BD Biosciences, New Jersey, USA) was thawed on ice, diluted 1:1000 with PBS and 200 µL diluted FVS575 was added to FVS, ISO and STAIN samples, with the US control re-suspended in PBS. Cells were incubated at RT, 10 minutes in the dark.

Following incubation, the plate was centrifuged at 400 x g, 4 minutes, 4°C to pellet cells, with supernatant discarded and cells washed in PBS. Human Fc Block (BD Biosciences, New Jersey, USA) was diluted 1:10 in Brilliant Stain Buffer (BD Biosciences, New Jersey, USA), with STAIN and ISO samples re-suspended in this medium. US and FVS controls were resuspended in Staining Media (SM) consisting of RPMI, 4% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich, Missouri, USA). Samples were incubated 10 minutes, 4°C in the dark. Following incubation, 100 µL of antibody mix (Table 2.7) was added to STAIN and ISO wells, SM was added to US and FVS controls and samples were incubated for 30 minutes, 4°C in the dark. Samples were washed twice with 200 µL SM and permeabilised with 1x Perm/Wash™ buffer (BD Biosciences, New Jersey, USA) and incubated for 45 minutes, 4°C in the dark.

Table 2.7. Antibody panel used for flow cytometry.

Antibody	**BD catalog #**	**Ab #**	**µL**	**Isotype**	**Iso #**	**µL**
Foxp3 PE	560046*	1	20*		13	20*
	Staining Media		51			65
CD25 PE-Cy7	557741	2	5	557872	16	5
CD107a APC	560664	3	5	555751	19	5
CD45RO PerCP-Cy5.5	560607	4	5	550927	4	2.5
CD45 BV510	563204	5	5	563204	Ab#5	5
CD3 BV711	563725	6	2.5	563725	Ab#6	2.5
CD4 BV650	563875	7	2.5	563875	Ab#7	2.5
CD8 APC-H7	560179	8	5	560179	Ab#8	5
CD56 PE-CF594	564849	9	5	564849	Ab#9	5
HLA-DR BB515	564516	10	5	564515	3	2.5
CD11b BV786	740965	11	1.5	563330	12	1.5
CD33 PE-Cy5	551377	12	10	555750	15	10
PD-1 (CD279) BV421	564323	24	5	562438	8	5
PD-L1 (CD274) APC-R700	565188	25	5	564974	21	1.5

** Transcription factor staining. Perm/Wash™ buffer used along with antibody and isotype.*

All antibodies were mouse anti-human monoclonal antibodies, unless otherwise stated.

Viability dye was Fixable Viability Stain 575 (BD Biosciences, New Jersey, USA)

2.2.7.3 Transcription Factor Staining for Flow Cytometry

Following permeabilisation, cells were washed twice with 1x Perm/Wash™ buffer and centrifuged at 350 x g, 4 minutes, 4°C. Cell pellets were resuspended in 100 µL of transcription factor (TF) monoclonal antibody/isotype mix and incubated for 30 minutes, 4°C in the dark. Cells were washed twice with 1x Perm/Wash and resuspended in 200 µL of SM and stored at 4°C. Cells were acquired on the BD LSR Fortessa™ X-20 within 48 hours of TF staining.

2.2.7.4 Cell Sorting

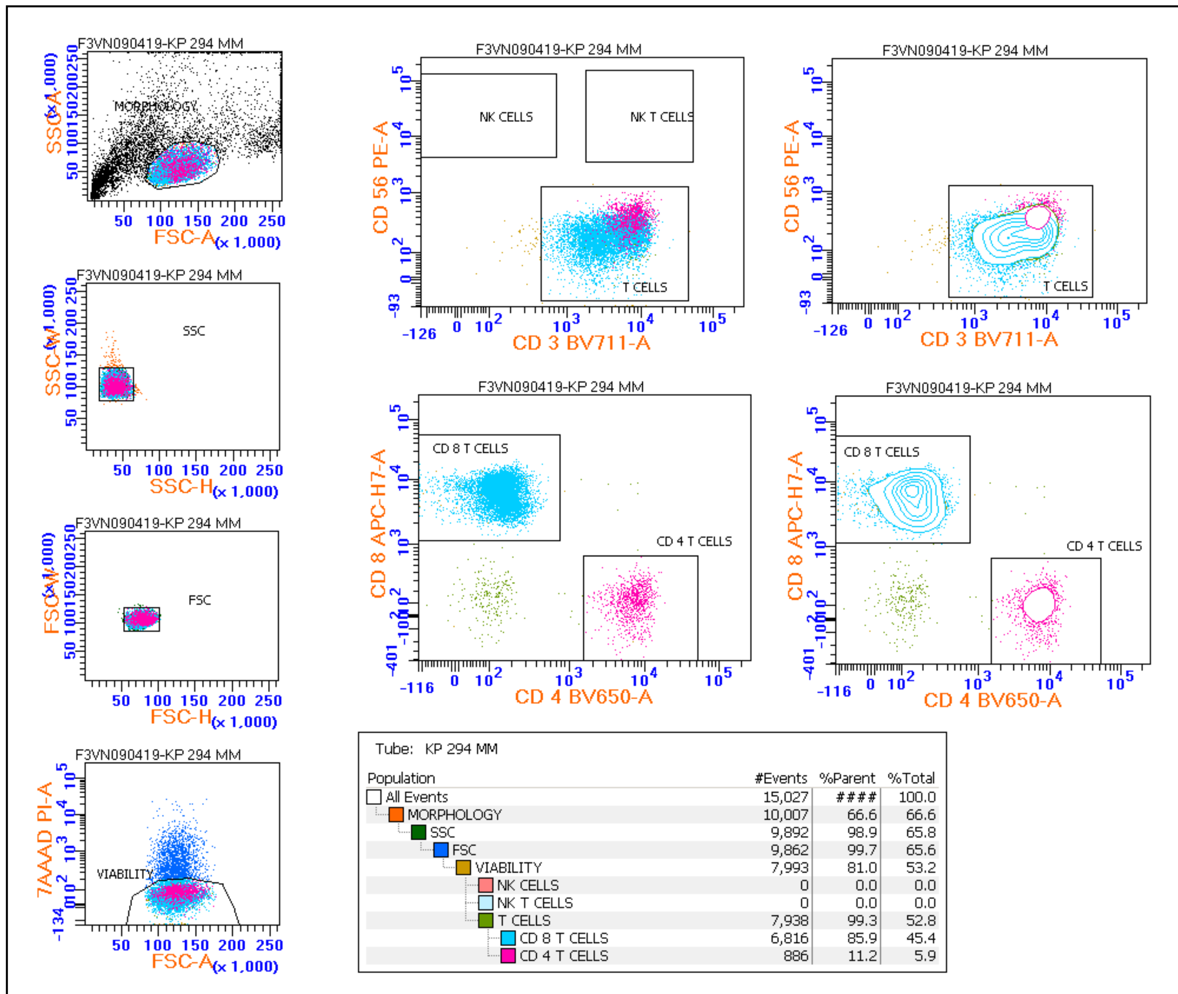
Cell sorting was performed on enriched TILs to evaluate function of specific cell types. Cells were counted using a haemocytometer and resuspended at 2 x10^8/mL in SM. Surface antibodies (Table 2.8) were added to the cell suspension along with a viability marker (7-AAD, 5 µL/100 µL) and incubated for 30 minutes in the dark at 4°C. Cells were washed with 5-10 mL of SM, centrifuged at 400 x g, 5 minutes, 4°C. The cell pellet was resuspended in 700 µL SM and acquired on the BD FACS Aria™ Fusion 3 or 5. Sorted cells were defined initially by double exclusion, lymphocyte morphology (as gated by SSC-A versus FSC-H) and viability. They were subsequently sorted based on phenotype as follows:

1) NK cells: $CD56^+$ $CD8^-$
2) NKT Cells: $CD3^+$ $CD56^+$
3) CD8: $CD3^+$ $CD8^+$
4) CD4: $CD3^+$ $CD4^+$

An example of the FACS sorting gating strategy is provided in Figure 2.7.

Table 2.8. Antibody panel used for cell sorting

Antibody number	Name	#BD	Volume of ab
6	BV711 Mouse Anti-Human CD3	563725	2.5µL/2 x 10^6 cells
7	BV650 Mouse Anti-Human CD4	563875	2.5µL/2 x 10^6 cells
8	APC-H7 Mouse Anti-Human CD8	560179	5µL/2 x 10^6 cells
9	PE-CF594 Mouse Anti-Human CD56	564849	5µL/2 x 10^6 cells



Population	#Events	%Parent	%Total
All Events	15,027	####	100.0
MORPHOLOGY	10,007	66.6	66.6
SSC	9,892	98.9	65.8
FSC	9,862	99.7	65.6
VIABILITY	7,993	81.0	53.2
NK CELLS	0	0.0	0.0
NK T CELLS	0	0.0	0.0
T CELLS	7,938	99.3	52.8
CD 8 T CELLS	6,816	85.9	45.4
CD 4 T CELLS	886	11.2	5.9

Figure 2.7. Gating strategy used in cell sorting to define the different cell populations. Doublets and non-viable cells were excluded. CD3 was gated against CD56 to identify NK (CD56$^+$), NKT (CD56$^+$CD3$^+$) and T cells (CD3$^+$). CD4$^+$ and CD8$^+$ T cells were identified within the CD3$^+$ population.

2.2.7.5 Flow Cytometry Analysis

Analysis of flow cytometry samples was performed using FlowJo, LLC, v X0.7 (BD Biosciences, New Jersey, USA). T-cells were defined initially by double exclusion, FVS 575-, lymphocyte morphology (as gated by SSC-A versus FSC-H), CD45$^+$ and subsequently CD3$^+$.

2.2.7.6 Multiplex Cytokine Bead Analysis (CBA)

2.2.7.6.1 Eight-plex cytokine analysis

Supernatant from all immune cytotoxic assays was collected for evaluation of cytokine release in duplicate or triplicate and frozen at -80°C. On the day of analysis, supernatant was thawed and used for cytokine bead analysis (CBA). Eight-plex cytokine array was performed using the CBA Flex Sets: IL-2, IL-6, IL-10, TNF-α, FasL/CD178, Granzyme-B, and IFN-γ according to manufacturers' instructions.

The highest concentration standards were prepared at 2500 pg/mL and serially diluted 1:2 to 10 pg/mL, with a blank control at 0 pg/mL. Briefly, capture beads were plexed and mixed 1:2 with standard/sample duplicates in 96-well plate. The plate was incubated in the dark at RT for one hour. PE-detection reagent was added to standard/sample wells, and re-incubated at RT in the dark for one hour. Wells were washed with wash buffer and centrifuged at 200 x g for 5 minutes. Wells were re-suspended in wash buffer and acquired on BD FACS Verse™. Acquisition of each cytokine included atleast 300 events. Results were analysed using FCAP Array™.

2.2.8 Immunohistochemistry

2.2.8.1 Immunohistochemistry: Chromogenic Staining

Formalin fixed paraffin embedded (FFPE) blocks were cut at 4 μm thickness and transferred onto Super frost Plus slides (Thermo fisher Scientific, Massachusetts, USA) and melted at 60°C for one hour. Slides were de-waxed in histolene and subsequently rehydrated in graded EtOH. Antigen retrieval was achieved by incubating slides in antigen retrieval buffer at 125°C for 3 minutes followed by 90°C for 10 seconds. Slides were subsequently cooled at RT for 30 minutes, and then blocked with 10% hydrogen peroxide (H_2O_2) (Merck-Millipore, Massachusetts, USA) for 10 minutes. Slides were washed with 1% Tri-Phosphate Buffer (TBS) Tween (Sigma Aldrich, Missouri, USA) and primary antibody was added to each slide covering tissue, and incubated either

overnight at 4°C or 1 hour at RT in humidified chamber. Following incubation, slides were washed three times in TBS-Tween and Impress® (Vector laboratories, California, USA) secondary antibody was added to tissue sections and incubated 30 minutes, RT in a humidified chamber. Slides were washed and developed using DAB solution (DAKO, Agilent Pathology, California, USA) visualised using a light microscope. Slides were counterstained using Meyer's haematoxylin, rehydrated in graded EtOH and cover-slipped using an automated coverslip machine (DAKO, Agilent Pathology, California, USA). List of antibodies used for chromogenic immunohistochemistry is detailed in Table 2.9.

To evaluate the immune profile of tumouroids, they were exposed to IFN-γ for 48 hours at 10 ng/mL. They were validated by flow cytometry and immunohistochemistry using MHC-I and PD-L1 antibodies.

Table 2.9. Antibodies used for chromogenic immunohistochemistry

Antibody	Antibody Details	Dilution	Antigen Retrieval	Secondary
Human CDX2	Millipore (EPR2764Y)	1: 500	Sodium Citrate	Anti-Rabbit
Human CK20	DAKO M7019	1: 200	Sodium Citrate	Anti-Mouse
Human anti-mitochondrial	Millipore (MAB1273)	1:500	EDTA	Anti-Mouse
Human MHC-I	Abcam (ab70328)	1:8000	EDTA	Anti-Mouse
Human PD-L1	Ventana SP263	Neat	EDTA	Anti-Rabbit

2.2.8.2 Multiplex Immunohistochemistry (mIHC/Opal)

Multiplex immunohistochemistry was performed on FFPE sections using the PerkinElmer Opal 6-colour kit (Massachusetts, USA) using the automated Leica bond RX opal multiplex protocol (Leica Biosystems, Wetzlar, Germany) as per manufacturer's instructions. The list of reagents is detailed in Table 2.10

Table 2.10. Antibodies used for multiplex immunohistochemistry

Marker	Primary Antibody	Dilution	Secondary Antibody	TSA Plus	Colour
AE1AE3	Leica (AE1 and AE3)	1: 200	Muse IgG	520	Magenta
CD8	Thermo Fisher (4B11)	1: 100	Mouse IgG (1:500)	540	Green
CD4	Spring Bioscience (SP35)	1: 100	Rabbit IgG (1: 1000)	570	Pink
PD-L1	Ventana (SP263)	Neat	Rabbit (neat)	620	Yellow
FoxP3	BioSB (Polyclonal)	1: 100	Rabbit IgG (1: 1000)	650	Orange
PD-1	Cell Marque (NAT105)	1: 100	Mouse (neat)	690	Red

2.2.8.3 Multispectral Imaging and Spectral Un-mixing and Phenotyping

Multiplex stained slides were scanned using the Vectra Multispectral Imaging System version 2 (PerkinElmer, Massachusetts, USA). High power multispectral images (20x) were acquired of the entire stained section. A spectral library containing the emitting spectral peaks of all fluorophores was created with the Inform Nuance Image Analysis software (PerkinElmer, Massachusetts, USA), using multispectral images obtained from single stained slides for each marker and associated fluorophore. This spectral library was used to separate each multispectral image into its individual components (spectral un-mixing), allowing for the colour-based identification of all six markers of interest into a single image using HALO image analysis software (PerkinElmer, Massachusetts, USA). Cells were phenotyped into one of five different classes as follows: tumour cells ($DAPI^{+}$ $AE1^{+}AE3^{+}$), cytotoxic T-cells ($DAPI^{+}$ $CD8^{+}$), T-helper cells ($DAPI^{+}$ $CD4^{+}$), T regs ($DAPI^{+}$ $CD4^{+}FOXP3^{+}$), and other nucleated cells ($DAPI^{+}$). All phenotyping and subsequent quantifications were performed blinded to the sample identity.

2.2.9 Molecular Techniques

2.2.9.1 DNA and RNA extraction from tumour tissue and tumouroids

Snap frozen samples were thawed on ice. The AllPrep DNA/RNA Mini (Qiagen, Hilden, Germany) kit was used to extract DNA/RNA as per manufacturer's instructions. Briefly, tissue was homogenised and placed in RLT buffer, and added to an AllPrep DNA/RNA spin column. Flow through was collected for DNA/RNA purification and added to an RNeasy column, washed in EtOH, subsequent buffers and eluted off the column as purified DNA/RNA. For DNA extraction, an additional step involving using RNAase to remove RNA was added. Quantification of DNA/RNA was analysed on a spectrophotometer.

2.2.9.2 RNAseq technique and analysis

2.2.9.2.1 Technique

Quantity and integrity of total RNA was assessed using the Agilent 4200 TapeStation (Agilent Technologies). Ribosomal RNA was removed from up to 1μg of total RNA using the NEBNext® rRNA Depletion Kit. Illumina compatible Next Generation Sequencing (NGS) libraries were prepared according to standard protocols (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, NEB). Briefly, the RNA was fragmented and first strand cDNA was reverse transcribed from RNA fragments using random primers. The second strand cDNA was synthesised using a dUTP mix, and hairpin adapters were ligated to the cDNA fragments. The dUTP sites were cleaved, linearising library molecules and retaining only anti-sense strands (directionality). The libraries were amplified using indexed primers. Indexed libraries were pooled and sequenced on an Illumine NextSeq 500 to generate 25-50 million paired-end 75 bp reads per sample

2.2.9.2.2 Analysis

Adapter sequence, primers, and too-short-reads sequences were removed using Cutadapt v1.9.1 (Marce Martin, Science for Life Laboratory). Reads were subsequently aligned to human (HG19) using the HISAT2 v2.0.4 read-mapping algorithm and HTSEQ v0.11.2 for gene expression quantification. The Voom-limma workflow was used for data normalisation and to compute statistical significance of differential gene expression.[302] Multidimensional scaling (MDS) plots were generated from limma R package. Heat maps were generated using ComplexHeatmap R package, with Euclidean distance on comparing across samples, and correlation distance on comparing across genes. Gene set enrichment analysis (GSEA) of functionally validated gene sets (obtained from the MSigDB repository) was performed using two group comparisons.[303]

2.2.9.2.3 Consensus Molecular Subtype calling and Cibersort

For accurate CMS calling,[33] RNA expression data from atleast 40 tumour samples is required.[304] Therefore, we selected a subset of publically available 378 The Cancer Genome Atlas (TCGA) samples to supplement the RNAseq data from the CRPM tumour tissue and tumouroids from our study. The TCGA samples were selected from across the four CMS categories as best representatives of each category (lowest predictive CMS false discovery rate). The non-normalised RNAseq count data were used as input for the R package 'CMScaller' (Version 0.99.1),[304] with a false detection rate threshold of 0.05 used. Gene Set Analysis (GSA) were carried out using 'CMSgsa()' function. Batch adjusted according to cohort (TCGA or our study) using the 'removeBatchEffect()' function implemented in limma.[305] All available genes in the CMS panels were used to graph the principle component analysis (PCA) scatter plot. Abundance of cell types estimation was performed using CIBERSORT[306] which is freely available online (https://cibersort.stanford.edu), using the LM22 signature file.

2.2.9.3 Whole exome sequencing (WES)

2.2.9.3.1 Technique

Whole exome sequencing (WES) libraries were prepared using the NEBNext DNA or KAPA DNA Kits using DNA from matched peripheral blood mononuclear cells (germline) or tumouroids. Exonic fragments were captured using the Roche SeqCap EZ Exome v3.0 kit and 75-bp paired-end sequencing was performed on an Illumina HiSeq2500 and NextSeq 500.

2.2.9.3.2 Small variant detection from whole exome sequencing data

Germline variants (SNPs and indels) were detected using the GATK Best Practices for variant discovery (HaplotypeCaller GVCF). The raw sequencing reads were aligned to the b37-decoy reference genome (GATK Resource bundle human_g1k_v37_decoy) using BWA-mem (v0.7.9a). Duplicate reads were marked with SAmbamba markdup. The identities of all germline and corresponding tumour samples were confirmed using BAM-matcher, which is a tool for comparing sample genotype at pre-determined genomic locations by pair-wise examination of BAM files. Germline variants were identified using GATK haplotype gVCF, then somatic variants were detected in matched tumour-germline WES datasets by first calling candidate somatic variants using seven somatic variant callers (MuTect 1.1.4, Seurat 2.6, somatic sniper 1.05, Strelka 2_2.9.0, Varscan 2.4.0, Virmid 1.1.1), followed by GATK IndelRealignment on all the identified candidate somatic variants. The degree of overlap of the identified somatic variants between the callers was used as a metric for variant confidence.

2.2.9.3.3 Copy number variation and structural variant detection

Copy number variations (CNVs) were detected from the WES data using an in-house method developed to sensitively detect CNVs at scales ranging from single exons to whole chromosomes. Germline samples were normalised together. Tumour samples were normalised individually against all germline samples and other archived background samples due to the presence of significant aneuploidy and structure

variation events. Panel CNV and Variant Allele Frequency (VAF) corrected normalization outputs were used to generate tumour ploidy estimates (PE). PE and VAF data were visualised using the Integrative Genomics Viewer. Tumour-associated variants were identified by searching for CNV and high confidence somatic and germline variants in genes found in the UW-ONCOPLEX[307] and/or MSK-IMPACT[308] panels.

2.2.10 *In-Vitro* Drug testing

2.2.10.1 Throughput drug testing

Throughput drug testing was performed in collaboration with SEngine, Seattle on a CLIA certified automated drug testing platform. For the first five samples, a broad 131 pan-cancer drug library was utilised. Tumouroids were passaged and dispensed into 384-well plates at 800 cells/well with 5% matrigel in triplicate. On the day after plating, the 131 pan-cancer drug library was acoustically administered (Labcyte Echo) as single agents using contactless, nano volume liquid transfers with drug concentrations ranging from 33ρM to 200µM. Doses were designed to capture previously reported Cmax values (serum) and the asymptotic response range. Therapeutic dose ranges for chemotherapies were derived from clinical dosage guidelines, one log above and two below an assumed 1.8m^2 average body surface area. After the first five patients, the drug library was refined to create a 36-38 drug CRC-focused panel to better reflect clinically available and Australian pharmaceutical benefits scheme approved options.

Tumouroids were challenged with drugs for six days, following which relative viability was determined by whole well ATP quantification using Cell-Titer-Glo 2.0 (Promega) and normalised to vehicle only controls (maximal DMSO concentration used was 0.2%). Quality control was ensured with the presence of a positive control (bortezomib) and negative control (vehicle only). Individual drug responses were assessed using a SEngine developed proprietary in-house multi-parameter analysis pipeline. Briefly, dose response curves were generated for each drug using a five-parameter logistic fit model. Area under the curve (AUC) and IC_{50} was calculated and dose response curves

were compared to the 'average response' among the SEngine precision medicine internal library of 300 primary tumor samples. This unique method of analysis allows for detection of exceptional responses as well as sample specific, unique sensitivities and is extensively validated semi-annually for both technically consistency and biological concordance with genomic biomarkers, as well as prospective and retrospective in-vivo drug responses as part of the CLIA certification process.

2.2.10.2 Replicate drug testing and combination testing

Replicate testing for Gemcitabine and combination FOLFOX and FOLFIRI testing was performed in collaboration with SAHMRI, Adelaide. Tumouroids were passaged and plated at 2000 cells/well in a 10μL matrigel dome in white-walled 96 well plates (Corning). Solidified matrigel domes were overlaid with 100 μL of CRC media. On the day after plating, media was removed and replaced with 100 μL of drug-containing CRC media. A seven-point ten-fold dilution series of each drug was assessed in quadruplicate. Drug concentrations ranged from 100 ρM to 100 μM for gemcitabine hydrochloride (Sigma) and 1 nM to 100 μM for 5-fluorouracil (Sigma). For *in-vitro* FOLFOX testing, drug doses were set according to the literature at 2 μM Oxaliplatin (SUN Pharmaceuticals) and 5 μM Leucovorin Calcium (Hospira). For *in-vitro* FOLFIRI testing, SN-38 (Selleck chemicals), the active metabolite of Irinotecan was used, in combination with 5 μM Leucovorin. In order to set the concentration of SN-38 for FOLFIRI testing, tumouroids were treated with a seven-point dilution series of SN-38 (10 ρM – 10 μM) or oxaliplatin (1 nM – 100 μM). 2 μM Oxaliplatin was equivalent to the IC_5 for multiple tumouroid lines; therefore the IC_5 or 1 nM for SN-38 was used. The efficacy of each drug combination to enhance the effect of 5-fluorouracil was assessed in multiple tumouroid lines. Drugs were dissolved in DMSO and all treatment wells were normalised to 0.1% DMSO content. Drug-containing media was changed every 2-3 days for 5 days of drug treatment. Tumouroid cell viability was quantitated with Cell-Titer-Glo 2.0 (Promega) using a GloMax microplate reader (Promega). Data analyses were performed using GraphPad Prism 7.02 software, which calculated values for IC_{50} and AUC by applying nonlinear regression (curve fit) and the equation log (inhibitor) versus response - variable slope (four parameters).

2.2.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 7 and 8; California, USA), IBM SPSS version 22 and Microsoft Excel. Categorical variables were reported as numbers with percentages. Continuous non-parametric variables were displayed as medians and inter-quartile ranges. Shapiro-Wilk test was used to evaluate normality. Student's *t* test was used to compare two continous parametric groups, or one-way/two-way ANOVA, with post hoc Bonferroni when analysing > 2 groups. Mann Whitney-U test was used to compare two non-parametric data sets, with Kruskal Wallis test used when analysing > 2 groups. Chi-Squared test was used to compare categorical variables. In Chapter 8, unless specified, data is presented as mean ± standard error of the mean (SEM).

3 Chapter 3: Perceptions in the management of peritoneal disease: A bi-national survey of colorectal surgeons

This chapter has been published as

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3.1 Introduction

Peritoneal metastases confer the worst survival among patients with metastatic colorectal cancer (CRC).[24] Historically, patients with peritoneal metastases had an overall survival of only six to nine months.[46,62] The greatest advance in the management of peritoneal metastases has been the advent and adoption of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC). Verwaal et al[94] demonstrated in a randomised trial that CRS and HIPEC offers a significantly superior survival of 22.3 months compared to 12.6 months with systemic chemotherapy. Since then, a number of other studies have reported favourable median survival of 30-58 months with CRS and HIPEC, with a 27-46% five-year survival.[26, 27,98, 104, 105, 107, 309]

Despite mounting evidence in favour of CRS and HIPEC, there remains ongoing skepticism about its role and efficacy among medical oncologists and surgeons alike. This is reflected in differing approaches to peritoneal disease by different hospitals and clinicians, as well as varying guidelines in management. However, it is unclear whether skepticism is due to lack of awareness and knowledge among clinicians in the management of peritoneal disease. Recent studies have shown that poor awareness of the value of the CRS and HIPEC in the management of colorectal peritoneal metastases (CRPM) contributes to reduced utilisation of CRS and HIPEC.[310, 311]

In Australia and New Zealand, management of peritoneal disease is restricted to selected centres, with most peritoneal units comprised of colorectal surgeons. This study aimed to gain a snapshot of perceptions in the management of peritoneal metastases from colorectal cancer among colorectal surgeons in Australia and New Zealand.

3.2 Methods

A structured ten-question online Survey Monkey survey was sent to all colorectal surgeons affiliated with the Colorectal Surgical Society of Australia and New Zealand (CSSANZ). Three questions were based on clinical experience and demographics, one on health economics and the remaining six were hypothetical scenarios evaluating management of peritoneal disease. These six scenarios were a range of classical cases involving management of isolated peritoneal metastases, synchronous and metachronous peritoneal disease, high-risk patients and risk factors for peritoneal recurrence. The survey design ensured that surveys could only be returned if all questions were completed.

After the survey was sent out, a reminder was sent to those who hadn't responded two weeks later. The survey was open for one month. Survey questions are presented as Appendix 1.1.

3.2.1 Statistical analyses

Data was collated and analysed using Microsoft Excel. Bar graphs were created to demonstrate the percentage of respondents choosing each management option.

Chi-square test was used to compare differences in responses based on surgical experience.

3.2.2 Ethics

This project was approved by the institutional research ethical review committee (LNR/19/PMCC/25).

3.3 Results

Surveys were sent to 224 colorectal surgeons affiliated with CSSANZ. Eighty-one (36.2%) surveys were successfully completed. Nineteen (23.5%) respondents were from New Zealand, with the remaining 62 (76.5%) being Australian colorectal surgeons.

Scores were collated based on classical Likert scales into five categories and reported accordingly.

3.3.1 Demographics, experience and health economics

The majority (88.9%) of colorectal surgeons were practicing in metropolitan cities. Of the 81 respondents, over two thirds (67.9%) had five or more years experience as a colorectal surgeon. The majority (65.4%) of all surgeons either agreed or strongly agreed with the existing model of a single state based service being the most effective (Figure 3.1a). The majority (48.1%) were not in favour of managing only complex cases at a state centre (Figure 3.1b). Most surgeons (66.7%%) strongly disagreed with offering CRS and HIPEC at all hospitals (Figure 3.1c).

3.3.2 Role of CRS and HIPEC over systemic chemotherapy

In treating pseudomyxoma peritonei from perforated appendiceal neoplasms, 87.7% of surgeons agreed or strongly agreed that CRS and HIPEC offered a higher survival benefit than systemic chemotherapy (Figure 3.2a). Comparatively, with CRPM, only 69.1% felt CRS and HIPEC offered improved survival over systemic chemotherapy. Notably, over a quarter of surgeons (27.1%) felt there was no difference in survival offered by CRS and HIPEC (Figure 3.2b). In gastric cancer, most (70.4%) felt there was no benefit from CRS and HIPEC over systemic chemotherapy (Figure 3.2c), while in ovarian cancer, 60.5% felt CRS and HIPEC offered an improved survival (Figure 3.2d).

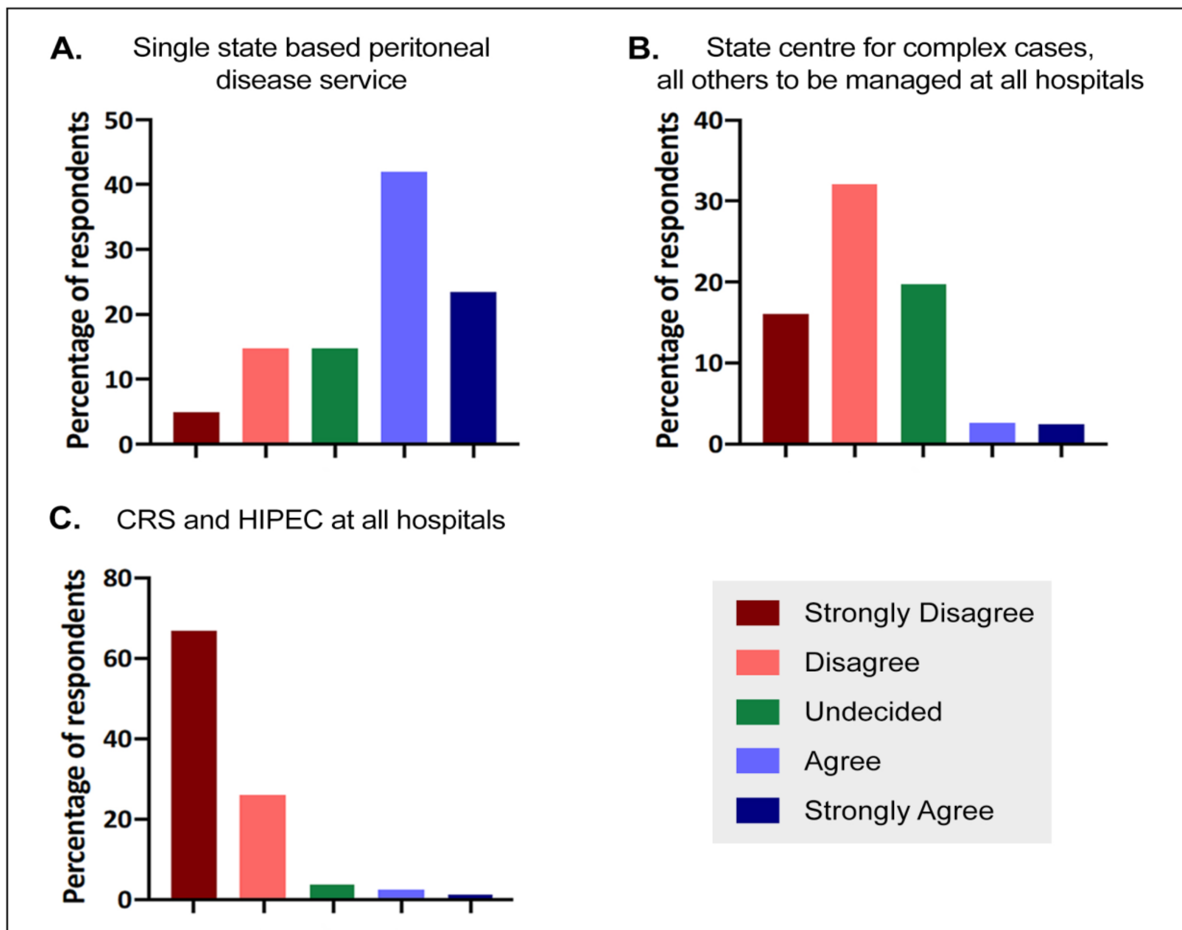

Figure 3.1a. Most surgeons agree with the existing model of a single state based peritoneal centre. Fig 3.1b. The majority disagree with the concept of only complex cases being managed at a state centre. Fig 3.1c. Almost all surgeons disagree with the concept of offering CRS and HIPEC at all hospitals.

3.3.3 Management of imaging detected isolated low volume peritoneal recurrence

Question 6 explored the management of an imaging detected 2cm isolated peritoneal recurrence, 18 months after primary colorectal cancer resection. There were mixed strategies among surgeons in managing this, with 45.7% favouring surgery to excise the isolated nodule (Figure 3.3a). Over three quarters (75.3%) disagreed or strongly disagreed with biopsing the nodule (Figure 3.3b). Over half (58.1%) favoured systemic therapy for treating low volume isolated peritoneal disease (Figure 3.3c). Almost three

quarters (72.8%) agreed or strongly agreed with referring such a case for consideration of CRS and HIPEC (Figure 3.3d).

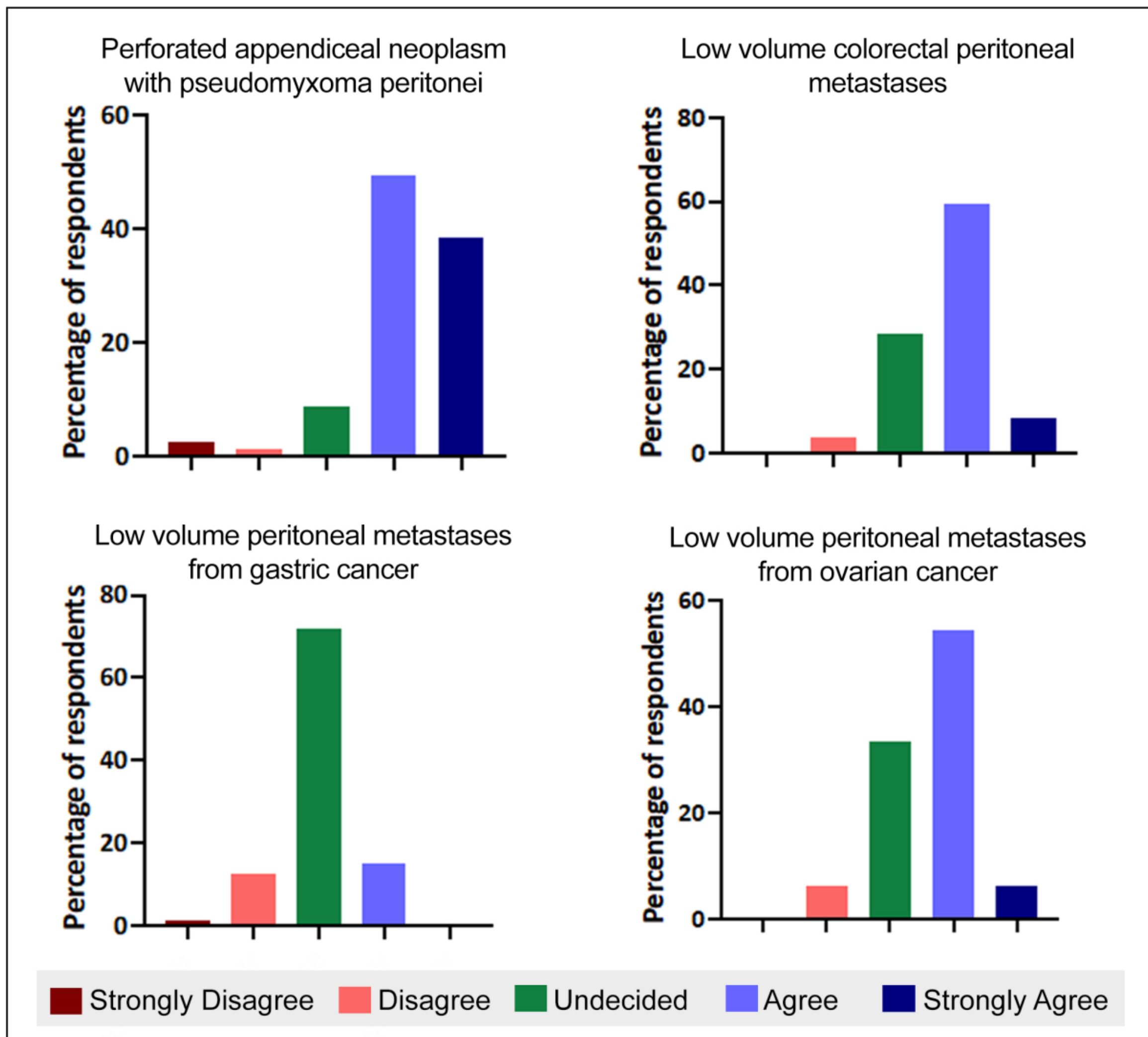

Figure 3.2. Improvement in survival with CRS and HIPEC compared to systemic chemotherapy in different situations. 3.2a. Pseudomyxoma peritonei from perforated appendiceal neoplasms; 3.2b. Low volume colorectal peritoneal metastases; 3.2c. Low volume gastric cancer peritoneal metastases; 3.2d. Low volume peritoneal metastases from ovarian cancer.

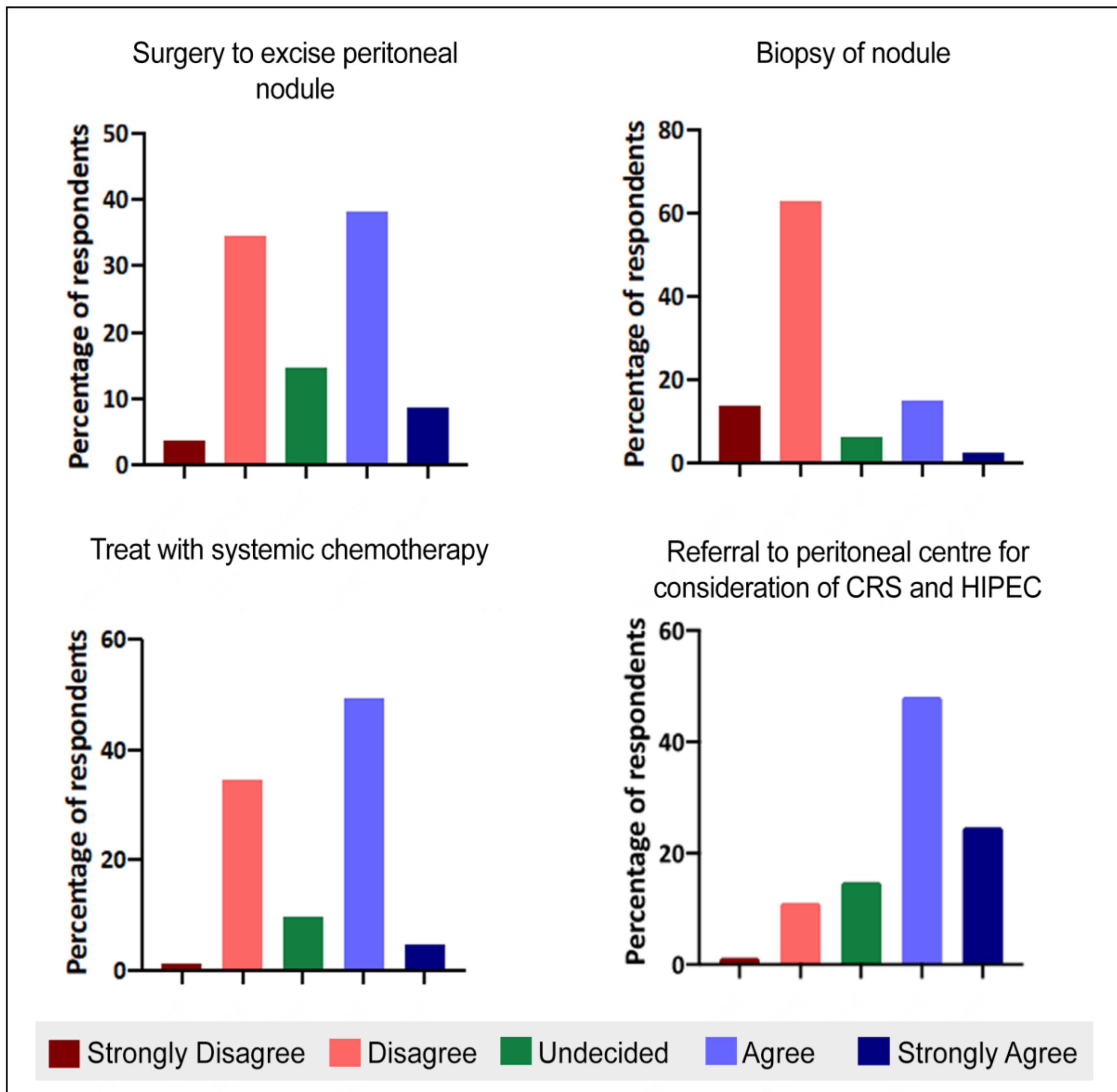

Figure 3.3a-d. Responses to different options in the management of an isolated 2 cm peritoneal recurrence.

3.3.4 Role of pro-active 'early relook' surgery

Question 7 evaluated the role of a pro-active "early relook" in a high-risk case of a T4a tumour with no imaging evidence of peritoneal recurrence. The majority (49.4%) of surgeons disagreed or strongly disagreed with offering a diagnostic laparoscopy at six months (Figure 3.4a). Almost three quarters (72.3%) would not refer this case to a peritoneal centre for consideration of "early relook" and HIPEC (Figure 3.4b), with the

majority of surgeons (81.5%) opting for standard national guideline based surveillance (Figure 3.4c).

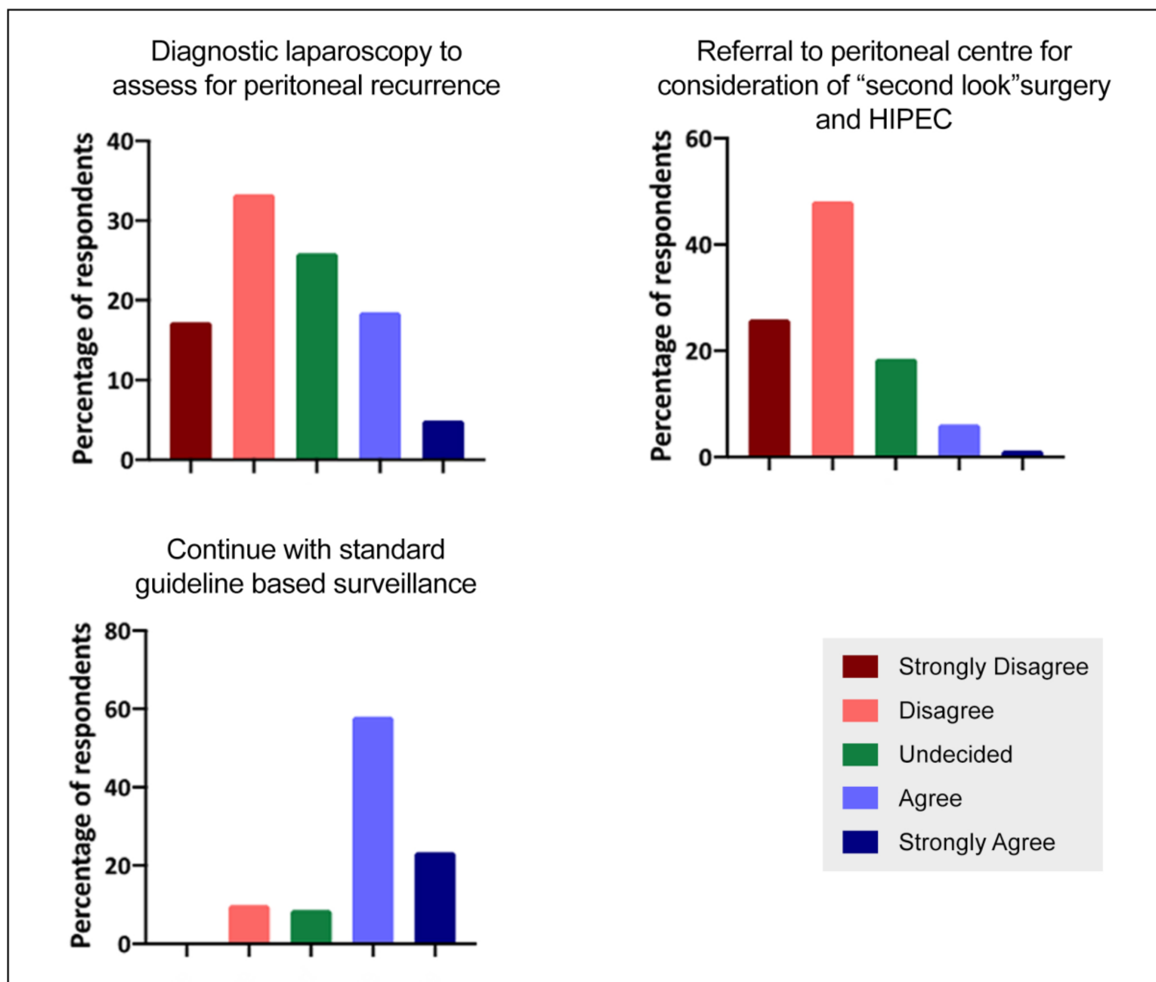

Figure 3.4a-c. Responses to options in managing a high-risk case for peritoneal recurrence with no imaging evidence of peritoneal recurrence.

3.3.5 Management of Krukenberg tumours

Question 8 evaluated responses to an imaging detected isolated left Krukenberg tumour nine months after primary colorectal tumour resection. Almost half (49.4%) agreed or strongly agreed to treat this case with systemic chemotherapy (Figure 3.5a). The majority (60.4%) agreed or strongly agreed with performing a diagnostic laparoscopy to evaluate PCI (Figure 3.5b). While the majority (64.2%) would not refer this case to gynae-oncology, over a quarter (25.9%) would refer a Krukenberg tumour to gynae-

oncology for an oophorectomy (Figure 3.5c). Most surgeons (63.0%) agreed or strongly agreed with referring this case to a peritoneal centre for consideration of CRS and HIPEC (Figure 3.5d).

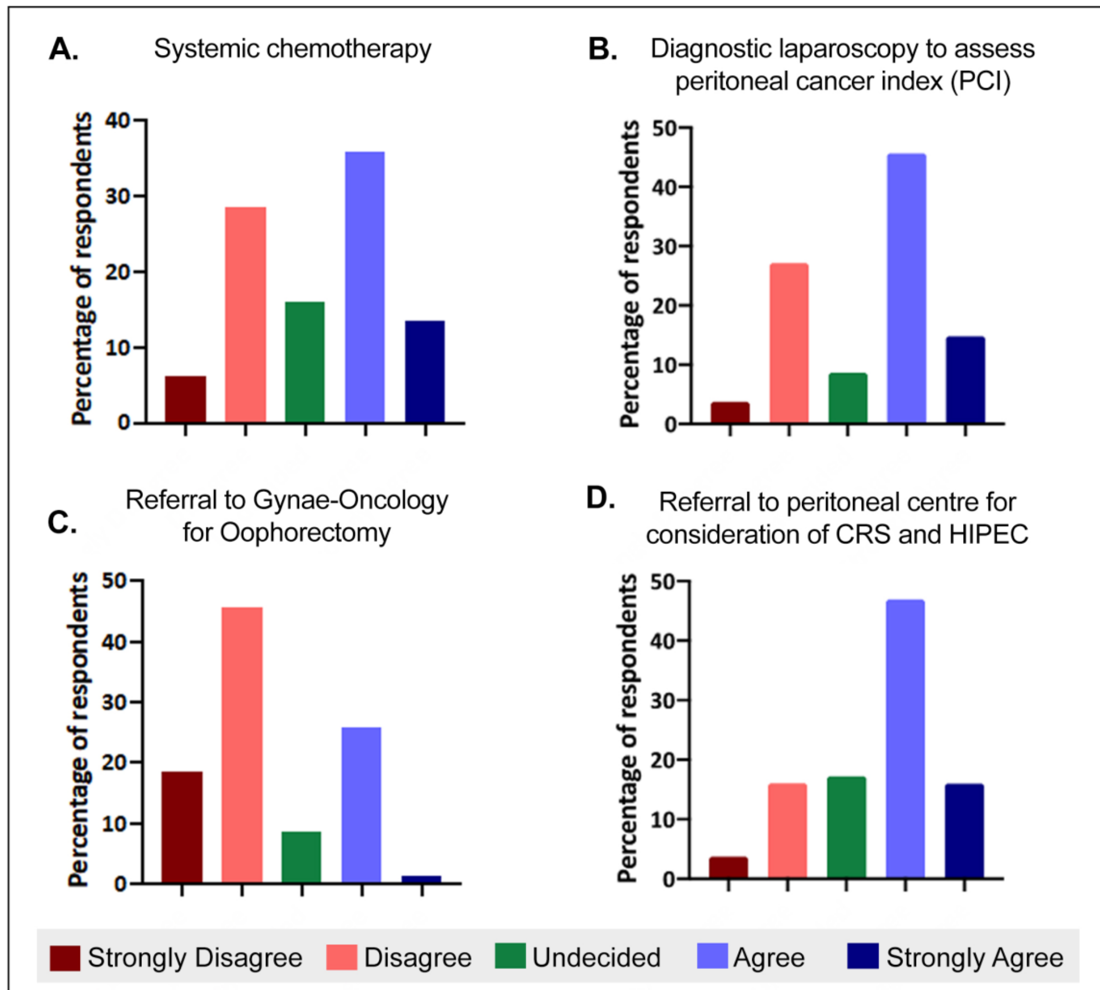

Figure 3.5a-d. Responses to options in managing an isolated left Krukenberg tumour.

3.3.6 Management of incidental synchronous peritoneal metastases

Question 9 evaluated the management strategy employed when resectable synchronous peritoneal metastases are incidentally encountered. The decision between proceeding with the right hemicolectomy or not was evenly divided with 46.7% of surgeons favouring completing surgery as planned, and approximately the same proportion not

in favour of completing surgery as planned (Figure 3.6a). Most (79%) felt an omentectomy should be performed with right hemicolectomy to complete the surgery (Figure 3.6b). Most surgeons (77.8%) disagreed or strongly disagreed with performing the right hemicolectomy and merely taking a biopsy of the omental metastases (Figure 3.6c). Almost a third (29.6%) favoured only taking a biopsy of an omental deposit and referring the patient for consideration of CRS and HIPEC (Figure 3.6d).

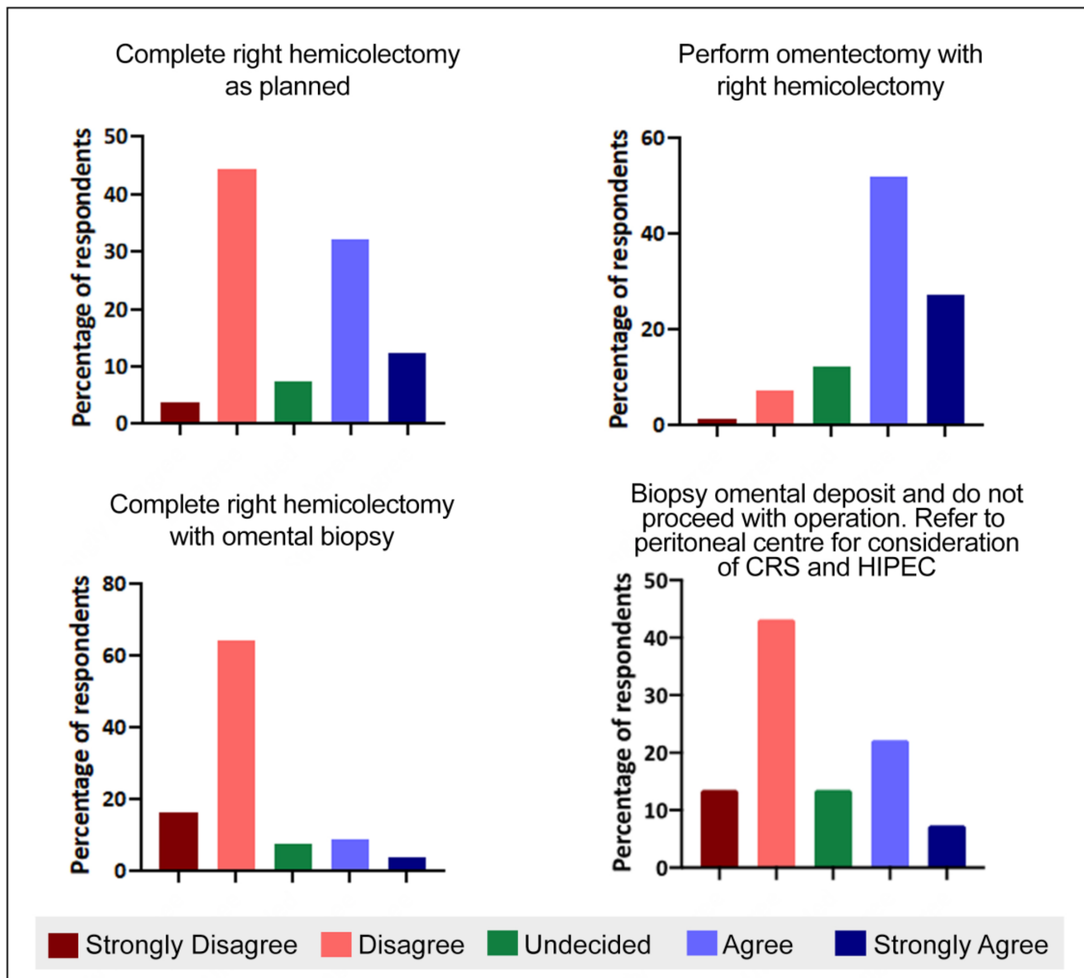

Figure 3.6a-d. Management options when incidental resectable synchronous omental metastases are found during a right hemicolectomy.

3.3.7 Risk factors for peritoneal recurrence

Question 10 evaluated surgeons' perceptions on risk factors for peritoneal recurrence. Over three quarters (76.5%) felt a non-perforated T4 cancer conferred a very high or above average risk for peritoneal recurrence. Almost all surgeons felt that a perforated

cancer (97.5%), ovarian metastases (97.5%) and isolated peritoneal metastases resected at index operation (100%) carried an above average or very high risk for peritoneal recurrence. Intra-operative tumour spillage was seen as an above average risk factor by two thirds (66.7%) of surgeons, with an obstructed tumour seen as average risk by the majority (54.3%).

3.3.8 Role of surgical experience on management of peritoneal metastases

Of the 81 respondents, 55 (67.9%) had five or more years' experience as a colorectal surgeon, with the remaining 26 (32.1%) having less than five years' experience.

Two questions yielded significantly different responses based on surgical experience, with all other questions having similar responses regardless of surgical experience.

In evaluating the role of a pro-active 'second look' surgery and HIPEC six months after a T4a resection, younger surgeons agreed with performing a diagnostic laparoscopy to assess PCI compared to more experienced surgeons (38.5% v 9.1%, $p = 0.036$) (Appendix 1.2a). Similarly, younger surgeons agreed to refer such a case to a peritoneal centre for consideration of 'second look' surgery and HIPEC compared to experienced surgeons (15.4% v 1.8%, $p=0.014$) (Appendix 1.2b).

3.4 Discussion

To our knowledge, this study provides the first snapshot of perceptions among colorectal surgeons in Australia and New Zealand in the management of CRPM. CRS and HIPEC is a complex procedure, with major morbidity and mortality rates ranging from 13.1-47.2% and 1.0-4.1% respectively.[105, 180] Numerous studies have demonstrated a consistent relationship between high volume centres and improved long-term survival after cancer surgery.[312, 313] Furthermore, there is a clear learning curve of approximately 140-220 cases that has to be overcome before achieving proficiency.[314, 315] Therefore, having a centralised system for management of peritoneal disease ensures selected centres can develop and maintain a high level of expertise in managing peritoneal disease.

While a number of international consensus guidelines support the role of CRS and HIPEC in the treatment of isolated CRPM,[110, 111] it must be noted that most of the data supportive of CRS and HIPEC is in the form of cohort studies that report a median survival of 30-58 months,[98, 104, 105, 309] with a paucity of randomised data.[94] Systemic chemotherapy can offer patients with CRPM a median survival of only 16 months,[24] with a five year survival of less than 5%.[155] In ovarian cancer, a recent RCT[316] demonstrated that CRS and HIPEC offered a significantly improved recurrence free and overall survival compared to CRS with systemic therapy alone. This trial did however demonstrate poorer survival in both arms compared to previous trials in ovarian peritoneal disease.[317] It is plausible that this landmark trial may lead to greater consideration for CRS and HIPEC in advanced ovarian cancer.[318]

While the superiority of CRS and HIPEC over systemic chemotherapy has been previously demonstrated in an RCT,[94] the role of systemic chemotherapy as an adjunct to CRS and HIPEC remains unexplored. Various studies[27,150] have reported an improved survival with the use of neoadjuvant or adjuvant systemic chemotherapy with CRS and HIPEC. However, based on current evidence, there is very limited role for systemic chemotherapy as mainstay of treatment for resectable isolated CRPM.[166] The role of

systemic chemotherapy as an adjunct to CRS and HIPEC, in the neoadjuvant or adjuvant setting is currently under investigation in the CAIRO 6 trial (NCT02758951).[148]

While CRS with HIPEC is viewed as the mainstay of treatment for isolated CRPM, recent evidence has raised questions about the efficacy of HIPEC. The recently completed PRODIGE 7 trial[106] demonstrated an impressive 41.2 months median overall survival following CRS alone, with the addition of HIPEC not offering a significant survival benefit (41.2 months v 41.7 months, HR 1.00; 95% CI: 0.73-1.37). Subgroup analysis however demonstrated a survival benefit with HIPEC in those with a PCI 11-15. This trial, while yet unpublished, reaffirmed the value of complete cytoreduction, but has raised doubts about the overall efficacy of oxaliplatin-based HIPEC.

The role of pro-active “second look” surgery in high-risk cases for peritoneal recurrence is controversial. In approximately 25% of primary CRC resections, there are clinical or pathological findings that indicate a high risk for peritoneal recurrence.[54] Given well-established factors that predict early peritoneal recurrence such as T4a pathology, ovarian metastases or perforated cancers, studies have evaluated the role of a pro-active approach in patients at high risk for peritoneal recurrence. Elias et al[175] demonstrated that asymptomatic peritoneal metastases were diagnosed in 55% of cases undergoing a second look laparotomy 13 months after resection of a high-risk primary colorectal cancer. These included cases of synchronous peritoneal metastases, ovarian metastases or perforated primary tumours. In a follow-up study,[59] peritoneal metastases were found in 56% of patients undergoing second look laparotomy for CRS and HIPEC 12 months following high-risk primary colorectal cancer resection. Following successful CRS and HIPEC at second look surgery, five year OS was 90%, with a 44% DFS.

Recent RCTs however, offer a different view. The recently completed French RCT (Prophylochip-NCT01226394)[176] explored the role of systematic second look surgery and oxaliplatin based HIPEC versus surveillance for asymptomatic patients six months following a high risk primary colorectal cancer resection. High risk was defined as minimal CRPM resected with the primary, ovarian metastases or perforated primary tumour. While peritoneal metastases were diagnosed in 52% of patients undergoing

second look surgery, there was no difference in 3 year DFS (44% v 51%, p=0.75) or 3 year OS (79% v 80%) in the second look or surveillance arms. Similarly, the COLOPEC trial (NCT02231086)[319] evaluated the role of adjuvant oxaliplatin based HIPEC after pT4 or perforated primary colorectal cancers. There was no difference in the primary endpoint of peritoneal metastases free survival (77% v 81%) by diagnostic laparoscopy at 18 months in the control and adjuvant HIPEC arms. Furthermore, there was no difference in DFS and OS at 18 months between the two arms.

All these studies confirm the validity of high risk factors for peritoneal recurrence. However, based on current evidence, early re-look with oxaliplatin based HIPEC or adjuvant oxaliplatin based HIPEC may not offer any survival benefit over surveillance. These trials are yet unpublished, and the final publications may offer further insight into why the results were unfavourable.

Krukenberg tumours are ovarian metastases commonly from cancers such as gastric, colorectal or appendiceal. The presence of a new ovarian mass, an elevated CEA and a previous CRC, is a Krukenberg tumour until proven otherwise. Ovarian metastases from CRC are seen in 4-19% of cases.[320] They are often chemo-resistant, associated with other peritoneal metastases, and confer a worse prognosis.[321, 322] Ovarian metastases from CRC are peritoneal disease and therefore should be treated with CRS and HIPEC.[323, 324] A laparoscopy may be performed to evaluate PCI as other peritoneal deposits may be present.

Synchronous CRPM are seen in 4-13% of patients[18, 47, 48]. There are currently no guidelines to direct care in patients with incidentally found resectable synchronous CRPM. A recent Dutch study[49] comparing outcomes after synchronous or metachronous management of CRPM with CRS and HIPEC demonstrated no difference in OS (34 v 33 months, p=0.819). Other studies have similarly demonstrated no difference in survival between metachronous and synchronous treatment of CRPM with CRS and HIPEC.[120, 179] Shida et al[325] recently demonstrated that a synchronous R0 resection for CRPM without HIPEC can offer a favourable median survival of 33 months and a five year survival of 28.7%. This is an area with only retrospective studies in the literature. If only low volume incidental CRPM are incidentally found, findings from PRODIGE 7[106] would also

suggest HIPEC may not add any survival benefit. CRS and HIPEC or alternatively ensuring an R0 resection without HIPEC both appear to be reasonable options supported by studies in the management of incidentally found synchronous CRPM.

One of the limitations inherent in all surveys is low response rate. However, our response rate of 36.2% is much higher that other similar international surveys in this field.[310, 311, 326] Furthermore, while most other surveys[310, 311, 327, 328] have assessed clinician views and awareness on effectiveness and safety of CRS and HIPEC, our survey and a recent Swiss survey[326] served to evaluate how clinicians view and manage commonly encountered cases with peritoneal disease, as timely, appropriate and evidence based care in such cases translates to better clinical outcomes. While this survey covered the use of HIPEC, it failed to evaluate the role of other intraperitoneal chemotherapy modalities such as early postoperative intraperitoneal chemotherapy (EPIC) or pressurised aerosolized chemotherapy (PIPAC). While EPIC and PIPAC are very infrequently used in Australia and New Zealand, it would have nonetheless been useful to evaluate its perceived role among colorectal surgeons. This survey also failed to capture general surgeons and medical oncologists. In Australia and New Zealand, CRCs are managed by both general and colorectal surgeons. In metropolitan centres with the majority of the population, they are managed largely by colorectal surgeons. It would have been challenging to capture gastrointestinal medical oncologists only, as medical oncologists not involved in colorectal cancer care would have been unlikely to respond, leading to a very low response rate. Furthermore, we believe that peritoneal disease is largely a surgical disease, therefore we elected to survey the most common surgical group involved in the care of these patients.

3.5 Conclusion

This survey provides the first snapshot of management strategies undertaken by colorectal surgeons in the management of CRPM in Australia and New Zealand. It demonstrates that while most colorectal surgeons have a similar view in the management of CRPM, there are some circumstances that lead to different management strategies. Regular published updates, and ongoing collaborations with peritoneal services would help ensure appropriate utilisation of CRS and HIPEC resources.

The message from this study extends beyond the borders of just the two countries and demonstrates that there is still a somewhat nihilistic attitude towards the treatment of peritoneal metastases. However, in selected patients, surgical management can offer a chance of a long-term survival. To evaluate how effective surgery is in treating peritoneal surface malignancies (PSM), the next chapter evaluates outcomes from CRS and HIPEC at a tertiary peritoneal disease centre in the management of all peritoneal surface malignancies.

4 Chapter 4: Evaluation of cytoreductive surgery and HIPEC for peritoneal surface malignancies: Analysis of 384 consecutive cases

This chapter has been published as

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4.1 Introduction

Peritoneal surface malignancy (PSM) was historically viewed as a terminal manifestation of gastrointestinal malignancies, with a median survival of 3.1 months.[62] Treatment with standard modalities such as systemic chemotherapy had poor efficacy in the treatment of peritoneal disease.[155, 329] Over the last two decades however, the concept of treating PSM as a loco-regional disease led to the introduction of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC).

The basis for CRS involves removal of all macroscopic intraperitoneal tumour deposits, with the addition of HIPEC aimed at eradicating any microscopic and isolated tumour cells. Hyperthermia to 41-43°C acts synergistically to enhance the efficacy of the chemotherapy. Pioneered by Sugarbaker in the 1990s in the treatment of pseudomyxoma peritonei (PMP),[88, 330] CRS and HIPEC soon became the standard of care for patients with PMP. Studies reported a highly favourable 43-83% five-year survival for patients undergoing CRS and HIPEC for PMP of appendiceal origin.[331-334]

Early studies also demonstrated survival benefit from aggressive CRS for low volume peritoneal metastases in colorectal cancer.[335, 336] Verwaal et al[94] in 2003 demonstrated in a randomised trial that CRS and HIPEC offered a significant overall survival benefit of 22.3 months compared to 12.6 months with systemic therapy for patients with isolated colorectal peritoneal metastases (CRPM). Further studies have since reported highly favourable 20-31% five-year survival, with a median overall survival of 32 to 41 months following CRS and HIPEC for CRPM.[98, 100, 104-106] This led to widespread adoption of CRS and HIPEC for CRPM, with CRS and HIPEC now part of the treatment pathway for CRPM in many countries.[110, 111] In other cancers such as mesothelioma, CRS and HIPEC form the mainstay of treatment, offering patients a 40 to 53 months overall survival.[337, 338]

Our institute has been the statewide peritoneal disease centre since 2009. In this study, we aim to evaluate our outcomes from CRS and HIPEC for PSM since the inception of the peritoneal disease service. In particular, we aim to explore the morbidity, mortality and survival following CRS and HIPEC and changes in our practice with increasing experience.

4.2 Methods

4.2.1 Patients and inclusion criteria

Data was retrospectively analysed from the prospectively maintained peritoneal disease database. All patients undergoing CRS and HIPEC from June 1, 2009 to 31 May 2018 were included in the study. Patient demographics, histology, operative details, complications and follow-up were recorded.

4.2.2 Peri-operative management

Patients were pre-operatively assessed with routine full blood counts, liver function tests, and coagulation studies. An FDG positron emission tomography based computed tomography (PET/CT) scan of the chest, abdomen and pelvis was performed in most cases. All patients were discussed at the peritoneal disease multidisciplinary meeting (MDT) involving peritoneal surgeons, gastrointestinal (GI) oncologists, GI pathologists, radiologists and nuclear medicine physicians.

4.2.3 Diagnoses

Pseudomyxoma peritonei (PMP): PMP is a clinical syndrome characterised by mucinous, gelatinous ascites. While PMP can arise from various pathologies, the most common is an appendiceal mucinous neoplasm. In this study, the term PMP refers to peritoneal disease originating from appendiceal mucinous neoplasms (low grade or high grade mucinous neoplasm) only.

Appendiceal Carcinoma/Cancer: peritoneal disease arising from appendiceal adenocarcinoma, mucinous adenocarcinoma (having clear pathological evidence of infiltrative invasion).

Colorectal peritoneal metastases (CRPM): Peritoneal metastases originating from colorectal adenocarcinoma

4.2.4 Management strategy based on pathology

PMP and appendiceal carcinoma: CRS and HIPEC was offered to all patients with PMP if they were medically fit. A high PCI was not a deterrent to offering CRS and HIPEC. The aim was always to achieve a macroscopically complete resection. If this was not possible due to areas of unresectable disease, maximal tumour debulking was performed, as debulking can also provide favourable survival.

CRPM: Selection for CRS and HIPEC was more selective in patients with CRPM. The unit policy and the MDT consensus were to offer CRS and HIPEC to patients with a PCI ≤15, as the benefit of CRS and HIPEC diminishes in those with a higher PCI. Patients are assessed with PET/CT to evaluate burden of disease. If disease is low volume on imaging and clearly resectable, they are offered CRS and HIPEC. If disease is very high volume, they are given systemic chemotherapy and not offered CRS and HIPEC. Patients with intermediate disease burden are evaluated further with a diagnostic laparoscopy. The main purpose of laparoscopy was to identify patients who may have unresectable disease that was not detected on imaging. If laparoscopy failed to identify any clearly unresectable disease, the patient is offered CRS and HIPEC.

Less common histologies (Ovarian, Mesothelioma, Sarcoma): These were discussed on a case-by-case basis at the MDT, before progressing to CRS and HIPEC.

4.2.5 Cytoreductive Surgery and HIPEC

Following laparotomy and adhesiolysis, the PCI was scored from 0-39. Cytoreductive surgery was performed in accordance with Sugarbaker's techniques[88, 339]. Organ resections involved segmental colectomy, proctectomy, small bowel resections, cholecystectomy, splenectomy, gastrectomy (partial or rarely total), segmental liver resection and hysterectomy with bilateral salpingo-oopherectomy in females. If nodules

were present on the organs, they were resected as long as an R0 resection could be achieved. Nodules on the small bowel serosa were excised with primary closure. If a large number of nodules were present on a segment of small bowel, a small bowel resection was performed. Nodules on the colon led to a segmental colectomy. Similarly, for nodules on the spleen, a splenectomy was performed. For nodules on the stomach, they were generally excised with the defect primarily closed. If a large number of nodules were present, partial or rarely total gastrectomy was performed. Stripping of pelvic and diaphragmatic peritoneum was recorded when performed. Cytoreduction score was recorded at the end of each operation. CC-0 implied no residual macroscopic disease. CC-1, 2 and 3 implied residual disease less than 2.5mm, 2.5mm-2.5cm, and greater than 2.5cm respectively. CC-0/1 was considered macroscopically complete resection, with subsequent administration of HIPEC. CC-2/3 cases were deemed incomplete cytoreduction and not given HIPEC, except in selected cases such as presence of intractable ascites. HIPEC was administered via open coliseum technique with a target intraperitoneal temperature of 41-43°C.

4.2.6 HIPEC drugs and dosage

Pseudomyxoma peritonei of appendiceal origin (PMP): Mitomycin C (15mg/m^2) for HIPEC, with intravenous 5-FU (400mg/m^2) for 90 minutes.

Appendiceal adenocarcinoma: Oxaliplatin (350mg/m^2) with intravenous 5-FU (400mg/m^2) for 30 minutes.

Colorectal peritoneal metastases (CRPM): Oxaliplatin (350mg/m^2) with intravenous 5-FU (400mg/m^2). If there was known insensitivity to Oxaliplatin, Mitomycin C (15mg/m^2) was used for 90 minutes.

Mesothelioma: Cisplatin (100mg/m^2) with Mitomycin C (12.5mg/m^2) for 90 minutes.

Ovarian Cancer: Cisplatin (100mg/m^2) with intravenous 5-FU (400mg/m^2) for 60 minutes.

Sarcoma (Desmoplastic Small Round Cell Tumour): Cisplatin (100mg/m^2) was used for 60 minutes.

Gastric cancer: Cisplatin (50mg/m^2), Doxorubicin (15mg/m^2) with intravenous 5-FU (400mg/m^2) for 60 minutes.

4.2.7 Systemic therapy

The use of systemic chemotherapy in both the neo-adjuvant setting (NAC) and adjuvant setting was an MDT based decision.

No patient with **PMP** received systemic chemotherapy.

Appendiceal carcinoma: NAC was given to patients when there were logistic reasons such as long wait times before an operation date. Adjuvant chemotherapy was generally given when a CC 0/1 resection was not achieved. Adjuvant chemotherapy was also given at the discretion of the medical oncologists in high-risk patients, such as young patients, high tumour burden, presence of signet ring cells.

CRPM: The unit is very supportive of the use of systemic chemotherapy in patients with CRPM, aiming for patients undergoing CRS and HIPEC to receive either neo-adjuvant or adjuvant chemotherapy. Neo-adjuvant chemotherapy was given for three months in most cases when possible. Adjuvant chemotherapy was generally only given when patients had unresectable or very high volume disease at CRS and HIPEC (CC 2/3 resection). For patients who had a CC 0/1 resection, adjuvant chemotherapy was only given if they had not received neo-adjuvant chemotherapy.

4.2.8 Post operative care and follow-up

Post operatively, most patients were transferred to intensive care. An enhanced recovery after surgery (ERAS) based postoperative care program was implemented in all patients. Patients were started on total parenteral nutrition (TPN) on day seven if there was ongoing evidence of postoperative ileus.

No patient received postoperative intraperitoneal chemotherapy (EPIC).

There are no consensus guidelines regarding how follow-up should be performed for patients with PSM. We utilise an institutional based follow-up program that was developed by the peritoneal service and is offered to all patients.

Following discharge, patients are seen in two to four weeks for clinical review. Subsequently, patients are followed up for clinical review at three, six and 12 months. Blood tests with tumour markers when appropriate are performed at six and 12 months. PET/CT imaging is performed at six and 12 months. From the second year onwards, patients are followed up six monthly for clinical review with blood tests and PET/CT.

4.2.9 Complications

Clavien Dindo grades III, IV and V were recorded[340].

4.2.10 Statistical analyses

Data was analysed with IBM SPSS version 22. Cases were divided into two cohorts based on the first half of cases performed and second half. Continuous parametric variables were expressed as means with standard deviations, with non-parametric variables expressed as medians with inter-quartile ranges. Categorical variables were expressed as numbers with percentages.

Differences between the two cohorts were evaluated by student t-test for continuous parametric variables, with Chi-Square or Mann-Whitney U test used for non-parametric categorical or continuous variables.

Kaplan- Meier survival analysis was performed to estimate the overall (OS) and disease free survival (DFS). Log rank test was used to assess statistical significance. Overall and disease free survival were calculated from date of cytoreductive surgery to date of last follow-up or death, and date of last follow-up or relapse, respectively.

Factors affecting survival were identified using Cox univariate analysis. Cox multivariate Hazards ratio model was developed to identify factors independently associated with overall survival. A p value < 0.05 was considered statistically significant.

4.2.11 Ethics

This project satisfied the PeterMacCallum Cancer Centre Ethics Committee and governance requirements and was granted ethical approval. Ethics approval number was 17/141R.

4.3 Results

4.3.1 Patient demographics

A total of 333 patients undergoing 384 CRS and HIPEC procedures were included in the study (Appendix 2.1).

Patient demographics are shown in Table 4.1. The median age of all patients was 56.0 years with a female preponderance (59.6%). Overall, the most common histology leading to CRS and HIPEC was PMP in 178 cases (46.4%). There was a significantly increased proportion of CRPM in the second cohort compared the first (40.6% v 21.4%, p<0.01), with a lower proportion of PMP cases in the second cohort compared to the first (36.5% v 56.3%, p<0.01). Mesothelioma (8 cases), ovarian cancer (5 cases), sarcoma (3 cases), gastric cancer (2 cases) and mucinous carcinoma of unknown primary (5 cases) accounted for only 23 cases in total, and therefore were classified together as “Others”. However, given the pathologies are significantly different, “others” have been excluded from the survival analysis.

The median PCI was 11 for the entire study population, with a lower PCI in the second cohort compared to the first (9 v 15, p<0.01). In particular, there was a much higher proportion of cases with PCI <10 in the second cohort (60.4% v 39.6%, p<0.01), and lower proportion of cases with a PCI >30 in the second cohort compared to the first (7.8% v 19.3%, p<0.01). Overall, there was a significantly higher rate of macroscopically complete resections (CC 0/1) in the second cohort compared to the first (82.3% v 67.7%, p <0.01). This was further reflected in a higher proportion of CC 0/1 resections in PMP (81.4% v 69.4%, p=0.05) and CRPM (83.3% v 63.4%, p=0.02) cases in the second cohort compared to the first. Consequently, there was a higher use of HIPEC in the second cohort compared the first (80.7% v 68.8%, p <0.01).

In keeping with improvement in practice, there were fewer maximal debulking (CC 2) and laparotomy and biopsy cases (CC 3) in the second cohort compared to the first (8.9% v 20.3% for CC 2 and 8.9% v 12.0% for CC 3 respectively).

Approximately one in four cases received a blood transfusion (24.1%). There was no difference in blood transfusion rates between the two cohorts.

Table 4.1. Demographics of the cohort

Characteristic		**Entire Cohort**	**Cases 1-192**	**Cases 193-384**	**P value**
Number of cases		384	192	192	
Median Age		56.0 (47.0-66.0)	57.0 (48.0-66.0)	55.0 (46.0-65.5)	0.64
Gender	*Male*	155 (40.4%)	85 (44.3%)	70 (36.5%)	0.07
	Female	229 (59.6%)	107 (55.7%)	122 (63.5%)	
Median LOS		11.0 (8.0-18.0)	11.0 (8.0-16.0)	12.0 (8.0-19.0)	0.24
Histology	*PMP*	178 (46.4%)	108 (56.3%)	70 (36.5%)	<0.01
	Appendix	64 (16.7%)	35 (18.2%)	29 (15.1%)	
	CRPM	119 (31.0%)	41 (21.4%)	78 (40.6%)	
	*Others**	23 (6.0%)	8 (4.2%)	15 (7.8%)	
Operation duration (min)		280 (240-360)	280 (220-360)	280 (240-320)	0.86
ASA		2 (1-3)	2 (1-3)	2 (1-3)	0.75
PCI	*<10*	192 (50.0%)	76 (39.6%)	116 (60.4%)	<0.01
	10-15	44 (11.5%)	24 (12.5%)	20 (10.4%)	
	16-20	28 (7.3%)	14 (7.3%)	14 (7.3%)	
	21-30	68 (17.7%)	41 (21.4%)	27 (14.0%)	
	>30	52 (13.5%)	37 (19.3%)	15 (7.8%)	
	Median	11	15	9	
CC score	*0/1*	288 (75.0%)	130 (67.7%)	158 (82.3%)	<0.01
	2	56 (14.6%)	39 (20.3%)	17(8.9%)	
	3	40 (10.4%)	23 (12.0%)	17 (8.9%)	
CC 0/1 based on histology	*PMP*	132/178 (74.2%)	75 (69.4%)	57 (81.4%)	0.05
	Appendix	51/64 (79.7%)	26 (74.3%)	25 (86.2%)	0.19
	CRPM	91/119 (76.5%)	26 (63.4%)	65 (83.3%)	0.02
	Others	14/23 (60.9%)	3 (37.5%)	11 (73.3%)	0.11
HIPEC	*Yes*	287 (74.7%)	132 (68.8%)	155 (80.7%)	<0.01
	No	97 (25.3%)	60 (31.2%)	37 (19.3%)	
Blood	*Yes*	93 (24.2%)	48 (25.0%)	45 (23.4%)	0.41
	No	291 (75.8%)	144 (75.0%)	147 (76.6%)	

LOS: length of stay; PMP: pseudomyxoma peritonei of appendiceal origin; appendix: appendiceal adenocarcinoma; CRPM: colorectal peritoneal metastases; ASA: American Society of Anesthesiologists; CC: completeness of cytoreduction
*** Others included mesothelioma (8), ovarian (5), sarcoma (3), gastric (2), mucinous carcinoma of unknown primary (5).*

4.3.2 Resections and Morbidity

Operative resections and morbidity are shown in Table 4.2. Organ resections were common, with a colectomy or proctectomy performed in 210 (54.7%) and 63 (16.4%) cases in the entire study. The majority of cases required upto two-organ resections (82.0%), with almost one in five cases requiring more than two organ resections (18.0%). Overall, there was no difference in the type of organ resections, number of anastomoses, stoma formation, or proportion of multi-visceral resections between the two cohorts.

Overall, one hundred and one cases (26.3%) had a major complication (Grade III/IV). Intra-abdominal collections occurred in 50 cases (13.0%), with most managed with percutaneous drainage. There were eighteen anastomotic leaks (4.7%) in the study. Renal failure requiring renal replacement therapy was rare, occurring in seven cases (1.8%). There were three peri-operative mortalities in the study (0.8%). There was no difference in morbidity or mortality rates between the two cohorts.

Table 4.2. Peri-operative details

Characteristic		**Entire Cohort**	**Cases 1-192**	**Cases 193-384**	**P value**
Number of cases		384	192	192	
Morbidity					
Grade III/IV	*Yes*	101 (26.3%)	48 (25.0%)	53 (27.6%)	0.32
	No	283 (73.7%)	144 (75.0%)	139 (72.4%)	
Collection	*Yes*	50 (13.0%)	21 (10.9%)	29 (15.1%)	0.14
	No	334 (87.0%)	171 (89.1%)	163 (84.9%)	
Anastomotic leak	*Yes*	18 (4.7%)	7 (3.6%)	11 (5.7%)	0.24
	No	366 (95.3%)	18(5 (96.4%)	181 (94.3%)	
*Major Respiratory**	*Yes*	59 (15.4%)	27 (14.0%)	32 (16.7%)	0.29
	No	325 (84.6%)	165 (86.0%)	160 (83.3%)	
*Renal Failure***	*Yes*	7 (1.9%)	3 (1.6%)	4 (2.1%)	0.71
	No	377 (98.1%)	189 (98.4%)	188 (97.9%)	
Return to theatre	*Yes*	21 (5.5%)	10 (5.2%)	11 (5.7%)	0.50
	No	363 (94.5%)	182 (94.8%)	181 (94.3%)	
Peri-operative Mortality	*Yes*	3 (0.8%)	2 (1.0%)	1 (0.5%)	0.50
	No	381 (99.2%)	190 (99.0%)	191 (99.5%)	
Resections					
Colectomy	*Yes*	210 (54.7%)	109 (56.8%)	101 (52.6%)	0.24
	No	174 (45.3%)	83 (43.2%)	91 (47.4%)	
Proctectomy	*Yes*	63 (16.4%)	28 (14.6%)	35 (18.2%)	0.20
	No	321 (83.6%)	164 (85.4%)	157 (81.8%)	
SB resection	*Yes*	70 (18.2%)	40 (20.8%)	30 (15.6%)	0.12
	No	314 (81.8%)	152 (79.2%)	162 (84.4%)	
Gastrectomy	*Yes*	11 (2.9%)	5 (2.6%)	6 (3.1%)	0.50
	No	373 (97.1%)	187 (97.4%)	186 (96.9%)	
HBSO	*Yes*	86 (37.6 %)	44 (41.1%)	42 (34.4%)	0.45
	No	143 (62.4%)	63 (58.9%)	80 (65.6%)	
Splenectomy	*Yes*	46 (12.0%)	28 (14.6%)	18 (4.7%)	0.08
	No	338 (88.0%)	164 (85.4%)	174 (95.3%)	
Number of anastomoses	*0*	140 (36.5%)	66 (34.4%)	74 (38.5%)	0.54
	1	196 (51.0%)	99 (51.6%)	97 (50.5%)	
	>1	48 (12.5%)	27 (14.1%)	21 (10.9%)	
Stoma	*Yes*	56 (14.6%)	28 (14.6%)	28 (14.6%)	0.56
	No	328 (85.4%)	164 (85.4%)	164 (85.4%)	
Organ resections	*≤2*	315 (82.0%)	157 (81.8%)	158 (82.3%)	0.50
	>2	69 (18.0%)	35 (18.2%)	34 (17.7%)	

HBSO: Hysterectomy, bilateral salpingo-oopherectomy. SB resection: Small bowel resection
** Major Respiratory includes pleural effusion, pneumonia, pneumothorax and respiratory failure*
***Renal Failure: those requiring renal replacement therapy*

4.3.3 Systemic therapy

4.3.3.1 Appendiceal carcinoma

Twelve cases (20%) received systemic chemotherapy. Four cases received NAC and eight received adjuvant chemotherapy

4.3.3.2 Colorectal peritoneal metastases

One hundred and four cases (87.4%) received systemic chemotherapy. 70 cases (67.3%) received NAC. Median OS was the same for those who had NAC compared to those who did not (34 months, log rank p=0.65).

Forty-nine cases (47.1%) received adjuvant chemotherapy. Of these, 27 cases received adjuvant chemotherapy after CC 0/1 resection (none of these had NAC).

Overall, of the 91 cases that achieved a CC 0/1 resection, 76 (83.5%) received either NAC or adjuvant chemotherapy. Median OS was 40 months for those who had systemic chemotherapy and 34 months for those who did not (log rank p=0.80).

4.3.4 Survival Outcomes

Median follow-up for all cases was 24 months (range 0-135 months), with median OS 85 months, with a 52% five-year survival (Figure 4.1a). Median DFS was 30 months, with a 37% five-year DFS (Figure 4.1b). There were significant differences in OS based on histology, with a median OS of 97 months for cases with PMP, and 34 months for CRPM (p<0.001) (Figure 4.2a). DFS was significantly better for PMP with a median DFS of 42 months, compared to 39 months for appendiceal cancer and 13 months for CRPM (p <0.001) (Figure 4.2b). Following macroscopically complete resection (CC 0/1), median OS was not reached for PMP and appendiceal cancers. Median OS for CRPM was 40 months (Appendix 2.2).

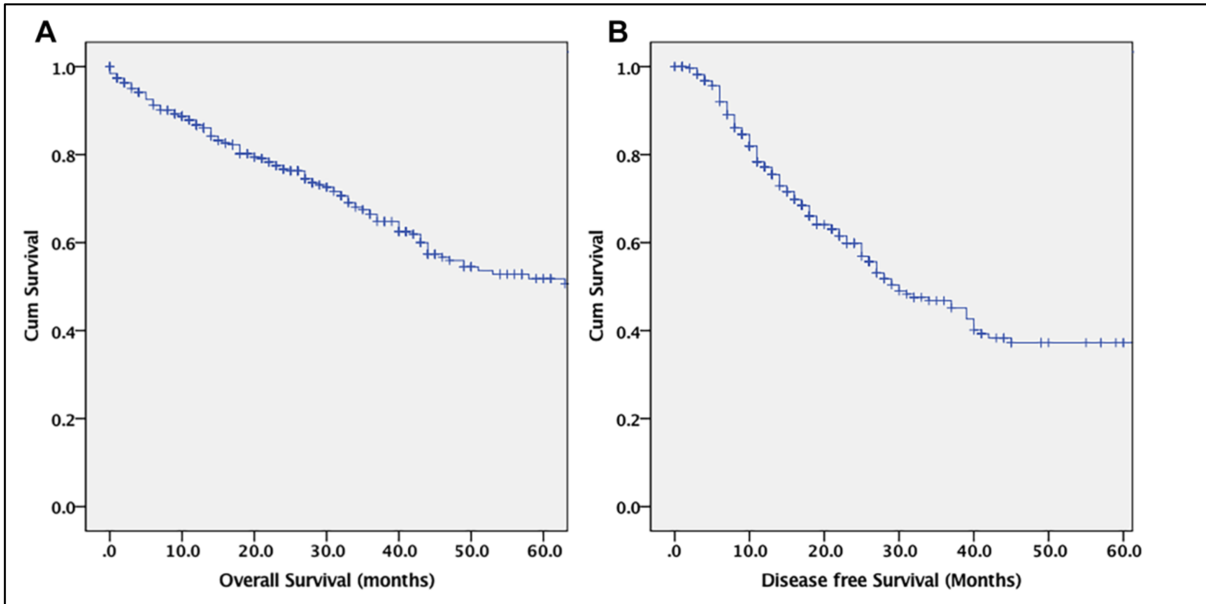


Figure 4.1a. Overall survival of all cases, with 85 months median OS and 52% 5-year survival.
Figure 4.1b. Median disease free survival of 30 months after complete cytoreduction for all cases

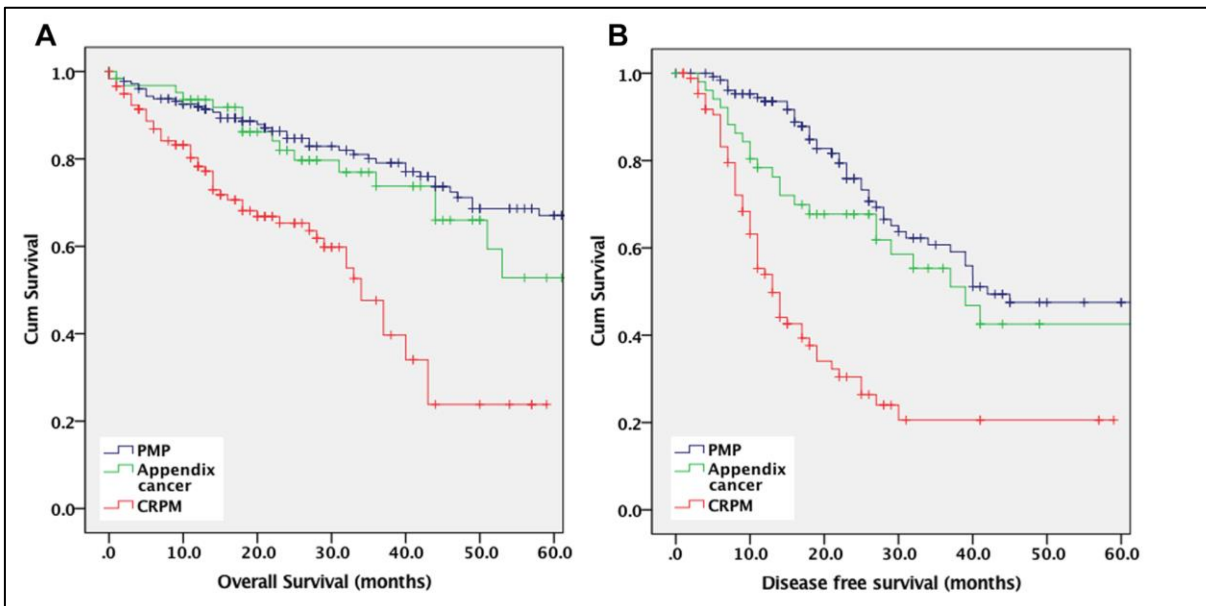


Figure 4.2a. Overall survival based on histology. Median OS of 97 months for PMP, median OS not reached for appendiceal cancers (loss to follow-up) and 34 months for CRPM (log rank p <0.001).
Figure 4.2b. Disease free survival based on histology. Median DFS of 42 months for PMP, 39 months for appendiceal cancers and 13 months for CRPM. (Log rank p <0.001).

4.3.5 Differences between first and second cohort

When comparing OS between the two cohorts, there was no discernible change in survival (log rank p =0.86). Median OS in the first cohort was 85 months, with a 3-year survival of 67%. Median OS was not reached in the second cohort, with a 3-year survival of 65% (Figure 4.3a). For PMP cases, 3-year survival was 80% in the first cohort compared to 83% in the second (log rank p=0.51) (Figure 4.3b). Similarly, for appendix cancers, 3-year survival was 72% compare to 82% in the second cohort (log rank p=0.69) (Figure 4.4a). For CRPM cases, there appeared to be a noticeable trend towards a difference, with median OS of 29 months in the first cohort compared to 37 months in the second cohort (log rank p=0.06) (Figure 4.4b).

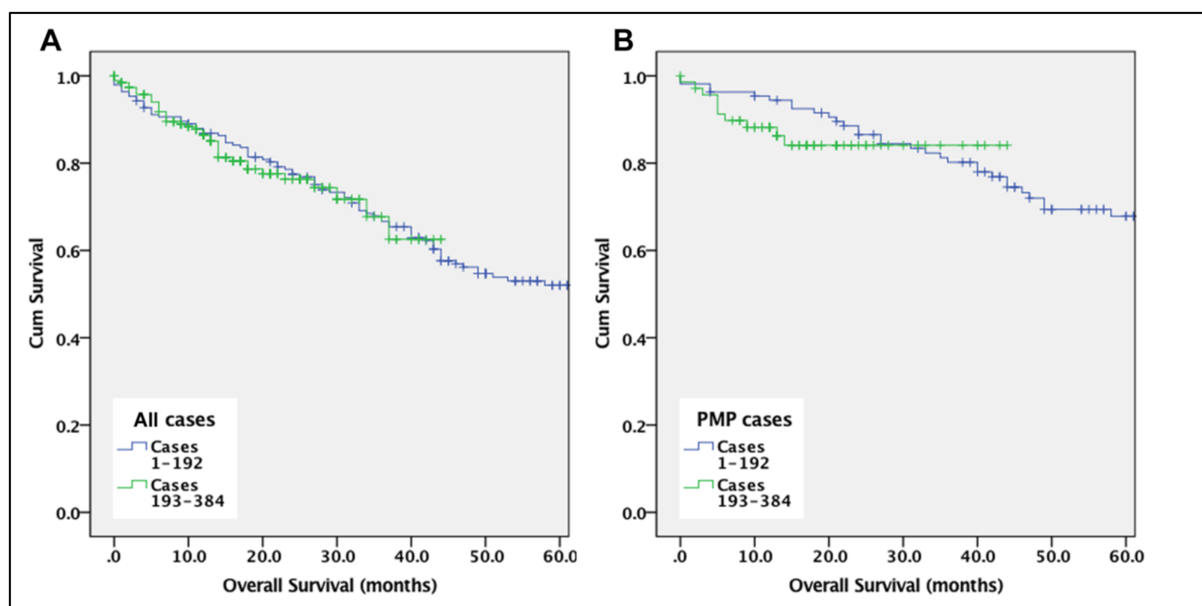


Figure 4.3a. Overall survival based on cohort. Median OS of 85 months in the first cohort, with a 3-year survival of 67%. Median OS not reached for second cohort, with 3-year survival of 65% (log rank p=0.86). Figure 4.3b. When comparing PMP cases, 3-year survival was 80% in the first cohort compared to 83% in the second cohort (log rank p=0.51).

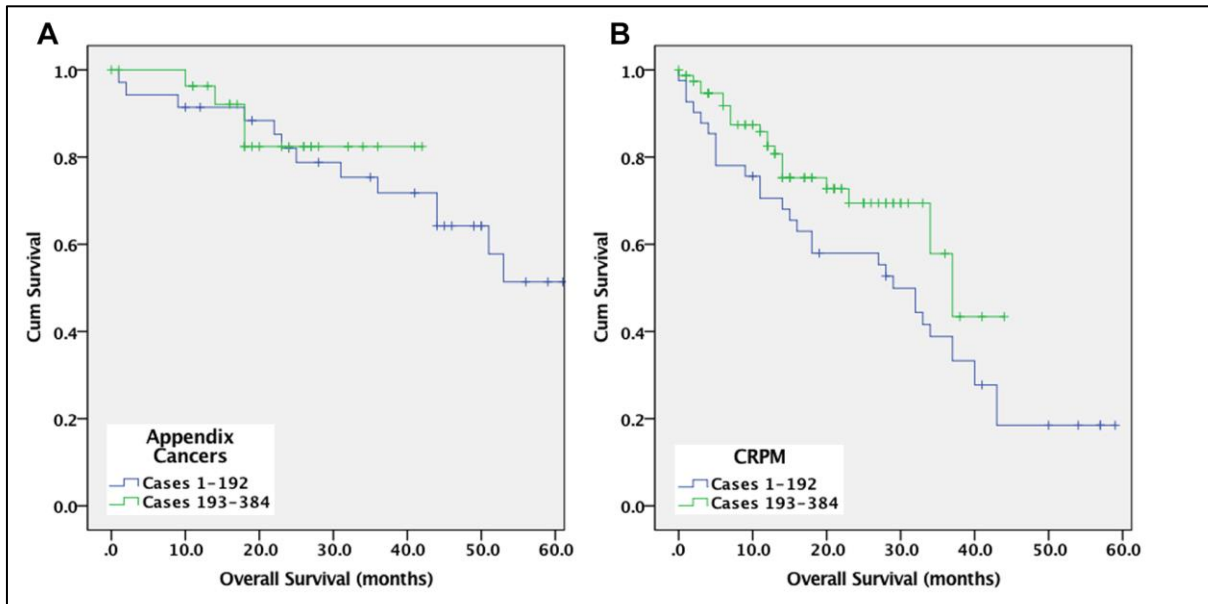


Figure 4.4a. When comparing appendix cancer cases, 3-year survival was 72% in the first cohort compared to 82% in the second cohort (log rank p=0.69).
Figure 4.4b. When comparing CRPM cases, median OS was 29 months in the first cohort compared to 37 months in the second cohort (log rank p=0.06).

4.3.6 PCI and completeness of cytoreduction

Macroscopically complete resections significantly impacted on survival with CC 0/1 resections having a much better survival compared to CC 2 or CC 3 resections (p<0.001) (Figure 4.5a). PCI also significantly impacted on survival, with a lower PCI having a significantly better survival compared to cases with a higher PCI (p<0.001) (Figure 4.5b).

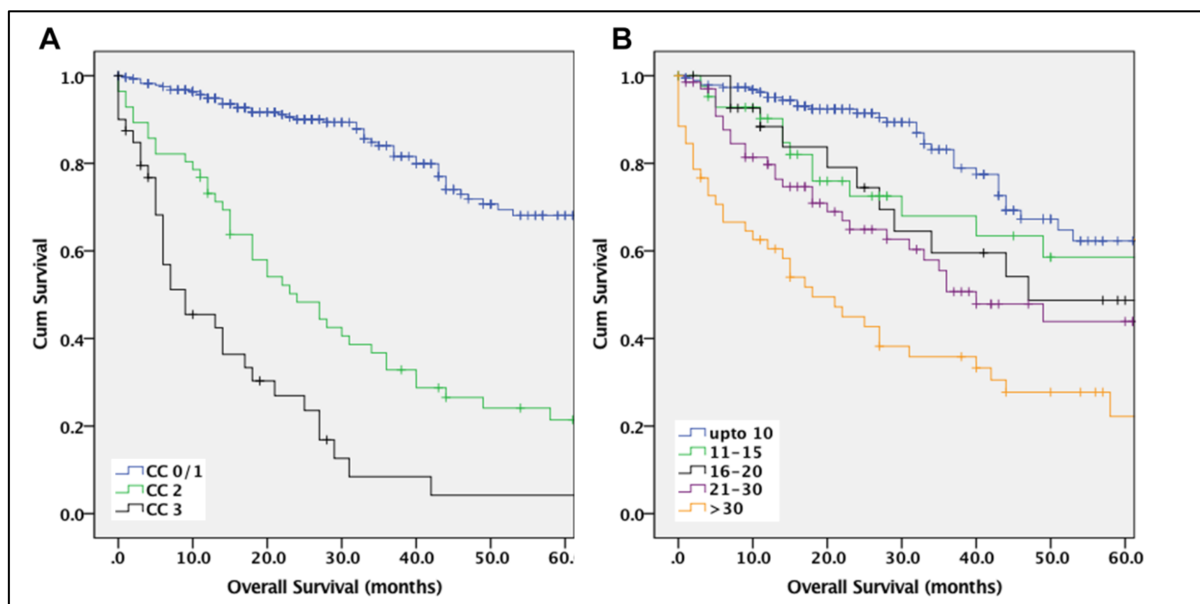


Figure 4.5a. Overall survival based on completeness of cytoreduction. Following CC 0/1 resection, Median OS not reached. Median OS of 24 months following CC 2 resection and 9 months following CC 3 resection (Log rank p <0.001).
Figure 4.5b. Overall survival based on PCI. Median OS not reached for PCI 0-10 and 11-15. Median OS of 47 months for PCI 16-20, 40 months for PCI 21-30, 18 months for PCI >30 (Log rank p <0.001).

4.3.7 Workload

There has been a significant increase in the number of CRS and HIPEC procedures performed annually since the start of the peritoneal disease service in 2009 (Appendix 2.3). Most notably, there has been a significant increase in the proportion of CRPM cases in the last few years.

4.3.8 Synchronous versus Metachronous resections

Forty-three patients underwent a synchronous resection of the primary with peritoneal disease. Median OS was 97 months with synchronous resection compared to 63 months with metachronous resection (log rank p=0.10) (Appendix 2.4a). Disease free survival was 40 months in those having a synchronous resection compared to 29 months in those with a metachronous resection (log rank p=0.25) (Appendix 2.4b).

4.3.9 Univariate and Multivariate Analysis

Increasing age, colorectal histology, increasing PCI, completeness of cytoreduction and use of HIPEC were associated with overall survival on univariate analysis. On multivariate analysis, histology (appendiceal and colorectal), increasing PCI, and incomplete cytoreduction were independent predictors of worse overall survival (Table 4.3)

Table 4.3. Cox Univariate and Multivariate Hazard ratio model evaluating factors associated with overall survival

Variable		Univariate HR (95% CI)	P Value	Multivariate HR (95% CI)	P value
Age at operation (Continuous variable)		1.02 (1.00-1.04)	**0.01***	1.01 (1.00-1.03)	0.17
Gender	*Male*				
	Female	0.75 (0.52-1.08)	0.12	0.95 (0.64-1.42)	0.81
Histology	*PMP*				
	Appendix	1.19 (0.68-2.09)	0.54	2.84 (1.47-6.50)	**<0.01****
	CRPM	3.31 (2.17-5.05)	**<0.01***	10.25 (5.59-18.78)	**<0.01****
Year group (cases)	*1-192*				
	193-384	1.04 (0.67-1.61)	0.86	1.13 (0.70-1.82)	0.61
PCI	*0-10*				
	11-15	1.78 (0.90-3.51)	0.10	2.07 (1.02-4.20)	**0.04****
	16-20	2.48 (1.26-4.88)	**0.01***	1.36 (0.56-3.32)	0.49
	21-30	3.16 (1.88-5.31)	**<0.01***	2.37 (1.10-5.07)	**0.03****
	>30	5.43 (3.28-8.99)	**<0.01***	6.76 (2.86-15.94)	**<0.01****
CC score	*CC 0/1*				
	CC 2	5.27 (3.45-8.05)	**<0.01***	5.04 (2.64-9.62)	**<0.01****
	CC 3	12.28 (7.54-19.97)	**<0.01***	5.49 (2.52-11.92)	**<0.01****
Grade III/IV	*No*				
complication	*Yes*	1.25 (0.84-1.86)	0.27	1.20 (0.79-1.84)	0.39
Organ resections	*≤2*				
	>2	0.61 (0.36-1.04)	0.07	1.02 (0.55-1.87)	0.96
Transfusion	*No*				
	Yes	1.06 (0.71-1.61)	0.77	0.79 (0.50-1.27)	0.33
Synchronous	*Yes*				
Resection	*No*	1.57 (0.92-2.70)	0.10	0.89 (0.47-1.67)	0.71

HR and 95% CI: hazard ratio and 95% confidence interval; PMP: pseudomyxoma peritonei of appendiceal origin; appendix: appendiceal adenocarcinoma; CRPM: colorectal peritoneal metastases; PCI: peritoneal carcinomatosis index; CC: completeness of cytoreduction;
*** Statistical significance on multivariate analysis*

4.4 Discussion

In its early years, the adoption of CRS and HIPEC was met with skepticism, largely due to concerns about morbidity, and the paucity of randomised evidence. However, with poor survival from standard systemic chemotherapy,[62] there was a strong need to pursue a more aggressive treatment for PSM. With increased experience in surgical practice, advances in radiological imaging and better patient selection, CRS and HIPEC can now offer highly favourable results for PSM from gastrointestinal cancers.[341-343] CRS and HIPEC form a critical part of the treatment pathway for PMP, appendiceal, ovarian cancer, CRPM, mesothelioma, and has an evolving role in the management of advanced gastric cancer and certain sarcomas.[108, 111, 338, 342, 344]

This study is the second largest series reporting on outcomes following CRS and HIPEC for PSM in Australia and New Zealand. Our study demonstrated CC score, PCI and histology as independent predictors of overall survival. This is in keeping with published literature where completeness of cytoreduction, increasing PCI, more aggressive tumour histology, major complications, and increasing age have been reported as factors independently influencing survival.[27, 331, 342, 345, 346] Our demonstrated OS of 97 months for PMP and 34 months for CRPM is similar to recently published series.[27, 98, 331, 341, 342, 345, 346] Following macroscopically compete resection, median OS is not reached for PMP and appendiceal cancer, and is 40 months for CRPM, emphasizing the importance of clearing all visible disease. The overall morbidity and mortality rates of 26.3% and 0.8% in the study are low, in keeping with other centres who have reported morbidity and mortality rates of 22.9-50% and 2-3.9% respectively.[98, 341, 342, 347, 348]

We believe there is evidence of improvement in our practice over the nine-year period of this study. This is reflected largely in the significantly increased complete cytoreduction rates in the second cohort compared to the first. Strikingly, there is an increase in complete cytoreduction rates for all histologies in the second cohort. The reasons for higher macroscopically complete resection (CC 0/1) rates in the second cohort are likely multifactorial. Having performed 192 cases in the first cohort, we

believe the unit had gained adequate experience as part of overcoming the learning curve. Larger studies have explored the learning curve more extensively and reported that approximately 130-220 procedures are required before gaining proficiency.[347, 349] This would have lead to better patient selection in the second cohort, along with improved technical expertise in clearing disease that may have been more challenging to clear early on in the experience. Better patient selection is a combination of improved pre-operative, and intra-operative decision-making. For example, all patients now get a PET/CT scan as part of staging, which helps staging better than a contrast CT scan alone. Furthermore, most cases are now performed as two-surgeon procedures, allowing for more balanced intra-operative support, decision-making and technical assistance. Furthermore, for CRPM, diagnostic laparoscopy is employed selectively to try to better select those who would benefit from CRS and HIPEC. All these factors together combine to lead to an improved macroscopically complete resection (CC 0/1) rate in the second cohort without increased morbidity.

Patient selection is critical in ensuring good outcomes following CRS and HIPEC. This is particularly true in the case of CRPM, where benefit from CRS and HIPEC is strongly linked to a lower PCI. With almost double the number of CRPM cases in the second cohort compared to the first, complete cytoreduction rates were still much higher in the second cohort (83.3% v 63.4%), which translates into improved median OS in the second cohort compared to the first. This again reflects better patient selection, particularly as there is well-established benefit for CRS and HIPEC in CRPM when the PCI is less than 15.[108, 109]

While widely accepted in many countries, certain aspects of CRS and HIPEC remain in question. With regard to the treatment of CRPM, the overall role of Oxaliplatin based HIPEC has been put in question based on the recent PRODIGE 7 trial.[106] This French randomised trial demonstrated an excellent 41.2 months median OS following complete cytoreduction for CRPM. The addition of Oxaliplatin based HIPEC following CRS did not improve survival for the entire cohort (41.2 months v 41.7 months, $p=0.995$). On subgroup analysis however, HIPEC did offer a significant survival benefit for those with a PCI of 11-15. This trial has raised some questions about the overall efficacy of Oxaliplatin based HIPEC. While there is a survival advantage with the addition of HIPEC

to those with a higher PCI, there is no reliable way to accurately select these patients pre-operatively. Furthermore, all patients in this trial received six months of peri-operative systemic chemotherapy. It is unknown what role this had on survival. It is possible that Oxaliplatin may not be the optimal agent for HIPEC, or alternatively that one dose of HIPEC in itself may not be adequate to treat microscopic disease. It is also conceivable that systemic chemotherapy in the form of neo-adjuvant or adjuvant therapy as an adjunct to CRS may be of benefit, both areas that haven't been well explored in the past. The CAIRO6 RCT[148] is currently recruiting to help evaluate this question further. This trial is evaluating the role of perioperative systemic therapy with CRS and HIPEC versus upfront CRS and HIPEC alone. At this stage, while PRODIGE 7 has generated a great deal of debate, we need to await the formal publication. It however, has highlighted a number of areas to focus further research on.

On the other hand, Van Driel et al[316] recently demonstrated that CRS and HIPEC does offer a significant overall and recurrence free survival over surgery alone in ovarian cancer. It is likely that this will contribute to more ovarian cancers referrals to peritoneal disease centres in the future.

Despite successful CRS and HIPEC, recurrence rates remain high. In PMP, over 25% recur.[350] In CRPM, up to 70% of patients undergoing complete cytoreduction recur, usually within 9-18 months.[132, 140, 351] While some can be offered iterative surgeries, there is a clear need to explore newer avenues of treatment. Newer treatment options like immunotherapy are changing the treatment landscape in many cancers yet remain largely unexplored in PSM.[263] Going forward, this is a rich area for collaborative translational research.

There are a number of limitations in this study that warrant mention. As a retrospective analysis, we remain reliant on the accuracy of the records previously kept. Thirty-nine patients in our cohort had more than one CRS and HIPEC procedure, with re-do procedures likely falling in the second cohort. Re-do procedures would be less likely to involve as many organ resections as the index procedure. This could contribute to the lack of a discernible difference in number of organ resections between the two cohorts. Selection bias is a major limitation that warrants mention. As the sole peritoneal service

for the state, almost all our referrals are from other hospitals. It is very likely that treating clinicians at the index hospital only refer patients who are medically fit for consideration of CRS and HIPEC. Therefore, there are possibly other patients with PSM who we would not be capturing in our cohort. Another point of selection bias is more specifically in the case of CRPM. Given initial skepticism in the role of CRS and HIPEC for CRPM, it is possible that patients with CRPM were not referred early on. In recent years, with greater acceptance of CRS and HIPEC, referrals are increasing. This is reflected in Supplementary figure 2, showing increasing workload at our service, particularly for CRPM. Furthermore, given we only offer CRS and HIPEC for patients with a PCI ≤ 15, contributing further to the patient selection bias. As a young peritoneal disease centre, our patient selection has been strict, to ensure safety and minimise morbidity. Furthermore, only 6% of our study population had mesothelioma, ovarian and other less common cancers. While this may be merely reflective of the referral patterns, we are unable to accurately report on outcomes of these cancers individually, owing to low numbers.

4.5 Conclusion

CRS and HIPEC can offer highly favourable survival for patients with PSM, with low morbidity. Histology, lower PCI and completeness of cytoreduction are factors that are associated with an improved overall survival.

This study has demonstrated that CRS and HIPEC is safe and can offer high favourable survival for PSM with low morbidity. The next chapter will focus specifically on the evolution of CRS and HIPEC for CRPM at a tertiary referral centre, evaluating outcomes that can be achieved with CRS and HIPEC.

5 Chapter 5: Evolution of cytoreductive surgery with HIPEC for colorectal peritoneal metastases: Single centre 8-year experience.

This chapter has been published as

<u>Narasimhan V</u>, Britto M, Pham T, Warrier S, Naik A, Lynch C, Michael M, Tie J, Ramsay R, Heriot A. Evolution of cytoreductive surgery and HIPEC for colorectal peritoneal metastases: 8-year Single Institutional Experience. *Dis Colon Rectum* 2019; 62(10):1195-1203

5.1 Introduction

Colorectal cancer (CRC) is the third most common cancer, and the second leading cause of cancer related mortality worldwide[5]. Up to half the patients with CRC will develop metastatic disease, with an associated five-year survival of 13.5%[197]. The peritoneum is the third most common site for CRC metastases and is associated with the worst prognosis among all metastatic sites[24]. Synchronous peritoneal metastases are seen in 5-7% of cases of CRC[109], with metachronous disease in 4-19%[109, 320, 352]. In up to 25% of patients with peritoneal metastases, the peritoneum is the only site of metastases[113]. Prognosis has historically been very poor from peritoneal metastases, with a median survival of less than six months[62]. With advances in chemotherapy drugs and biologic agents, median survival has improved in recent years to 16 months[24], but remains the lowest among all sites of metastases. If treated with chemotherapy alone, only 4.1% of patients with CRPM are alive at five years[155].

Surgical management in the form of cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) can offer selected patients the possibility of long-term survival. Initially pioneered and popularised by Sugarbaker [88, 353] for the treatment of disseminated appendiceal neoplasms, CRS and HIPEC has since been expanded to cases of colorectal peritoneal metastases. In recent years, various centres have reported highly favourable outcomes with a 22-63 months median overall survival and 19-51% five-year survival in selected patients[94, 98, 104, 155] with isolated CRPM treated with CRS and HIPEC.

CRS and HIPEC was formally implemented into surgical practice in various parts of the world following a consensus statement strongly supporting its use in 2007[108]. Since then, there has been growing acceptance for CRS and HIPEC as part of standard treatment guidelines for CRPM. There, however, remains concern over the utility of CRS and HIPEC and the associated morbidity. The aim of this study was to assess the utilisation of CRS and HIPEC for colorectal peritoneal metastases following its introduction at a tertiary centre, and evaluate the associated morbidity, mortality and long-term survival.

5.2 Materials and Methods

5.2.1 Patients and inclusion criteria

Data was prospectively collected on the peritoneal disease database and analysed retrospectively. All patients undergoing CRS and HIPEC for CRPM from June 1, 2009 to 31 December 2017 were included. Patient age, gender, American Society of Anaestheologists score (ASA), tumour marker levels, site and stage of index primary cancer, histopathology details, peri-operative chemotherapy use and previous operation details were recorded.

5.2.2 Peri-operative management

Patients were pre-operatively assessed with a full history and examination. Routine full blood count, carcinoembryonic antigen (CEA), liver function tests, coagulation studies, along with an FDG positron emission tomography based computed tomography (PET/CT) scan of the chest, abdomen and pelvis were performed. All patient cases were reviewed at the peritoneal disease multidisciplinary meeting (MDT). Patients with concurrent extra-abdominal disease or very high volume disease on imaging were not offered CRS and HIPEC. Those with clearly very low volume disease on imaging were offered CRS and HIPEC. Diagnostic laparoscopy was selectively performed if there was moderate volume disease on imaging. The policy of the MDT was to offer CRS and HIPEC to patients with a peritoneal carcinoma index (PCI) of 15 or below. Patients with liver metastases were offered CRS and HIPEC if the PCI was less than 10, with less than three liver metastases.

At operation, all patients had an in dwelling urinary catheter placed and received mechanical DVT prophylaxis intra-operatively along with antibiotics.

Patients were managed in intensive care in the immediate postoperative period. Chest physiotherapy along with an early recovery after surgery (ERAS) based postoperative

care program was utilised. This included an early upgrade of diet as tolerated, without routine nasogastric tube (NGT) use. Total parenteral nutrition (TPN) was started on day seven if there was ongoing evidence of postoperative ileus.

Postoperative intraperitoneal chemotherapy (EPIC) is not used at our institution.

5.2.3 Operative Details

Following laparotomy and adhesiolysis, an assessment of the PCI was conducted. This was scored from 0-39. Cytoreductive surgery was performed in keeping with Sugarbaker's techniques[88, 353]. Curative intent was usually only pursued if the PCI was less than 15. Organ resections involved segmental colectomies, small bowel resections, and hysterectomy with bilateral salpingo-oopherectomy, cholecystectomy, splenectomy and partial gastrectomy. Peritoneal stripping of the pelvis and diaphragm were recorded when performed. The cytoreduction score (CC score) was recorded at the end of each operation. CC-0 reflected no remaining visible disease. CC-1, 2 and 3 implied remaining disease less than 2.5mm, 2.5mm-2.5cm, and greater than 2.5cm respectively. CC-0/1 was deemed complete cytoreduction, with CC-2/3 cases considered incomplete cytoreduction. HIPEC was generally administered only when a complete cytoreduction was achieved. HIPEC administration was via open coliseum technique. Oxaliplatin (350mg/m^2) or Mitomycin C (15mg/m^2) was used for intraperitoneal chemotherapy, based on clinician preference along with intravenous 5-FU (400mg/m^2). Mitomycin C was always used when there was previous intolerance to Oxaliplatin.

5.2.4 Complications

Major complications (Grade III and IV) were recorded in accordance with the Clavien-Dindo classification.

5.2.5 Data analysis

Data was de- identified and analysed with IBM SPSS version 22 and Microsoft Excel. Categorical variables were reported as numbers with percentages. Continuous non-parametric variables were displayed as medians and inter-quartile ranges. Overall and relapse free survival were calculated from date of cytoreductive surgery to date of last follow-up or death, and date of last follow-up or relapse, respectively. Kaplan- Meier analysis with log rank test were conducted to estimate the overall (OS) and relapse free survival (RFS). Cox univariate analysis was utilised to identify factors affecting overall survival. . Pearson's correlation was used to assess for prognostic factors having strong correlation. Cox multivariate Hazards ratio model was developed to identify factors independently associated with overall survival. A p value < 0.05 was considered statistically significant.

5.2.6 Ethics

Institutional ethics was provided to undertake this study (PMCC 18/227R).

5.3 Results

5.3.1 Demographic and operative data

Ninety-six patients underwent 101 cytoreductive surgeries during the time period of the study. A flowchart showing the breakdown of all cases is shown in Figure 5.1. Patient characteristics are shown in Table 5.1. The median patient age was 60 years with 55.2% being female. The majority of patients with peritoneal metastases had a previous primary tumour that was T4 (60.4%). Median follow-up was 13 months (range 0-57 months). 93 (92.1%) of cases received prior chemotherapy for metastatic disease before proceeding to CRS and HIPEC.

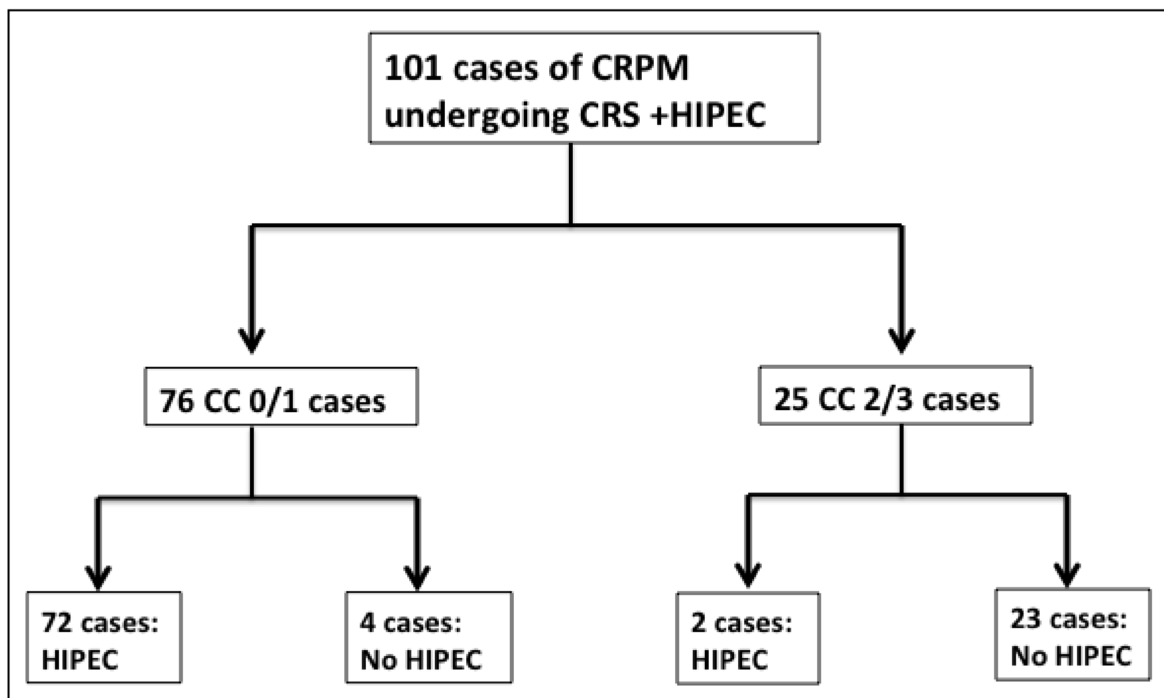


Figure 5.1. Flowchart summarizing patients undergoing CRS and HIPEC

Table 5.1. Demographics of the cohort

Demographic		**Median (Range or Percent)**
Total Patients		96
Total CRS and HIPEC procedures		101
Median Age		60 (27.2-80.5)
Sex	*Male* *Median age*	43 (44.8%) 65.6 (53.3-80.5)
	Female *Median Age*	53 (55.2%) 55 (29.4-75.6)
Median Pre-Operative CEA		5.3 (0.5-110)
Peri-operative Chemotherapy	*Yes* *No*	93 (92.1%) 8 (7.9%)
Location of Primary	*Colon*	84 (87.5%)
	Rectum	12 (12.5%)
T stage of Primary	*T2*	1 (1%)
	T3	29 (30.2%)
	T4	58 (60.4%)
	Unknown	8 (8.3%)

CRS and HIPEC: cytoreductive surgery and hyperthermic intraperitoneal chemotherapy; CEA: carcinoembryonic antigen; T stage: American Joint Committee on Cancer tumour stage

The median PCI was nine (range 0-39), with 78 cases (77.3%) having a PCI less than fifteen. The remaining 23 cases had a PCI over 15, which was only determined at time of surgery. There were 13 cases that had a laparotomy and biopsy only, owing to high volume of disease.

HIPEC was given in 74 (73%) cases, with Oxaliplatin used in forty (54.1%) and Mitomycin C in the remainder (45.9%). A complete cytoreduction was achieved in 76 (75.2%) cases, with an incomplete cytoreduction in the remaining 25 (24.8%) cases. HIPEC was given in 72 (94.7%) cases where a complete cytoreduction was achieved, and in two cases (8%) where cytoreduction was incomplete. HIPEC was not given in four cases where complete cytoreduction was achieved. In two cases, the decision not to give HIPEC was made pre-operatively owing to patient co-morbidities. HIPEC was not given in one case due to intra-operative bleeding. In one case, symptomatic debulking of a large krukenberg tumour was planned in a patient with presumed high volume disease. Operatively, the mass was fully resected, with minimal peritoneal disease that was completely cleared, leading to an unexpected CC 0 resection. As the institute runs a perfusionist based HIPEC service needing pre-planning, HIPEC could not be given. HIPEC was also given in two cases with incomplete cytoreduction. Both patients had very isolated areas of unresectable disease, and therefore were given HIPEC after intraoperative consultation with a second surgeon.

The median length of operations was 270 minutes (range 75 – 600 minutes). Systemic chemotherapy was almost universally given with 93 (92.1%) of cases receiving peri-operative chemotherapy.

5.3.2 Resections and Morbidity

Perioperative details and morbidity are shown in Tables 5.2 and 5.3. The median length of stay was 12 days (range 3 -58 days). A blood transfusion was required in approximately a quarter of cases (25.7%). Visceral resections were common with colectomies and proctectomies performed in 44 (43.6%) and 19 (18.8%) of cases respectively. A synchronous liver resection was performed with CRS and HIPEC in three cases. A palliative bypass was performed in five (5%) cases. Twenty-two (21.8%) cases involved formation of a stoma, with 61 cases (60.4%) having one or more anastomosis.

Grade III/IV complications occurred in 26 (25.7%) of cases. There were sixteen (15.8%) intra-abdominal collections, most of which were managed with percutaneous drainage. There were seven (6.9%) anastomotic leaks and two (2%) postoperative in-hospital deaths. One death was following an anastomotic leak and subsequent multi-organ failure. The second was from inoperable small bowel obstruction from high tumour burden and subsequent palliation.

Table 5.2. Peri-operative details of all cases

Detail			Number of Cases (or median with percentage)
Median Operation time (minutes)			270 (75-600)
Median ASA			2 (1-3)
Length of stay (days)			12 (3-58)
PCI	0-10		64 (63.4%)
	11-15		14 (13.9%)
	16-20		7 (6.9%)
	>20		16 (15.8%)
	Median PCI		9
Use of HIPEC	Yes		74 (73.3%)
	No		27 (26.7%)
HIPEC drug used	Oxaliplatin		40 (54.1%)
	Mitomycin C		34 (45.9%)
Cytoreduction Score	CC 0/1		76 (75.2%)
	HIPEC used		72 (94.7%)
	CC 2/3		25 (24.8%)
	HIPEC used		2 (8%)
Blood transfusion	Yes		26 (25.7%)
	No		75 (74.3%)
Resections	Colectomy		44 (43.6%)
	Proctectomy		19 (18.8%)
	Small Bowel Resection		26 (25.7%)
	Hysterectomy/oophorectomy		22 (41.5%)
	Omentectomy		68 (67.3%)
	Bypass		5 (5.0%)
	Liver Segmental resection		3 (3.0%)
	Others*		14 (13.9%)
	Stoma	Ileostomy	15 (14.9%)
		Colostomy	7 (6.9%)
	Number of Anastomoses	0	40 (39.6%)
		1	49 (48.5%)
		>1	12 (11.9%)

HIPEC: hypothermic intraperitoneal chemotherapy; PCI: peritoneal carcinomatosis index; CC: completeness of cytoreduction

**Others include cystectomy (2), gastrectomy (2), nephrectomy (2), splenectomy (1), cholecystectomy (2), ureter excision (2), and duodenal resection (2).*

5.3.3 Survival Outcomes

Median survival for all cases was 32 months, with a three-year survival rate of 38% (Figure 5.2a). The median overall survival in patients who achieved a complete cytoreduction was 37 months, with a three-year survival rate of 54% (Figure 5.3a) and a median relapse free survival of 13 months (Figure 5.2b), compared to a median overall survival of 11 months for those with incomplete cytoreduction (HR 3.93, 95% CI

2.11-7.34, p<0.05). Similarly, if the PCI was less than 15, median survival was significantly better at 37 months with a three-year survival of 52%, compared to a median survival of 12 months for cases with PCI greater than 15 (HR 4.45, 95% CI 2.29-8.63, p<0.05, Figure 5.3b).

Table 5.3. Morbidity and Mortality from CRS and HIPEC

Complication	Number (percentage)
Grade III/IV	26 (25.7%)
Bleeding	7 (6.9%)
Intra-abdominal Collection	16 (15.8%)
Anastomotic leak	7 (6.9%)
Wound Complication	20 (19.8%)
*Respiratory Complication**	20 (19.8%)
Deep Vein Thrombosis/Pulmonary Embolism	9 (8.9%)
Renal Failure	6 (5.9%)
Peri-Operative Mortality	2 (2%)
Return to theatre	5 (5%)
*Others ***	6 (5.9)

** Respiratory includes pleural effusion, pneumonia, pneumothorax and respiratory failure*
*** Others include cardiac complications (2), enterocutaneous fistula (1), post-operative small bowel obstruction (2), bladder injury (1).*

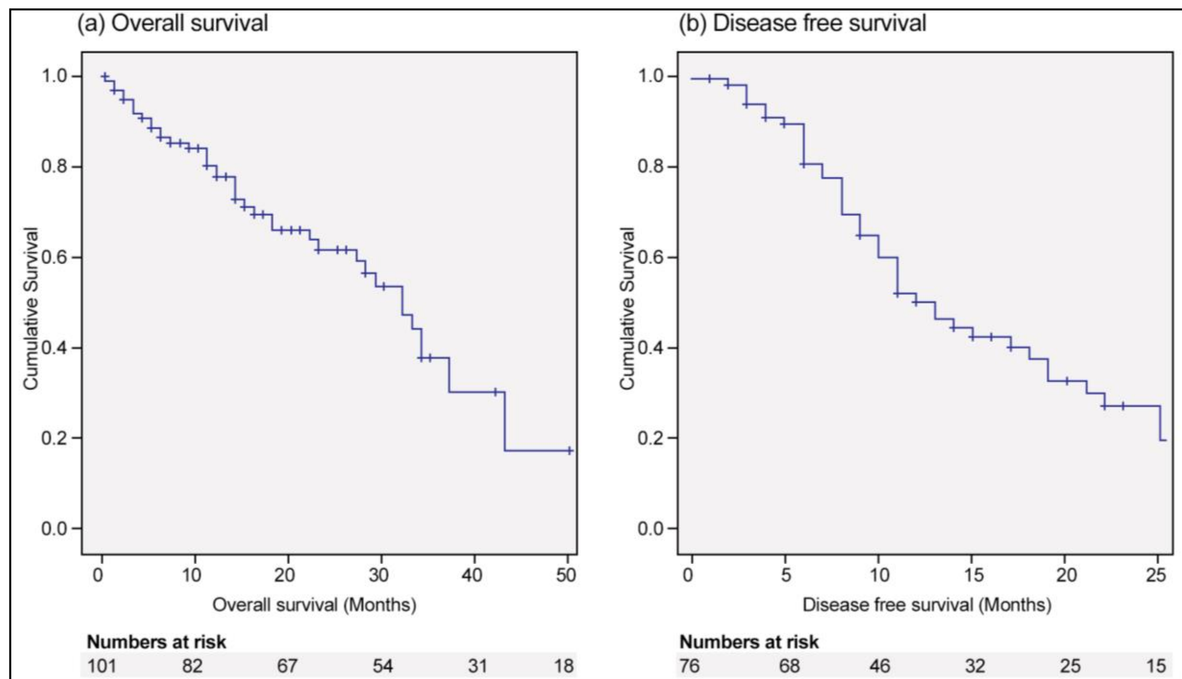


Figure 5.2a. Overall survival of all cases, with 32 months median survival and 34% 3-year survival.
Figure 5.2b. Median relapse-free survival of 13 months after complete cytoreduction.

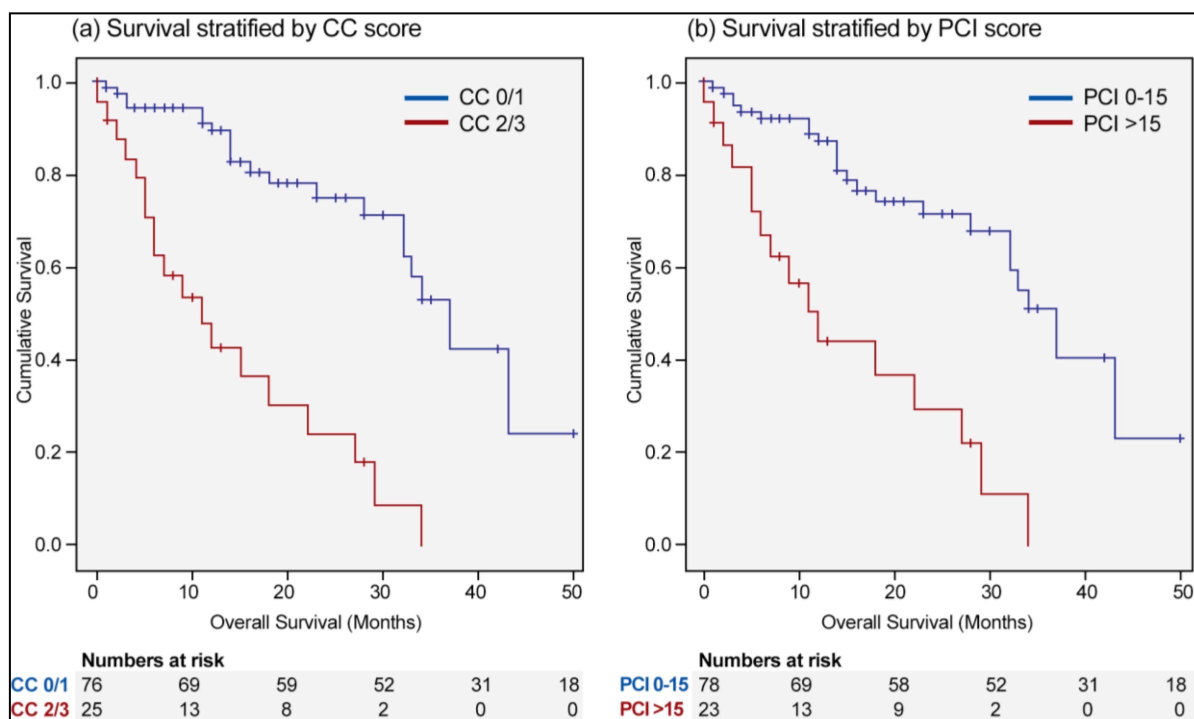


Figure 5.3a. Overall survival based on completeness of Cytoreduction. With CC 0/1, median survival of 37 months compared to 11 months for CC 2/3 (HR 3.93 (2.11-7.34), p <0.05). Figure 5.3b. Overall survival based on PCI. Median overall survival with PCI 0-15 was 37 months, compared to 12 months with PCI >15 (HR 4.45 (2.29-8.63) p <0.05)

5.3.4 Univariate and Multivariate analysis

Cox univariate regression analysis identified non-mucinous histology, PCI <15 and completeness of cytoreduction as factors associated with improved overall survival (Table 5.4). PCI and completeness of cytoreduction were closely related with a Pearson correlation of 0.856. Cox multivariate hazards model revealed non-mucinous histology and completeness of cytoreduction as independent predictors of improved overall survival.

Table 5.4. Cox univariate and multivariate regression model

Variable		**Univariate HR (95% CI)**	**P value**	**Multivariate HR (95% CI)**	**P value**
Age at operation		0.99 (0.98-1.02)	0.919	1.01 (0.98-1.04)	0.592
Gender	*Female*				
	Male	1.22 (0.65-2.32)	0.536	1.15 (0.58-2.23)	0.697
Blood transfusion		0.77 (0.38-1.58)	0.477		
Grade III/IV complication		1.34 (0.67-2.67)	0.414		
Site of original	*Colon*				
	Rectum	0.53 (0.16-1.74)	0.293		
Histology	*Adenocarcinoma*				
	Mucinous	3.093 (1.63-5.88)	**0.001***	2.75 (1.39-5.45)	**0.004****
Tumour stage	*T2*				
	T3	1.08 (0.54-2.17)	0.826		
	T4	7.43 (0.95-58.30)	0.056		
Nodal stage	*N0*				
	N+	1.51 (0.70-3.27)	0.294		
Chemotherapy	*No*				
	Yes	0.60 (0.31-1.16)	0.127	0.64 (0.22-1.83)	0.405
CC Score	*0/1*				
	2/3	3.93 (2.11-7.34)	**<0.001***	6.94 (1.86-25.95)	**0.004****
PCI	*0-15*				
	>15	4.45 (2.29-8.63)	**<0.001***	0.69 (0.18-2.57)	0.577

PCI: peritoneal carcinomatosis index; CC: completeness of cytoreduction; HR: Hazard ratio; CI: confidence interval
** Statistical significance on univariate analysis*
*** Statistical significance on multivariate analysis*

5.3.5 Workload

There has been a significant increase in workload since the initiation of the peritonectomy service as shown in Figure 5.4.

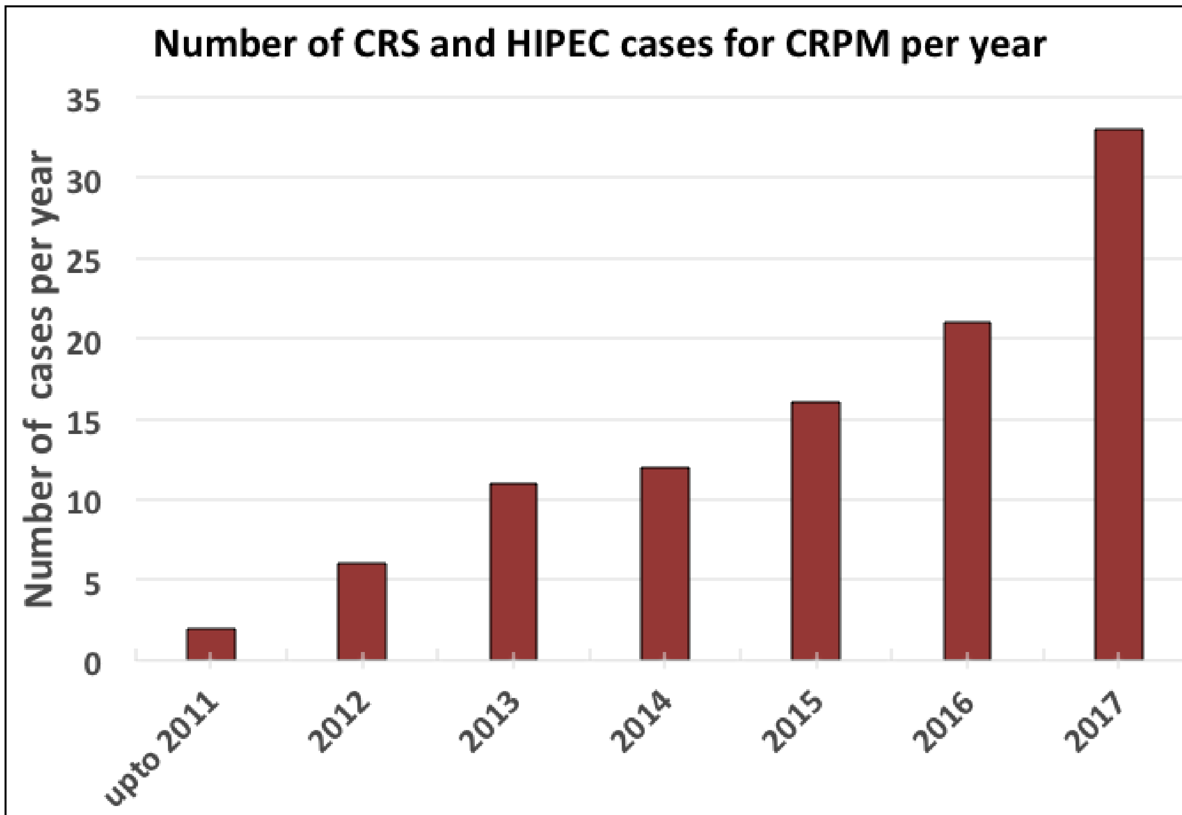


Figure 5.4. Increase in workload of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for colorectal peritoneal metastases per year

5.4 Discussion

CRS and HIPEC is fast becoming the standard of care for isolated low volume colorectal peritoneal metastases. The results demonstrate an overall median survival of 32 months for the entire cohort. If a complete cytoreduction is achieved, median survival improves to 37 months with a relapse free survival of 13 months. Completeness of cytoreduction and non-mucinous histology were the main factors found to be independently associated with improved overall survival.

These findings are largely consistent with other published studies. In the first randomised controlled trial, Verwaal et al[94] demonstrated a median overall survival of 22.3 months with CRS and HIPEC compared to 12.6 months with systemic chemotherapy only in the treatment of CRPM. Ihemelandu et al[102] further demonstrated in a two decade experience a median survival of 36.6 months if a complete cytoreduction was achieved. Other large reported cohorts[98, 99, 104, 354-356] have shown similar results with a 32 to 63 months median overall survival. Huang et al, in a recent meta-analysis[357] of 76 studies reported a median overall survival of 29 months after CRS and HIPEC. The recently concluded French PRODIGE 7 randomised controlled trial[106] provided further evidence that high quality cytoreductive surgery performed at specialised peritonectomy centres can offer a median survival of 41.2 months with a 36.7% 5-year survival in patients with CRPM. The use of Oxaliplatin based HIPEC did not offer any added survival benefit to CRS among the entire cohort, but did demonstrate a survival benefit in a subgroup analysis among those with PCI 11-15 on subgroup analysis.

The utility of CRS and HIPEC for isolated CRPM is supported by numerous international consensus guidelines such as the American society of Colon and Rectal Surgeons[111] and the European Society of Medical Oncology[110] and has become standard of care for low volume CRPM in a number of other countries. The National Health Service Commissioning Board in the United Kingdom includes CRS and HIPEC as part of the treatment guidelines for patients with isolated low volume CRPM[358]. In Australia, the

Cancer Council recommends referral to a peritonectomy centre for consideration of CRS and HIPEC for patients with low volume CRPM[359].

The peritoneal service at our centre has been active since 2009, with an increasing workload especially for CRPM in recent years. This is further reflective of the overall greater acceptance in the oncologic community of the growing role of CRS and HIPEC in the treatment of low volume CRPM.

While effective, CRS and HIPEC does carry significant morbidity. Grade III/IV complications occurred in 25.7% of cases. The peri-operative mortality rate in the cohort was 2%. These figures are comparable to larger volume studies with reported morbidity and mortality rates of 28.8-60% and 2.9-8.3% respectively[98, 100, 112, 129, 360]. The recent PRODIGE 7 trial also demonstrated grade III/IV complication rates of 40.6% and 31.1% in the HIPEC and non-HIPEC groups respectively[106].

There are a number of prognostic factors that have been found to be associated with overall survival. We found non-mucinous histology and completeness of cytoreduction to be independently associated with improved overall survival. Numerous other studies have found completeness of cytoreduction, PCI, positive lymph nodes, histology, use of systemic peri-operative chemotherapy and experience of the centre as prognostic factors influencing overall survival[27, 98, 104, 361, 362].

There are a few limitations that warrant discussion. As a retrospective review, it is reliant on the accuracy of pre-recorded data. All patient records were thoroughly reviewed to ensure data accuracy. There was a 13% rate of laparotomy and biopsy only, due to high disease burden. This is likely reflective of the surgical learning curve and may reduce with increasing experience. Furthermore, this also highlights limitations in current staging modalities. Current imaging methods such as CT and PET/CT scans offer 72.4-87% sensitivity in detection of peritoneal metastases[80, 81]. Going forward, a significant challenge remains the ability to accurately predict a pre-operative PCI, thereby maximizing the chance of gaining a complete cytoreduction. Improved imaging modalities and use of routine staging laparoscopy may help better select patients for CRS and HIPEC in the future.

5.5 Conclusion

CRS and HIPEC can offer long-term survival for selected patients with low volume CRPM, with acceptable morbidity. Completeness of cytoreduction, and non-mucinous histology are factors independently associated with improved overall survival.

This study demonstrates that CRS and HIPEC can offer favourable outcomes to selected patients with CRPM. However, one of the more topical issues currently debated in the oncologic community is the role of HIPEC, whether it is required, and if oxaliplatin is indeed the optimal HIPEC agent. The next chapter aims to evaluate the difference in outcomes between the use of oxaliplatin and mitomycin C as HIPEC agents at a tertiary referral centre.

6 Chapter 6: Oxaliplatin versus Mitomycin C following complete cytoreduction for colorectal peritoneal metastases: A comparative study

This chapter has been published as

Narasimhan V, Warrier S, Michael M, Ramsay R, Heriot A. Oxaliplatin versus Mitomycin C following complete cytoreduction for colorectal peritoneal metastases: A comparative study. *J Gastrointest Surg* 2019, DOI: 10.1007/s11605-019-04447-y (In press)

6.1 Introduction

Colorectal cancer (CRC) is the third most common cancer, and the second leading cause of cancer related mortality worldwide,[5] with an overall five year survival of 68.7%.[363] Upto half the patients with CRC develop metastatic disease, with a five year survival of only 14%.[265] The peritoneum is a common site for metastases, but confers the worst prognosis among all sites of colorectal cancer metastases.[24] Approximately five percent of patients present with synchronous peritoneal metastases, and a further 10-15% develop metachronous disease.[18, 48, 62, 124] Historically, median survival for patients with peritoneal metastases was approximately three months.[62] Advances in systemic therapies and biologic agents in recent years has improved overall survival in patients with colorectal peritoneal metastases (CRPM) to 16.3 months[24]

The greatest advance in the management of CRPM has been the adoption of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC). In a randomised trial, Verwaal et al[94] demonstrated that CRS and HIPEC can offer a significantly improved survival of 22.3 months compared to 12.6 months with systemic chemotherapy alone. Various studies have subsequently shown that in selected patients, CRS and HIPEC can offer a favorable long-term survival of 32 to 61 months with a five-year survival of 25-50%.[27, 98, 104, 129, 364] CRS and HIPEC has since been integrated into treatment guidelines for CRPM published by various oncologic societies.[109-111, 365]

The concept of successful cytoreductive surgery is dependent on the clearance of all macroscopic disease. HIPEC is administered after complete cytoreduction, aiming to clear any microscopic tumour deposits and free tumour cells. While surgical techniques in cytoreduction are fairly standardized across the world, there remains great variability in the drug regimens used for HIPEC. In CRPM, Mitomycin C and oxaliplatin are the drugs commonly utilized for intraperitoneal chemotherapy. They are both large molecules, with pharmacologic profiles conducive to their use as intraperitoneal chemotherapies with minimal systemic absorption and toxicity.[366] They are both cell cycle independent, and shown in pre-clinical studies to have improved efficacy in the

presence of hyperthermia. They both have well documented dose related toxicities such as hematological toxicities in the case of oxaliplatin, and myelosuppression with Mitomycin C.[367, 368] Despite their widespread use as HIPEC agents, there is no randomised data that has explored the efficacy of one over the other. The choice of HIPEC drug is based largely on clinician and hospital practice and experience rather than on drug efficacy or toxicity profile. Recent results from the Prodige 7 trial[106] has posed some questions over the overall utility of HIPEC, particularly as the use of oxaliplatin based HIPEC in this trial failed to demonstrate an improvement in survival over complete cytoreduction alone.

In this study, we aimed to compare the outcomes following the use of Oxaliplatin or Mitomycin C as HIPEC after successful complete cytoreduction for colorectal peritoneal metastases at a statewide peritoneal disease centre.

6.2 Methods

6.2.1 Patients and inclusion criteria

Data was prospectively collected on the peritoneal disease database from September 1, 2011 to 30 April 2018, and analysed retrospectively. Only patients with CRPM, achieving a complete cytoreduction (CC 0/1) with HIPEC were included in the study. Patient sex, age, American Society of Anaestheologists (ASA) score, stage and site of primary cancer and nodal status, tumour grade, histological subtypes, use of neo-adjuvant or adjuvant chemotherapy, intra-operative peritoneal carcinoma index (PCI), operation details and complications were recorded.

6.2.2 Pre-operative workup

Along with routine blood tests, all patients underwent a pre-operative FDG positron emission tomography based computed tomography (PET/CT) scan of the chest, abdomen and pelvis. All cases were discussed at the peritoneal disease multidisciplinary meeting (MDT).

The policy of the peritoneal service is to offer CRS and HIPEC to patients with a PCI less than 15. This is based on a number of studies,[27, 129, 154, 369] including our experience wherein benefit of CRS and HIPEC for CRPM diminishes with increasing tumour burden. If disease burden is very high on imaging, CRS and HIPEC is not offered and the patient is referred for palliative chemotherapy. Conversely, if disease burden is low on imaging, patients are recommended for CRS and HIPEC. If disease burden is intermediate or unclear, patients are referred for staging laparoscopy. Staging laparoscopy is performed to mainly help identify those patients with high volume miliary type disease that was undetected on imaging and would not benefit from CRS and HIPEC. The efficacy of staging laparoscopy can sometimes be limited due to significant adhesions and poor views.

6.2.3 Cytoreductive Surgery and HIPEC

All four surgeons in the unit follow a common, standardized technique for CRS and HIPEC.

At laparotomy, an assessment of the peritoneal carcinoma index (PCI) was conducted in all cases. Cytoreductive surgery was performed in keeping with Sugarbaker's described techniques.[88, 353] Organ resections and peritoneal stripping of the pelvis and diaphragm were recorded when performed. The aim of cytoreductive surgery in CRPM is to achieve an R0 resection. The unit policy is to administer HIPEC only if a complete cytoreduction (CC 0/1) is achieved. HIPEC was administered via open coliseum technique with a target intraperitoneal temperature of 41-43° C. Anastomoses were performed after HIPEC.

6.2.4 HIPEC agents and dosage

The HIPEC agent used is based on medical oncologist preference. Early in our experience, Mitomycin C was the HIPEC agent used in CRPM. This was largely because oncologists had experience in Mitomycin C use, as it is the routine HIPEC drug used for patients with pseudomyxoma peritonei. More recently, Oxaliplatin based chemotherapy regimens (FOLFOX) have become the standard of care in first line systemic treatment for CRC. Furthermore, with studies[104, 356] demonstrating the safety and efficacy of Oxaliplatin as a HIPEC agent, oncologists begun to change the HIPEC agent to Oxaliplatin as it had proven benefit in CRC. In recent years, oxaliplatin has been the preferred HIPEC agent prescribed by oncologists.

Mitomycin C: 15mg/m^2 for 90 minutes with concurrent intravenous bolus 5-FU (400mg/m^2) and folininc acid 20mg/m^2.

Oxaliplatin: 350mg/m^2 for 30 minutes with intravenous bolus 5-FU (400mg/m^2) and folininc acid 20mg/m^2. Patients were grouped based on the chemotherapy agent used for HIPEC.

6.2.5 Peri-operative systemic chemotherapy

The unit is supportive of peri-operative systemic chemotherapy utilization. While there is no randomised data supporting the use of systemic chemotherapy as an adjunct to CRS and HIPEC, a number of single institute series have demonstrated an improvement in survival associated with the use of neo-adjuvant or adjuvant chemotherapy.[27, 121, 150, 370] At our institute, neo-adjuvant chemotherapy was more often given partly for logistic reasons, if for example a patient has had recent abdominal surgery or if there is a long wait time before CRS and HIPEC operation date.

6.2.6 Post operative management and follow up

Post operatively, most patients are initially managed in intensive care in accordance with an early recovery after surgery (ERAS) based postoperative care program. This involved early mobilization, diet upgrade with selective nasogastric tube (NGT) use. Patients who had intra-operative concerns were not managed with the ERAS pathway. Similarly, patients who failed to progress or had significant peri-operative complications were managed on a case-by-case basis and not on an ERAS based postoperative care program. Grade III/IV complications were recorded in keeping with the Clavien-Dindo classification.

Early postoperative intraperitoneal chemotherapy (EPIC) is not used at our institute.

The unit based follow up policy involves review in outpatient clinic two to four weeks after discharge. Subsequently, patients are reviewed three monthly with blood tests and tumour markers for the first year, with a PET/CT six monthly. From the second year onwards, patients are seen six monthly with blood tests, tumour markers and PET/CT. As we are a state based referral centre, many patients sometimes elect to follow up with clinicians at their local provincial hospitals.

6.2.7 Statistical analyses

Statistical analysis was performed using IBM SPSS version 22. Continuous non-parametric variables were expressed as medians with inter-quartile ranges, with categorical variables expressed as numbers with percentages. Differences between the Mitomycin C and Oxaliplatin group were assessed using the Chi-squared test for categorical variables and Student t-test or Mann-Whitney U test for continous variables, depending on whether the variable was normally distributed or not. Overall and disease free survival were calculated from date of CRS and HIPEC to date of last follow up or death, and date of last follow-up or recurrence, respectively. Kaplan- Meier analysis was used to estimate the overall (OS) and disease free survival (DFS). Log rank test was used to assess statistical significance. A p value of < 0.05 was considered statistically significant.

6.2.8 Ethics

Peter MacCallum Cancer Centre institutional ethics was provided to conduct this comparative study.

6.3 Results

6.3.1 Patient Selection and characteristics

One hundred and eight patients underwent CRS and HIPEC for CRPM during this time. Of these, seventy-eight patients (72.2%) were included in the study who achieved successful complete cytoreduction with HIPEC.

The median age of all patients in the study was 58.5 years (range 29.0-81.0) with 57.7% being female. Forty-six patients (59.0%) received Oxaliplatin for HIPEC, with the remaining 32 receiving Mitomycin C. The median PCI of patients in the Mitomycin C group was 7 (range 0-14) compared to 5 (range 0-17) in the Oxaliplatin group (p=0.31). Peri-operative systemic chemotherapy use was common, with 62.8% receiving neo-adjuvant and 28.2% receiving adjuvant chemotherapy. There was no difference in peri-operative chemotherapy use between the two groups. Over 85% of the index tumours were colonic in origin, with no difference in the site of the original tumour between the two groups. The majority of the index primary tumours that led to peritoneal metastases were T4 (57.7%). Over a third of all cancers were mucinous in nature (33.3%), with a small proportion being signet ring cell histology (6.4%). Overall, there were no significant differences in baseline patient characteristics between the Mitomycin C and Oxaliplatin groups (Table 6.1).

Table 6.1. Baseline patient characteristics based on HIPEC drug used

Patient characteristics		**All Cases (N=78)**	**Mitomycin C N=32 (41.0%)**	**Oxaliplatin N=46 (59.0%)**	**P Value**
Median age (with range)		58.5 (29.0-81.0)	59.5 (29.0-74.0)	56 (35.0-81.0)	0.68
Gender	*Male* *Female*	33 (42.3%) 45 (57.7%)	14 (43.8%) 18 (56.3 %)	19 (44.2 %) 27 (62.8%)	0.51
Length of stay in days (range)		12.5 (4-171)	11 (4-58)	13 (4-171)	0.11
PCI (median)		6 (0-18)	7 (0-14)	5 (0-18)	0.31
Neo-adjuvant Chemotherapy	*Yes* *No*	49 (62.8%) 29 (37.2%)	22 (68.8%) 10 (31.2%)	27 (58.7%) 19 (41.3%)	0.25
Adjuvant Chemotherapy	*Yes* *No*	22 (28.2%) 56 (71.8%)	8 (25.0%) 24 (75.0%)	14 (30.4%) 32 (69.6%)	0.40
Median ASA (Range)		2 (1-3)	2 (1-3)	2 (1-3)	0.94
Operation Duration in minutes (median and range)		280 (150-600)	282.5 (200-600)	265 (150-520)	0.47
Blood transfusion	*Yes* *No*	23 (29.5%) 55 (70.5%)	10 (31.3%) 22 (68.7%)	13 (28.3%) 33 (71.7%)	0.49
Blood transfusion (Number of units)	*0-1 unit* *>1 unit*	58 (74.4%) 20 (25.6%)	23 (71.9%) 9 (28.1%)	35 (76.1%) 11 (23.9%)	0.44
Site of original tumour	*Right/Transverse* *Left colon* *Rectum*	37 (47.4%) 29 (37.2%) 12 (15.4%)	16 (50.0%) 11 (34.4%) 5 (15.6%)	21 (45.7%) 18 (39.1%) 7 (15.2%)	0.91
Tumour stage	*T4* *T3* *NA*	45 (57.7%) 23 (29.5%) 10 (12.8%)	16 (50.0%) 13 (40.6%) 3 (9.4%)	29 (63.0%) 10 (21.7%) 7 (15.2%)	0.08
Nodal status	*N0* *N+* *NA*	54 (69.2%) 17 (21.8%) 7 (9.0%)	23 (71.9%) 9 (28.1%) 0 (0.0%)	31 (67.4%) 8 (17.4%) 7 (15.2%)	0.32
Tumour Differentiation	*Well/moderate* *Poor* *NA*	54 (69.2%) 18 (23.1%) 6 (7.7%)	26 (81.3%) 6 (18.7%) 0 (0.0%)	28 (60.9%) 12 (26.1%) 6 (13.0%)	0.48
Mucinous histology	*Yes* *No*	26 (33.3%) 52 (66.7%)	11 (34.4%) 21 (65.6%)	15 (32.6%) 31 (67.4%)	0.53
Signet ring cell histology	*Yes* *No*	5 (6.4%) 73 (93.6%)	2 (6.2%) 30 (93.8%)	3 (6.5%) 43 (93.5%)	0.67

PCI: peritoneal carcinoma index; ASA: American Society of Anesthesiology score; NA: not available

6.3.2 Resections and Morbidity

Resections were common, with colectomies or proctectomies performed in 59.0% and 26.9% of patients in the entire cohort. Gastrectomies and splenectomy were uncommon with only two (2.6%) of each in the entire cohort. Similarly, parenchymal liver resections were uncommon, with only three (3.8%) in the entire cohort. Parietal peritonectomies in the form of stripping of the diaphragmatic or pelvic peritoneum were performed in 19.2% and 46.2% of cases respectively. Over two thirds of the entire cohort had at least one anastomosis (69.2%). Less than a quarter of all patients had a

stoma (24.4%). There were no differences in organ resections between the Mitomycin C and Oxaliplatin groups (Table 6.2).

Complications were not infrequent, with anastomotic leaks occurring in 7.7% of cases. An infected intra-abdominal collection occurred in 16.7% of cases. These were usually managed successfully with percutaneous drainage. Overall, grade III/IV complications occurred in 32.1% of cases. Superficial wound infections were significantly higher in the Mitomycin C group (37.5% versus 15.2%, p=0.02). These were usually managed successfully with antibiotics and wound dressings. There was one mortality in the entire cohort in a patient who suffered an anastomotic leak, with subsequent fulminant sepsis and multi-organ failure. Apart from superficial wound infections, there were no significant differences in morbidity between the Mitomycin C and Oxaliplatin groups (Table 6.2).

Table 6.2. Peri-operative details of all cases

		All cases (N=78)	Mitomycin C (n=32)	Oxaliplatin (n=46)	P value
Resections:					
Colectomy		46 (59.0%)	17 (53.1%)	29 (63.0%)	0.26
Proctectomy		21 (26.9%)	7 (21.9%)	14 (30.4%)	0.28
Splenectomy		2 (2.6%)	0	2 (4.3%)	0.35
Partial Gastrectomy		2 (2.6%)	2 (6.3%)	0	0.17
Small Bowel Resection		23 (29.5%)	13 (40.6%)	10 (21.7%)	0.06
Parenchymal liver resection		3 (3.8%)	2 (6.3%)	1 (2.2%)	0.37
Hysterectomy/Oophorectomy		18 (40.0%)	8 (44.4%)	10 (37.0%)	0.47
Number of organ resections	*0-2*	42 (53.8%)	17 (53.1%)	25 (54.3%)	0.55
	>2	36 (46.2%)	15 (46.9%)	21 (45.7%)	
Diaphragmatic peritoneum		15 (19.2%)	8 (25.0%)	7 (15.2%)	0.22
Pelvic Peritoneum		36 (46.2%)	19 (59.4%)	17 (37.0%)	0.21
Stoma		19 (24.4%)	9 (28.1%)	10 (21.7%)	0.35
Anastomoses	*0*	24 (30.8%)	9 (28.1%)	15 (32.6%)	0.42
	1	42 (53.8%)	16 (50.0%)	26 (56.5%)	
	>1	12 (15.4%)	7 (21.9%)	5 (10.9%)	
Complications:					
Bleeding		8 (10.3%)	3 (9.4%)	5 (10.9%)	0.57
Intra-abdominal infected collection		13 (16.7%)	5 (15.6%)	8 (17.4%)	0.55
Anastomotic leak		6 (7.7%)	2 (6.3%)	4 (8.7%)	0.52
DVT/PE		5 (6.4%)	2 (6.3%)	3 (6.5%)	0.67
Superficial wound infection		19 (24.4%)	12 (37.5%)	7 (15.2%)	0.02*
Enterocutaneous fistula		2 (2.6%)	1 (3.1%)	1 (2.2%)	0.66
*Respiratory**		14 (17.9%)	6 (18.8%)	8 (17.4%)	0.55
Mortality		1 (1.3%)	1 (3.1%)	0	0.41
Total Grade III/IV complications		25 (32.1%)	9 (28.1%)	16 (34.8%)	0.36
Return to theatre		6 (7.7%)	2 (6.3%)	4 (8.7%)	0.52

DVT/PE: Deep vein thrombosis or Pulmonary embolus.

** Respiratory includes pleural effusion, pneumonia, pneumothorax and respiratory failure*

6.3.3 Survival outcomes

The median follow up for the entire cohort was 22 months (range 1-59). Median overall survival for the entire cohort was 40 months (95% CI: 35.2-44.8), with a 92% one year, 83% two year and 66% three year survival (Figure 6.1a). Median disease free survival for all patients was 14 months (95% CI: 11.8-16.2), with a 55% one year and 31% two year survival (Figure 6.1b).

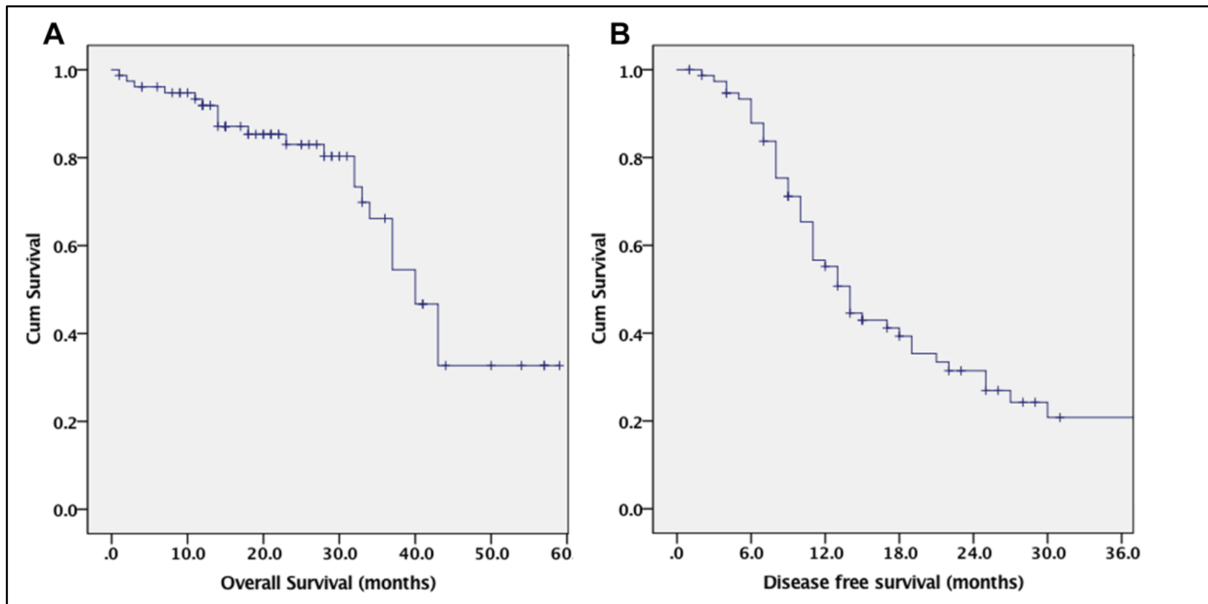


Figure 6.1a. Median overall survival of all cases was 40 months. Fig 6.1b. Median disease free survival for all cases was 14 months.

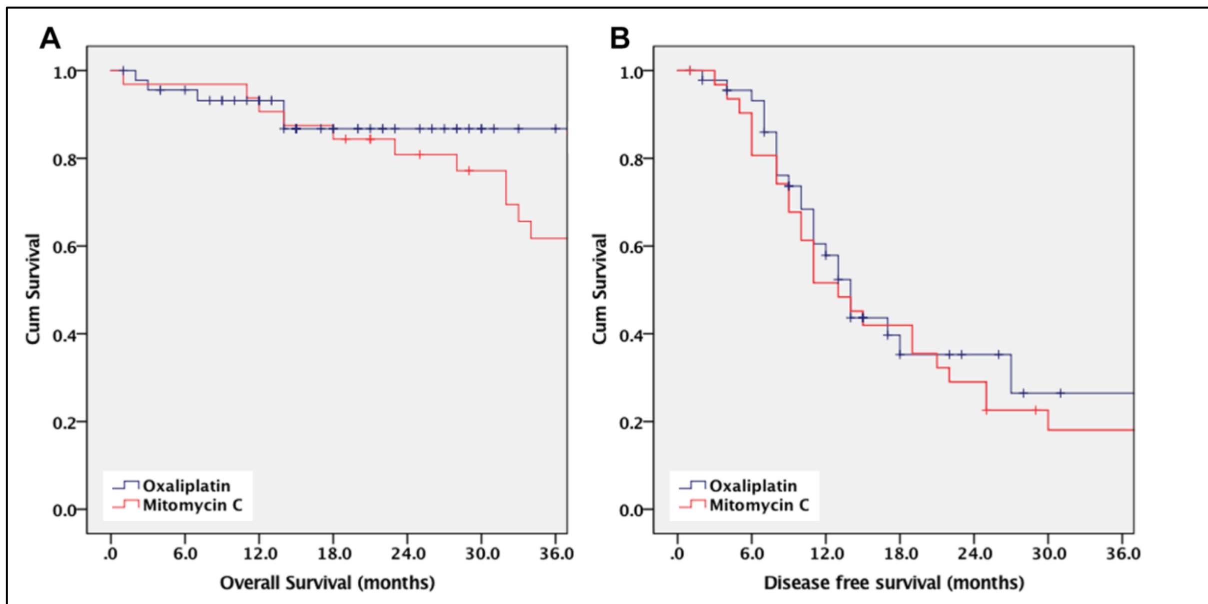


Figure 6.2a. Median survival was 40 months for Mitomycin C and was not reached for Oxaliplatin (log rank p =0.35). Fig 6.2b. Median disease free survival was 13 months for Mitomycin C and 14 months for Oxaliplatin (log rank p=0.59).

Median follow up was 33.5 months for the Mitomycin C group and 15 months for the Oxaliplatin group (p=0.01). This was because Oxaliplatin has been used in more recent years, while Mitomycin C was the HIPEC agent used when the peritoneal service started. On comparing survival based on HIPEC agent, there was no difference in OS with the use

of Mitomycin C or Oxaliplatin (40 months versus not reached for Oxaliplatin, log rank p=0.35). One and two year survival was similar as well (91% v 93% and 81% v 86%) (Figure 6.2a). Similarly, there was no difference in DFS between the Mitomycin C and Oxaliplatin groups (13 months (95% CI: 8.5-17.5) v 14 months (95% CI: 11.8-16.2, log rank p=0.59) (Figure 6.2b).

After adjusting for extent of disease (PCI), there remained no difference in overall survival with the use of Mitomycin C or Oxaliplatin (HR 1.60; 95% CI: 0.53-4.84, p=0.40). Similarly, there was no difference in disease free survival with the use of either HIPEC agent (HR 1.12; 95% CI: 0.64-1.97, p=0.70).

6.4 Discussion

This study demonstrates that in patients achieving a successful complete cytoreduction, CRS and HIPEC as a combination treatment offers a favorable long-term survival of 40 months, with a 14 month DFS. There was no difference in survival with the use of Mitomycin C or Oxaliplatin as the HIPEC agent. Notably, the median PCI in both groups was low, suggesting that the choice of HIPEC agent especially in the low PCI cohort does not influence survival. Apart from superficial wound infections, there was no difference in morbidity between the Mitomycin C and Oxaliplatin groups. Superficial wound infections were not grade III/IV complications, and were largely managed with antibiotics and wound dressings, and did not adversely affect patient outcome. The choice of HIPEC agent at our institute is purely based on medical oncology preference, with Mitomycin C used early on in our service, and Oxaliplatin being the drug of choice in more recent years. Oxaliplatin is commonly used in FOLFOX regime and has proven benefit in CRC as systemic therapy. Oncologists are therefore more comfortable with Oxaliplatin and its pharmacodynamics and pharmacokinetic profile, preferentially choosing it as the HIPEC agent. Furthermore, Oxaliplatin was possibly more appealing for surgeons as well, as it is used for only 30 minutes as HIPEC instead of 90 minutes in the case of Mitomycin C.

While the use of HIPEC is conceptually very rational, with the potential synergistic activity of heat and the concentrated effect of the drug in direct contact with tumour cells and minimising systemic toxicity, it is interesting that the use of HIPEC was adopted universally without any randomised data until recently to evaluate its efficacy in addition to surgery. The role of intraperitoneal chemotherapy in CRPM was first evaluated in a randomised trial by Elias et al[96] where early postoperative intraperitoneal chemotherapy (EPIC) in addition to CRS and systemic chemotherapy was evaluated against CRS and systemic chemotherapy alone. This trial closed early due to poor accrual, with EPIC not shown to add any additional survival benefit. The recent PRODIGE 7 trial[106] demonstrated that the addition of oxaliplatin based HIPEC did not improve survival compared to complete cytoreduction alone. Subgroup analysis however, did demonstrate a survival benefit with HIPEC in patients with a PCI 11-15.

This trial, while yet unpublished, reaffirmed the value of complete cytoreduction, but has raised doubts about the overall efficacy of oxaliplatin based HIPEC, and more broadly, whether HIPEC itself is even required, given the exceptional results from complete cytoreduction alone.

To date, there have been no randomised trials that have evaluated the use of Oxaliplatin or Mitomycin C as the preferred HIPEC drug. Most centres in the United States use Mitomycin C, while much of Europe and Australia use Oxaliplatin. It is noteworthy that Mitomycin C was the HIPEC agent used in the index RCT that demonstrated superiority of CRS and HIPEC over systemic chemotherapy.[94] It is plausible that similar to our centre, oncologists elsewhere are more comfortable using Oxaliplatin as HIPEC given increased familiarity with the drug in systemic regimens, with the reduced HIPEC duration of 30 minutes being an added incentive to use it.

There are only a handful of studies in the literature that have compared the difference between Oxaliplatin and Mitomycin C as HIPEC following complete cytoreduction. Hompes et al[371] in a comparative study reported no survival difference between the use of Oxaliplatin or Mitomycin C as HIPEC agents. Mitomycin C however, was associated with a significantly higher rate of complications. In another study, Leung et al[372] found a significant survival benefit of 56 versus 29 months with the use of Oxaliplatin compared to Mitomycin C. This survival benefit was particularly notable in females, those with non-signet ring cell pathology and a PCI of 10-15. Prada-Villeverde et al[107] in the largest study to date involving a multicentre review of 539 patients also demonstrated no overall survival difference between the use of Mitomycin C and Oxaliplatin. However, in patients with low volume disease, Mitomycin C offered a significant survival benefit over Oxaliplatin. Other comparative studies have demonstrated an increase risk of electrolyte disturbances such as hyponatraemia, hyperglycemia, metabolic acidosis and haematologic toxicities such as neutropenia with Oxaliplatin, without any survival benefit over Mitomycin C.[107, 367, 373]

Based on current evidence, there appears to be no difference in survival based on the HIPEC drug used. This clearly highlights the urgent need to explore the efficacy of the different HIPEC agents in a prospective trial. While challenging to conduct, the strong

collaborations evident between peritoneal centres in recent years leading to multi-centre studies with large patient numbers present an ideal opportunity to conduct a multi-centre, prospective randomised study in patients with comparable peritoneal surface disease severity scores (PSDSS) to evaluate the efficacy of different HIPEC agents. Such collaborations would also enable research into other important issues such as the overall efficacy of intraperitoneal chemotherapy itself in the form of HIPEC or EPIC over systemic chemotherapy after complete cytoreduction, especially as systemic therapies have evolved dramatically over the last two decades, whereas HIPEC drugs have remained stagnant with only Oxaliplatin and Mitomycin C as options. Furthermore, the optimal dose and duration for oxaliplatin is unknown,[374, 375] with no consensus in the literature. The use of hyperthermia itself over normothermia has not been evaluated in human studies. Further HIPEC options such as Cisplatin or combinations such as Cisplatin with Mitomycin C have been used with favorable results in studies,[376] and need further evaluation in prospective studies. In more recent years, advances in translational research with pre-clinical modeling of disease and development of throughput drug testing platforms can be integrated to help personalise therapy to identify the ideal HIPEC agent or systemic therapy based on each patient tumours unique sensitivities. This would have far greater efficacy than currently used generic regimens of treatment that have a high risk of failure.

As a relatively young peritoneal disease centre, this study has a small sample size. Therefore, while we could not demonstrate a survival difference between the two groups, the lack of survival difference could possibly be due to the small sample size. However, a larger sample size from our institute would be heavily skewed with Oxaliplatin cases given its almost exclusive use now. Furthermore, given that no difference between the two agents was seen, an alternative plausible hypothesis can be also be considered that neither agent has efficacy, or that another HIPEC drug may be better or that HIPEC itself as a single hit may not offer any added efficacy over surgery alone. These are also important issues that need a prospective trial to address effectively. As a retrospective analysis, the study is inherently reliant on the accuracy of pre-recorded data. Lastly, while there remains no consensus on the preferred HIPEC drug, there is similarly a need to consider forming a consensus on other aspects of HIPEC use such as the optimal dose of each drug, delivery system (open or closed) or

duration, factors that could alter the pharmacokinetics of the drugs and alter its overall efficacy.[91]

6.5 Conclusion

Complete cytoreduction with HIPEC as a combination treatment can offer a favorable survival in selected patients with CRPM. Notably, the choice of Oxaliplatin or Mitomycin C as HIPEC had no influence on overall survival. Further prospective studies are urgently needed to evaluate the ideal agent used in HIPEC.

This study has highlighted that the choice of HIPEC agent requires further evaluation in prospective trials, at a time when the efficacy of HIPEC itself is being questioned. The last two chapters have demonstrated that while CRS and HIPEC can offer favourable outcomes in CRPM, optimal patient selection is critical. While completeness of cytoreduction and PCI are commonly accepted prognostic factors, there may be other factors that may impact survival in patients undergoing CRS and HIPEC. Identification of clinicopathologic factors that impact survival can help improve patient selection and peri-operative treatment. To evaluate these factors, the next chapter is a systematic review and meta-analysis that aims to identify key prognostic factors that influence survival in patients undergoing CRS and HIPEC for isolated CRPM.

7 Chapter 7: Prognostic Factors influencing survival in patients undergoing CRS and HIPEC for isolated CRPM: A systematic review and meta-analysis

This chapter has been published as

Narasimhan V, Tan S, Kong J, Pham T, Michael M, Ramsay R, Warrier S, Heriot A. Prognostic factors influencing survival in patients undergoing cytoreductive surgery with HIPEC for isolated colorectal peritoneal metastases: A systematic review and meta-analysis. *Colorectal Disease* 2019 (In press)

7.1 Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, and second leading cause of cancer related mortality.[1] The peritoneum confers the worst prognosis among all sites of CRC metastases.[24] Historically, patients with colorectal peritoneal metastases (CRPM) had a median survival of three to six months.[46, 62] Approximately 8-10% of patients present with synchronous peritoneal metastases, while up to 40% develop metachronous peritoneal metastases.[18, 46, 364]

Over the last two decades, peritoneal metastases have begun to be viewed as loco-regional disease, with cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) as an aggressive loco-regional treatment option. In a randomised trial, Verwaal et al[94] demonstrated that CRS and HIPEC led to a significantly improved survival of 22.3 months compared to 12.6 months with systemic therapy in patients with CRPM. Further studies have since demonstrated that CRS and HIPEC can offer selected patients with low volume isolated CRPM a median survival of up to 62.7 months[104] with a 22-51% five year survival.[104, 117, 309, 377, 378] With increasing acceptance in recent years, CRS and HIPEC has been integrated into treatment pathways for CRPM in many countries.[109-111]

Prognostic factors such as completeness of cytoreduction (CC score) and peritoneal carcinoma index (PCI) have been shown to influence survival following CRS and HIPEC.[105, 118, 122, 369, 379] However, there remains significant variability in patient selection and prognostic factors reported by investigators. In this study, we aim to conduct a systematic review and meta-analysis to evaluate key peri-operative prognostic factors that influence survival in patients undergoing CRS and HIPEC for isolated CRPM.

7.2 Methods

7.2.1 Search strategy and inclusion/exclusion criteria

This systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A comprehensive search was undertaken on Ovid MEDLINE, EMBASE database and the Cochrane collaboration library. The following keywords were used: colorectal; peritoneum; cytoreductive surgery; hyperthermic intraperitoneal chemotherapy; prognostic factor; and combinations thereof. The search strategy is supplied as Appendix 3.1

7.2.2 Inclusion criteria

Only full text English studies with over 30 patients, reporting on prognostic factors influencing overall survival following CRS and HIPEC for isolated peritoneal metastases published between 1 January 2008 to 30 November 2018 were included. Manual cross-referencing from bibliographies of papers in the initial search was done to include additional papers that were not previously identified. Two reviewers performed the search and data extraction, while the senior author independently evaluated any discrepancies in study inclusion or exclusion.

7.2.3 Exclusion criteria

Studies reporting on outcomes from appendiceal or non-colorectal cancers were excluded. Similarly, studies reporting on multiple cancers were screened and excluded if data specific to CRPM was unable to be extracted, studies on patients with synchronous liver resections and studies with inadequate or no survival outcomes or prognostic factors were excluded.

Papers were excluded from the meta-analysis if hazard ratios with 95% confidence intervals were not reported or could not be extracted from the study results.

7.2.4 Data extraction and analysis

An electronic datasheet with pre-determined criteria was used to ensure all pertinent data was collected from each study. Data analysis was performed using general inverse variance statistical methods, with random effects model on Review Manager (RevMan) Version 5.3 (Copenhagen: The Nordic Cochrane Centre, Cochrane Collaboration, 2014). The parameters of interest were prognostic factors influencing survival, with the outcome of interest being overall survival. Hazard ratios with 95% CI were extracted from included papers. A minimum of three papers had to report the same variable for a pooled analysis to be performed. The in-built RevMan software reported pooled P values for overall effect and forest plots with 95% CI for each variable. P value <0.05 was considered significant. Heterogeneity was calculated by the I^2 test. Heterogeneity can be interpreted as: 0-30%: minimal; 30-60%: moderate; 60-90%: substantial and >90%: very high heterogeneity.

7.3 Results

Thirty-three studies were eligible and included in the systematic review, with 25 studies included in the meta-analysis (Figure 7.1).

The Systematic review was registered with Prospero (CRD42019138094).

Eight studies were excluded from the meta-analysis due to inadequate statistical data available (Appendix 3.2).

7.3.1 Study Quality and Risk of Bias assessment

Study quality was assessed using the Newcastle Ottawa Score (Appendix 3.3). There was one prospective Phase II trial,[377] with the remaining 32 being retrospective studies. There were no randomised trials published during the defined search period. Nine studies were multi-centre, with 24 being single institution studies. All studies had similar methodology and interventions and were of fair quality. Risk of bias assessment was performed using the Modified Cochrane Tool for cohort studies and EPOC data collection form (Appendix 3.4).

7.3.2 Follow up and Assessment of Survival

Median follow up ranged from 13.3-88 months.[380, 381] Median overall survival ranged from 16.0-51 months,[377, 382-384] with a median disease free survival of 10.9-34.5 months.[382, 385] One, three and five year survival ranged from 70.5-91%,[377, 380] 22-65%[377, 385] and 22-43.2%[118, 377] respectively. Study characteristics and survival data are summarised in Table 7.1 and Table 7.2.

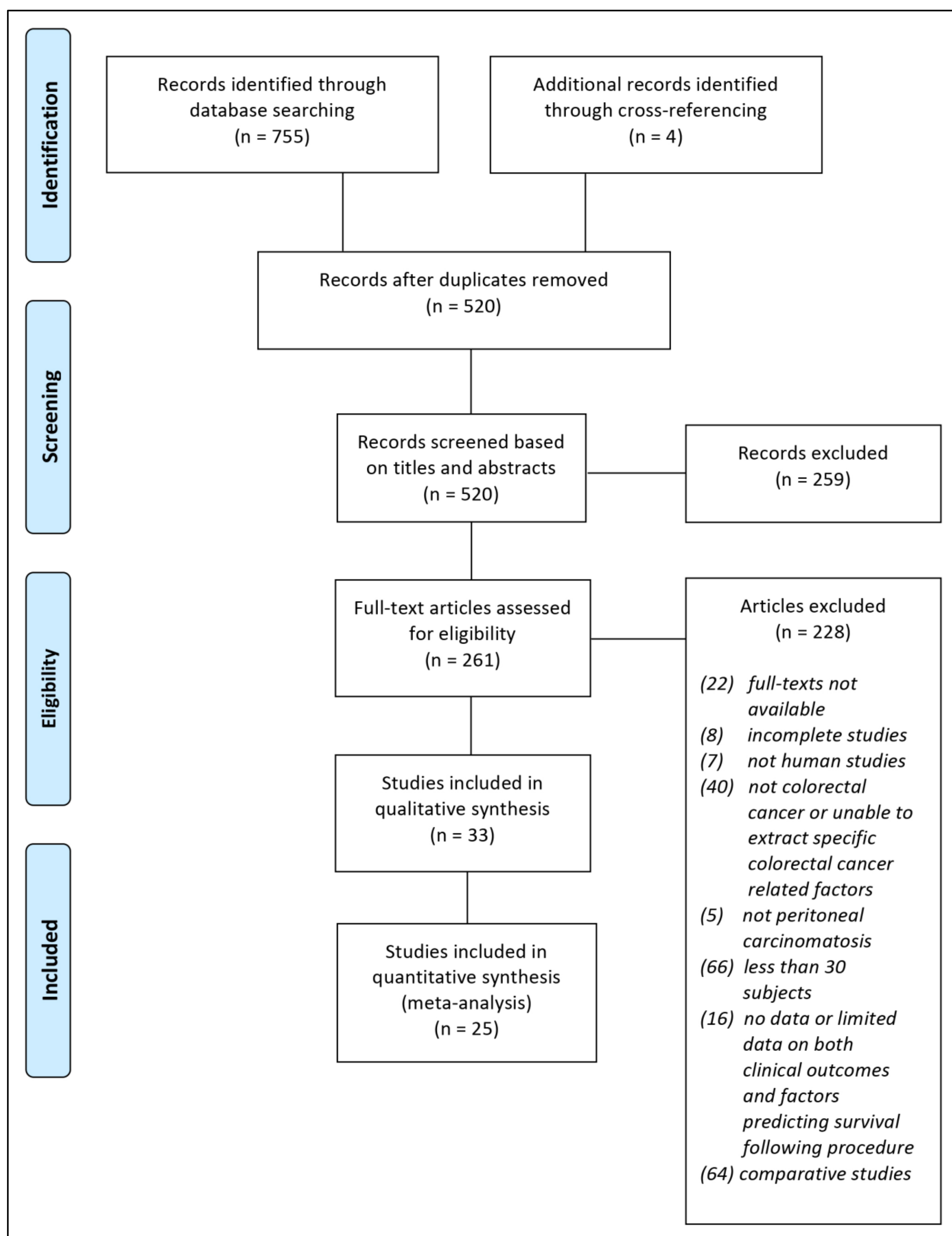


Figure 7.1. PRISMA flow chart demonstrating identification of relevant studies

Table 7.1. Study Characteristics

No.	Author (year)	No. of Sites (country)	Study Period	Study type	No. of Patients	Intraperitoneal Chemotherapy Used	Systemic Chemotherapy (dosage)		Median Follow-up (months)
							NAC	AC	
1	Yan (2008)[386]	1 (Australia)	1997-2007	Cohort	50	MMC (HIPEC) and 5-FU (EPIC)	NR	NR	14
2	Shen (2009)[380]	1 (USA)	1991-2005	Cohort	121	NR	NR	NR	86
3	Chua (2010)[309]	2 (multiple countries)	1997-2008	Cohort	56	MMC (HIPEC)	NR	NR	20**
4	Franko (2010)[387]	1 (USA)	2001-2007	Case control	105	5-FU + IRI or OXL (HIPEC)	NR	NR	NR
5	Elias (2010)[27]	23 (France)	1990-2007	Cohort	523	MMC ± CIS or OXL ± IRI (HIPEC) and MMC + 5-FU (EPIC)	NR	NR	45
6	Cavaliere (2011)[369]	5 (Italy)	1995-2007	Cohort	146	CIS ± MMC or OXL (HIPEC)	NR	NR	19
7	Quenet (2011)[105]	2 (France)	1998-2007	Cohort	146	OXL ± IRI (HIPEC)	NR	NR	48.5
8	Benizri (2012)[382]	1 (France)	2000-2007	Cohort	49	MMC (HIPEC)	NR	NR	27**
9	Cashin (2012)[388]	1 (Sweden)	2005-2008	Cohort	107	NR	NR	NR	NR
10	Passot (2012)[379]	1 (France)	1991-2010	Cohort	120	MMC ± IRI or MMC ± OXL (HIPEC)	Various *	NR	58.5
11	Gervais (2013)[378]	1 (Canada)	2004-2011	Cohort	40	OXL (HIPEC)	NR	NR	22.8
12	Ung (2013)[370]	1 (Australia)	2000-2012	Cohort	125	MMC (HIPEC) and 5-FU (EPIC)	NR	NR	23.3

13	Votanopoulos (2013)[384]	1 (USA)	1993-2011	Cohort	217	NR	NR	NR	88.1 (colon); 40.1 (rectal)
14	Yonemura (2013)[117]	1 (USA)	1993-2011	Cohort	142	MMC + CIS (HIPEC)	Various †	NR	NR
15	Baratti (2014)[118]	1 (Italy)	2004-2012	Cohort	101	CIS + MMC (HIPEC)	NR	NR	44.9
16	Ceelen (2014)[150]	1 (Belgium)	2002-2012	Cohort	166	MMC or OXL (HIPEC)	FOLFOX or FOLFIRI or FOLFIRI + BEVA	NR	18
17	Elias (2014)[115]	1 (France)	2000-2009	Cohort	139	OXL ± IRI (HIPEC)	5-FU and LV	NR	NR
18	Hompes (2014)[389]	2 (Netherlands and Belgium)	2004-2010	Cohort	95	MMC or OXL (HIPEC)	NR	NR	5.1 (MMC) 2.8 (OXL)
19	Huang (2014)[377]	1 (Japan)	2005-2013	Prospective	60	MMC (HIPEC) and 5-FU (EPIC)	NR	FOLFOX or FOLFIRI + Carboplatin	29.9
20	Passot (2014)[151]	1 (France)	2005- 2012	Cohort	115	OXL (HIPEC)	Various #	NR	18.6
21	Prada-Villaverde (2014)[107]	15 (multiple countries)	2000-2012	Cohort	584	MMC or OXL (HIPEC)	NR	NR	NR
22	Rivard (2014)[179]	1 (Canada)	2003-2011	Cohort	68	NR	NR	NR	30.3
23	Faron (2016)[119]	1 (France)	2003-2012	Cohort	173	OXL + IRI (HIPEC)	5-FU and LV	NR	48.5
24	Frøysnes (2016)[385]	1 (Norway)	2004-2013	Cohort	119	MMC (HIPEC)	NR	NR	45

25	Maillet (2016)[120]	4 (France)	2004-2012	Cohort	231	MMC or OXL (HIPEC)	NR	Various §	NR
26	Ng (2016)[370, 381]	1 (Singapore)	2003-2014	Cohort	50	MMC or OXL (HIPEC)	NR	NR	13.3
27	Sluiter (2016)[390]	2 (Netherlands)	2007-2010	Cohort	65	MMC (HIPEC)	NR	NR	21
28	Ihemelandu (2017)[391]	1 (USA)	1990-2015	Cohort	318	MMC (HIPEC) and 5-FU (EPIC)	NR	NR	15
29	Leung (2017)[392]	1 (Australia)	1996-2015	Cohort	202	MMC or OXL (HIPEC)	NR	NR	NR
30	Massalou (2017)[383]	1 (France)	1999-2013	Cohort	84	MMC (HIPEC)	NR	NR	NR
31	Kozman (2018)[121]	1 (Australia)	1996-2016	Cohort	260	MMC or OXL (HIPEC)	NR	NR	NR
32	Sluiter (2018)[122]	2 (Netherlands)	2008-2016	Cohort	175	MMC or OXL (HIPEC)	NR	OXL or Capecitabine or CAPOX or FOLFOX	NR
33	Tonello (2018)[393]	1 (Spain)	2004-2015	Cohort	36	MMC or OXL (HIPEC)	NR	NR	NR

*HIPEC: hyperthermic intraperitoneal chemotherapy; EPIC: early post-operative intraperitoneal chemotherapy; NAC: neo-adjuvant chemotherapy; AC: adjuvant chemotherapy; NR: not reported; MMC: mitomycin-C; OXL: oxaliplatin; CIS: cisplatin; IRI: irinotecan; 5-FU: 5-fluorouracil; LV: leucovorin; BEVA: bevacizumab; CTX: cetuximab; NA: Neoadjuvant chemotherapy; **: mean value*

** : 5-FU, LV, OXL, IRI, BEVA or CTX*

† : FOLFOX + BEVA or FOLFIRI + BEVA or CTX or Xeloda + OXL or BEVA or S1 + Paclitaxel or IRI + Paclitaxel or Taxol + CBDCA or S1 + OXL

 # : FOLFOX or FOLFIRI or FOLFIRI + BEVA or FOLFIRI + CTX or FOLFOX + BEVA or FOLFOX + CTX

§ : FOLFOX or FOLFIRI or FOLFIRI + BEVA or FOLFIRI + CTX or FOLFOX + BEVA or FOLFOX + CTX or 5-FU + BEVA or LV5FU2 or FOLFIRINOX

Table 7.2. Survival data from all included studies

No.	Author (year)	Survival Rates (%)			Median DFS (months)	Median OS (months)	Overall Mortality (%)	Grade III/IV Morbidity (%)
		1-year	3-year	5-year				
1	Yan (2008)[386]	79	39	NR	NR	29	NR	NR
2	Shen (2009)[380]	91	48	26	NR	34	5.5	NR
3	Chua (2010)[309]	85	48	NR	NR	38	NR	NR
4	Franko (2010)[387]	90*	49*	25*	NR	34.7	NR	NR
5	Elias (2010)[27]	81	41	27	NR	30.1	3.3	31
6	Cavaliere (2011)[369]	80*	30*	NR	NR	21	2.7	NR
7	Quenet (2011)[105]	95*	53*	41.8	NR	41	4.1	NR
8	Benizri (2012)[382]	82*	52*	40	34.5	51	0	20.5
9	Cashin (2012)[388]	NR	NR	NR	NR	NR	NR	NR
10	Passot (2012)[379]	77	NR	33	NR	36.2	NR	NR
11	Gervais (2013)[378]	88	61	36	NR	NR	4	NR
12	Ung (2013)[370]	84 (colon)*; 80 (rectal)	53 (colon)*; 42 (rectum)*	33 (colon); 20 (rectal)	12.6 (colon); 19.0 (rectal)	37.1 (colon); 29.6 (rectal)	NR	48.8 (colon); 6.8 (rectal)
13	Votanopoulos (2013)[384]	63 (colon); 83 (rectal)	25.1 (colon); 28.2 (rectal)	NR	NR	17.3 (colon); 14.6 (rectal)	5.7 (colon); 0 (rectal)	18 (colon); 15 (rectal)
14	Yonemura (2013)[117]	68*	25*	23.4	NR	24.4	0.7	17.6
15	Baratti (2014)[118]	NR	NR	43.2	NR	32	3	NR
16	Ceelen (2014)[150]	95*	70*	NR	NR	27	2.4	35
17	Elias (2014)[115]	NR	NR	39	NR	39	5.5	21.5
18	Hompes (2014)[389]	NR	NR	NR	NR	26.5 (MMC); 37.1 (OXL)	0	41.1
19	Huang (2014)[377]	70.5	22	22	NR	16	0	NR
20	Passot (2014)[151]	NR	NR	NR	NR	36	4	NR
21	Prada-Villaverde (2014)[107]	NR	NR	NR	NR	32.6	NR	NR
22	Rivard (2014)[179]	NR	30.8	NR	10.9	30.8	NR	23.5
23	Faron (2016)[119]	NR	NR	42	NR	41	4.6	NR
24	Frøysnes (2016)[385]	93*	65	36	14	47	0	15.1
25	Maillet (2016)[120]	NR	58	34	NR	43.3	4	NR
26	Ng (2016)[381]	87.6	NR	NR	NR	28.8	NR	NR
27	Sluiter (2016)[390]	NR	NR	NR	NR	34.4**	NR	NR
28	Ihemelandu (2017)[391]	NR	35	25	NR	21.5	NR	NR
29	Leung (2017)[392]	NR	NR	NR	20 (MMC); 19 (OXL)	26 (MMC); 56 (OXL)	NR	NR
30	Massalou (2017)[383]	NR	NR	NR	NR	39.7	NR	13
31	Kozman (2018)[121]	NR	NR	30.3	NR	35	0.77	35
32	Sluiter (2018)[122]	NR	NR	NR	12	27	NR	25.7
33	Tonello (2018)[393]	NR	60	34	15.7	40.5	8.3	19.4

7.3.3 Prognostic factors influencing overall survival

7.3.3.1 Location of primary tumour

Three studies[179, 385, 387] with 254 patients reported the impact of primary tumour location. On pooled analysis, a rectal primary leading to peritoneal metastases was associated with a significantly worse survival than a colonic primary (HR 1.93, 95% CI 1.10-3.37, p=0.02), with no heterogeneity (I^2=0%). (Figure 7.2).

7.3.3.2 Timing of onset of peritoneal metastases

Three studies[120, 179, 387] with 366 patients evaluated the difference in survival between synchronous or metachronous presentation of peritoneal metastases. On pooled analysis, there was no difference in survival based on timing of onset of peritoneal metastases (HR 1.02, 95% CI 0.59-1.73, p=0.96), with moderate heterogeneity (I^2=50%). (Figure 7.2)

7.3.3.3 Grade III/IV morbidity

Four studies[118, 120, 121, 370] with 717 patients evaluated the effect of peri-operative Grade III/IV morbidity on overall survival. On pooled analysis, the presence of Grade III/IV morbidity was independently associated with worse overall survival (HR 1.59, 95% CI 1.17-2.16, p=0.003), with low heterogeneity (I^2=18%). (Figure 7.2)

7.3.3.4 Completeness of Cytoreduction (CC 0/1)

Eight studies[27, 107, 117, 118, 150, 377, 382, 386] with 1675 patients evaluated completeness of cytoreduction on survival after CRS and HIPEC. On pooled analysis, incomplete cytoreduction was significantly associated with a worse survival (HR 2.21, 95% CI 1.57-3.10, p<0.001), with moderate heterogeneity (I^2=46%). To evaluate the heterogeneity

further, the largest weighted study (Elias et al[27]) was removed and re-analysed, with a similar significant outcome, with low heterogeneity (I^2=21%). (Figure 7.2)

7.3.3.5 Peritoneal Carcinoma Index (PCI)

PCI was evaluated as a continuous variable in eight studies[27, 119, 122, 370, 381, 382, 385, 390] with 1279 patients. Increasing PCI was associated with worse survival, with each unit increase in PCI contributing to a 10% increased hazard of death (HR 1.10, 95% CI 1.05-1.15). Heterogeneity was high (I^2=78%). (Figure 2)

7.3.3.6 Tumour differentiation

Three studies[117, 121, 386] evaluated tumour differentiation as a prognostic factor. On pooled analysis, poor tumour differentiation did not appear not appear to confer a worse survival (HR 1.91, 95% CI 0.72-5.07). Heterogeneity was substantial (I^2=72%). (Figure 7.3)

7.3.3.7 Lymph node positivity

Nine studies[27, 105, 117, 120, 121, 151, 179, 370, 390] with 1675 patients evaluated the effect of lymph node involvement of the primary tumour on overall survival after CRS and HIPEC. On pooled analysis, lymph node involvement was significantly associated with worse overall survival (HR 1.33, 95% CI 1.04-1.72, p =0.03). Heterogeneity was moderate (I^2=31%). When Ung et al[370] was excluded, the overall outcome remained the same, with no heterogeneity (I^2=0%). (Figure 7.3)

7.3.3.8 Signet ring cell histology

Three studies[121, 388, 391] with 643 patients evaluated the role of signet ring cell histology on overall survival. Pooled analysis revealed no survival difference based on signet ring cell histology (HR 1.11, 95% CI 0.65-1.90, p=0.71), with no heterogeneity (I^2=0%). (Figure 7.3)

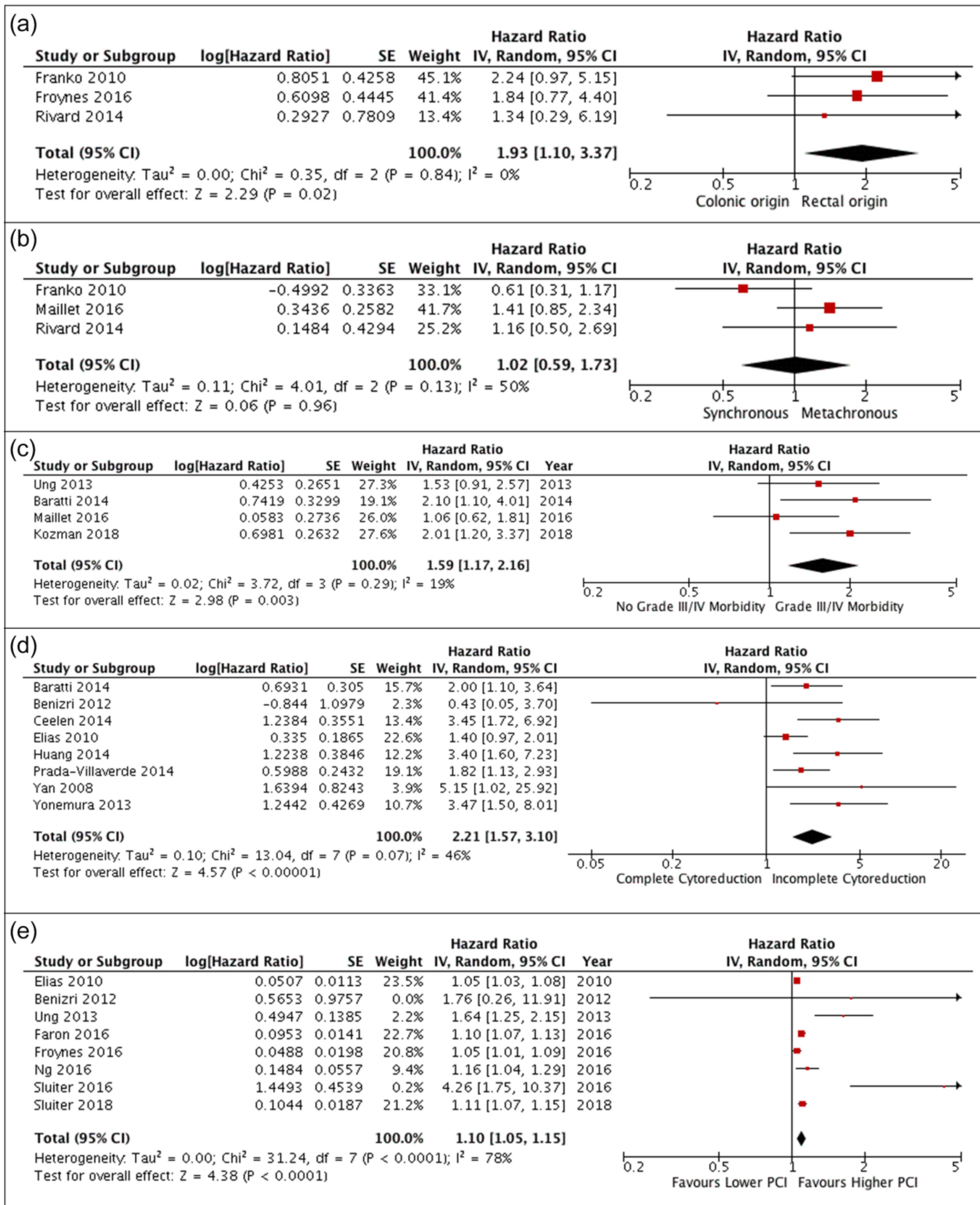


Figure 7.2 (a-e) Forest plots demonstrating prognostic factors influencing survival in patients undergoing CRS and HIPEC

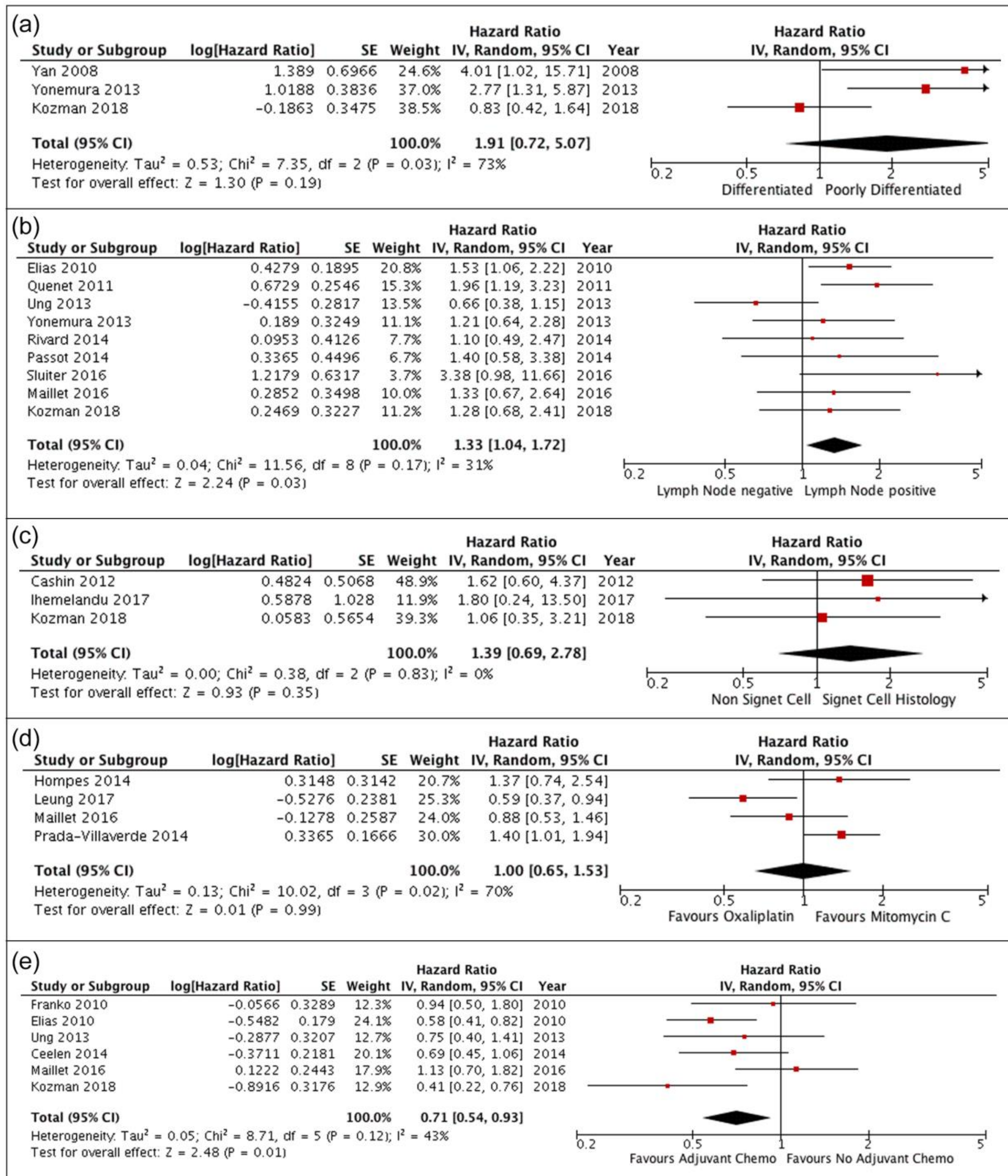


Figure 7.3 (a-e) Forest plot demonstrating prognostic factors influencing survival in patients undergoing CRS and HIPEC

7.3.3.9 HIPEC drug

Four studies[107, 120, 389, 392] with 1112 patients evaluated the survival benefit based on HIPEC drug used. On pooled analysis, there was no difference in survival based on

HIPEC drug used (HR 1.00, 95% CI 0.65-1.53, p=0.99). There was substantial heterogeneity (I^2=70%). (Figure 7.3)

7.3.3.10 Adjuvant systemic chemotherapy

Six studies[27, 120, 121, 150, 370, 387] with 1372 patients evaluated the impact of adjuvant systemic chemotherapy following CRS and HIPEC. Use of adjuvant systemic chemotherapy was associated with an improved overall survival (HR 0.71, 95% CI 0.54-0.93, p=0.01), with moderate heterogeneity (I^2=43%). (Figure 7.3)

Figure 7.4 summarises all evaluated prognostic factors and their influence on OS.

7.3.3.11 Other factors

Age and gender did not influence survival on pooled analysis (Appendix 3.5)

7.3.4 Sensitivity Analysis

Sensitivity was evaluated on all variables using fixed effects model. The outcomes remained the same for all variables except tumour differentiation, where poorly differentiated tumours were associated with a significantly worse survival (HR 1.62, 95% CI 1.01-2.60, p=0.01), with substantial heterogeneity (I^2=73%) (Appendix 3.6-3.8).

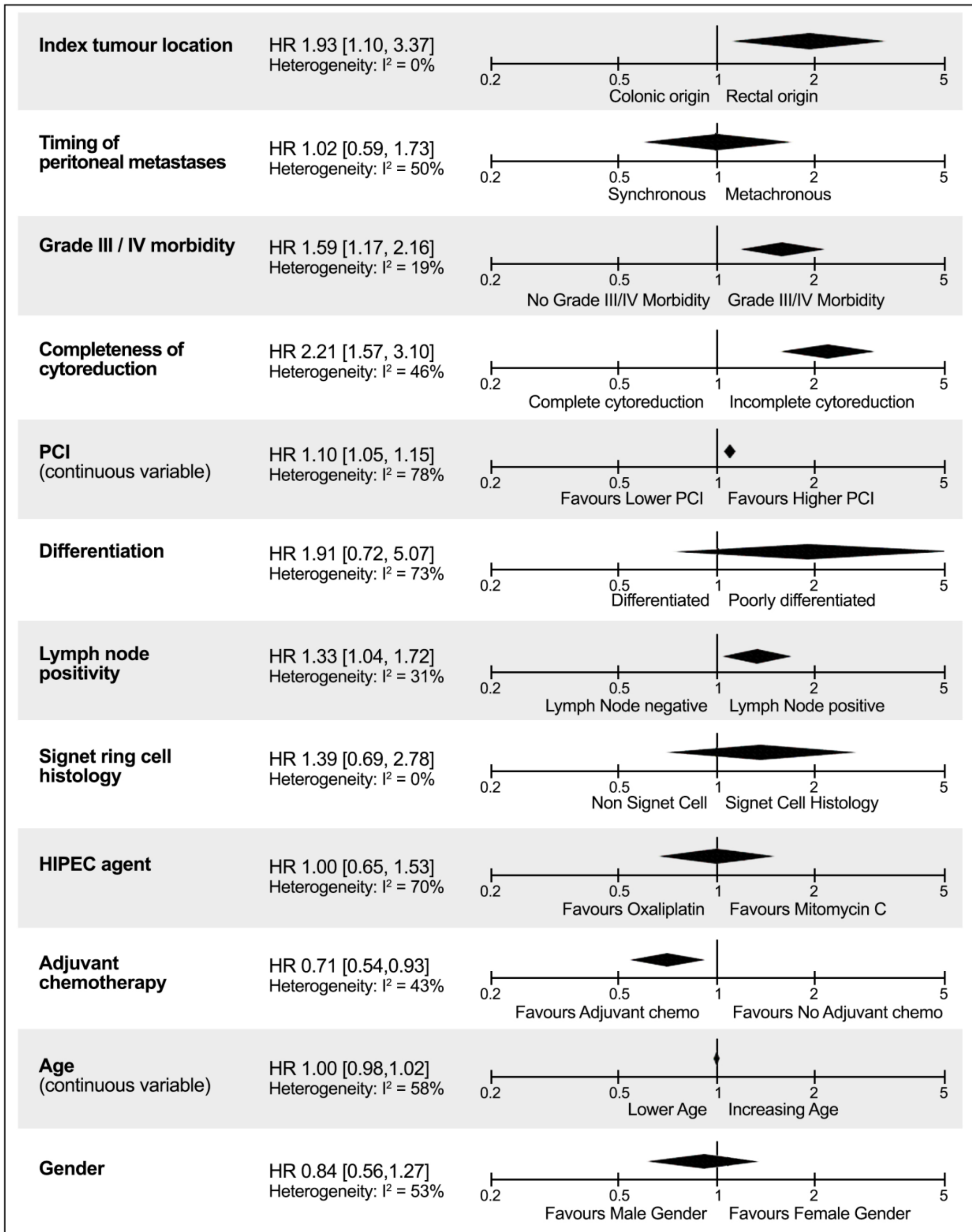


Figure 7.4. Summary forest plot with all evaluated prognostic factors influencing survival in patients undergoing CRS and HIPEC

7.4 Discussion

In addition to completeness of cytoreduction, increasing PCI and lymph node involvement, this meta-analysis also demonstrates that primary tumor location, adjuvant chemotherapy and grade III/IV morbidity are key prognostic factors influencing survival in patients undergoing CRS and HIPEC for isolated CRPM.

This is the first meta-analysis to demonstrate that a rectal primary leading to peritoneal metastases in patients undergoing CRS and HIPEC confer a significantly worse survival than patients with a colonic primary. Rectal cancers less commonly spread to the peritoneum than colonic cancers.[17] Therefore, rectal cancers metastasising to the peritoneum may be a manifestation of more advanced disease, or aggressive tumour biology, with other possible sites of undetected micro-metastatic disease. This cohort of patients may benefit from more aggressive multi-modal treatment including incorporation of longer duration of peri-operative systemic treatment, an area for further research.

The timing of peritoneal metastases and its impact on survival has not been well described in the literature. While some consider the presence of synchronous metastases as a more aggressive disease manifestation, we found no difference in survival based on synchronous or metachronous presentation. Recently, a large multi-centre study also evaluated the impact of timing of metastases and found no difference in survival.[49]

Along with completeness of cytoreduction, PCI has long been the backbone in the assessment of the value of CRS and HIPEC, as it is both predictive and prognostic, by stratifying tumour burden and potential resectability. While this meta-analysis confirms that increasing PCI is associated with a worse survival, it does not inform us of a cut-off PCI above which CRS and HIPEC would offer no benefit. This is largely due to variability in studies reporting the effect of PCI. While a PCI >15[394] confers a worse prognosis, there remains significant variability in practice between institutions. The recent Prodige 7 trial for example included patients with a PCI up to 25.[106] Such variability in practice

highlights the need for a consensus on a cut off PCI for offering CRS and HIPEC. Furthermore, PCI does not take into account important factors such as tumour biology,[122] histologic subtype[121] or small bowel involvement,[382] all of which are known to influence survival. In contrast, composite tools such as the peritoneal surface disease severity score (PSDSS) incorporate some of these factors and may help stratify patients better than the use of PCI.[364] The development of a multi-faceted prognostic tool incorporating patient and tumour related factors, with imaging findings that help predict resectability is an area of research that would greatly help this field.

The favourable results achieved with CRS and HIPEC have been obtained in studies utilising peri-operative systemic chemotherapy.[94, 106] A recent study reported that in patients suffering recurrence after CRS and HIPEC, 31% recur at distant sites.[395] Adjuvant chemotherapy use may help reduce this distant failure rate. Additionally, adjuvant chemotherapy can help treat residual micro-metastatic disease, helping reduce loco-regional or distant failure. However, the use of systemic chemotherapy in patients undergoing CRS and HIPEC remains variable across the world, with centres in France[27, 394] using peri-operative systemic chemotherapy routinely, while those in Netherlands offer upfront CRS and HIPEC[100] without systemic therapy. To date, there have been no prospective studies that have evaluated outcomes with CRS and HIPEC without use of peri-operative systemic chemotherapy. The CAIRO 6 RCT[148] is currently recruiting, aiming to evaluate the role of peri-operative systemic chemotherapy with CRS and HIPEC versus upfront CRS and HIPEC alone. This trial will hopefully provide more insight to help direct the use of systemic chemotherapy.

Lymph node involvement of the primary tumour was found to significantly influence survival. Predictive nomograms have previously described nodal status as an independent risk factor for peritoneal recurrence.[57] Some studies have also suggested that evidence of lymph node metastases, along with other poor prognostic factors should be a contraindication to offering CRS and HIPEC.[27] This is possibly because lymph node metastases are a portal to haematogenous spread of tumour cells to other sites such as the lung or liver.[76, 118]

While CRS and HIPEC as a combined treatment has been demonstrated to be superior to systemic chemotherapy in CRPM,[94] very few studies have evaluated the role of CRS alone without HIPEC. In an RCT, Elias et al[96] compared the use of EPIC plus systemic chemotherapy versus systemic chemotherapy alone, in patients undergoing complete cytoreduction. While this trial closed early due to patient dissatisfaction with the inclusion criteria, it did report a 60% two year survival in both groups, with no survival benefit seen with the use of EPIC. In a single centre study, Desolneux et al[396] evaluated outcomes following CRS alone with peri-operative systemic chemotherapy, and reported a favourable median survival of 32.4 months if a CC 0/1 resection was achieved. More recently, the PRODIGE 7 RCT[106] evaluated the role of Oxaliplatin based HIPEC by comparing CRS and HIPEC versus CRS alone in patients undergoing complete cytoreduction. Median survival was 41.2 months for patients undergoing CRS alone, with no survival advantage seen with the addition of Oxaliplatin based HIPEC. Additionally, the HIPEC group had a significantly higher complication rate. Sub group analysis however, did demonstrate a survival advantage with HIPEC in those with a PCI 11-15. This trial has contributed to international debate about whether HIPEC is indeed needed, with some proposing that HIPEC be dropped altogether.[397] While yet unpublished, this trial has reaffirmed the value of high quality cytoreductive surgery, but has raised doubts among the international community about the efficacy of oxaliplatin based HIPEC.

While cytoreductive procedures are relatively standardised around the world,[84] there remains variability in HIPEC drugs used, with the choice of Mitomycin C or Oxaliplatin largely based on country and institutional preference, rather than efficacy of the drug.[107, 389] Additionally, comparative retrospective studies have not clearly determined either to be superior, a finding confirmed in this meta-analysis. Importantly, there are no prospective trials to date that have compared outcomes between the two agents. With results from PRODIGE 7 demonstrating no overall benefit from Oxaliplatin HIPEC, it would be a logical step to stop using Oxaliplatin and consider preferentially use Mitomycin C, especially as the sentinel RCT[94] that demonstrated a survival benefit with CRS and HIPEC over systemic chemotherapy utilised Mitomycin C. The findings from PRODIGE 7 highlight other important issues that more prospective studies are urgently required to compare the efficacy of CRS alone, and with other HIPEC agents, which may

provide valuable insight into whether any HIPEC agent offers survival benefit, or if CRS alone is adequate.

Peri-operative complications are associated with a worse survival in a number of studies.[398, 399] Complications such as anastomotic leaks lead to an immunosuppressive effect on the host immune system, coupled with an inflammatory cascade and release of cytokines and pro-inflammatory mediators that contribute to tumour cell dissemination.[118, 400] This emphasises the importance of careful patient selection, along with meticulous operative technique and safe decision-making.

It is plausible that studies have an inherent degree of selection bias with regard to age, with only younger patients with favorable functional status being offered CRS and HIPEC. This is reasonable, given CRS and HIPEC carries a 12.5-35% and 1.0-8.3% risk of major morbidity[130, 150] and mortality,[393] respectively. Gender has not been shown to influence survival in previous studies. Apart from ovarian metastases, there is no difference in sites of peritoneal metastases in either sex.

While the lack of RCTs is a limitation in our study, it reflects the existing literature, with only one RCT[94] completed successfully. Despite careful study selection, differences in the quality of the underlying included studies contributed to an overall moderate degree of heterogeneity. Studies had different prognostic factors evaluated, with discordance in how factors such as PCI were reported. Variable sample sizes, with heterogeneous populations, and selection bias within each study owing to individual institutional policies for offering CRS and HIPEC, that were not reported in some studies contributed further to heterogeneity. Additionally, important prognostic factors such as tumour biology and histologic subtypes were not reported in many studies. Tumour biology is an important prognostic factor, with RAF/RAS mutations and high-grade tumours recently shown to be independently associated with worse cancer specific survival in patients undergoing CRS and HIPEC.[401] The presence of RAS/RAF mutations render anti-EGFR antibodies ineffective, thereby limiting treatment options for these patients. Similarly, aggressive histologic subtypes such as mucinous and signet ring cell histology have also previously been shown to confer a worse prognosis.[402, 403] However, these are relatively rare, with signet ring cell histology for instance accounting for less

than 1-2% of all CRCs.[404] With limited studies reporting on tumour biology, small study populations, patient selection bias in study samples and low incidence of features such as signet ring cell histology, it is plausible that we were unable to accumulate an adequate sample size to demonstrate a survival difference. Chemotherapy protocols similarly, were variable, and not fully reported in many studies, along with different HIPEC agents, delivery techniques (open or closed) and drug dosages used in studies. Furthermore, studies were over different time frames, with variable follow up, with possibly different levels of experience among surgeons. Eight studies were excluded for not having adequate survival data; this may have contributed to study inclusion bias. Publication bias is likely an issue, especially as unpublished data is not accessible. With wider acceptance of CRS and HIPEC in recent years, we felt it more representative to evaluate recent evidence in the last decade as opposed to all studies published since inception. Liver metastases are a notable exclusion in our study, as they have been described to influence survival in patients with CRPM.[362] However, we chose to limit our study to isolated CRPM only, aiming to render studies more comparable.

7.5 Conclusion

Apart from completeness of cytoreduction, PCI and lymph node involvement, this meta-analysis demonstrates that adjuvant chemotherapy use, rectal primary and peri-operative grade III/IV morbidity are important prognostic factors influencing survival in patients undergoing CRS and HIPEC for CRPM.

8 Chapter 8: Exploring the immune landscape of CRPM

Material from this chapter has led to contributing authorships in:

1) Kong J, Guerra G, Millen R, Roth S, Xu H, Neeson P, Darcy P, Kershaw M, Sampurno S, Malaterre J, Liu D, Pham T, **Narasimhan V**, Wang M, Huang YK, Visvanathan K, McCormick J, Lynch A, Warrier S, Michael M, Desai J, Murray W, Mitchell C, Ngan S, Phillips W, Heriot A, Ramsay R. Tumor Infiltrating Lymphocyte Function Predicts Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer. *JCO Precision Oncology* 2018. DOI: 10.1200/PO.18.00075

2) Michie J, Beavis P, Freeman A, Vervoot, S, Ramsbottom K, **Narasimhan V**, Lelliott E, Lalaoui N, Ramsay R, Johnstone R, Silke J, Darcy P, Voskoboinik I, Kearney C, Oliaro J. Antagonism of IAPs enhances CAR T-Cell efficacy. *Cancer Immunol Res* 2019. 7(2): 183-192

3) Ceelan W, Ramsay R, **Narasimhan V**, Heriot A, De Wever O. Targeting the tumor microenvironment in colorectal peritoneal metastases. *Trends in Cancer* 2020, DOI: 10.1016/j.trecan.2019.12.008

8.1 Introduction

Since Jass[236] demonstrated in 1986 that a pronounced lymphocytic infiltrate strongly correlated with improved survival in primary rectal cancer, various studies have further evaluated the role of the immune system in CRC. Galon et al[40] reaffirmed in a sentinel study that an increased presence of CD3+/CD8+/CD45RO cells in the invasive margin and centre of the tumour was strongly associated with an improved disease free and overall survival. This was independent of the AJCC TNM stage. More recently, the development of the Immunoscore[42] based on quantification of tumour infiltrating lymphocytes (TILs) in the form of CD3+ and CD8+ cells in the tumour centre and invasive margin using automated immunohistochemistry has led to a more clinically translatable measure of the influence of the immune system. In terms of prognostic capacity, the Immunoscore has been shown in studies to be superior to the established TNM classification for stage I-III CRC.[39, 40] In addition, the role of the entire tumour microenvironment (TUME), and its interactions with immune cells in determining the fate of the tumour and its invasive capabilities is an area of intense interest.

In patients with cancer, metastases have been estimated to be responsible for approximately 90% of deaths.[405] This has changed very little in recent decades.[406] While the role of the immune infiltrate and the Immunoscore have been shown to have prognostic and predictive value in Stage I-III cancer,[407] its role in Stage IV CRC, and especially in CRPM, which confer the worst prognosis in metastatic CRC remains unexplored.

Immunohistochemistry techniques used to determine the Immunoscore provides a static snapshot in time of the presence of TILs, without any assessment of the functionality of the TILs. Recent studies have demonstrated that T cells in the TUME might be dysfunctional[408] or merely bystander T cells without any specificity for tumour antigens.[409] Evaluating functionality of TILs is also particularly important with the emergence of immunotherapy, in particular checkpoint blockade as a treatment modality for many types of cancers. However, despite the promise of immunotherapy, the majority of patients do not respond to this therapy.[410] In CRC currently, the only

reliable biomarker to predict response to checkpoint blockade is microsatellite instability (MSI-H),[411] which is most commonly evaluated using IHC. Within the MSI-H cohort as well, Le et al demonstrated that only 53% of patients demonstrate an objective response to checkpoint antibodies.[198]

Recently, Chen et al[271] compared the use of durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) to supportive care in an RCT with MSS patients with standard treatment refractory metastatic CRC. They reported that use of dual checkpoint blockade led to an improved OS of 6.6 months compared to 4.4 months (HR 0.72; 90% CI: 0.54-0.97). This study lends further weight to the hypothesis that other factors apart from MSI-H status alone influence response to immunotherapy. Thus, evaluation of the functionality of the TILs in the TUME may help direct more efficient use of checkpoint blockade therapies.

The emergence of the CMS subtypes of CRC is another area that lends itself to help develop and direct personalised therapy based on the unique TUME of each tumour.[33] This again requires a thorough assessment and understanding of the TUME at metastatic sites of CRC.

While the immune contexture of normal peritoneal tissue has been previously described,[258] very little information exists on the immune infiltrate and TUME of CRPM. Additionally, as previously discussed in Chapter 1, there are currently no studies that have explored the efficacy of checkpoint blockade in patients with CRPM. Given the significant limitations in current treatment options in CRPM, it is imperative to explore newer avenues of treatment in this poor prognosis cohort.

Broadly, this chapter is a descriptive evaluation of the immune landscape of CRPM. Understanding the role of the immune system in CRPM may provide more insight into why the immune system is unable to fight peritoneal metastases, and help develop new strategies in targeting this disease.

In particular, this chapter aims to:

1) Evaluate the immune infiltration, in particular the T cell infiltration and expression profile in CRPM using a combination of Flow Cytometry (FACS) and Multiplex IHC (OPAL)

2) Expand on a recently developed immune cytotoxic assay (Tumouroid-TIL co-culture) to evaluate the specificity of the assay, functionality of TILs in CRPM and the potential role of checkpoint antibodies

3) Utilise RNASeq from CRPM tissue to evaluate the CMS classification of CRPM, gene expression and pathway differences between primary tumours and CRPM.

8.2 Methods

The methods utilised for the various techniques used in the study are outlined in detail in **Chapter 2**.

8.3 Results

8.3.1 Flow Cytometry (FACS)

8.3.1.1 Immune cell infiltration in normal peritoneum and CRPM

Flow cytometry was performed on 26 fresh CRPM tissue with matched normal peritoneum. Myeloid cells ($CD45^{+}$ $CD3^{-}$ $CD56^{-}$$CD33^{+}$ $CD11b^{+}$) were the predominant leukocyte cell type in normal peritoneum accounting for 65.8 ± 7.45% of all $CD45^{+}$ leukocytes, with T cells accounting for 11.0 ± 3.9% (Figure 8.1A). In CRPM however, there were significantly more T cells compared to normal peritoneum (32.0 ± 3.68% v 11.0 ± 3.9%, p =0.002), suggesting that there is an adaptive immune response into the TUME of CRPM. Myeloid lineage cells were reduced in CRPM compared to normal peritoneum (20.7 ± 4.4 v 65.8 ± 7.45%, p<0.001) (Figure 8.1B)

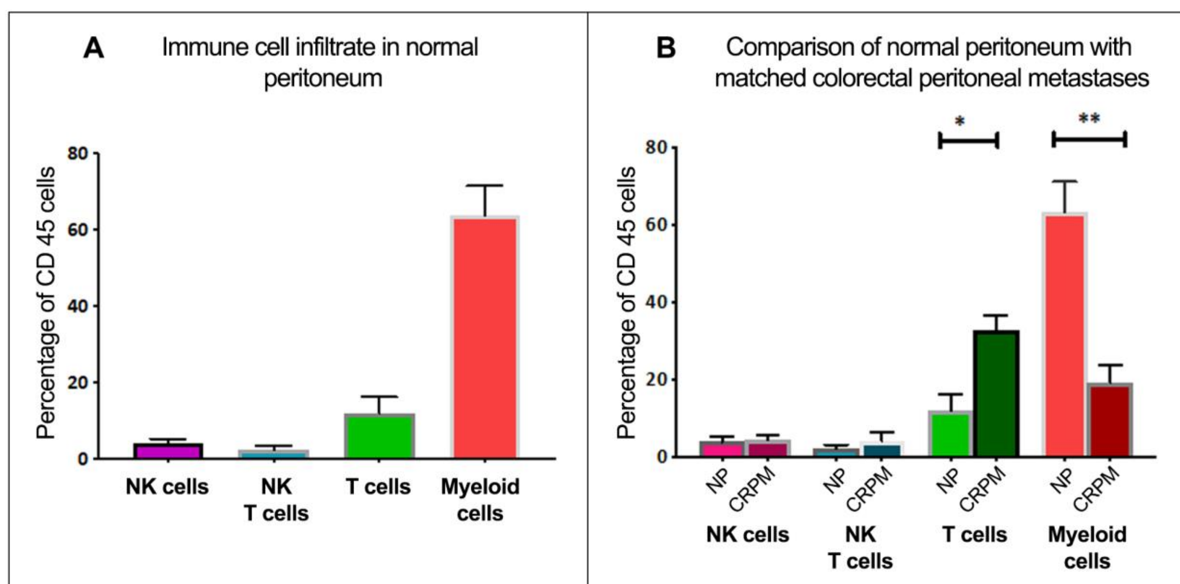


Figure 8.1. Immune cell infiltrate in normal peritoneum (NP) and colorectal peritoneal metastases (CRPM). Fig 8.1A. Myeloid cells were the predominant leucocyte cell type in normal peritoneum. Fig 8.1B. T cells were significantly higher in CRPM compared to normal peritoneum.

*N=26 patients; means and standard error of the mean; * p=0.002 on Student T test (parametric data); **p<0.001 on Mann-Whitney U test (non-parametric data)*

8.3.1.2 Lymphocyte phenotypes and expression status in CRPM

Upon phenotyping T cells in CRPM further, $CD4^+$ T cells accounted for 45.1 ± 4.62%, with $CD8^+$ T cells accounting for 36.4 ± 3.09% of all T cells. Tregs were classified as $CD4^+$ $CD25^+$ $FoxP3^+$ and accounted for 3.90 ± 0.67% of all T cells (Figure 8.2A). Proportionally, T cell subsets were similar across normal peritoneum and CRPM. However, the overall T cell population was substantially lower in normal peritoneum compared to CRPM as seen in Figure 8.1. In CRPM, there were large proportions of effector memory $CD45RO^+CD4^+$ (39.3 ± 6.6%) and $CD45RO^+CD8^+$ (43.0 ± 5.18%). Approximately a fifth of T cells in CRPM demonstrated a degree of activation, being HLA-DR^+. Interestingly, approximately a fifth of all T cells (20.0 ± 3.16%) were PD-1^+, suggesting a potential role of anti-PD-1 antibodies (Figure 8.2B).

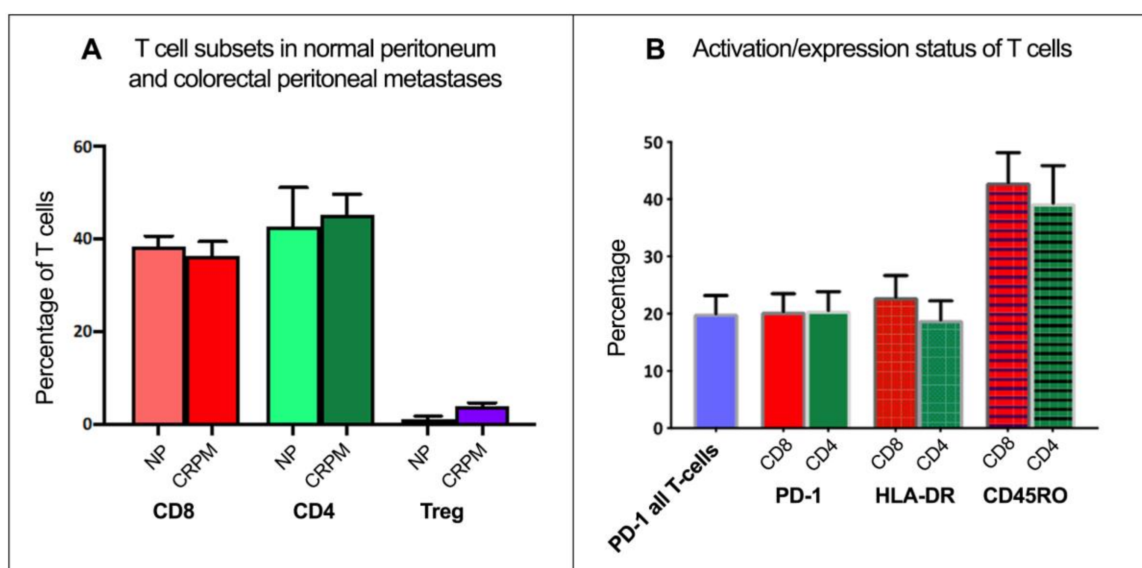


Figure 8.2A. T cell subsets in normal peritoneum and colorectal peritoneal metastases. Figure 8.2B. Expression status of T cells in CRPM. Over a fifth of all T cells were PD-1^+, with a significant proportion of $CD45RO^+$ effector memory cells.

8.3.1.3 Comparison of primary and matched CRPM

There were three matched primary tumours with CRPM. Overall, there was a non-significant trend towards an increased T cell infiltrate (26.8 v 38.7%, p=0.65) in CRPM compared to the primary tumours (Figure 8.3A). However, this was with a very small sample size, and therefore no robust conclusions can be drawn.

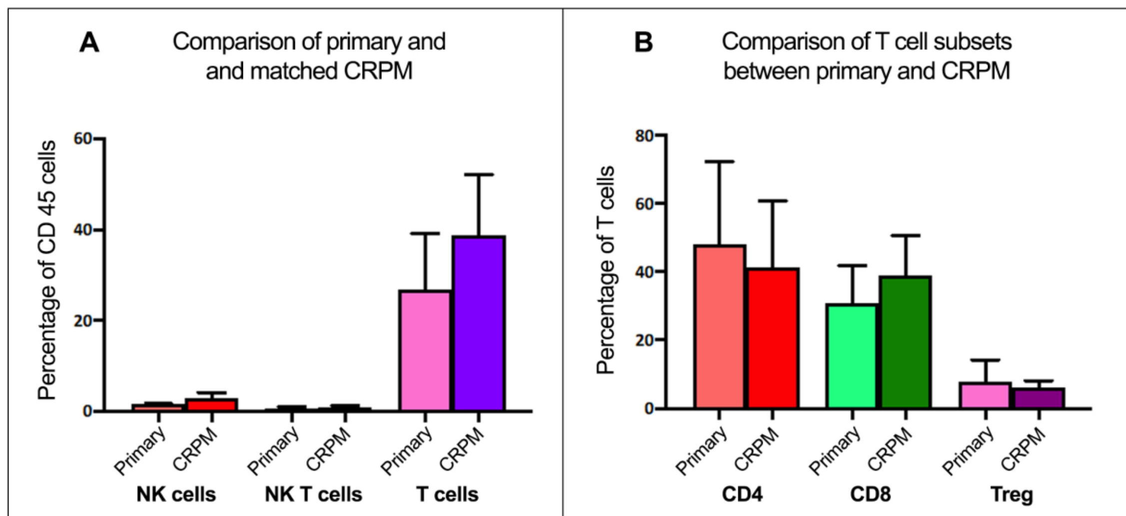


Figure 8.3A. Comparison of immune infiltrate between the primary tumour and matched synchronous CRPM. Figure 8.3B. There was no difference in T cell subsets between the primary tumour and matched synchronous CRPM.

N=3 matched patients with synchronous primary and CRPM

8.3.1.4 Flow Cytometry summary

FACS analysis clearly demonstrates that there are T cells in the TUME in CRPM. However, it is plausible that the adaptive immune system is ineffective in CRPM, given the dismal prognosis conferred by the presence of peritoneal metastases. Furthermore, merely the presence of T cells does not inform us of its function as dysfunctional T cells could explain why the immune system is unable to fight the tumour at the site of CRPM. Assessment of function required adaptation of an immune cytotoxic assay recently developed in the Ramsay lab.

8.3.2 Immune cytotoxic assay (Tumouroid-TIL co-culture)

The Ramsay lab recently developed an immune cytotoxic assay wherein functionality and kinetics of TILs and their cytotoxic ability on tumouroids can be measured using mean fluorescence intensity (MFI).[412] In patients with locally advanced rectal cancer, this tumouroid-TIL co-culture assay has shown promising results in predicting complete pathological response after neo-adjuvant chemo-radiotherapy based on the

MFI differences in complete responders compared to incomplete responders. However, the specificity of the T cells grown *in-vitro* for this co-culture has previously not been evaluated. If the T cells are indeed specific towards tumour antigen, this assay can help evaluate functionality of TILs in CRPM. To evaluate specificity and functionality, we had to:

1) Confirm that tumouroids retained the immune profile of the native tumour with regard to immune ligand expression.

2) Confirm *in-vitro* enriched T cells could kill tumouroids and not normal colonic epithelium organoids

3) Confirm cytotoxicity was $CD8^+$ mediated, and could be suppressed by blocking function of $CD8^+$ cells.

8.3.2.1 Tumouroids retain the immune profile of the native tumour

Native CRPM tumour tissue expressed MHC-I and did not express PD-L1 in most cases. CRPM tumouroids accurately recapitulated the immune profile of the native tumour. Additionally, tumouroids up regulated expression of MHC-I and PD-L1 upon exposure to IFN-γ (Figure 8.4).

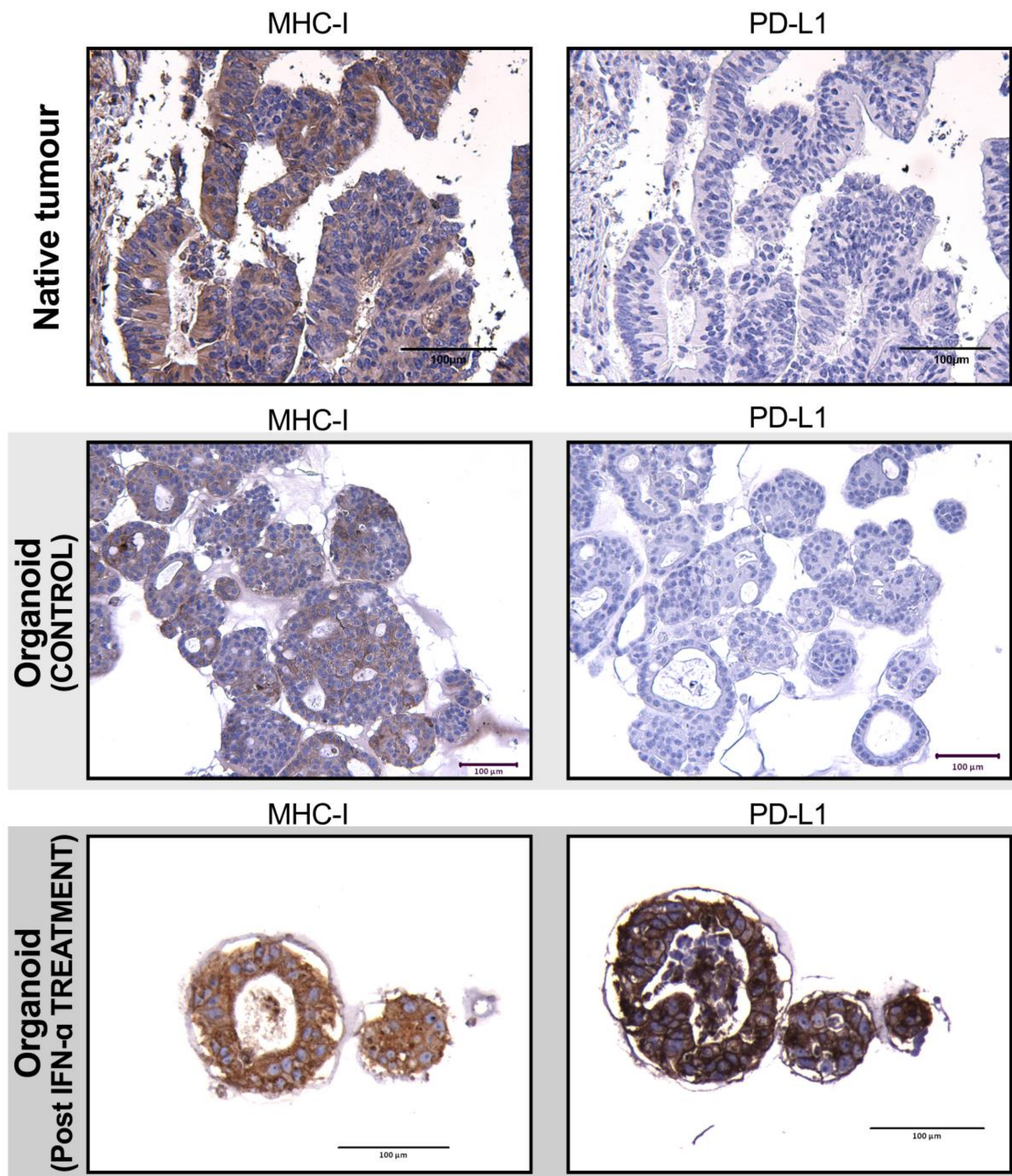


Figure 8.4. Tumouroids accurately recapitulate the immune profile of the native tumour. Tumouroids up regulate MHC-I and PD-L1 ligand expression on exposure to interferon gamma at 10ng/mL over a 48hour period.

Interferon exposure was found to be directly cytotoxic to tumouroids, with disruption of tumouroid architecture as seen in Figure 8.5. Tumouroid up regulation of MHC-I and PD-L1 upon exposure to IFN-γ was confirmed, with both, immunohistochemistry (Figure 8.5) and FACS (Figure 8.6)

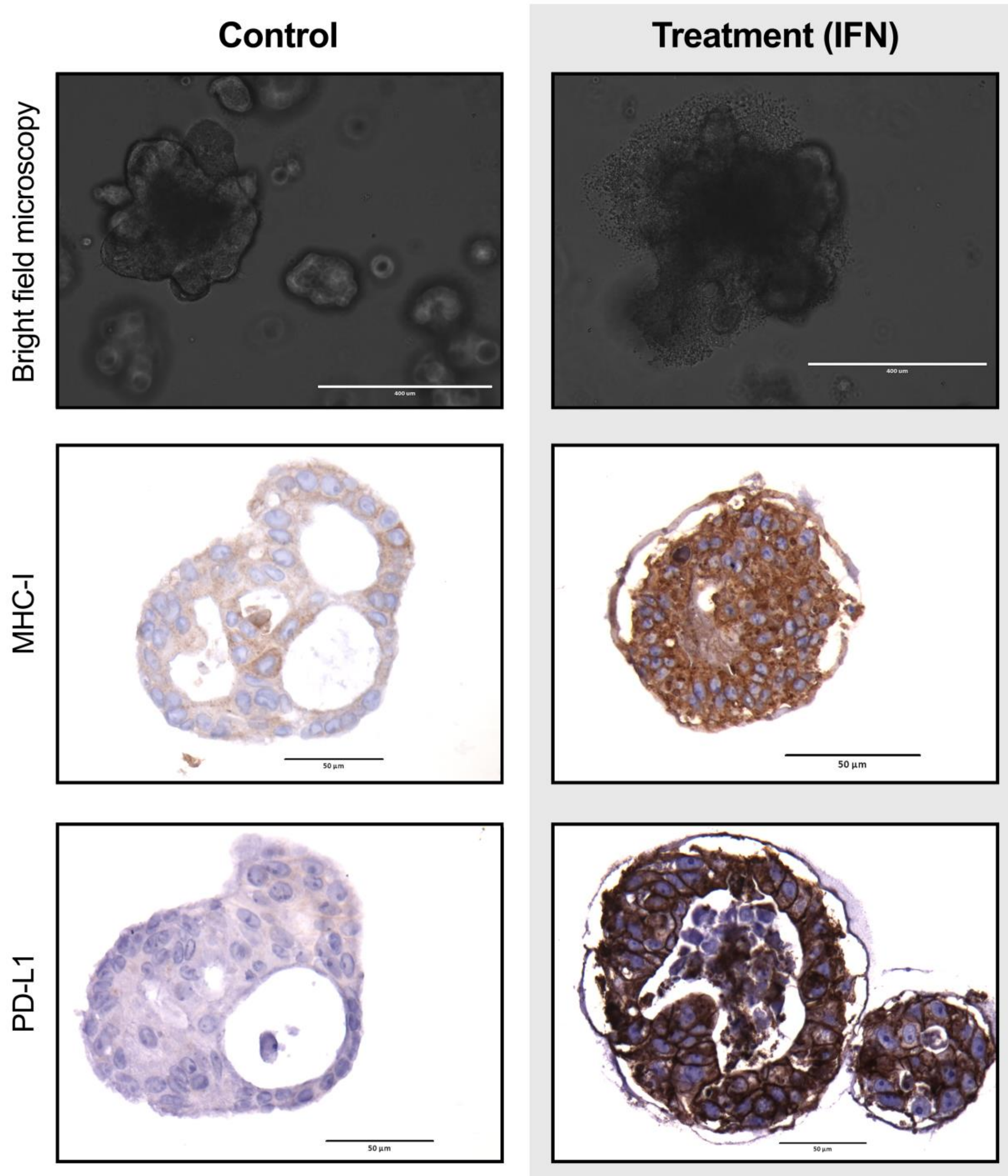


Figure 8.5. The top row demonstrates bright field microscope evidence of disruption of tumouroid architecture on exposure to interferon. The second and third row demonstrates that at baseline, tumouroids express MHC-I but not PD-L1. After 48 hours of 10ng/mL interferon gamma exposure, tumouroids up regulated MHC-I and PD-L1.

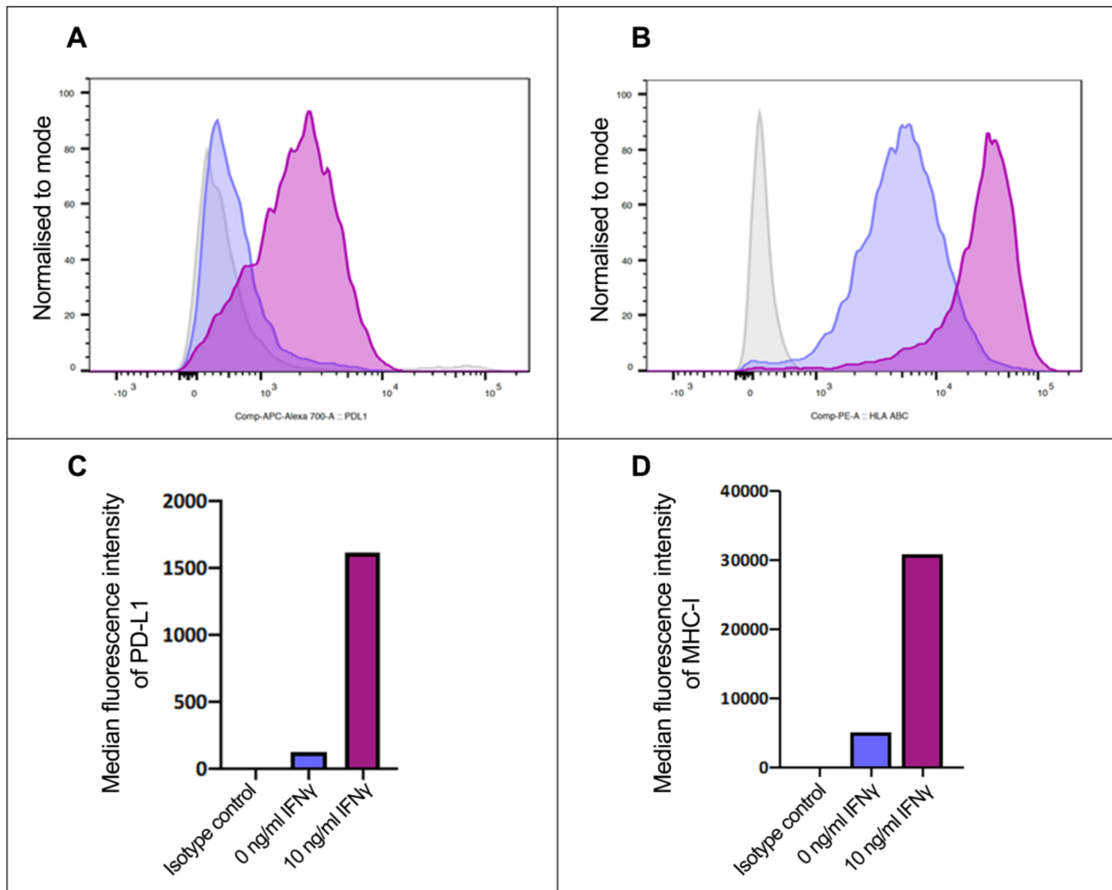


Figure 8.6A-D. One example with flow cytometry demonstrating that at baseline there is almost no PD-L1 expression (baseline blue is same as isotype grey on tumouroids. However, following interferon exposure, PD-L1 expression is up regulated as seen in 8.6A and 8.6C. In 8.6B, it is evident that tumouroids express MHC-I at baseline (baseline blue is higher than isotype grey), which increases further after interferon exposure.

8.3.2.2 Evaluating specificity of the immune cytotoxic assay

8.3.2.2.1 TILs do not kill normal colonic organoids

As a proof of concept, matched normal colonic epithelium organoids and matched colonic tumouroids were grown and validated from three separate patients. Morphologically, normal colonic organoids appeared to have a more cystic appearance compared to tumouroids (Figure 8.7).

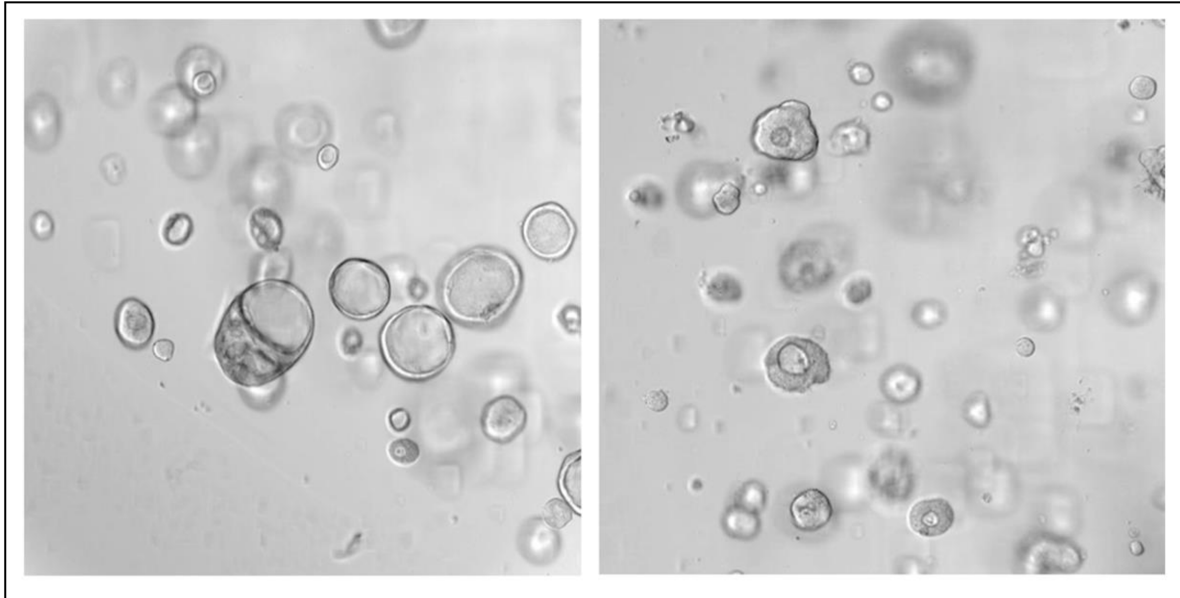


Figure 8.7. Normal colonic epithelium organoids on the left and matched colonic tumouroids on the right. Normal colon organoids can be seen to have a more cystic appearance.

When tumouroids were co-cultured with TILs, there was clear evidence that TILs were able to kill tumouroids (Mean MFI 13606 v 3397, $p < 0.001$). However, when the TILs were co-cultured with matched normal colonic epithelium organoids, there was no TIL mediated cytotoxicity (Mean MFI 2644 v 2279, $p = 0.71$), confirming that TILs preferentially kill tumouroids and not normal cells (Figure 8.8).

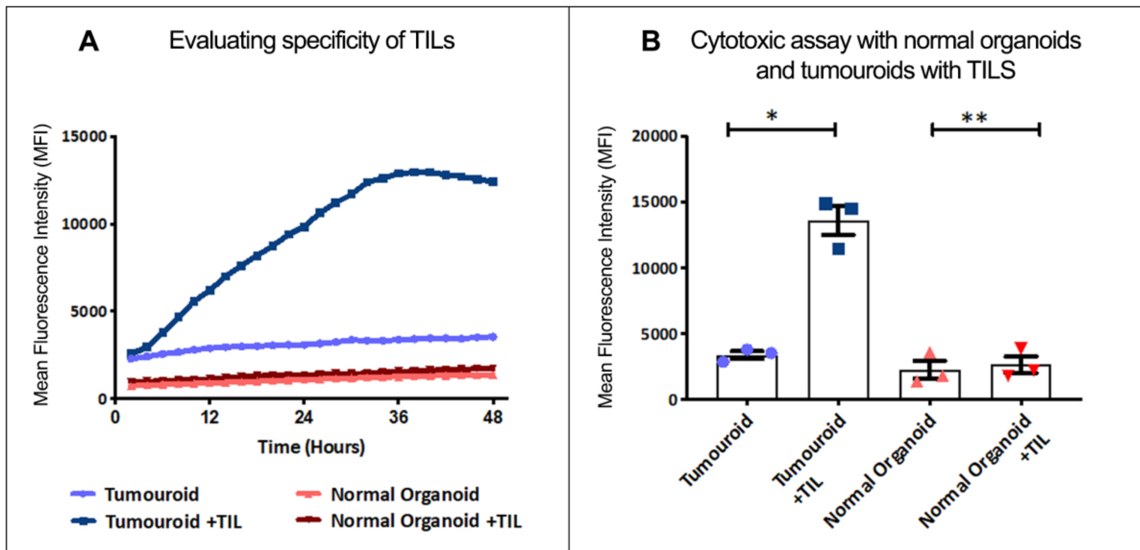


Figure 8.8A. Kinetics over 48 hours demonstrating that TILs kill matched patient tumouroids but not matched patient normal colonic epithelium organoids, confirming firstly that TILs are functional and secondly that they are specific against tumour antigen and can differentiate foreign (tumour antigen) from self (normal colon organoids). Fig 8.8B. Collation of three independent samples demonstrating that TILs are able to kill tumouroids but not normal cells (n=3 patient samples).

**: p<0.001; **: p=0.71(student t test)*

8.3.2.2.2 Cytotoxicity is dependent on CD8+ mediated killing

When FACS sorted $CD8^+$ and $CD4^+$ TILs were independently co-cultured with tumouroids, only $CD8^+$ T cells were responsible for cytotoxicity, with minimal tumouroid death with $CD4^+$ T cells alone. Furthermore, when tumouroids were incubated with MHC-I antibody, and then co-cultured with $CD8^+$ T cells, cytotoxicity was significantly reduced, suggesting that the enriched $CD8^+$ T cells remained specific to tumour antigen, as blocking their function with MHC-I antibody reduced their cytotoxic activity. Additionally, addition of anti-PD-1 antibody along with MHC-I antibody showed no increase in cytotoxicity, confirming that reduction in cytotoxicity was likely because $CD8^+$ T cell function was suppressed by blocking MHC-I and unlikely to be due to PD-1/PD-L1 interaction (Figure 8.9A). Pooled results from three samples show that tumouroid death is $CD8^+$ mediated and that $CD8^+$ T cell action can be suppressed by blocking MHC-I antibody (Figure 8.9B).

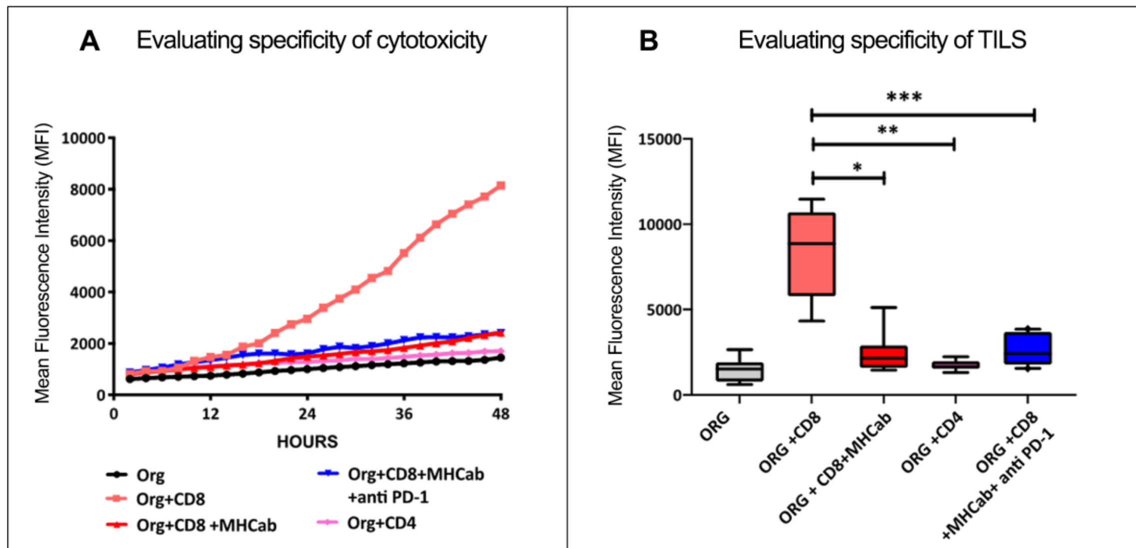


Figure 8.9A. Tumouroids co-cultured independently with T cell subsets demonstrating that CD8⁺ T cells are responsible for cytotoxicity, and can be suppressed by blocking CD8⁺ function with MHC-I antibody. Fig 8.9B.Summary of three samples demonstrating CD8⁺ T cell mediated cytotoxicity responsible for tumouroid death.

, **, *: Student t test $p<0.001$, n=3 patient sample)*

When cytokines from the co-culture assays were analysed, it was evident that IFN-γ and Granzyme B were only secreted by TILs when they were exposed to tumouroids and not with normal colonic organoids (Figure 8.10A). Similarly, when FACS sorted T cell subsets were co-cultured with tumouroids, IFN-γ and Granzyme B release was highest with $CD8^+$ T cells, and was successfully suppressed with MHC-I antibody (Figure 8.10B)

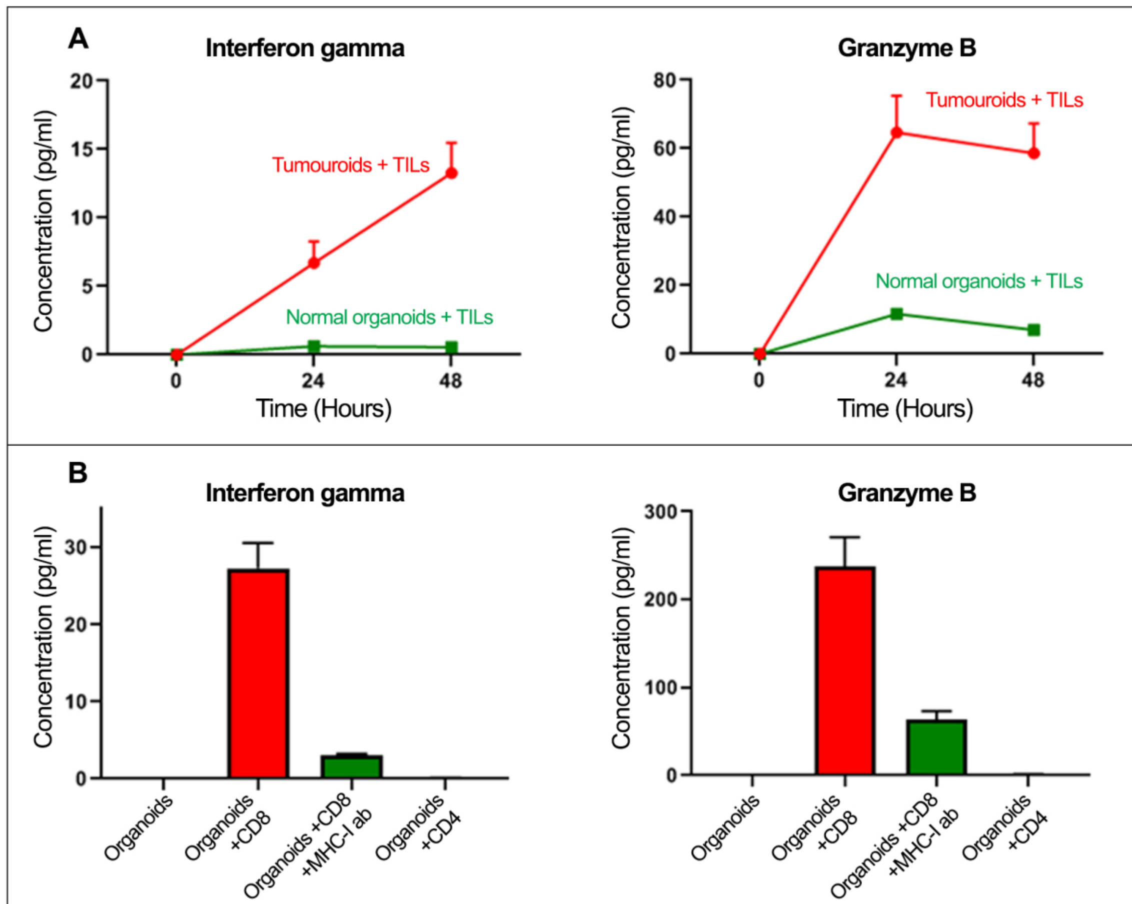


Figure 8.10A. TILs release interferon and granzyme in contact with tumouroids, but not normal colonic organoids. Fig 8.10B. Interferon and Granzyme release suppressed by blocking CD8⁺ T cell function with MHC-I antibody.

8.3.2.3 Functionality of TILs and role of immune checkpoint antibodies

As TILs were cultured *in-vitro* and expanded for four to six weeks before they were suitable for the co-culture assay, FACS was performed on the enriched TILs before running the co-culture assay. There was no significant difference in T cell phenotype of expression markers between fresh tissue TILs and *in-vitro* enriched TILs (Figure 8.11).

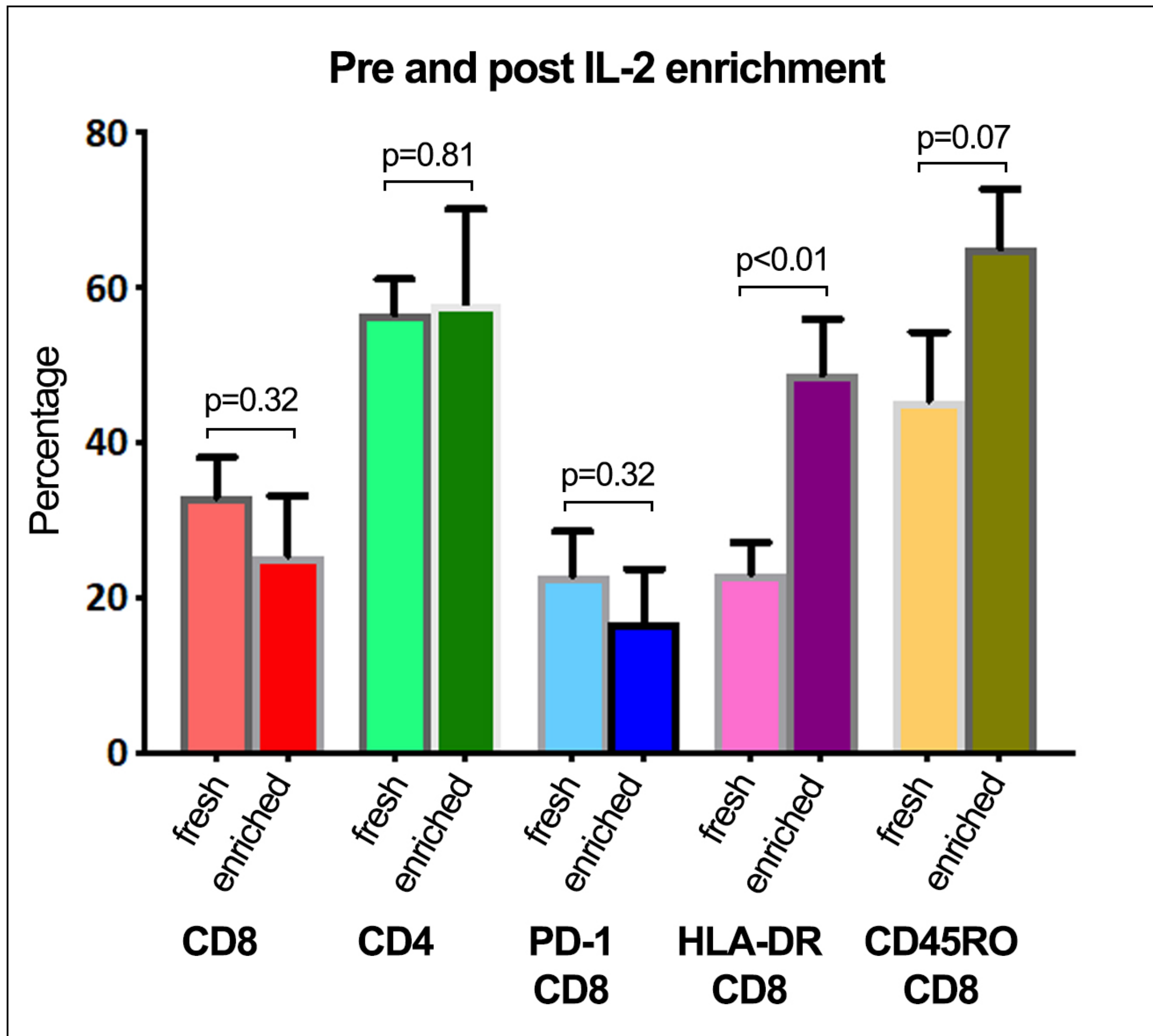


Figure 8.11. No significant difference between fresh and enriched TILs with regard to CD8/CD4 proportions or PD-1 expression after culturing in-vitro. However, expression markers such as HLA-DR increased significantly post-culturing, possibly because T cells are cultured in the presence of tumour tissue, providing a constant source of antigen for T cell activation.

(non parametric data, Mann Whitney U test for significance, n= ten independent samples)

Upon co-culturing CRPM tumouroids and matched TILs, TILs were able to kill tumouroids, confirming that they were functional. The addition of anti-PD-1 antibody to the co-culture revealed significantly improved killing in selected samples (Figure 8.12).

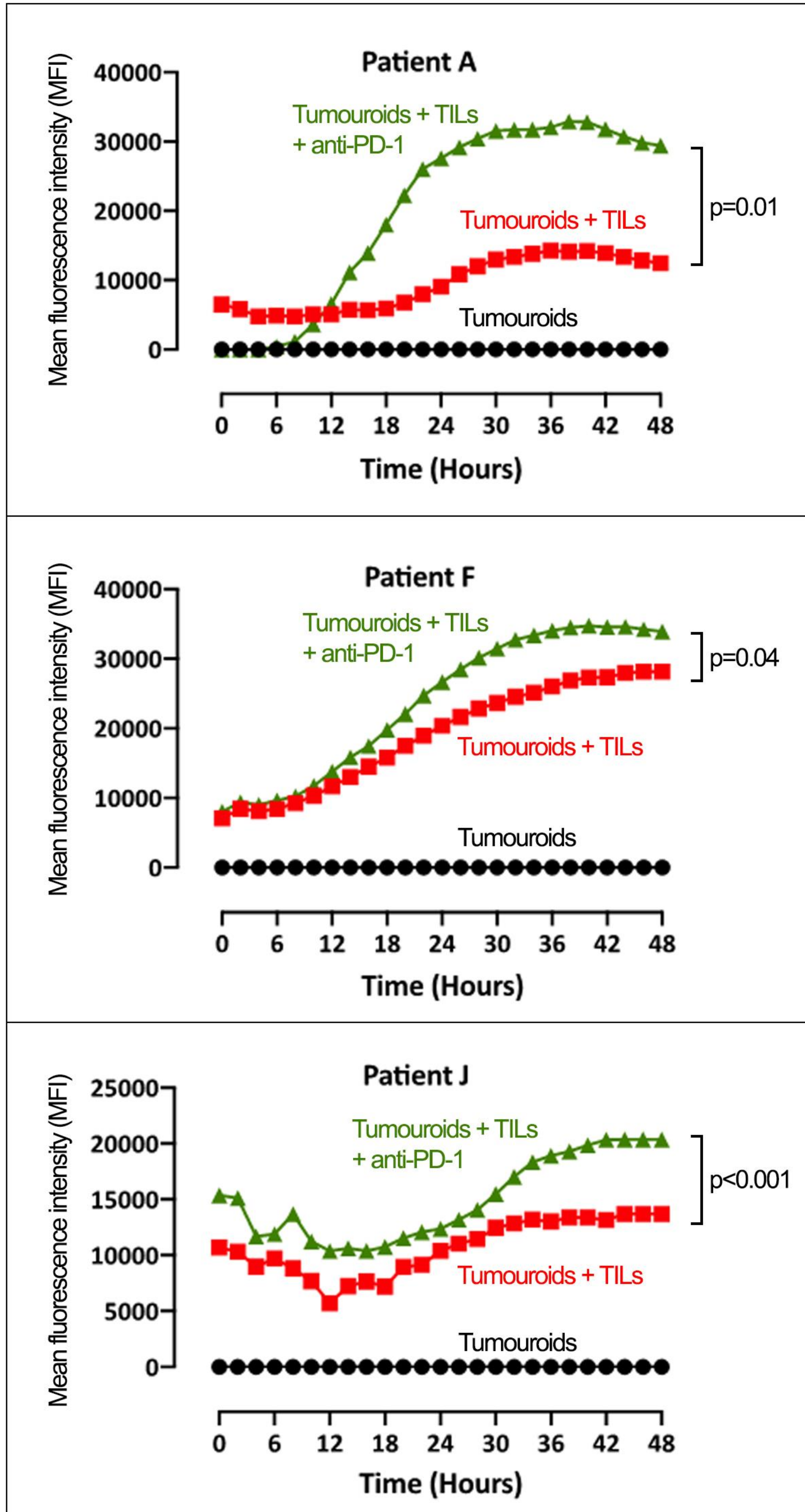


Figure 8.12. In the three samples shown above, there was significantly higher cytotoxicity with the addition of anti-PD-1 antibody compared to TILs alone. (Mann Whitney U test to evaluate significance)

In the remaining seven samples however, the addition of anti-PD-1 antibody did not have any effect on improving cytotoxicity (Figure 8.13).

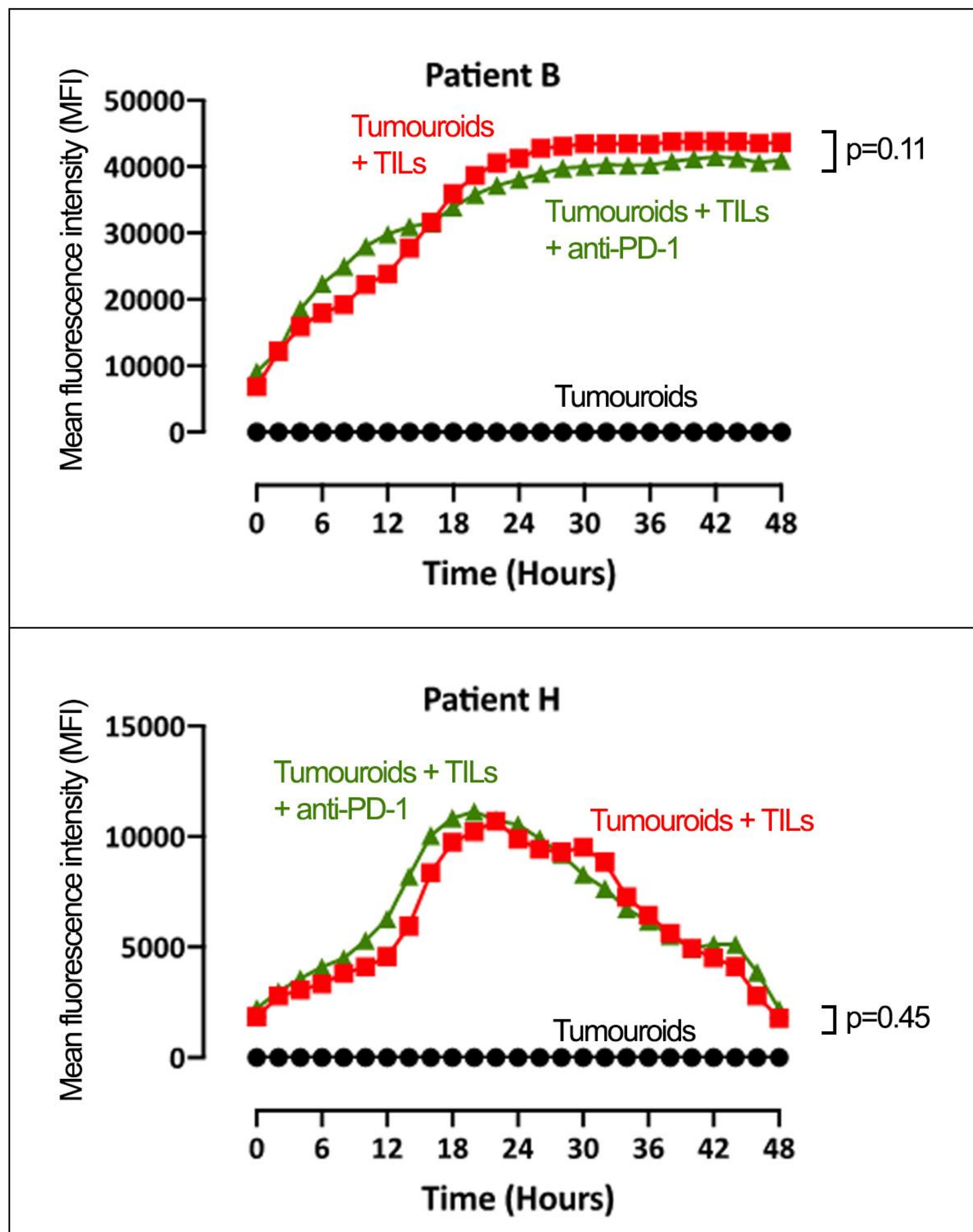


Figure 8.13. Example of two samples where addition of anti-PD-1 antibody had no effect on TIL cytotoxicity. In Patient H, the maximal MFI peaked at only 10,700 over the 48 hours, which was the lowest among all samples, suggesting that TILs from this sample may have been dysfunctional.

When evaluating the relationship between PD-1+ status on CD8+ T cells and increased cytotoxicity with the addition of anti-PD-1 antibody, Pearson correlation demonstrated that a higher PD-1 status on enriched CD8+ T cells contributed to greater cytotoxicity with the addition of anti-PD-1 antibody ($r=0.8385$, $p=0.0024$).

Evaluation of cytokines from the assays confirmed IFN-γ and granzyme B release with the addition of TILs and anti-PD-1 antibody, again confirming functionality of TILs through cytokine release. Furthermore, TNF-α, FasL and IL-6 release was also detected in the co-culture assays (Figure 8.14).

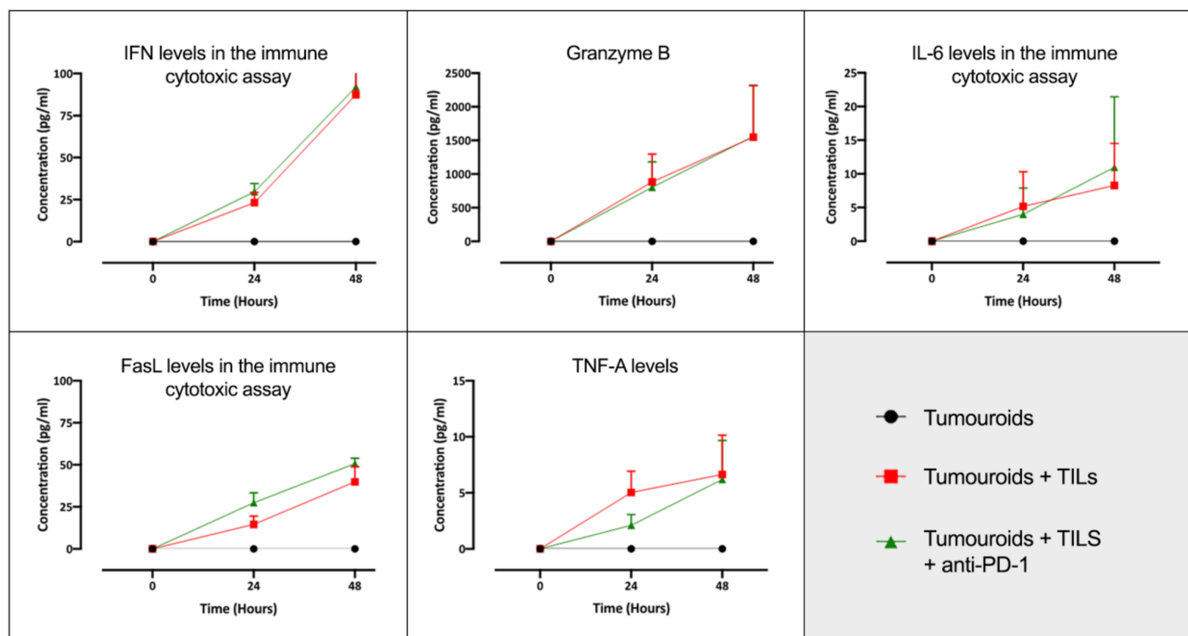


Figure 8.14. Collated summary of various cytokines such as Interferon, Granzyme B, IL-6, FasL and TNF released during the co-culture assays. The release of Interferon, Granzyme B and TNF by the TILs provide further evidence to show that the TILs are functional.

8.3.2.4 Summary of Immune cytotoxic assay

The immune cytotoxic assay firstly demonstrated that tumouroids retained the immune profile of the native tumour tissue. Secondly, the assay demonstrated that *in-vitro* cultured TILs were functional and could kill tumouroids, and retained specificity, as they did not kill normal colonic organoids. The addition of anti-PD-1 therapy significantly improving cytotoxic capability of TILs in selected cases, offering this assay

as a potential platform to personalise use of immunotherapy in patients with CRPM. However, the immune system *in situ* in CRPM is clearly unable to effectively fight the tumour, which contributes to the dismal prognosis associated with CRPM. A possible explanation for this discordance between a clinically poor outcome despite the detection of immune cells through FACS and functional TILs demonstrated in this assay could be the location of the T cells within the TUME. Therefore, OPAL analysis was undertaken to spatially evaluate T cells within the TUME.

8.3.3 Spatial distribution of TILs in the tumour microenvironment (OPAL)

On evaluating the density of T cells based on location, there was no significant difference in $CD4^+$ numbers between the tumour and stroma. However, $CD8^+$ T cells and Tregs were significantly more in the stroma compared to the tumour (Figure 8.15).

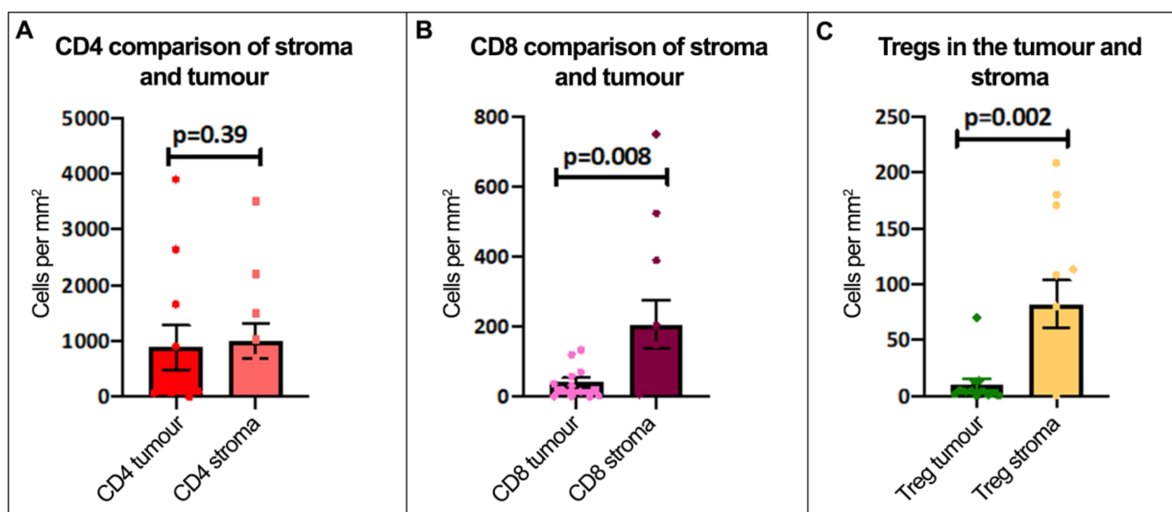


Figure 8.15A-C. Distribution of T cell subsets in the tumour and stroma (Mann Whitney U test for significance).

Opal analysis revealed a spectrum with regard to immune infiltrate, with some samples having a strong peri-tumoural infiltrate (Figure 8.16). Here, there was marked evidence of peri-tumoural and stromal PD-L1 expression, demonstrating possible immunosuppressive mechanisms in the TUME to suppress $CD8^+$ T cell function.

Checkpoint blockade in the form of anti-PD-1/anti-PD-L1 therapy may have a role to help re-activate the T cells suppressed by the PD-1/PD-L1 axis.

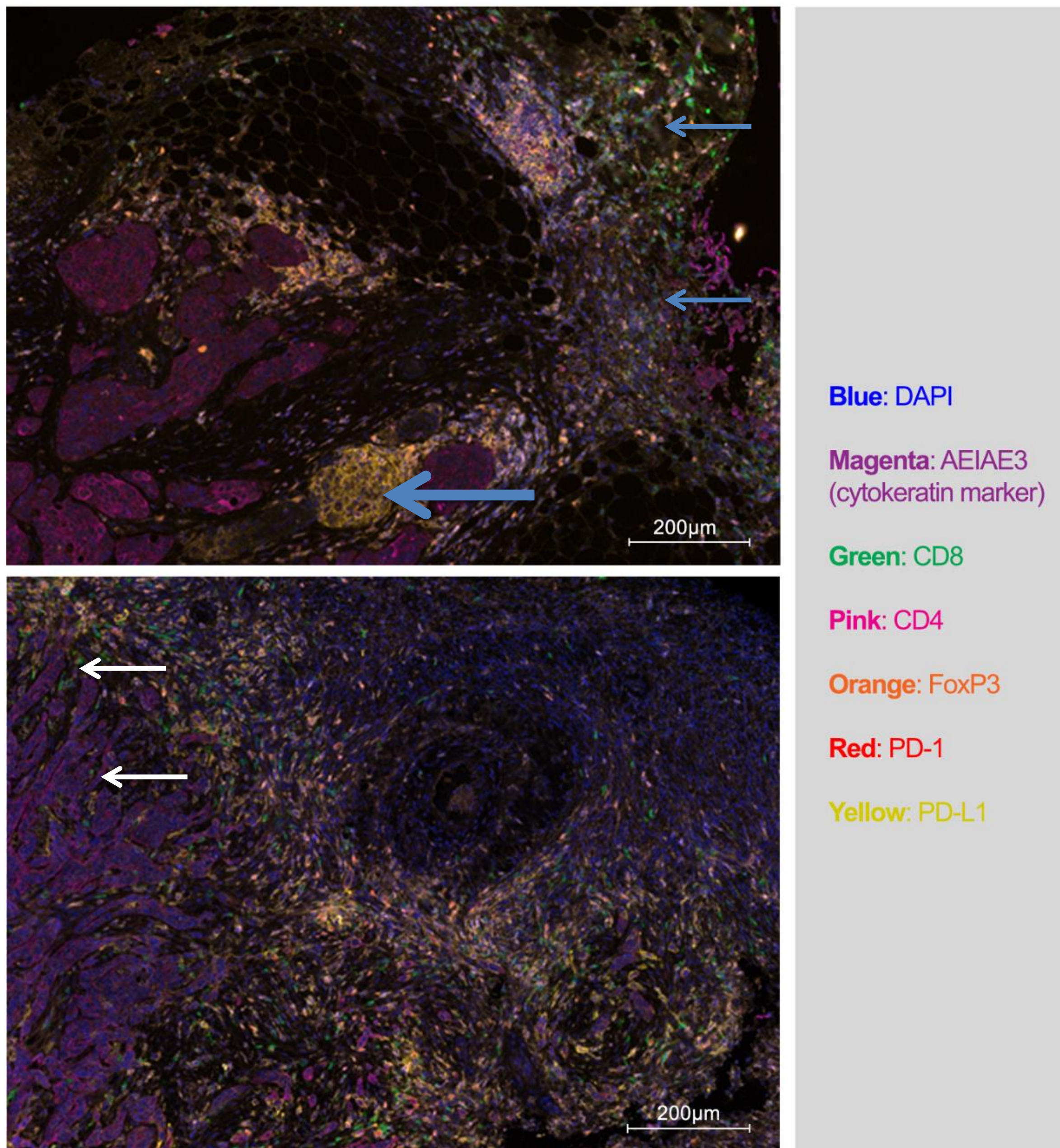


Figure 8.16. Two samples with a peri-tumoural infiltrate, in particular a significant stromal infiltrate. In the top sample, there is a strong stromal infiltrate with evidence of CD8⁺ and other immune cells, with the areas demonstrated by the two thin blue arrows. Additionally, there is evidence of peri-tumoural PD-L1 positivity, demonstrated by the thick blue arrow. Similarly, in the lower sample, there is a strong stromal infiltrate, with some scant intra-tumoural CD8⁺ cells present as well, demonstrated by the white arrows. There are a number of DAPI⁺ cells that do not co-stain with CD4⁺/CD8⁺. These may be other stromal cells such as macrophages, myeloid derived suppressor cells or cancer-associated fibroblasts.

Similarly, others had an immune excluded appearance (Figure 8.17), where there was clearly minimal immune activation within the TUME.

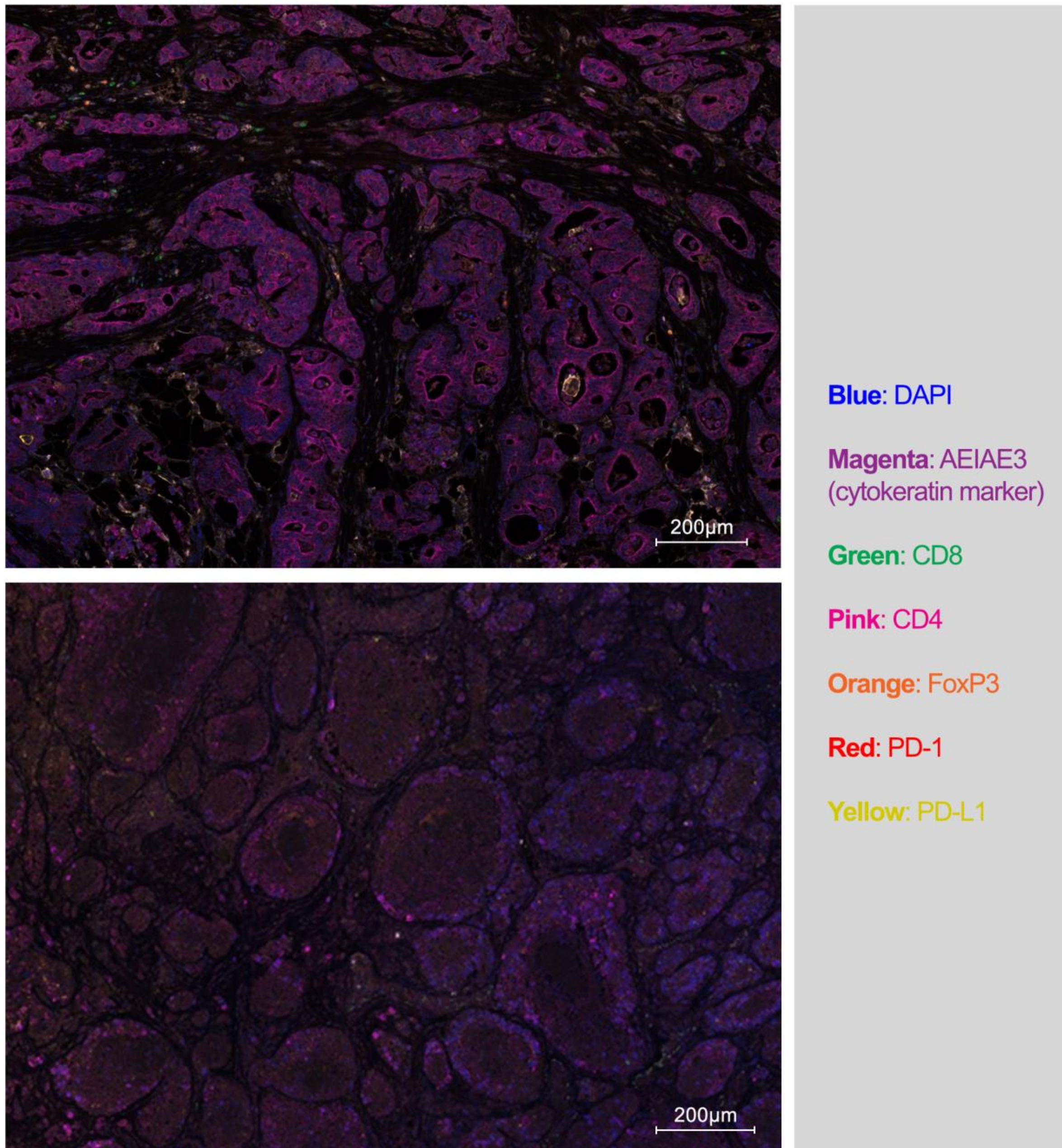


Figure 8.17. Two samples that demonstrate an 'immune desert'. Both these samples demonstrate almost no immune cell infiltrate within the TUME, commonly referred to as 'cold' tumours.

Figure 8.18 presents another example with a significant stromal immune infiltrate.

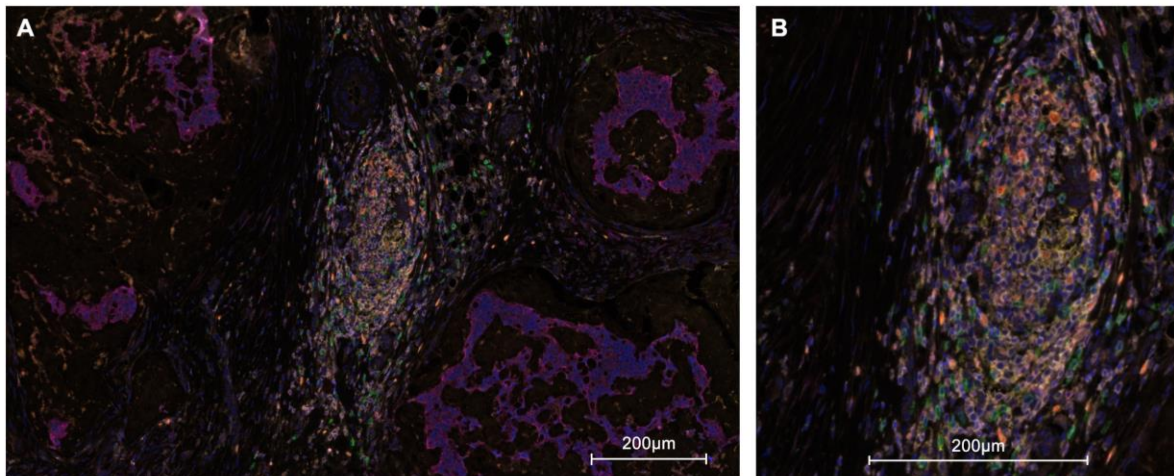


Figure 8.18. There is significant stromal immune cell infiltration evident in the image on the left. On the right, a magnified view of the immune infiltrate demonstrating evidence of PD-1, PD-L1 as well as $CD4^+$, $CD8^+$ T cells. There are also other $DAPI^+$ cells that are $PD\text{-}L1^+$ positive, which may represent immunosuppressive stromal cells such as macrophages, myeloid derived suppressor cells or cancer-associated fibroblasts.

8.3.3.1 Correlation between FACS and OPAL findings

As FACS and Opal are different methods to evaluate cellular infiltrate, a comparative analysis between FACS and Opal was performed using the CD8/CD4 and CD8/Treg ratio. The findings were concordant (Figure 8.19).

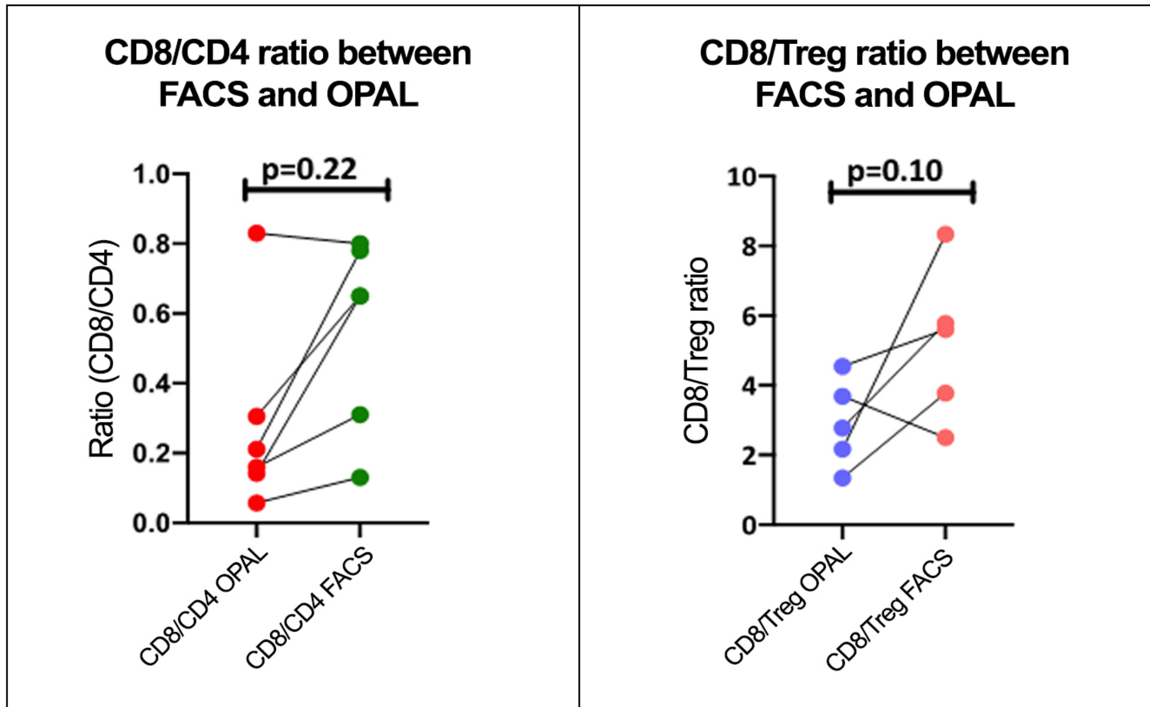


Figure 8.19. Comparative analysis between FACS and Opal (tumour and stromal components combined) of all CRPM samples reveal largely similar findings using FACS and Opal (Mann Whitney U test for significance). Black lines represent the corresponding ratios for the same sample on FACS and OPAL.

8.3.3.2 Comparison of normal peritoneum to CRPM

When comparing normal peritoneum and matched CRPM, findings were largely concordant with those found with FACS, with numbers of $CD4^+$ and $CD8^+$ T cells being significantly higher in CRPM compared to normal peritoneum (Figure 8.20).

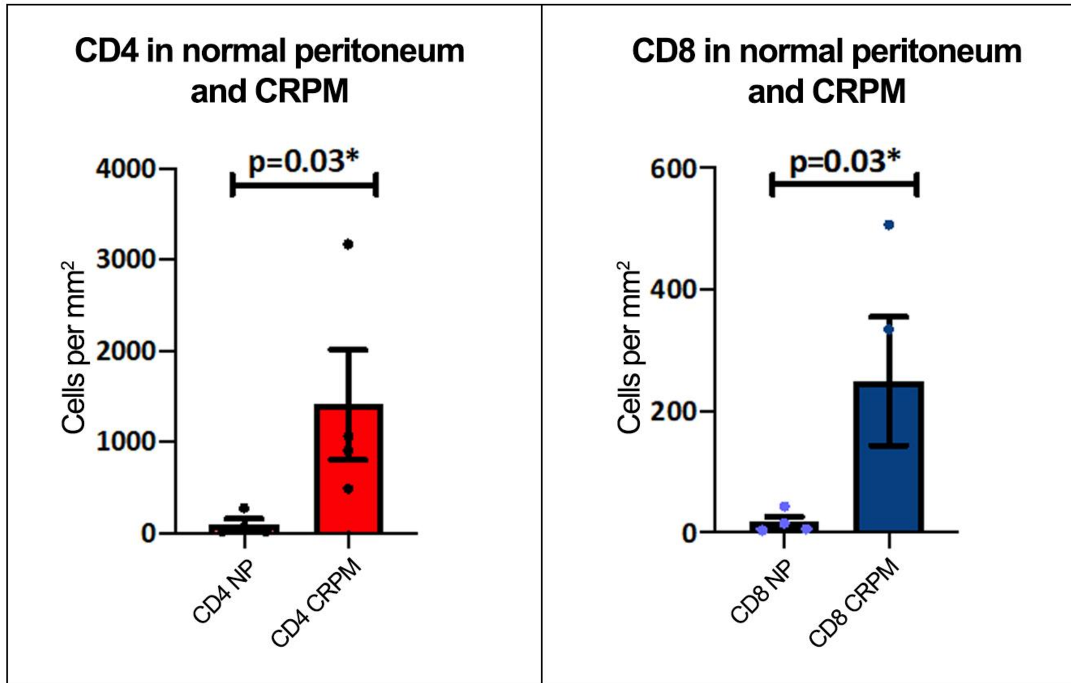


Figure 8.20. Comparing T cell subsets between normal peritoneum and colorectal peritoneal metastases (Mann Whitney U test for significance)

NP: normal peritoneum; CRPM: colorectal peritoneal metastases

8.3.3.3 Comparison of primary and matched synchronous CRPM

There were three matched primary and synchronous CRPM samples. In these, there was a non-significant trend towards higher $CD4^+$, $CD8^+$ and Treg in CRPM compared to the primary (Figure 8.21). A larger cohort is required to further evaluate the difference in T cell infiltrate between synchronous sites.

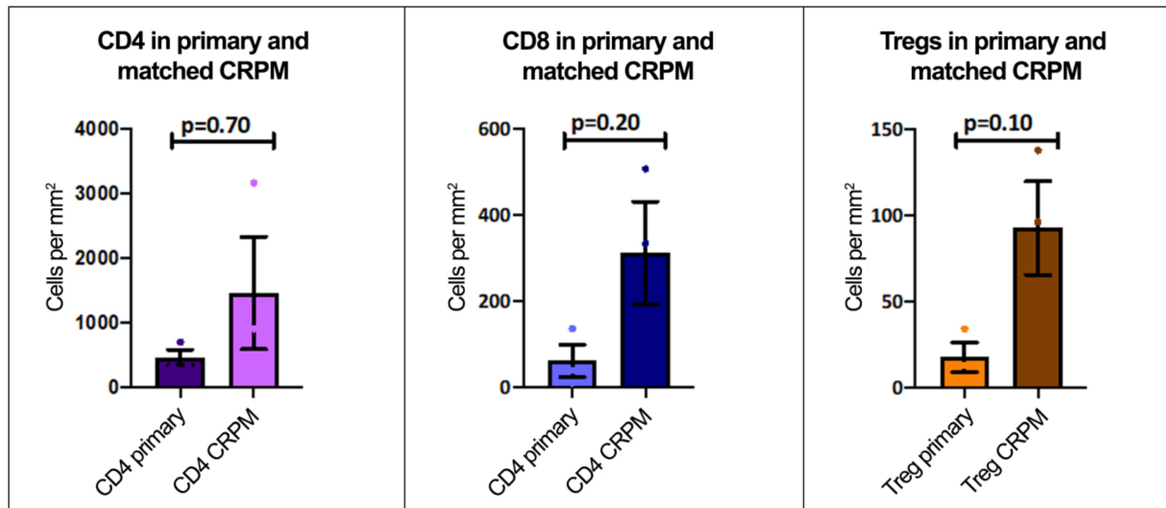


Figure 8.21. Comparison of T cell subsets between primary and synchronous CRPM (Mann Whitney U test for significance).

8.3.3.4 Distance between cells

Opal analysis revealed that on average, $CD8^+$ T cells were 194.14 ± 37.91μm from a tumour cell (Figure 8.22A). Additionally, a moderately strong correlation was seen between the distance from a $CD8^+$ T cell to a tumour cell and the distance from a Treg to a $CD8^+$ T cell, suggesting that as $CD8^+$ T cells traffic closer to a tumour cell, Tregs are more likely to appear in close proximity to the $CD8^+$ T cells (Pearson correlation R=0.71, p=0.0067) (Figure 8.22B). Further research is required to explore this finding to evaluate whether this is merely an incidental findings or whether the proximity of Tregs contributes to an immunosuppressive effect on the $CD8^+$ T cells.

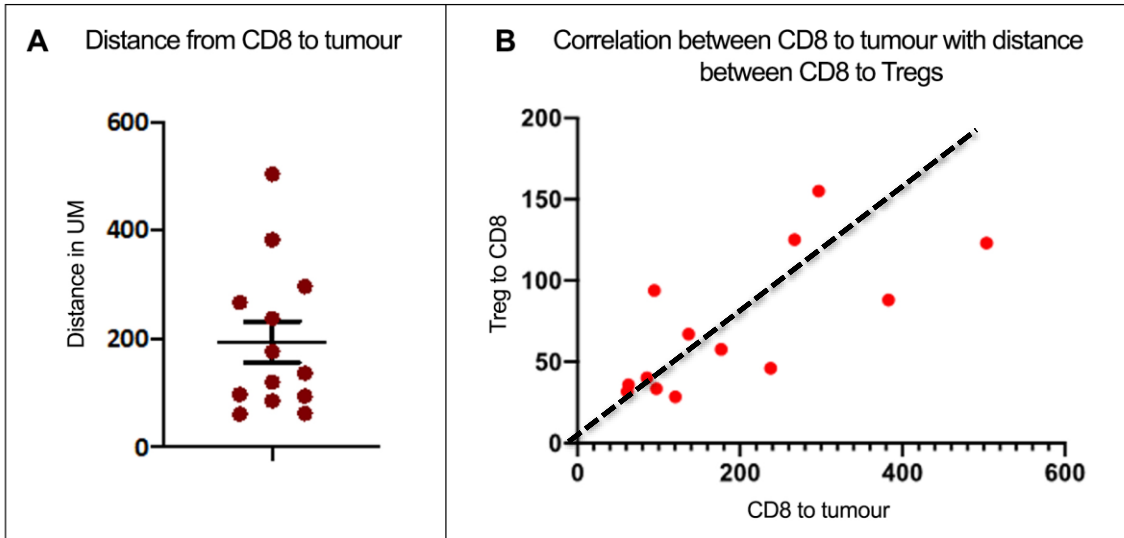


Figure 8.22A. Distance from CD8+ T cells to tumour cells. Fig 8.22B. Correlation between CD8⁺ T cell-tumour in association with CD8⁺ T cell-Treg, R=0.71, p=0.0067.

8.3.3.5 Summary of OPAL analysis

Opal analysis confirmed a spectrum of T cell infiltrate within the TUME of CRPM, with some having a T cell infiltrate, and others being immune deserts. However, in cases that had a strong T cell infiltrate, most of the T cells were largely stromal, with minimal intra-tumoural T cell infiltrate. Additionally, the presence of stromal PD-L1$^+$ cells suggests that the PD-1/PD-L1 axis may be one immunosuppressive mechanism at play in suppressing T cell function in the TUME. Another dimension to explore the immune landscape was to evaluate the gene expression profiles seen within the samples using RNASeq and CMS calling. Given the strong stomal infiltrate seen in many samples, we hypothesised that CRPM may be CMS 4, in keeping with stromal infiltration, EMT, inflammation and immunosuppressive pathways such as TGF-β.

8.3.4 RNASeq and differential gene expression

Principal component analysis (PCA) following RNASeq revealed a clear distinction in overall gene expression between tumour tissue (CRPM and matched primary tumour tissue RNA), normal peritoneum tissue RNA and CRPM tumouroid RNA (Figure 8.23).

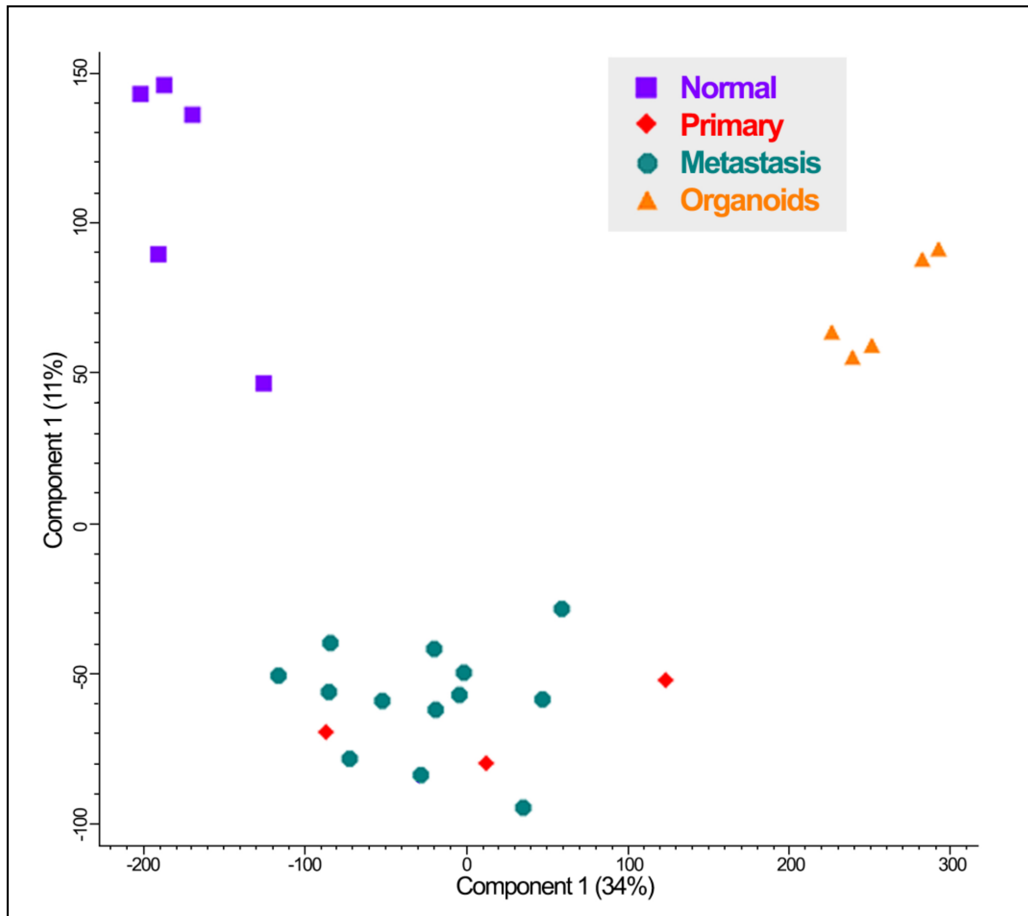


Figure 8.23. Principal component analysis demonstrating clear distinction in overall gene expression between normal peritoneum, tumour tissue (primary and colorectal peritoneal metastases) and tumouroids.

Normal: normal peritoneum; primary: primary tumour; metastasis: CRPM, organoids: CRPM tumouroids

8.3.4.1 Consensus Molecular Subtyping (CMS)

RNASeq was performed on 17 tumour samples, which included 14 CRPM and three matched synchronous primary cancers. CMS calling revealed that 10 out of 14 CRPM (71.4%) were CMS 4, with two being CMS 2, one being CMS 3 and one indeterminate (Figure 8.24A). Of the three matched pairs of primary tumours and peritoneal metastases, two of the primary tumours were CMS 2 and CMS 3, whereas their matched CRPM was CMS 4, suggesting that as the tumours metastasised, the stromal signature became predominant. One primary tumour with its matched CRPM were both CMS 4. The key pathways differentiating CMS 2, 3 and 4 are summarised in Figure 8.24B, with activation of the TGF-β and EMT genes hallmark features in the CMS 4 samples, an

epithelial differentiation signature in the CMS 3 and Wnt signature in CMS 2, consistent with expression signatures previously described for each subtype.[33] As expected, there were no CMS 1 samples, as only MSS cancers were recruited in the study.

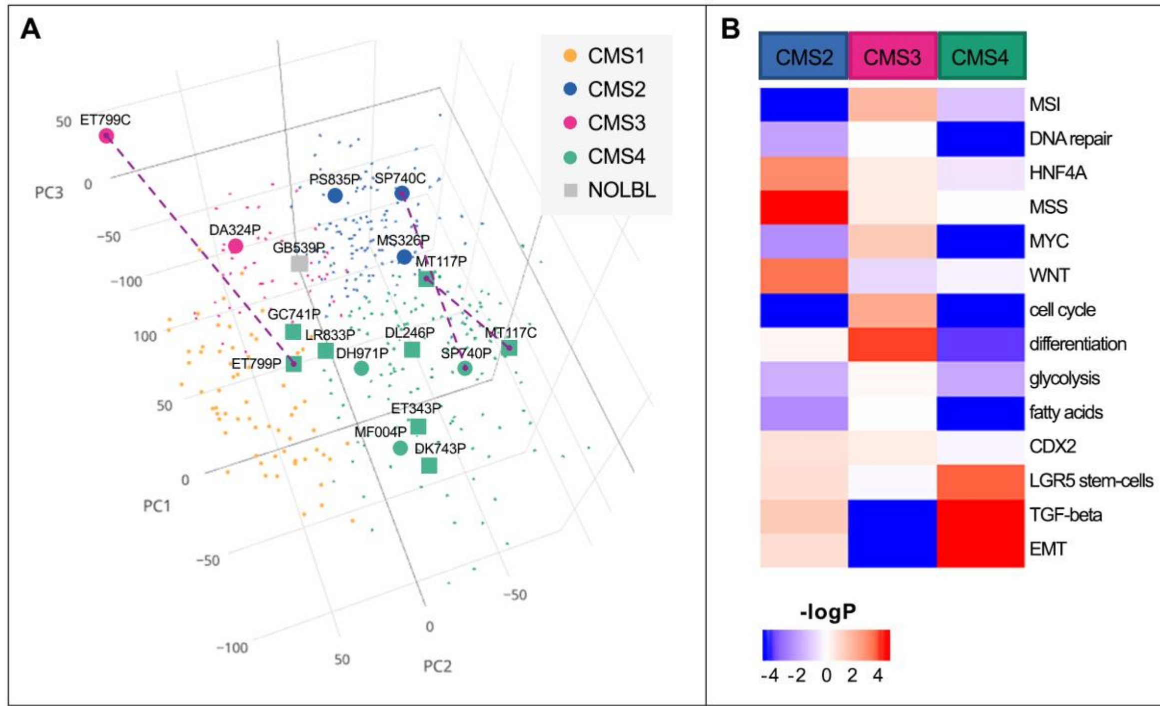


Figure 8.24A. CMS classes. Each large square or circle represents a sample, with the colour based on the CMS type. The small dots in the background are a larger cohort of RNASeq samples extracted from the publically available TCGA database to enable greater accuracy in CMS calling. Dotted lines represent primary tumours with their matched CRPM. Fig 8.24B. Key pathways differentiating CMS classes. CMS 4 for example has up regulation of EMT and TGF-β pathways compared to CMS 2 and CMS 3. There were no CMS 1 in our cohort, as all our samples were microsatellite stable.

NOLBL: indeterminate class

CMS classes were also compared between ten tumouroids and matched CRPM tissue. There was no CMS 4 among the tumouroids, with the majority of the matched CRPM tissue exhibiting a CMS 4 class. This was likely because tumouroids lack stromal elements, which is the predominant feature of CMS 4 (Table 8.1).

Table 8.1. Comparison of CMS classes between tumouroids and matched tumour tissue.

Sample	**Tumouroid CMS**	**CRPM tissue CMS**
DH971	3	4
DK743	2	4
DL246	2	4
ET343	2	4
GB539	Indeterminate	Indeterminate
GC741	3	4
MF004	1	4
MS326	2	2
MT117P	2	4
PS835	2	2

8.3.4.2 Concordance between FACS and Cibersort

RNASeq signatures for immune cell types were extracted using a newer publicly available program called 'Cibersort'.[306] Overall proportions of NK cells, T cells and myeloid cells were similar in CRPM samples between FACS and Cibersort, providing further validation of our findings by using different techniques (Figure 8.25).

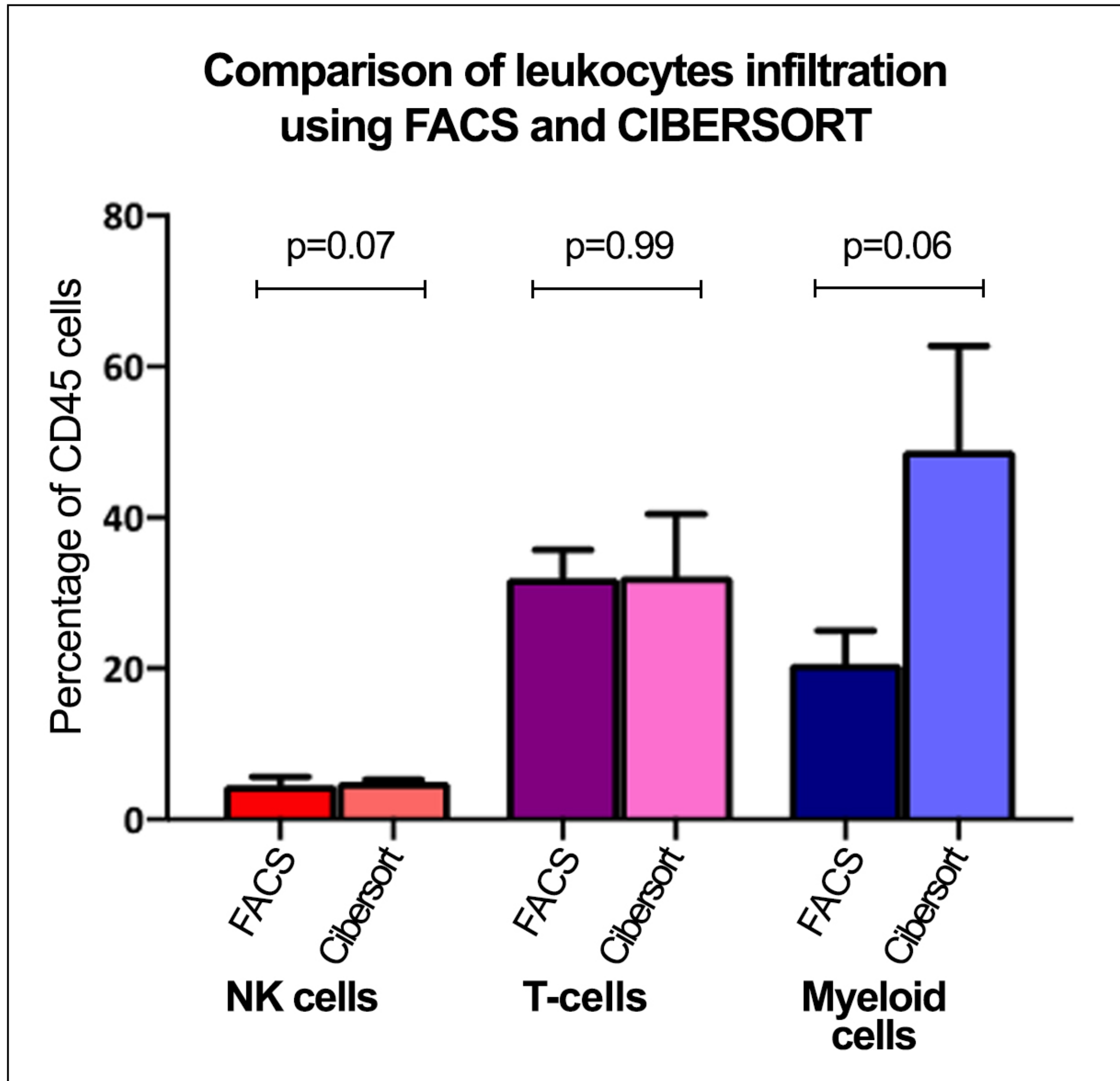


Figure 8.25. Comparison between leucocyte populations between FACS and Cibersort show no significant difference in proportions of NK cells, T cells or myeloid cells. Whilst not significant, myeloid appear more abundant based on Cibersort compared to FACS. This difference may be due to the expression markers used to capture myeloid lineage cells using the two techniques. With FACS, our panel defines myeloid lineage cells as $CD45^{+}CD3^{-}CD56^{-}Cd33^{+}CD11b^{+}$. With Cibersort, macrophages, monocytes and dendritic cells may have been incorporated under the myeloid cell lineage label. The difference in expression markers used to identify myeloid lineage cells may account for the increased myeloid cells seen with Cibersort.

Non-parametric data: Mann Whitney U test for significance

8.3.4.3 Pathway differences between primary tumours and CRPM

Differential gene expression was evaluated between primary tumours (n=3) and CRPM (n=14). Gene set enrichment analysis (GSEA) revealed numerous pathways such as

TNF-α signalling, epithelial mesenchymal transformation (EMT), IFN-γ response, angiogenesis, TGF- β, inflammation and apoptosis pathways that were significantly up regulated in the CRPM compared to primary tumours, with a number of differentially expressed genes (DEGs) within each pathway. As three examples, Figure 8.26 demonstrates a heat map of DEGs in the TGF- β pathway, with figure 8.27 and 8.28 demonstrating DEGs in the EMT and IL-6/JAK-STAT pathway respectively. All three heat maps demonstrate that most genes in these pathways are up regulated in CRPM.

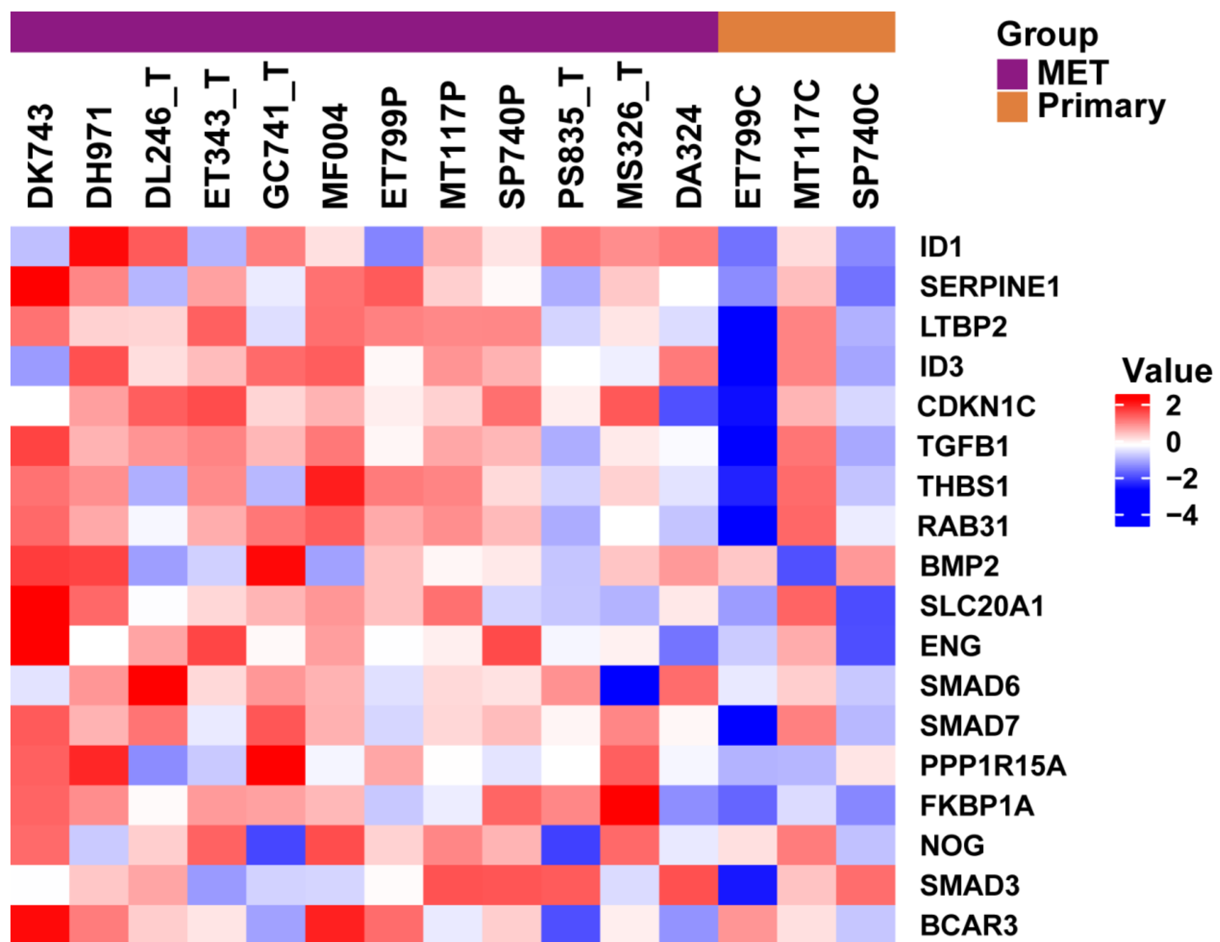


Figure 8.26. Differential gene expression between CRPM and primary tumours in the TGF- β pathway.

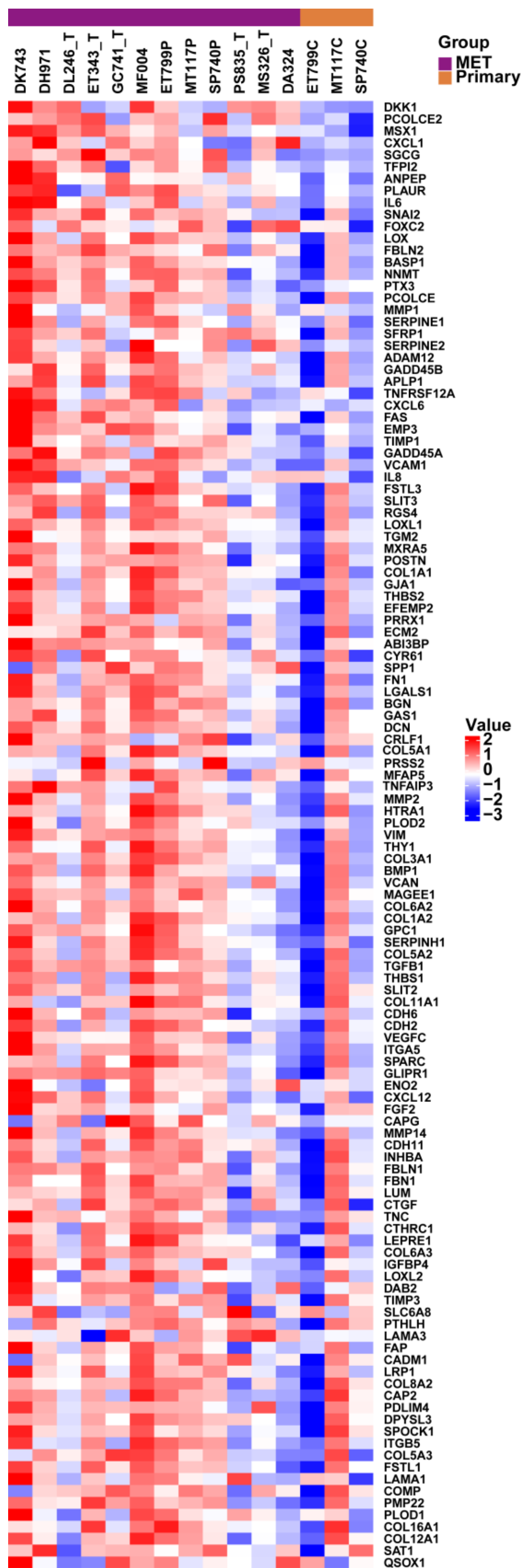


Figure 8.27. Heat map demonstrating differentially expressed genes in the EMT pathway. This demonstrates up regulation of many genes in the CRPM that shift expression towards a mesenchymal phenotype.

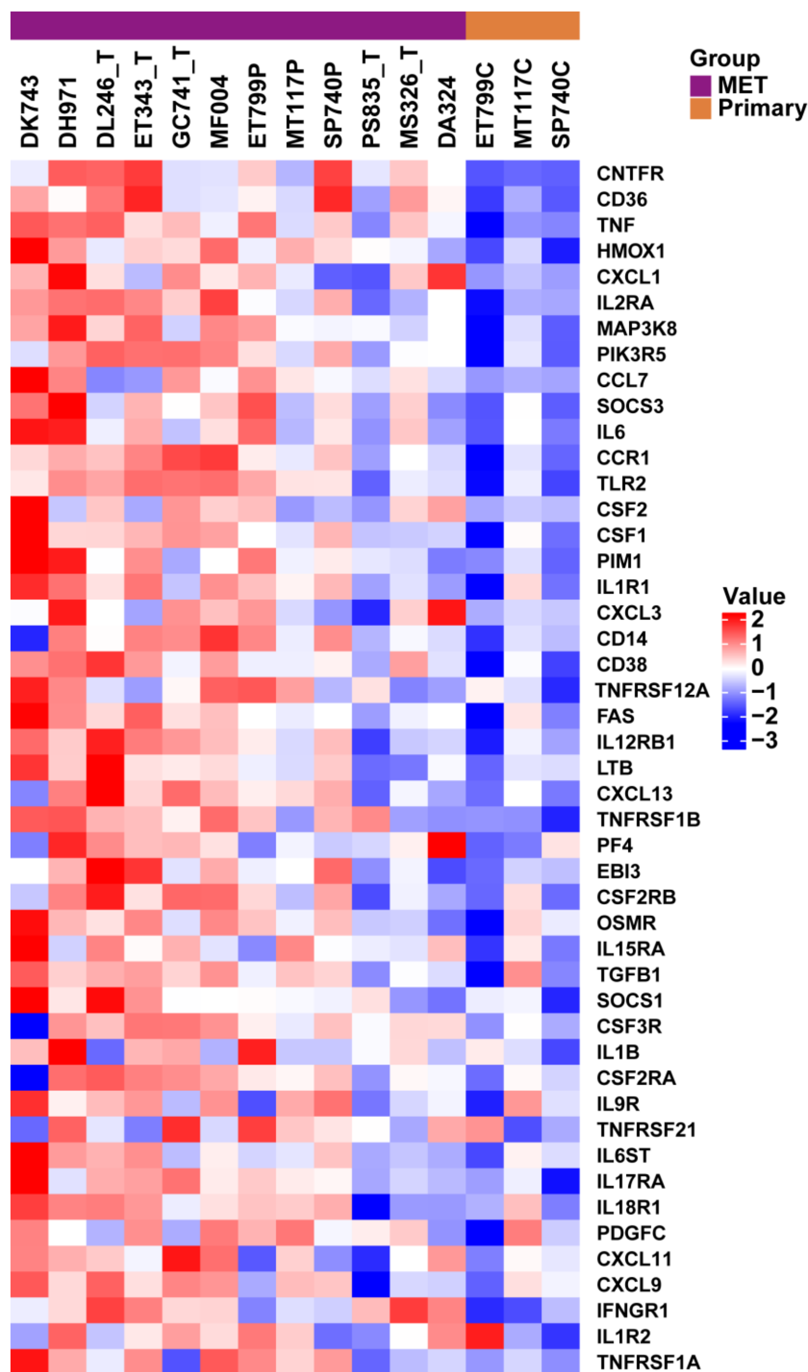


Figure 8.28. Comparison of differentially expressed genes in the IL-6/JAK-STAT pathway also showing up regulation among CRPM compared to primary tumours

8.3.4.3.1 Immune differences

To specifically profile the immune landscape, a curated immune gene panel[413] was used to evaluate differences between CRPM and primary tumours (Figure 8.29).

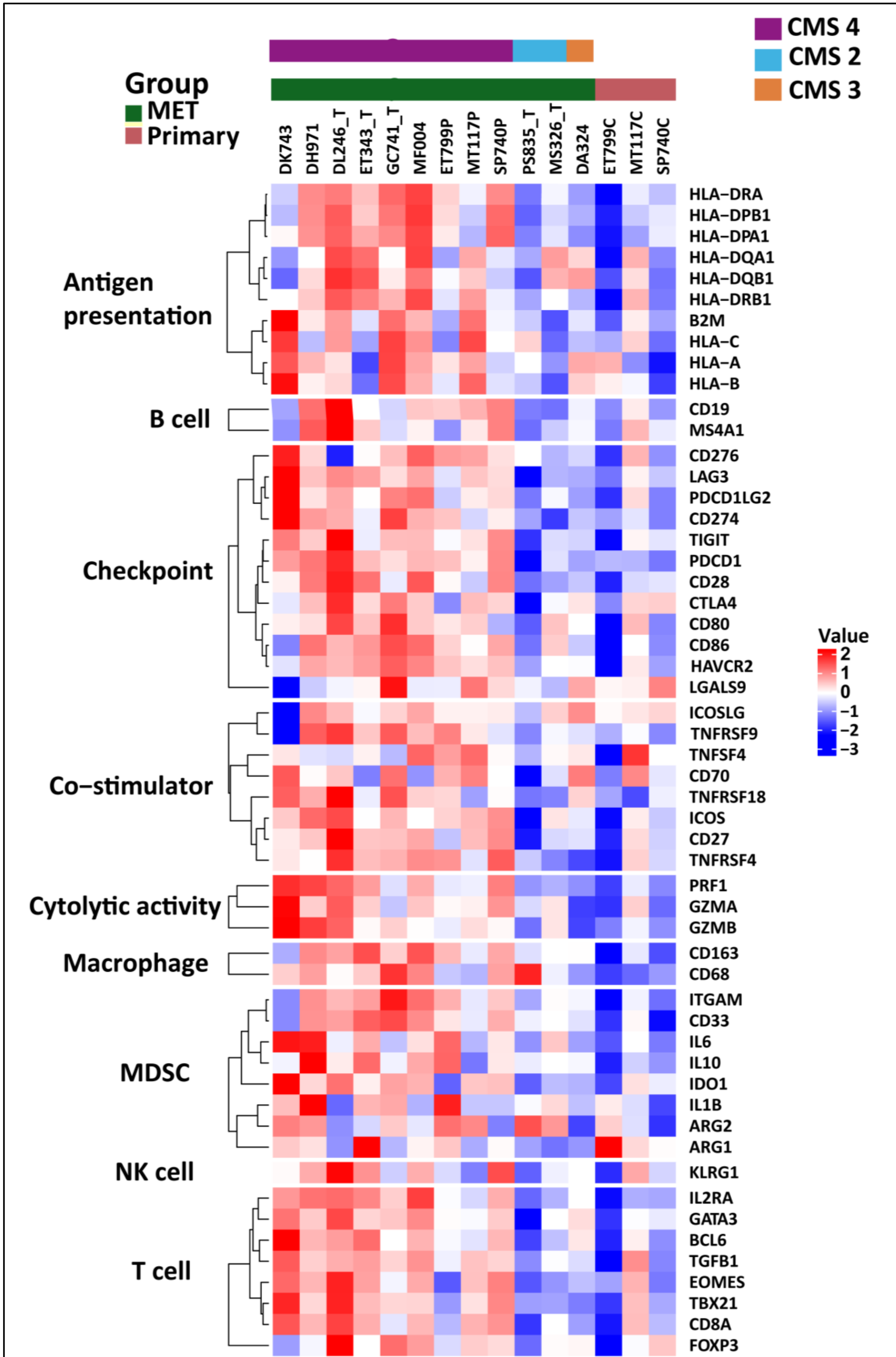


Figure 8.29. Comparative analysis of the immune profile of CRPM with primary tumours

As seen in Figure 8.29, there appears to be a difference in the immune profile among the cohort of samples evaluated. This analysis once again demonstrates that there is up regulation of T cell gene expression, along with cytolytic activity, features that are in keeping with T cell functionality. In addition, checkpoint blockade markers such as CTLA-4, CD274 (PD-1) are up regulated in many of the CRPM. Macrophages, MDSCs and B cells are similarly up regulated in many of the CRPM. While this sample size is small, it does demonstrate that there possibly is an immune difference between the primary tumours and CRPM.

An alternative approach to evaluating differences in immune signatures among samples was to group them based on CMS classes as seen in Figure 8.30. Here, a more visible difference in immune cell types, cytolytic activity and checkpoint blockade molecules being up regulated in CMS 4 compared to CMS 2 and CMS 3 is seen. Additionally, in Figure 8.12, of the three samples showing a significantly improved TIL cytotoxicity with anti –PD-1, two samples were CMS 4 (Patient J did not have adequate tissue for RNASeq to be performed). It must be emphasised that these findings are largely hypothesis generating and cannot be used to draw strong conclusions, especially as the sample size is small (total of n= 16 with defined CMS types, one sample indeterminate CMS). These findings however do suggest that CMS classes are perhaps more important than location of the tumour in determining the immune profile of the tumour, highlighting that the molecular expression of the tumours (CMS classes) may have a growing role to influence a personalised model of care in the future.

8.3.4.4 Summary of RNASeq and differential gene expression

The key finding from this section was that most CRPM were CMS 4, offering a molecular rationale for why they may confer a poor prognosis. In keeping with CMS 4, many of the CRPM had up regulation of immunosuppressive pathways such as the TGF- β pathway. Going forward, CMS subtypes may be recognised as a more important feature, apart from merely location or mutational status, and may offer a molecular basis for a personalised approach to therapy.

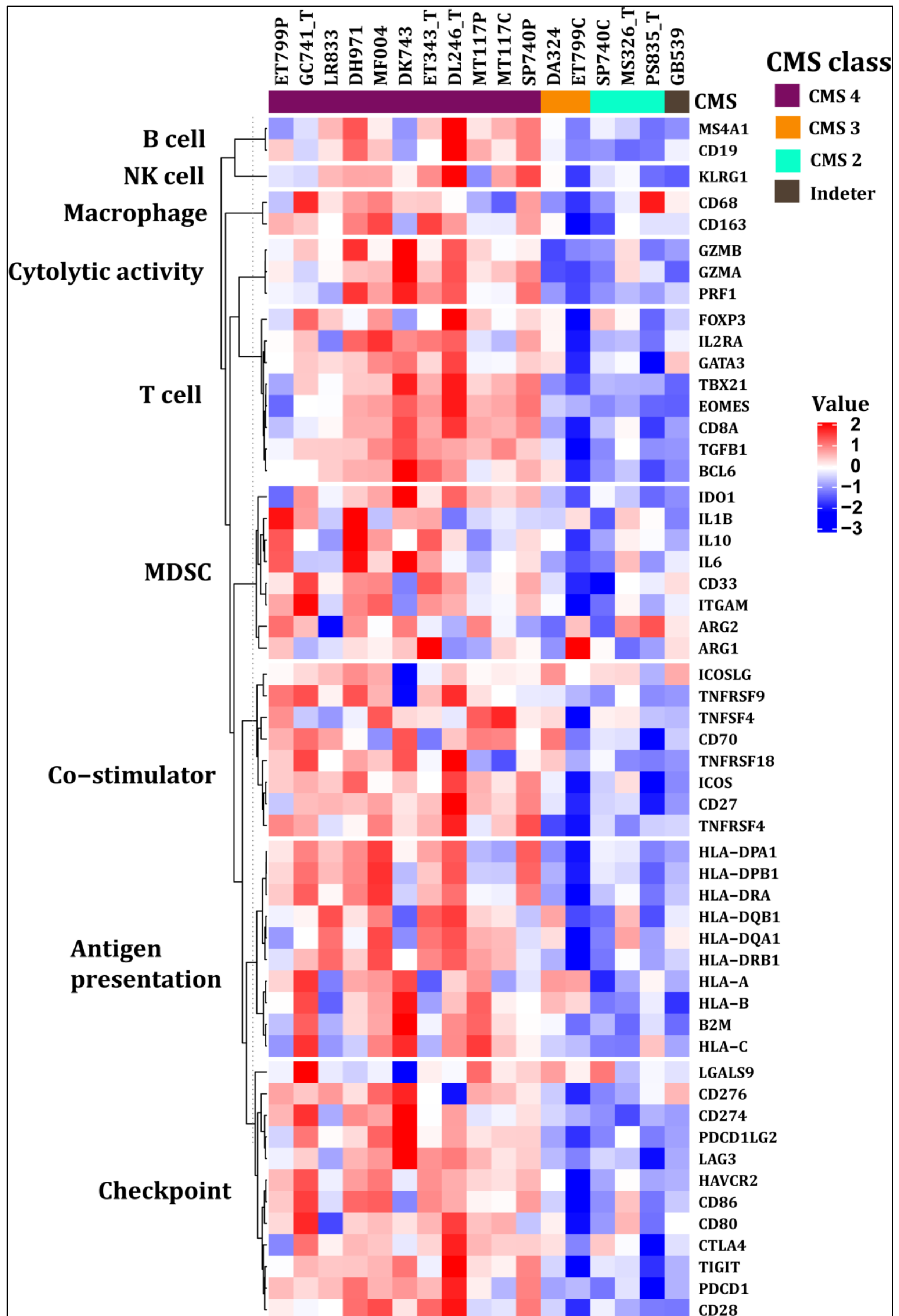


Figure 8.30. Comparative analysis of all 17 samples (primary and peritoneal) based on CMS types

8.4 Discussion

To our knowledge, this is the first study to have explored the immune landscape of CRPM utilising multiple static and functional modalities. Firstly, we demonstrated that CRPM do indeed have an immune infiltrate, with a T cell population that is significantly higher than adjacent normal peritoneum. There was good concordance in the proportions of immune cell infiltrates found using FACS, Opal and Cibersort, providing reassurance that there is indeed a notable immune, specifically T cell infiltrate in CRPM. Additionally, almost a fifth of T cells express PD-1, suggesting a possible role for anti-PD-1 therapy.

Using a novel tumouroid-autologous TIL co-culture system, we firstly demonstrated that TILs from CRPM can be successfully cultured *in-vitro* and are functional in killing tumour cells, with cytotoxicity remaining tumour specific and $CD8^+$ T cell mediated. RNASeq data also demonstrated upregulation of cytolytic activity in the form of granzyme A and B, in keeping with functional T cell activity. Additionally, checkpoint blockade molecules such as CD274 (PD-1) were up regulated in many of the CRPM. While PD-1 expression was previously considered a marker of T cell exhaustion, Clarke et al recently demonstrated that many T cells expressing PD-1, especially tissue resident memory cells expressing PD-1 were enriched for superior functionality.[414] Despite being MSS, anti-PD-1 therapy led to significantly improved cytotoxicity of TILs in selected cases, demonstrating a possible role for such an assay to be used clinically to evaluate the efficacy of anti-PD-1 therapy in a personalised fashion.

While a T cell infiltrate was present and was shown to be functional, T cells are unable to eradicate the tumour *in-vivo*. Therefore, Opal analysis was utilised to explore the spatial distribution of cells in the TUME and demonstrated that in most cases, the immune infiltrate was stromal in nature, with many cases being an immune excluded phenotype, with minimal intra-tumoural T cell infiltrate. This suggests that there may be various immunosuppressive mechanisms such as PD-L1 expression on MDSCs, CAFs, or macrophages within the stromal environment preventing T cells from trafficking to the tumour cells. Additionally, RNASeq revealed most CRPM to be CMS 4, consistent

with a strong stromal infiltration, with a highly immunosuppressive TUME associated with an inflamed phenotype and TGF- β activation. This was further confirmed on RNASeq GSEA, which revealed pathways such as the EMT, inflammation and TGF- β to be up regulated in many CRPM.

While the immune landscape of primary CRC has been explored extensively, the role of the immune system and TUME in CRPM remains poorly defined. There are few studies that have explored the role of the immune system in CRPM. In evaluating ascites in patients with CRC, Heo et al[415] found that high levels of IL-10, IL-6, VEGF, CCL20, TGF-β correlated with intraperitoneal CRC growth. In the study, CCL 2, associated with recruitment of monocytes and dendritic cells and CCL 20, associated with recruitment of Tregs that promote chemoresistance were up regulated in CRPM compared to primary tumours.[416] Additionally, the IL-6 and angiogenesis pathways were up regulated in CRPM. Seebauer et al[417] examined the difference in proliferation, senescence and neovascularization between CRPM and the primary tumour based on IHC and found that CRPM surprisingly had lower proliferation rates than the primary tumour with lower Ki-67 and cyclin-D1 expression, along with increased senescence marked by tri-methyl-histone H3, $p21^{Cip1}$ and CDKN2A. They postulated that the lower proliferation rates could partly explain the poor response of CRPM to chemotherapy, which is reliant on cells being highly proliferative. They also reported CRPM to have higher levels of TGF-β, VEGF-A, IFN-γ and TNF-α, in keeping with increased neo-angiogenesis. We similarly found that the TGF-β, IFN-γ response and TNF-α pathways were up regulated in many CRPM. Additionally, a number of IFN-γ mediated chemokines such as CXCL 9 and CXCL11 associated with the presence of $CD8^+$ and Th_1 $CD4^+$ T cells were up regulated in CRPM in our cohort.[418]

While immunotherapy has a defined role in MSI-H CRC, its role in MSS CRC has not been established. Additionally, there have been no studies involving checkpoint blockade in patients with CRPM.[419] Currently, apart from MSI-H status, there are no other promising biomarkers that predict an increased likelihood of response to checkpoint blockade in CRC. Other promising areas such as the T cell repertoire (TCR),[420] tumour mutational burden,[267] and emerging immune markers such as indoleamine 2,3-dioxygenase (IDO),[420] are still under investigation. More recently, the development of models to

evaluate T cell dysfunction revealed *SERPINB9*, a regulatory gene that inactivates granzyme B, was found to be highly expressed in patients who do not respond to immunotherapy.[421] However, *SERPINB9* was not found to be highly expressed in our cohort. Additionally, the immune assay described in this study is an opportunity to functionally evaluate the T cell responses *in-vitro* and personalise the use of checkpoint blockade to patients with CRPM who may benefit from its use. The assay can be performed within six to eight weeks of receiving suitable tumour tissue, making it a highly clinically relevant and applicable utility. The expression of PD-1 on approximately a fifth of T cells suggests an exhausted T cell phenotype.[422, 423] An exhausted phenotype along with stromal PD-L1 staining on Opal suggests that anti-PD-1/PD-L1 therapy may be a plausible treatment option to re-activate cytotoxic T cells, and has been shown in studies to enhance anti-tumour immunity.[422] The use of this assay can also extend beyond merely checkpoint blockade, and can be utilised in other avenues utilising immunomodulating therapies. Recently, this assay was successfully utilised to evaluate the role of inhibitors of apoptotic proteins using CAR-T cells and CRPM tumouroids.[424]

One of the key drivers believed to contribute to the immunosuppressive TUME is TGF-β. The TGF-β pathway is an important pathway in regulating cellular homeostasis. In the gastrointestinal tract, TGF-β works as a promoter of fibrosis, immune modulator, and plays a critical role in cell growth, differentiation and apoptosis. TGF-β functions as a tumour suppressor and paradoxically also as a tumour promoter.[425] Three TGF-β isoforms are expressed in mammals (TGF-β1, TGF-β2 and TGF-β3), with TGF-β1 the most abundant and well studied.[426] Apart from the very early stages of tumourogenesis where TGF-β acts as a tumour suppressor, it is one of the key drivers in promoting tumour growth and the development of an immunosuppressive TUME.[427] It is a key inducer of EMT, immune evasion and metastatic tumour growth during cancer progression. In addition to its direct effect on epithelial tumour cells, TGF-β also controls tumour development by regulating growth factor production by stromal components. It is known to promote tumour immune escape and resistance to immunotherapy.[421] Apart from cancer cells themselves, TGF-β is also secreted by M_2 macrophages, MDSCs, CAFs and Tregs,[425] and has been shown to exist at high levels in blood samples of patients with advanced stages of cancer.[428] Furthermore, TGF-β

suppresses immune cells by inhibiting $CD8^+$ and $CD4^+$ T cells, along with preventing IL-2 production,[429] while stimulating immunosuppressive Tregs and Th_{17} cells.[255] The TGF-β pathway, with many of its associated genes such as *TGF-β1*, *SMAD* genes and chemokines such as CCL2 which has been described to foster Treg and MDSC accumulation and shape a tumour permissive microenvironment,[430] were up regulated in CRPM in this study, suggesting that these agents play an important role in the TUME in CRPM. Further research in exploring avenues to target the TGF-β pathway may help reduce the immunosuppressive effects within the TUME.[426]

The CMS classes of CRC help to stratify CRCs based on their clustering of gene pathway aberrations, stromal composition, immune infiltrate, extent of hypoxia and vascularisation.[33] As the four CMS classes cannot be determined based on the presence of specific mutations alone, it suggests that the genetic makeup is only partly responsible for the gene expression and overall behaviour of each tumour.[431] Similar to Ubink et al,[432] this study lends further support to the use of the CMS classes, demonstrating that the majority of CRPM are CMS 4, requiring the exploration of targeted therapies that are specific to each subtype.

In this study, we were unable to expand TILs from some samples, despite tumour pieces being placed in the presence of IL-2 *in-vitro*. It is possible that owing to tumour heterogeneity, these were samples with low TIL infiltrate, or with TILs that may have been dysfunctional. Alternatively, this could be a limitation in the TIL expansion protocol, which would need to be optimised further if this assay is to be considered in clinical practice. Additionally, the immune assay is an artificial assay, with different effector to target ratios seen *in-vivo*. Furthermore, while tumouroids recapitulate the native tumour well, they fail to recapitulate other aspects of the TUME such as the stroma and vasculature, the effects of which require further study. The relatively small sample size and short follow up of all recruited patients in this study limits any survival analysis and correlation with immune infiltrate. This study was designed as an exploratory study, given the significant paucity of data on the immune landscape in CRPM. In view of the highly insightful findings, it would warrant further validation in a larger cohort. Additionally, the role of other stromal cells such as TAMs, particularly the

M_2 macrophages, CAFs and MDSCs warrant further study as they are may be important players contributing to the immunosuppressive environment in CRPM.

8.5 Conclusion

This study has provided substantial insight into the immune landscape and TUME of CRPM. It confirms both the presence of a functional adaptive immune system, along with a complex and intricate immunosuppressive environment that allows cancer cells to thrive. Immunotherapy may improve the efficacy of the adaptive immune system in selected cases; however, further research in a larger cohort is required to explore other aspects of the TUME.

While this chapter has explored the immune landscape and the role of checkpoint blockade, the next chapter will focus on the development and use of a novel tumouroid based platform to help deliver personalised therapies to patients with CRPM.

9 Chapter 9: Precision medicine for CRPM: Can it be done?

Material from this chapter has been submitted as:

Narasimhan V, Wright J, Churchill M, Wang T, Rosati R, Lannagan T, Vrbanac L, Richardson A, Price T, Tye G, Marker J, Irvine T, Hewett P, Pereira S, Michael M, Tie J, Mukherjee S, Grandori C, Heriot A, Worthley D, Ramsay R, Woods S. High throughput drug sensitivity screening of patient derived organoids for colorectal peritoneal metastases can be used to guide therapy *Clin Cancer Res* (Under review)

9.1 Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related mortality worldwide.[1] The peritoneum is a common site for metastases, but confers the worst survival among all sites in patients with metastatic CRC.[24] Four to seven percent of patients present with synchronous peritoneal metastases,[50] while up to 19% developing metachronous disease.[320]

Historically, PM were viewed with nihilism, with a median survival of only three to six months.[46, 62] Advances in systemic chemotherapy and biological agents have since improved survival to 12-16 months.[24, 155] Despite advances, standard chemotherapy regimens are less efficacious at treating PM compared to other sites of metastases.[433] Treated with systemic chemotherapy alone, five-year survival is less than five percent for patients with CRPM.[155]

The advent of cytoreductive surgery (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) can offer selected patients with CRPM a favourable 27 to 41 months median survival with a 23 to 42% five-year survival.[150, 392] Despite successful CRS and HIPEC, recurrences are common, occurring in up to 80% of patients within 12-18 months.[140, 348, 434] Presently, there are no new established treatment options on the horizon for patients with recurrent CRPM. Recurrence after CRS and HIPEC is treated on a case by case basis, but is largely centered on systemic chemotherapy, fraught with high failure rates. The role of iterative CRS and HIPEC is evolving, and may benefit a highly selected group of patients.[140] Novel drug delivery methods such as pressurised aerosolised chemotherapy (PIPAC) are showing early promise as a palliative surgical option in patients with recurrent or unresectable CRPM, but require further evaluation in clinical trials.[173, 435]

The concept of precision medicine is garnering significant interest in the oncologic community, with the aim to rationally assign drug treatments based not just on cancer type, but on the unique molecular features of each cancer- a tumour agnostic model of care. Advances in molecular analyses have revealed that while tumours such as CRCs share the same broad histology, they are a highly heterogeneous entity,[295] with multiple

molecular subtypes,[33] each with their own unique alterations in molecular pathways and tumour microenvironments.[33] This would therefore make a precision medicine model of care more logical, especially with more advanced cancers, compared to current generic treatment regimens.

Advances in next generation sequencing have led to the discovery of several actionable targets, leading to the generation of rationally designed therapeutic agents. These targeted therapies have now become standard therapy across several cancer types.[286, 436] However, clinical outcomes led purely by genomics have overall been disappointing.[437] Previous analyses by targeted next-generation sequencing of over 1000 patients with metastatic cancer found that only 11% could be matched with an on-indication, FDA-approved drug and an additional nine percent could be matched when off-label use of targeted FDA-approved drugs were considered.[438]

Genomic sequencing alone is a static measure of evaluating genomic alterations in the tumour, and fails to provide any functional assessment of whether the tumours are actually sensitive to specific targeted drugs. Furthermore, sequencing may not detect important tumour-related factors such as tumour heterogeneity, which has been postulated to be one of the key reasons for treatment failures.[295, 439] The ability to functionally evaluate tumour responses to drugs, in addition to genomic sequencing may offer unique potential to identify therapeutic options for patients who progress on standard chemotherapy regimens, and lack established biomarkers to successfully select treatments based on genomic data alone.

One of the foremost advances in translational oncology in recent years has been the development of tumour-derived organoids or tumouroids as a newer pre-clinical model of disease. Tumouroids are three-dimensional models of tumours grown *in-vitro*, that recapitulate tumour heterogeneity and molecular integrity better than traditionally used cell lines or murine models.[440, 441] Furthermore, tumouroids can be readily grown from percutaneous biopsies or operative specimens with high success rates.[442] Early studies have demonstrated that tumouroid drug responses *in-vitro* successfully mirror patient drug responses *in-vivo*,[443, 444] supporting the use of tumouroids as a novel pre-clinical model for personalising therapy at a functional level.

In this exploratory study, we aimed to firstly establish an *in-vitro* tumouroid-based platform to evaluate the efficacy of patient-derived tumouroids (PDTs) in predicting response to standard of care therapies in patients with CRPM. We subsequently aimed to integrate functional drug testing with genomic profiling to identify novel therapies in patients with treatment-refractory CRPM.

9.2 Methods

This study was conducted in collaboration with South Australia Health and Medical Research Institute (SAHMRI), Adelaide (Australia) and Sengine, Seattle (USA)

9.2.1 Ethics

All patients were recruited in accordance with HREC approval at both institutions (PMCC 15/76, HREC/16/SAC/344, SSA/17/TQEH/291).

The methods utilised for the various techniques used in the study are outlined in detail in **Chapter 2**.

9.3 Results

9.3.1 Establishment and validation

Twenty-one (67.7%) CRPM tumouroids were established and validated across both sites (Figure 9.1). Tumour samples were from patients undergoing CRS and HIPEC, staging laparoscopy or percutaneous biopsy. While previous studies had raised concerns about the ability to grow tumouroids from pre-treated (prior chemotherapy) tissue,[300] we had reasonable success in growing tumouroids (67.7%), especially as the majority of the patients in this cohort had been pre-treated with various chemotherapy agents. Methods of characterisation and validation are described in detail in ***Chapter 2***. Of note, two tumouroid samples (synchronous primary and peritoneal) were found to have a significantly lower allele frequency than germline and matched native tumour tissue on STR analysis, signifying genomic drift in the tumouroids and therefore lack of genomic concordance. These two tumouroid samples were deemed 'unsuccessful' and excluded from tumouroid based testing and assays. Overall genomic concordance between tumouroids with native tumour was 90.5% (21/23).

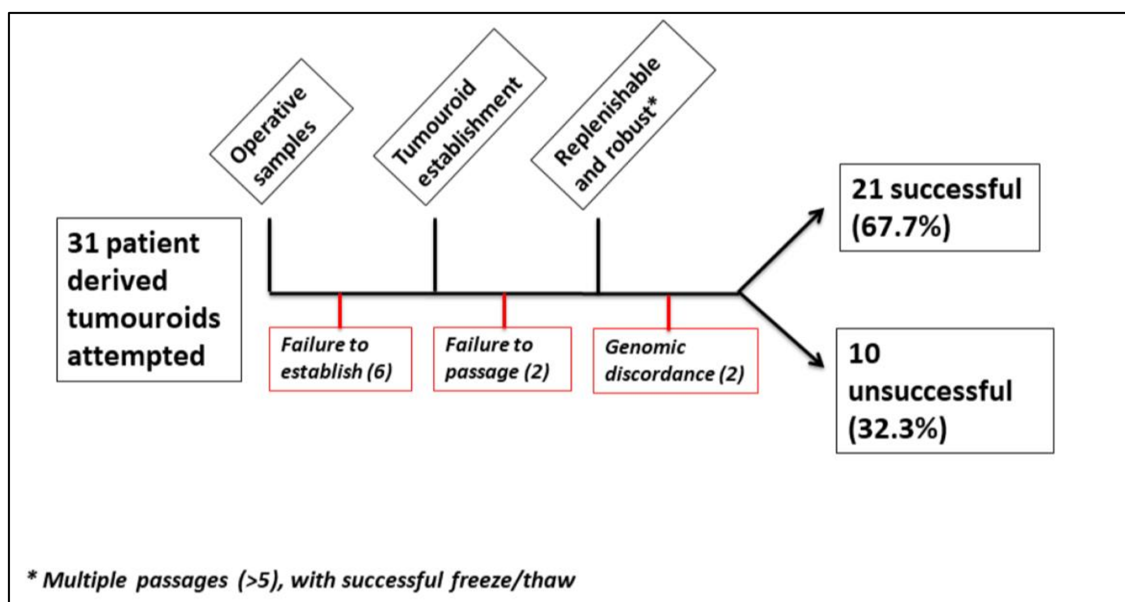


Figure 9.1. Success rates in establishment of patient derived tumouroids. Two tumouroids had only 70% genomic concordance compared to native tumour tissue, and were excluded from any further tumouroid based testing. All other tumouroids had atleast 85% concordance with native tissue

9.3.2 Tumouroid responses to standard of care chemotherapies

9.3.2.1 Single agents: 5-flurouracil, oxaliplatin and SN-38

In collaboration with SEngine and SAHMRI, drug sensitivity was evaluated on tumouroids firstly against 5-fluoruracil, oxaliplatin and SN-38 as single agents. Irinotecan is a pro-drug that is converted *in-vivo* by carboxyl esterase enzymes to the active metabolite SN-38, which is approximately 1000x more potent than irinotecan.[445] SN-38 was used as a surrogate for irinotecan efficacy, as irinotecan itself is inactive *in-vitro.* Evaluating the unique sensitivity of a tumouroid to a specific drug requires comparison of the tumouroid drug response to an "average" drug response. SEngine have an established library of over 300 tumour samples that have been drug tested against single agents, which is constantly updated with every new sample tested. This therefore allowed us to compare the drug sensitivity of each tumouroid against an "average" response of over 300 samples, giving us greater confidence in unique tumouroid sensitivities when they demonstrated significant sensitivity or resistance to a drug compared to the average response. Figure 9.2 demonstrates a heterogenous response to the three drugs in three different patient tumouroids. The red line is the tumouroid response, with the grey line the average response based on over 300 samples in the SEngine library.

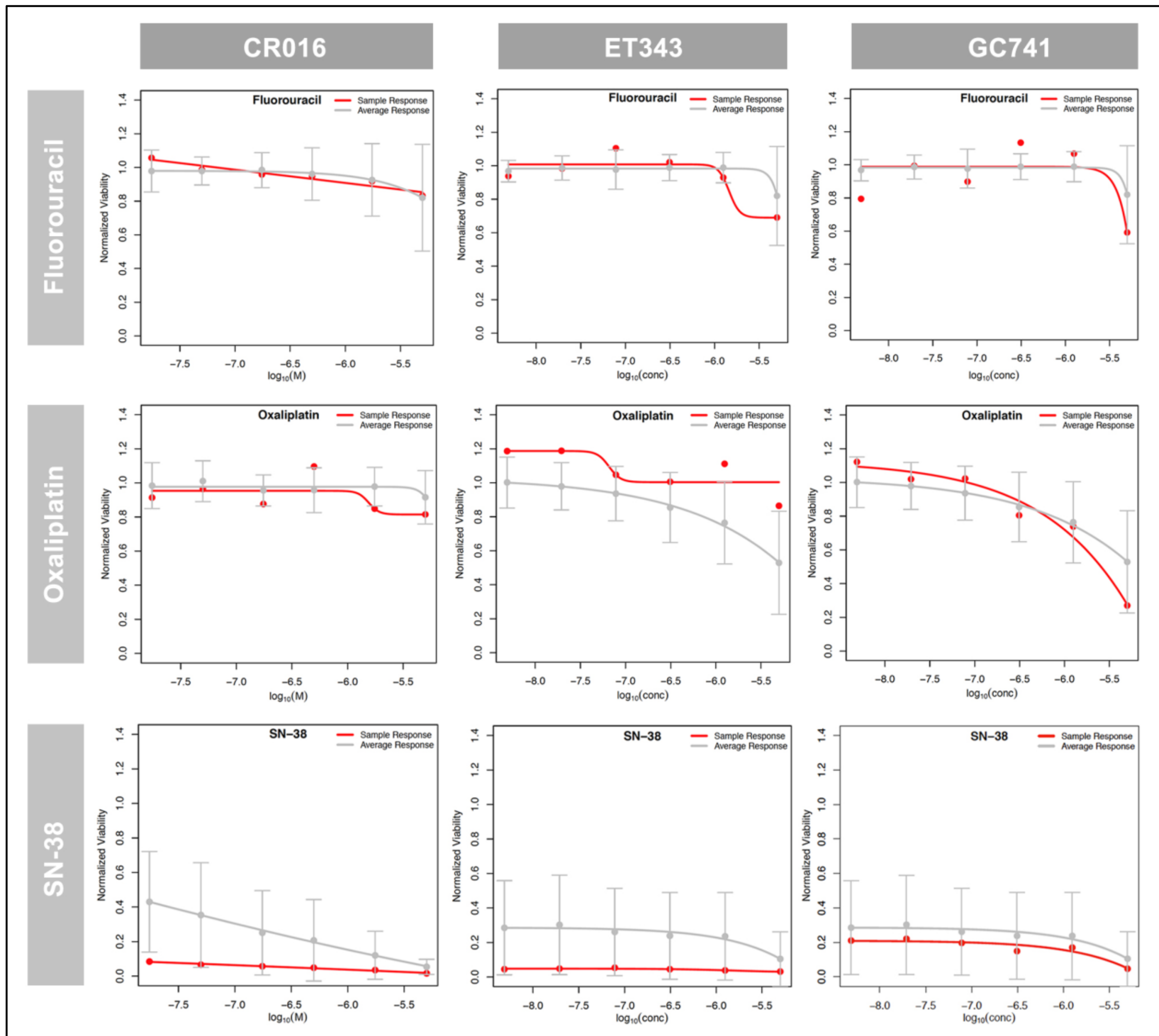


Figure 9.2. Three tumouroid lines demonstrating different sensitivities to 5-fluorouracil, oxaliplatin and SN-38. Red line refers to the tumouroid drug response. Grey line refers to average response across approximately 300 samples. As is evident, all three lines appear to have relatively average sensivity to 5-FU. All three lines have either an average response or are resistant to oxaliplatin, as in the case of ET343. With SN-38, all three lines demonstrate increased sensitivity compared to the average.

9.3.2.2 Combination regimens: FOLFOX and FOLFIRI

Eight tumouroid lines were evaluated against standard of care regimens of FOLFOX and FOLFIRI. Figure 9.3 below demonstrates heterogenous responses among eight tumouroid lines to FOLFOX and FOLFIRI. The heterogeneity in drug response to the same regimen parallels that observed in the clinical setting.

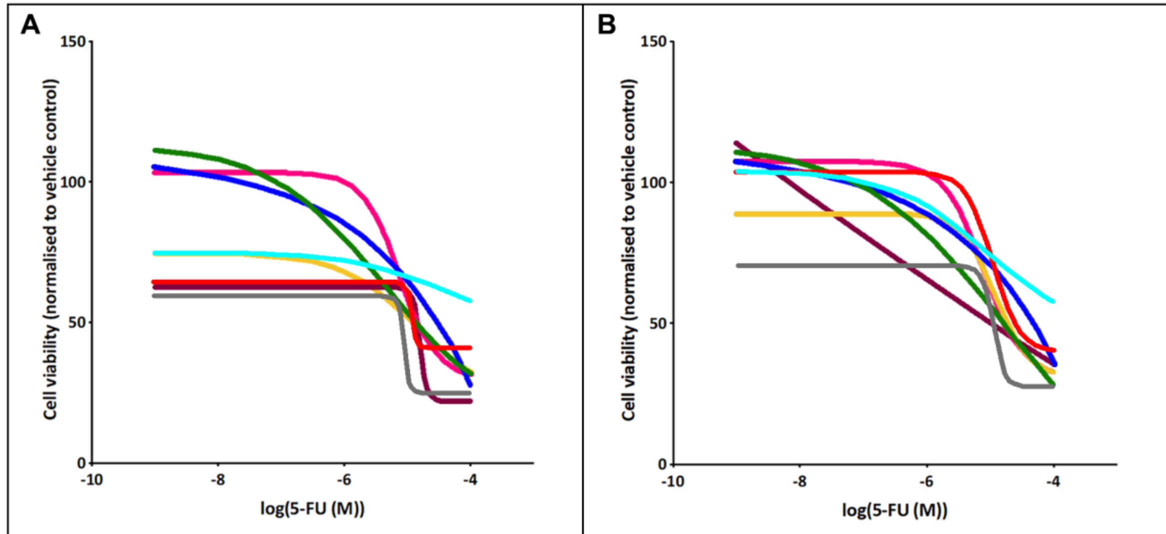


Figure 9.3. Eight samples showing heterogenous responses to A. FOLFOX and B. FOLFIRI regimens. The y-axis is the cell viability in percentage, while the x-axis is the logarithmic concentration of 5-FU. As described in Chapter 2, combination testing involved keeping the dosages of oxaliplatin and leucovorin constant in FOLFOX, and SN-38 in FOLFIRI, with titration of 5-FU, consistent with previously published methods. Data was graphed using nonlinear regression curve fitting – log (inhibitor) vs. response – variable slope, which was felt to be the best model. We elected not to use a normalised fit from 100% to 0%, as a normalised curve cannot fit a sigmoidal curve when samples are sensitive to low doses of drugs.

9.3.3 Tumouroid drug responses *in-vitro* correlation with patient responses *in-vivo*

Evaluating how sensitive a specific tumour is to a drug requires assessment of the tumours sensitivity to the drug in comparison to an average response. An average response requires a large cohort of tumours to be tested against the specific drug. As we had only eight samples challenged against FOLFOX and FOLFIRI, we were unable to confidently determine what an average response might be (given the small sample size). However, tumouroid lines that were highly sensitive *in-vitro* or conversely highly resistant were found to correlate very well with patient responses *in-vivo*. Figure 9.4 below demonstrates very good correlation between drug testing and patient response in a line that was highly sensitive to FOLFOX with almost complete metabolic response *in-vivo* on FDG-PET/CT to FOLFOX for patient 8 (grey line on figure 9.3).

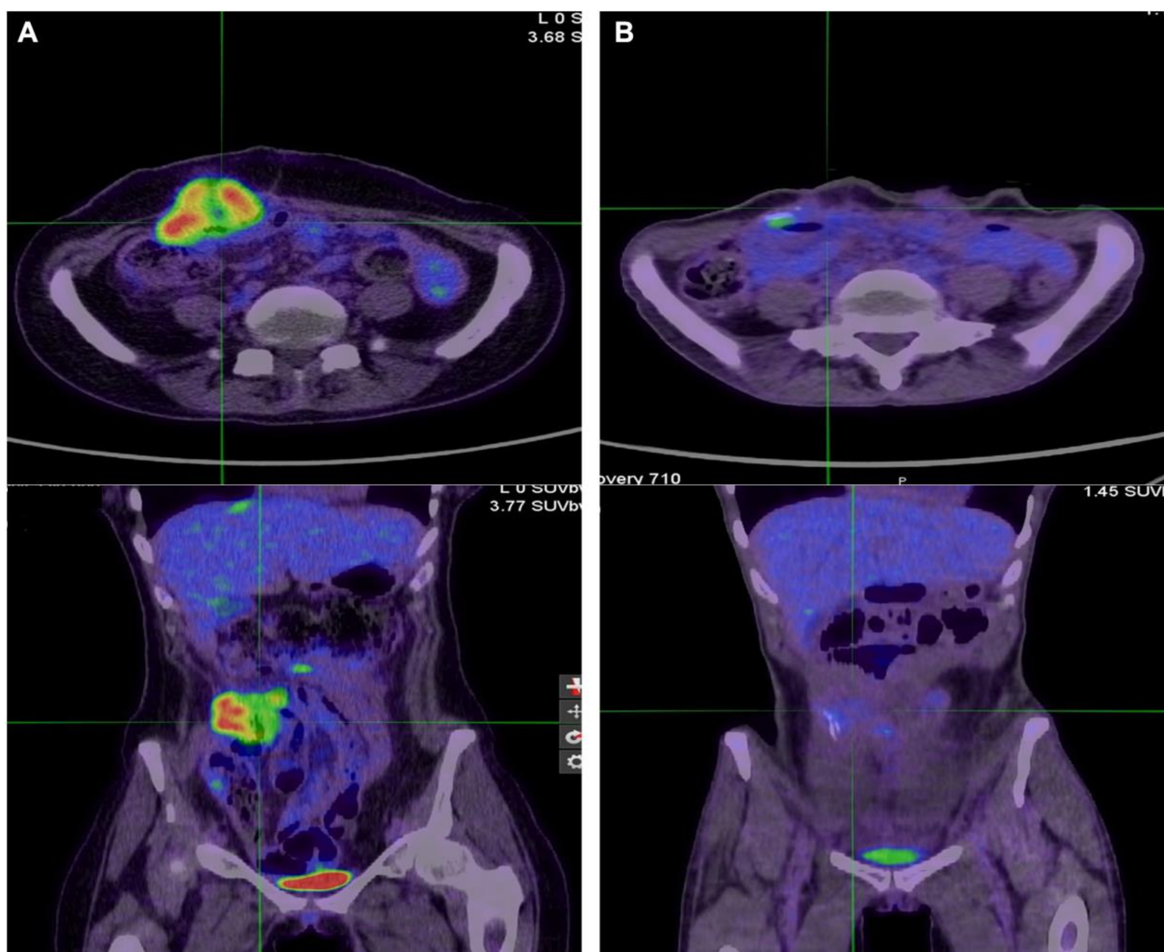


Figure 9.4. This was a patient who initially had what was deemed an unresectable mass (Fig 9.4A) involving the right abdominal wall, and was started on FOLFOX. Tumouroid drug response in-vitro showed this line to be one of the most sensitive of the eight lines to FOLFOX. Following three months of FOLFOX, there was almost complete metabolic response of the abdominal wall mass (Fig 9.4B) demonstrating that tumouroid drug response in-vitro correlated well with patient drug response in-vivo.

In another example, a tumouroid line was sensitive to FOLFOX combination therapy, but relatively resistant to 5-FU as monotherapy compared to the average response to 5-FU. In the patient, disease remained stable on FOLFOX for three cycles. However, the patient subsequently developed peripheral neuropathy, likely secondary to oxaliplatin, leading to FOLFOX being stopped and put on 5-FU monotherapy. On 5-FU monotherapy, there was evidence of disease progression as seen in Figure 9.5.

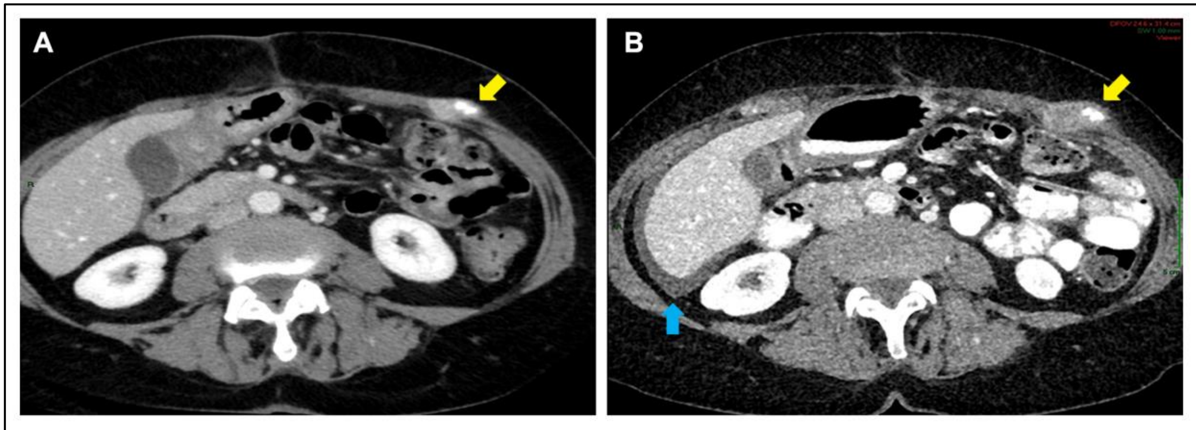


Figure 9.5. Example of tumouroid drug resistance to 5-FU monotherapy. Abdominal wall nodule remained stable in size on FOLFOX (Fig 9.5A). However, following change to 5-FU monotherapy, follow up scan demonstrated progression on 5-FU monotherapy, with increase in the size of the abdominal wall nodule and new ascites (Fig 9.5B).

In another instance, a tumouroid line (light blue line on figure 9.3) demonstrated resistance to FOLFOX and FOLFIRI compared to the other lines. As evident in Figure 9.3, the IC_{50} was not reached at the concentration ranges evaluated for this tumouroid line. In Figure 9.6 below, the patient experienced rapid disease progression on FOLFIRI.

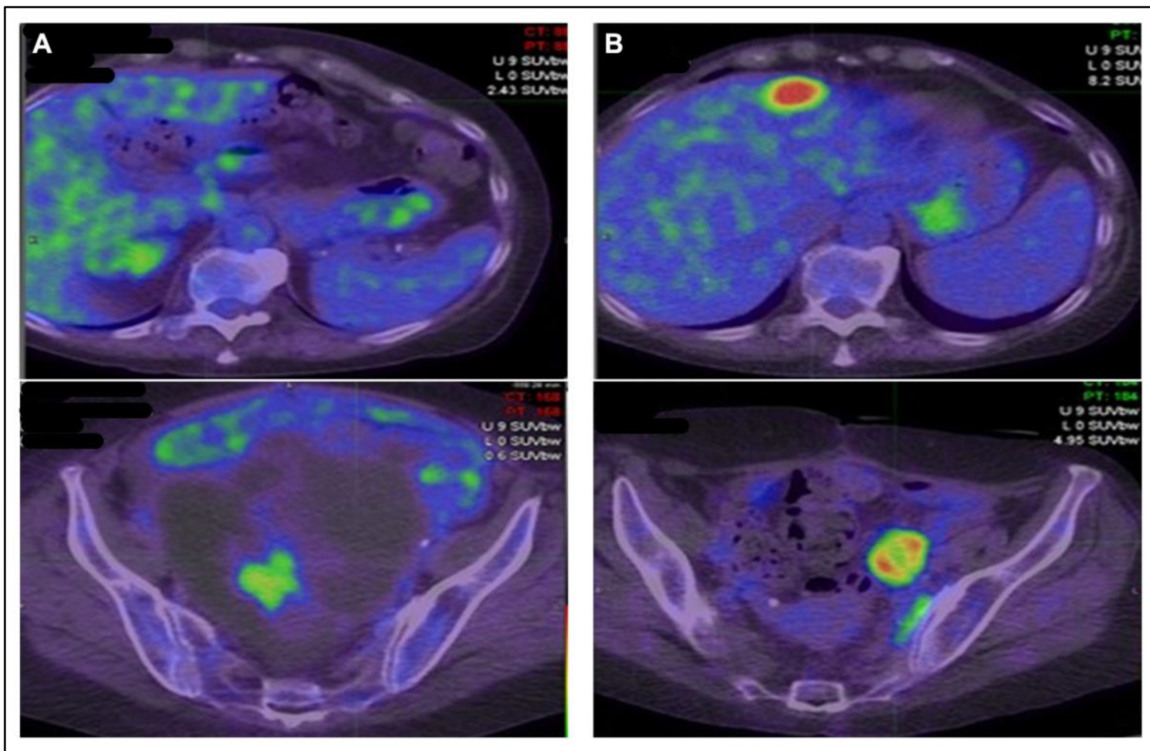


Figure 9.6. Fig 9.6A demonstrates pre-FOLFIRI, with evidence of a hepatic metastasis and a pelvic mass developing while on FOLFIRI (Fig 9.6B)

Overall, given the small sample size, we found that the *in-vitro* strong responders and non-responders correlated well with patient responses *in-vivo*. However, we cannot confidently comment on the drug curves in the middle with such a small sample size. A larger drug response library would be required to help ascertain an "average response" to FOLFOX and FOLFIRI before can we aim to predict sensivity of every line to FOLFOX or FOLFIRI.

9.3.4 Response to therapy may be different based on site of disease

There were three chemo-naïve patients who underwent synchronous resections of primary tumours with PM. Primary cancer and CRPM tumouroids were successfully validated and drug tested against FOLFOX and FOLFIRI for two of these patients. Figure 9.7 and 9.8 below demonstrates that in both cases, the PM was inherently more resistant to both chemotherapy regimens compared to the primary tumour.

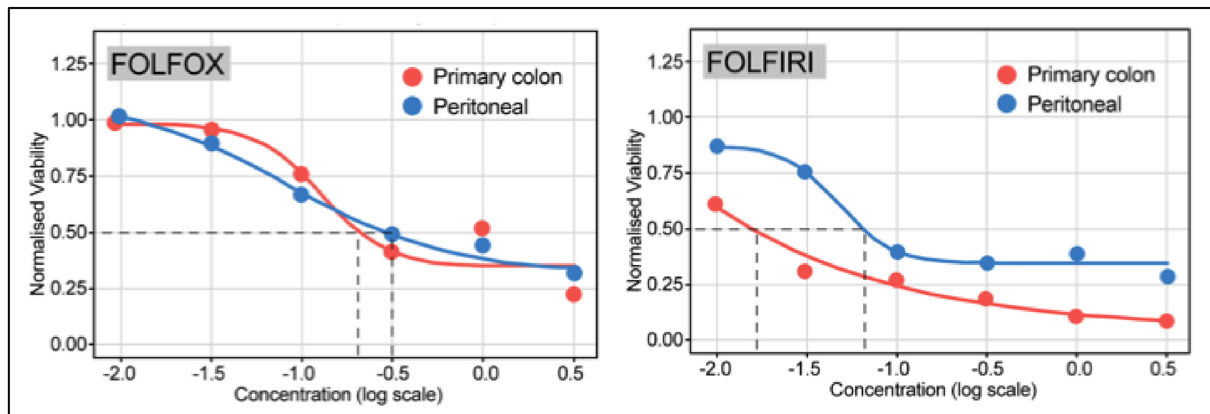


Figure 9.7.In patient 6, the peritoneal metastasis was inherently more resistant than the primary, particularly with FOLFIRI.

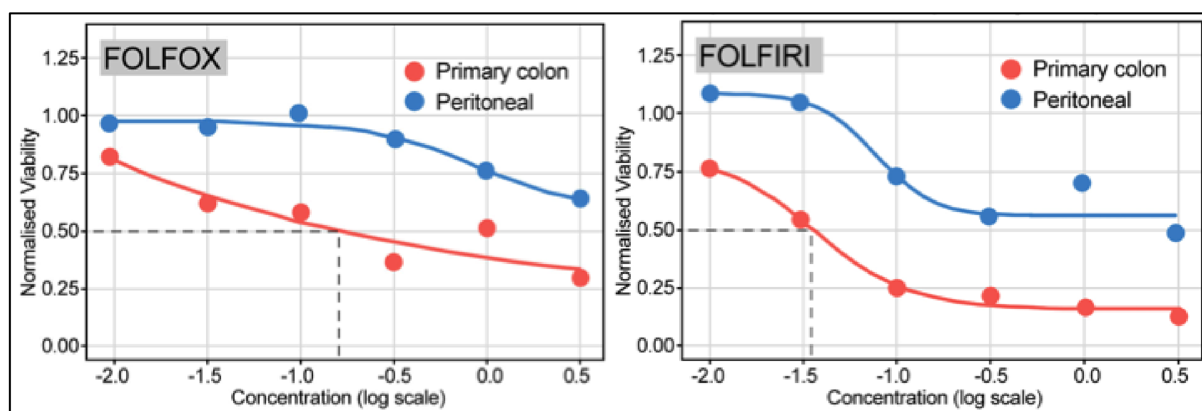


Figure 9.8. In patient 7, the peritoneal metastasis was significantly more resistant than the primary to FOLFOX and FOLFIRI.

The reasons for such inherent resistance in the PM are unclear. While a small cohort of two samples is difficult to draw any conclusions from, it is an interesting observation. There were no significant genomic differences between the primary and PM that would render increased resistance in the PM. On RNASeq, both PM were CMS 4, with the two primaries being CMS 2 and CMS 4 for patient 6 and 7 respectively. A larger cohort size is needed to further explore the reasons for variable resistance at different sites.

9.3.5 Genomic profiling of CRPM

Whole exome sequencing (WES) was undertaken on tumouroids from 14 patients to evaluate DNA alterations that may predict sensitivity or resistance to targeted therapies. The samples had a mean somatic mutation rate of 1.8/Mb, in line with previous reports of mutation rates in MSS CRC[446] (Table 9.1). No sample had a high tumour mutation burden (≥ 10 mutations/Mb) that may indicate potential sensitivity to immunotherapy.[447] Summary of WES data on all samples is presented as Appendix 4.1

Table 9.1. Tumour mutational burden (somatic mutations in protein coding regions of genes/Mb, normalised to the breadth of WES coverage for each sample) across the cohort

Patient-derived tumouroid	Somatic mutations in protein coding regions of genes/Mb
Patient 1	1.66
Patient 2	0.59
Patient 3	1.44
Patient 4	0.97
Patient 5	1.58
Patient 6C	3.00
Patient 6P	1.61
Patient 7C	2.13
Patient 7P	1.75
Patient 8	1.27
Patient 9	2.24
Patient 10	1.74
Patient 11	1.22
Patient 12	1.28
Patient 13	1.14
Patient 14	1.51

C and P: primary and peritoneal samples from synchronous cases

The high confidence genomic variant calls and copy number variations (CNVs) were similar to those commonly associated with CRC.[448, 449] To evaluate commonly altered pathways in CRC, publically available sequencing data from CRC samples covering 341 cancer genes was accessed.[448] This dataset contained 1011 tumours (478 primaries and 533 metastases) from 979 patients with metastatic CRC and 123 tumours from 120 patients with early stage CRC. Mutant gene frequency from this study was compared to frequencies in our cohort of 14 CRPM. This analysis focused mainly on gene targets with drugs that were either FDA approved or in clinical trials for solid tumours (Table 9.2).

Table 9.2. The mutational landscape of CRPM, with relation to potential therapeutics.

Pathway	Gene	MSK-IMPACT Mutated	Pathway Percent altered	Our study Mutated	Drugs
EGFR/MAPK	*KRAS*	44%	48%	36%	MEKi
	NRAS	4%		7%	
	BRAF	12%	12%	7%	BRAFi
	ERBB2	5%	19%	0	EGFRi
	ERBB3	5%		14%	
	ERBB4	6%		21%	
	EGFR	3%		14%	
PI3K/mTOR	*PIK3CA*	20%	35%	14%	PI3K/ mTORi
	PIK3R1	4%		7%	
	PTEN	6%		7%	
	mTOR	5%		7%	
DNA Repair	*POLE*	5%	18%	7%	PARPi
	BRCA1	3%		14%	
	BRCA2	6%		0	
	ATRX	4%		14%	
FLT family	*FLT1*	3%	10%	7%	FLTi
	FLT3	2%		14%	
	FLT4	5%		7%	
Rare Actionable Mutations	*TGFBR2*	4%	4%	0	Galuni sertib
	ALK	4%	4%	7%	ALKi

Mutational frequency in our cohort was compared to the publically available MSK- IMPACT cohort of over 1000 cancers. Mutant gene frequency in our cohort of 14 CRPM was similar to that seen in the MSK-IMPACT cohort.

The ploidy estimate plots from WES demonstrated that some patients had relatively normal chromosomal copy numbers across the genome, but the majority had widespread variation in ploidy (Appendix 4.2).

The COSMIC DNA mutation signature that can suggest the molecular aetiology of DNA alterations in each sample was also evaluated in the context of therapeutics that can target specific mechanisms of DNA alterations (for example the use of PARP inhibitors for BRCA mutant cancers) (Appendix 4.3).

9.3.6 Throughput drug testing to evaluate novel therapies

With limited efficacy of standard chemotherapies in treating patients with CRPM, functional drug testing on tumouroids was performed using a CLIA-certified PARIS™ *in-vitro* platform in collaboration with SAHMRI, Adelaide and SEngine, Seattle. Fifteen patients in total were subjected to drug testing. The first five samples were subjected to a 131 pan-cancer drug panel comprising a combination of chemotherapies, targeted therapies and Phase I/II drugs. Upon recognising that many of these drugs were not available in Australia, a curated panel of 37 agents was selected for the remaining samples. This curated panel of agents was established based on drugs that were listed by the Pharmaceutical Benefits Scheme (PBS) for any indication or in clinical trials in Australia (Appendix 4.4).

Drugs were classified based on drug response and area under the curve (AUC) to evaluate sensitivity. All drug testing was performed at least in duplicate. Tumouroid response to each drug was compared to an "average" response based on the SEngine drug response library of over 300 samples. Comparison to an average response allowed us to evaluate for unique sensitivities to specific drugs. A heat map depicting unsupervised clustering of tumouroid sensitivities to 35 drugs that were largely common to the pan-cancer and curated drug panel, along with limited clinical and genomic information is shown in Figure 9.9.

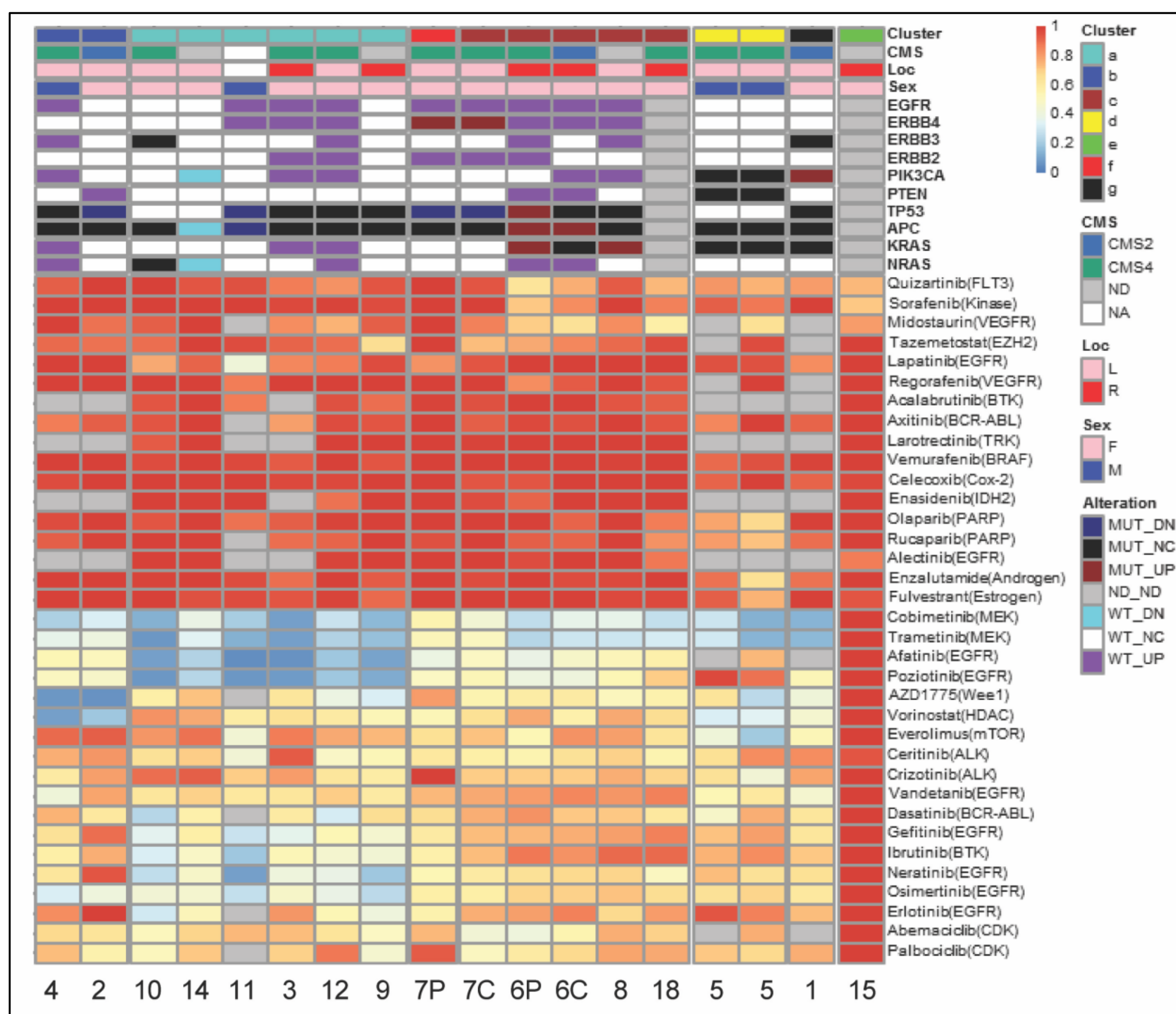


Figure 9.9. High throughput drug screening reveals shared and unique drug sensitivities to targeted agents. Heat map demonstrating unsupervised clustering of area under the curve data from high throughput drug screening (Red: 100% cells viable, i.e- insensitive; blue 0% viable, i.e highly sensitive). Patient numbers at the bottom of each column.

CMS subtype: blue CMS2; green CMS4; Genomic alterations: navy –mutant and copy number loss; black-mutant; maroon-mutant and copy number gain; grey-not available; aqua-copy number loss; white-wild type; purple- copy number gain; Cluster: based on unsupervised clustering; Loc: Location; L-left sided; R-right sided. Grey boxes in the drug sensitivity column indicate drugs not tested against that sample.

The heat map demonstrates that drugs with common mechanisms of action largely generated similar responses across all tested tumouroids. For example, almost all tumouroids were sensitive to the MEK inhibitors trametinib and cobimetinib. Conversely, almost all tumouroids were resistant to drugs such as fulvestrant (estrogen receptor degrader) and celecoxib (COX-2 inhibitor). Importantly, unique drug sensitivities can be seen in seen in patients such as high sensitivity to the Phase II drug

AZ1775 (Wee1 inhibitor) in patients 4 and 2 compared to the rest of the cohort. Likewise, unique patient specific resistance is seen towards selected drugs such as resistance to Erlotinib in patient 2 or poziotinib in patient 5, while the rest of the cohort appear sensitive to these drugs. Tumouroids from patient 15 appear generally resistant to almost all therapies, highlighting potentially highly resistant tumour biology in this patient. Such patient specific unique findings along with common drug sensitivities and resistance across the cohort underpin the value of functional drug testing.

9.3.7 Genomic predicted sensitivity consistent with functional drug responses

Genomic alterations previously shown to influence response to targeted agents could explain some but not all tumouroid drug sensitivities as seen in Figure 9.9, emphasising the value of functional testing.

The presence of a *BRCA* mutation in patient 5 was significantly associated with sensitivity to PARP inhibitors (olaparcib and rucaparcib) (one way ANOVA $p<0.005$, figure 9.10A).

Two patients (patient 5 and 15) had aberrations in *PIK3CA*. Patient 5 harbored a frameshift mutation (N1068fs) alongside the truncating mutation in *PTEN*, and responded exceptionally well to both PIK3CA kinase inhibitors (alpelisib and taselisib) (Figure 9.10B).

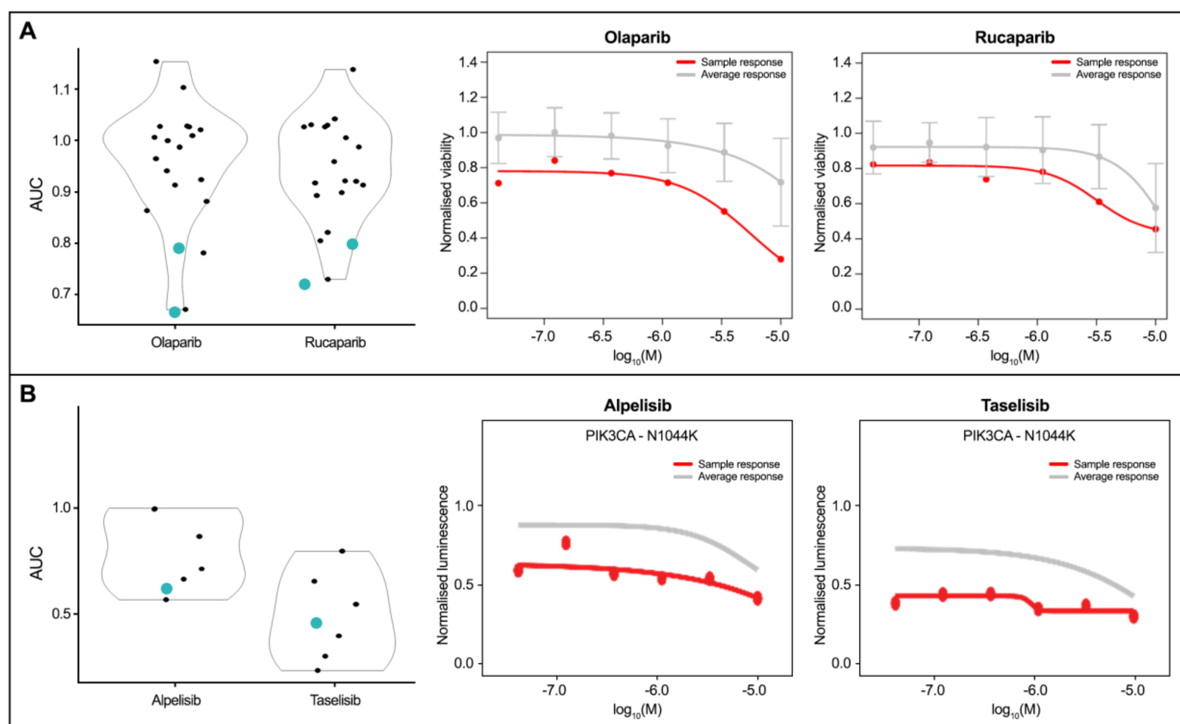


Figure 9.10. Fig 9.10A. On the left, the violin plots demonstrate that tumouroids from patient 5 (highlighted in blue), with a BRCA mutation, were among the most sensitive to the PARP inhibitors. The drug response curve for patient 5 on the right similarly demonstrates significant sensitivity compared to the average response. Similarly, in Fig 9.10B, the two lines among the more sensitive to PIK3CA kinase inhibitors, were again those with a PIK3CA mutation showing concordance between functional testing and genomics.

Patient 11 harbored aberrations in *EGFR*, *ERBB2* and *ERBB4* and demonstrated sensitivities to all *EGFR* inhibitors tested, with exceptionally strong sensivity to afatinib and poziotinib, two kinase receptor inhibitors with *ERBB4* activity (Figure 9.11A). Many tumouroids had at least partial responses to one or more *EGFR* family inhibitors, consistent with the known dependency on *EGFR* signalling in CRC.[450] Activating mutations in *KRAS* were significantly associated with decreased sensitivity to five of the seven *EGFR* inhibitors, consistent with resistance to *EGFR* inhibition in *KRAS* mutant states (Figure 9.11B).[450]

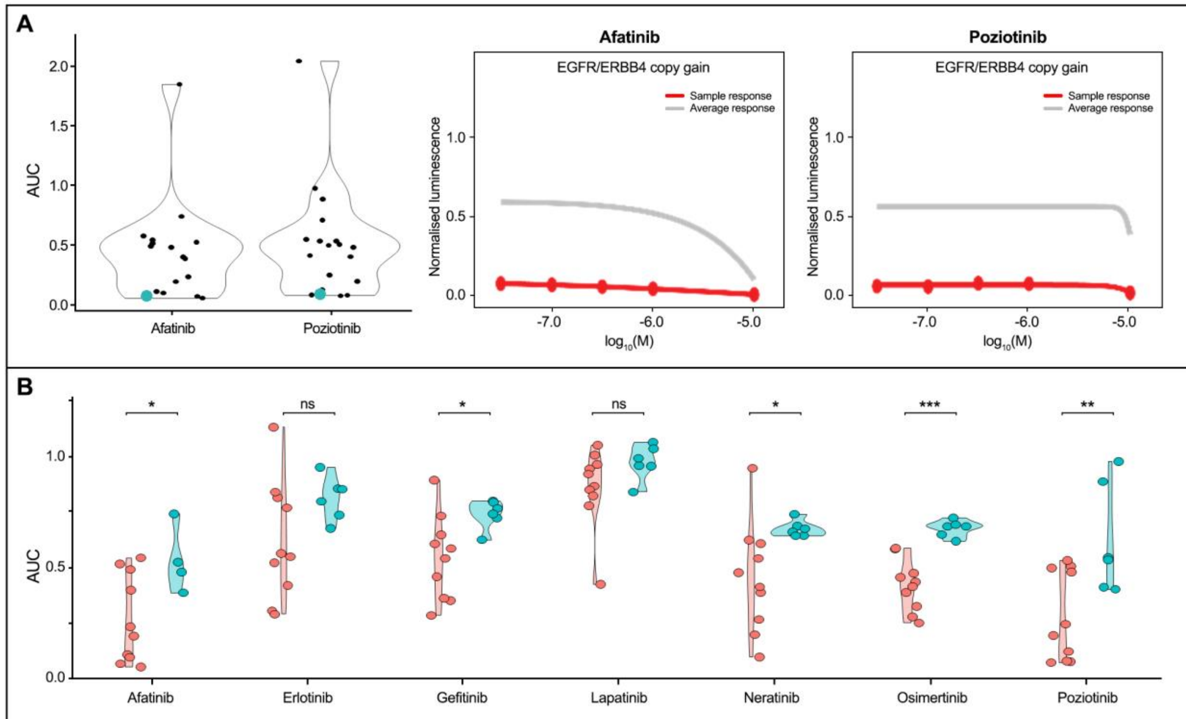


Figure 9.11. Fig 9.11A. Tumouroids from patient 11 demonstrating exceptional sensitivity compared to the average to afatinib and poziotinib, kinase inhibitors with ERBB4 activity, in keeping with known aberrations in EGFR, ERBB2 and ERBB4. Figure 9.11B demonstrates that KRAS wild type tumouroids (red) show significantly greater sensitivity (lower AUC) to five out of seven EGFR inhibitors compared to KRAS mutant tumouroids (blue)

*AUC: area under the curve; *, **, ***: p<0.05; ns: not significant*

9.3.7.1 Novel therapies with no genomic markers

Whilst tumouroids confirmed that genomics predicted drug sensitivities and resistance, novel drug sensitivities were also uncovered which could not be explained by any specific genomic aberration. For example, ibrutinib (BTK small molecule inhibitor used in mantle cell lymphoma) demonstrated strong sensitivity in 10/15 (66.7%) patients with no known genomic drivers to predict such sensitivity (Figure 9.12).

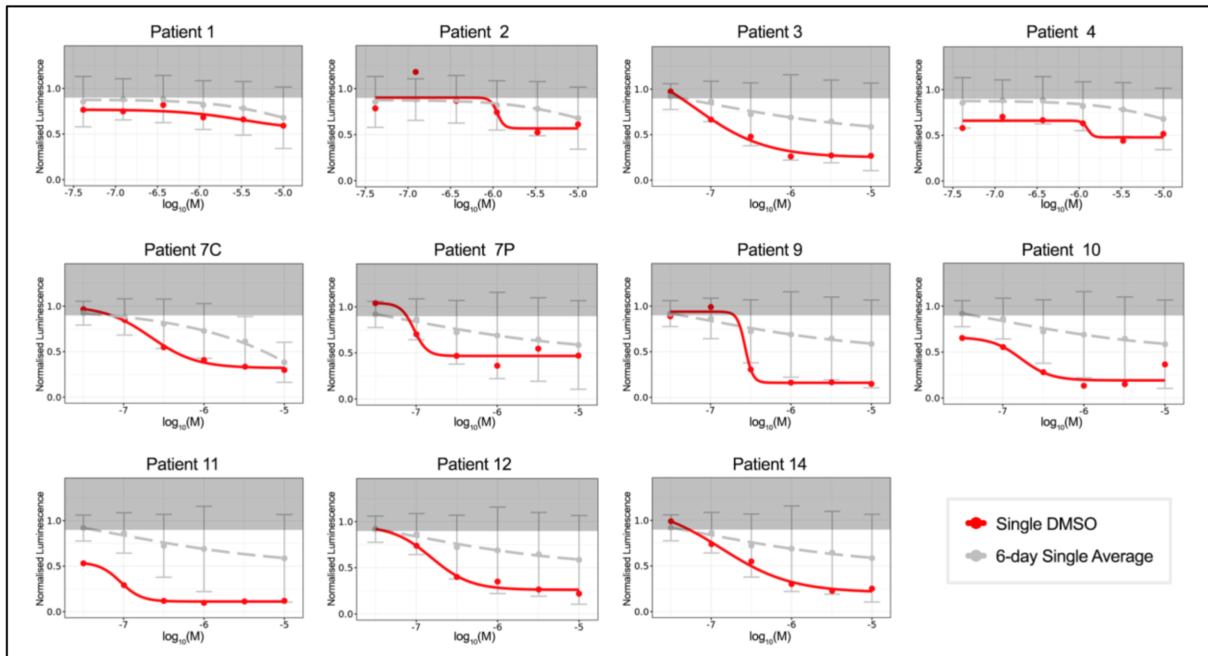


Figure 9.12 demonstrating eleven samples (10 patients with patient 7C and 7P being synchronous tumours) that demonstrated significant sensitivity to ibrutinib, despite no known genomic drivers, highlighting the value of functional drug testing to help uncover unique sensitivities when no genomic links are known.

Red line demonstrates patient tumouroid response; grey line represents the average response.

Similarly, while *RAS* mutations are known to render resistance to *EGFR* based targeted therapies, functional testing showed that in some *RAS* mutant cases, a partial response could be demonstrated to *EGFR* based targeted therapies. Figure 9.13 demonstrates strong sensitivity to *EGFR* based targeted therapies can be seen in two *KRAS* wild type lines while resistance to *EGFR* based therapy is seen in one *KRAS* mutant line. However, in two *KRAS* mutant lines, a partial response to *EGFR* based therapy is seen, underscoring the added importance of functional testing over genomics alone. Clearly, just having a *KRAS* mutation may not necessarily imply blanket insensitivity as genomic mutational data does not inform us of downstream pathway activation or gene expression which may impact on sensivity to specific drugs.

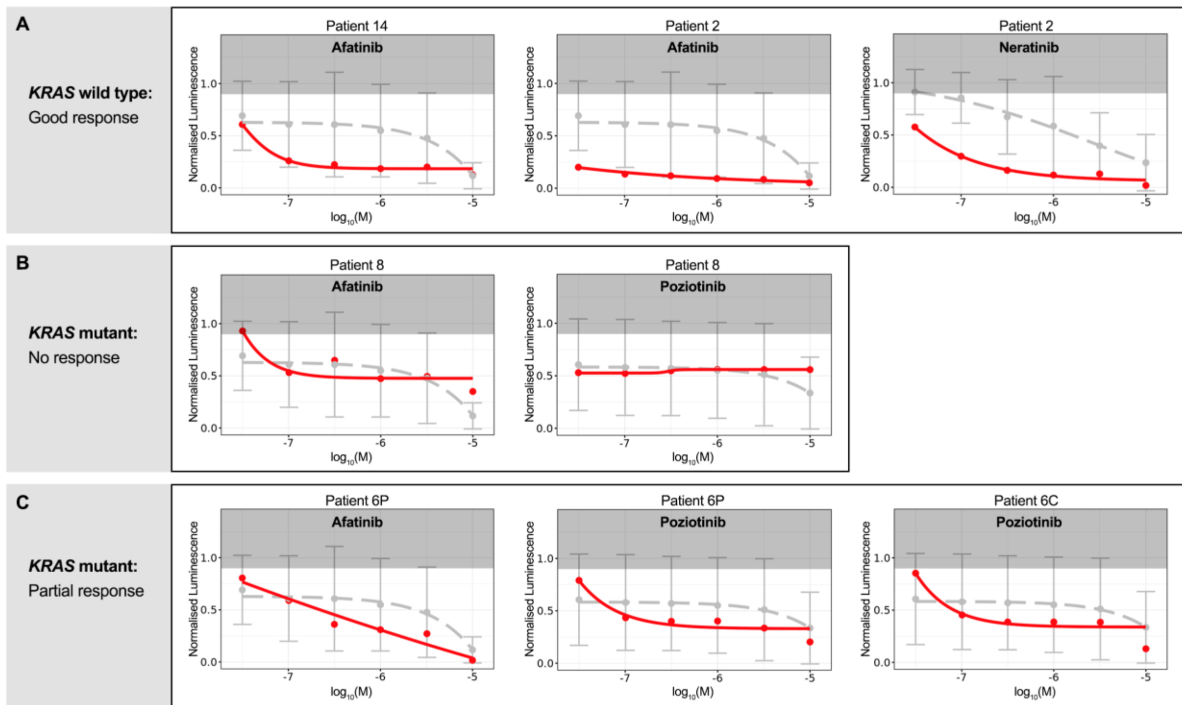


Figure 9.13. Fig 9.13A demonstrates sensivity to two EFGR inhibitors among three KRAS wild type tumouroid lines. Fig 9.13B demonstrates resistance to two EGFR inhibitors in a KRAS mutant line, in keeping with expected resistance to EGFR based therapy in the setting of KRAS mutations. Fig 9.13C demonstrates a partial response to two EGFR inhibitors in two tumouroid lines despite being KRAS mutant.

MEK inhibitors are known to be efficacious in *RAS/RAF* mutant states,[451]with MEK inhibitors licenced for use in Australia in *BRAF*^{V600E} mutant metastatic melanoma. Functional testing confirmed that *KRAS* mutant tumouroid lines were highly sensitive to MEK inhibitors. However, many *KRAS* wild type lines also demonstrated significant sensitivity to trametinib and cobimetinib, the commonly used MEK inhibitors, highlighting how functional testing can reveal unexpected drug sensitivities that may offer patients with novel therapeutic options (Figure 9.14).

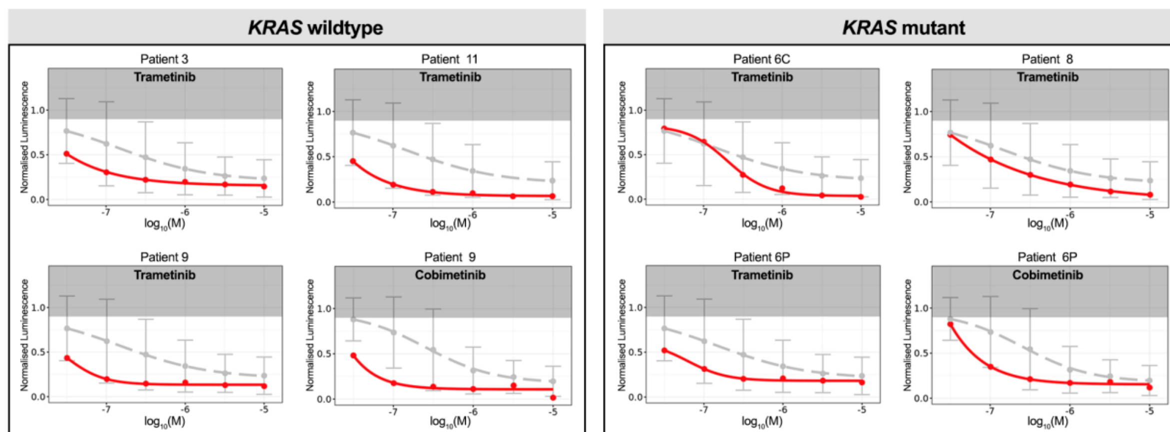


Figure 9.14 demonstrating sensitivity to two MEK inhibitors Trametinib and Cobimetinib, in both KRAS wild type and KRAS mutant tumouroids.

9.3.8 Using functional testing to offer novel therapies in treatment refractory cases

Many patients in this study failed standard lines of chemotherapy. For example, Patient 3 was started on regorafenib, a multikinase inhibitor, recently approved on PBS for treatment refractory metastatic CRC after progressing on standard lines of therapy. Tumouroid testing demonstrated insensitivity to regorafenib, with no difference compared to the average response. Clinically, this patient failed to respond to regorafenib, consistent with *in-vitro* findings.

Patient 2 similarly failed multiple lines of standard treatment, with no further therapeutic options available. Tumouroids from patient 2 displayed striking sensitivity to AZD1775 (Avadosertib), a Wee1 inhibitor currently in clinical trials in Europe and USA for patients with metastatic CRC (NCT02906059 and ISRCTN 90061546).[452]. However, we were unable to get access to AZD1775 despite liaising with the European clinical trial team. Other high sensitivity options from the drug screen such as osimertinib, afatinib and vorinostat were explored, but access to off label use of these drugs was restricted by funding, with pharmaceuticals unwilling to provide these drugs off label for compassionate access. Gemcitabine, an anti-metabolite chemotherapy, normally used in the treatment of hepatobiliary cancers was offered to the patient as a

novel therapy based on good efficacy from drug screening and ease of access to the drug (Figure 9.15).

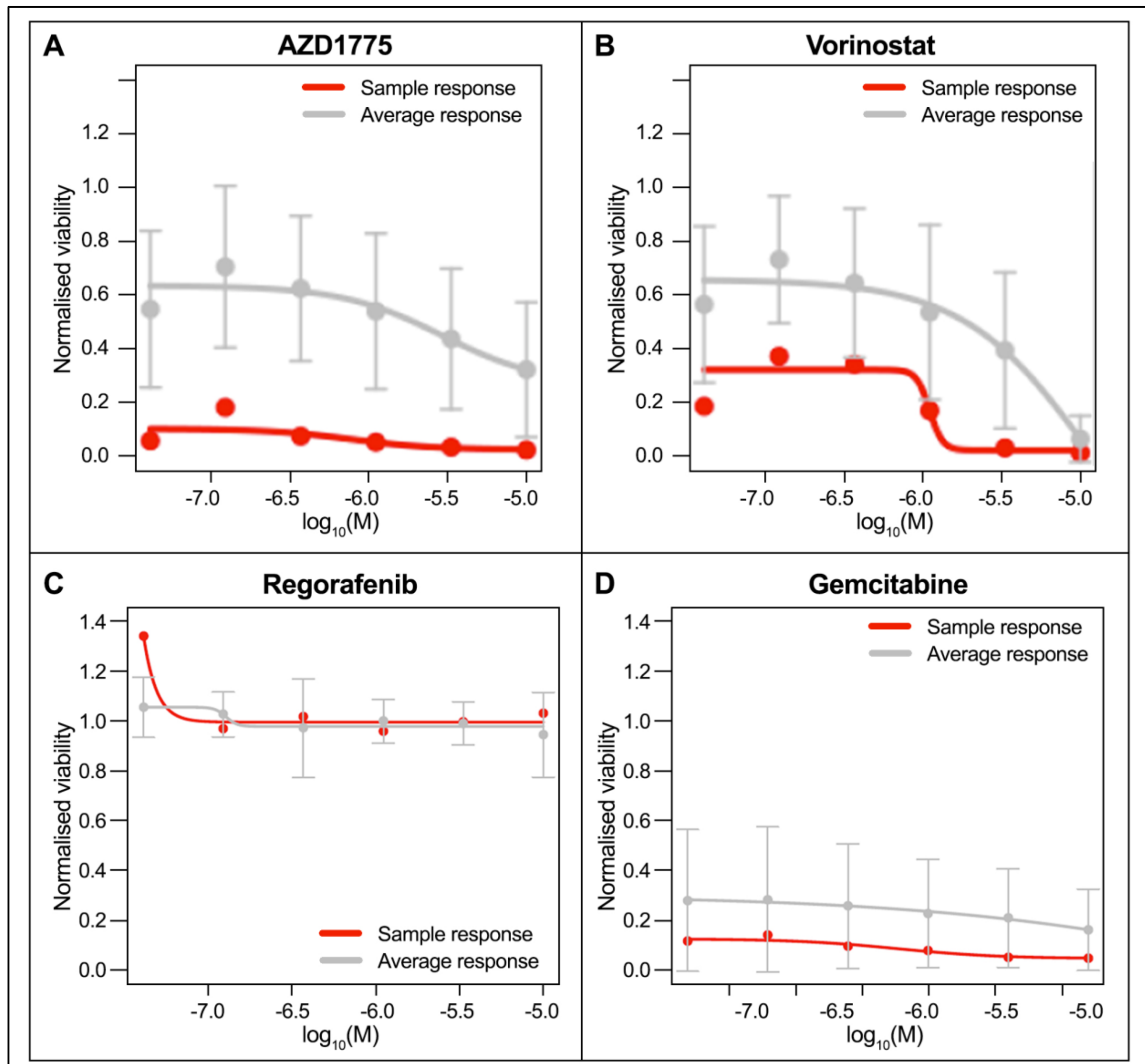


Figure 9.15. Drug sensitivity curves for tumouroids from patient 2 demonstrating a unique strong sensitivity to AZD1775. Vorinostat, an HDAC inhibitor, normally used in cutaneous T cell lymphoma was also found to have good sensitivity compared to the average response. Regorafenib, which was an option via PBS for treatment refractory metastatic colorectal cancer, was predicted to have minimal response. Gemcitabine had good in-vitro sensitivity and was accessible.

Gemcitabine had lower efficacy relative to the Wee1 inhibitor (AZD1775), afatinib, osimertinib and vorinostat. However, it had better efficacy than oxaliplatin, SN-38 and regorafenib, and was accessible at low cost. Furthermore, given its favourable toxicity profile, the medical oncologists offered it as a novel therapy to the patient. Six weeks after treatment change to gemcitabine, follow up FDG PET/CT imaging demonstrated

partial metabolic response of the pre-sacral metastasis and complete metabolic response of an abdominal wall nodule (Figure 9.16), demonstrating that *in-vitro* sensitivity in this case correctly predicted response *in-vivo*. Ongoing monitoring will continue to evaluate durability of this response.

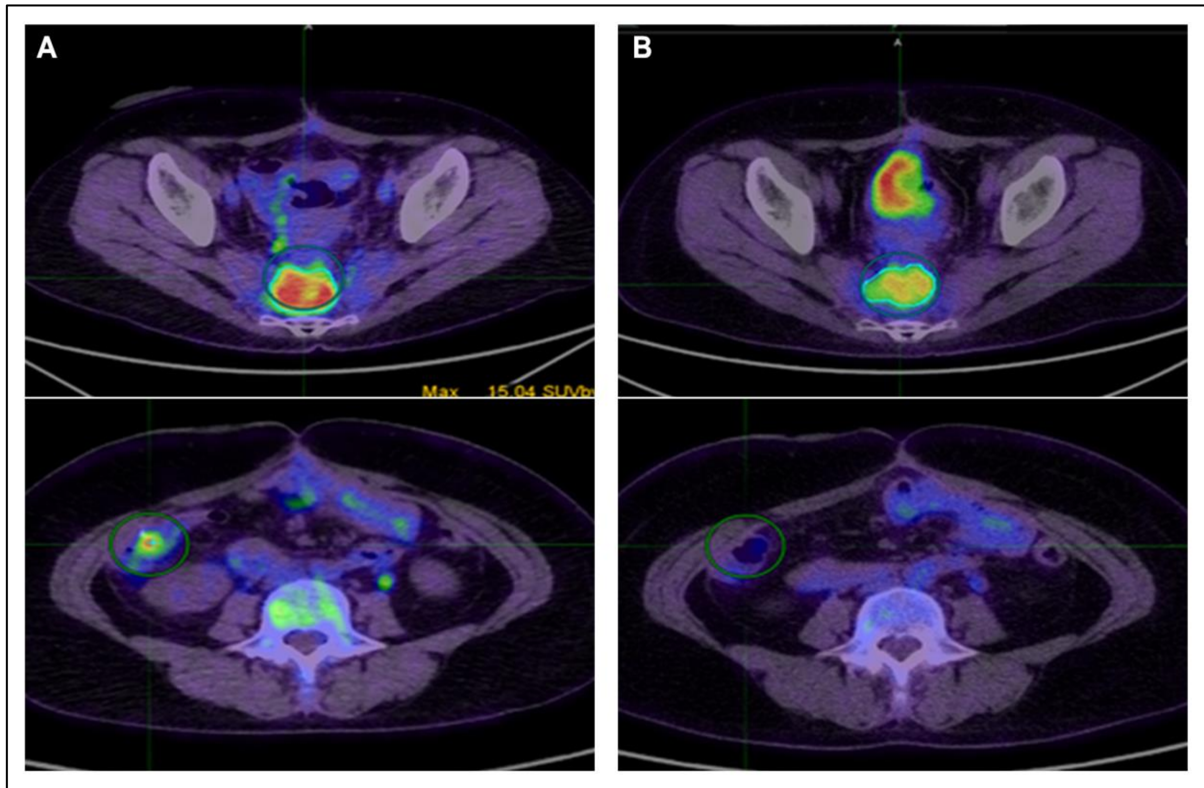


Figure 9.16. Fig 9.16A demonstrates pre- treatment axial slices demonstrating a FDG PET- avid pre-sacral nodule and a right sided abdominal wall nodule. Fig 9.16B demonstrates complete metabolic response of the abdominal wall nodule, with partial metabolic response in the pre-sacral nodule following treatment with gemcitabine.

9.4 Discussion

Colorectal cancer is a heterogenous disease, with several unique molecular subtypes that may explain the variable biological behaviour and drug efficacies among patients. For example, *RAS* mutations are present in 40-45% of CRCs, *BRAF* mutations in 9%, *HER2* amplification in 4%, *PTEN* and *PIK3CA* mutations in 10-15%.[453] Additional knowledge on CMS subtypes in CRC suggests that there are distinct differences in gene expression and pathway activation in the four subtypes,[33] that may lead to potentially different targetable agents between the four subtypes. It would therefore seem logical to offer a tailored approach to treatment given the current tools available.

In this multicentre, prospective study, we have successfully established an *in-vitro* patient derived tumouroid based platform that can be used to integrate functional drug sensitivity testing with genomic profiling to identify suitable therapeutic options in patients with CRPM in a clinically relevant and timely manner. Additionally, early results from this *in-vitro* platform reliably mirror patient drug responses *in-vivo*, and can be utilised to identify novel therapeutic options in patients who fail standard treatment options.

To highlight the utility of functional testing, we firstly demonstrated that sensitive and conversely resistant tumouroid lines correlated well with *in-vivo* findings in patients receiving FOLFOX or FOLFIRI. Subsequently, we evaluated the *in-vitro* response of tumouroids to specific drugs with known genomic biomarkers to evaluate the utility of integrating genomics with functional testing, demonstrating that this approach was reliable and predictable. Along with functional screening demonstrating concordance with genomic driven sensitivities, additional drug sensitivities were detected with throughput screening, suggesting that alternative drivers within the tumour may be present, owing to tumour heterogeneity. The additional drugs could be offered as potential combination therapy along with genomic concordant drugs or as an alternative treatment option. The MSK-Impact study of 1134 matched primary and metastatic CRC (including ~50 CRPM) revealed that CRPM were enriched for alterations to the phosphoinositide 3-kinase (PI3K) and mitogen-activated kinase (MAPK)

pathways compared to other metastatic sites.[448] These common genomic alterations in MAPK and PI3K pathway genes were reflected in our CRPM cohort. Concordantly, tumouroids were found to be broadly sensitive to MEK inhibitors (69% had mutation and/or copy gain to *KRAS, NRAS* or *BRAF*) and EGFR inhibitors (54% had *EGFR* copy gain), highlighting again the critical importance of EGFR/MAPK signalling for CRC. Acting on these drug sensitivity data for MAPK targets will require, as yet non-standard, combination treatment to constrain feedback loops that reactivate MAPK signalling in CRC, as trialed for *BRAF* mutant CRC[454] or in combination with immune checkpoint modulation in the future. Hence, such findings can help identify and lead to the utilisation of newer drugs or drug combinations to improve outcomes in this poor prognostic cohort.

Precision medicine has traditionally been synonymous with genomic profiling of a patients tumour to inform treatment choices. Recent clinical trials have evaluated the use of real time molecular analysis of tumours to guide treatment in patients with hard to treat or treatment refractory cancer. The MOSCATO 01 trial[286] was a prospective clinical trial that explored the use of molecular analysis of tumour biopsies to guide treatment. Objective responses were seen in only 11% (95% CI: 7% to 17%) of patients. However, of all included patients, only 7% of screened patients benefited from this approach. Other studies have similarly demonstrated that only a small proportion of patients gain any significant benefit from a sequencing guided drug selection.[455] [287] More recently, the NCI-MATCH trial[436] is an ongoing precision medicine trial currently recruiting patients with hard to treat or rare cancers. It aims to offer a molecular analysis based treatment to patients who have progressed on standard treatment in a variety of solid tumours. However, unlike many other cancers, the most prominent oncogenic drivers of CRC such as *MYC, RAS, and TP53* do not have any known targetable drugs, limiting the use of a purely genomic-based approach for advanced CRC.

In the metastatic setting, *KRAS* mutations occur in approximately 40% of cases.[456] *KRAS* mutation status in the metastatic setting is important to guide treatment, as it renders resistance to anti-EGFR mediated therapies based on clinical trials.[457, 458] However, what large clinical trials fail to explore is how every individual patient with a *KRAS* mutation behaves. While genomics may demonstrate the presence of a mutation, the

functionality of each patients tumour may be different despite the presence of the mutation. This could be due to pathway changes downstream of *KRAS*, altered signalling, or variable gene expression, none of which are identified from genomic sequencing alone. Evaluation of response on an individual patient basis is where functional drug testing offers an added dimension. While the presence of a *KRAS* mutation led to resistance to EGFR based therapy in most *KRAS* mutant patients in this study, there were selected patients where EGFR based therapy offered a partial response. Similarly, functional drug testing unearthed drugs such as ibrutinib, which many tumouroid lines were sensitive to, without any known genomic link. *MEK* inhibitors likewise, were found to have efficacy against most tumouroid lines regardless of *KRAS* mutation status. These unique sensitivities to which there were no validated biomarkers or discernible genomic links, underscore the true value of functional drug testing, which can potentially offer novel therapies to a patient cohort with limited treatment options.

Historically, the inability to replicate tumour heterogeneity with the use of cell lines and murine models was postulated to be one of the key reasons limiting the ability to advance the field of precision medicine. Tumour heterogeneity is present in all cancers, whereby tumour cells develop genomic aberrations through different mutational pathways, leading to temporal and spatial genomic diversity within the tumour.[459] One of the inherent limitations with genomic sequencing is the inability to accurately capture tumour heterogeneity in many instances.[460] Targeting an actionable mutation based on genomics alone may prove effective only if the mutation is truncal and present in most regions of the tumour.[461] The development and use of tumouroids now offer a robust pre-clinical model that can recapitulate tumour heterogeneity, and retains genomic integrity of the native tumour better than previous pre-clinical models. Recent studies have explored the genomic integrity of organoids at depth, and found that patient derived organoids successfully recapitulate primary and metastatic disease at the level of somatic mutations with 88-92% concordance.[300, 440] The utility of organoids also offers the ability to create living biobanks of tumour. Cancer consortiums such as The Cancer Genome Atlas and the Cancer Genome Consortium represent specimens of primary tumour and are a wealth of genomic information on the primary cancer. However, most patients who succumb to their cancers do so from metastases, which are

underrepresented in these consortiums. With the ability to successfully grow organoids from needle biopsies, an organoid biobank of metastatic disease along with the primary can be developed.

A number of studies have now successfully shown correlation between *in-vitro* tumouroid drug responses to clinical outcomes across multiple solid cancers [462-466], albeit with fairly small patient cohorts to date. For any such platform to be clinically relevant, results need to be offered in a timely manner. To that end, we were able to integrate tumouroid growth, genomics and throughput drug testing within eight weeks, making it highly clinical applicable. Furthermore, with this platform it is entirely feasible to re-biopsy an accessible tumour deposit in the event of disease progression to re-evaluate genomic changes and drug sensitivities.

There are a number of limitations in this study that warrant discussion. *In-vitro* testing of combination therapies such as FOLFOX and FOLFIRI is new and we have not accumulated adequate sensitivity data to be able to develop what may be considered an “average response”. It is possible that due to being a combinational regime, dosages of the different drugs in combination may not have been optimised. This will need further samples to evaluate. However, it was reassuring that *in-vitro* highly sensitive and resistant lines to FOLFOX/FOLFIRI correlated well with clinical findings. This study was designed as an exploratory study to evaluate if an *in-vitro* platform can be feasible, and if novel therapeutic options can be evaluated in a timely manner, not to evaluate if we could alter patient care. However, we found that this model of care can be feasibly delivered within eight weeks from receiving a tumour sample, making it highly clinically relevant. Additionally, we were fortunate to have had the opportunity to offer one patient a novel therapy that so far appears to have good efficacy. Durability of treatment response is unknown at this stage, and will become apparent with further follow up. While some oncogenic targets, such as *BCR-ABL* in chronic myeloid leukemia respond to targeted therapies for prolonged periods, pathways such as *RAS-RAF-MEK-ERK* or *HER2-PI3K-AKT-mTOR* are known to demonstrate an adaptive response, wherein a short-lived benefit may be seen before outright resistance develops.[467, 468] If only an adaptive response is found in this patient, further optimisation of the platform to develop the use of combinational therapy to target overlapping pathways may be

required. Novel combination therapies were not explored in this study, however this represents a focus for further research. Additionally, other agents used clinically such as cetuximab were not used in this study, and may offer more insight once its use is optimised into this platform. The sample size in this study is small, owing largely to the exploratory nature of the study.

In summary, this study explores a clinically unmet need to evaluate newer treatment options for patients with CRPM. Our *in-vitro* platform can offer novel therapies beyond solely genomic drivers in a clinically relevant time frame. Given these favourable findings, we believe the next logical step would be to translate this into a Phase II multi arm clinical trial targeting patients who are currently on final lines of standard treatment. One of the challenges we anticipate is access to novel drugs, which would require lobbying and engagement with pharmaceuticals to acquire off label use. Compassionate use of any drug is difficult to acquire, and remains a significant challenge for clinicians. Even in this study, while a patient was changed to a high sensitivity agent, we were unable to access the screening platform predicted most active agents due to lack of access. Overcoming this hurdle would greater help evaluate the true potential of our platform, along with offering the best available agents to patients.

9.5 Conclusion

In this study, we have successfully established an *in-vitro* tumouroid-based platform to evaluate the efficacy of patient derived tumouroids in predicting response to standard of care therapies in patients with CRPM. Additionally, we have integrated the use of genomics and functional testing to identify genomic concordant treatment as well as novel therapies for patients with treatment refractory CRPM

10 Chapter 10: Summary and future directions

10.1 Summary of thesis findings

Peritoneal metastases from CRC are a common occurrence and confer a dismal survival. Historical survival from PM was approximately six months, with the condition generally viewed with nihilism.[62] Systemic chemotherapy has relatively poor efficacy compared to other metastatic sites, with attempts at palliative surgery associated with high morbidity and mortality rates.[469]

The adoption of CRS and HIPEC can offer selected patients with low volume CRPM a favourable 27 to 41 months median survival with a 23 to 42% five-year survival.[150, 392] However, optimal patient selection for CRS and HIPEC remains key, as it carries a 12.5-35% and 1.0-8.3% risk of major morbidity[130, 150] and mortality[393], respectively. Additionally, only 20-25% of patients with CRPM are eligible candidates for CRS and HIPEC owing to disease burden.[470] Thus, systemic chemotherapy still remains the mainstay of treatment for the majority of patients. Advances in systemic therapies with combination regimens and biologic agents has led to an improvement in median survival to 12-16 months in patients with CRPM, still significantly lower than those with liver or lung metastases.[24] Treatment resistance and disease progression remain common in those treated with systemic chemotherapy. In the latest edition of the AJCC TNM classification, PM have been designated an independent category of M1c, highlighting the poor prognostic nature of this disease.[29]

Despite mounting evidence in favour of CRS and HIPEC, there remains ongoing skepticism about its role and efficacy among medical oncologists and surgeons alike. This is reflected in differing approaches to peritoneal disease by different hospitals and clinicians, varying uptake for CRS and HIPEC, as well as varying guidelines in management. However, it is unclear whether skepticism is due to lack of awareness and knowledge among clinicians in the management of peritoneal disease. Recent studies have shown that poor awareness of the value of the CRS and HIPEC in the management of CRPM contributes to reduced utilisation of CRS and HIPEC.[310, 311] To that effect, in Chapter 3, we evaluated perceptions among colorectal surgeons in the management of CRPM through an online survey. The survey demonstrated that there still appears to be

some skepticism associated with the role of CRS and HIPEC in offering an improved survival compared to systemic chemotherapy for patients with CRPM. Most surgeons would consider CRS and HIPEC for resectable CRPM, although the role of second look surgery was not supported. Some would also consider systemic chemotherapy as a stand-alone treatment option for peritoneal metastases, along with referral to gynae-oncology for a krukenberg tumour, suggesting that there are deficiencies in awareness and knowledge regarding the management of peritoneal disease. Overall, the survey painted a picture of local decision making largely in keeping with current international evidence, whereby there remains skepticism and variable uptake even among local colorectal surgeons.

To evaluate the efficacy of surgery, Chapter 4 evaluated outcomes from CRS and HIPEC for all PSM at a statewide peritoneal disease centre. This study firstly demonstrated that across 384 cases of PSM, CRS and HIPEC was safe, with a 26.3% morbidity and 0.8% peri-operative mortality. Not surprisingly, pseudomyxoma peritonei from perforated appendiceal mucinous neoplasms was the most common indication for CRS and HIPEC and conferred the best survival. The rates of CRS and HIPEC for CRPM were increasing, in keeping with increasing acceptance, case referrals and uptake.

Chapter 5 subsequently specifically evaluated outcomes from CRS and HIPEC for CRPM at a statewide referral centre, demonstrating that incomplete cytoreduction and mucinous histology were the main factors independently associated with a poor survival. Overall, median survival from CRS and HIPEC was 32 months, with a three-year survival of 38%. Additionally, if a complete cytoreduction was achieved, median survival increased to 37 months, with a median relapse free survival of 13 months. Major morbidity was 25.7% with a 2% peri-operative mortality rate. This study demonstrated that CRS and HIPEC was safe and could offer a favorable survival for selected patients with CRPM. Optimal patient selection was critical in ensuring a favorable outcome.

Results from the recent PRODIGE 7 trial[106] has garnered significant interest amongst the peritoneal community. Firstly, many are advocating for HIPEC to be abandoned,[397] with CRS alone to become the standard of care for resectable CRPM. Others argue that

there were many deficiencies in the trial that warrant further study,[419] whilst others conclude that mitomycin C should be the HIPEC drug used instead of oxaliplatin.[471] The latter especially as the pivotal RCT supporting the use of CRS and HIPEC used mitomycin C as HIPEC.[94] This trial, while no doubt a landmark study in the field is yet to be published despite being presented over 18 months ago.

With our unit having used both mitomycin C and oxaliplatin as HIPEC agents in our practice for CRPM, we sought to compare the difference in survival between both HIPEC agents in Chapter 6. We found no difference in overall or disease free survival between the two HIPEC agents (HR 0.50, 95% CI 0.11-2.28). Superficial wound infections were higher with the use of mitomycin C (37.5% v 15.2%, p=0.02), which were managed with dressings and antibiotics. There was however no difference in major morbidity (Grade III/IV) or mortality between the use of either agent. This study, while having small numbers demonstrated no survival difference between the use of either HIPEC agent.

While prognostic factors such as completeness of cytoreduction (CC score) and peritoneal carcinoma index (PCI) have been shown to influence survival following CRS and HIPEC[105, 118, 122, 369, 379] for CRPM, there remains significant variability in patient selection and prognostic factors reported by different studies. Therefore, in Chapter 7, we performed a systematic review and meta-analysis to evaluate key prognostic factors that influence survival in patients undergoing CRS and HIPEC for isolated CRPM. We found that in addition to completeness of cytoreduction and PCI, lymph node involvement, rectal primary, grade III/IV morbidity and adjuvant chemotherapy use were factors that influenced survival in patients undergoing CRS and HIPEC for CRPM. There was an overall moderate degree of heterogeneity amongst all included studies, highlighting the lack of standardisation in the use of CRS and HIPEC in the literature. This study helped identify some key factors that may improve patient selection for CRS and HIPEC. However, it also highlighted the paucity of high quality studies in the field of CRS and HIPEC and the urgent need to explore important areas such as the role of peri-operative systemic chemotherapy, role of HIPEC and the optimal HIPEC agent in prospective studies.

In those undergoing successful CRS and HIPEC, recurrences are common, occurring in up to 80% of patients within 12-18 months.[140, 348, 434] There are no established treatment guidelines for patients with recurrent CRPM, with cases managed on a case-by-case basis. If recurrent disease appears resectable, re-do CRS and HIPEC may be an option, with an evolving role for iterative CRS and HIPEC.[140] In most patients, systemic chemotherapy would become the mainstay of treatment. Novel drug delivery methods such as PIPAC may become a feasible palliative surgical option in patients with recurrent or unresectable CRPM, but needs further evaluation in clinical trials.[173, 435]

Overall, advances in treatment and palliative options have been limited in the field of CRPM, especially when compared to other sites of metastases such as liver or lung metastases. This highlighted a significant unmet need in clinical practice to explore newer modalities of treatment options for patients with CRPM.

The role of the immune system and the tumour microenvironment in anti-tumour immunity has garnered significant interest in recent years. Additionally, the use of immune modulating antibodies in the form of anti-PD-1 and anti CTLA-4 antibodies has revolutionised the treatment landscape for various immunogenic cancers like melanoma, renal cell carcinoma and non-small cell lung cancer in recent years.[263] The introduction of anti-CTLA-4 (ipilimumab) in advanced melanoma for example, has led to improved survival, with a 10-year survival rate of 20%.[264] In CRC, use of immune checkpoint inhibitors has shown significant efficacy only in MSI-H CRC. However, even in the MSI-H CRC cohort, durable responses to checkpoint antibodies were only seen in 53% of patients,[198] highlighting that MSI status alone may not predict response to checkpoint antibodies. Checkpoint antibodies remain unexplored in peritoneal metastases. On a more important note, the immune landscape of peritoneal metastases remains poorly explored.

Chapter 8 therefore, was an exploratory study on exploring the immune landscape of CRPM using a wide range of laboratory based techniques. Firstly, we demonstrated that CRPM do indeed have an immune infiltrate, with a T cell population that is significantly higher than adjacent normal peritoneum, with almost a fifth of T cells expressing PD-1, suggesting a possible role for anti-PD-1 therapy. Using a novel tumouroid-autologous

TIL co-culture system, we firstly demonstrated that TILs from CRPM can be successfully cultured *in-vitro* and are functional in killing tumour cells, with cytotoxicity remaining tumour specific and $CD8^+$ T cell mediated. Functionality was further confirmed by demonstrating IFN-γ, granzyme B and TNF-α release by T cells in the co-culture assays. Additionally, RNASeq data also demonstrated upregulation of cytolytic activity in the form of granzyme A and B, in keeping with functional cytotoxic T cell activity. Additionally, RNASeq showed that checkpoint blockade molecules such as CD274 (PD-1) were up regulated in many of the CRPM. While PD-1 expression was previously considered a marker of T cell exhaustion, recent studies such as Clarke et al demonstrated that many T cells expressing PD-1, especially tissue resident memory cells expressing PD-1 were enriched for superior functionality.[414] Despite being MSS, anti-PD-1 therapy led to significantly improve cytotoxicity of TILs in selected cases in our cohort, demonstrating a possible role for such an assay to be used clinically to evaluate the efficacy of anti-PD-1 therapy in a personalised fashion.

However, despite finding T cells in the TUME, they are unable to clear the tumour *in-vivo*. We further demonstrated that one possible reason for this was that in most cases, the immune infiltrate was stromal in nature, with minimal intra-tumoural T cell infiltrate. Additionally, RNASeq demonstrated that immunosuppressive pathways such as TGF- β pathway were up regulated in many CRPM, suggesting immuno-suppressive methods deployed by the TUME to inhibit T cells from clearing the tumour. PD-L1 was also found on many stromal cells, which may have prevented T cells from trafficking to the tumour cells. RNASeq also revealed most CRPM to be CMS 4, consistent with a strong stromal infiltration, with a highly immunosuppressive TUME associated with an inflamed phenotype and TGF- β activation. These findings greatly extend the current understanding of the immune landscape of CRPM, and suggest that despite being MSS, checkpoint antibodies may have a role in highly selected patients with CRPM. In others, further research using other modalities such as vaccines to stimulate T cells to traffic to the tumour in combination with checkpoint antibodies to overcome the PD-1/PD-L1 axis may hold promise in the treatment of CRPM. The MYPHISMO Phase I/II trial (NCT03287427) is currently recruiting using this combinational approach with vaccines and checkpoint blockade in patients with metastatic MSS CRC.

Another avenue currently attracting interest is the field of personalised medicine, wherein drugs can be rationally assigned, based not just on cancer type, but on the unique molecular features of each cancer- a tumour agnostic model of care. A purely genomics based approach to personalising care has had limited success, possibly because genomics offers a static representation of the tumour, without any knowledge of a tumours functional sensitivities to drugs. Advances in translational oncology with the development of three dimensional pre-clinical models of cancer called tumouroids can help bridge this divide by offering the opportunity to functionally evaluate a tumours sensitivities to drugs, thereby maximising efficacy, and minimising potential side effects or toxicities if a drug is unlikely to work.

In Chapter 9, in a multicentre, prospective study, we successfully established an *in-vitro* patient derived tumouroid-based platform that can be used to integrate functional drug sensitivity testing with genomic profiling to identify suitable therapeutic options in patients with CRPM in a clinically relevant and timely manner. We firstly showed that this *in-vitro* platform can reliably mirror patient drug responses *in-vivo*, and that functional testing based drug sensitivities are concordant with genomic drivers. Additionally, this platform can help identify novel therapeutic options in patients who fail standard treatment options and have no genomic-guided biomarkers.

Advances in molecular analyses have revealed that while tumours such as CRCs share the same broad histology, they are a highly heterogeneous entity,[295] with multiple molecular subtypes,[33] each with their own unique alterations in molecular pathways and tumour microenvironments.[33] This would therefore make a precision medicine model of care a more logical approach, especially with more advanced cancers, compared to current generic treatment regimens with their limited efficacy.

10.2 Future directions

10.2.1 Immune based studies

This thesis has provided some insight into the immune landscape of CRPM, and offered some reasons for the generally poor intra-tumoural infiltrate seen in many CRPM. Studies such as Galon et al[40] and the Immunoscore[42, 43] have focused on Stage I-III CRC with no clear understanding of the role of the immune system in Stage IV disease. A worthwhile study to undertake in the future is a comparative analysis of immune cell infiltrate, in particular $CD8^{+}$, Tregs and macrophages, and its correlation with survival.

Additionally, comparing and contrasting the immune response at different sites would provide insight into any discernible immunological reasons for the poor survival seen with PM. This would involve comparison of synchronous resections involving the primary tumour and PM; peritoneal and liver metastases, primary and liver metastases, and primary, liver and PM. A curated nanostring based RNASeq on FFPE samples from synchronous resections can provide valuable immune specific information that may further our understanding of the different tumour microenvironments present at different sites of disease.

As demonstrated in Chapter 8, the organoid-TIL co-culture platform to evaluate the role of checkpoint antibodies has significant potential in offering checkpoint antibody therapy as a personalised therapy to MSS patients who may not otherwise qualify for immunotherapy. One of the important limitations of this current co-culture is that it is manually analysed. Further research is required to improve this co-culture system by automating it, thereby removing any potential human error. Given we currently do not have any reliable biomarkers to predict responders to immunotherapy, automating this platform to ensure consistency and reproducibility should be a key area for further research. This would allow patients who fail standard lines of treatment to be offered immunotherapy if there is functional evidence based on the assay for benefit from checkpoint antibodies.

10.2.2 Personalised medicine clinical trial

The use of tumouroids for personalising therapy in patients is arguably one of the most exciting avenues for precision medicine. Our *in-vitro* platform can offer novel therapies beyond solely genomic drivers in a clinically relevant time frame. Given these favourable findings, we believe the next logical step would be to translate this into a Phase II multi arm clinical trial targeting patients who are currently on final lines of standard treatment. One of the challenges we anticipate is access to novel drugs, which would require lobbying and engagement with pharmaceuticals to acquire off label use. Compassionate use of any drug is difficult to acquire, and remains a significant challenge for clinicians. Even in our study, while one patient was changed to a higher sensitivity agent, we were unable to access the screening platform predicted top agents due to lack of access. Overcoming this hurdle would greater help evaluate the true potential of our platform, along with offering the best available agents to patients. To date, there have been no prospective reports of patient treatment being altered based on tumouroid drug sensitivity results. These promising results hence provide the impetus for such prospective clinical trials.

11 Appendices

11.1 Appendix 1 (Chapter 3)

11.1.1 Survey questions sent to all colorectal surgeons affiliated with CSSANZ

1. Your level of experience

1. Consultant for >15 years
2. Consultant 5-15 years
3. Consultant <5 years
4. Fellow

2. Location of practice

1. Metropolitan
2. Provincial

3. State/Region of practice

1. Victoria
2. New South Wales
3. Western Australia
4. South Australia/ACT/NT
5. Queensland
6. New Zealand

4. From a health economics point of view, how do you feel peritoneal disease should be managed at a state level?

1. CRS and HIPEC facilities at all hospitals

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

2. Single state based centre for highly complex and re-do cases. However, CRS and HIPEC facilities at all tertiary hospitals for other cases

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

3. Single state based referral centre for all peritoneal disease referrals

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

5. In the following circumstances, what do you feel is the change in survival when CRS and HIPEC is used, compared to systemic chemotherapy alone?

1. Perforated appendiceal neoplasm and subsequent pseudomyxoma peritoneii

1 Much lower	2 Lower	3 About the same	4 Higher	5 Much higher

2. Low volume colorectal peritoneal metastases

1 Much lower	2 Lower	3 About the same	4 Higher	5 Much higher

3. Ovarian cancer with low volume peritoneal metastases

1 Much lower	2 Lower	3 About the same	4 Higher	5 Much higher

4. Gastric cancer with low volume peritoneal metastases

1 Much lower	2 Lower	3 About the same	4 Higher	5 Much higher

The following questions utilise a short hypothetical scenario involving detection of peritoneal metastases or peritoneal recurrence. For each case, please choose the option that you feel is most appropriate.

6. A surveillance CT scan shows a new solitary 2 cm peritoneal nodule suggestive of a peritoneal recurrence 18 months after a right hemicolectomy for a T3N1 caecal cancer. How do you feel about each of the following management options?

1) Surgery to excise this solitary nodule

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

2) Biopsy of the nodule

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

3) Refer to medical oncology for systemic chemotherapy

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

4) Refer to a peritoneal disease centre for consideration of CRS and HIPEC

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

7. Surveillance scans are clear six months after resection for a T4aN0 transverse colon cancer. On a scale of 1 to 5, how do you feel about each of the following?

1) Diagnostic laparoscopy to assess for evidence of peritoneal recurrence

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

2) Referral to peritoneal centre for consideration of "second look" laparotomy and HIPEC

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

3) Continue with standard CEA/ staging CT scans and surveillance as per National NHMRC guidelines

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

8. CT scan 6 months following anterior resection for T3N1 sigmoid cancer shows a new 6 cm right adnexal mass. The CEA is also elevated at 15 (normal 0-5). On a scale of 1-5, how strongly would you agree with each of the following as the next step in management?

1) Further systemic chemotherapy

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

2) Diagnostic laparoscopy to assess peritoneal carcinoma index (PCI).

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

3) Referral to Gynae-Oncology for consideration of oophorectomy

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

4) Referral to peritoneal disease centre for consideration of CRS and HIPEC.

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

9. At laparoscopy for a right hemicolectomy for caecal cancer, three peritoneal deposits are seen in the greater omentum. There is no other metastatic disease. How do you proceed? (On a scale of 1 to 5, how do you feel about each of the following?)

1) Complete right hemicolectomy as planned.

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

2) Perform omentectomy with right hemicolectomy

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

3) Complete right hemicolectomy and only biopsy an omental deposit for histological confirmation of metastatic disease.

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

4) Biopsy omental deposit if possible and do not proceed with the operation. Discuss case at multidisciplinary meeting (MDT) and refer to a peritoneal disease centre for consideration of CRS and HIPEC.

1 Strongly Disagree	2 Disagree	3 Undecided	4 Agree	5 Strongly agree

10. On a scale of 1-5, what do you feel is the risk of developing peritoneal recurrence in the following situations?

1. T4 tumour (Non perforated)

1 Very low	2 Low	3 Average	4 High	5 Very High

2. Perforated cancer

1 Very low	2 Low	3 Average	4 High	5 Very High

3. Ovarian metastases evident at time of index operation

1 Very low	2 Low	3 Average	4 High	5 Very High

4. Isolated peritoneal metastases that were removed at the time of the index operation

1 Very low	2 Low	3 Average	4 High	5 Very High

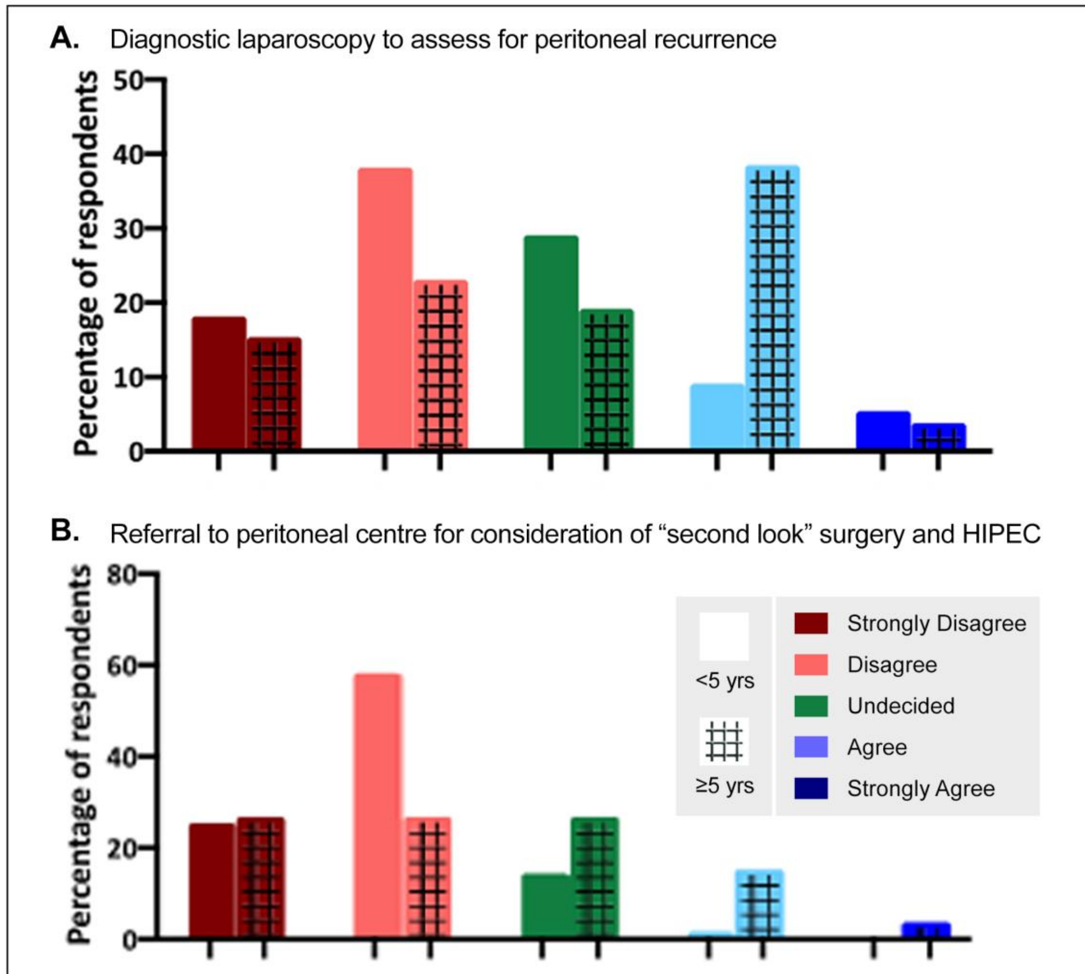
5. Obstructed cancer

1 Very low	2 Low	3 Average	4 High	5 Very High

6. Tumour spillage intra-operatively

1 Very low	2 Low	3 Average	4 High	5 Very High

11.1.2 Appendix 1.2. Role of surgical experience on management of peritoneal metastases



Appendix 1.2a. Younger surgeons were more supportive of a laparoscopy to assess PCI compared to experienced surgeons (38.5% v 9.1%, p = 0.036). Similarly, younger surgeons agreed more than experienced surgeons with referring such a case to a peritoneal centre (15.4% v 1.8%, p=0.014) (Appendix 1.2bb)

11.2 Appendix 2 (Chapter 4)

11.2.1 Appendix 2.1. Flowchart of all patients undergoing CRS and HIPEC for PSM

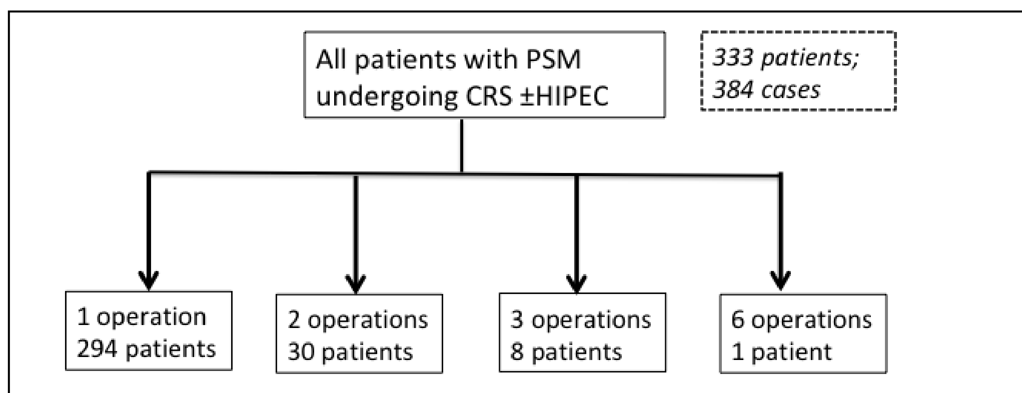
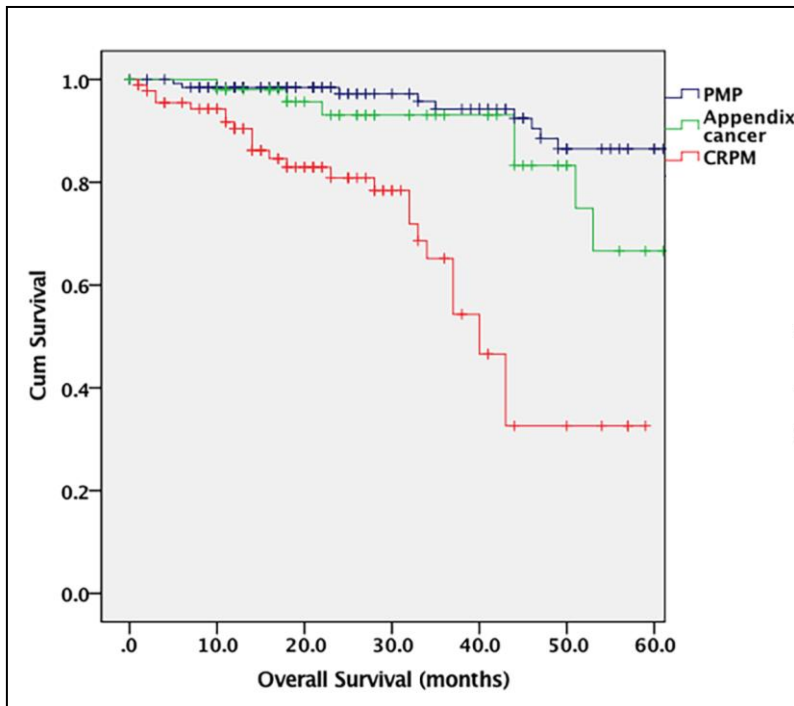


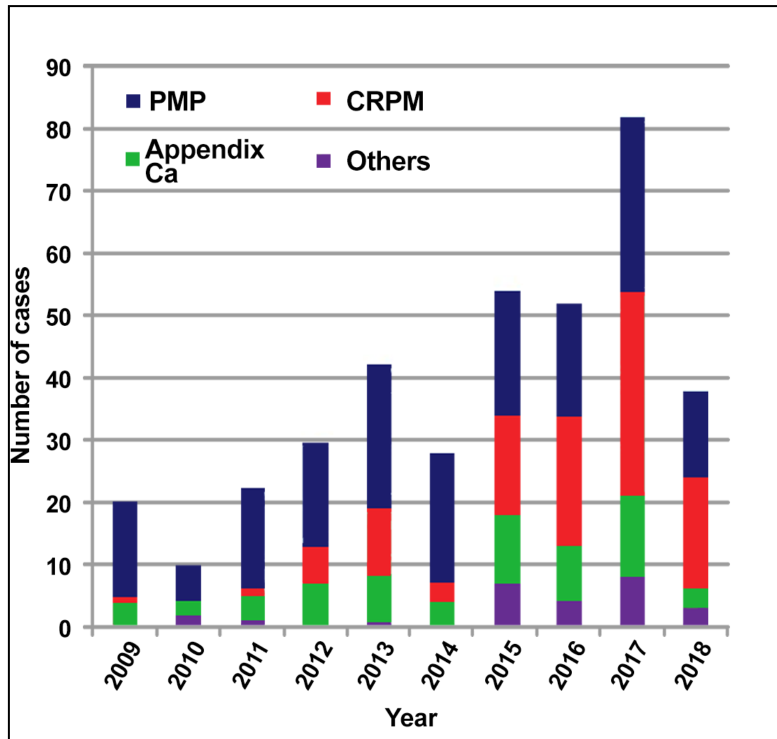
Figure 4.1. Flowchart of all patients undergoing CRS and HIPEC.

11.2.2 Appendix 2.2. Median OS after complete cytoreduction



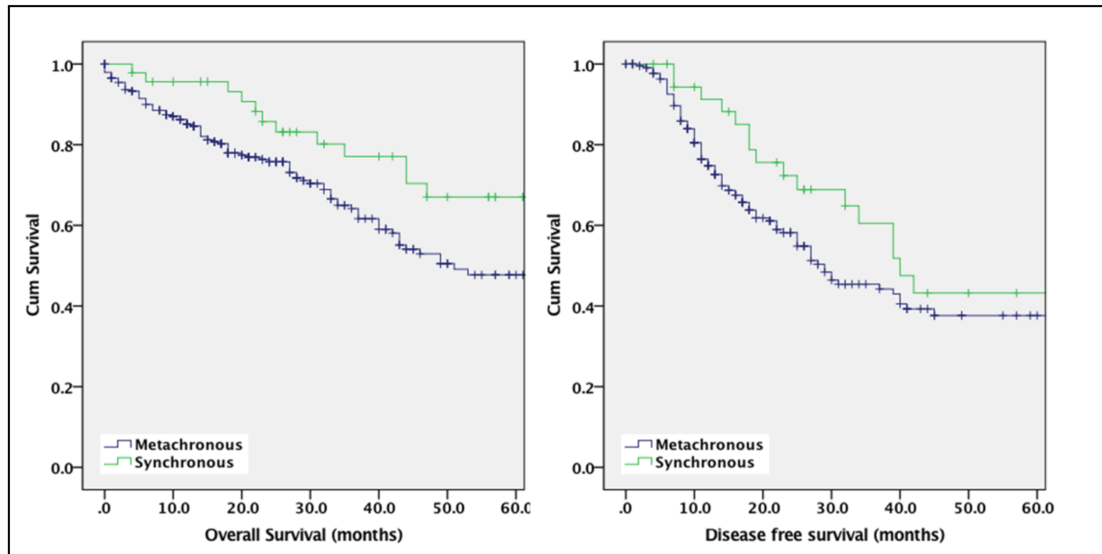
Appendix 2.2. Overall survival following complete cytoreduction based on histology. Median OS not reached for PMP and appendiceal cancers and 40 months for CRPM (log rank p <0.001)

11.2.3 Appendix 2.3. Workload from CRS and HIPEC for PSM from 2009-2018



Appendix 2.3. Bar graph demonstrating CRS and HIPEC cases since the institution of the peritoneal disease service. 2018 numbers are upto 31 May 2018

11.2.4 Appendix 2.4. Synchronous versus metachronous resections



Appendix 2.4a. Median OS was 97 months in those having a synchronous resection, compared to 63 months for those having a metachronous resection (log rank p=0.10). Appendix 2.4b. Median DFS months was 40 months in those having a synchronous resection, compared to 29 months for those having a metachronous resection (log rank p=0.25)

11.3 Appendix 3 (Chapter 7)

11.3.1 Appendix 3.1: Search Strategy

1. (colorectal cance* or colorectal neoplas* or colorectal tumo* or colorectal carcinom*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
2. (CRS or cytoreductive surger*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
3. (HIPEC or hyperthermic intraperitoneal chemotherap* or intraperitoneal chemohyperthermia or IPCH).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
4. (peritoneal or peritoneum).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary

concept word, rare disease supplementary concept word, unique identifier, synonyms]

5. 1 and 2 and 3 and 4
6. limit 5 to (yr="2008 - 2018" and english)

11.3.2 Appendix 3.2: Studies excluded from meta-analysis

Number	Study	Reason for exclusion
1	Shen et al. (2009)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
2	Chua et al. (2010)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
3	Cavaliere et al. (2011)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
4	Passot et al. (2012)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
5	Gervais et al. (2013)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
6	Votanopoulos et al. (2013)	Inadequate data to allow hazard ratios and confidence intervals to be extracted
7	Massalou et al. (2017	Inadequate data to allow hazard ratios and confidence intervals to be extracted
8	Tonello et al. (2018))	Inadequate data to allow hazard ratios and confidence intervals to be extracted

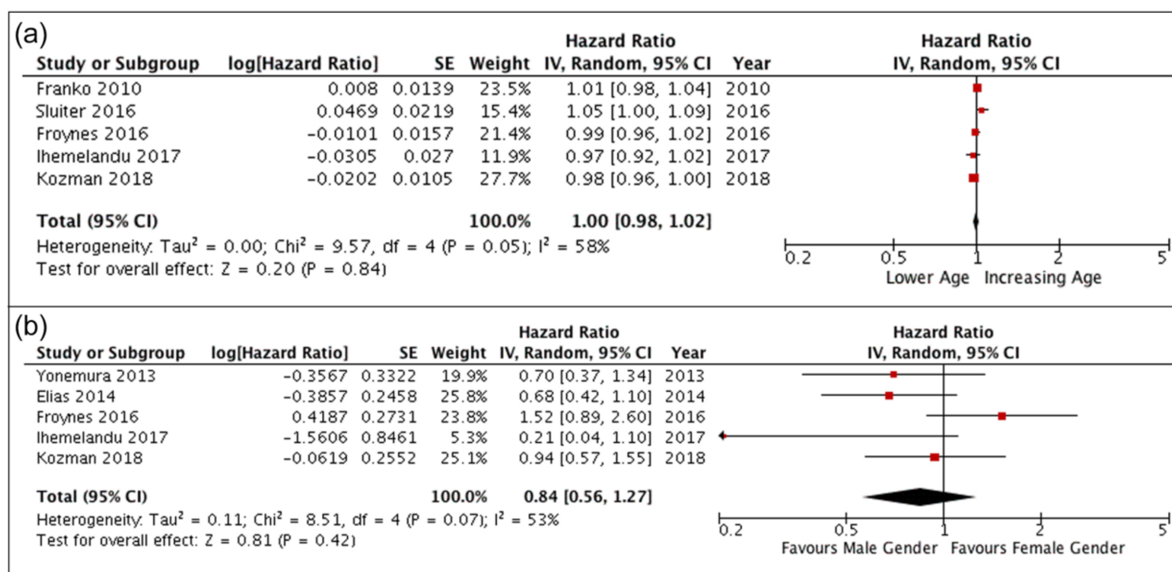
11.3.3 Appendix 3.3: Study Quality assessment

No.	Study	Selection	Comparability	Outcome	Total
1	Yan et al. (2008)	***		**	*****
2	Shen et al. (2009)	***		***	******
3	Chua et al. (2010)	***		***	******
4	Franko et al. (2010)	***		**	*****
5	Elias et al. (2010)	***		***	******
6	Cavaliere et al. (2011)	***		***	******
7	Quenet et al. (2011)	***		***	******
8	Cashin et al. (2012)	***		**	****
9	Benizri et al. (2012)	***		***	******
10	Passot et al. (2012)	***		***	******
11	Gervais et al. (2013)	***		***	******
12	Ung et al. (2013)	***		***	******
13	Votanopoulos et al. (2013)	***		***	******
14	Yonemura et al. (2013)	***		**	*****
15	Baratti et al. (2014)	***		***	******
16	Ceelen et al. (2014)	***		***	******
17	Elias et al. (2014)	***		**	*****
18	Hompes et al. (2014)	***		***	******
19	Huang et al. (2014)	***		***	******
20	Passot et al. (2014)	***		***	******
21	Prada-Villaverde et al. (2014)	***		**	*****
22	Rivard et al. (2014)	***		***	******
23	Faron et al. (2016)	***		***	******
24	Frøysnes et al. (2016)	***		***	******
25	Maillet et al. (2016)	***		**	****
26	Ng et al. (2016)	***		***	******
27	Sluiter et al. (2016)	***		***	******
28	Ihemelandu et al. (2017)	***		***	******
29	Leung et al. (2017)	***		**	*****
30	Massalou et al. (2017)	***		***	******
31	Kozman et al. (2018)	***		**	*****
32	Sluiter et al. (2018)	***		***	******
33	Tonello et al. (2018)	***		**	*****

11.3.4 Appendix 3.4: Risk of bias assessment

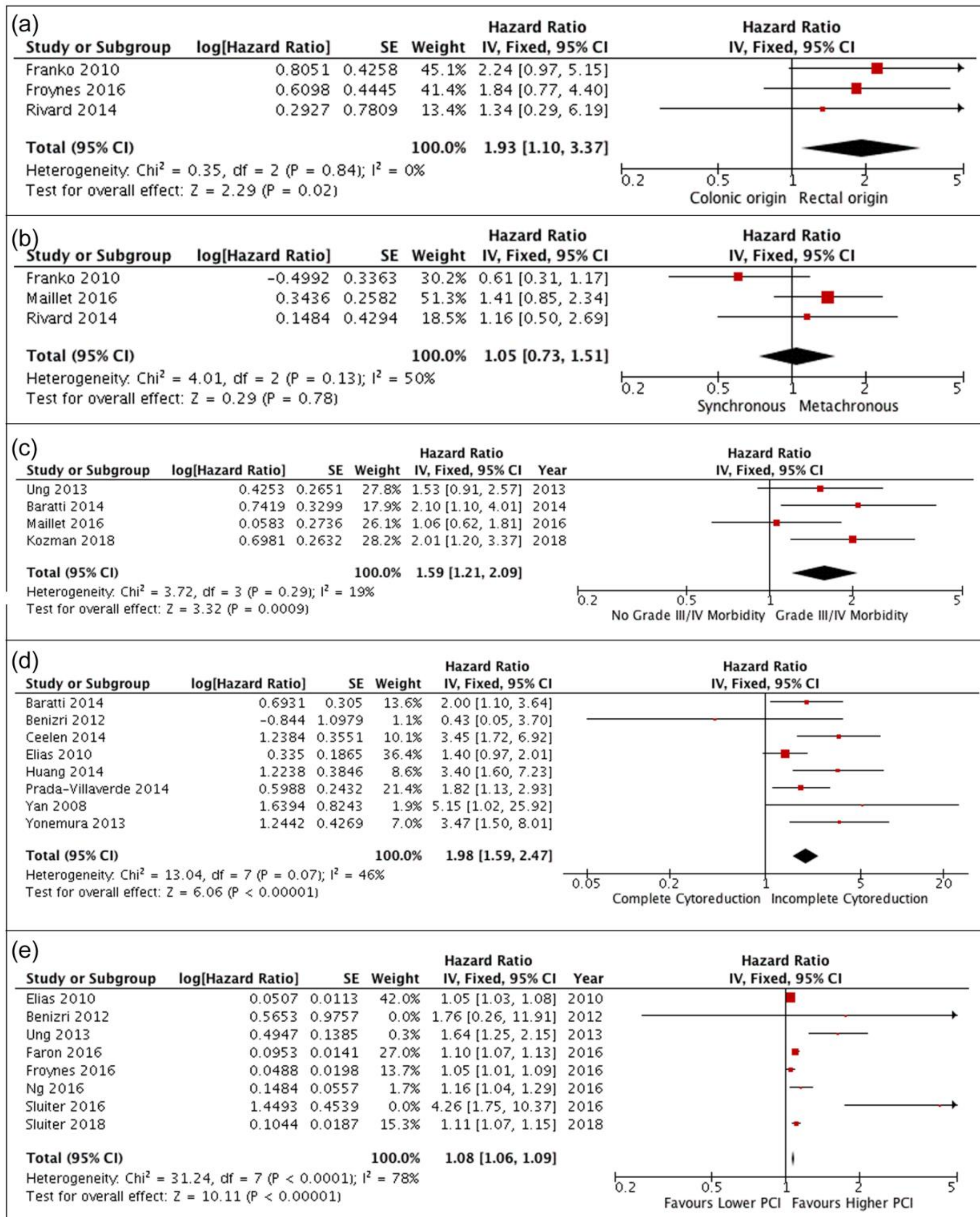
No.	Study	Allocation Concealment (Selection Bias)	Assessment of Exposure (Self-report)	Outcome of Interest Present at Beginning	Confounding Bias	Incomplete Data	Selective Reporting (Reporting bias)	Total*
1	Yan et al. (2008)	?	+	+	+	?	+	4
2	Shen et al. (2009)	?	+	+	+	?	+	4
3	Chua et al. (2010)	?	+	+	-	?	+	3
4	Franko (2010)	?	+	+	?	?	+	3
5	Elias et al. (2010)	?	+	+	+	?	+	4
6	Cavaliere et al. (2011)	?	+	+	+	?	+	4
7	Quenet et al. (2011)	?	+	+	+	?	+	4
8	Benizri et al. (2012)	?	+	+	+	?	+	4
9	Cashin et al. (2012)	?	+	+	?	?	+	3
10	Passot et al. (2012)	?	+	+	+	?	+	4
11	Gervais et al. (2013)	?	+	+	+	?	+	4
12	Ung et al. (2013)	?	+	+	+	?	+	4
13	Votanopoulos et al. (2013)	?	+	+	-	?	+	3
14	Yonemura et al. (2013)	?	+	+	+	?	+	4
15	Baratti et al. (2014)	?	+	+	+	?	+	4
16	Ceelen et al. (2014)	?	+	+	+	?	+	4
17	Elias et al. (2014)	?	+	+	?	?	+	3
18	Hompes et al. (2014)	?	+	+	?	?	+	3
19	Huang et al. (2014)	?	+	+	+	?	+	4
20	Passot et al. (2014)	?	+	+	+	?	+	4
21	Prada-Villaverde et al. (2014)	?	+	+	+	?	+	4
22	Rivard et al. (2014)	?	+	+	+	?	+	4
23	Faron et al. (2016)	?	+	+	+	?	+	4
24	Frøysnes et al. (2016)	?	+	+	+	?	+	4
25	Maillet et al. (2016)	?	+	+	+	?	+	4
26	Ng et al. (2016)	?	+	+	+	?	+	4
27	Sluiter et al. (2016)	?	+	+	+	?	+	4
28	Ihemelandu et al. (2017)	?	+	+	+	?	+	4
29	Leung et al. (2017)	?	+	+	?	?	+	3
30	Massalou et al. (2017)	?	+	+	+	?	+	4
31	Kozman et al. (2018)	?	+	+	+	?	+	4
32	Sluiter et al. (2018)	?	+	+	+	?	+	4
33	Tonello et al. (2018)	?	+	+	-	?	+	3

11.3.5 Appendix 3.5: Forest plot for age and gender



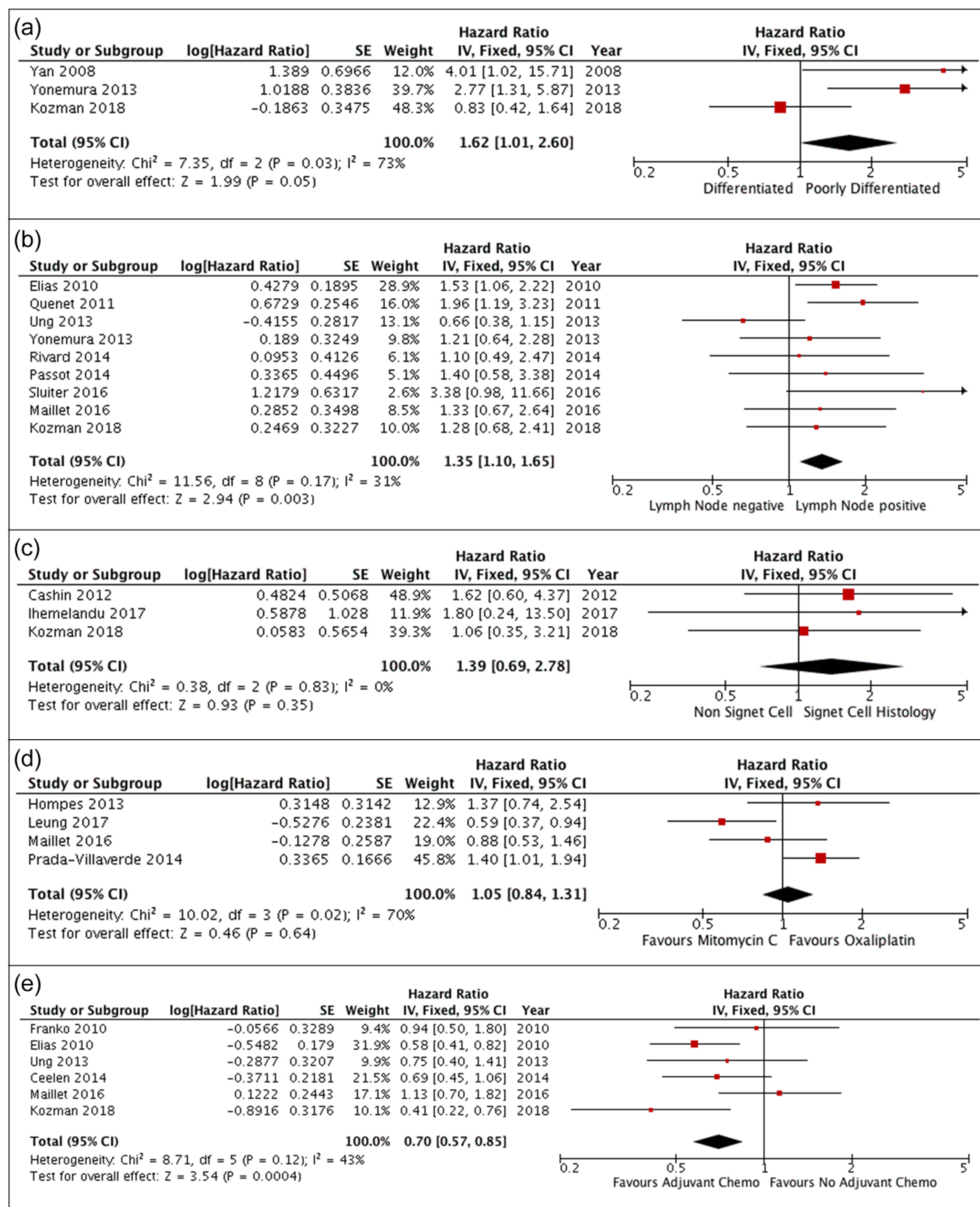
Appendix 3.5 (a-b). Forest plot demonstrating role of age and gender in influencing survival in patients undergoing CRS and HIPEC

11.3.6 Appendix 3.6: Forest plot using fixed effects model



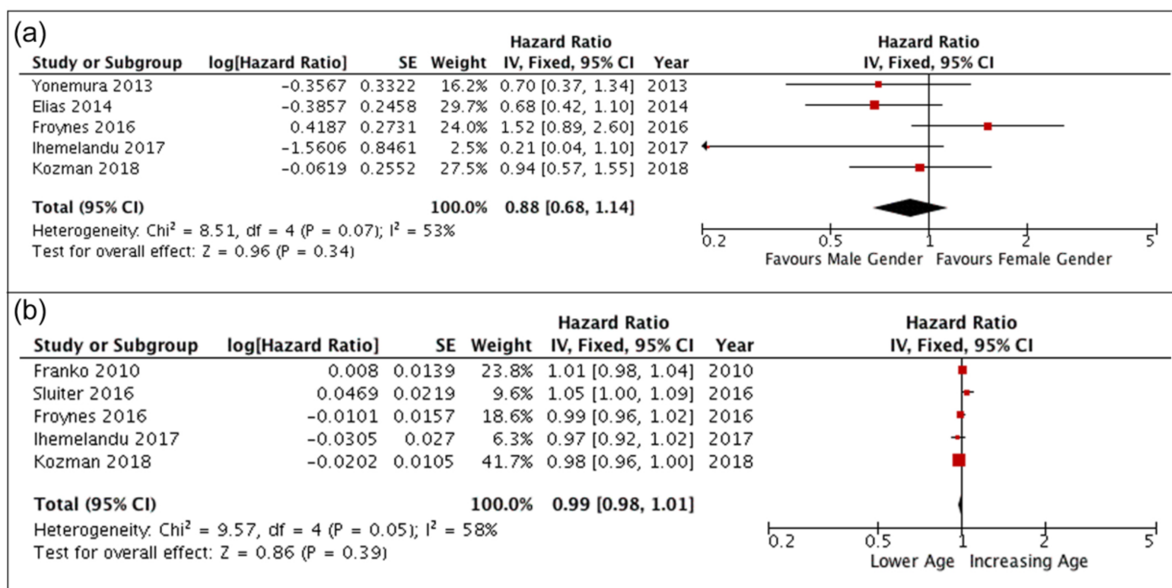
Appendix 3.6 (a-e) Forest plot demonstrating prognostic factors influencing survival following CRS and HIPEC using fixed effects model

11.3.7 Appendix 3.7: Forest plot using fixed effects model



Appendix 3.7 (a-e). Forest plot demonstrating prognostic factors influencing survival following CRS and HIPEC using fixed effects model

11.3.8 Appendix 3.8: Forest plot using fixed effects model


(a)

Study or Subgroup	log[Hazard Ratio]	SE	Weight	Hazard Ratio IV, Fixed, 95% CI	Year
Yonemura 2013	−0.3567	0.3322	16.2%	0.70 [0.37, 1.34]	2013
Elias 2014	−0.3857	0.2458	29.7%	0.68 [0.42, 1.10]	2014
Froynes 2016	0.4187	0.2731	24.0%	1.52 [0.89, 2.60]	2016
Ihemelandu 2017	−1.5606	0.8461	2.5%	0.21 [0.04, 1.10]	2017
Kozman 2018	−0.0619	0.2552	27.5%	0.94 [0.57, 1.55]	2018
Total (95% CI)			**100.0%**	**0.88 [0.68, 1.14]**	

Heterogeneity: $Chi^2 = 8.51$, df = 4 (P = 0.07); $I^2 = 53\%$
Test for overall effect: Z = 0.96 (P = 0.34)

(b)

Study or Subgroup	log[Hazard Ratio]	SE	Weight	Hazard Ratio IV, Fixed, 95% CI	Year
Franko 2010	0.008	0.0139	23.8%	1.01 [0.98, 1.04]	2010
Sluiter 2016	0.0469	0.0219	9.6%	1.05 [1.00, 1.09]	2016
Froynes 2016	−0.0101	0.0157	18.6%	0.99 [0.96, 1.02]	2016
Ihemelandu 2017	−0.0305	0.027	6.3%	0.97 [0.92, 1.02]	2017
Kozman 2018	−0.0202	0.0105	41.7%	0.98 [0.96, 1.00]	2018
Total (95% CI)			**100.0%**	**0.99 [0.98, 1.01]**	

Heterogeneity: $Chi^2 = 9.57$, df = 4 (P = 0.05); $I^2 = 58\%$
Test for overall effect: Z = 0.86 (P = 0.39)

Appendix 3.8 (a-e). Forest plot demonstrating prognostic factors influencing survival following CRS and HIPEC using fixed effects model

11.4 Appendix 4 (Chapter 9)

11.4.1 Appendix 4.1: Whole exome data summary

Table of whole exome data summary of 14 CRPM samples

Patient number	Gene symbol	Panel	Chrom	Position	Tier	TYPE	Impact	Effect	AA Change	Protein length	Confidence
1	APC	MSK.IMPACT OncoPlex	5	112175475	1	INDEL	HIGH	frameshift_variant	p.Phe1396fs/c.4188delT	2843	4
1	ERBB3	MSK.IMPACT OncoPlex	12	56482537	1	SNP	MODERATE	missense_variant	p.Glu332Lys/c.994G>A	1342	7
1	HIST1H3F	MSK.IMPACT	6	26250721	1	SNP	MODERATE	missense_variant	p.Lys38Thr/c.113A>C	136	7
1	SOX9	MSK.IMPACT	17	70119857	1	INDEL	HIGH	frameshift_variant	p.Phe289fs/c.860_866dupAGACC TT	509	4
1	STAT5B	MSK.IMPACT	17	40375417	1	SNP	MODERATE	missense_variant	p.Glu178Gly/c.533A>G	787	6
1	KRAS	MSK.IMPACT OncoPlex	12	25398284	2	SNP	MODERATE	missense_variant	p.Gly12Asp/c.35G>A	189	6
1	PIK3CA	MSK.IMPACT OncoPlex	3	178952077	2	SNP	MODERATE	missense_variant	p.Asn1044Lys/c.3132T>A	1068	7
1	TP53	MSK.IMPACT OncoPlex	17	7577094	2	SNP	MODERATE	missense_variant	p.Arg282Trp/c.844C>T	393	6
1	ASXL1	MSK.IMPACT OncoPlex	20	31017738	3	SNP	LOW	synonymous_variant	p.Gly200Gly/c.600C>T	1541	7
1	EPHA3	MSK.IMPACT OncoPlex	3	89390819	5	SNP	MODIFIER	intron_variant	c.971-86C>A	983	6
1	GNAS	MSK.IMPACT OncoPlex	20	57415854	5	SNP	LOW	synonymous_variant	p.Pro231Pro/c.693G>A	245	7
1	PTPRT	MSK.IMPACT	20	40713294	5	SNP	MODIFIER	intron_variant	c.4136+28C>A	1441	7
1	ABCC4	OncoPlex	13	95705614	6	SNP	MODIFIER	intron_variant	c.3367-176C>T	1325	4
1	ARID2	MSK.IMPACT	12	46205492	6	SNP	MODIFIER	intron_variant	c.418+158C>A	1835	5
1	ARID5B	MSK.IMPACT	10	63845700	6	SNP	MODIFIER	intron_variant	c.1398+41G>T	1188	7
1	ATR	MSK.IMPACT	3	142281046	6	SNP	MODIFIER	intron_variant	c.1170+28A>T	2644	7
1	CCND1	MSK.IMPACT OncoPlex	11	69457677	6	SNP	MODIFIER	intron_variant	c.199-122A>T	295	5
1	HDAC4	OncoPlex	2	240111474	6	SNP	MODIFIER	intron_variant	c.339+55C>T	1084	6

1	MLH1	MSK.IMPACT OncoPlex	3	37089840	6	INDEL	MODIFIER	intron_variant	c.1897-162delT	756	3
1	NOTCH1	MSK.IMPACT OncoPlex	9	139412002	6	SNP	MODIFIER	intron_variant	c.1442-165G>A	2555	7
1	NOTCH4	MSK.IMPACT	6	32191544	6	SNP	MODIFIER	intron_variant	c.73+89G>A	2003	7
1	RSPO2	OncoPlex	8	108995261	6	SNP	MODIFIER	intron_variant	c.283+6023G>C	243	5
1	SUFU	MSK.IMPACT OncoPlex	10	104269079	6	SNP	MODIFIER	intron_variant	c.317+19T>G	484	7
1	TEK	MSK.IMPACT	9	27168432	6	SNP	MODIFIER	intron_variant	c.365-61A>G	1124	5
2	TCF7L2	MSK.IMPACT	10	114911559	1	SNP	MODERATE	missense_variant	p.Leu359Phe/c.1077G>C	602	5
2	YES1	MSK.IMPACT	18	751792	1	INDEL	MODERATE	disruptive_inframe_deletion	p.Thr94del/c.281_283delCTA	543	3
2	APC	MSK.IMPACT OncoPlex	5	112154826	2	INDEL	HIGH	frameshift_variant	p.Ser367fs/c.1100_1101delCT	2843	4
2	APC	MSK.IMPACT OncoPlex	5	112116592	2	SNP	HIGH	stop_gained	p.Arg213*/c.637C>T	2843	7
2	MITF	MSK.IMPACT OncoPlex	3	70014280	2	SNP	MODERATE	missense_variant	p.Asp482Asn/c.1444G>A	520	7
2	TP53	MSK.IMPACT OncoPlex	17	7578212	2	SNP	HIGH	stop_gained	p.Arg213*/c.637C>T	393	7
2	DPYD	OncoPlex	1	97770763	5	INDEL	MODIFIER	intron_variant	c.2299+51_2299+52insC	1025	4
2	ATR	MSK.IMPACT	3	142232189	6	SNP	MODIFIER	intron_variant	c.4641+154T>A	2644	6
2	CARM1	MSK.IMPACT	19	11032442	6	SNP	MODIFIER	3_prime_UTR_variant	c.*9C>A	608	7
2	CARM1	MSK.IMPACT	19	11032443	6	SNP	MODIFIER	3_prime_UTR_variant	c.*10C>A	608	7
2	DDR2	MSK.IMPACT OncoPlex	1	162730863	6	SNP	MODIFIER	intron_variant	c.856-138A>G	855	6
2	DDR2	MSK.IMPACT OncoPlex	1	162730864	6	SNP	MODIFIER	intron_variant	c.856-137G>T	855	6
2	NOTCH2	MSK.IMPACT OncoPlex	1	120572496	6	SNP	MODIFIER	intron_variant	c.155+33C>T	2471	4
2	RET	MSK.IMPACT OncoPlex	10	43590052	6	SNP	MODIFIER	intron_variant	c.74-5855G>A	1114	4
2	TCF3	MSK.IMPACT	19	1627188	6	SNP	MODIFIER	intron_variant	c.366+170C>T	654	6
2	TERT	MSK.IMPACT	5	1254106	6	SNP	MODIFIER	intron_variant	c.3296-160C>T	1132	7
3	APC	MSK.IMPACT OncoPlex	5	112175753	1	INDEL	HIGH	frameshift_variant	p.Leu1488fs/c.4464_4465delAT	2843	4
3	BMPR1A	MSK.IMPACT	10	88679142	1	SNP	MODERATE	missense_variant	p.Arg361Pro/c.1082G>C	532	6
3	CHD1	OncoPlex	5	98217814	1	SNP	MODERATE	missense_variant	p.Arg911His/c.2732G>A	1710	7
3	FUBP1	MSK.IMPACT	1	78414922	1	SNP	HIGH	stop_gained	p.Trp615*/c.1844G>A	644	7
3	RASA1	MSK.IMPACT	5	86672857	1	SNP	MODERATE	missense_variant+ splice_region_variant	p.Asp782Asn/c.2344G>A	1047	7
3	APC	MSK.IMPACT OncoPlex	5	112164616	2	SNP	HIGH	stop_gained	p.Arg564*/c.1690C>T	2843	7
3	TP53	MSK.IMPACT OncoPlex	17	7577022	2	SNP	HIGH	stop_gained	p.Arg306*/c.916C>T	393	7
3	SMARCB1	MSK.IMPACT OncoPlex	22	24129401	5	SNP	LOW	synonymous_variant	p.Val15Val/c.45G>T	385	7

3	ATR	MSK.IMPACT	3	142222305	6	SNP	MODIFIER	intron_variant	c.5197-10A>T	2644	4
3	BAP1	MSK.IMPACT OncoPlex	3	52442354	6	SNP	MODIFIER	intron_variant	c.255+136C>T	729	6
3	CENPA	MSK.IMPACT	2	27014927	6	SNP	MODIFIER	intron_variant	c.101-72A>G	140	4
3	CHEK1	MSK.IMPACT OncoPlex	11	125505576	6	SNP	MODIFIER	intron_variant	c.718+148T>A	476	6
3	ESR1	MSK.IMPACT OncoPlex	6	152129574	6	SNP	MODIFIER	intron_variant	c.452+75G>A	595	4
3	FANCA	MSK.IMPACT OncoPlex	16	89807094	6	SNP	MODIFIER	intron_variant	c.3828+118G>A	1455	6
3	HGF	MSK.IMPACT	7	81355062	6	SNP	MODIFIER	intron_variant	c.1168+144A>G	728	4
3	LRP2	OncoPlex	2	170129371	6	SNP	MODIFIER	intron_variant	c.2116+66T>G	4655	4
3	NF1	MSK.IMPACT OncoPlex	17	29654886	6	SNP	MODIFIER	intron_variant	c.5609+29G>C	2839	6
3	NF2	MSK.IMPACT OncoPlex	22	30079213	6	SNP	MODIFIER	intron_variant	c.1737+1623T>C	595	4
3	NF2	MSK.IMPACT OncoPlex	22	30091020	6	SNP	MODIFIER	3_prime_UTR_variant	c.*229T>A	595	5
3	NKX2-1	MSK.IMPACT OncoPlex	14	36986296	6	INDEL	MODIFIER	3_prime_UTR_variant	c.*186_*187insCCACCC	401	3
3	NKX2-1	MSK.IMPACT OncoPlex	14	36986303	6	INDEL	MODIFIER	3_prime_UTR_variant	c.*176_*179delTTTT	401	3
3	NTRK3	MSK.IMPACT OncoPlex	15	88420004	6	INDEL	MODIFIER	3_prime_UTR_variant	c.*161dupA	825	3
3	RB1	MSK.IMPACT OncoPlex	13	48941610	6	SNP	MODIFIER	intron_variant	c.940-20A>G	928	5
3	SDHA	MSK.IMPACT	5	231228	6	SNP	MODIFIER	intron_variant	c.895+113C>G	664	7
3	STAT3	MSK.IMPACT	17	40475534	6	SNP	MODIFIER	intron_variant	c.1653+57G>A	770	7
3	SUZ12	MSK.IMPACT OncoPlex	17	30267579	6	INDEL	MODIFIER	intron_variant	c.386+85dupA	739	3
3	TYR	OncoPlex	11	88960972	6	INDEL	MODIFIER	intron_variant	c.1037-10dupT	529	3
4	APC	MSK.IMPACT OncoPlex	5	112175215	1	INDEL	HIGH	frameshift_variant	p.Glu1309fs/c.3925delG	2843	4
4	INHBA	MSK.IMPACT	7	41729375	1	SNP	MODERATE	missense_variant	p.Ala385Val/c.1154C>T	426	7
4	SMAD4	MSK.IMPACT OncoPlex	18	48604787	1	SNP	MODERATE	missense_variant	p.Asp537Tyr/c.1609G>T	552	7
4	SOS1	MSK.IMPACT	2	39262360	1	SNP	MODERATE	missense_variant	p.Leu356Arg/c.1067T>G	1333	7
4	APC	MSK.IMPACT OncoPlex	5	112151261	2	SNP	HIGH	stop_gained	p.Arg302*/c.904C>T	2843	7
4	ATM	MSK.IMPACT OncoPlex	11	108196831	2	SNP	MODERATE	missense_variant	p.Ser2285Ile/c.6854G>T	3056	7
4	GNAS	MSK.IMPACT OncoPlex	20	57484420	2	SNP	MODERATE	missense_variant	p.Arg844Cys/c.2530C>T	1037	6
4	GNAS	MSK.IMPACT OncoPlex	20	57484421	2	SNP	MODERATE	missense_variant	p.Arg844His/c.2531G>A	1037	4
4	NTRK3	MSK.IMPACT OncoPlex	15	88680705	2	SNP	MODERATE	missense_variant	p.Ser184Arg/c.552C>A	839	7
4	POLE	MSK.IMPACT OncoPlex	12	133249223	2	SNP	MODERATE	missense_variant	p.Arg559Gln/c.1676G>A	2286	7

4	TP53	MSK.IMPACT OncoPlex	17	7578529	2	SNP	MODERATE	missense_variant	p.Phe134Cys/c.401T>G	393	7
4	DROSHA	MSK.IMPACT	5	31483406	5	SNP	MODIFIER	intron_variant	c.2071+255G>A	1374	5
4	HIST1H3D	MSK.IMPACT	6	26198997	5	SNP	MODIFIER	intron_variant	c.-20+90G>C	136	6
4	IKZF1	MSK.IMPACT OncoPlex	7	50454939	5	INDEL	MODIFIER	intron_variant	c.590-4478delT	477	3
4	KMT2A	MSK.IMPACT OncoPlex	11	118351035	5	INDEL	MODIFIER	intron_variant	c.3634+92dupT	3972	4
4	RASA1	MSK.IMPACT	5	86670294	5	SNP	MODIFIER	intron_variant	c.1934+157T>C	1047	6
4	CARD11	MSK.IMPACT	7	2976575	6	SNP	MODIFIER	intron_variant	c.1341+96A>G	1154	5
4	ERCC4	MSK.IMPACT	16	14016357	6	SNP	MODIFIER	intron_variant	c.388+289G>A	916	7
4	HIF1A	OncoPlex	14	62164550	6	INDEL	MODIFIER	5_prime_UTR_variant	c.-9dupT	850	3
4	HNF1A	MSK.IMPACT OncoPlex	12	121435756	6	SNP	MODIFIER	intron_variant	c.1501+288A>G	631	5
4	INPP4B	MSK.IMPACT	4	143129525	6	SNP	MODIFIER	intron_variant	c.1072+53C>T	924	6
4	LRP2	OncoPlex	2	170025198	6	INDEL	MODIFIER	intron_variant	c.11498-13delT	4655	4
4	NTRK3	MSK.IMPACT OncoPlex	15	88575787	6	SNP	MODIFIER	intron_variant	c.1585+301T>G	839	5
4	TET1	MSK.IMPACT OncoPlex	10	70412454	6	SNP	MODIFIER	intron_variant	c.4461+103T>C	2136	7
5	APC	MSK.IMPACT OncoPlex	5	112173324	1	INDEL	HIGH	frameshift_variant	p.Ser678fs/c.2034delT	2843	4
5	ATRX	MSK.IMPACT OncoPlex	X	76944315	1	SNP	MODERATE	missense_variant	p.Cys197Phe/c.590G>T	2492	7
5	HSPH1	OncoPlex	13	31719822	1	INDEL	HIGH	frameshift_variant	p.Gly488fs/c.1461dupT	858	4
5	KDM5C	MSK.IMPACT	X	53227029	1	SNP	MODERATE	missense_variant	p.Thr849Ile/c.2546C>T	1560	7
5	PHF6	OncoPlex	X	133547854	1	SNP	MODERATE	missense_variant +splice_region_variant	p.Arg196Thr/c.587G>C	365	5
5	PTEN	MSK.IMPACT OncoPlex	10	89711902	1	SNP	MODERATE	missense_variant	p.Tyr174His/c.520T>C	403	7
5	PTEN	MSK.IMPACT OncoPlex	10	89717762	1	SNP	HIGH	stop_gained	p.Lys263*/c.787A>T	403	7
5	AMER1	MSK.IMPACT	X	63412090	2	SNP	MODERATE	missense_variant	p.Ser359Arg/c.1077T>G	1135	5
5	BRCA1	MSK.IMPACT OncoPlex	17	41246559	2	SNP	MODERATE	missense_variant	p.Asp330Val/c.989A>T	1884	4
5	KRAS	MSK.IMPACT OncoPlex	12	25398281	2	SNP	MODERATE	missense_variant	p.Gly13Asp/c.38G>A	189	7
5	NPRL3	OncoPlex	16	138792	2	SNP	MODERATE	missense_variant	p.Thr483Met/c.1448C>T	568	4
5	PIK3CA	MSK.IMPACT OncoPlex	3	178952146	2	INDEL	HIGH	frameshift_variant	p.Asn1068fs/c.3203dupA	1068	4
5	PML	OncoPlex	15	74327655	2	INDEL	HIGH	frameshift_variant	p.Ala621fs/c.1859dupC	829	4
5	PTPRT	MSK.IMPACT	20	40710553	2	SNP	MODERATE	missense_variant	p.Arg1414His/c.4241G>A	1441	7
5	EPHA5	MSK.IMPACT OncoPlex	4	66201754	3	SNP	LOW	synonymous_variant	p.Gln916Gln/c.2748G>A	1037	4
5	HLA-B	MSK.IMPACT	6	31323128	4	SNP	MODERATE	missense_variant	p.His287Gln/c.861T>A	362	6
5	RSPO3	OncoPlex	6	127476293	5	SNP	MODIFIER	intron_variant	c.437-93G>A	272	6

5	TCF7L2	MSK.IMPACT	10	114849554	5	SNP	MODIFIER	intron_variant	c.552+49669G>T	602	6
5	AKT3	MSK.IMPACT OncoPlex	1	243708771	6	SNP	MODIFIER	intron_variant	c.1251+41G>T	479	7
5	ATR	MSK.IMPACT	3	142238304	6	SNP	MODIFIER	intron_variant	c.4382+207G>A	2644	7
5	CUX1	OncoPlex	7	101891513	6	INDEL	MODIFIER	intron_variant	c.3921-178delT	1516	4
5	GNAS	MSK.IMPACT OncoPlex	20	57480340	6	SNP	MODIFIER	intron_variant	c.2362-98G>A	1037	7
5	JAK2	MSK.IMPACT OncoPlex	9	5021879	6	SNP	MODIFIER	intron_variant	c.-25-84C>T	1132	7
5	LYN	MSK.IMPACT	8	56863553	6	SNP	MODIFIER	intron_variant	c.487+210A>C	512	4
5	NTRK3	MSK.IMPACT OncoPlex	15	88423336	6	INDEL	MODIFIER	intron_variant	c.2334+164delA	839	4
5	RAD54L	MSK.IMPACT	1	46739749	6	INDEL	MODIFIER	intron_variant	c.1611-59delA	747	4
5	STAT5B	MSK.IMPACT	17	40371530	6	SNP	MODIFIER	intron_variant	c.682-49C>A	787	5
6	APC	MSK.IMPACT OncoPlex	5	112102894	1	INDEL	HIGH	frameshift_variant	p.Asp78fs/c.233_236delATAG	2843	4
6	APC	MSK.IMPACT OncoPlex	5	112175951	1	INDEL	HIGH	frameshift_variant	p.Thr1556fs/c.4666dupA	2843	4
6	GNAS	MSK.IMPACT OncoPlex	20	57485789	1	SNP	MODERATE	missense_variant	p.Thr1007Ala/c.3019A>G	1037	5
6	JUN	MSK.IMPACT	1	59248324	1	INDEL	HIGH	frameshift_variant	p.Ala140fs/c.417_418dupGG	331	4
6	PREX2	MSK.IMPACT	8	68956739	1	SNP	MODERATE	missense_variant	p.Lys286Arg/c.857A>G	1606	7
6	XIAP	MSK.IMPACT	X	123020112	1	SNP	HIGH	stop_gained	p.Cys200*/c.600C>A	497	7
6	APC	MSK.IMPACT OncoPlex	5	112173917	2	SNP	HIGH	stop_gained	p.Arg876*/c.2626C>T	2843	7
6	ARID1A	MSK.IMPACT OncoPlex	1	27023007	2	INDEL	MODERATE	disruptive_inframe_deletion	p.Ala43del/c.126_128delGGC	2285	3
6	KRAS	MSK.IMPACT OncoPlex	12	25398284	2	SNP	MODERATE	missense_variant	p.Gly12Asp/c.35G>A	189	7
6	SMAD4	MSK.IMPACT OncoPlex	18	48604749	2	SNP	MODERATE	missense_variant	p.Trp524Leu/c.1571G>T	552	7
6	TP53	MSK.IMPACT OncoPlex	17	7577094	2	SNP	MODERATE	missense_variant	p.Arg282Trp/c.844C>T	393	7
6	ARID1A	MSK.IMPACT OncoPlex	1	27023468	3	SNP	LOW	synonymous_variant	p.Leu192Leu/c.574C>T	2285	3
6	DICER1	MSK.IMPACT	14	95599795	3	SNP	LOW	initiator_codon_variant	p.Met1?/c.1A>T	1922	3
6	PIK3C3	MSK.IMPACT	18	39570409	3	INDEL	LOW	splice_region_variant +intron_variant	c.619-4delT	887	2
6	RSPO2	OncoPlex	8	109001480	3	SNP	LOW	splice_region_variant +intron_variant	c.95-8T>G	243	6
6	SOX17	MSK.IMPACT	8	55370722	3	SNP	LOW	synonymous_variant	p.Tyr8Tyr/c.24C>T	414	7
6	AR	MSK.IMPACT OncoPlex	X	66765207	5	SNP	LOW	synonymous_variant	p.Gln73Gln/c.219G>A	920	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27022984	5	SNP	LOW	synonymous_variant	p.Gln30Gln/c.90G>A	2285	4
6	ARID1A	MSK.IMPACT OncoPlex	1	27023065	5	SNP	LOW	synonymous_variant	p.Glu57Glu/c.171A>G	2285	4

6	ARID1A	MSK.IMPACT OncoPlex	1	27023246	5	SNP	MODERATE	missense_variant	p.Thr118Pro/c.352A>C	2285	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27023269	5	SNP	LOW	synonymous_variant	p.Gly125Gly/c.375T>C	2285	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27023386	5	SNP	LOW	synonymous_variant	p.Ala164Ala/c.492C>G	2285	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27023482	5	SNP	LOW	synonymous_variant	p.Ala196Ala/c.588G>C	2285	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27023500	5	SNP	LOW	synonymous_variant	p.Ser202Ser/c.606T>G	2285	3
6	ARID1A	MSK.IMPACT OncoPlex	1	27023812	5	SNP	LOW	synonymous_variant	p.Arg306Arg/c.918G>T	2285	3
6	ATR	MSK.IMPACT	3	142215179	5	INDEL	MODIFIER	intron_variant	c.5898+23_5898+24insAG	2644	2
6	ATR	MSK.IMPACT	3	142184047	5	SNP	LOW	synonymous_variant	p.Lys2311Lys/c.6933G>A	2644	6
6	ATR	MSK.IMPACT	3	142297614	5	SNP	MODIFIER	5_prime_UTR_variant	c.-68G>T	2644	7
6	CEBPA	MSK.IMPACT OncoPlex	19	33792283	5	SNP	LOW	synonymous_variant	p.Pro346Pro/c.1038A>T	358	3
6	CEBPA	MSK.IMPACT OncoPlex	19	33792322	5	SNP	LOW	synonymous_variant	p.Arg333Arg/c.999C>T	358	3
6	CSF1R	MSK.IMPACT OncoPlex	5	149460355	5	SNP	LOW	synonymous_variant	p.Ser94Ser/c.282C>T	972	7
6	EP300	MSK.IMPACT	22	41556546	5	SNP	MODIFIER	intron_variant	c.3591-100C>T	2414	3
6	MAX	MSK.IMPACT	14	65568998	5	SNP	MODIFIER	intron_variant	c.36+24C>T	160	3
6	MDC1	MSK.IMPACT	6	30673105	5	SNP	LOW	synonymous_variant	p.Val1285Val/c.3855G>C	2089	3
6	MDC1	MSK.IMPACT	6	30673236	5	SNP	MODERATE	missense_variant	p.Pro1242Thr/c.3724C>A	2089	3
6	MDC1	MSK.IMPACT	6	30673315	5	SNP	LOW	synonymous_variant	p.Thr1215Thr/c.3645C>A	2089	3
6	MYCN	MSK.IMPACT OncoPlex	2	16085838	5	SNP	LOW	synonymous_variant	p.Tyr338Tyr/c.1014C>T	464	6
6	ATM	MSK.IMPACT OncoPlex	11	108188266	6	INDEL	MODIFIER	intron_variant	c.6347+31delT	3056	2
6	DUSP4	MSK.IMPACT	8	29197529	6	SNP	MODIFIER	intron_variant	c.579+86G>A	394	5
6	E2F3	MSK.IMPACT	6	20482821	6	INDEL	MODIFIER	intron_variant	c.726-163_726-162delAA	465	2
6	EPHA3	MSK.IMPACT OncoPlex	3	89528682	6	SNP	MODIFIER	3_prime_UTR_variant	c.*30A>C	983	6
6	HDAC4	OncoPlex	2	240008258	6	SNP	MODIFIER	intron_variant	c.2429+997T>A	1084	4
6	KDM5A	MSK.IMPACT	12	427165	6	INDEL	MODIFIER	intron_variant	c.2897+106dupT	1690	2
6	LATS2	MSK.IMPACT	13	21562482	6	INDEL	MODERATE	disruptive_inframe_insertion	p.Pro479_Ala480insAlaPro/c.1431_1436dupCGCCCC	1088	2
6	MET	MSK.IMPACT OncoPlex	7	116409675	6	INDEL	MODIFIER	intron_variant	c.2638-9delT	1408	2
6	NSD1	MSK.IMPACT	5	176636529	6	INDEL	MODIFIER	intron_variant	c.1237-92dupA	2696	2
6	PTPRS	MSK.IMPACT	19	5216896	6	INDEL	MODIFIER	intron_variant	c.4049-119dupC	1948	2
6	PTPRT	MSK.IMPACT	20	40714592	6	INDEL	MODIFIER	intron_variant	c.3847-100dupA	1441	2
6	RXRA	MSK.IMPACT	9	137328250	6	SNP	MODIFIER	intron_variant	c.1242-63C>T	462	7
6	SESN3	MSK.IMPACT	11	94906606	6	SNP	MODIFIER	intron_variant	c.1393-101G>A	492	7
6	SF1	OncoPlex	11	64534762	6	SNP	MODIFIER	intron_variant	c.1718-39C>A	673	4

6	SULT1A1	OncoPlex	16	28617930	6	INDEL	MODIFIER	intron_variant	c.594+151_594+152insC	295	2
6	XPO1	MSK.IMPACT	2	61726050	6	INDEL	LOW	splice_region_variant +intron_variant	c.591-3dupT	1071	2
6	APC	MSK.IMPACT OncoPlex	5	112102894	1	INDEL	HIGH	frameshift_variant	p.Asp78fs/c.233_236delATAG	2843	3
6	APC	MSK.IMPACT OncoPlex	5	112175951	1	INDEL	HIGH	frameshift_variant	p.Thr1556fs/c.4666dupA	2843	4
6	JUN	MSK.IMPACT	1	59248324	1	INDEL	HIGH	frameshift_variant	p.Ala140fs/c.417_418dupGG	331	4
6	XIAP	MSK.IMPACT	X	123020112	1	SNP	HIGH	stop_gained	p.Cys200*/c.600C>A	497	7
6	APC	MSK.IMPACT OncoPlex	5	112173917	2	SNP	HIGH	stop_gained	p.Arg876*/c.2626C>T	2843	7
6	KRAS	MSK.IMPACT OncoPlex	12	25398284	2	SNP	MODERATE	missense_variant	p.Gly12Asp/c.35G>A	189	7
6	TP53	MSK.IMPACT OncoPlex	17	7577094	2	SNP	MODERATE	missense_variant	p.Arg282Trp/c.844C>T	393	6
6	GNAS	MSK.IMPACT OncoPlex	20	57429964	3	SNP	MODERATE	missense_variant	p.Arg485His/c.1454G>A	625	7
6	MDC1	MSK.IMPACT	6	30673457	3	SNP	MODERATE	missense_variant	p.Leu1168Pro/c.3503T>C	2089	3
6	ATR	MSK.IMPACT	3	142184047	5	SNP	LOW	synonymous_variant	p.Lys2311Lys/c.6933G>A	2644	6
6	ATR	MSK.IMPACT	3	142297614	5	SNP	MODIFIER	5_prime_UTR_variant	c.-68G>T	2644	7
6	CUX1	OncoPlex	7	101839856	5	INDEL	MODIFIER	intron_variant	c.1256-49dupT	1516	2
6	MDC1	MSK.IMPACT	6	30672165	5	SNP	MODERATE	missense_variant	p.Cys1599Gly/c.4795T>G	2089	3
6	MDC1	MSK.IMPACT	6	30673236	5	SNP	MODERATE	missense_variant	p.Pro1242Thr/c.3724C>A	2089	3
6	ATM	MSK.IMPACT OncoPlex	11	108121410	6	INDEL	HIGH	splice_acceptor_variant +intron_variant	c.1236-3dupT	3056	2
6	BTK	MSK.IMPACT	X	100614965	6	INDEL	MODIFIER	intron_variant	c.839+109_839+110delTA	659	2
6	CDH1	MSK.IMPACT OncoPlex	16	68843129	6	INDEL	MODIFIER	intron_variant	c.687+394delT	882	2
6	DNAJB1	MSK.IMPACT OncoPlex	19	14629293	6	INDEL	MODIFIER	intron_variant	c.-89-1436dupG	240	2
6	DUSP4	MSK.IMPACT	8	29197529	6	SNP	MODIFIER	intron_variant	c.579+86G>A	394	5
6	IKZF1	MSK.IMPACT OncoPlex	7	50444181	6	INDEL	MODIFIER	intron_variant	c.161-40dupT	477	2
6	KDM5A	MSK.IMPACT	12	427165	6	INDEL	MODIFIER	intron_variant	c.2897+106dupT	1690	2
6	MTOR	MSK.IMPACT OncoPlex	1	11187618	6	INDEL	MODIFIER	intron_variant	c.6216+62delA	2549	2
6	PTPRS	MSK.IMPACT	19	5216896	6	INDEL	MODIFIER	intron_variant	c.4049-119dupC	1948	2
6	PTPRS	MSK.IMPACT	19	5218213	6	INDEL	MODIFIER	intron_variant	c.4048+217dupT	1948	2
6	TERT	MSK.IMPACT	5	1272549	6	SNP	MODIFIER	intron_variant	c.2287-154G>A	1132	5
7	BRCA1	MSK.IMPACT OncoPlex	17	41267759	1	SNP	MODERATE	missense_variant	p.Asp40Tyr/c.118G>T	1884	7
7	FAT1	MSK.IMPACT	4	187522571	1	SNP	MODERATE	missense_variant	p.Ser3831Cys/c.11492C>G	4588	6
7	SMAD4	MSK.IMPACT OncoPlex	18	48591892	1	SNP	MODERATE	missense_variant	p.Gly352Val/c.1055G>T	552	7
7	TP53	MSK.IMPACT	17	7579316	1	INDEL	HIGH	frameshift_variant	p.Cys124fs/c.370delT	393	4

		OncoPlex									
7	APC	MSK.IMPACT OncoPlex	5	112164616	2	SNP	HIGH	stop_gained	p.Arg564*/c.1690C>T	2843	7
7	APC	MSK.IMPACT OncoPlex	5	112175423	2	SNP	HIGH	stop_gained	p.Gln1378*/c.4132C>T	2843	7
7	ERBB4	MSK.IMPACT OncoPlex	2	212251716	2	SNP	MODERATE	missense_variant	p.Val1115Met/c.3343G>A	1308	4
7	GNAS	MSK.IMPACT OncoPlex	20	57428698	2	SNP	MODERATE	missense_variant	p.Gly63Val/c.188G>T	625	3
7	SMAD4	MSK.IMPACT OncoPlex	18	48591919	2	SNP	MODERATE	missense_variant	p.Arg361His/c.1082G>A	552	7
7	CUL3	MSK.IMPACT	2	225338878	5	SNP	MODIFIER	3_prime_UTR_variant	c.*84C>T	768	7
7	DOCK7	OncoPlex	1	63010806	5	SNP	MODIFIER	intron_variant	c.2767-1390T>C	2109	6
7	EML4	OncoPlex	2	42556123	5	SNP	LOW	synonymous_variant	p.Val813Val/c.2439C>T	981	4
7	PLCG2	MSK.IMPACT	16	81990506	5	SNP	MODIFIER	intron_variant	c.3755+22C>A	1265	7
7	SF3B1	MSK.IMPACT OncoPlex	2	198263661	5	SNP	MODIFIER	intron_variant	c.3014-356T>C	1304	4
7	ABCG2	OncoPlex	4	89053658	6	SNP	MODIFIER	intron_variant	c.263+70T>G	655	4
7	ATM	MSK.IMPACT OncoPlex	11	108100105	6	INDEL	MODIFIER	intron_variant	c.331+69dupT	3056	2
7	ATM	MSK.IMPACT OncoPlex	11	108114661	6	INDEL	LOW	splice_region_variant +intron_variant	c.497-4delT	3056	3
7	BCL2L11	MSK.IMPACT OncoPlex	2	111886180	6	INDEL	LOW	splice_region_variant +intron_variant	c.215-6dupT	112	2
7	BMPR1A	MSK.IMPACT	10	88679356	6	INDEL	MODIFIER	intron_variant	c.1166+137delG	532	2
7	CCND3	MSK.IMPACT	6	41905146	6	SNP	MODIFIER	intron_variant	c.415-14C>T	292	4
7	CENPA	MSK.IMPACT	2	27016302	6	INDEL	MODIFIER	intron_variant	c.*47+110dupT	140	2
7	CENPA	MSK.IMPACT	2	27016259	6	SNP	MODIFIER	intron_variant	c.*47+65A>G	140	4
7	CIC	MSK.IMPACT	19	42788719	6	INDEL	MODIFIER	intron_variant	c.2795-2193delG	2514	2
7	CUX1	OncoPlex	7	101847868	6	INDEL	MODIFIER	intron_variant	c.3106+39dupC	1516	2
7	DNMT1	MSK.IMPACT	19	10277400	6	INDEL	MODIFIER	intron_variant	c.804-40dupC	1632	4
7	E2F3	MSK.IMPACT	6	20481790	6	SNP	MODIFIER	intron_variant	c.725+134T>C	465	6
7	FYN	MSK.IMPACT	6	112029331	6	SNP	MODIFIER	intron_variant	c.345-108C>T	537	4
7	HIF1A	OncoPlex	14	62194112	6	INDEL	MODIFIER	intron_variant	c.643-50dupT	850	2
7	IGF1R	MSK.IMPACT OncoPlex	15	99192754	6	INDEL	MODIFIER	5_prime_UTR_variant	c.-41_-33delTTTTTTTTT	1367	2
7	KMT2A	MSK.IMPACT OncoPlex	11	118373018	6	INDEL	MODIFIER	intron_variant	c.6506-81_6506-80delTA	3972	2
7	MLH3	OncoPlex	14	75497428	6	INDEL	MODIFIER	intron_variant	c.3828-24delT	1453	3
7	MSH6	MSK.IMPACT OncoPlex	2	48032613	6	SNP	MODIFIER	intron_variant	c.3557-144G>A	1360	3
7	NTRK3	MSK.IMPACT OncoPlex	15	88428810	6	SNP	MODIFIER	intron_variant	c.2175+115A>T	839	5
7	ZRSR2	MSK.IMPACT OncoPlex	X	15836525	6	SNP	MODIFIER	intron_variant	c.772-185G>C	482	3

7	BRCA1	MSK.IMPACT OncoPlex	17	41267759	1	SNP	MODERATE	missense_variant	p.Asp40Tyr/c.118G>T	1884	7
7	FAT1	MSK.IMPACT	4	187522571	1	SNP	MODERATE	missense_variant	p.Ser3831Cys/c.11492C>G	4588	6
7	TP53	MSK.IMPACT OncoPlex	17	7579316	1	INDEL	HIGH	frameshift_variant	p.Cys124fs/c.370delT	393	4
7	APC	MSK.IMPACT OncoPlex	5	112164616	2	SNP	HIGH	stop_gained	p.Arg564*/c.1690C>T	2843	7
7	APC	MSK.IMPACT OncoPlex	5	112175423	2	SNP	HIGH	stop_gained	p.Gln1378*/c.4132C>T	2843	7
7	ERBB4	MSK.IMPACT OncoPlex	2	212251716	2	SNP	MODERATE	missense_variant	p.Val1115Met/c.3343G>A	1308	7
7	SMAD4	MSK.IMPACT OncoPlex	18	48591919	2	SNP	MODERATE	missense_variant	p.Arg361His/c.1082G>A	552	7
7	CUL3	MSK.IMPACT	2	225338878	5	SNP	MODIFIER	3_prime_UTR_variant	c.*84C>T	768	7
7	DOCK7	OncoPlex	1	63010806	5	SNP	MODIFIER	intron_variant	c.2767-1390T>C	2109	5
7	PLCG2	MSK.IMPACT	16	81990506	5	SNP	MODIFIER	intron_variant	c.3755+22C>A	1265	7
7	ATR	MSK.IMPACT	3	142215120	6	INDEL	MODIFIER	intron_variant	c.5898+82_5898+83insAT	2644	2
7	BMPR1A	MSK.IMPACT	10	88679356	6	INDEL	MODIFIER	intron_variant	c.1166+137delG	532	2
7	CENPA	MSK.IMPACT	2	27016302	6	INDEL	MODIFIER	intron_variant	c.*47+110dupT	140	2
7	CENPA	MSK.IMPACT	2	27016259	6	SNP	MODIFIER	intron_variant	c.*47+65A>G	140	4
7	CUX1	OncoPlex	7	101847868	6	INDEL	MODIFIER	intron_variant	c.3106+39dupC	1516	2
7	DNMT1	MSK.IMPACT	19	10277400	6	INDEL	MODIFIER	intron_variant	c.804-40dupC	1632	4
7	E2F3	MSK.IMPACT	6	20481790	6	SNP	MODIFIER	intron_variant	c.725+134T>C	465	7
7	EIF3A	OncoPlex	10	120795795	6	INDEL	MODIFIER	intron_variant	c.3920-16delT	1382	3
7	EZH1	MSK.IMPACT	17	40856573	6	INDEL	MODIFIER	intron_variant	c.2017+46delT	747	2
7	FYN	MSK.IMPACT	6	112029331	6	SNP	MODIFIER	intron_variant	c.345-108C>T	537	3
7	IGF1R	MSK.IMPACT OncoPlex	15	99192754	6	INDEL	MODIFIER	5_prime_UTR_variant	c.-41_-33delTTTTTTTTT	1367	2
7	MLH3	OncoPlex	14	75497428	6	INDEL	MODIFIER	intron_variant	c.3828-24delT	1453	2
7	NTRK3	MSK.IMPACT OncoPlex	15	88428810	6	SNP	MODIFIER	intron_variant	c.2175+115A>T	839	6
7	STK11	MSK.IMPACT OncoPlex	19	1219443	6	INDEL	MODIFIER	intron_variant	c.464+40_464+46dupGGGGGCC	433	2
7	TPMT	OncoPlex	6	18139973	6	SNP	MODIFIER	intron_variant	c.367-25T>A	245	3
7	U2AF1	MSK.IMPACT OncoPlex	21	44513110	6	INDEL	MODIFIER	3_prime_UTR_variant	c.*101dupA	240	2
7	ZRSR2	MSK.IMPACT OncoPlex	X	15836525	6	SNP	MODIFIER	intron_variant	c.772-185G>C	482	3
8	APC	MSK.IMPACT OncoPlex	5	112173992	1	SNP	HIGH	stop_gained	p.Gln901*/c.2701C>T	2843	7
8	ATRX	MSK.IMPACT OncoPlex	X	76952100	1	SNP	MODERATE	missense_variant	p.Ser112Leu/c.335C>T	2492	6
8	ALK	MSK.IMPACT OncoPlex	2	29416301	2	SNP	MODERATE	missense_variant	p.Pro1551Leu/c.4652C>T	1620	6
8	APC	MSK.IMPACT OncoPlex	5	112175576	2	SNP	HIGH	stop_gained	p.Gln1429*/c.4285C>T	2843	5

8	BRIP1	MSK.IMPACT OncoPlex	17	59876594	2	SNP	MODERATE	missense_variant	p.Arg403Trp/c.1207C>T	1249	7
8	KRAS	MSK.IMPACT OncoPlex	12	25398281	2	SNP	MODERATE	missense_variant	p.Gly13Asp/c.38G>A	189	7
8	TP53	MSK.IMPACT OncoPlex	17	7578272	2	SNP	MODERATE	missense_variant	p.His193Tyr/c.577C>T	393	7
8	JAK1	MSK.IMPACT OncoPlex	1	65306997	5	SNP	MODERATE	missense_variant	p.Lys860Asn/c.2580A>C	1154	7
8	MEN1	MSK.IMPACT OncoPlex	11	64572160	5	SNP	LOW	synonymous_variant	p.Pro498Pro/c.1494G>A	615	7
8	SMO	MSK.IMPACT OncoPlex	7	128828925	5	SNP	LOW	5_prime_UTR_premature _start_codon_gain_variant	c.-68G>A	787	4
8	BAP1	MSK.IMPACT OncoPlex	3	52435477	6	SNP	MODIFIER	3_prime_UTR_variant	c.*827C>T	729	6
8	CUX1	OncoPlex	7	101883019	6	SNP	MODIFIER	intron_variant	c.3920+155T>C	1516	5
8	DEPDC5	OncoPlex	22	32229812	6	SNP	MODIFIER	intron_variant	c.2105-89G>T	1594	7
8	IDH1	MSK.IMPACT OncoPlex	2	209116069	6	SNP	MODIFIER	intron_variant	c.122+85T>G	414	5
8	IKZF1	MSK.IMPACT OncoPlex	7	50358845	6	SNP	MODIFIER	intron_variant	c.40+148C>A	477	7
8	MED12	MSK.IMPACT OncoPlex	X	70354811	6	SNP	MODIFIER	intron_variant	c.4863+113T>C	2177	7
8	MTHFR	OncoPlex	1	11851514	6	SNP	MODIFIER	intron_variant	c.1633-131G>T	656	7
8	NCOR1	MSK.IMPACT	17	15984135	6	SNP	MODIFIER	intron_variant	c.3178-94T>A	2440	6
8	PLK4	OncoPlex	4	128819545	6	SNP	MODIFIER	intron_variant	c.2811-49C>A	970	4
8	PMS2	MSK.IMPACT OncoPlex	7	6017523	6	SNP	MODIFIER	intron_variant	c.2276-135T>C	862	5
8	PREX2	MSK.IMPACT	8	69103886	6	SNP	MODIFIER	intron_variant	c.4347-71C>A	1606	5
8	SOX17	MSK.IMPACT	8	55370547	6	SNP	MODIFIER	5_prime_UTR_variant	c.-152G>A	414	7
9	EPHA7	MSK.IMPACT	6	93956637	1	SNP	MODERATE	missense_variant	p.Gln867Glu/c.2599C>G	998	7
9	FLT3	MSK.IMPACT OncoPlex	13	28588617	1	SNP	MODERATE	missense_variant	p.Gly944Ala/c.2831G>C	993	7
9	LRP2	OncoPlex	2	170011002	1	SNP	MODERATE	missense_variant	p.Asp4088Gly/c.12263A>G	4655	7
9	PTPN11	MSK.IMPACT OncoPlex	12	112939969	1	SNP	HIGH	stop_gained	p.Glu541*/c.1621G>T	593	7
9	SOX9	MSK.IMPACT	17	70119943	1	SNP	HIGH	stop_gained	p.Tyr315*/c.945C>G	509	7
9	TP53	MSK.IMPACT OncoPlex	17	7577535	1	SNP	MODERATE	missense_variant	p.Arg249Met/c.746G>T	393	7
9	TRRAP	OncoPlex	7	98565210	1	SNP	MODERATE	missense_variant	p.Glu2460Asp/c.7380G>T	3859	5
9	APC	MSK.IMPACT OncoPlex	5	112151204	2	SNP	HIGH	stop_gained	p.Arg283*/c.847C>T	2843	7
9	CBLB	OncoPlex	3	105377828	2	SNP	MODERATE	missense_variant	p.Arg979Cys/c.2935C>T	982	7
9	RECQL4	MSK.IMPACT	8	145743013	2	SNP	MODERATE	missense_variant	p.Val31Leu/c.91G>T	1208	7
9	KEAP1	MSK.IMPACT	19	10602378	3	SNP	LOW	synonymous_variant	p.Thr400Thr/c.1200C>T	624	5
9	HNF1A	MSK.IMPACT OncoPlex	12	121437227	5	SNP	MODERATE	missense_variant	p.Cys553Tyr/c.1658G>A	662	5

9	HNF1A	MSK.IMPACT OncoPlex	12	121437228	5	SNP	MODERATE	missense_variant	p.Cys553Trp/c.1659T>G	662	6
9	LATS2	MSK.IMPACT	13	21565454	5	SNP	LOW	synonymous_variant	p.Pro144Pro/c.432G>T	1088	7
9	NOTCH1	MSK.IMPACT OncoPlex	9	139401869	5	SNP	LOW	synonymous_variant	p.Gly1177Gly/c.3531G>C	2555	7
9	BCOR	MSK.IMPACT OncoPlex	X	39916361	6	SNP	MODIFIER	intron_variant	c.4595+47T>C	1755	5
9	BCR	OncoPlex	22	23610503	6	SNP	MODIFIER	intron_variant	c.1753-92G>T	1271	7
9	DNMT1	MSK.IMPACT	19	10265253	6	SNP	MODIFIER	intron_variant	c.1832+9G>T	1632	7
9	EGFR	MSK.IMPACT OncoPlex	7	55322402	6	SNP	MODIFIER	intron_variant	c.*29-1545A>T	657	7
9	GLI1	MSK.IMPACT OncoPlex	12	57859183	6	SNP	MODIFIER	intron_variant	c.534+145C>G	1106	4
9	NKX2-1	MSK.IMPACT OncoPlex	14	36988928	6	INDEL	MODIFIER	intron_variant	c.77+328_77+329dupCT	401	3
9	NTRK1	MSK.IMPACT OncoPlex	1	156837710	6	SNP	MODIFIER	intron_variant	c.429-186C>T	796	4
9	RASA1	MSK.IMPACT	5	86629061	6	INDEL	MODIFIER	intron_variant	c.829-12dupT	1047	3
10	APC	MSK.IMPACT OncoPlex	5	112175745	1	INDEL	HIGH	frameshift_variant	p.Asp1486fs/c.4455delT	2843	4
10	BRIP1	MSK.IMPACT OncoPlex	17	59885907	1	SNP	MODERATE	missense_variant	p.Asp280Val/c.839A>T	1249	7
10	ELF3	MSK.IMPACT	1	201984335	1	SNP	HIGH	splice_acceptor_variant +intron_variant	c.1002-2A>C	371	7
10	ERBB3	MSK.IMPACT OncoPlex	12	56478854	1	SNP	MODERATE	missense_variant	p.Val104Leu/c.310G>T	1342	7
10	FLT4	MSK.IMPACT OncoPlex	5	180047965	1	SNP	MODERATE	missense_variant	p.Arg737His/c.2210G>A	1363	7
10	PAK5	MSK.IMPACT	20	9520130	1	SNP	MODERATE	missense_variant	p.Met713Ile/c.2139G>C	719	7
10	PIK3R1	MSK.IMPACT OncoPlex	5	67589219	1	SNP	HIGH	stop_gained	p.Glu403*/c.1207G>T	724	7
10	SMAD2	MSK.IMPACT OncoPlex	18	45375011	1	SNP	MODERATE	missense_variant	p.Ala278Pro/c.832G>C	467	7
10	SMAD3	MSK.IMPACT OncoPlex	15	67358628	1	INDEL	MODERATE	disruptive_inframe_deletion	p.Gly46_Lys53delinsGlu/c.137_157delGGCAGCTGGACGAGCTGGAGA	425	4
10	SMAD4	MSK.IMPACT OncoPlex	18	48581322	1	SNP	MODERATE	missense_variant	p.Thr209Ile/c.626C>T	552	7
10	APC	MSK.IMPACT OncoPlex	5	112173917	2	SNP	HIGH	stop_gained	p.Arg876*/c.2626C>T	2843	7
10	FBXW7	MSK.IMPACT OncoPlex	4	153249385	2	SNP	MODERATE	missense_variant	p.Arg465Cys/c.1393C>T	707	7
10	GATA1	MSK.IMPACT OncoPlex	X	48650871	2	SNP	MODERATE	missense_variant	p.Arg247His/c.740G>A	413	4
10	IRS1	MSK.IMPACT	2	227659794	2	SNP	MODERATE	missense_variant	p.Arg1221Cys/c.3661C>T	1242	7
10	MAP2K1	MSK.IMPACT OncoPlex	15	66727442	2	SNP	MODERATE	missense_variant	p.Phe53Ser/c.158T>C	393	7
10	NRAS	MSK.IMPACT OncoPlex	1	115258747	2	SNP	MODERATE	missense_variant	p.Gly12Asp/c.35G>A	189	7
10	PAK5	MSK.IMPACT	20	9523270	2	SNP	MODERATE	missense_variant	p.Arg656Gln/c.1967G>A	719	5

10	PLK3	OncoPlex	1	45268710	2	SNP	MODERATE	missense_variant	p.Thr278Met/c.833C>T	646	7
10	POLD1	MSK.IMPACT OncoPlex	19	50910264	2	SNP	MODERATE	missense_variant	p.Arg507Cys/c.1519C>T	1107	7
10	SMAD3	MSK.IMPACT OncoPlex	15	67479810	2	SNP	MODERATE	missense_variant	p.Arg373Cys/c.1117C>T	425	7
10	LATS2	MSK.IMPACT	13	21557442	5	SNP	LOW	synonymous_variant	p.Leu801Leu/c.2403G>A	1088	4
10	ROS1	MSK.IMPACT OncoPlex	6	117706985	5	SNP	MODERATE	missense_variant	p.Thr722Met/c.2165C>T	2347	7
10	RRAS	MSK.IMPACT	19	50138985	5	SNP	LOW	splice_region_variant +intron_variant	c.572+6C>A	218	7
10	AXIN1	MSK.IMPACT	16	354186	6	SNP	MODIFIER	intron_variant	c.1254+118T>A	862	7
10	BRAF	MSK.IMPACT OncoPlex	7	140448947	6	SNP	MODIFIER	intron_variant	c.1992+140A>G	766	7
10	CYP2C19	OncoPlex	10	96534779	6	SNP	MODIFIER	intron_variant	c.169-36G>T	490	7
10	FLT3	MSK.IMPACT OncoPlex	13	28635909	6	INDEL	MODIFIER	intron_variant	c.368+94delT	993	4
10	FLT4	MSK.IMPACT OncoPlex	5	180040153	6	SNP	MODIFIER	intron_variant	c.3332-43G>A	1363	7
10	KMT2C	MSK.IMPACT	7	151949944	6	INDEL	MODIFIER	intron_variant	c.1300-145delA	4911	3
10	LATS2	MSK.IMPACT	13	21620222	6	INDEL	MODIFIER	5_prime_UTR_variant	c.-58dupT	1088	3
10	PIK3CD	MSK.IMPACT	1	9776854	6	SNP	MODIFIER	intron_variant	c.781-163C>T	1044	7
10	PTPRT	MSK.IMPACT	20	40827745	6	SNP	MODIFIER	intron_variant	c.2491+135G>T	1441	5
10	RBM10	MSK.IMPACT	X	47038994	6	SNP	MODIFIER	intron_variant	c.901+100T>A	930	7
11	APC	MSK.IMPACT OncoPlex	5	112175350	1	INDEL	HIGH	frameshift_variant	p.Ser1355fs/c.4063dupT	2843	4
11	APC	MSK.IMPACT OncoPlex	5	112175322	1	SNP	HIGH	stop_gained	p.Ser1344*/c.4031C>A	2843	7
11	CUX1	OncoPlex	7	101747708	1	SNP	MODERATE	missense_variant	p.Lys178Gln/c.532A>C	1516	7
11	ELF3	MSK.IMPACT	1	201982979	1	SNP	HIGH	stop_gained	p.Trp276*/c.828G>A	371	7
11	HSPH1	OncoPlex	13	31732957	1	SNP	MODERATE	missense_variant	p.Ala51Ser/c.151G>T	858	7
11	MSH3	MSK.IMPACT	5	80024755	2	SNP	MODERATE	missense_variant	p.Phe513Leu/c.1539C>G	1137	7
11	SMC3	OncoPlex	10	112343967	2	SNP	MODERATE	missense_variant	p.Thr373Met/c.1118C>T	1217	7
11	TP53	MSK.IMPACT OncoPlex	17	7577539	2	SNP	MODERATE	missense_variant	p.Arg248Trp/c.742C>T	393	7
11	ARID5B	MSK.IMPACT	10	63810912	5	INDEL	MODIFIER	intron_variant	c.846+155delC	1188	4
11	CREBBP	MSK.IMPACT OncoPlex	16	3819233	5	SNP	MODERATE	missense_variant	p.Thr1001Asn/c.3002C>A	2442	7
11	MED12	MSK.IMPACT OncoPlex	X	70345711	5	SNP	MODIFIER	intron_variant	c.2422+148C>T	2177	5
11	TRRAP	OncoPlex	7	98574141	5	SNP	LOW	synonymous_variant	p.Ser2658Ser/c.7974C>T	3859	7
11	ASXL2	MSK.IMPACT	2	26017168	6	SNP	MODIFIER	intron_variant	c.403+5086A>G	1435	4
11	BCR	OncoPlex	22	23605262	6	SNP	MODIFIER	intron_variant	c.1752+1535G>T	1271	6
11	CRLF2	MSK.IMPACT OncoPlex	X	1331182	6	SNP	MODIFIER	intron_variant	c.79+267G>A	256	4
11	EML4	OncoPlex	2	42513608	6	SNP	MODIFIER	intron_variant	c.1122+89G>A	981	7

11	ERBB4	MSK.IMPACT OncoPlex	2	212495412	6	SNP	MODIFIER	intron_variant	c.1947-93G>A	1308	5
11	ERBB4	MSK.IMPACT OncoPlex	2	212522828	6	SNP	MODIFIER	intron_variant	c.1872-275A>G	1308	7
11	MED12	MSK.IMPACT OncoPlex	X	70345698	6	SNP	MODIFIER	intron_variant	c.2422+135C>T	2177	6
11	MGA	MSK.IMPACT	15	41961003	6	INDEL	MODIFIER	intron_variant	c.-67-14dupT	3065	4
11	PRKAR1A	MSK.IMPACT	17	66521719	6	SNP	MODIFIER	intron_variant	c.550-176C>G	381	7
11	PTPRD	MSK.IMPACT OncoPlex	9	8485169	6	SNP	MODIFIER	intron_variant	c.3153+58C>T	1912	7
11	PTPRT	MSK.IMPACT	20	40827845	6	SNP	MODIFIER	intron_variant	c.2491+35G>A	1441	7
11	TET3	OncoPlex	2	74273384	6	SNP	MODIFIER	intron_variant	c.82-21C>T	737	7
12	APC	MSK.IMPACT OncoPlex	5	112175226	1	INDEL	HIGH	frameshift_variant	p.Thr1313fs/c.3937delA	2843	4
12	CTNNB1	MSK.IMPACT OncoPlex	3	41280804	1	SNP	HIGH	stop_gained	p.Gln773*/c.2317C>T	781	7
12	KDM5C	MSK.IMPACT	X	53227817	1	SNP	MODERATE	missense_variant +splice_region_variant	p.Leu791Phe/c.2371C>T	1560	7
12	MAP3K13	MSK.IMPACT	3	185167719	1	SNP	MODERATE	missense_variant	p.Thr348Pro/c.1042A>C	966	6
12	SF3B1	MSK.IMPACT OncoPlex	2	198269877	1	SNP	MODERATE	missense_variant	p.Ser488Cys/c.1462A>T	1304	7
12	APC	MSK.IMPACT OncoPlex	5	112128191	2	SNP	HIGH	stop_gained	p.Arg232*/c.694C>T	2843	7
12	BRAF	MSK.IMPACT OncoPlex	7	140481411	2	SNP	MODERATE	missense_variant	p.Gly466Val/c.1397G>T	766	5
12	PLCG2	MSK.IMPACT	16	81888075	2	SNP	MODERATE	missense_variant	p.Arg74Cys/c.220C>T	1265	7
12	TP53	MSK.IMPACT OncoPlex	17	7577121	2	SNP	MODERATE	missense_variant	p.Arg273Cys/c.817C>T	393	7
12	ERCC5	MSK.IMPACT	13	103498694	3	SNP	LOW	synonymous_variant	p.Ile26Ile/c.78C>T	1186	7
12	NFE2L2	MSK.IMPACT	2	178098829	3	SNP	LOW	synonymous_variant	p.Ala72Ala/c.216T>G	605	7
12	EIF3E	OncoPlex	8	109245894	5	SNP	MODERATE	missense_variant	p.Ser158Phe/c.473C>T	170	7
12	AXIN2	MSK.IMPACT	17	63556247	6	SNP	MODIFIER	intron_variant	c.-117+1321C>T	843	7
12	BTK	MSK.IMPACT	X	100615025	6	SNP	MODIFIER	intron_variant	c.839+51C>A	659	6
12	EGFR	MSK.IMPACT OncoPlex	7	55223709	6	SNP	MODIFIER	intron_variant	c.1006+70C>T	1210	6
12	EIF3E	OncoPlex	8	109227000	6	SNP	MODIFIER	intron_variant	c.952-55G>A	445	7
12	IKZF1	MSK.IMPACT OncoPlex	7	50459588	6	INDEL	MODIFIER	intron_variant	c.724+36delA	477	4
12	LRP2	OncoPlex	2	169999428	6	SNP	MODIFIER	intron_variant	c.12989-125G>A	4655	7
12	PDGFRA	MSK.IMPACT OncoPlex	4	55147825	6	SNP	MODIFIER	intron_variant	c.2323+1176C>T	1089	7
12	RSPO2	OncoPlex	8	109001198	6	SNP	MODIFIER	intron_variant	c.283+86A>T	243	6
13	SMAD4	MSK.IMPACT OncoPlex	18	48575671	1	SNP	HIGH	stop_gained	p.Ser144*/c.431C>A	552	6
13	TP53	MSK.IMPACT OncoPlex	17	7577566	1	INDEL	HIGH	frameshift_variant	p.Cys238fs/c.714delT	393	4

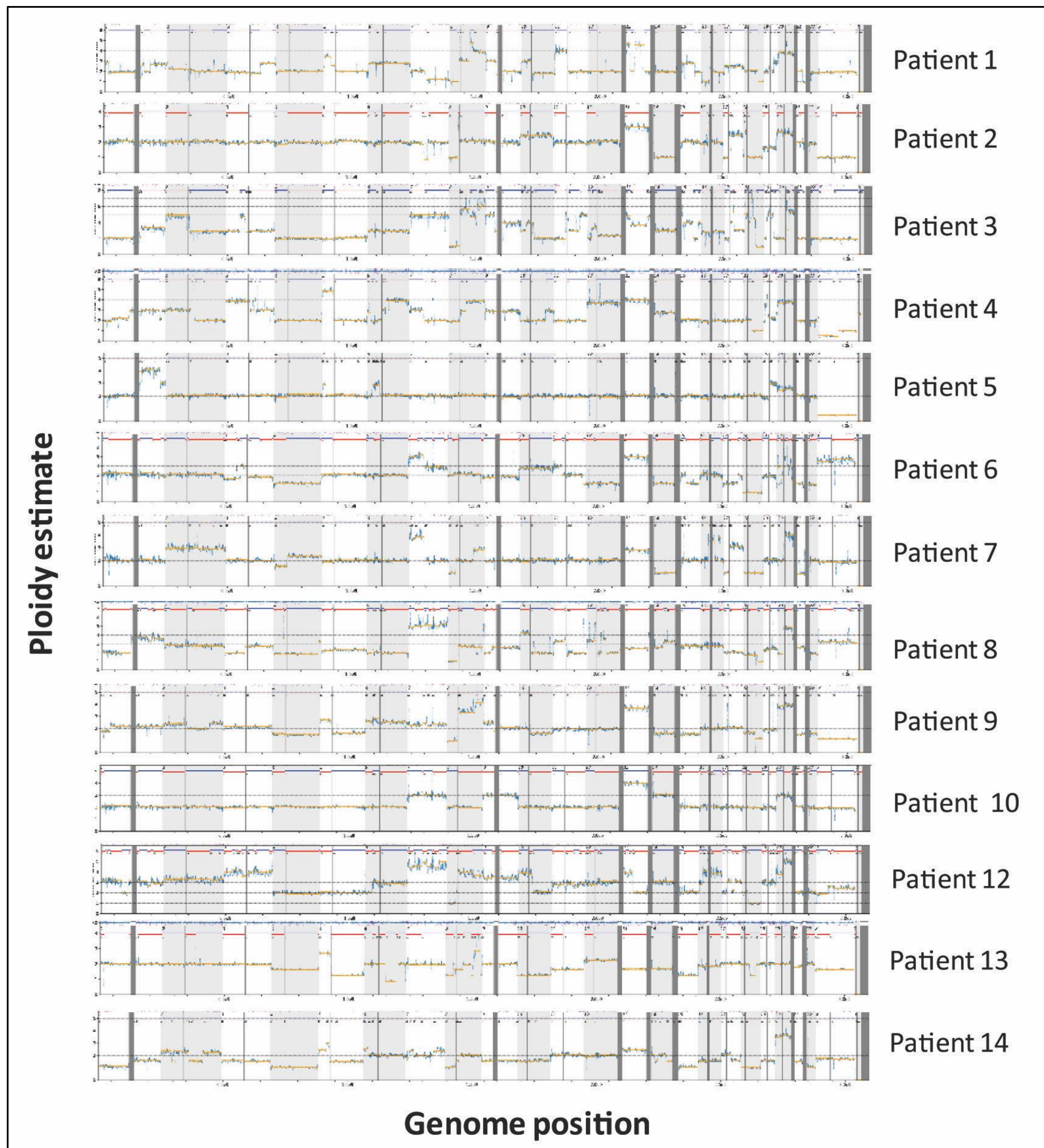
13	ARID1B	MSK.IMPACT	6	157522410	2	SNP	MODERATE	missense_variant	p.Arg1561His/c.4682G>A	2249	5
13	BRAF	MSK.IMPACT OncoPlex	7	140453136	2	SNP	MODERATE	missense_variant	p.Val600Glu/c.1799T>A	766	6
13	HDAC4	OncoPlex	2	240056254	2	SNP	MODERATE	missense_variant	p.Thr355Met/c.1064C>T	1084	6
13	MEF2B	MSK.IMPACT	19	19256682	2	SNP	MODERATE	missense_variant	p.Ala344Gly/c.1031C>G	368	4
13	TNFRSF14	MSK.IMPACT	1	2493152	2	SNP	MODERATE	missense_variant	p.Ser198Cys/c.592A>T	283	6
13	TP53	MSK.IMPACT OncoPlex	17	7578203	2	SNP	MODERATE	missense_variant	p.Val216Met/c.646G>A	393	6
13	ERBB4	MSK.IMPACT OncoPlex	2	212530131	3	SNP	LOW	synonymous_variant	p.Gly596Gly/c.1788C>T	1308	4
13	MYOD1	MSK.IMPACT	11	17741935	5	SNP	LOW	synonymous_variant	p.Pro202Pro/c.606G>A	320	5
13	TSHR	MSK.IMPACT	14	81574914	5	SNP	LOW	synonymous_variant	p.Ser238Ser/c.714G>A	274	5
13	AR	MSK.IMPACT OncoPlex	X	66941535	6	SNP	MODIFIER	intron_variant	c.2319-140C>T	920	5
13	ATM	MSK.IMPACT OncoPlex	11	108157971	6	SNP	MODIFIER	intron_variant	c.3994-356G>C	3056	6
13	AXIN1	MSK.IMPACT	16	341012	6	INDEL	MODIFIER	intron_variant	c.2294+175_2294+177delCCT	862	4
13	BCR	OncoPlex	22	23631827	6	SNP	MODIFIER	intron_variant	c.2707+19G>A	1271	6
13	DEPDC5	OncoPlex	22	32188130	6	SNP	MODIFIER	intron_variant	c.694+42C>T	1594	4
13	GRIN2A	MSK.IMPACT OncoPlex	16	10205676	6	SNP	MODIFIER	intron_variant	c.414+68179C>A	1464	6
14	DPYD	OncoPlex	1	97839196	1	SNP	MODERATE	missense_variant	p.Ser660Tyr/c.1979C>A	1025	7
14	JUN	MSK.IMPACT	1	59247811	1	INDEL	HIGH	frameshift_variant	p.Lys311fs/c.930_931delGA	331	4
14	SOS1	MSK.IMPACT	2	39281778	1	SNP	MODERATE	missense_variant	p.Asn233Tyr/c.697A>T	1333	7
14	TET1	MSK.IMPACT OncoPlex	10	70360737	1	SNP	HIGH	splice_acceptor_variant +intron_variant	c.1915-1G>A	2136	7
14	MED12	MSK.IMPACT OncoPlex	X	70356308	2	SNP	MODERATE	missense_variant	p.Arg1735Cys/c.5203C>T	2177	7
14	TP53	MSK.IMPACT OncoPlex	17	7577018	2	SNP	HIGH	splice_donor_variant +intron_variant	c.919+1G>A	393	7
14	APC	MSK.IMPACT OncoPlex	5	112151184	3	SNP	LOW	splice_region_variant +intron_variant	c.835-8A>G	2843	7
14	NOTCH4	MSK.IMPACT	6	32188942	3	SNP	LOW	synonymous_variant	p.Pro204Pro/c.612C>A	2003	7
14	PIK3C3	MSK.IMPACT	18	39600596	3	SNP	LOW	splice_region_variant +intron_variant	c.1417-6T>G	887	7
14	SYK	MSK.IMPACT	9	93627379	3	SNP	LOW	splice_region_variant +synonymous_variant	p.Ala282Ala/c.846G>A	635	7
14	FLT1	MSK.IMPACT OncoPlex	13	29012465	5	SNP	MODERATE	missense_variant	p.Val136Ile/c.406G>A	1338	7
14	KMT2B	MSK.IMPACT	19	36223716	5	SNP	MODERATE	missense_variant	p.Gly2089Val/c.6266G>T	2715	7
14	MLH3	OncoPlex	14	75515327	5	SNP	LOW	synonymous_variant	p.Val344Val/c.1032G>T	1453	7
14	BRD4	MSK.IMPACT	19	15366688	6	SNP	MODIFIER	intron_variant	c.1751+187C>T	1362	6
14	CUX1	OncoPlex	7	101713590	6	SNP	MODIFIER	intron_variant	c.223-29T>C	1516	6

14	DROSHA	MSK.IMPACT	5	31504762	6	INDEL	MODIFIER	intron_variant	c.1588-21dupA	1374	4
14	FGFR3	MSK.IMPACT OncoPlex	4	1795609	6	SNP	MODIFIER	5_prime_UTR_variant	c.-53G>A	808	5
14	GATA2	MSK.IMPACT OncoPlex	3	128200459	6	SNP	MODIFIER	intron_variant	c.1143+203A>G	480	4
14	IGF2	MSK.IMPACT	11	2156526	6	SNP	MODIFIER	intron_variant	c.325+71C>T	236	7
14	NOTCH2	MSK.IMPACT OncoPlex	1	120609053	6	INDEL	MODIFIER	intron_variant	c.73+2894dupA	2471	3
14	NOTCH2	MSK.IMPACT OncoPlex	1	120612223	6	INDEL	MODIFIER	5_prime_UTR_variant	c.-206_-204delGGC	2471	3
14	PIK3C3	MSK.IMPACT	18	39600586	6	SNP	MODIFIER	intron_variant	c.1417-16C>A	887	7
14	PTPRS	MSK.IMPACT	19	5218960	6	INDEL	MODIFIER	intron_variant	c.3924-152delA	1948	3
14	STAT3	MSK.IMPACT	17	40476630	6	SNP	MODIFIER	intron_variant	c.1600+99C>A	770	5
14	SYK	MSK.IMPACT	9	93640973	6	SNP	MODIFIER	intron_variant	c.1392-73G>A	635	7
14	TGFBR1	MSK.IMPACT	9	101906880	6	SNP	MODIFIER	intron_variant	c.974-134C>T	503	4
14	TRAF2	MSK.IMPACT	9	139781114	6	INDEL	MODIFIER	intron_variant	c.-29+133_-29+155delAGAGGCCGCGGGGCGGGGGCGGG	501	3

High confidence (identified by 3 or more callers) genomic variants from WES data in 551 cancer associated or actionable genes listed in the UW-Oncoplex[307] and/or MSK-IMPACT[308] panels.

Gene symbol: The HGNC gene symbol for the selected gene; Chrom and Pos: Chromosome and (reference) position; Tier-Annotated variants are ranked into tiers, with tier 1 as the highest based on: how rare the variant is in the general population (Tier 1-5 have minor allele frequency (MAF) of <1% in the general population); whether there is evidence showing the variant is predictively functional, and how strong the evidence is; whether there is evidence showing the variant is potentially artificial, and how strong the evidence is; AA_Change: Amino acid change, in HGVS standard; Protein length: Protein length; Confidence is the number of variant caller algorithmns that identified the variant out of a total of 7. All variants in this table were somatic changes not found in the germline WES from the patient.

11.4.2 Appendix 4.2: Ploidy estimate plots



Ploidy estimate plots show that while some CRPM in the cohort have relatively normal copy numbers across the genome, the majority have widespread variation in ploidy.

11.4.3 Appendix 4.3: Cosmic DNA mutation signature

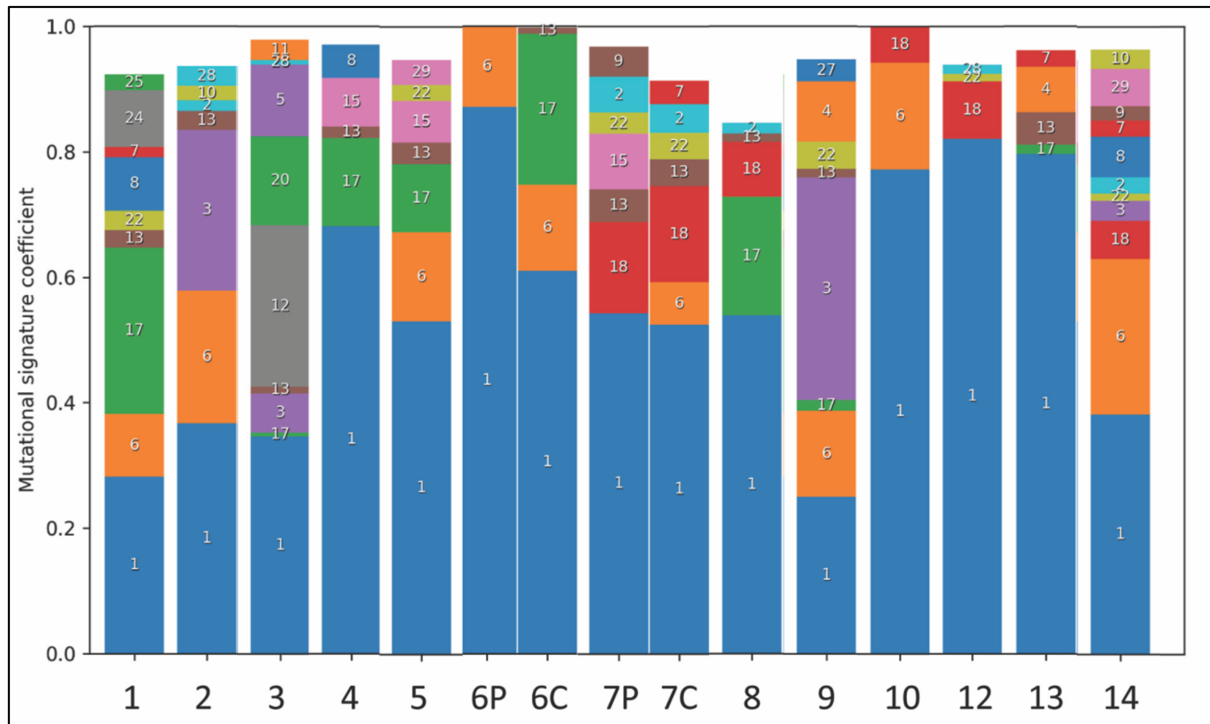


Figure 9.10. COSMIC DNA mutation signature, with the most common signature across all sequenced samples being ageing.

Patient numbers lie on the X-axis; Signature 1: ageing; 3: BRCA; 6: mismatch repair/microsatellite instability; 20: mismatch repair fault; 5, 12, 17, 18: unknown

11.4.4 Appendix 4.4. Curated panel of drugs

Table of curated panel of drugs (based on availability in Australia)

Drug	Type	Status	Main Target	All targets	Licenced in Australia
Abemaciclib	Targeted	Phase III	CDK	Cdk4, Cdk6	HER2-negative metastatic breast cancer
Acalabrutinib	Targeted	Approved	BTK		Mantle cell lymphoma
Afatinib	Targeted	Approved	EGFR	EGFR, HER2	Non small cell lung cancer
Alectinib	Targeted	Approved	ALK	ALK tyrosine kinase	Non small cell lung cancer
Axitinib	Targeted	Approved	BCR-ABL	VEGFR1, VEGFR2, VEGFR3 KIT, PDGFR	Metastatic renal cell carcinoma
AZD1775	Targeted	Phase II	Wee1	Wee1	In trials for metastatic CRC and other cancers
Celecoxib	Targeted	Phase III	Cox-2	cox-2, cadherin-11	Non-steroidal anti-inflammatory
Ceritinib	Targeted	Approved	ALK	ALK, IGF-1R, ROS1	Non small cell lung cancer
Cetuximab	mAb	Approved	EGFR	EGFR	RAS wild type metastatic CRC
Cobimetinib	Targeted	Approved	MEK	MEK	Metastatic melanoma
Crizotinib	Targeted	Approved	ALK	ALK, ROS1, MET	Non small cell lung cancer

Dasatinib	Targeted	Approved	BCR-ABL	Bcr-Abl, Src	Chronic myeloid leukemia
Enasidenib	Targeted	Approved	IDHi	isocitrate dehydrogenase-2 inhibitor	Acute myeloid leukemia
Enzalutamide	Targeted	Approved	Androgen	Androgen receptor antagonist	Metastatic prostate cancer
Erlotinib	Targeted	Approved	EGFR	EGFR, ALK, JAK2 mutant (JAK2V617F)	Non small cell lung cancer
Everolimus	Targeted	Approved	mTOR	mTORC1	Neuroendocrine tumours
Fulvestrant	Targeted	Approved	Estrogen	selective estrogen receptor degrader	Metastatic breast cancer
Gefitinib	Targeted	Approved	EGFR	EGFR	Non small cell lung cancer
Ibrutinib	Targeted	Approved	BTK	BTK	Chronic lymphocytic leukemia
Lapatinib	Targeted	Approved	EGFR	EGFR, HER2	Metastatic breast cancer
Larotrectinib	Targeted	Phase II	Trk	TrkA, TrkB, TrkC receptor antagonist	Non hodgkins lymphoma
Midostaurin	Targeted	Approved	VEGFR	FLT3, PKCα, PKCβ, PKCγ, Syk, Flk-1, Akt, PKA, c-Kit, FGFR, SRC, PDFRβ, VEGFR1, VEGFR2	Acute myeloid leukemia
Neratinib	Targeted	Approved	EGFR	EGFR, HER1, HER2, HER4	HER2 pos breast cancer
Olaparib	Targeted	Approved	PARP	PARP	BRCA mutant cancers
Osimertinib	Targeted	Approved	EGFR	EGFR	Non small cell lung cancer

Palbociclib	Targeted	Approved	CDK	CDK4, CDK6	ER/PR pos, HER2 neg metastatic breast cancer
Poziotinib	Targeted	Phase II	EGFR	EGFR, HER2, HER4	Recurrent platinum resistant ovarian cancer
Quizartinib	Targeted	Phase III	FLT3	FLT3	Acute myeloid leukemia
Regorafenib	Targeted	Approved	VEGFR	VEGFR2, TIE2 (multikinase inhibitor)	Metastatic CRC
Rigosertib	Targeted	Phase III	Plk1	Plk1, PI3K	Myelodysplastic syndrome
Rucaparib	Targeted	Approved	PARP	PARP1, PARP2, PARP3	Recurrent ovarian cancer
Sorafenib	Targeted	Approved	Kinase	VEGFR, PDGFR, Raf	Hepatocellular carcinoma, renal cell carinoma, thyroid cancer
Tazemetostat	Targeted	Phase II	EZH2	EZH2	B-cell lymphoma
Trametinib	Targeted	Approved	MEK	MEK1, MEK2	BRAF V600E mutant metastatic melanoma
Vandetanib	Targeted	Approved	EGFR	VEGFR2, EGFR, RET	Medullary thyroid cancer
Vemurafenib	Targeted	Approved	BRAF	BRAF	BRAF V600E mutant metastatic melanoma
Vorinostat	Targeted	Approved	HDAC	histone deacetylase	Cutaneous T cell lymphoma

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